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Ministry of Food and Drug Safety

MFDS NAMs Report: Regulatory Acceptance and Research Outcomes

MINISTRY OF FOOD AND DRUG SAFETY
National Institute
of Food and Drug Safety Evalution

KoCVAM
Korean Center for the
Validation of Alternative Methods

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Ⅰ KoCVAM and R&D in NAMs

1 KoCVAM: past, present and future

- Korean Center for the Validation of Alternative Methods (KoCVAM) has been operated within the Ministry of Food and Drug Safety (MFDS) since 2009 in order to introduce alternative test methods or new approach methodologies (NAMs) in safety assessment, which conventionally relies on animal testing, and to support the promotion and utilization of NAMs.

- MFDS has provided support for the promotion of NAMs since when they were not much popular and nor studied by, for example, conducting research and providing education on the methods. MFDS is the only government agency in Korea that has developed NAMs adopted in OECD Test Guidelines. As a member of the OECD, Korea has adhered to the Mutual Acceptance of Data (MAD), a multilateral agreement on recognition of the safety assessment data of chemicals including cosmetics, and mutually accepts the safety assessment data with other participating countries. According the the OECD, the MAD saves around 300 million euros (300M €) of testing cost and sacrifices of over 10,000 laboratory animals each year. MFDS has helped the national industry and GLP facilities to increase their competitiveness in the world by helping domestic technologies to be adopted by the OECD, which the MAD system is based on.

- This is attributed to over 14 years of KoCVAM's commitment to international cooperation with the OECD and the International Cooperation on Alternative Test Methods (ICATM).

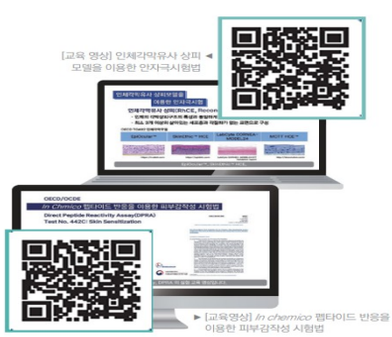
- KoCVAM signed the Memorandum of Cooperation (MOC) to join the ICATM in 2011 and has participated in the development and validation of over 30 NAMs led by the ICATM member countries.

- MFDS has focused on regulatory acceptance and development of NAMs for evaluation of medical products including medical devices as well as cosmetics ingredients. In particular, an *in vitro* skin irritation test for evaluation of medical devices developed by MFDS has been approved unanimously as an internationally standardized test method at the ISO/TC194* Meeting held in October 2023. This is the first and the only accomplishment made by a national organization relating to ISO 10993-23** skin irritation test.

  * Technical Committee of ISO (International Organization for Standardization) dealing with biological and clinical evaluation of medical devices

  ** International standard ISO 10993-23:2021 (Biological evaluation of medical devices-Part 23: Tests for irritation)

- Also, KoCVAM has provided full support for distribution of NAMs in Korea to promote the use of the methods by organizing workshops and training programs (including video training) for academia and industry including non-clinical research institutions as well as co-organizing international symposium each year since 2013



- KoCVAM has been committed to supporting the development of NAMs and promoting practical use and regulatory acceptance of advanced new methods through active international cooperation.

2 Regulatory acceptance of NAMs

- The world has tightened ban on animal testing for safety assessment of chemicals including cosmetic ingredients and medical devices, and the ban has increasingly spread to medical products. Therefore, it is imperative to develop and accept internationally harmonized NAMs.

- Since the EU's complete ban on animal testing for cosmetics, more and more countries have also prohibited animal testing. With the revision of Cosmetics Act in 2016, Korea has also banned sales and marketing of cosmetics and their ingredients tested on animals.

- The domestic companies exporting cosmetics and medical devices need NAMs for safety assessment, and failing to perform one will make them experience a huge financial loss. The current Korean laws relating to NAMs are; *Laboratory Animal Act*; *Cosmetics Act; Regulation on Evaluation of Functional Cosmetics*; and *Standards for Biological Safety Evaluation of Medical Devices. Pursuant to the Laboratory Animal Act.* MFDS is responsible to establish and implement the policy to support development and acceptance of NAMs able to replace animal testing. Accordingly, the Minister has established KoCVAM that carries out tasks related to development, validation, and dissemination of NAMs. Reticently, "*the draft bill for promotion of development, dissemination and utilization of alternative test method* (proposed by law maker In-soon Nam, Dec. 2020)" and "*the draft bill for encouraging development, distribution and use of alternative test methods* (proposed by law maker Jung-ae Han, Dec. 2022)" have been submitted to the national assembly in order to strengthen the role of KoCVAM and to promote the use of NAMs in Korea.

- In 2023, MFDS revised the Korean Pharmacopoeia to accept NAMs for quality control of pharmaceutical products. With the revision, the test methods using recombinant factor C have newly been included to replace the use of horseshoe crab blood to detect endotoxins in pharmaceutical products.

- With the establishment of the FDA Modernization Act 2.0 in December 2022, FDA allows the use of NAMs in new drugs and biologics application. In other words, the law concerning foods, drugs and cosmetics has permitted the use of the data generated using NAMs as non-clinical test data.

- To keep up with the change in the U.S., MFDS has made an administrative notice on implementation of the revised "*Regulation on Pharmaceuticals Approval, Notification and Review*". The revised regulation allows submission of data from non-animal methods or human biology-based tests (cell-based test, MPS, bioprinting, computaional model, etc.) that are produced in accordance with the Good Laboratory Practice.

- To proactively respond to the changes in the world where NAMs are expanding their realm from cosmetics including cosmetic ingredients and medical devices to pharmaceuticals, MFDS has worked hard to promote NAMs in Korea for the last 14 years. With the knowledge and know-hows accumulated in KoCVAM, MFDS will be able to take lead nationally and internationally in the field of NAMs.

3 MFDS R&D budget relating to NAMs

- The Toxicological Evaluation and Research Department at the National Institute of Food and Drug Safety Evaluation (NIFDS), an affiliation of MFDS, has conducted researches on development of advanced safety assessment methods since 1998 as part of MFDS research and development project for food and drug safety.

- The project is intended to establish base technologies for safety prediction and evaluation including development of toxicological, pharmacological and clinical tests, advanced analytic methods, laboratory animals and alternative test methods, which can serve as a scientific basis for the policies relating to safety management of foods, drugs, etc. As a part of the project for development of safety assessment methods, the Toxicological Evaluation and Research Department has developed and validated NAMs and developed novel toxicity assessment and narcotics analysis methods.

- KoCVAM and Toxicological Screening and Testing Division of the Department has developed and validated NAMs for safety assessment of cosmetics, medical devices, etc. in the project.

Table 1. R&D project for development of NAMs for safety assessment conducted by Toxicological Screening and Testing Division

Title	Main topic
Establishment of foundation for development and validation of NAMs	○ Development of internationally harmonized test guidelines for NAMs reflecting animal welfare and their acceptance in Korea

- **Scientific outcome of the project**

  - The R&D project for development of NAMs for safety assessment is divided into 8 studies, which are carried out by each division in Toxicological Evaluation and Research Department.

  - Many remarkable papers have been able to be published from the project, leading to establishment of scientific basis for the regulations on safety management of foods and drugs.

    * 139 SCI(E)-grade papers were published through this project for the past 3 years (2020 to 2023), exhibiting average Impact Factor of 4.17

- **Social outcome of the project**

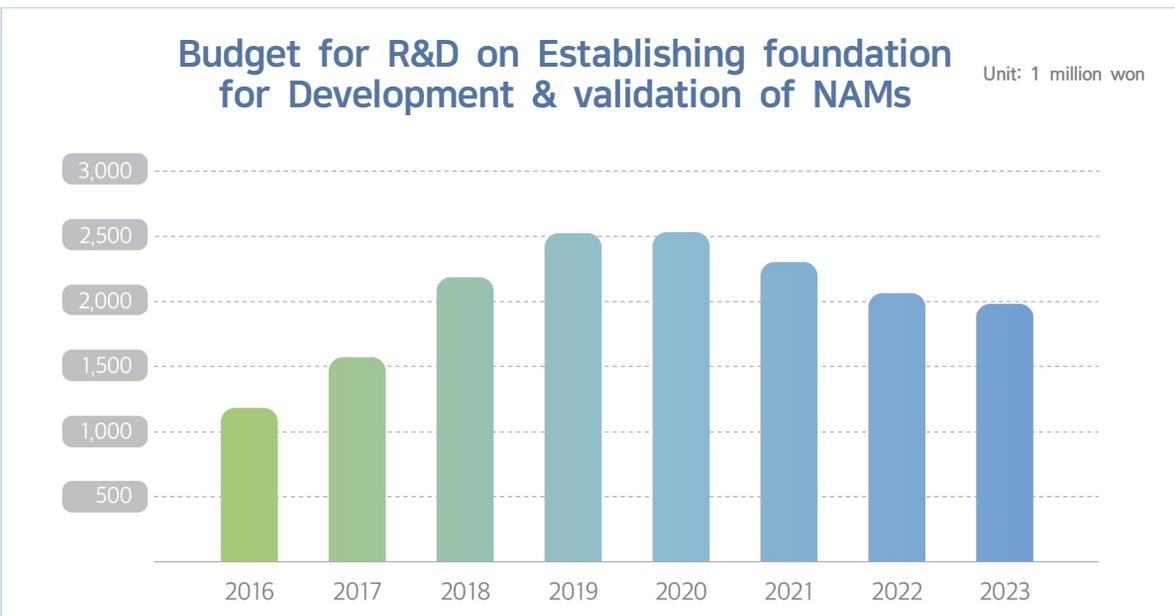
  - Establishment of the system to control new narcotics and to prevent their introduction in Korea by rapidly responding to them.

    * Selected as one of the 100 best national R&D projects in 2020

- Reduction of public fear over hazardous chemicals by performing toxicity evaluation on consumer chemical products and medicinal substances and establishing regulations on hazardous ingredients that might threaten public health.
- Protection of public health from corona virus by preparing the draft guideline for efficacy evaluation of drug candidates and by providing information on clinical trials in order to facilitate rapid development of COVID-19 treatments

● **Technical outcome of the project**

- Development of internationally harmonized alternative test methods adopted in OECD Test Guidelines
- Application of 14 patents in Korea and in other countries for the technologies developed by internal R&D project
  * Out of the 14 patents applied for the past 3 years (2020 to 2023) through the project, 3 applications have been filed in other countries
  * A qualitatively remarkable outcome with 5.00 of patent rating in SMART



● The project for research and development of safety assessment technology will continue to serve as a platform for strengthening the country's global profile and competitiveness by developing safety assessment technology meeting international standards. ❶ In order to actively respond to global bans on animal testing, research for development of NAMs using advanced technologies will be performed to obtain original technology for next

generation safety assessment method and to enhance economic feasibility of the domestic industry by using the technologies and products produced within the country. ❷ Confidence in the policy for safe management of narcotics and safe use of foods and drugs will be maintained by preemtively responding to new narcotics, foods and drugs. ❸ The public will continue to trust MFDS as it is able to rapidly execute political countermeasures to any issues relating to toxic substances in foods and drugs.

- Research project: establishment of foundation for advanced toxicity evaluation technology (2022~2025)
  - Toxicological Research Division of NIFDS has conducted the study aiming to establish foundation for regulatory science by preemtively obtaining advanced toxicity evaluation technologies developed in Industry 4.0 (budget: 8.38 billion won).
  - The Division has been working to propose a toxicity evaluation platform using liver organoid as an international standard and to create new bioindustry by performing an independent validation study and peer-review.
- New research project: standardization of NAMs for practical implementation (2024~2028)
  - Toxicological Research Division of NIFDS will launch 5 year project in 2024 budgeted at 47.5 billion. The project aims to improve coherence of regulatory science and to encourage practical use of NAMs by developing, optimizing and standardizing NAMs for safety, efficacy and quality evaluation of drugs.
  - The project consists of research on development of alternatives to animal testing especially for safety evaluation and for specification testing of drugs. It will focus on standardization of toxicity and efficacy evaluation technologies including organoid, organ-on-chips and 3D tissue models and fostering their regulatory acceptance.

    * ❶ heart ❷ kidney ❸ nerve system ❹ respiratory system ❺ skin ❻ digestive system
  - MFDS will build intergovernmental cooperation system to facilitate development of toxicity and safety evaluation methods based on standardized organoids and organ-on-chips and to propose the methods as OECD Test Guidelines with support from KoCVAM.

Table 2. Main topics of the research project "standardization of alternative test methods for practical implementation"

	Main Topics		
Project A	Project Title	Development of alternatives to animal testing for safety evaluation of drugs	
	Detailed subject	Timeline	budget
	① International harmonization and standardization of alternative test methods	5 years	30.875 billion won
	② International harmonization and standardization of safety and efficacy evaluation technologies based on heart organoid and (or) tissue chip		
	③ Optimization and standardization of safety and efficacy evaluation technologies based on nerve system organoid and (or) tissue chip		
	④ Optimization and standardization of safety and efficacy evaluation technologies based on skin organoid and (or) tissue chip		
	⑤ Optimization and standardization of safety and efficacy evaluation technologies based on respiratory system organoid and (or) tissue chip		
	⑥ Optimization and standardization of safety and efficacy evaluation technologies based on kidney organoid and (or) tissue chip		
	⑦ Optimization and standardization of safety and efficacy evaluation technologies based on digestive system organoid and (or) tissue chip		
	⑧ Development of AI-based alternative test methods		
Project B	Project title	Development of alternative test methods for specification testing of drugs	
	Detailed subject	timeline	budget
	① Development of alternative test methods for quality evaluation of plasma-derived medicinal products	5 years	16.625 billion won
	② Development of alternative test methods for evaluation of oral mucosa irritation potential of medical devices		
	③ Development and optimization of alternative test methods using zebrafish and C. elegans		
	④ Establishment of biological resource bank		

Ⅱ Development and validation of internationally harmonized alternative test methods recognized by the OECD

1. The first domestically-developed skin sensitisation test method adopted in OECD TG
2. Development of an alternative eye irritation test method adopted fourth in the OECD Test Guideline 492
3. Development and OECD adoption of androgen receptor screening assay
4. Development and OECD adoption of an alternative skin irritation test method

Ⅱ Development and validation of internationally harmonized alternative test methods recognized by the OECD

☐ Development and validation of alternative test methods

- Test Guidelines (TGs) issues by the OECD are adopted by governments, industries and research institutions in the world for hazard and safety evaluation of chemicals including cosmetic ingredients. Use of the Test Guidelines that are based on validated test methods help obtain reliable safety assessment data for protection of human and animal health.

- Most NAMs for evaluation of hazardous substances should be validated by national and (or) international organizations. The size of validation studies can range from small to large, international scale.

- Validation of a test method (or an approach) is a research process that is based on scientific principles to establish reliability and relevance.

  - Reliability refers to measures of the extent that a test method can be performed reproducibility within and between laboratories over time, when performed using the same protocol.

  - Relevance refers to description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which a test correctly measures or predicts the biological effect of interest.

- KoCVAM has supported the OECD adoption of the NAMs developed by NIFDS' R&D projects in Test Guidelines. The test methods in OECD TGs are regulatorily accepted in Korea under *Cosmetics Act*, *Regulation on Evaluation of Functional Cosmetics*, and *Standards for Biological Safety Evaluation of Medical Devices* and utilized in safety assessment of not only drugs that MFDS is concerned but also chemicals and pesticides.

1 The first domestically developed skin sensitisation test method adopted in OECD TG

Supervising division	Toxicological Screening and Testing Division of NIFDS
type	Internal project
title	Study on international harmonization for development of test guidelines based on alternative test methods (I)(17181MFDS486)

☐ Background for the study

- Since the 3R principles (reduction, refinement and replacement) suggesting ethical use of animals in science were proposed, demand for NAMs for safety assessment has been increased.
- There should be an alternative to the existing Local Lymph Node Assay (LLNA) (OECD TG 429) that uses lab animals and radioisotope.
  - A KoCVAM-developed "*me-too*" method to TG 442B (alternative to TG 429) is able to overcome some of the limitations of LLNA. The method was included in the OECD workplan in April 2016 and adopted in the TG 429 in June 2018 following a successful validation study and an independent peer-review.
- There has been an increasing demand on alternatives to animal testing in biological safety assessment of medical devices. An alternative skin sensitisation test method for safety assessment of medical devices should be developed.

☐ Study in brief

- The objectives of the research were to propose the revised OECD TG 442B including LLNA: BrdU-FCM developed in Korea; to conduct follow-up study based on the comments from the OECD expert group; and to perform research on the skin sensitisation test method for safety assessment of medical devices.
- LLNA:BrdU-FCM
  - LLNA:BrdU-FCM, a "*me-too*" method to OECD TG 429, has advantages over OECD TG406 that requires use of guinea pigs. The method is able to detect skin sensitisation potential without using radioisotope, to reduce the number of animals used, and to alleviate pain in animals as it does not require the use of an adjuvant.

- **Principle of the method:** A local lymph node assay is able to detect skin sensitisation potential of test substances according to the UN GHS[1)] by evaluating T-cell activation and proliferation, the fourth key event of skin sensitisation Adverse Outcome Pathway (AOP).
  - The method is able to evaluate skin sensitisation potential of test substances using a flow cytometry that measures proliferation of auricular lymph node cells during the induction phase of skin sensitisation.

☐ Overview of the Research Outcome

- **Major accomplishment:** LLNA:BrdU-FCM developed by NIFDS has become the first domestically-developed toxicity test method adopted in OECD Test Guideline (OECD TG 442B)
  - **Validation study:** KoCVAM worked with international stakeholders and operated a validation management team to coordinate the validation study in accordance with the guidance suggested by the OECD, and prepared the validation study report.
  - **OECD recognition:** The scientific rationale of the method was proved during the peer review, and the method was officially included in OECD TG 442B in June 2018 following the OECD WNT approval in April 2018.
    * WNT: OECD Working Group of National Co-ordinators of the TGs programme who take decisions on TGs (approve and update of TGs) and decide on project proposals to include in the work plan. The group consists of representative from the member countries that adhere to MAD and meets annually.
- **Academic accomplishment**
  - Awarded as one of the best MFDS R&D projects in 2017
  - Publication: 1 paper in a SCI-grade journal*
    * Regulatory Toxicology and Pharmacology

☐ Excellence and advantages

- **Excellence:** LLNA: BrdU-FCM developed in Korea has been recognized as a reliable and relevant alternative to OECD TG 442B and a "*me-too*" method to OECD TG 442B during the KoCVAM-coordinated international peer review consisting of experts from the U.S., Switzerland and Japan.

1) UN GHS: United Nations Globally Harmonized System of classification and labelling of chemicals

- **Advantages:** Compared to Local Lymph Node Assay (LLNA) that often faces issues relating to radiation exposure or disposal of radioactive waste due to use of radioisotope, LLNA:BrdU-FCM is able to evaluate skin sensitisation potential without using radioisotope and to perform mechanistic research including B-cell and T-cell.

☐ Utilization of the Outcome and Its Impact

- The outcome has proven that KoCVAM plays a leading role in the field of alternative test methods in Korea.
  * Countries developed test methods recognized in OECD TGs: the U.S., 5 European Countries (Germany, Italy, Netherlands, Belgium, and France), Japan, Canada and Korea.

- **International relation:** International exchange and cooperation has been promoted (e.g. participation and presentation in the general meeting of ISO/TC194, Biological and clinical evaluation of medical devices).
  * Countries joining in the ISO/TC194 general meeting: 29 participating countries including Germany (host), Australia, Austria, Belgium, Canada, the U.S. Japan Brazil and 19 observing countries

- The related industry has grown thanks to introduction and dissemination of a new alternative test method, which has helped nurture professional personnel, and technical support provided by the government.
  - Guidance on the use of LLNA:BrdU-FCM, has been published, education workshops have been organized; and training programs have been run to transfer the techniques required to use the method to those working in the domestic GLP facilities and related academic area.

☐ Performance of the research

	Title	MFDS performance evaluation standard	
		type	performance indicators
1	Proposal to add a method in the OECD Test Guideline (OECD TG442B)	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
2	Press release: Alternative test method developed in Korea soon to be added in OECD TG	Education and promotion of the research outcome	Number of the education or promotion materials about the outcome
3	Publication: Evaluation of skin sensitization potential of chemicals by local lymph node assay using 5-bromo-2-deoxyuridine with flow cytometry	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journal

	Title	MFDS performance evaluation standard	
		type	performance indicators
4	Publication: Development of the LLNA: BrdU-FCM for Skin Sensitization Evaluation	Building on expertise through academic accomplishment	Number of presentations and publications in international workshops
5	Publication in academic journal: Evaluation of Skin Sensitization Potency Using the LLNA: BrdU-FCM and the *Ex vivo* Cytokine Assay	Building on expertise through academic accomplishment	Number of presentations and publications in national workshops
6	Publication: Evaluation of Skin Sensitization Potential of Chemicals Using the LLNA: BrdU-FCM	Building on expertise through academic accomplishment	Number of presentations and publications in national workshops
7	Publication: Predictive Possibility of the *Ex vivo* Cytokine Assay in LNCs on Skin Sensitization Potency	Building on expertise through academic accomplishment	Number of presentations and publications in international workshops
8	Addition in OECD Test Guideline (OECD TG 442B)	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
9	Awarded as an excellent R&D project	Utilization and dissemination of research outcome	Number of the education or promotion materials about the outcome
10	Press release: MFDS-developed skin sensitisation test method adopted in OECD TG	Utilization and dissemination of research outcome	Number of the education or promotion materials about the outcome
11	Making presentation in 2018 Education Workshop on Alternative Test Mehtod	Utilization and dissemination of research outcome	Level of mutual exchange of information for dissemination of technology
12	Hosting 2019 Training Workshop on Alternative Test method	Utilization and dissemination of research outcome	Level of mutual exchange of information for dissemination of technology
13	SOP-sensitisation-19: Local Lymph Node Assay Using Medical Devices	Improvement of ability in testing, research and experiment	Total count of test methods developed
14	Making oral presentation in the 29th ISO/TC194 general meeting	Improving national competitiveness	Performance in international exchange (e.g. signing MOU etc.)
15	Publication: Evaluation of the Local Lymph Node Assay: BrdU-FCM to investigate Skin Sensitizers	Encouragement of practical use by policy improvement, etc.	Number of presentations and publications in international workshops
16	Publication: Utilization of the LLNAL: BrdU-FCM and the *Ex vivo* Cytokine Assay to evaluation of Skin Sensitization Potency.	Building on expertise through academic accomplishment	Number of presentations and publications in national workshops

☐ Major accomplishment

1. Adoption in OECD Test Guideline (OECD TG442B)

OECD/OCDE 442B

Adopted:
25June 2018

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Local lymph node assay: BRDU-ELISA or –FCM

GENERAL INTRODUCTION

1. A skin sensitiser refers to a substance that will lead to an allergic response following repeated skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1).

2. There is general agreement regarding the key biological events underlying skin sensitisation. The current knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised in the form of an Adverse Outcome Pathway (AOP) (2), starting with the molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. This AOP focuses on chemicals that react with thiol (i.e. cysteine) and primary amines (i.e. lysine) such as organic chemicals. In this instance, the molecular initiating event (i.e. the first key event) is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The first key event can be addressed using the in chemico Direct Peptide Reactivity Assay (DPRA) TG 442C (3). The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. This key event can be addressed using the in vitro ARE-Nrf2 Luciferase Test Methods (KeratinoSensTM or LuSens) TG 442D (4). The third key event is the activation of dendritic cells (DC), typically assessed by expression of specific cell surface markers, chemokines and cytokines, and can be addressed using either the in vitro Human Cell Line Activation Test (h-CLAT), the in vitro U937 Cell Line Activation Test (U-SENS™) or the Interleukin-9 Reporter Gene assay (IL-8 Luc assay) as described in TG 442E (5). The fourth key event is T-cell proliferation, which is indirectly assessed in the in vivo murine Local Lymph Node Assays (LLNA) (6).

3. The first Test Guideline (TG) for the determination of skin sensitisation in the mouse, the Local Lymph Node Assay (LLNA: TG 429) was adopted in 2002, and has since then been revised (7). The details of the validation of the LLNA and a review of the associated work have been published (8) (9) (10) (11) (12) (13) (14) (15) (16). In the LLNA, radioisotopic thymidine or iodine is used to measure lymphocyte proliferation and therefore the assay has limited use in regions where the acquisition, use, or disposal of radioactivity is problematic.

In accordance with the decision of the Council on a delegation of authority to amend Annex I of the decision of the council on the Mutual Acceptance of Data in the assessment of chemicals [C(2018)49], this Guideline was amended by the OECD's Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology by written procedure on 25 June 2018.

3. Publication: Evaluation of skin sensitization potential of chemicals by local lymph node assay using 5-bromo-2-deoxyuridine with flow cytometry

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Evaluation of skin sensitization potential of chemicals by local lymph node assay using 5-bromo-2-deoxyuridine with flow cytometry

Byeol-I. Han[a,1], Jung-Sun Yi[a,1], Souk Jin Seo[a], Tae Sung Kim[a], Ilyoung Ahn[a], Kyungyuk Ko[a], Joo Hwan Kim[a], SeungJin Bae[b], Jong Kwon Lee[c,*]

[a] Toxicological Screening and Testing Division, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Cheongju-si, 28159, Republic of Korea
[b] College of Pharmacy, Ewha Womans University, Seoul, 03760, Republic of Korea
[c] Toxicological Research Division, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Cheongju-si, 28159, Republic of Korea

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ABSTRACT

The local lymph node assay using 5-bromo-2-deoxyuridine with flow cytometry (LLNA: BrdU-FCM) is a modified LLNA used to identify skin sensitizers. This assay measures the proliferation of auricular lymph node cells (LNCs) during the induction phase of skin sensitization and the number of BrdU-positive LNCs using flow cytometry. We determined if LLNA: BrdU-FCM can evaluate the skin sensitization potential of 20 substances, including 16 sensitizers and 4 non-sensitizers, that were tested using LLNA: DA and LLNA: BrdU-ELISA but not listed in OECD TG 429. After selecting appropriate vehicles and conducting pre-screen tests in 2 phases, solvents and test concentrations for the main test were determined. In the main study, we measured changes in LN weight, the number of LNCs, and the proportion of BrdU incorporated into LNCs to calculate stimulation indexes (SI). SI was calculated based on the total number of LNCs and BrdU incorporation in LNCs. We found that all substances were correctly classified as sensitizers or non-sensitizers. Overall, we confirmed that the LLNA: BrdU-FCM can evaluate skin sensitization potential of the 20 substances. Additionally, our results of combining 22 reference substances listed in OECD TG 429 and 20 additional substances showed that concordance of LLNA: BrdU-FCM with the LLNA was higher than before.

1. Introduction

The murine local lymph node assay (LLNA) for evaluating the skin sensitization potential of substances has been used worldwide as an alternative test method to the conventionally used guinea pig test (OECD TG 406, 1992); it was adopted as OECD TG 429 (Skin Sensitization: Local Lymph Node Assay) in 2002 (OECD, 2010a). The LLNA is a skin sensitization test in mice that measures the proliferation of murine local auricular lymph node cells (LNCs) after topical exposure to test substances. However, the use of LLNA has been limited due to the employment of radioisotope-labeled ^{3}H-methyl thymidine and the resultant difficulty in the disposal of radioactive waste in some countries. For this reason, LLNA was modified to replace ^{3}H-methyl thymidine, and two non-radio isotopic LLNA methods (LLNA: DA as OECD TG 442A and LLNA: BrdU-ELISA as OECD TG 442B) were adopted in 2010. LLNA: DA quantifies the ATP content of LNCs and LLNA: BrdU-ELISA measures the incorporation of BrdU, an analogue of thymidine, into lymph nodes (OECD TG 442A, 2010b; OECD TG 442B, 2010c,d) to evaluate LNC proliferation. *In Chemico* Skin Sensitization (DPRA) as OECD TG 442C and *In Vitro* Skin Sensitization (KeratinoSens) as OECD TG 442D were adopted in 2015 and *In Vitro* Skin Sensitization (h-CLAT) as OECD TG 442E was adopted in 2017 (OECD TG 442C, 2015a; OECD TG 442D, 2015b; OECD TG 442E, 2017).

The LLNA: BrdU- FCM is a novel non-radioisotopic version of the LLNA that performs similar to existing LLNA methods. This test measures the proliferation of auricular LNCs during the induction phase of skin sensitization by determining the number of BrdU-positive LNCs by flow cytometry. The LLNA: BrdU-FCM has been optimized and validated (Yang et al., 2015; Kim et al., 2016; Ahn et al., 2016). LLNA: BrdU-FCM can be used to evaluate the skin sensitization potency of test substances in the same way as the conventional LLNA, LLNA: BrdU-ELISA, and LLNA: DA. The LLNA: BrdU-FCM has several additional advantages. First, the proliferation of living LNCs is quantitatively measured in the LLNA: BrdU-FCM, whereas cells are indirectly scored

* Corresponding author.
E-mail address: jkleest@korea.kr (J.K. Lee).
[1] These authors contributed equally to do this work.

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0273-2300/

2 Development of an alternative eye irritation test method adopted fourth in the OECD Test Guideline 492

Supervising division	Toxicological screening and testing division of NIFDS
Project type	Entrusted project (prof. Kyung-min Lim, Ewha Womans University)
Title	Validation study on the eye irritation test method using RhCE (16182MFDS522)

☐ Background for the study

- Local toxicity tests are an integral part of cosmetics safety assessment. In particular, an eye irritation test requires use of animals (rabbits) and includes procedures causing pain to the animals, and thus should be replaced by an alternative method.
- With the global ban in European countries on animal testing for cosmetics and their ingredients, Korea has revised the Cosmetics Act. In this sense, development of a new alternative test method that can be utilized by national industry and non-clinical test facilities was considered necessary.

☐ Study in brief

- The study aimed to develop an *in vitro* eye irritation test method using RhCE (reconstructed human cornea-like epidermis), MCTT HCE™, as an alternative to Rabbit Eye Draize Test (OECD TG405) that uses rabbits.
- An *in vitro* eye irritation test method using MCTT HCE™ developed in Korea was studied further, and an international validation study was performed to verify the feasibility of the method as an internationally harmonized one accepted by the OECD.
- MCTT HCE™ is a 3D human cornea model that is manufactured with primary cultured cornea epithelium using residual skin tissues from cornea transplant. Since human primary cornea cells are used, its morphological microstructure and biomarker expressions are similar to those of human epithelium.
- **Principle of the method:** This method evaluates an eye irritation potential of a test chemical by measuring reduction in cell viability of MCTT HCE™ following local exposure to the test chemical using WST-1 assay.

- Serious eye damage and (or) eye irritation (corneal opacity, iritis, conjunctival redness and/or conjunctical chemosis) are the results of the responses initiated by penetration of a test chemical through cornea and/or conjunctiva and following cell damage.

- **Development and validation of the test method:** A validation study on the eye irritation test method using MCTT HCE™ was conducted in accordance with OECD guidance. KoCVAM led the validation study in cooperation with the method developer.

☐ Overview of the study outcome

- **Major accomplishment:** An eye irritation test method using the model developed in Korea, MCTT HCE™ EIT, has become the fourth test method adopted in the OECD TG 492 as one of the internationally harmonized methods.
  * MCTT HCE™ is the only model in the world that is manufactured with human primary cultured epithelium.

- **Validation study:** KoCVAM was in charge of assuring that the validation study fulfilled OECD guidance by cooperating with international partners including OECD Secretariat and operating a validation management team.

- **OECD recognition:** The method was approved to be included in the OECD Test Guideline 492 at the WNT Meeting in April 2019 and the revised TG was officially issued in June 2019.
  * WNT: OECD Working Group of National Co-ordinators of the TGs programme who take decisions on TGs (approve and update of TGs) and decide on project proposals to include in the work plan. The group consists of representative from the member countries that adhere to MAD and meets annually.

- **Major accomplishment**

- **Academic accomplishment:** publication of 4 papers in SCI and non-SCI grade journals
  * Food and Chemical Toxicology, Toxicology *in vitro* and Toxicological Research

☐ Excellence and advantages

- **Excellence:** Compared to the other OECD-adopted models that are manufactured with human keratinocytes (EpiOcular™) or corneal epithelial cell line (SkinEthic HCE), MCTT HCE™ used in this test method is manufactured with human primary cultured corneal cells and thus able to effectively mimic human corneal responses.

- **Advantages:** Other methods using EpiOcular™ or SkinEthic™ HCE use separate protocols for liquids and solids and require a lot of models because each protocol requires inclusion of both positive and negative controls. In this regard, the test method using MCTT HCE™ has advantages over the two methods.

- **Economic advantage:** Foreign Models including EpiOcular™ and SkinEthic™ HCE are unlikely to be shipped directly to the Korean users. Further, shipment takes a long time due to shipping or customs process. Since MCTT HCE™ is domestically-developed model, it does not require long shipment period and tends to cost less compared to the foreign models.

☐ Utilization of the outcome and its impact

- The outcome has proven that KoCVAM plays a leading role in the field of alternative test methods in Korea.

  * Countries having test methods recognized in OECD TGs: the U.S., 5 European Countries (Germany, Italy, Netherlands, Belgium, and France), Japan, Canada and Korea.

- **Increasing export:** MCTT HCE™ is the first domestically-developed and manufactured ocular model that has been internationally recognized and can be exported to overseas test facilities conducting eye irritation testing.

- **Development of related industry:** Eye irritation test was highly dependent on foreign models in the past. However, the development of MCTT HCE™), will help increase the sales of the domestic manufacturer.

- Introduction and dissemination of the alternative test method helps train professional personnel and transfer necessary techniques and thus promote the related industry.

  - Following development of MCTT HCE™ eye irritation test method, standard operating procedure and guideline have been prepared and the method have been transferred to the people at the national GLP test facilities and academia in education workshops, etc.

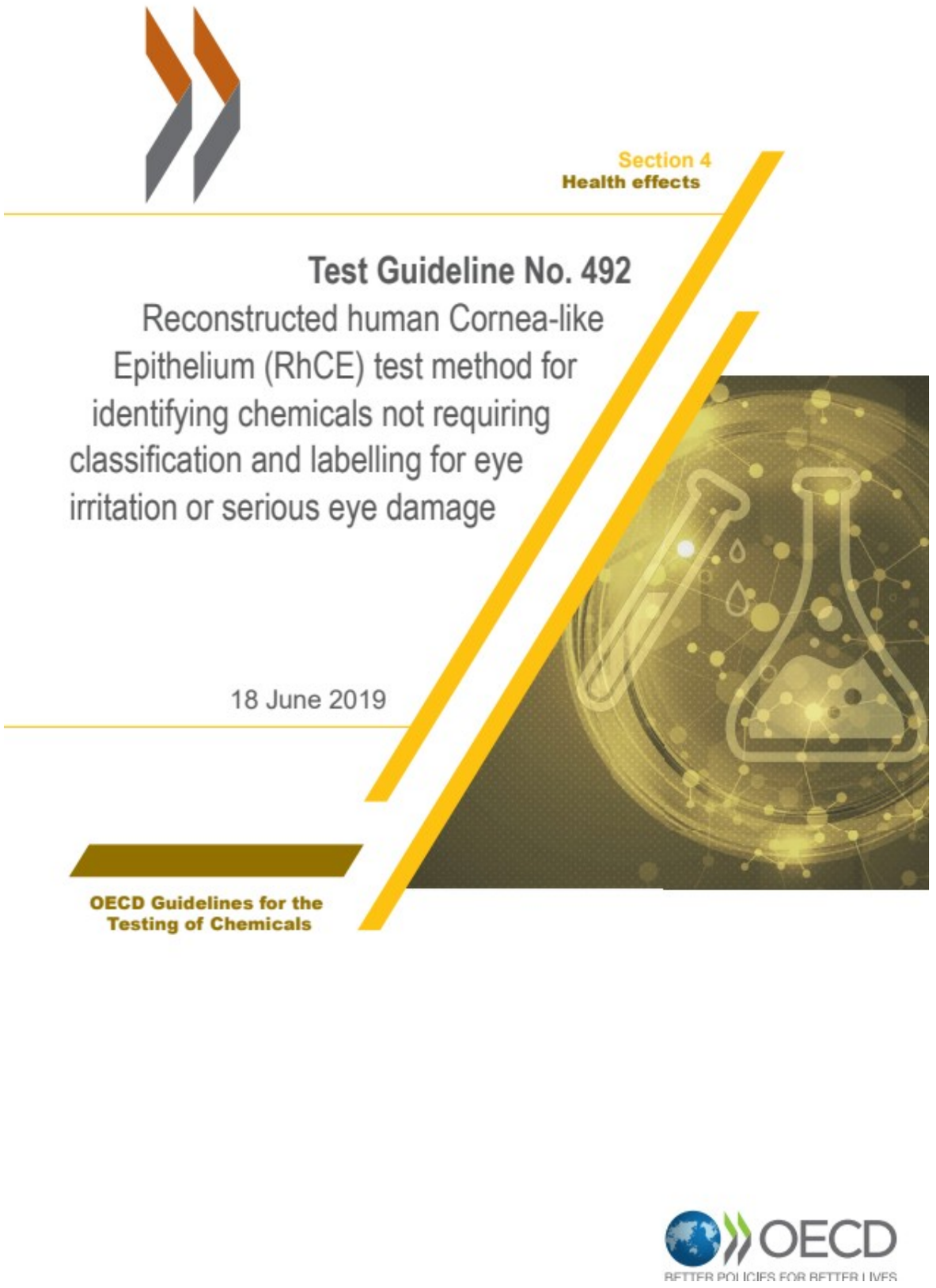
☐ Performance of the study

	Title	MFDS performance evaluation standard	
		type	performance indicators
1	Presentation: Identification of ezrin expression as a possible biomarker of in-vitro eye irritation using a 3D reconstructed human corneal model MCTT HCE™ and immortalized human corneal cells	Building on expertise through academic accomplishment	Number of presentations and publications in national workshops
2	Presentation at the industry, academia and government workshop for promotion of alternative test methods development and advancement of validation management: Global trends in development of alternative test methods and future demand	Utilization and dissemination of research outcome	Number of education or promotion materials about the outcome
3	Presentation at the 1st Cosmetics forum organized by Cosmetics ICC: Overview of current research on alternative test methods	Utilization and dissemination of research outcome	Number of education or promotion materials about the outcome
4	Intensive course in non-clinical education program hosted by KOHI: International validation study and global trends	Utilization and dissemination of research outcome	Number of education or promotion materials about the outcome
5	Presentation: A new 3D reconstructed human cornea-like epithelium, MCTT HCETM and its application for alternative to draize rabbit eye irritation test	Building on expertise through academic accomplishment	Number of presentations and publications in national workshops
6	Presentation: Development of 3D reconstructed human cornea-like epithelium, MCTT HCETM from primary limbal cells, and its application for alternative to animal test	Building on expertise through academic accomplishment	Number of presentations and publications in international workshops
7	Producing specialists with a master's degree	Nurturing professional personnel	Manpower training performance
8	Producing specialists with a master's degree	Nurturing professional personnel	Manpower training performance
9	Producing specialists with a master's degree	Nurturing professional personnel	Manpower training performance
10	Digital PCR assay	Improvement of ability in testing, research and experiment	Number of test methods development
11	SOP: Ezrin reporter assay-based eye toxicity evaluation method using immortalized corneal cells	Improvement of ability in testing, research and experiment	Number of test methods development
12	Presentation: Assessment of the Availability of Ezrin Expression in 3D Reconstructed Human Cornea Models (MCTT HCE™)and Immortalized Human Corneal Cell Lines as Biomarkers of *In Vitro* Eye Stimulation Tests	Building on expertise though academic accomplishment	Number of presentations and publications in national workshops

	Title	MFDS performance evaluation standard	
		type	performance indicators
13	Presentation: An *in-vitro* test to indentify eye irritants using a stable human corneal epithelial-derived EZR reporter cell line	Building on expertise though academic accomplishment	Number of presentations and publications in national workshops
14	Presentation: Application of the EZR repoter assay as an *in vitro* test	Building on expertise though academic accomplishment	Number of presentations and publications in national workshops
15	Presentation: Investigation of ezrin expression as a potential biomarkers for *in-vitro* eye irritation using a 3D reconstructed human corneal model MCTT HCE™ and immortalized human corneal cells	Building on expertise though academic accomplishment	Number of presentations and publications in national workshops
16	Presentation: Development of the EZR luc assay as an *in vitro* test	Building on expertise though academic accomplishment	Number of presentations and publications in international workshops
17	Press release: 2017 Rush Prize Awarded	Utilization and dissemination of research outcome	Number of education or promotion materials about the outcome
18	Publication: Evaluation of ocular irritancy of coal-tar dyes used in cosmetics employing reconstructed human cornea-like epithelium and short time exposure tests	Building on expertise though academic accomplishment	Number of papers published in SCI-grade journal
19	Publication: Nervonoylceramide (C24:1Cer), a lipid biomarker for ocular irritants released from the 3D reconstructed human cornea-like epithelium, MCTT HCE™	Building on expertise though academic accomplishment	Number of papers published in SCI-grade journal
20	Publication: Prevalidation trial for a novel *in vitro* eye irritation test using the reconstructed human cornea-like epithelial model, MCTT HCE™	Building on expertise though academic accomplishment	Number of papers published in SCI-grade journal
21	Publication: Alternatives to *In Vivo* Draize Rabbit Eye and Skin Irritation Tests with a Focus on 3D Reconstructed Human Cornea-Like Epithelium and Epidermis Models.	Building on expertise though academic accomplishment	Number of papers published in non-SCI-grade journal
22	Presentation: OECD TG492 Performance standard based multi-laboratory validation study for a new *in vitro* eye irritation test using the reconstructed human corneal epithelial model, MCTT HCE™.	Building on expertise though academic accomplishment	Number of presentations and publications in international workshops
23	Presentation: Validation study of alternative *in vitro* eye irritation test with 3D-reconstructed human cornea epithelium, MCTT HCE™ for OECD Test guideline	Building on expertise though academic accomplishment	Number of presentations and publications in national workshops

☐ Major accomplishment

1. Adoption in OECD TG492



4. Publication: Evaluation of ocular irritancy of coal-tar dyes used in cosmetics employing reconstructed human cornea-like epithelium and short time exposure tests

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Evaluation of ocular irritancy of coal-tar dyes used in cosmetics employing reconstructed human cornea-like epithelium and short time exposure tests

Miri Lee [a, 1], Ki Taek Nam [b, 1], Jungah Kim [a], Song E. Lim [a], Sang Hyeon Yeon [c], Buhyun Lee [b], Joo Young Lee [c, **], Kyung-Min Lim [a, *]

[a] College of Pharmacy, Ewha Womans University, Seoul 37060, Republic of Korea
[b] Severance Biomedical Science Institute, Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul, Republic of Korea
[c] BK21 Plus Team, College of Pharmacy, The Catholic University of Korea, Bucheon 14662, Republic of Korea

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Short time exposure test
STE

ABSTRACT

Coal-tar dyes in cosmetics may elicit adverse effects in the skin and eyes. Countries, like the US, have banned the use of coal-tar dyes in cosmetics for the eye area due to the potential for ocular irritation. We evaluated the eye irritation potential of 15 coal-tar dyes permitted as cosmetic ingredients in reconstructed human cornea-like epithelium (RhCEs [EpiOcular™ and MCTT HCE™]) tests and the short time exposure (STE) test. Eosin YS, phloxine B, tetrachlorotetrabromofluorescein, and tetrabromofluorescein were identified as irritants in RhCEs; dibromofluorescein and uranine yielded discrepant results. STE enabled further classification in accordance with the UN Globally Harmonized System of Classification and Labelling of Chemicals, as follows: eosin YS as Cat 2; phloxine B, Cat 1; and tetrachlorotetrabromofluorescein and tetrabromofluorescein, Cat 1/2. STE indicated dibromofluorescein (irritant in EpiOcular™) and uranine (irritant in MCTT HCE™) as No Cat, resulting in the classification of "No prediction can be made." based on bottom-up approach with each model. These results demonstrated that *in vitro* eye irritation tests can be utilized to evaluate the potential ocular irritancy of cosmetic ingredients and provide significant evidence with which to determine whether precautions should be given for the use of coal-tar dyes in cosmetics or other substances applied to the eye area.

1. Introduction

Colorants or dyes are widely used in foods, cosmetics, beauty supplies, and personal care products to increase the appeal of the product to consumers. Colorants are prepared from natural sources or are artificially synthesized. Coal-tar dyes, representative artificial colorants, are widely used as dye agents in color cosmetics and face paints, owing to their lasting, vivid, and appealing colors (Keck-Wilhelm et al., 2015), intense hues, bright tints and relatively low cost (Kanekar and Khale, 2014). Coal-tar dyes are chemically synthesized utilizing various aromatic hydrocarbons, such as toluene, xylene, or benzene, chemicals that are acquired through the distillation of bituminous coal or petroleum (Harp and Barrows, 2015; Pérez-Ibarbia et al., 2016). Coal-tar dyes have heteroaromatic, azo, or aromatic amines in their chemical structures, which may be associated with a variety of adverse health effects, ranging from dermatitis (Bonamonte et al., 2014; Sugai et al., 1977) to carcinogenesis (Andrew et al., 2004; Møller and Wallin, 2000). Accordingly, there are increasing concerns regarding the safety of cosmetics containing coal-tar dyes for human use.

Case reports suggest that blindness can occur after the application of color cosmetics for dyeing eyebrows and eyelashes. The color cosmetics therein were suspected of deriving their color from coal-tar dyes (Gettings et al., 1992). Chemical injury to the human eye, mainly manifesting as eye irritation, can also cause blindness, and some visual loss might be attributable to use of products containing color derived from coal-tar dye (Wagoner, 1997). Some coal-tar dyes, such as phloxine B (Foster et al., 2004), and synthetic organic color additives (Burnett and Opdyke, 1971) have been

* Corresponding author. College of Pharmacy, Ewha Womans University, 52 Ewhayeodae-gil, Seodaemun-gu, Seoul 03760, Republic of Korea.
** Corresponding author. College of Pharmacy, The Catholic University of Korea, Bucheon 14662, Republic of Korea.
E-mail addresses: joolee@catholic.ac.kr (J.Y. Lee), kmlim@ewha.ac.kr (K.-M. Lim).
[1] These authors contributed equally to this work.

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5. Publication: Nervonoylceramide (C24:1Cer), a lipid biomarker for ocular irritants released from the 3D reconstructed human cornea-like epithelium, MCTT HCE™

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Nervonoylceramide (C24:1Cer), a lipid biomarker for ocular irritants released from the 3D reconstructed human cornea-like epithelium, MCTT HCE™

Miri Lee[a,1], Kyung-Mi Joo[b,1], Seunghye Choi[a], Su-Hyon Lee[c], Seol Yeong Kim[c], Young-Jin Chun[d], Dalwoong Choi[e,*], Kyung-Min Lim[a,**]

[a] College of Pharmacy, Ewha Womans University, Seoul 03760, Republic of Korea
[b] AMOREPACIFIC Corp. R&D Center, Yongin 17074, Republic of Korea
[c] Biosolution Co., Seoul 101811, Republic of Korea
[d] College of Pharmacy, Chung-Ang University, Seoul 06974, Republic of Korea
[e] Department of Public Health Science, Graduate School, Korea University, Seoul 02841, Republic of Korea

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ABSTRACT

Due to invasive and painful procedures during *in vivo* rabbit eye irritation test, *in vitro* alternative methods have been widely investigated. Recently, 3D reconstructed human cornea-like epitheliums (RhCEs) garner a huge attention. RhCEs employ the tissue viability as a primary endpoint to determine ocular irritancy but additional biomarkers may improve its predictive capacity. Here, we explored lipid biomarkers for ocular irritants in MCTT HCE™ RhCE model. Three irritants; sodium lauryl sulfate, benzalkonium chloride and triton X-100 were selected to represent anionic, cationic and non-ionic detergent respectively. After treating MCTT HCE™ with irritants, the alteration of lipids in the treated tissues was examined with Nile Red staining, which revealed the depletion of corneal lipids. We further quantitated the release of ceramides and free fatty acids, major lipid components of cornea, into the medium during the post-treatment incubation, employing a sensitive UPLC-MS/MS method. Among 44 lipid species, nervonoylceramide (C24:1Cer) was found to be released commonly by all three irritants in a concentration-dependent manner. Tests with 10 additional reference substances further supported that C24:1Cer release was significantly correlated with viability. Examination of the genes involved in the biosynthetic pathway for C24:1Cer revealed that stearoylCoA desaturase (SCD) and elongase1 (ELOVL1) were upregulated, suggesting that lipids and related genes may be employed as biomarkers for ocular irritants.

1. Introduction

Cosmetics, contact lenses and personal care products are frequently being placed in direct contacts with human eyes during daily use. Accordingly the evaluation of eye irritation is mandated by regulation (Ng et al., 2012; Ng et al., 2015). Rabbit draize eye test has long been a gold standard method (Draize et al., 1944; OECD, 2002) but with the increasing awareness of animal welfare, demands for the establishment of non-animal-based alternative test methods are escalating. Especially, due to the painful and invasive test procedure, and long restraints of animals during *in vivo* eye irritation test, *in vitro* methods to replace the draize eye irritation test have been explored actively (Wilson et al., 2015; Lee et al., 2017).

There are several alternatives to the draize test: *in vitro* methods using rabbit cornea epithelial cells (Matsuda et al., 2009; OECD, 2015b; Takahashi et al., 2008), and organ culture methods such as bovine corneal opacity permeability (BCOP) and the isolated chicken eye test (ICE) (Gautheron et al., 1992; OECD, 2013a, 2013b; Prinsen, 1996). Another *in vitro* alternative test method using reconstructed human cornea-like epithelium (RhCE), has drawn increasing attention, which may resolve the issues of species difference and enable the identification of weak or moderate irritants. At least four RhCE models have been reported; EpiOcular™ (MatTeck, USA) (Kaluzhny et al., 2015; OECD, 2015c), SkinEthic™ HCE (SkinEthic, France) (Alepee et al., 2010; Pfannenbecker et al., 2013), MCTT HCE™ (Biosolution, Korea) (Jang et al., 2015; Jung et al., 2011) and Labcyte Cornea model (JTE, Japan)

* Correspondence to: D.W. Choi, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Republic of Korea.
** Correspondence to: K.M. Lim, College of Pharmacy, Ewha Womans University, 52 Ewhayeodae-gil, Seodaemun-gu, Seoul 03760, Republic of Korea.
E-mail addresses: aahhhh@paran.com (D. Choi), kmlim@ewha.ac.kr (K.-M. Lim).
[1] These authors contributed equally to this work.

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6. Publication: Pre-validation trial for a novel *in vitro* eye irritation test using the reconstructed human cornea-like epithelial model, MCTT HCE™

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Prevalidation trial for a novel *in vitro* eye irritation test using the reconstructed human cornea-like epithelial model, MCTT HCE™

Hyeri Yang [a], Da-eun Kim [a], Won-Hee Jang [b], Susun An [b], Sun-A Cho [b], Mi-Sook Jung [c], Ji Eun Lee [c], Kyung-Wook Yeo [d], Sang Bum Koh [d], Tae-Cheon Jeong [e], Mi-Jeong Kang [e], Young-Jin Chun [f], Su-Hyon Lee [g], Kyung-Min Lim [a,*], SeungJin Bae [a,*]

[a] College of Pharmacy, Ewha Womans University, Seoul 03760, Republic of Korea
[b] AmorePacific Corp. R&D Center, Yongin 17074, Republic of Korea
[c] Pharmacology Efficacy Team, Biotoxtech Co., Ltd., Osong 18115, Republic of Korea
[d] Healthcare R&D Center, Korea Testing & Research Institute, Hwa-Soon 58141, Republic of Korea
[e] College of Pharmacy, Yeungnam University, Gyeongsan 38541, Republic of Korea
[f] College of Pharmacy, Chung-Ang University, Seoul 06911, Republic of Korea
[g] R&D Institute, Biosolution Co., Ltd., Seoul 01811, Republic of Korea

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ABSTRACT

Here, we report the results of a prevalidation trial for an *in vitro* eye irritation test (EIT) using the reconstructed human cornea-like epithelium, MCTT HCE™. The optimal cutoff to determine irritation in the prediction model was established at 35% with the receiver operation characteristics(ROC) curve for 126 substances. Within-lab(WL) and between-lab(BL) reproducibility was tested for 20 reference substances by 3 participating laboratories. Viability data described by mean ± SD or ± 1/2 difference between duplicate wells, and scatter plots, demonstrated the WL/BL consistency. WL/BL concordance with the binary decision, whether non-irritant or irritant was estimated to be 85–95% and 95%, respectively. WL/BL reproducibility of viability data was further supported by a strong correlation(ICC, $r > 0.9$). WL/BL agreement of binary decisions was also examined by Fleiss' Kappa statistics, which showed a strong level of agreement (>0.78), nevertheless weaker than the reproducibility of the viability. The EIT with MCTT HCE™ exhibited a sensitivity of 82.2% (60/73), a specificity of 81.1% (43/53), and an accuracy of 81.8% (103/126) for 126 reference substances (for liquids; a sensitivity of 100% (47/47), a specificity of 70.6% (24/34), and an accuracy of 87.7% (71/81), and for solids, a sensitivity of 50% (13/26), a specificity of 100% (19/19), and an accuracy of 71.1% (32/45), suggesting that the accuracy is satisfactory but the sensitivity needs improvement, which shall be addressed through correcting the poor sensitivity for solid substances in future full validation trials.

1. Introduction

Animals have long been exploited to assess the effects of chemicals on human health, however, concerns regarding animal welfare and species differences are escalating. In particular, the Draize *in vivo* eye irritation test in rabbits (Draize et al., 1944; OECD, 2002; Reshma et al., 2015) has been criticized by animal welfare activists due to its cruel procedure. In addition, other problems have also been raised such as low reproducibility (Weil and Scala, 1971) and species differences (Roggeband et al., 2000) with respect to identifying ocular irritants that conform to the UN global harmonization system (GHS) classification, namely serious eye damage (UN GHS Category 1), eye irritation (UN GHS Categories 2A and 2B), and non-classified (UN GHS No Category).

In view of a replacement, numerous alternative *in vitro* tests have been developed, including organ-culture methods using isolated animal eyes, *in vitro* cytotoxicity tests employing monolayer cell lines, and reconstructed human cornea-like epithelium (RhCE) (Wilson et al., 2015). Of these, RhCEs such as EpiOcular™ (Kaluzhny et al., 2015), SkinEthic™ HCE (Alépée et al., 2013; Alepee et al., 2016; Cotovio et al., 2010), MCTT HCE™ (Jang et al., 2015; Jung et al., 2011), and the Labcyte Cornea model (Katoh et al., 2013) have garnered tremendous interest, since they are expected to provide a high level of structural and physiological similarity to those of real human corneal epithelium (Choi et al.,

Abbreviations: EIT, eye irritation test; RhCE, reconstructed human cornea-like epithelium; OECD TG, OECD Test guidelines; GHS, global harmonization system; WL/BL, within-/between-laboratory; ROC, receiver operating characteristics; SD, standard deviation; AUC, area under curve; ICC, intra-class correlation; PS, performance standard.

* Corresponding authors at: College of Pharmacy, Ewha Womans University, 52 Ewhayeodae-gil, Seodaemun-gu, Seoul 03760, Republic of Korea.
E-mail addresses: kmlim@ewha.ac.kr (K.-M. Lim), sjbae@ewha.ac.kr (S. Bae).

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0887-2333/

7. Publication: Alternatives to *In Vivo* Draize Rabbit Eye and Skin Irritation Tests with a Focus on 3D Reconstructed Human Cornea-Like Epithelium and Epidermis Models.

Review Article

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Alternatives to *In Vivo* Draize Rabbit Eye and Skin Irritation Tests with a Focus on 3D Reconstructed Human Cornea-Like Epithelium and Epidermis Models

Miri Lee†, Jee-Hyun Hwang† and Kyung-Min Lim

College of Pharmacy, Ewha Womans University, Seoul, Korea

Human eyes and skin are frequently exposed to chemicals accidentally or on purpose due to their external location. Therefore, chemicals are required to undergo the evaluation of the ocular and dermal irritancy for their safe handling and use before release into the market. Draize rabbit eye and skin irritation test developed in 1944, has been a gold standard test which was enlisted as OECD TG 404 and OECD TG 405 but it has been criticized with respect to animal welfare due to invasive and cruel procedure. To replace it, diverse alternatives have been developed: (i) For Draize eye irritation test, organotypic assay, *in vitro* cytotoxicity-based method, *in chemico* tests, *in silico* prediction model, and 3D reconstructed human cornea-like epithelium (RhCE); (ii) For Draize skin irritation test, *in vitro* cytotoxicity-based cell model, and 3D reconstructed human epidermis models (RhE). Of these, RhCE and RhE models are getting spotlight as a promising alternative with a wide applicability domain covering cosmetics and personal care products. In this review, we overviewed the current alternatives to Draize test with a focus on 3D human epithelium models to provide an insight into advancing and widening their utility.

Key words: Eye irritation, Skin irritation, Alternative to animal tests, 3D reconstructed human cornea-like epithelium (RhCE) models, 3D reconstructed human epithelium (RhE) models

INTRODUCTION

Chemicals can be exposed to human accidentally or intentionally, and toxicity tests of chemicals are essential to ensure human safety against chemicals. Especially there are high probabilities of ocular and dermal exposure to pharmaceuticals, cosmetics and personal care products. It is required therefore to test the ocular and dermal irritancy of chemicals whereupon, the irritancy of chemicals are classified and labeled properly according to the severity. UN GHS categorization provides a universal standard for labeling the ocular irritancy of chemicals, which categorizes chemicals into Category 1, Category 2A/2B and No category, and the dermal irritancy of chemicals are categorized into Category 1A/1B/1C, Category 2, Category 3, and No category according to the severity and irreversibility of irritation (1). To obtain relevant information for labeling and classification, eye and skin irritation tests are mandatory. Before 2009, Draize *in vivo* rabbit irritation test developed in 1940, has been only officially accepted test method by OECD (2). Draize rabbit test procedure is composed of forced application of test substance to the eye or skin of a non-anesthetized rabbit in a restrainer and subsequent scoring of signs of irritation including redness, swelling, cloudiness, edema, hemorrhage, and discharge (3). Due to this cruel and invasive procedure, experimental animals are imposed severe pain and discomfort (4,5). As the concern for animal welfare increases throughout the world, the testing of finished cosmetics on animals has been banned in EU since 2004 and it enters full into force in 2013. Here, we present an overview of several types of alternatives to Draize test, with a focus on 3D reconstructed human cornea-like epithelium (RhCE) and 3D reconstructed human epithelium (RhE) models and suggest future direction for

Correspondence to: Kyung-Min Lim, College of Pharmacy, Ewha Womans University, 52 Ewhayeodae-gil, Seodaemun-gu, Seoul 03760, Korea
E-mail: kmlim@ewha.ac.kr

†These authors contribute equally to this work.

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3 Development and OECD adoption of androgen disruptor screening assay

Supervising division	Food Safety Risk Assessment Division of NIFDS
Project type	Internal project
Title	Study I on development of internationally harmonized (OECD) estrogen/androgen receptor interaction test method using novel technique (18161MFDS108)

☐ Background for the study

- With increasing development and use of chemicals, potential endocrine disruptor, also known as environmental hormone, has become a global issue. Therefore, it is necessary to develop an internationally harmonized test method (OECD Test Guideline) that can be used to screen existence of substances with potential endocrine disrupting effect that people consume or are exposed to in daily life.

☐ Study in brief

- The study aimed to develop an alternative method to animal testing for evaluation of androgen interaction by endocrine disruptors. The method can reduce sacrifices of lab animals by screening and selecting test chemicals at the cell experiment stage.

- **Principle of the method:** This assay can evaluate existence of endocrine disruptors relating to androgen hormone at a cell-level by qualitatively measuring the expression of the genes in a cell line that specifically interacts with androgen receptor - test chemical conjugate in the cell line.

- **Development and validation:** The androgen disruptor screening assay was developed using a prostate cancer cell line, and validated in collaboration with KTR (Korea Testing & Research Institute) and Dongguk University.

  - In the study, the existing human prostate cancer cell line (22Rv1/MMTV)* was optimized by minimizing false positive responses by removing glucocorticoid receptors that have the same hormone response elements with androgen receptors. The assay was developed using the optimized cell line (22Rv1/MMTV-GR-KO)**.

    * Study on the development of endocrine disruptors screening assay – Androgen receptor and transcriptional activation assay (11162MFDS728, Prof. Mi-sook Dong, Korea University)

    ** International validation study on development of standardized androgen screening assay using a cell line (for OECD recognition) (16161MFDS115, Food Safety Risk Assessment Divison)

☐ Overview of the study outcome

- **Major accomplishment:** OECD Recognition of the androgen disruptor screening assay using human prostate cancer cell line (OECD TG 458).

- **Summary**

  - **OECD recognition:** Approval of inclusion of the assay in the OECD TG 458 by the OECD WNT in April 2020, and the revised TG was officially issued in June 2020.

☐ Excellence and advantages

- **Excellence:** An androgen transcriptional activation assay using a human prostate caner cell line for detection of androgen receptors. This is the first national and the second OECD adopted test method for evaluation of endocrine disruption activation. This shows that our domestic technology is recognized internationally as advanced technology.

  * Compared to the existing androgen screening test using the cell line that does not include activated androgen receptors (developed by Japan in 2016), the assay exhibits improved accuracy and sensitivity.

- **Advantages:** This is an alternative method having improved accuracy and sensitivity for detection of androgenic substances. The method can address ethical issues relating to use of laboratory animals by reducing their sacrifices. The method brings economic benefit as well since it can detect a large number of substances with endocrine activation in a short period (4 days).

  * Comparison with the existing technology

	(cell line test) Androgen screening assay using a human prostate cancer cell line	(animal test) Reproductive and developmental toxicity screening assay *
Summary	An assay for detection of endocrine active chemicals that can interact with androgen receptors using human prostate cancer cell line expressing androgen receptors	A screening assay for determination of developmental and (or) reproductive toxic does. It aims to obtain initial information about the impact of test chemicals on reproduction and development.
test chemical number/ repetition	4 test chemicals/96-well plate	1 test chemical
Test period	4 days	at least 50 days
Number of animals used	-	at least 40 male mouse at least 48 female mouse (including at least 40 pregnant mouse)
Other experimental condition	Should be performed in a laboratory with Bio safety level 2 (BL2) for 22R1V1 cell line experiment	Should be performed in a GLP test facility for animal experiment

* Chapter 5 of the Regulation on Testing of Chemicals: tests on health effects, paragraph 16: reproductive and developmental toxicity screening test

☐ Utilization of the Outcome and Its Impact

- The assay was developed to support the safety management of the chemicals with endocrine disrupting potential and has been recognized as an internationally harmonized method. This shows that MFDS plays a leading role in development of NAMs.

- **Support for industry, academia and research institute:** The know-hows accumulated during the development of the first successful endocrine disruptor assay in Korea is expected to bring about a leap ahead in a follow-up study aiming international standardization.

  - 22Rv1/MMTV_GR-KO cell line developed with our own technology has been donated to the KCTC (Korean Collection for Type Cultures) for national and overseas researchers. The cell line can be obtained with no charge. This will contribute to utilization and development of the related technology and field.

  - Access to the information on the assay has been expanded. OECD TG 458 was translated in Korean and published in July 2020, uploaded on the NIFDS website and distributed to related organizations. Also, press release on the publication was sent.

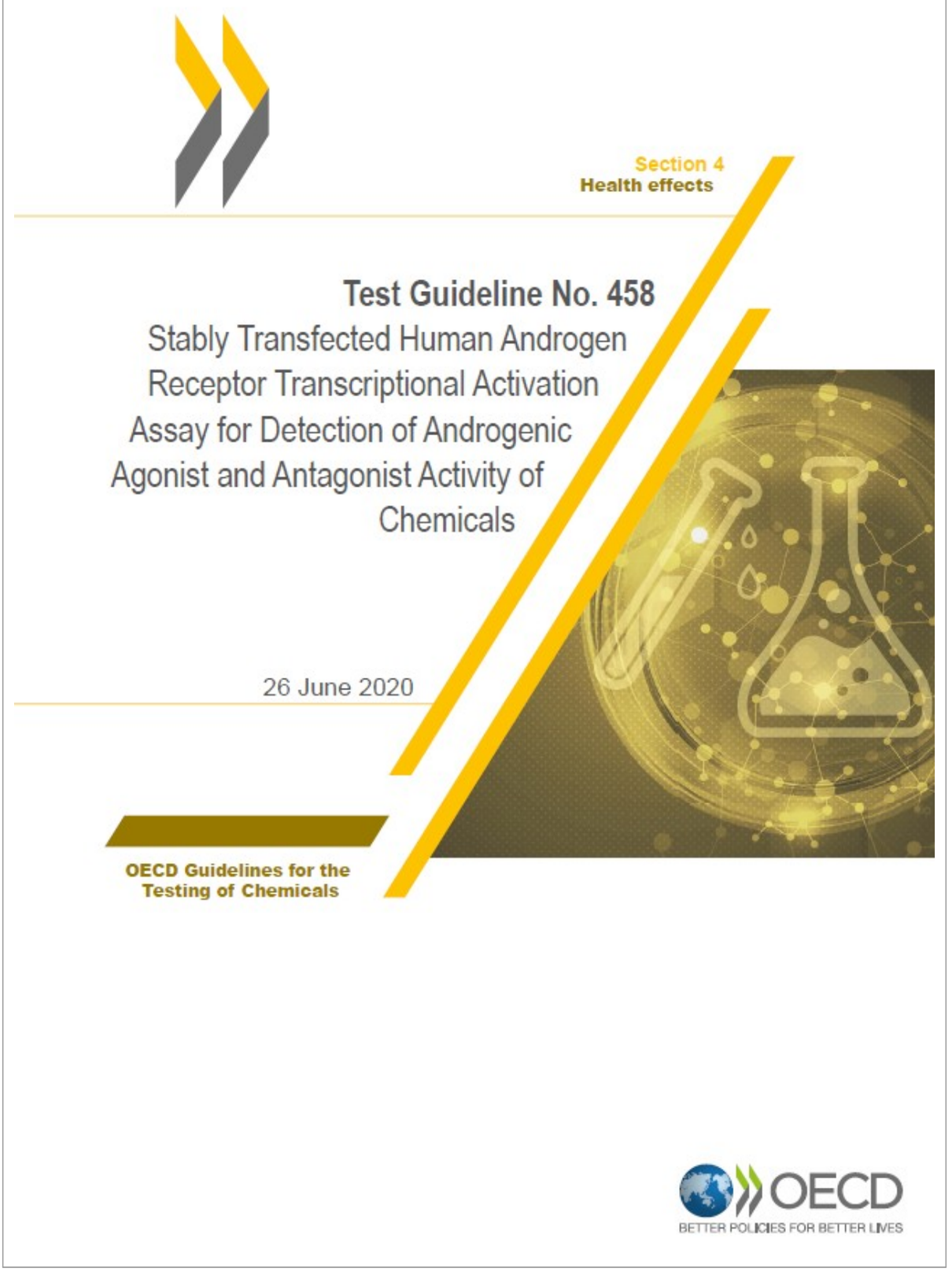
  - **Minimization of sacrifices of lab animals:** Countries in the world have been focusing on developing alternatives to animal testing in order to reduce use of lab animals. The assay developed in this study is expected not only to reduce the cost for tests but also to address ethical issues relating to lab animals by minimizing their sacrifices.

☐ Performance of the study

	Title	MFDS Performance Evaluation Standard	
		type	Performance Indicators
1	Adoption in OECD Test Guideline (OECD TG 458)	system improvement and utilization of policy	Proposal and inclusion in OECD TG
2	(Establishment of standardized product) Development and donation of 22Rv1/MMTV_GR-KO cell line	Establishment of basis for standardization	Number of development (donation) of standardized products
3	Guideline on Endocrine disruptor screening assay (Korean translation of OECD TG 458)	system improvement and utilization of policy	Publication and revision of guidelines
4	Publication in academic journal: Assessment of androgen receptor agonistic antagonistic effects on 25 chemicals in household applicants by OECD *in vitro* stably transfected transcriptional activation assays	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journal
5	Publication in academic journal: Enhancement of androgen transcriptional activation assay based on genome edited glucocorticoid knock out human prostate cancer cell line	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journal

☐ Major accomplishment

1. Adoption in OECD Test Guideline



4. (Publication in academic journal) Assessment of androgen agonistic/antagonistic effect on 25 chemicals in household applicants by OECD *in vitro* stably transfected transcriptional activation assays

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Assessment of androgen receptor agonistic/antagonistic effects on 25 chemicals in household applicants by OECD *in vitro* stably transfected transcriptional activation assays

Hee-Seok Lee [a, *], Eun-Jung Park [a], Songyi Han [a], Gyeong-Yong Oh [a], Hui-Seung Kang [a], Jin-Hyang Suh [a], Min-Ki Shin [a], Hyun-Suk Oh [a], Myung-Sil Hwang [a], Guiim Moon [a], Young-Ho Koh [a], Yooheon Park [b], Jin-Hwan Hong [a], Yong Eui Koo [a, **]

[a] Food Safety Risk Assessment Division, National Institute of Food and Drug Safety Evaluation, Korean Ministry of Food and Drug Safety, Chungcheongbuk-do 28159, South Korea
[b] Dongguk University Research Institute of Biotechnology & Medical Converged Science, Dongguk University, Gyeonggi 10325, South Korea

HIGHLIGHTS

- AR agonistic/antagonistic effects on 25 chemicals in household applicants are assessed.
- Two *in vitro* stably transfected transcriptional activation assays are applied.
- α-Dodecyl-ω-hydroxypoly (oxyethylene) has been determined as AR antagonist.
- 3-Iodo-2-propynyl butylcarbamate also exhibited a weak AR antagonistic effect.
- This report firstly provides information about their AR agonist/antagonistic effects.

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Antagonist

ABSTRACT

The aim of this study is to assess the androgen receptor (AR) agonistic/antagonistic effects on various chemicals, which are used in household products including cleaning agents and wetted tissues by *in vitro* OECD test guideline No. 458 (using AR-EcoScreen™ cell line) and the me-too test method (using 22Rv1cell line), which was adopted as OECD project No. 4.99. All chemicals were not determined as AR agonists. However α-dodecyl-ω-hydroxypoly (oxyethylene) and 3-iodo-2-propynyl butylcarbamate have shown a weak AR antagonistic effects with IC_{50} values of 2.18 ± 0.12 and 4.26 ± 0.17 μg/ml via binding affinity to AR in only 22Rv1/mouse mammary tumor virus using AR transcriptional activation assay, because of their different cytotoxicity on each applied cell line. This report firstly provides information about agonistic/antagonistic effects against human AR of various chemicals including surfactants and biocides by OECD *in vitro* stably transfected transcriptional activation assays. However, further *in vivo* and human model studies are needed to confirm their adverse effects.

1. Introduction

During the past several decades, a number of synthetic chemicals were developed to meet a wide variety of industrial needs, and they were easily released into the environment (UNEP, 2004). Unfortunately, various chemicals such as bisphenols, phthalates and parabens are considered endocrine disrupting chemicals (EDCs), since they can exhibit the adverse effect on the mammalian endocrine system (Schug et al., 2011). Especially, nonylphenolis the most abundant derivatives of alkylphenol polyethoxylate compounds that mimics the biological activity of estrogens by binding to estrogen receptor affecting reproduction (Brown and Reinhard, 2003). Alkylphenol polyethoxylate compounds are widely used as non-ionic surfactants in detergents, emulsifiers and cosmetics (Cevdet et al., 2009). EDCs can be exposed to human by oral consumption of food and water or by using cosmetics and various household applicants including cleaning agents (Giulivo et al.,

* Corresponding author. Tel: +82 437194515; fax: +82 437194500.
** Corresponding author.
E-mail addresses: hslee0515@korea.kr (H.-S. lee), kye2020@korea.kr (Y.E. Koo).

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0045-6535/

5. (Publication in academic journal) Enhancement of androgen transcriptional activation assay based on genome edited glucocorticoid knock out human prostate cancer cell line

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Enhancement of androgen transcriptional activation assay based on genome edited glucocorticoid knock out human prostate cancer cell line

Hee-Seok Lee[a,1], Seok-Hee Lee[b,1], Yooheon Park[b,*]

[a] National Institute of Food and Drug Safety Evaluation, Osong 28159, Republic of Korea
[b] Department of Food Science and Biotechnology, Dongguk University, Goyang 10326, Republic of Korea

ARTICLE INFO

Keywords:
22Rv1 cell lines
Androgen transactivation assay
RNA-guided engineered nuclease
Glucocorticoid receptor

ABSTRACT

Endocrine-disrupting chemicals (EDCs) interfere with the biological activity of hormones. Among EDC's, (anti-) androgenic compounds potentially cause several androgen-related diseases. To improve the accuracy of an in vitro transactivation assay (TA) for detection of (anti-)androgenic compounds,

We established the glucocorticoid receptor (GR) knockout 22Rv1/MMTV cell line by using an RNA-guided engineered nuclease (RGEN)-derived CRISPR/Cas system. The 22Rv1/MMTV GRKO cell line was characterized and validated by androgen receptor (AR)-mediated TA assay compared with the AR-TA assay using 22Rv1/MMTV. In conclusion, the AR-TA assay with the 22Rv1/MMTV GRKO cell line was more accurate, excluding the misleading signals derived from glucocorticoids or equivalent chemicals, and might be an effective method for screening potential (anti-)androgenic compounds.

1. Introduction

Over recent decades, concrete scientific conclusions have provided evidence that many industrial chemicals, including agricultural chemicals including pesticides, have disruptive effects on endocrine systems (Colborn et al., 1993; Jabłońska-Trypuć et al., 2017; Naz, 2004; Tyler et al., 1998). In response to the concerns of society, in 1998, the Organization for Economic Cooperation and Development (OECD) initiated an activity based on the OECD conceptual framework to revise existing and develop new test guidelines (TGs) for screening and testing potential endocrine-disrupting chemicals. This comprises five levels, each level corresponding to a different level of biological complexity (OECD, 2005, 2007).

The transcriptional activation (TA) assay, a level 2 'in vitro assay providing mechanistic information', is a constructed stable cell line for the detection of agonist/antagonist activities mediated through endocrine receptors. TA assays should be validated by demonstrating the relevance and reliability of the assay for its intended purpose. In vitro TA assays are based on binding of the candidate chemical to a specific receptor that functions as a transcription factor that regulates expression of a reporter gene, the product of which can be readily quantitated. Numerous in vitro TA assays have been proposed to screen estrogenicity as well as androgenicity (ICCVAM, 2003; Jefferson et al., 2002; Sonneveld et al., 2006).

Androgenicity is one of four important endocrinological endpoints classified by OECD to distinguish it from other endocrine disruptors (EDs). A considerable number of chemicals components, including pesticides (Kojima et al., 2004), flame retardants (Hamers et al., 2006), packaging chemicals (Satoh et al., 2004), and industrial chemicals (Araki et al., 2005a, 2005b), have been identified as androgenic or antiandrogenic. In terms of TA assays, only one assay employing a stably transfected HeLa cell line for detecting estrogenicity has been validated and adopted as OECD TG455 in 2009. Other assays are currently under development, consideration, and validation (Araki et al., 2005a; Satoh et al., 2004; Sun et al., 2016). One of the proposed TA assays, the Androgen Receptor (AR)-EcoScreen system, is a CHO cell-based reporter assay (Satoh et al., 2004). CHO cells have been stably transfected with human androgen receptor (hAR) (Lee et al., 1981) as well as hormone response element (HRE) from the C3 gene of prostatic binding protein (Kojima et al., 2003), which regulates the reporter luciferase. Another TA assay, the AR-TA, which comprises the 22Rv1/MMTV cell line stably transfected with a pGL4 reporter plasmid containing the luciferase gene under the control of mouse mammary tumor virus (MMTV), has also been considered (Sun et al., 2016). Among the various prostate cancer cell lines, the 22Rv1 cells might be an appropriate model cell line for the screening of (anti-)androgenic endocrine

* Correspondence to: Department of Food Science and Biotechnology, Dongguk University, 32, Dongguk-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 10326, Republic of Korea.
E-mail address: ypark@dongguk.edu (Y. Park).
[1] These authors contributed equally to this work.

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4 Development and OECD adoption of an alternative skin irritation test method

Supervising division	Toxicological screening and testing division of NIFDS
Project type	Entrusted project (prof. Kyung-min Lim, Ewha Womans University)
Title	Pre-validation of an alternative to skin irritation test using RhE tissue model developed in Korea (18182MFDS463)

☐ Background for the study

- With enforcement of Cosmetics Act in Korea and global bans on animal testing for cosmetics and their ingredient including the EU and other countries, development of alternatives to animal testing that can be utilized in the national industry and GLP facilities considers necessary.

- In particular, an *in vitro* test method that can replace *in vivo* Draize rabbit skin irritation test (OECD TG 404) is needed.

☐ Study in brief

- A pre-validation study on KeraSkin™ SIT (skin irritation test), which closely mimics human skin and generates more precise results while reducing sacrifices of experimental rabbits, was conducted.

  - The pre-validation was performed in collaboration with KoCVAM

- **KeraSkin™:** KeraSkin™ is 3D reconstructed human epidermis (RhE) model manufactured with human-derived keratinocytes isolated from foreskin. It owns biochemically and morphologically similar features to human skin.

  - KeraSkin™ is produced by culturing it until it forms highly differentiated, multi-layered RhE. It consists of multi-layered stratum corneum composed of basal layer, spinous layer, and lamellar lipid layer representing main lipid classes analogous to those found *in vivo.*

- **Principle of the method:** The method evaluates skin irritation potential of test chemical-induced cell viability of KeraSkin™ using MTT assay following local exposure of the chemical to the RhE model.

  - Skin irritants may damage the underlying layers of keratinocytes by penetrating through stratum corneum. The damaged cells may induce an inflammatory cascade

which also acts on the cells in the dermis, particularly the stromal and endothelial cells of the blood vessels.

- **Development and validation:** Validation study on KeraSkin™ SIT using the domestically developed RhE model, KeraSkin™, was set up by KoCVAM and performed in collaboration with national researchers
  * Pre-validation study on KeraSkin™ SIT (18182MFDS463, Prof. Kyung-min Lim, Ewha Womans University)

☐ Major outcome

- **Summary:** KeraSkin™ SIT using the domestically developed RhE model was approved and adopted in OECD Test Guideline (OECD TG 439)
  - KeraSkin™ is the world's first and only model that is manufactured with residual foreskin separated after circumcision.
- **Overview of the outcome**
  - **Validation study:** KoCVAM coordinated the validation study and ensured that it fulfilled the OECD guidance by operating a validation management team and cooperating with the OECD.
  - **OECD approval:** Inclusion of the method to OECD TG 439 was approved by the OECD WNT in April 2021 and the updated TG was officially issued in June 2021.
    * WNT: OECD Working Group of National Co-ordinators of the TGs programme who take decisions on TGs (approve and update of TGs) and decide on project proposals to include in the work plan. The group consists of representative from the member countries that adhere to MAD and meets annually.
  - **Academic accomplishment:** Publication of 2 papers in SCI-grade journal
    * Toxicology *in vitro* and Regulatory Toxicology and pharmacology

☐ Excellence and advantages

- **Excellence:** An international peer-review panel consisting of experts from the U.S., Switzerland and Japan organized by KoCVAM concluded that KeraSkin™ SIT is scientifically valid "*me-too*" method to OECD TG 439.
- **Advantages:** KeraSkin™ used in this test method is manufactured in Korea. With OECD recognition, the data generated using this domestic model can be accepted by regulatory bodies in the world.
  * The RhE-based assays in OECD TG 439 are the only alternatives to *in vivo* skin irritation test and are essential in cosmetic toxicity testing.

☐ Utilization of the outcome and its impact

- The outcome has proven that KoCVAM plays a leading role in the field of alternative test methods in Korea.
  * Countries developed test methods recognized in OECD TGs: the U.S., 5 European Countries (Germany, Italy, Netherlands, Belgium, and France), Japan, Canada and Korea.

- **Increasing export:** International recognition of the RhE model for evaluation of skin irritation that is developed and produced in Korea enables export of the model to foreign test facilities conducting skin irritation test.

- **Supporting the related industry:** Utilization of domestically-developed RhE models in the skin irritation tests that highly depend on oversea models is expected to promote the sales of the manufacturer.

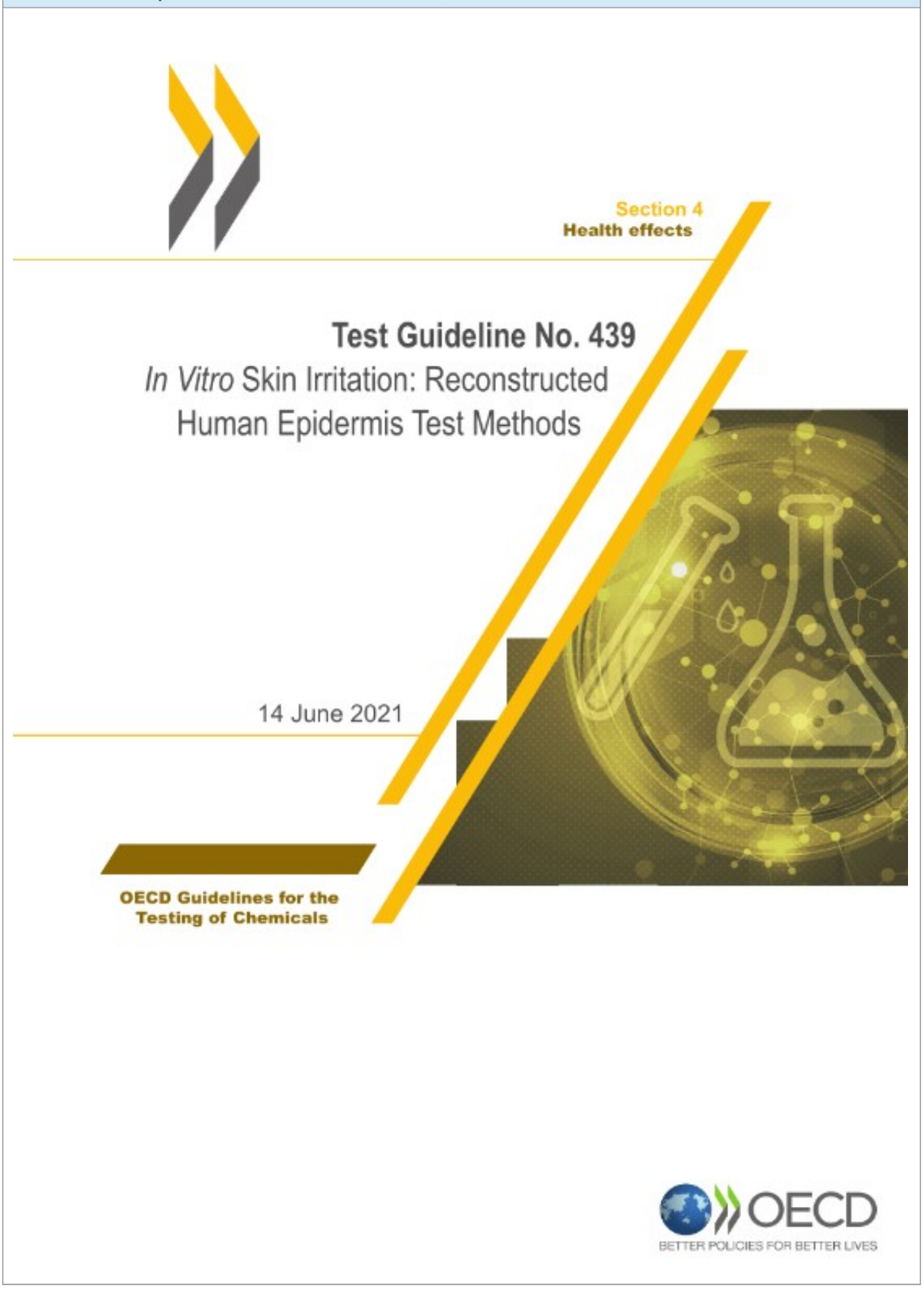
- The related industry is expected to grow thanks to introduction and dissemination of a new alternative test method because it will help nurture professional personnel and support for new technique will be provided.
  - Training on the techniques needed to perform KeraSkin™ SIT has been provided to the people working in GLP test facilities and the related academic field by publishing and distributing the guideline on KeraSkin™ SIT; by organizing workshops; and by servicing the video demonstrating the method.

☐ Performance pf the study

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
1	Adoption in OECD Test Guideline	Encouragement of practical use including policy improvement	Number of TG proposals approved
2	Preparation of the draft protocol for *in vitro* skin irritation test with a 3D-reconstructed human skin model, KeraSkin™.	Improvement of ability in testing, research and experiment	Number of test method development
3	Publication: Employment of cytology for *in vitro* skin irritation test using a reconstructed human epidermis model, Keraskin™.	Encouragement of practical use including policy improvement	Number of publication in SCI-grade journal
4	Publication: Me-too validation study for *in vitro* skin irritation test with a reconstructed human epidermis model, KeraSkin™ for OECD test guideline 439.	Encouragement of practical use including policy improvement	Number of publication in SCI-grade journal
5	Presentation: Employment of cytological evaluation of skin irritancy for Keraskin™, 3D reconstructed human epidermis model.	Encouragement of practical use including policy improvement	Number of presentations (publications) in national workshops
6	Presentation: Global trends in alternatives to animal tests and regulatory uptake.	Encouragement of practical use including policy improvement	Number of publications in national workshops
7	Presentation: *In vitro* toxicity evaluation of biocide using RhE models	Encouragement of practical use including policy improvement	Number of publications in national workshops

☐ Major accomplishment

1-1. OECD Adoption in OECD Test Guideline (OECD TG 439)



1-2. OECD Adoption in OECD Test Guideline (OECD TG 439)

ANNEX 2 - TEST METHODS INCLUDED IN THIS TG

Pre-validation, optimisation and validation studies have been completed for seven commercially available *in vitro* test methods (10) (11) (12) (13) (14) (15) (16) (17) (18) (19) (20) (21) (22) (23) (24) (25) (26) (27) (28) (43) (45) (50) (51) based on the RhE test system with the following minimum of predictive capacity: (80% sensitivity, 70% specificity, and 75% accuracy). These seven test methods are included in this TG and are listed below, together with the type of validation study used to validate the respective test methods. The VRMs that have been used to develop the present TG and the PS (8) are EpiSkin™ and EpiDerm™ SIT (EPI-200).

Nr.	Test method name	Validation study type	References
1	**EpiSkin™ (VRM)**	Full prospective validation study (2003-2007). The test method components of this method were used to define the essential test method components of the original and updated ECVAM PS (39) (40) (21)*. Moreover, the method's data relating to identification of non-classified vs classified substances formed the main basis for defining the specificity and sensitivity values of the original PS*.	(2) (10) (11) (14) (15) (16) (17) (18) (19) (20) (21) (23) (32) (39) (40)
2	**EpiDerm™ SIT (EPI-200) (VRM)**	EpiDerm™ (*original*): Initially the test method underwent full prospective validation together with Nr. 1. from 2003-2007. The test method components of this method were used to define the essential test methods components of the original and updated ECVAM PS (39) (40) (21)*.	(2) (10) (12) (13) (15) (16) (17) (18) (20) (21) (23) (33) (39) (40)
		EpiDerm™ SIT (EPI-200): A modification of the original EpiDerm™ was validated using the original ECVAM PS (21) in 2008*	(2) (21) (22) (23) (33)
3	**SkinEthic™ RHE**	Validation study based on the original ECVAM Performance Standards (21) in 2008*.	(2) (21) (22) (23) (31)
4	**LabCyte EPI-MODEL24 SIT**	Validation study (2011-2012) based on the Performance Standards (PS) of OECD TG 439 (8) which are based on the updated ECVAM PS* (39) (40).	(24) (25) (26) (27) (28) (35) (39) (40) and PS of this TG (8)*
5	**epiCS®**	Performance Standards based Validation Study for SIT according to OECD GD 220 (8) following ESAC opinion in 2016 (45) and independent peer review in 2018 (43)	(1) (8) (23) (39) (40) (44) (43) (45)
6	**Skin+ ®**	Performance Based Validation Study for SIT according to OECD GD 220 (8) following ECVAM opinion in 2016 (46) and independent peer review in 2018 (43)	(1) (8) (23) (39) (40) (42) (43) (46)
7	**KeraSkin™ SIT**	Performance Standards based Validation Study according to OECD GD 220 (8) followed by independent peer-review in 2020.	(48) (49) (50) (51)

3. Publication: Employment of cytology for *in vitro* skin irritation test using a reconstructed human epidermis model, Keraskin™.

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Employment of cytology for *in vitro* skin irritation test using a reconstructed human epidermis model, Keraskin™

Jee-hyun Hwang[a], Haengdueng Jeong[b], Sumin Hur[b], Ki Taek Nam[b], Kyung-Min Lim[a,*]

[a] College of Pharmacy, Ewha Womans University, Seoul 03760, Republic of Korea
[b] College of Medicine, Yonsei University, Seoul 03722, Republic of Korea

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Reconstructed human epidermis model
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ABSTRACT

Skin irritation tests using reconstructed human epidermis (RhE) employ viability as an endpoint, but color interference or borderline results are often problematic. We examined whether the cytology of cells from treated RhE could determine skin irritancy. Six chemicals (three irritants; DnP, 1-B, PH, three non-irritants; DP, APA, HS) were evaluated in a RhE, Keraskin™. DP, HS, and PH were clearly classified with viability, but DnP, 1-B, and APA were often falsely determined, due to borderline values falling near the cutoff, 50%. In histology, the tissues treated with DnP, 1-B, and PH showed erosion of the stratum corneum, vacuolization, and necrosis in the basal layer. DP- and HS-treated tissues showed relatively normal morphology but APA induced necrosis similar to irritants. Cytology revealed that DnP, 1-B or PH depleted cells and induced irregular and abnormal cell shapes. In contrast, relatively regular and normal shapes and clear distinction between the nucleus and cytoplasm was observed for DP, APA and HS. To further confirm it, additional 10 substances, including false positives from OECD TG 439, were tested. Overall (16 substances in total), cytology: total area predicted the skin irritancy of test chemicals with the highest accuracy (87.5%) followed by cytology: cell count (81.3%), histology (75%) and viability (68.8%), confirming the utility of cytology as an alternative endpoint in the skin irritation test using RhE.

1. Introduction

The Draize skin irritation test has been replaced by the *in vitro* skin irritation test (SIT) using reconstructed human epidermis (RhE) (Lee et al., 2017a; Lemper et al., 2014). OECD Test Guideline (TG) 439 (OECD, 2015b) is being widely used to determine the skin irritancy of chemicals either as a stand-alone test or in combination with other *in vitro* test methods within a frame of IATA - Integrated Approaches to Testing and Assessment (OECD, 2017; Tollefsen et al., 2014). TG 439 addresses skin irritation by using RhE, which closely mimics the biochemical and physiological properties of the human epidermis. Human derived non-transformed keratinocytes are cultured to form a multi-layered, highly differentiated model, RhE.

In most of tissue-based irritation test methods including RhE, irritancy of a test chemical is determined by the viability of the treated tissues measured after the chemical application (Joo et al., 2019; Jung et al., 2014; Lim et al., 2019). In SIT using RhE, viability is measured by the enzymatic conversion of a vital dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a blue formazan salt (Mosmann, 1983). The optical density of formazan extracted from the treated tissues is then measured and calculated into % viability relative to the negative control. Classification of skin irritancy is made using a prediction model based on a viability cutoff value (Alepee et al., 2010).

Incidentally, test chemicals with strong-color or MTT-reducing activity may interfere with MTT assay, often leading to a false estimate of viability. To avoid color interference, OECD TG 439 advises that additional controls should be used to correct the viability measurement. Alternatively, HPLC/UPLC-spectrophotometry is being employed to measure reduced formazan directly, circumventing color interference (Alepee et al., 2015). But the cost for the instrumental analysis is often expensive and prohibitive. Furthermore, it is pre-requisite to conducting independent analytical validation before doing an instrumental analysis in GLP tests. Histology has been explored to supplement viability assays in tissue models (Hwang et al., 2018; Lee et al., 2017b; Park et al., 2018; Zanetti et al., 2016), which can examine the micro-anatomy of cells, tissues, and organs under a microscope. Especially,

Abbreviations: DP, diethyl phthalate; APA, allyl phenoxyacetate; HS, hexyl salicylate; DnP, di-n-propyldisulphide; 1-B, 1-bromohexane; PH, 5% potassium hydroxide

* Corresponding author at: College of Pharmacy, Ewha Womans University, 52 Ewhayeodae-gil, Seodaemun-gu, Seoul 03760, Republic of Korea.
E-mail address: kmlim@ewha.ac.kr (K.-M. Lim).

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4. Publication: *Me-too* validation study for *in vitro* skin irritation test with a reconstructed human epidermis model, KeraSkin™ for OECD test guideline 439.

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Me-too validation study for *in vitro* skin irritation test with a reconstructed human epidermis model, KeraSkin™ for OECD test guideline 439

Juhee Han [a], Seolyeong Kim [b], Su-Hyun Lee [b], Jin-Sik Kim [c], Yu Jin Chang [d], Tae-Cheon Jeong [e], Mi-Jeong Kang [e], Tae-Sung Kim [f], Hae Seong Yoon [f], Ga Young Lee [f], SeungJin Bae [a,**], Kyung-Min Lim [a,*]

[a] College of Pharmacy, Ewha Womans University, Seoul, Republic of Korea
[b] Biosolution Co, Seoul, Republic of Korea
[c] COSMAX Korea, Seongnam-si, Republic of Korea
[d] Korea Conformity Laboratories, Incheon, Republic of Korea
[e] College of Pharmacy, Yeungnam University, Gyeongsan, Republic of Korea
[f] National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong, Republic of Korea

ARTICLE INFO

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OECD TG 439
Reproducibility
Predictive capacity
Skin irritation

ABSTRACT

We conducted a me-too validation study to confirm the reproducibility, reliability, and predictive capacity of KeraSkin™ skin irritation test (SIT) as a me-too method of OECD TG 439. With 20 reference chemicals, within-laboratory reproducibility (WLR) of KeraSkin™ SIT in the decision of irritant or non-irritant was 100%, 100%, and 95% while between-laboratory reproducibility (BLR) was 100%, which met the criteria of performance standard (PS, WLR≥90%, BLR≥80%). WLR and BLR were further confirmed with intra-class correlation (ICC, coefficients >0.950). WLR and BLR in raw data (viability) were also shown with a scatter plot and Bland-Altman plot. Comparison with existing VRMs with Bland-Altman plot, ICC and kappa statistics confirmed the compatibility of KeraSkin™ SIT with OECD TG 439. The predictive capacity of KeraSkin™ SIT was estimated with 20 reference chemicals (the sensitivity of 98.9%, the specificity of 70%, and the accuracy of 84.4%) and additional 46 chemicals (for 66 chemicals [20 + 46 chemicals, the sensitivity, specificity and accuracy: 95.2%, 82.2% and 86.4%]). The receiver operating characteristic (ROC) analysis suggested a potential improvement of the predictive capacity, especially sensitivity, when changing cut-off (50% → 60–75%). Collectively, the me-too validation study demonstrated that KeraSkin™ SIT can be a new me-too method for OECD TG 439.

1. Introduction

The European Commission has prohibited animal experiments for cosmetics in 2013 and has been working on developing alternative test methods to replace animal testing based on the '3R's Principle' (Adler et al., 2011). Since the EU ban on animal testing for cosmetics, more than 37 countries worldwide have legally prohibited animal experiments for the development of cosmetics (Akbarsha and Mascarenhas, 2019). In addition, there is an increasing demand for new approach methodologies (NAMs) to evaluate the safety of numerous chemicals on human health in various sectors (Parish et al., 2020). *In vitro* skin irritation test, OECD TG 439, has been developed to replace OECD TG 404 (OECD, 2002) in which rabbits are used as a test species to evaluate the skin irritancy of chemicals and cosmetic products (Kose et al., 2018; Park et al., 2018). OECD TG 439 uses a reconstructed human epidermis (RhE) (OECD, 2015a) and can be used as a stand-alone to identify UN GHS No category chemical, *i.e.*, non-irritant or in combination with other replacement methods, such as OECD TG 435 "*In Vitro* Membrane Barrier Test Method for Skin Corrosion" (OECD, 2015b) to further classify the hazard on skin in the framework of Integrated Approach on

Abbreviations: OECD, The Organisation for Economic Co-operation and Development; TG, Test guideline; PS, Performance Standards; VRM, Validated Reference Method; SIT, skin irritation test; WLR, Within-laboratory reproducibility; BLR, Between-laboratory reproducibility; RhE, Reconstructed human Epidermis; CV, cell viability; I, irritant; NI, non-irritant; GHS, Globally Harmonized System of Classification and Labelling of Chemicals.

* Corresponding author.
** Corresponding author.
E-mail addresses: sjbae@ewha.ac.kr (S. Bae), kmlim@ewha.ac.kr (K.-M. Lim).

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Available online 5 August 2020

Ⅲ Development and validation of NAMs based on advanced technologies

1. Development and standardization of organoid platform for toxicity assessment
2. Development of next-generation toxicity prediction and evaluation method
3. Study on creation of basis for next-generation toxicologic pathology diagnosis
4. Survey on the latest technology trends of stem cells and organoid
5. Study on next-generation safety pharmacology evaluation of cardiovascular effects
6. Study on next-generation neurotoxicity evaluation method
7. Study on development of a method for evaluation of (new) narcotics pharmacology and toxicity in central nervous system

Ⅲ Development & validation of NAMs based on advanced technologies

- Countries around the world have developed alternatives to animal testing or new approach methodologies (NAMs) in order to address various issues including ethical issues related to laboratory animals and interspecies differences.
- The definition of NAMs has evolved from the methods reflecting the 3R principles to the methods based on advanced technologies.

The methods **replace** and **reduce** the use of animals or **refine** the treatment of animals based on the **3R principles**	→	Comprehensive term embracing NAMs (New Aproach Methodologies) using innovative non-animal methods (approaches) which can improve predictivity against human

- The U.S. FDA enacted the FDA Modernization ACT 2.0 in December 2022 that allows the use of NAMs for new drug and biologic applications. The data generated with NAMs using advanced technologies including MPS and bioprinting are able to be submitted as non-clinical data.
- Also, MFDS has made administrative notice on the revision of Regulations on pharmaceuticals approval, notification and review, and allows submission of the data generated with non-animal or human biology based methods (e.g. cell-based assays, MPS, bioprinting, computational model, etc.) that are conducted in accordance with the GLP.
- MFDS and KoCVAM has focused on developing NAMs using advanced technologies in order to actively respond to such a global change.
- Different government agencies have conducted research on advanced technologies including organoid in order to obtain source technology. However, regulatory acceptance of organoid requires validation of assays using the source technology.
  - MFDS, as a regulatory body, has focused on development and international harmonization of safety assessment methods using organoid.

1 Development and standardization of organoid platform for toxicity assessment

Supervising division	Toxicological Research Division of NIFDS
Type	Entrusted project
Title	Development of an organoid platform for toxicity assessment (22213MFDS386, Myung-Jin Son, KRIBB)
	Validation study on an organoid platform for toxicity assessment (23213MFDS489, Gun-Hwa Kim, NST)

☐ Background for the study

- There has been an urgent need to develop an toxicity evaluation model able to make an accurate toxicity prediction and support rapid decision-making during new drug development that require enormous cost and time.
  - The toxicity evaluation model will help prevent waste of money incurred from withdrawal of drug candidates from the market by supporting rapid and accurate selection of the candidates passing non-clinical stage.
- For the last decade, a lot of money has been invested into R&D led by different government agencies and some achievements have been made. However, none of them has feasibility in actual utilization for evaluation.
- With development of advanced technologies, countries are competing harshly with each other to hold a lead in the competition concerning the technologies. However, such advanced technologies are rarely accepted by regulatory bodies. Stakeholders from industry, academia and research institutes have urged the regulatory authorities to accept the technologies.
  - Since it was demonstrated that Korea has technological prowess in organoid, MFDS has determined to take the lead to develop and propose an international standard for organoid.

☐ Study in brief

- Development of organoid models (liver and intestine) for toxicity assessment and selection of test methods
- Pre-validation and transfer of the organoid-based toxicity assessment methods

- Optimization of the toxicity assessment methods based on the organoids (liver and intestine) through pre-validation
- Proposal of an international standardization through establishment of the organoid-based toxicity assessment method and a between-laboratory validation study (using liver organoid, proposal of an OECD SPSF)
- **Final goal:** Development of a toxicity assessment method using the standardized organoid
  * Study on the development of an organoid platform for toxicity assessment (22213MFDS386, Myung-Jin Son, KRIBB) Validation study on an organoid platform for toxicity assessment (23213MFDS489, Gun-Hwa Kim, NST)

☐ Overview of the study outcome

- **Major accomplishment:** Development of organoid models (liver and intestine) for toxicity assessment and standardized organoid through between-laboratory validation and establishment of assessment the method.
- **Main outcome**
  - Development of organoid models for toxicity assessment using human-derived stem cells
    * A standard organoid pool categorized by races, genders and ages has been established.
  - **Establishment of the organoid models for toxicity assessment:** A Quality Control (QC) method for standard organoids have been established. Following selection of a toxicity assessment method (evaluation of within-laboratory reproducibility and accuracy) and reference substances, the method has been validated by confirming reproducibility and accuracy.
  - **Standardization of the organoid model**

    1) Reliability and relevance of the toxicity assessment method has been demonstrated by performing within-laboratory pre-validation and between-laboratory validation. The standardized method has been established by transferring it to other laboratories.

    2) Proposal of the standardized organoid model to the OECD for international standardization

    * MFDS presented the toxicity assessment method using the organoid model at the WNT 35 (April 2023). Many countries made positive response and offered joint research.
    * MFDS presented the toxicity assessment method using the organoid model at the OECD ESCA meeting in June 2023 and received positive response. With guidance by the OECD secretariat, MFDS has been focusing on standardization of the method for international acceptance.

☐ Excellence and advantages

- Excellence
  - The organoid-based toxicity assessment method has been developed with our own technology
  - The model owns various advantages* that can compensate some of the limitations of the existing models and able to produce more accurate results in a short period.
    * The model is able to be customized for each patient, passaged over 90 times, and be frozen and thawed repeatedly. Further, it enables toxicity evaluation based on mechanism and produces more accurate results compared to animal testing.
  - Drugs that passed non-clinical and clinical studies but were withdrawn from the market due to hepatotoxicity have been tested with our organoid model. The model exhibited over 80% of accuracy, which is higher than the accuracy from the non-clinical trials using animals.
- Advantages
  - This toxicity assessment method is not a "*me-too*" method that is developed by modifying exiting method. Rather, it is developed using our original technology based on the organoid developed in Korea
  - Among many nations researching organoids, Korea is the first to announce international standardization of organoids and presented study outcome. Now, MFDS of Korea is leading the international standardization of organoid.
    * MFDS presented the toxicity assessment method using the organoid model at the WNT 35 (April 2023). Many countries made positive response and offered joint research.
    * MFDS presented the toxicity assessment method using the organoid model at the OECD ESCA meeting in June 2023 and received positive response. With guidance by the OECD secretariat, MFDS has been focusing on standardization of the method for international acceptance.

☐ Utilization of the outcome and its impact

- **Development of national industry:** Development of OECD Test Guideline for organoid-based toxicity assessment method accepted internationally is expected to help create a new bio-industry relating to organoid.
- **Contribution to new drugs development:** With increased accuracy in replicating human response, the toxicity evaluation model is expected to reduce time and cost for drug development.

☐ Performance of the research

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
1	MFDS researching toxicity assessment method using organoid	Promotion and dissemination of research outcome	Number of evaluation and promotion materials about the outcome
2	Publication: Advances in liver organoids: Model systems for liver disease	Academic outcome	Number of papers published in SCI-grade journals
3	Publication: Limosilactobacillus reuteri DS0384 promotes intestinal epithelial maturation via the postbiotic effect in human intestinal organoids and infant mice	Academic outcome	Number of papers published in SCI-grade journals
4	Publication: Advanced human liver models for assessment of drug-induced liver injury	Academic outcome	Number of papers published in non-SCI-grade journals
5	Publication: Recent advancements of multicellular human liver models	Academic outcome	Number of papers published in non-SCI-grade journals
6	Presentation: 2022 International Conference: Korean Society for Molecular and Cellular Biology	Academic outcome	Number of presentations (publications) in workshops
7	Preparation of draft: Draft SOP on liver organoid passaging	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
8	Preparation of draft: Draft SOP on liver organoid Indocyanine Green absoption and release test	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
9	Preparation of draft: Draft SOP on liver organoid freezing	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
10	Preparation of draft: Draft SOP on orgaonid differentiation induction	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
11	Preparation of draft: Draft SOP on measurement of cell count on orgaonid	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
12	Preparation of draft: Draft SOP on organoid thawing	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
13	Preparation of draft: Draft SOP on measurement of organoid CYP3A4 activation level	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
14	Preparation of draft: Draft SOP on measurement of bile acid pool size on oraganoid	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
15	Preparation of draft: Draft SOP on determination of liver function marker expression on organoid using IF staining	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
16	Preparation of draft: Draft SOP on quantitative analysis of albumin secretion on organoid	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
17	Preparation of draft: Draft SOP on quantitative analysis of alpha-1-antitrypsin on organoid	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
18	Preparation of draft: Draft SOP on mycoplasma test method	Encouragement of practical use by policy improvement, etc.	Number of TG proposals submitted
19	Organization of industry, academia and research institutes meeting for standardization of organoid	Promotion and dissemination of research outcome	Number of education and promotion materials about the outcome
20	Attending and making presentation at the OECD WNT35	Promotion and dissemination of research outcome	Performance in international exchange
21	Attending and making presentation at the OECD ESCA	Promotion and dissemination of research outcome	Performance in international exchange

☐ Major accomplishment

2. Publication: Advances in liver organoids: Model systems for liver disease

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REVIEW

Advances in liver organoids: model systems for liver disease

Jaeseo Lee[1] · Seon Ju Mun[1,2] · Yongbo Shin[1,2] · Seonbhin Lee[1,2] · Myung Jin Son[1,2]

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Abstract Reliable in vitro models with human-derived cells that recapitulate in vivo-like physiologies are required for drug discovery and development to reduce the gap between the results of cell-based drug testing, animal testing, and human clinical trials. Liver organoid models have emerged as novel tools for hepatotoxicity evaluation, liver disease modeling, and drug screening. Liver organoids can be generated from biopsies of liver tissues or pluripotent stem cells and can be applied to various liver diseases, including metabolic associated fatty liver disease, infectious liver disease, genetic liver disease, and liver cancer. This review focuses on recent studies on organoids to model human liver diseases and discusses the advantages and limitations of current liver organoids for translational applications.

Keywords Liver · Organoids · Disease model · Drug screening

Introduction

New drug discoveries and developments are complex, with a huge failure rate in which one FDA-approved drug can start with 10,000 candidate compounds, and take over 10 years (Harrer et al., 2019). This discrepancy arises from the gap between in vitro and in vivo animal testing and human responses. In vitro testing cannot fully reflect the complex responses to drugs, and animal testing is not sufficient to replace the human system owing to species differences. Therefore, there is an urgent need for model systems that accurately reflect human responses to drugs. In the case of liver models, primary human hepatocytes (PHHs) have been the gold standard for drug testing and toxicity prediction; however, the limited availability of cellular sources and long-term maintenance have been major obstacles to their use in various in vitro applications, including disease modeling. With recent advances in stem cell research and three-dimensional (3D) tissue engineering, the limitations of conventional models have gradually been resolved. In particular, human cell-based organoid models are expected to substitute conventional in vitro models in terms of accessibility to cellular sources and similarity to in vivo physiology. Therefore, recent progress in organoid technology is in the spotlight as a bridge that recapitulates complex responses in non-clinical drug development research and provides reliable human cell-based ex vivo models (Fig. 1).

Organoids are self-organized 3D structures that recapitulate aspects of native tissue architecture and function in vitro (Marsee et al., 2021), thus, they have attracted attention as highly advanced models with in vivo-like features. Liver organoids have emerged as novel platforms for drug development. The liver is the main organ for drug metabolism, and drug-induced liver injury is the primary cause of drug toxicity and market withdrawal owing to unexpected side effects. Furthermore, no proper models represent a patients' actual pathology of fatal liver diseases. Liver organoids can be generated from patient-derived liver biopsies or patient-derived induced pluripotent stem cells (iPSCs), and have great advantages in that it is possible to implement personalized drug testing platforms and disease models to recapitulate the

✉ Myung Jin Son
mjson@kribb.re.kr

[1] Stem Cell Convergence Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Yuseong-gu, Daejeon 34141, Republic of Korea

[2] Department of Functional Genomics, Korea University of Science & Technology (UST), 217 Gajungro, Yuseong-gu, Daejeon 34113, Republic of Korea

3. Publication: Limosilactobacillus reuteri DS0384 promotes intestinal epithelial maturation via the postbiotic effect in human intestinal organoids and infant mice

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RESEARCH PAPER

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Limosilactobacillus reuteri DS0384 promotes intestinal epithelial maturation via the postbiotic effect in human intestinal organoids and infant mice

Hana Lee[a§], Kwang Bo Jung[a§], Ohman Kwon[a§], Ye Seul Son[a], Eunho Choi[a,b], Won Dong Yu[a,b], Naeun Son[a,b], Jun Hyoung Jeon[c], Hana Jo[c], Haneol Yang[c], Yeong Rak Son[c], Chan-Seok Yun[c], Hyun-Soo Cho[b,e], Sang Kyu Kim[d], Dae-Soo Kim[b,e], Doo-Sang Park[c], and Mi-Young Son[a,b]

[a]Stem Cell Research Convergence Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Republic of Korea; [b]KRIBB School of Bioscience, Korea University of Science and Technology (UST), Daejeon, Republic of Korea; [c]Korean Collection for Type Cultures, Biological Resource Center, KRIBB, Jeongeup, Republic of Korea; [d]Laboratory of Efficacy Research, Korea Ginseng Corp., Daejeon, Republic of Korea; [e]Digital Biotech Innovation Center, KRIBB, Daejeon, Republic of Korea

ABSTRACT

Little is known about the modulatory capacity of the microbiota in early intestinal development. We examined various intestinal models that respond to gut microbial metabolites based on human pluripotent stem cell-derived human intestinal organoids (hIOs): physiologically relevant *in vitro* fetal-like intestine, intestinal stem cell, and intestinal disease models. We found that a newly isolated *Limosilactobacillus reuteri* strain DS0384 accelerated maturation of the fetal intestine using 3D hIO with immature fetal characteristics. Comparative metabolomic profiling analysis revealed that the secreted metabolite N-carbamyl glutamic acid (NCG) is involved in the beneficial effect of DS0384 cell-free supernatants on the intestinal maturation of hIOs. Experiments in an intestinal stem cell spheroid model and hIO-based intestinal inflamed model revealed that the cell-free supernatant from DS0384 comprising NCG promoted intestinal stem cell proliferation and was important for intestinal protection against cytokine-induced intestinal epithelial injury. The probiotic properties of DS0384 were also evaluated, including acid and bile tolerance and ability to adhere to human intestinal cells. Seven-day oral administration of DS0384 and cell-free supernatant promoted the intestinal development of newborn mice. Moreover, NCG exerted a protective effect on experimental colitis in mice. These results suggest that DS0384 is a useful agent for probiotic applications and therapeutic treatment for disorders of early gut development and for preventing intestinal barrier dysfunction.

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Human intestinal organoid; intestinal development; epithelial maturation; gut microbiota; probiotic; postbiotic; *Limosilactobacillus reuteri*; N-carbamyl glutamic acid

Introduction

Postnatal intestinal maturation plays an important role in the normal development and physiological function of the intestine, including establishment of the epithelial barrier and immune system as well as colonization and stabilization of the microbiota during the first two years of life.[1] A key aspect of intestinal maturation is the development of barrier integrity, which is critical not only for nutrient absorption but also for preventing the entry of pathogenic bacteria and toxic substances.[2] The initial colonization of gut microbiota and their interactions with the host can influence intestinal development and epithelial maturation by promoting the proliferation and differentiation of intestinal epithelial cells, vascularization, production of mucus, and maintenance of epithelial junctions.[3,4] Failure of the intestine to mature normally has been implicated in the pathogenesis of neonatal intestinal diseases, such as necrotizing enterocolitis and early-onset inflammatory bowel disease (IBD). Very early onset IBD (VEO-IBD) is characterized by not only the common symptoms of adult IBD, such as rectal bleeding and diarrhea due to inflammation and intestinal epithelial disruption, but also growth failure.[5–7]

One strategy for reinforcing intestinal epithelial functions is administration of probiotics, which are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on

CONTACT Mi-Young Son myson@kribb.re.kr Stem Cell Research Convergence Center, KRIBB, Daejeon, 34141, Republic of Korea; Doo-Sang Park dspark@kribb.re.kr Korean Collection for Type Cultures, Biological Resource Center, KRIBB, Jeongeup, 56212, Republic of Korea; Dae-Soo Kim kds2465@kribb.re.kr Digital Biotech Innovation Center, KRIBB, Daejeon, 34141, Republic of Korea
[§]These authors contributed equally to this work.
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4. Publication: Advanced human liver models for assessment of drug-induced liver injury

Review Article

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Organoid

Advanced human liver models for the assessment of drug-induced liver injury

Seon Ju Mun[1,2], Jaeseo Lee[1], Yongbo Shin[1,2], Myung Jin Son[1,2]

[1]Stem Cell Convergence Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea
[2]Department of Functional Genomics, Korea University of Science & Technology (UST), Daejeon, Korea

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Correspondence to:
Myung Jin Son, PhD
Stem Cell Convergence Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Yuseong-gu, Daejeon 34141, Korea
E-mail: mjson@kribb.re.kr

Drug safety issues continue to occur even with drugs that are approved after the completion of clinical studies. Drug-induced liver injury (DILI) is a major obstacle to drug development, because the liver is the primary site of drug metabolism, and injuries caused during this process are severe. Conventional *in vitro* human liver models, such as 2-dimensional hepatic cell lines, lack *in vivo* physiological relevance, and animal studies have limitations in the form of species differences and regulatory restrictions. To resolve this issue, an increasing number of 3-dimensional human liver systems, including organoids, are being developed. In this review, we provide an overview of recent assessments of DILI prediction, approaches for *in vitro* hepatotoxicity evaluation, and a variety of advanced human liver models. We discuss the advantages, limitations, and future perspectives of current human liver models for accurate drug safety evaluations.

Keywords: Liver; Organoids; Chemical and drug induced liver injury; Toxicity

Introduction

Drug development requires complex steps, including drug discovery, *in vitro* and *in vivo* nonclinical trials, clinical trials, and the United States Food and Drug Administration (FDA) approval. The entire process typically takes more than 10 years and costs $3 billion [1]. Additionally, the success rate of clinical trials is lower than 10%, and drugs are withdrawn from the market owing to safety issues even after their approval. Drug-induced liver injury (DILI), one of the leading causes of drug development failure, accounts for 18% of all drug withdrawals from the market between 1953 and 2013 [2]. Statistically, only approximately 50% of compounds exhibiting liver toxicity in humans have been identified through animal studies, which have recently been restricted due to ethical concerns [3]. Therefore, more accurate model systems are urgently required to reduce these discrepancies and concerns, as well as to accurately predict human responses.

In this regard, primary human hepatocytes (PHHs) obtained from human liver tissue are considered the gold standard for the evaluation of hepatotoxicity. However, the function of PHHs *in vitro* rapidly decreases in conventional 2-dimensional (2D) culture formats; therefore, 3-dimensional (3D) culture systems have been extensively studied to overcome this limitation. PHHs in 3D culture maintain their function *in vitro* for over 2 weeks and exhibit accurate toxicity prediction results compared to those in 2D culture [4,5]. Furthermore, 3D organoids have recently emerged as a novel alternative source for human liver models with advanced native tissue architecture and function [6–8]. As a mechanistic understanding of DILI-related adverse

5. Publication: Recent advancements of multicellular human liver models

Review Article

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Organoid 2022;2:e26 • https://doi.org/10.51335/organoid.2022.2.e26

Organoid

Recent advances in multicellular human liver models

Seon Ju Mun[1,2,*], Jaeseo Lee[1,*], Yongbo Shin[1,2], Vincent Eun[3], Youngmi Ji[3], Myung Jin Son[1,2]

[1]Stem Cell Convergence Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea
[2]Department of Functional Genomics, Korea University of Science & Technology, Daejeon, Korea
[3]National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA

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Correspondence to:
Myung Jin Son, PhD
Stem Cell Convergence Research Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon 34141, Korea
E-mail: mjson@kribb.re.kr

*These authors contributed equally.

The liver is the most important metabolic organ in the body. Model systems that recapitulate the complex organ structure and cell composition of the human liver are insufficient to study liver biology and to test toxicity and efficacy during new drug development. Recently established 3-dimensional liver models, including spheroids and organoids, organs-on-a-chip, bioprinting, and the decellularization/recellularization technique, have provided platforms that emulate the structural and functional characteristics of the human liver better than conventional 2-dimensional cell culture models and animal models. This review summarizes the architecture and cell compositions of human liver tissue, focusing on recent studies of multicellular human liver models that recapitulate *in vivo*-like physiologies with morphological and functional advances by the cellular communication of parenchymal and non-parenchymal cells. We discuss the applications, limitations, and future perspectives of advanced multicellular human liver models.

Keywords: Liver; Organoids; Coculture Techniques; Tissue engineering; Growth & development

Introduction

The liver is a central organ in various metabolic processes, and its primary functions are (1) metabolism of carbohydrates, lipids, amino acids, and bile acids; (2) detoxification; (3) synthesis of plasma proteins such as albumin and clotting factors; and (4) storage of glycogen, vitamins, and minerals [1]. Hepatic failure arises due to the deterioration of liver function, mainly owing to xenobiotics or disease progression, and is often fatal. Unfortunately, reliable liver models for predicting the risk of hepatotoxicity during new drug development and evaluating the efficacies of drugs to target liver diseases are insufficient.

Three-dimensional (3D) liver models, including spheroids and organoids, have recently been developed, and their advantages were highlighted with respect to recapitulation of the complexities of cell compositions and tissue structure in comparison with 2-dimensional (2D) cell culture models. Primary human hepatocytes (PHHs) isolated from human liver tissue have high functionality and are therefore generally used as the gold standard for an *in vitro* drug testing platform. However, their viability and functionality are not sufficiently maintained in conventional 2D culture formats. Approaches to manage the 3D microenvironment by controlling stiffness using extracellular matrix (ECM), such as Matrigel and hydrogel, have been implemented and improved the viability of PHHs until 40 days in culture [2,3]. In addition, interactions between hepatocytes and non-parenchymal cells, such as endothelial cells and hepatic stellate cells (HSCs), in 3D hepatic spheroids improved viability and the liver phenotype [4,5]. Importantly, ameliorated liver functions, including high cytochrome P450 (CYP) enzyme ac-

2 Development of next-generation toxicity prediction and evaluation method

Supervising division	Toxicological Research Division of NIFDS
Type	Entrusted project
Title	Study on the development of next-generation toxicity prediction and evaluation method using advanced technologies

☐ Background for the study

- Every year, thousands of new chemicals are introduced and used as ingredients in foods and drugs, cosmetics, medical devices, etc. Humans are exposed many new alien chemicals as well as known existing chemicals that are flown into air and water.
- It is a universal task for countries around the world to evaluate the effects of the chemicals on humans, diagnose their risks and introduce necessary regulations. However, the current approaches (or methodologies) are unlikely to rapidly investigate their mechanism and their interaction with humans or environment.
- In particular, the existing toxicity test methods relied on laboratory animals tend to have issues concerning energy consumption and environmental pollution. Also, the existing methods have limitations including differences incurring during interspecies extrapolation and dose extrapolation that evaluates biological toxicity of chemicals based on the results following high-volume exposure of the chemicals to parenchyma cells in a single organ.
- This increases the need for use of laboratory animals. Further, for lots of chemicals that are used nowadays, it is difficult to obtain their safety data in a rapid and efficient manner.

☐ Study in brief

- **Goal:** Development of an intelligent toxicity prediction and evaluation model based on Industry 4.0. technologies
- Big data were collected to support the development of a toxicity prediction model
- A plan for development and utilization of an intelligent toxicity prediction model was proposed
- Hepatotoxicity prediction system was developed based on deep-learning technique with the big data related to toxicology
- A two-stage* deep learning model for toxicity prediction was built based on the big data
  * Deep learning algorithm for toxicity prediction based on chemical structure, physical and chemical properties, and atomic information
  * Deep learning algorithm for toxicity network prediction based on integrated data of transcriptome and bionetwork.
- Confirming and validating feasibility of the toxicity prediction model

☐ Overview of the study outcome

- **Major accomplishment:** Development of a hepatotoxicity prediction system using a deep learning technique by creating hepatotoxicity big data.
- **Main outcome**

(1) Collecting data on compounds and creating big data related to hepatotoxicity

- Data on compounds including their hepatotoxicity potential were collected from Tox-21 (EPA), LTKB (FDA) and LiverTox
- Structure and physical and chemical property of the compounds were collected from PubChem, ChEMBL and ZINC, and atomic information from RDKit.
- Information on the transcriptome before and after application of the compounds were collected from cMAP, LINCS (Human) and Drug Matrix (Mouse/Rat).

(2) Development of a deep learning model for the big data

- A positive and negative compound set for heapatotoxicity was defined.
- A positive and negative compound set was defined using compound structure, physical and chemical properties and atomic information
- A deep learning model for toxicity prediction was created based on transcriptome

(3) Validation of the performance of the deep learning model for hepatotoxicity prediction

- The deep learning model was applied to the compounds with no toxicity information to identify the compounds with hepatotoxic potential.
- A toxicity evaluation platform applied with the toxicity prediction model was established and 17 reference substances were evaluated with the platform.
- Producing real cases of toxicity prediction based on the developed model: The toxicity prediction model was validated with substances selected specifically for the validation using *in vivo* and *in vitro* toxicity test platform.

☐ Excellence and Advantages

- **Excellence**
  - Development of AI-based deep learning algorithm for toxicity prediction using our own technology
  - Laying the foundation for the development of an alternative to animal testing by building a database containing information on over 5000 chemicals and developing a hepatotoxicity prediction model
    * An evidence-based hepatotoxicity prediction model developed based on the database applied with AI technology
  - The AI-based toxicity prediction model is able to identify chemicals with hepatotoxic potential, which is expected to help reduce time and cost of drug development.
  - With the development of the toxicity prediction model, hepatotoxicity prediction of newly developed chemicals* used in consumer chemical products is possible, and base technology for risk and toxicity prediction of chemicals that humans are exposed to is obtained.
    * Perfluorinated compounds (e.g. PFOS, PFAS, PFOA, F53B, etc.) and Bisphenol are reported to induce hepatotoxicity
- **Advantages**
  - Development of the nation's first AI-based toxicity prediction model and chemical database
  - Risk and (or) toxicity information on over 5000 chemicals scattered across various national or foreign databases have been collected and standardized to create big data for development of a two-stage toxicity prediction model.

- The database* has been developed based on the standardized big data and the two-stage prediction model, which is expected to support the new drug development and (or) chemical developers by providing information on toxicity prediction.

  * HepatoToxicity portal based on AI and applied with hepatotoxicity prediction technology

☐ Utilization of the outcome and its impact

- **Safety assessment:** In the non-clinical study of compounds, the model can be utilized as a hepatotoxicity screening system before performing animal experiment
  - The model can provide basis for a next-generation safety assessment technology adhering to the 3R principles
- **Mechanism prediction:** toxicity mechanism can be predicted by generating transcriptome data on compounds

☐ Performance of the Study

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
1	Publication: A review on compound-protein interaction prediction methods: Data, format, representation and model	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journals
2	Publication: On modeling and utilizing chemical compound information with deep learning technologies; A task-oriented approach	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journals
3	Publication: Drug-likeness scoring based on unsupervised learning	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journals
4	Publication: Subnetwork representation learning for discovering network biomarkers in predicting lymph node metastasis in early oral cancer	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journals
5	Publication: Risk stratification for breast cancer patient by simultaneous learning of molecular subtype and survival outcome using genetic algorithm-based gene set selection	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journals
6	Publication: Improved drug response prediction by drug target data integration via network-based profiling	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journals
7	Publication: Supervised chemical graph mining improves drug-induced liver injury prediction	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journals

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
8	Publication: DRPreter: interpretable anticancer drug response prediction using knowledge-guided graph neural networks and transformer	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journals
9	Presentation: A Supervised Markovian Random Walk Model for Investigating Hepatotoxicity Signatures of Chemical Drugs with Structural Alerts	Building on expertise through academic accomplishment	Number of presentations (publications) in national workshops
10	Presentation: Identification of toxic signature in transcriptomic space	Building on expertise through academic accomplishment	Number of presentations (publications) in national workshops
11	Presentation: Integrating Drug Target Information via Network Propagation for Drug Response Prediction	Building on expertise through academic accomplishment	Number of presentations (publications) in national workshops
12	Presentation: Leveraging biological knowledge as guide for random walk on biomedical heterogeneous network for drug repurposing	Building on expertise through academic accomplishment	Number of presentations (publications) in national workshops
13	Presentation: Leveraging hierarchical tree data as guide for random walk on heterogeneous network for drug repurposing	Building on expertise through academic accomplishment	Number of presentations (publications) in national workshops
14	Presentation: 1H NMR-based metabolomics of brain, urine and serum in rat model of valproic acid-induced autism spectrum disorders (ASD)	Building on expertise through academic accomplishment	Number of presentations (publications) in national workshops
15	DB creation: HepatoToxicity Portal https://www.kobic.re.kr/htp/	Creation of website (database)	Number of DB creation or updates

☐ Major outcome

1. Publication: A review on compound–protein interaction prediction methods: Data, format, representation and model

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A review on compound-protein interaction prediction methods: Data, format, representation and model

Sangsoo Lim [a,1], Yijingxiu Lu [b], Chang Yun Cho [d], Inyoung Sung [d], Jungwoo Kim [b], Youngkuk Kim [b], Sungjoon Park [b], Sun Kim [a,b,c,d,*]

[a] Bioinformatics Institute, Seoul National University, Seoul, Republic of Korea
[b] Department of Computer Science and Engineering, College of Engineering, Seoul National University, Seoul, Republic of Korea
[c] Institute of Engineering Research, Seoul National University, Seoul, Republic of Korea
[d] Interdisciplinary Program in Bioinformatics, College of Natural Sciences, Seoul National University, Seoul, Republic of Korea

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ABSTRACT

There has recently been a rapid progress in computational methods for determining protein targets of small molecule drugs, which will be termed as compound protein interaction (CPI). In this review, we comprehensively review topics related to computational prediction of CPI. Data for CPI has been accumulated and curated significantly both in quantity and quality. Computational methods have become powerful ever to analyze such complex the data. Thus, recent successes in the improved quality of CPI prediction are due to use of both sophisticated computational techniques and higher quality information in the databases. The goal of this article is to provide reviews of topics related to CPI, such as data, format, representation, to computational models, so that researchers can take full advantages of these resources to develop novel prediction methods. Chemical compounds and protein data from various resources were discussed in terms of data formats and encoding schemes. For the CPI methods, we grouped prediction methods into five categories from traditional machine learning techniques to state-of-the-art deep learning techniques. In closing, we discussed emerging machine learning topics to help both experimental and computational scientists leverage the current knowledge and strategies to develop more powerful and accurate CPI prediction methods.

Contents

* Corresponding author.
E-mail address: sunkimbioinfo@snu.ac.kr (S. Kim).
[1] Equal contribution.

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2. Publication: On modeling and utilizing chemical compound information with deep learning technologies; A task-oriented approach

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Mini review

On modeling and utilizing chemical compound information with deep learning technologies: A task-oriented approach

Sangsoo Lim [a,1], Sangseon Lee [b,1], Yinhua Piao [c], MinGyu Choi [d,g], Dongmin Bang [e], Jeonghyeon Gu [f], Sun Kim [c,f,g,h,*]

[a] Bioinformatics Institute, Seoul National University, Gwanak-ro 1, Gwanak-gu, Seoul 08826, South Korea
[b] Institute of Computer Technology, Seoul National University, Gwanak-ro 1, Gwanak-gu, Seoul 08826, South Korea
[c] Department of Computer Science and Engineering, Seoul National University, Gwanak-ro 1, Gwanak-gu, Seoul 08826, South Korea
[d] Department of Chemistry, Seoul National University, Gwanak-ro 1, Gwanak-gu, Seoul 08826, South Korea
[e] Interdisciplinary Program in Bioinformatics, Seoul National University, Gwanak-ro 1, Gwanak-gu, Seoul 08826, South Korea
[f] Interdisciplinary Program in Artificial Intelligence, Seoul National University, Gwanak-ro 1, Gwanak-gu, Seoul 08826, South Korea
[g] AIGENDRUG Co., Ltd., Gwanak-ro 1, Gwanak-gu, Seoul 08826, South Korea
[h] MOGAM Institute for Biomedical Research, Yong-in 16924, South Korea

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ABSTRACT

A large number of chemical compounds are available in databases such as PubChem and ZINC. However, currently known compounds, though large, represent only a fraction of possible compounds, which is known as chemical space. Many of these compounds in the databases are annotated with properties and assay data that can be used for drug discovery efforts. For this goal, a number of machine learning algorithms have been developed and recent deep learning technologies can be effectively used to navigate chemical space, especially for unknown chemical compounds, in terms of drug-related tasks. In this article, we survey how deep learning technologies can model and utilize chemical compound information in a task-oriented way by exploiting annotated properties and assay data in the chemical compounds databases. We first compile what kind of tasks are trying to be accomplished by machine learning methods. Then, we survey deep learning technologies to show their modeling power and current applications for accomplishing drug related tasks. Next, we survey deep learning techniques to address the insufficiency issue of annotated data for more effective navigation of chemical space. Chemical compound information alone may not be powerful enough for drug related tasks, thus we survey what kind of information, such as assay and gene expression data, can be used to improve the prediction power of deep learning models. Finally, we conclude this survey with four important newly developed technologies that are yet to be fully incorporated into computational analysis of chemical information.

Contents

* Corresponding author.
[1] Equal contribution.

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3. Publication: Drug-likeness scoring based on unsupervised learning

Chemical Science

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Drug-likeness scoring based on unsupervised learning†

Kyunghoon Lee,‡[a] Jinho Jang,‡[a] Seonghwan Seo,‡[a] Jaechang Lim *[b] and Woo Youn Kim *[abc]

Drug-likeness prediction is important for the virtual screening of drug candidates. It is challenging because the drug-likeness is presumably associated with the whole set of necessary properties to pass through clinical trials, and thus no definite data for regression is available. Recently, binary classification models based on graph neural networks have been proposed but with strong dependency of their performances on the choice of the negative set for training. Here we propose a novel unsupervised learning model that requires only known drugs for training. We adopted a language model based on a recurrent neural network for unsupervised learning. It showed relatively consistent performance across different datasets, unlike such classification models. In addition, the unsupervised learning model provides drug-likeness scores that well separate distributions with increasing mean values in the order of datasets composed of molecules at a later step in a drug development process, whereas the classification model predicted a polarized distribution with two extreme values for all datasets presumably due to the overconfident prediction for unseen data. Thus, this new concept offers a pragmatic tool for drug-likeness scoring and further can be applied to other biochemical applications.

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1 Introduction

Prediction of the biochemical properties of molecules is essential for efficient drug development. Simulations based on physicochemical principles can be used for this purpose. However, these methods are often not practical, especially if either target properties are associated with a number of different biological causes or those mechanisms are unclear. Data-driven approaches can be applied to such cases thanks to their convenience of making predictions from data alone without efforts to know the underlying biological mechanisms, as have been shown by successful examples of deep learning methods for accelerated drug developments.[1–7]

One example of such biochemical properties is drug-likeness. It can be used to remove compounds in advance that are likely to fail in clinical trials, which is important to enhance the success rate and reduce the economic costs of drug development.[8,9] It is presumably associated with the whole set of essential characteristics to pass through clinical trials such as bioactivity, metabolic stability, toxicity, and so on. As those numerous factors can affect drug-likeness, it cannot be directly measured as a single-valued quantity. Therefore, various drug-likeness expressions have been suggested using data-driven approaches as a result of studies for more than two decades.[10]

In the beginning, the drug-likeness has been defined based on the certain physicochemical properties of the known drug molecules.[11] In general, human experts select those physicochemical properties that seem to be closely associated with the drug-likeness and have been analyzed their distribution from drug databases. According to the result, a certain drug-likeness method is determined and used in a virtual screening scenario. Most methods developed in the early days were a classifier type based on the rules derived from the property distribution analysis.[12–20] It is designed to determine whether a query molecule has drug potential or not. The representative example is the rules of five (Ro5) proposed by Lipinski *et al.*, which introduced the criteria of the number of hydrogen bond donors, the number of hydrogen bond acceptors, the molecular weight, and the octanol–water partition coefficient for being drug-like.[21,22] Though these rule-based filters have been widely used thanks to the convenience, their inflexibility provokes a substantial possibility of screening out good drug candidates. For instance, 16% of the oral drugs violate at least one of the Ro5, and 6% of them violate more than two.[23,24] Thus, these rules have been used to predict the bioavailability of molecules.

[a]Department of Chemistry, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 34 141, Republic of Korea. E-mail: wooyoun@kaist.ac.kr
[b]HITS Incorporation, 124 Teheran-ro, Gangnam-gu, Seoul 06 234, Republic of Korea. E-mail: jaechang@hits.ai
[c]KI for Artificial Intelligence, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 34 141, Republic of Korea

† Electronic supplementary information (ESI) available: The algorithm and implementation details of all the models used in the experiments. See DOI: 10.1039/d1sc05248a

‡ These authors contributed equally to this work.

4. Publication: Subnetwork representation learning for discovering network biomarkers in predicting lymph node metastasis in early oral cancer

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Subnetwork representation learning for discovering network biomarkers in predicting lymph node metastasis in early oral cancer

Minsu Kim[1], Sangseon Lee[2], Sangsoo Lim[3], Doh Young Lee[4✉] & Sun Kim[3,5,6,7,8✉]

Cervical lymph node metastasis is the leading cause of poor prognosis in oral tongue squamous cell carcinoma and also occurs in the early stages. The current clinical diagnosis depends on a physical examination that is not enough to determine whether micrometastasis remains. The transcriptome profiling technique has shown great potential for predicting micrometastasis by capturing the dynamic activation state of genes. However, there are several technical challenges in using transcriptome data to model patient conditions: (1) An Insufficient number of samples compared to the number of genes, (2) Complex dependence between genes that govern the cancer phenotype, and (3) Heterogeneity between patients between cohorts that differ geographically and racially. We developed a computational framework to learn the subnetwork representation of the transcriptome to discover network biomarkers and determine the potential of metastasis in early oral tongue squamous cell carcinoma. Our method achieved high accuracy in predicting the potential of metastasis in two geographically and racially different groups of patients. The robustness of the model and the reproducibility of the discovered network biomarkers show great potential as a tool to diagnose lymph node metastasis in early oral cancer.

Oral tongue squamous cell carcinoma (OTSCC) is one of the most common malignant tumors in the oral cavity[1]. Cervical lymph node metastasis is a major factor in a poor prognosis for OTSCC and also occurs even in early stages[2]. Currently, clinical diagnosis relies on physical examinations such as palpation, ultrasonography, computed tomography (CT-scan), and magnetic resonance imaging (MRI). Unfortunately, these physical examinations are not accurate enough to determine if micrometastasis remains in the lesion. Micrometastasis indicates that a small number of cancer cells that have spread from the primary tumor to other parts of the body are too few to be detected by screening or physical examination. For this reason, clinicians recommend lymphadenectomy for patients who do not require resection[3]. Lymphadenectomy refers to surgery to remove lymph nodes, which can cause serious side effects. Therefore, being able to detect micrometastases with molecular-level data could be of significant benefit to patients with OTSCC.

Transcriptome data are whole genome-scale molecular profiles generated by high-throughput RNA profiling techniques such as microarrays and RNA sequencing (RNA-seq), which are known to have great potential to identify micrometastasis in cancer patients[4–6]. There are several challenges in modeling patient conditions using transcriptome data. First, despite advances in high-throughput RNA profiling technology, the cost of production per sample is still at a non-negligible level, and the number of genes to consider is relatively large compared to the number of samples, which is a challenge for many researchers. This problem is also referred to as the low sample high dimension problem[7]. In addition, cellular proteins rarely act individually and generally cooperate to perform specific functions and express a specific phenotype[8]. Therefore, the complex dependence between genes due to protein interactions should also be considered. Finally, heterogeneity between patient samples is

[1]Computer Science and Mathematics Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA. [2]Institute of Computer Technology, Seoul National University, Seoul 08826, Korea. [3]Bioinformatics Institute, Seoul National University, Seoul 08826, Korea. [4]Seoul National University College of Medicine, Seoul 03080, Korea. [5]Department of Computer Science and Engineering, Seoul National University, Seoul 08826, Korea. [6]Interdisciplinary Program in Bioinformatics, Seoul National University, Seoul 08826, Korea. [7]Interdisciplinary Program in Artificial Intelligence, Seoul National University, Seoul 08826, Korea. [8]AIgenDrug, Co., Ltd, Seoul, Korea. ✉email: gedo0212@naver.com; sunkim.bioinfo@snu.ac.kr

5. Publication: Risk stratification for breast cancer patient by simultaneous learning of molecular subtype and survival outcome using genetic algorithm-based gene set selection

cancers

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Article

Risk Stratification for Breast Cancer Patient by Simultaneous Learning of Molecular Subtype and Survival Outcome Using Genetic Algorithm-Based Gene Set Selection†

Bonil Koo [1], Dohoon Lee [2,3], Sangseon Lee [4], Inyoung Sung [1], Sun Kim [1,5,6,7] and Sunho Lee [8,*]

1 Interdisciplinary Program in Bioinformatics, Seoul National University, Seoul 08826, Korea
2 Bioinformatics Institute, Seoul National University, Seoul 08826, Korea
3 BK21 FOUR Intelligence Computing, Seoul National University, Seoul 08826, Korea
4 Institute of Computer Technology, Seoul National University, Seoul 08826, Korea
5 Department of Computer Science and Engineering, Seoul National University, Seoul 08826, Korea
6 Interdisciplinary Program in Artificial Intelligence, Seoul National University, Seoul 08826, Korea
7 MOGAM Institute for Biomedical Research, Yongin-si 16924, Korea
8 AIGENDRUG Co., Ltd., Seoul 08826, Korea
* Correspondence: sunholee@aigendrug.com
† This paper is an extended version of our paper published in ICIBM 2022.

Simple Summary: Patient stratification is clinically important because it allows us to understand the characteristics and establish treatment strategies for a group. Transcriptomic data play an important role in determining molecular subtypes and predicting survival. In the case of breast cancer, although the order of prognosis according to molecular subtypes is well known, there is heterogeneity even within a subtype. Therefore, patient stratification considering both molecular subtypes and survival outcomes is required. In this study, a methodology to handle this problem is presented. A genetic algorithm is used to select a set of genes, and a risk score is assigned to each patient using their expression level. According to the risk score, patients are ordered and stratified considering molecular subtypes and survival outcomes. Consequently, informative genes for patient stratification with respect to both aspects could be nominated, and the usefulness of the risk score was shown through comparison with other indicators.

Abstract: Patient stratification is a clinically important task because it allows us to establish and develop efficient treatment strategies for particular groups of patients. Molecular subtypes have been successfully defined using transcriptomic profiles, and they are used effectively in clinical practice, e.g., PAM50 subtypes of breast cancer. Survival prediction contributed to understanding diseases and also identifying genes related to prognosis. It is desirable to stratify patients considering these two aspects simultaneously. However, there are no methods for patient stratification that consider molecular subtypes and survival outcomes at once. Here, we propose a methodology to deal with the problem. A genetic algorithm is used to select a gene set from transcriptome data, and their expression quantities are utilized to assign a risk score to each patient. The patients are ordered and stratified according to the score. A gene set was selected by our method on a breast cancer cohort (TCGA-BRCA), and we examined its clinical utility using an independent cohort (SCAN-B). In this experiment, our method was successful in stratifying patients with respect to both molecular subtype and survival outcome. We demonstrated that the orders of patients were consistent across repeated experiments, and prognostic genes were successfully nominated. Additionally, it was observed that the risk score can be used to evaluate the molecular aggressiveness of individual patients.

Keywords: patient stratification; molecular subtype; survival outcome; genetic algorithm; gene set selection

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6. Publication: Improved drug response prediction by drug target data integration via network-based profiling

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Problem Solving Protocol

Improved drug response prediction by drug target data integration via network-based profiling

Minwoo Pak[†], Sangseon Lee[†], Inyoung Sung, Bonil Koo and Sun Kim

Corresponding author: Sun Kim, Department of Computer Science and Engineering, Seoul National University, 1 Gwanak-ro, Gwanak-gu, 08826, Seoul, South Korea. Tel.: +82-2-880-7280; E-mail: sunkim.bioinfo@snu.ac.kr.

[†]Minwoo Pak and Sangseon Lee contributed equally to this work.

Abstract

Drug response prediction (DRP) is important for precision medicine to predict how a patient would react to a drug before administration. Existing studies take the cell line transcriptome data, and the chemical structure of drugs as input and predict drug response as IC50 or AUC values. Intuitively, use of drug target interaction (DTI) information can be useful for DRP. However, use of DTI is difficult because existing drug response database such as CCLE and GDSC do not have information about transcriptome after drug treatment. Although transcriptome after drug treatment is not available, if we can compute *the perturbation effects by the pharmacologic modulation of target gene*, we can utilize the DTI information in CCLE and GDSC. In this study, we proposed a framework that can improve existing deep learning-based DRP models by effectively utilizing drug target information. Our framework includes NetGP, a module to compute gene perturbation scores by the network propagation technique on a network. NetGP produces genes in a ranked list in terms of gene perturbation scores and the ranked genes are input to a multi-layer perceptron to generate a fixed dimension vector for the integration with existing DRP models. This integration is done in a model-agnostic way so that any existing DRP tool can be incorporated. As a result, our framework boosts the performance of existing DRP models, in 64 of 72 comparisons. The performance gains are larger especially for test scenarios with samples with unseen drugs by large margins up to 34% in Pearson's correlation coefficient.

Keywords: drug response, drug target, network propagation, deep learning

Introduction

The whole premise of precision medicine is to provide individualized treatment for patients. A recent study reported that only about 5% of the patients are actually benefiting from precision medicine [1]. This is largely due to the lack of data that is required to analyze variations in responses of individual patients to the same treatment [2, 3]. Since clinical data are often not publicly available due to privacy and ethical issues, most studies for drug response rely on pre-clinical data such as cell lines, patient-derived xenografts (PDX) or organoids. There are several large consortiums that provide large pre-clinical data such as CCLE [4], GDSC [5] and CTRP [6]. Now that such large amounts of molecular profiles of cell lines and diverse drug screening data are available, development of computational methods for individualized drug response prediction (DRP) is getting more important.

Related work

Recent studies show that it is possible to predict drug response with chemical structure and cell line gene expression data. [7] developed an interpretable DRP framework, DEERS, that uses two autoencoders, one for encoding cell line data and another for drug data. The latent vectors produced by each autoencoder contain interpretable information that represent biological processes associated with the drug response. SRDFM is a DRP framework developed by [8]. SRDFM utilizes a siamese network to predict relative drug response so that the model can recommend best-suited drugs for a cell line. One of the main components of SRDFM is a module called response unit, which adaptively weights important genes based on drug properties. DeepTTA presented by [9] predicts drug response using a transformer-based model [10] for encoding SMILES representation of a drug as well as a

Minwoo Pak is a Ph.D. student at the Department of Computer Science and Engineering, Seoul National University. His research interests include bioinformatics, network analysis, artificial intelligence and drug discovery.

Sangseon Lee is a postdoctoral fellow at Institute of Computer Technology, Seoul National University. He received a Ph.D. degree in Computer Science & Engineering from Seoul National University in 2020. His research area focuses on developing machine learning algorithms and deep learning models for analyzing graph-structured data and multi-omics data.

Inyoung Sung is a Ph.D. student at the Interdisciplinary Program in Bioinformatics, Seoul National University. She received a M.S. degree in the Department of Mathematical Science from Seoul National University in 2018. Her research interests focus on developing deep learning models for drug discovery and safety, and analyzing biological networks.

Bonil Koo is a Ph.D. student at the Interdisciplinary Program in Bioinformatics, Seoul National University. His research interests include artificial intelligence, multi-omics and drug discovery.

Sun Kim received the PhD degree in computer science from the University of Iowa, in 1997. He is currently a professor with the School of Computer Science and Engineering, Director of Bioinformatics Institute, and an affiliated faculty for the Interdisciplinary Program in Bioinformatics at Seoul National University. Before joining SNU, he was chair of Faculty Division C; director of Center for Bioinformatics Research, an associate professor in School of Informatics and Computing, Indiana University (IU) Bloomington. Prior to joining IU, in 2001, he worked at DuPont Central Research from 1998 to 2001, and with the University of Illinois at Urbana-Champaign from 1997 to 1998. His research interests include bioinformatics, computational biology, machine learning, and data mining. He is actively contributing to the bioinformatics community, serving on the editorial board for journals including editors for the METHODS Journal and the International Journal of Data Mining and Bioinformatics, associate editor-in-chief for the ACM/IEEE Transactions on Computational Biology and Bioinformatics, having served on the board of directors for ACM SIG Bioinformatics and for education for the IEEE Computer Society Technical Committee on Bioinformatics.

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6. Publication: Improved drug response prediction by drug target data integration via network-based profiling

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Article

Supervised chemical graph mining improves drug-induced liver injury prediction

Sangsoo Lim,[1] Youngkuk Kim,[2] Jeonghyeon Gu,[3] Sunho Lee,[4] Wonseok Shin,[2] and Sun Kim[2,3,4,5,*]

SUMMARY

Drug-induced liver injury (DILI) is the main cause of drug failure in clinical trials. The characterization of toxic compounds in terms of chemical structure is important because compounds can be metabolized to toxic substances in the liver. Traditional machine learning approaches have had limited success in predicting DILI, and emerging deep graph neural network (GNN) models are yet powerful enough to predict DILI. In this study, we developed a completely different approach, supervised subgraph mining (SSM), a strategy to mine explicit subgraph features by iteratively updating individual graph transitions to maximize DILI fidelity. Our method outperformed previous methods including state-of-the-art GNN tools in classifying DILI on two different datasets: DILIst and TDC-benchmark. We also combined the subgraph features by using SMARTS-based frequent structural pattern matching and associated them with drugs' ATC code.

INTRODUCTION

Drug-induced liver injury (DILI) has been the leading cause of drug withdrawal or the discontinuation of newly approved drugs from the market in the US since 1970.[1,2] Only 4.7% of drug candidates progress from preclinical trials to the clinical stage due to safety concerns.[1–3] Thus, DILI is a major hurdle in drug development. While direct DILI can be detected in preclinical/clinical studies in a dose-related manner, idiosyncratic DILI is detected only at an incidence rate of 0.1% or below during clinical studies.[2,4–6] What makes DILI identification even more difficult in clinical trials is the low concordance between DILI outcomes in animal and human models, with 63% and 43% in non-rodents and rodents, respectively.[2,7–9] Thus, a number of research projects have been launched to characterize DILI from multiple perspectives.

Evaluating DILI directly from small-molecule drugs in terms of clinical outcomes is very difficult, and it was necessary to design new approaches to fill in a large gap in how chemical structures translate to hepatotoxic risk in humans.[10] Thus, additional biochemical experiments are performed to explore the mechanism of DILI using high-throughput screening or pharmacogenomics[11,12] in projects designed to bridge the gap between structural information and the DILI outcome. Tox21 is a seminal project to broadly define the chemical toxicity of more than 10,000 chemical compounds.[13,14] Tox21 includes 14 major projects designed to improve our understanding of how chemical drugs reflect biochemical mechanisms and affect downstream pathways, and Tox21 data have been used for further investigation. Wu et al.[15] divided drug modes of action into 17 different assays to investigate whether a drug affects individual targets. This approach demonstrated how to integrate assay data into DILI prediction to improve our knowledge of the mechanistic details of DILI. Follow-up methods adopted a similar strategy by leveraging deep learning (DL) architecture[16] or gene expression profiles[17] to bridge the gap between chemical structure and DILI outcome using biological/biochemical measurements.

On the other hand, we also need to understand the toxicity of small-molecule drugs directly from the chemical structure perspective because projects such as Tox21 require careful experimental design and expert knowledge on how to integrate and interpret the vast amount of experimental data, which is expensive and time consuming. For more than 90% of orally administered drugs, the liver is the main site of structure-dependent metabolism. Thus, the detection of DILI at the compound structure level requires the identification of substructures of toxic drugs. A structural alert (SA) is a substructure of a compound that contributes to a specific chemical property and determines the metabolic process of a compound. Examples of SAs include functional groups such as aromatic amines, carboxylic acids, and benzene moieties. Some chemical moieties are reported to be related to chemical toxicity, such as arylacetic acid,

[1]Bioinformatics Institute, Seoul National University, Gwanak-ro 1, Seoul 08826, South Korea

[2]Department of Computer Science and Engineering, Seoul National University, Gwanak-ro 1, Seoul 08826, South Korea

[3]Interdisciplinary Program in Artificial Intelligence, Seoul National University, Gwanak-ro 1, Seoul 08826, South Korea

[4]AIGENDRUG Co., Ltd., Gwanak-ro 1, Seoul 08826, South Korea

[5]Lead contact

*Correspondence: sunkim.bioinfo@snu.ac.kr

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8. Publication: DRPreter: interpretable anticancer drug response prediction using knowledge-guided graph neural networks and transformer

International Journal of *Molecular Sciences*

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Article

DRPreter: Interpretable Anticancer Drug Response Prediction Using Knowledge-Guided Graph Neural Networks and Transformer

Jihye Shin [1,†], Yinhua Piao [2,†], Dongmin Bang [1,3], Sun Kim [1,2,4,5] and Kyuri Jo [6,*]

1 Interdisciplinary Program in Bioinformatics, Seoul National University, Seoul 08826, Korea
2 Department of Computer Science and Engineering, Institute of Engineering Research, Seoul National University, Seoul 08826, Korea
3 AIGENDRUG Co., Ltd., Seoul 08826, Korea
4 Interdisciplinary Program in Artificial Intelligence, Seoul National University, Seoul 08826, Korea
5 MOGAM Institute for Biomedical Research, Yongin-si 16924, Korea
6 Department of Computer Engineering, Chungbuk National University, Cheongju 28644, Korea
* Correspondence: kyurijo@chungbuk.ac.kr
† These authors contributed equally to this work.

Abstract: Some of the recent studies on drug sensitivity prediction have applied graph neural networks to leverage prior knowledge on the drug structure or gene network, and other studies have focused on the interpretability of the model to delineate the mechanism governing the drug response. However, it is crucial to make a prediction model that is both knowledge-guided and interpretable, so that the prediction accuracy is improved and practical use of the model can be enhanced. We propose an interpretable model called DRPreter (drug response predictor and interpreter) that predicts the anticancer drug response. DRPreter learns cell line and drug information with graph neural networks; the cell-line graph is further divided into multiple subgraphs with domain knowledge on biological pathways. A type-aware transformer in DRPreter helps detect relationships between pathways and a drug, highlighting important pathways that are involved in the drug response. Extensive experiments on the GDSC (Genomics of Drug Sensitivity and Cancer) dataset demonstrate that the proposed method outperforms state-of-the-art graph-based models for drug response prediction. In addition, DRPreter detected putative key genes and pathways for specific drug–cell-line pairs with supporting evidence in the literature, implying that our model can help interpret the mechanism of action of the drug.

Keywords: transcriptomics; artificial intelligence; pharmacogenomics; human health; cancer; drug sensitivity; graph neural networks; Explainable AI; precision medicine; drug discovery

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1. Introduction

The advances in technology and scientific capability enable the acquisition of large amounts of personal omics data at a reduced cost [1]. Consequently, there is a growing interest in using individualized health data for precision medicine, leading to a number of data-driven healthcare models [2]. Pharmacogenomics, one of the branches of precision medicine, is the study of how a person's genetic profile influences their response to medications [3,4]. Prediction of drug response or efficacy using the omics data of patients before the actual treatment is crucial because it can help increase clinical success and minimize adverse drug effects by modifying dosages or selecting alternative medications based on predicted value for personalized chemotherapy. However, obtaining patients' tumor tissues via surgical procedure or biopsy involves safety issues [5], and performing animal experiments for clinical trials to infer human drug efficacy leads to ethical and financial concerns [6]. In addition, even though correlating the drug response and omics data can help improve understanding the drugs' mechanisms of action [7], many candidate drugs

3 Study on creation of basis for next-generation toxicologic pathology diagnosis

Supervising division	Toxicological Research Division of NIFDS
Type	Entrusted project lead by Jae-Wooo Cho, KIT
Title	Creation of basis for next-generation toxicologic pathology diagnosis (20183MFDS411)

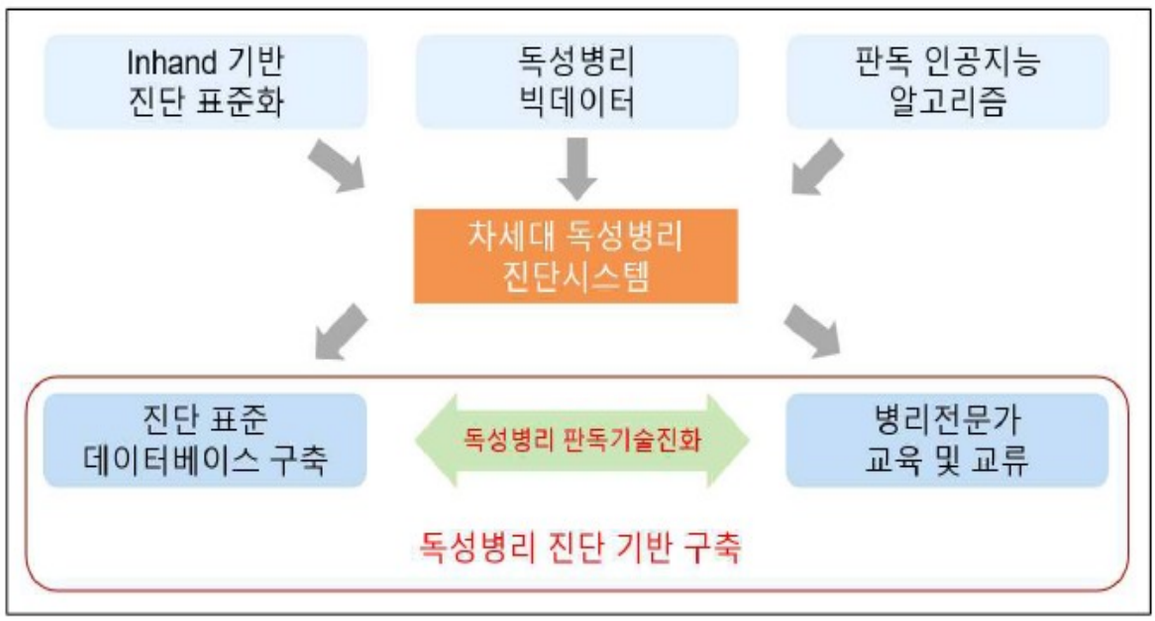
☐ Background for the study

- As new types of cancers are introduced to human body, AI-based pathology system that can rapidly and accurately diagnoses cancers have emerged.
  - With adoption of a digital pathology software, rapid and accurate diagnosis of human pathology data become possible.
- In toxicologic pathology that is used to make comprehensive toxicological decision in the non-clinical safety assessment, a standardized program for toxicologic pathology diagnosis using big data and AI is necessary.
  - With the emergence of the Industry 4.0, demand from research institute and industry for digital pathology technologies in non-clincal safety assessment has increased, and the need to build a digital system for pathology diagnosis led by the government has been increasing.

☐ Study in brief

- Collecting toxicologic pathology data relating to a digital pathology technology and creating big data
- Standardizing toxicologic pathology diagnosis on main organs
  - Diagnostic terminology of lesions in main organs (standardization of INHAND-based diagnostic terminologies and KNTP toxicologic pathology terminologies) were standardized.
  - Intensity of toxicologic pathology diagnosis was standardized.
- Developing and validating toxicologic pathology diagnosis technology based on digital pathology technology
  - Database for learning lesions in main organs was created and learning algorithm was created.
  - Learning algorithm for intensity of lesions was generated using AI: Toxicologic pathology diagnosis technology was developed.

- Final Goal



☐ Overview of the study outcome

- **Major accomplishment:** Establishment of basis for next-generation toxicologic pathology diagnosis by converging big data containing large image files of toxicologic pathology slides and AI.

- Summary of the outcome

(1) Creation of toxicologic pathology big data for digital pathology technology

- Existing pathology diagnosis models and technologies in Korea and in the world were investigated.

- Toxicologic pathology data were obtained and WSI (Whole Slide Image) archive was constructed.

  · data includes multi-site slides. The WSI archive is accessible on-line and utilized as education material for researchers in the related field.

- Data set for AI diagnosis was created.

  · Images of over 20 multi-site lesions in liver, kidney and lung have been obtained and over 10,000 data set has been created for each organ.

(2) Standardization of terminologies and intensity of toxicologic pathology diagnosis

- Terminologies for toxicologic pathology diagnosis were standardized.

· Terminologies for diagnosing lesions in main organs has been standardized based on INHAND

· Consultation group consisting of pathologists from GLP facilities was organized to create the system for validation of standard terminologies for diagnosis

· The standard terms has been disseminated and an education system created.

· The terms are available on KNTP as standard terminologies for toxicologic pathology

· Standardized terminologies for AI-based diagnosis system and the guideline for reading were provided.

(3) Development of toxicologic pathology diagnosis program

- Based on the database for learning lesions in liver, kidney and lung, the algorithms for learning the lesions and the intensity of the lesions by using AI were created and toxicologic pathology diagnosis program was developed.

- **Toxicologic pathology viewer was developed:** The viewer that supports management of tissue reading history was created and linked to auto-documentation of reading results and diagnosis standardization files. All these greatly enhance convenience of pathologists

☐ Excellence and advantage

● **Excellence**

- The AI-based system and toxicologic pathology diagnosis has been developed with our own technology.

- The AI model selected is appropriate for toxicologic pathology and exhibits over 90% of average accuracy.

  * Digital slide images can be analyzed by developing a program viewer equipped with AI technology

- Time and accuracy of diagnosis by pathologists have greatly improved thanks to automation using AI

● **Advantages**

- The nation's first AI-based toxicologic diagnosis (reading) program

- By upgrading the learning contents on the program format, the program can be expanded and customized program for individual research can be supplied.

- AI accuracy is expected to be improved by giving access of the toxicologic pathology data sets to the AI model researchers

☐ Utilization of the outcome and its impact

- Development of the nation's first AI-based toxicologic pathology diagnosis program will lead to development of the overall domestic industry.
- Enhanced diagnostic speed and accuracy of toxicologic pathology, an essential part of non-clinical study, will eventually improve the speed and accuracy of drug development.

☐ Performance of the study

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
1	Participated in 2023 KALAS winter symposium as an exhibitor	Promotion and dissemination of research outcome	Number of education and promotion materials about the outcome
2	Presentation: Possibility and reality of toxicologic pathology evaluation through AI model	Academic accomplishment	Number of presentations (publications) in national symposium
3	Toxicologic pathology diagnosis terminology explanation program	Intellectual property	Number of patent applications(In Korea)
4	Authomatic pathology screening program	Intellectual property	Number of patent applications(In Korea)
5	Region extraction method of medical image data	Intellectual property	Number of patent applications(In Korea)
6	Meeting to report research the outcome of the project on establishing basis for next generation toxicologic pathology diagnosis	Promotion and dissemination of research outcome	Number of education and promotion materials about the outcome
7	Publication: A comparative study for the implementation of deep learning algorithms to analyze hepatic necrosis in toxicity studies.	Academic accomplishment	Number of papers published in SCI-grade journals
8	Presentation: A comparative study of artificial intelligence analysis for diagnosis of liver fibrosis in rats	Academic accomplishment	Number of presentations (publications) in national symposium
9	Presentation: Artificial Intelligence(AI)-assisted Histopathology Analysis of Acetaminophen-induced Rat Liver Injury in Preclinical Study	Academic accomplishment	Number of presentations (publications) in national symposium
10	Publication: Artificial intelligence-assisted image analysis of acetaminophen-induced acute hepatic injury in Sprague-Dawley rats	Academic accomplishment	Number of papers published in SCI-grade journals
11	Presentation: Evaluation on seatosis using AI model learning steatosis in digital slide image	Academic accomplishment	Number of presentations (publications) in national symposium

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
12	Presentation: Hepatic steatosis screening analysis study applying artificial intelligence to rats	Academic accomplishment	Number of presentations (publications) in national symposium
13	Publication: Image analysis for acetaminophen (APAP)-induced liver necrosis in SD rat using classification algorithm	Academic accomplishment	Number of presentations (publications) in national symposium
14	Publication: Preparing pathological data to develop an artificial intelligence model in the nonclinical study	Academic accomplishment	Number of papers published in SCI-grade journals
15	Presentation: Segmentation algorithm can be used for detecting hepatic fibrosis in SD rat	Academic accomplishment	Number of presentations (publications) in national symposium
16	Presentation: Study on effect of staining condition in H&E slide on prediction performance of AI model	Academic accomplishment	Number of presentations (publications) in national symposium
17	Presentation: How to create lesion image data set for AI-based tissue slide reading	Academic accomplishment	Number of presentations (publications) in national symposium
18	Database for toxicologic pathology diagnosis terminology	Creation and utilization of DB	Creation of information basis (e.g. database)
19	Patent application: 10-2021-0072051_extraction method of medical image data	Intellectual property	Number of patent applications(In Korea)
20	Patent application: 10-2022-0049876_ region extraction method of medical image data	Intellectual property	Number of patent applications(In Korea)
21	Patent registration: 10-2383495_ extraction method of medical image data	Intellectual property	Number of patent applications(In Korea)
22	TPX 2022 Series EP2	Promotion and dissemination of research outcome	Number of education and promotion materials about the outcome
23	Publication: Implementation and Practice of Deep Learning-Based Instance Segmentation Algorithm for Quantification of Hepatic Fibrosis at Whole Slide Level in Sprague-Dawley Rats	Academic accomplishment	Number of papers published in SCI-grade journals
24	Presentation: Application of object detection deep learning algorithms for automated detection of infiltration of mononuclear cell in SD rat liver	Academic accomplishment	Number of presentations (publications) in national symposium
25	Presentation: Artificial intelligence-assisted histopathology analysis of APAP-induced rat liver injury in preclinical study	Academic accomplishment	Number of presentations (publications) in national symposium

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
26	Presentation: Automated detection using the deep-learning method of liver lesions induced by acetaminophen in Sprague-Dawley rats	Academic accomplishment	Number of presentations (publications) in national symposium
27	Presentation: Preliminary study for developing renal injury diagnosis system in SD rat using deep learning method	Academic accomplishment	Number of presentations (publications) in national symposium
28	Presentation: Quantification of hepatic fibrosis in SD rats using deep learning instance segmentation focused on H&E staining whole slide level	Academic accomplishment	Number of presentations (publications) in international symposium
29	Presentation: Segmentation of histology images of mononuclear cell infiltration in liver of SD rat through deep learning algorithms	Academic accomplishment	Number of presentations (publications) in national symposium
30	Presentation: Segmentation of histology images of the portal triad in liver of SD rat through deep learning algorithms	Academic accomplishment	Number of presentations (publications) in national symposium
31	Presentation: Deep Learning and the Application Trend in Veterinary Medicine Research	Academic accomplishment	Number of presentations (publications) in national symposium
32	Patent application: 10-2021-0072051_extraction method of medical image data	Academic accomplishment	Number of patent applications(In Korea)
33	Press release: Developing AI-based toxicity prediction technology	Promotion and dissemination of research outcome	Number of education and promotion materials about the outcome
34	Presentation: Current status and practice of toxicologic pathology evaluation using and artificial intelligence model.	Academic accomplishment	Number of presentations (publications) in national symposium
35	Presentation: Pixel level detection of rat liver fibrosis using Mask R-CNN	Academic accomplishment	Number of presentations (publications) in national symposium
36	Presentation: Research on a Proper Deep Learning Algorithm for Automated Detection of Coagulative Necrosis in SD Rat	Academic accomplishment	Number of presentations (publications) in national symposium

☐ Major outcome

7. Publication: A comparative study for the implementation of deep learning algorithms to analyze hepatic necrosis in toxicity studies.

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ORIGINAL ARTICLE

A comparative study on the implementation of deep learning algorithms for detection of hepatic necrosis in toxicity studies

Ji-Hee Hwang[1] · Minyoung Lim[1] · Gyeongjin Han[1] · Heejin Park[1] · Yong-Bum Kim[2] · Jinseok Park[3] · Sang-Yeop Jun[3] · Jaeku Lee[3] · Jae-Woo Cho[1]

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Abstract

Deep learning has recently become one of the most popular methods of image analysis. In non-clinical studies, several tissue slides are generated to investigate the toxicity of a test compound. These are converted into digital image data using a slide scanner, which is then studied by researchers to investigate abnormalities, and the deep learning method has been started to adopt in this study. However, comparative studies evaluating different deep learning algorithms for analyzing abnormal lesions are scarce. In this study, we applied three algorithms, SSD, Mask R-CNN, and DeepLabV3+, to detect hepatic necrosis in slide images and determine the best deep learning algorithm for analyzing abnormal lesions. We trained each algorithm on 5750 images and 5835 annotations of hepatic necrosis including validation and test, augmented with 500 image tiles of 448 × 448 pixels. Precision, recall, and accuracy were calculated for each algorithm based on the prediction results of 60 test images of 2688 × 2688 pixels. The two segmentation algorithms, DeepLabV3+ and Mask R-CNN, showed over 90% of accuracy (0.94 and 0.92, respectively), whereas SSD, an object detection algorithm, showed lower accuracy. The trained DeepLabV3+ outperformed all others in recall while also successfully separating hepatic necrosis from other features in the test images. It is important to localize and separate the abnormal lesion of interest from other features to investigate it on a slide level. Therefore, we suggest that segmentation algorithms are more appropriate than object detection algorithms for use in the pathological analysis of images in non-clinical studies.

Keywords Deep learning · Hepatic necrosis · Histopathology · Image analysis · Toxicology

Introduction

In recent years, artificial intelligence (AI) methods involving the use of convolutional neural networks (CNN), also known as deep learning algorithms, have been applied in various fields. Particularly in computer vision tasks, deep learning methods deconvolute the image content into thousands of prominent features and select or aggregate the most meaningful features to identify the complex characters of the image. This process shows high accuracy in image analysis and has, therefore, been actively applied to fields that use image data, such as medical imaging. Within this application, computational analysis of histopathology has recently shown significant advancement with the introduction of slide scanners. A slide scanner generates a whole-slide image (WSI) by combining multiple captured images of entire tissue sections on the slide. This procedure enabled the transition from classical pathology to digital pathology [1, 2] and has been applied to clinical as well as non-clinical studies. According to the Food and Drug Administration (FDA) guidelines, many tissue slides are generated to assess the toxicity of test compounds in non-clinical studies. For example, when following FDA guidelines in rodent subchronic toxicity tests, over 3,000 tissue slides are produced based on 40 different tissues from 20 animals of each sex in each treatment group, for the control and high-dose groups. [3, 4]. Therefore, several studies have attempted to adapt deep learning methods for

✉ Jae-Woo Cho
cjwoo@kitox.re.kr

1 Toxicologic Pathology Research Group, Department of Advanced Toxicology Research, Korea Institute of Toxicology, Daejeon 34114, Republic of Korea

2 Department of Advanced Toxicology Research, Korea Institute of Toxicology, Daejeon 34114, Republic of Korea

3 Research & Development Team, LAC Inc, Seoul 07807, Republic of Korea

10. Publication: Artificial intelligence-assisted image analysis of acetaminophen-induced acute hepatic injury in Sprague-Dawley rats

diagnostics

MDPI

Article

Artificial Intelligence-Assisted Image Analysis of Acetaminophen-Induced Acute Hepatic Injury in Sprague-Dawley Rats

Eun Bok Baek [1,†], Ji-Hee Hwang [2,†], Heejin Park [2], Byoung-Seok Lee [2], Hwa-Young Son [1], Yong-Bum Kim [3], Sang-Yeop Jun [4], Jun Her [4], Jaeku Lee [4] and Jae-Woo Cho [2,*]

1 College of Veterinary Medicine, Chungnam National University, Daejeon 34134, Korea; baekeunbok@hanmail.net (E.B.B.); hyson@cnu.ac.kr (H.-Y.S.)
2 Toxicologic Pathology Research Group, Department of Advanced Toxicology Research, Korea Institute of Toxicology, Daejeon 34114, Korea; jihee.hwang@kitox.re.kr (J.-H.H.); hpark@kitox.re.kr (H.P.); bslee@kitox.re.kr (B.-S.L.)
3 Department of Advanced Toxicology Research, Korea Institute of Toxicology, Daejeon 34114, Korea; ybkim@kitox.re.kr
4 Research & Development Team, LAC Inc., Seoul 07807, Korea; syjun@lacin.co.kr (S.-Y.J.); hjun@lacin.co.kr (J.H.); jklee@lacin.co.kr (J.L.)
* Correspondence: cjwoo@kitox.re.kr; Tel.: +82-42-610-8023
† These authors contributed equally to this work.

Abstract: Although drug-induced liver injury (DILI) is a major target of the pharmaceutical industry, we currently lack an efficient model for evaluating liver toxicity in the early stage of its development. Recent progress in artificial intelligence-based deep learning technology promises to improve the accuracy and robustness of current toxicity prediction models. Mask region-based CNN (Mask R-CNN) is a detection-based segmentation model that has been used for developing algorithms. In the present study, we applied a Mask R-CNN algorithm to detect and predict acute hepatic injury lesions induced by acetaminophen (APAP) in Sprague-Dawley rats. To accomplish this, we trained, validated, and tested the model for various hepatic lesions, including necrosis, inflammation, infiltration, and portal triad. We confirmed the model performance at the whole-slide image (WSI) level. The training, validating, and testing processes, which were performed using tile images, yielded an overall model accuracy of 96.44%. For confirmation, we compared the model's predictions for 25 WSIs at 20× magnification with annotated lesion areas determined by an accredited toxicologic pathologist. In individual WSIs, the expert-annotated lesion areas of necrosis, inflammation, and infiltration tended to be comparable with the values predicted by the algorithm. The overall predictions showed a high correlation with the annotated area. The R square values were 0.9953, 0.9610, and 0.9445 for necrosis, inflammation plus infiltration, and portal triad, respectively. The present study shows that the Mask R-CNN algorithm is a useful tool for detecting and predicting hepatic lesions in non-clinical studies. This new algorithm might be widely useful for predicting liver lesions in non-clinical and clinical settings.

Keywords: drug-induced liver injury; acute hepatic injury; deep neural network; mask region-based convolutional neural network; artificial intelligence; deep learning

Citation: Baek, E.B.; Hwang, J.-H.; Park, H.; Lee, B.-S.; Son, H.-Y.; Kim, Y.-B.; Jun, S.-Y.; Her, J.; Lee, J.; Cho, J.-W. Artificial Intelligence-Assisted Image Analysis of Acetaminophen-Induced Acute Hepatic Injury in Sprague-Dawley Rats. *Diagnostics* **2022**, *12*, 1478. https://doi.org/10.3390/diagnostics12061478

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1. Introduction

In recent years, artificial intelligence (AI)-assisted digital pathology has made rapid progress owing to the success of deep learning [1,2]. Some trials have applied deep-learning techniques in clinical and non-clinical fields of digital pathology, as they may be used to accomplish tasks that could not be automated using classical imaging analysis methods [3,4]. Deep-learning-based techniques are being increasingly applied in many

14. Publication: Preparing pathological data to develop an artificial intelligence model in the nonclinical study

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Preparing pathological data to develop an artificial intelligence model in the nonclinical study

Ji-Hee Hwang[1], Minyoung Lim[1], Gyeongjin Han[1], Heejin Park[1], Yong-Bum Kim[2], Jinseok Park[3], Sang-Yeop Jun[3], Jaeku Lee[3] & Jae-Woo Cho[1]✉

Artificial intelligence (AI)-based analysis has recently been adopted in the examination of histological slides via the digitization of glass slides using a digital scanner. In this study, we examined the effect of varying the staining color tone and magnification level of a dataset on the result of AI model prediction in hematoxylin and eosin stained whole slide images (WSIs). The WSIs of liver tissues with fibrosis were used as an example, and three different datasets (N20, B20, and B10) were prepared with different color tones and magnifications. Using these datasets, we built five models trained Mask R-CNN algorithm by a single or mixed dataset of N20, B20, and B10. We evaluated their model performance using the test dataset of three datasets. It was found that the models that were trained with mixed datasets (models B20/N20 and B10/B20), which consist of different color tones or magnifications, performed better than the single dataset trained models. Consequently, superior performance of the mixed models was obtained from the actual prediction results of the test images. We suggest that training the algorithm with various staining color tones and multi-scaled image datasets would be more optimized for consistent remarkable performance in predicting pathological lesions of interest.

Histopathological images include influential information referring to the cell anatomy and tissues of organisms, which can be crucial for the final decision procedure of effective therapeutics for diseases such as cancer[1,2]. Traditional pathological diagnosis is performed by observing the stained specimen on a glass slide using a microscope[3]. The development of whole-slide scanners has allowed for the digitization of histopathological images by generating whole-slide images (WSI), which have facilitated the pathologist's workflow through digital pathology[4]. In addition, a large number of WSIs can be accumulated, which accelerates the adaptation of digital image analysis methods to aid in pathology-related tasks, including diagnosis[5].

After the dissemination of WSIs, digital approaches to histopathological image analysis in digital pathology have focused primarily on low-level image analysis tasks, such as staining normalization, nuclear segmentation, and feature extraction, followed by the construction of classification models using classical machine learning methods[6]. As a result of low-level image analysis, computer-aided diagnosis (CAD) using histopathological images has become a standard clinical diagnostic procedure for cancer detection, and it is now one of the major stages in the histopathological imaging and diagnosis process[6]. The first stage of the diagnosis process is categorizing a WSI or multiple WSIs for a disease, which is essential for supervised learning tasks. The classification accuracy of the machine learning system is different from that of a human pathologist[7]; therefore, it can be improved using CAD and could prevent oversight by investigating all pixels within WSIs[8]. After categorizing the WSIs, the other diagnosis tasks are the detection or segmentation of regions of interest (ROI) such as the tumor region in WSI[9], scoring of immunostaining[9,10], cancer staging[7,11,12], mitosis detection[7,13], gland segmentation[14,15], and detection and quantification of vascular invasion[16]. These are the labeling stages of AI algorithm training. The performance of the supervised learning AI model is greatly affected by data preparation for training and testing, which could be the key to overcoming the obstacles to applying AI in pathological diagnosis[17].

There are various obstacles to overcome in preparation for training an AI algorithm with WSI of organ tissue, such as the large size of the image and insufficiently labeled images. Numerous researchers have attempted to solve these problems by increasing the efficiency of label data, utilizing weak labels or unlabeled information, or utilizing models/parameters for other tasks. However, the magnification and staining variation of the image are

[1]Toxicologic Pathology Research Group, Department of Advanced Toxicology Research, Korea Institute of Toxicology, Daejeon 34114, Korea. [2]Department of Advanced Toxicology Research, Korea Institute of Toxicology, Daejeon 34114, Korea. [3]Research and Development Team, LAC Inc., Seoul 07807, Korea. ✉email: cjwoo@kitox.re.kr

23. Publication: Implementation and Practice of Deep Learning–Based Instance Segmentation Algorithm for Quantification of Hepatic Fibrosis at Whole Slide Level in Sprague–Dawley Rats

Original Article

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Implementation and Practice of Deep Learning-Based Instance Segmentation Algorithm for Quantification of Hepatic Fibrosis at Whole Slide Level in Sprague-Dawley Rats

Ji-Hee Hwang[1], Hyun-Ji Kim[1,2], Heejin Park[1], Byoung-Seok Lee[1], Hwa-Young Son[2], Yong-Bum Kim[3], Sang-Yeop Jun[4], Jong-Hyun Park[4], Jaeku Lee[4], and Jae-Woo Cho[1]

Abstract

Exponential development in artificial intelligence or deep learning technology has resulted in more trials to systematically determine the pathological diagnoses using whole slide images (WSIs) in clinical and nonclinical studies. In this study, we applied Mask Regions with Convolution Neural Network (Mask R-CNN), a deep learning model that uses instance segmentation, to detect hepatic fibrosis induced by N-nitrosodimethylamine (NDMA) in Sprague-Dawley rats. From 51 WSIs, we collected 2011 cropped images with hepatic fibrosis annotations. Training and detection of hepatic fibrosis via artificial intelligence methods was performed using Tensorflow 2.1.0, powered by an NVIDIA 2080 Ti GPU. From the test process using tile images, 95% of model accuracy was verified. In addition, we validated the model to determine whether the predictions by the trained model can reflect the scoring system by the pathologists at the WSI level. The validation was conducted by comparing the model predictions in 18 WSIs at 20× and 10× magnifications with ground truth annotations and board-certified pathologists. Predictions at 20× showed a high correlation with ground truth (R^2 = 0.9660) and a good correlation with the average fibrosis rank by pathologists (R^2 = 0.8887). Therefore, the Mask R-CNN algorithm is a useful tool for detecting and quantifying pathological findings in nonclinical studies.

Keywords

deep learning, Mask R-CNN, liver fibrosis, NASH, steatohepatitis, NDMA, cirrhosis

Introduction

With the development of medical imaging techniques over the past few decades, research has been actively conducted on diagnosis and prediction in the clinical field, using the data derived from imaging.[1] Artificial intelligence (AI) methods, including traditional machine learning and deep learning, have offered opportunities to apply medical imaging data, such as radiological and histopathological data, in clinical prediction to reduce the human effort in diagnostics.[2] Since the advent of deep learning, which uses convolutional neural networks (CNNs), computer vision techniques have enabled breakthrough developments in accuracy, which could not be accomplished by traditional machine learning.[3,4]

There are 2 main tasks in image analysis where deep learning can be used. One is image classification, which treats each image as an identical category. The other is object detection, which refers to object localization and recognition. In addition to object detection, segmentation classifies the categories of images using pixel-level prediction.[5] Image segmentation has been widely used in the medical field, even before the advent of deep learning, using machine learning methods to detect and track medical instruments in surgical operations,[6] to analyze brains and their tumors from magnetic resonance imaging (MRI),[7] and to visualize colon crypts.[8] After the introduction of deep learning, the implementation of

[1] Toxicologic Pathology Research Group, Department of Advanced Toxicology Research, Korea Institute of Toxicology, Daejeon, Korea
[2] College of Veterinary Medicine, Chungnam National University, Daejeon, Korea
[3] Department of Advanced Toxicology Research, Korea Institute of Toxicology, Daejeon, Korea
[4] Research & Development Team, LAC Inc, Seoul, Korea

Corresponding Author:
Jae-Woo Cho, Korea Institute of Toxicology, 141 Gajeong-ro Yuseong-gu, Daejeon, Chungcheongnam-do 34114, Republic of Korea.
Email: cjwoo@kitox.re.kr

4 Survey on the latest technology trends of stem cells and organoid

Supervising division	Clinical Research Division of NIFDS
Type	Entrusted project (led by Prof. Si-woon Kim, Konkuk University)
Title	Study on utilization of stem cell-based translational medicine – focusing on stem cells and organoid (21182MFDS293)

☐ Background for the study

- Research on translational technology has been actively performed globally in order to better predict the reactions of human body to drugs in the process of drug development.
  - In order to address limitations of *in vivo* animal models and ethical issues related to animal experiment, many are attempt to assess pharmaceuticals's efficacy and safety using induced pluripotent stem cells (iPSCs) or organoid including 3D culture mimicking the structure and function of human organs and tissues.

☐ Study in brief

- ○ Survey and analysis of research trends of pharmaceutical evaluation using stem cells and organoid (translational technology)
  - Changes in national and international environment and megatrend (of R&D and industry) were analyzed.
  - Studies published in national and international academic journals were surveyed.
- Mid to long-term study plan on pharmaceutical evaluation technology using stem cells and organoid (translational technology) was established.
  - Based on analysis of SWOT and GAP, key strategies, mid to long-term research road-map and new projects were proposed.

☐ Overview of the study outcome

- **Major accomplishment:** Providing information on the most updated technology trends on the use of stem cells and organoid

- **Main outcome**
  - **Publication:** Providing the most updated information on the market trend of the advanced assessment technology including organoid, research and development, and related patent status in collaboration with KRIBB.
  - **Publication and promotion of casebook:** Providing the most updated information including the cases creating organoid specific to an organ/cancer type and and efficacy evaluation models using the organoid.
  - **Presentation in workshops:** Promoting and disseminating the research outcome by presenting in professional workshops.

☐ Excellence and advantage

- **Excellence:** Establishment of mid to long term research plan on pharmaceuticals efficacy and safety evaluation technology using advanced technologies.
- **Advantage:** Providing R&D strategy of an assessment system using organoid and the direction for future research (multi-site validation, need for establishment of technology infra structure including exper training, etc.)from the perspective of regulatory organization

☐ Utilization of the outcome and its impact

- The publications prepared in the study can be referred by drug developers and researchers when establishing a research plan and strategy for safety and efficacy evaluation of drugs.
- Also, it provides basis for developing and utilizing NAMs using iPSCs and organoid.

☐ Performance of the study

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
1	BioINdustry No. 160(2021-06) - Global market status and prospect of organoids	Promotion and dissemination of research outcome	publications
2	Publication of casebook of creation and utilization of organ-specific organoids and upload on the official website	Promotion and dissemination of research outcome	publications
3	Presentation: Establishment of advanced multi-organoid model based on functional characteristics	Building on expertise through academic accomplishment	Number of presentations (publications) in national workshops
4	Presentation: Generation of Human iPSCs Derived Heart Organoids Structurally and Functionally Similar to Heart	Building on expertise through academic accomplishment	Number of presentations (publications) in national workshops

5 Study on next-generation safety pharmacology evaluation of cardiovascular effects

Supervising division	Pharmacological Research Division of NIFDS
Type	Internal project
Title	Study on next-generation safety pharmacology evaluation of cardiovascular effects (21181MFDS276)

☐ Background for the study

- The paradigm of regulatory science of safety pharmacology evaluation has shifted to development of human-relevant models and the ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) also is adapting to the shift.
- Since inclusion of a new test method using a stem cell line in ICH S7B, a guideline for cardicovascular safety pharmacology, is currently being discussed, it is necessary to establish in Korea a next generation safety pharmacology technology for evaluation of cardiovascular effects.
- Therefore, the goal of this study was to establish next generation safety pharmacology technology for evaluation of cardiovascular effects using human hiPSC and to understand electrophygiology of cardiotoxicity.

☐ Study in brief

- Development of cardiotoxicity evaluation models for drugs causing deep vein thrombosis
  - Drugs causing deep vein thrombosis (high/intermediate/low risk) were selected.
  - Sensitivity of each drug in cells with cardiac ion channel expression and hiPSC-CMs ion channels was compared.
- Mechanism study on cardiotoxicity induction of durgs (test drugs) causing cardiotoxicity
  - Mechanism inhibiting cardiac ion channel was investigated.
  - Pathway effects related to cardiovascular system caused by test drugs were compared and analyzed.

☐ Overview of the study outcome

- *In vitro* safety pharmacology test methods for high/intermediate/low TdP-risk drugs on cardiovascular system
  - Development of a multiple cardiac ion channel test method using CiPA-based cell line
  - Development of a test method for repolarization delay using CiPA-based hiPSC-CMs
  - Development of a test method for cardiac action potential measurement using stem cells
- Study on a next-generation multiple cardiac ion channel test method using commercialized national hiPSC-CMs
  - Development of test methods for cardiac action potential and multiple cardiac ion channel using hiPSC-CMs
  - Similarity was observed from the test results using cell lines and hiPSC-CMs
- Cardiovascular safety pharmacology assay
  - Development of a test method for prediction of TdP risks based on the results from the test method for cardiac repolization delay

☐ Excellence and advantage

- Overcoming current limitations of cardiovascular safety pharmacology methods
  - It was demonstrated that the existing methods have limitations of low specificity in prediction of TdP induction risks due to their dependency on hERG channel.
  - In this study, a cardiovascular safety pharmacology method for high/intermediate/low TbP risk drugs was developed, which would help overcome some of the limitations of the existing methods with higher accuracy and reliability.
- Proposal of the need for multiple cardiac ion channel evaluation
  - This study has proposed the need for novel methods for multiple cardiac ion channel evaluation to assess the effects of drugs.
- Utilization of an hiPSC-CMs model
  - This study demonstrated the possibility of various applications of hiPSC-CMs as human-relevant models and reliability and accuracy compared to existing *in vitro* cell culture models

☐ Utilization of the outcome and its impact

- Responding to the updated ICH (S7B) that can replace hERG assay and telemetry
  - This study has contributed to the revision of ICH (S7B) by developing and proposing novel methods for cardiovascular safety pharmacology that are able to replace hERG assay and telemetry.
- Inclusion of various ion channel evaluation
  - While the existing method rely on hERG channel, the methods developed in this study are able to conduct more sophisticated evaluation by including various ion channels including action potential measurement of calcium and sodium channel as well as potassium channel (hERG).
- Utilization of hiPSC-derived cardiomyocytes
  - This study demonstrated the reliability and usefulness of the new method by developing human-relevant cardiovascular model using hiPSC-derived cardiomyocyte models
- Provision of scientific basis for prediction of adverse drug reaction
  - This study has contributed to pharmaceutical development and safety evaluation by providing scientific basis for prediction of adverse drug reaction using a new cardiovascular safety pharmacology method.

☐ Performance of the research

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
1	New *in vitro* multiple cardiac ion channel screening system for preclinical Torsades de Pointes risk prediction under the Comprehensive *in vitro* Proarrhythmia Assay concepta	academic outcome	Number of papers published in SCI-grade journals
2	*In vitro* cardiac safety and pharmacology assessment of the 3 drugs categorized as TdP low risk, intermediate risk and high risk	academic outcome	Number of papers published in non-SCI-grade journals
3	Assessment of Drug Proarrhythmic Potential in Human iPSC-Dereived Cardiomyocytes using Microelectrode Arrays (Korean Society for Stem Cell Research)	academic outcome	Number of publications and presentations in national workshops

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
4	*In vitro* cardiac safety and pharmacology assessment of the 3 drugs categorized as TdP low risk, intermediate risk and high risk (Korean Society for Stem Cell Research)	academic outcome	Number of publications and presentations in national workshops
5	*In vitro* electrophysiological assessment for proarrhythmia risk prediction under CiPA initiative (Korean Physiological Society)	academic outcome	Number of publications and presentations in national workshops
6	Cardiotoxicity Screening for Proarrhythmic Potential of Drugs in Human iPSC-Dereived Cardiomyocytes using Microelectrode Arrays (Korea Society of Toxicology)	academic outcome	Number of publications and presentations in national workshops
7	A Novel Approach in the Area of Preclinical Cardiac Safety Testing via the CiPA Concept(Korea Society of Toxicology)	academic outcome	Number of publications and presentations in national workshops
8	Outstanding poster award: A Novel Approach in the Area of Preclinical Cardiac Safety Testing via the CiPA Concept (awarded by Korea Society of Toxicology)	social assessment	Number of educational and promotion materials about the study outcome (e.g. social assessment, rewards, etc.)
9	Guidance on cardiovascular safety pharmacology assessment methods	Promotion and dissemination of the study outcome	Number of educational and promotion materials about the study outcome
10	Q&A book on comprehensive deep vein thrombosis evaluation	Promotion and dissemination of the study outcome	Number of educational and promotion materials about the study outcome

☐ Major accomplishment

1. Publication: New *in vitro* multiple cardiac ion channel screening system for preclinical Torsades de Pointes risk prediction under the Comprehensive *in vitro* Proarrhythmia Assay concepta

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KJPP

Original Article

New *in vitro* multiple cardiac ion channel screening system for preclinical Torsades de Pointes risk prediction under the Comprehensive *in vitro* Proarrhythmia Assay concepta

Jin Ryeol An[1], Seo-Yeong Mun[2], In Kyo Jung[1], Kwan Soo Kim[1], Chan Hyeok Kwon[1], Sun Ok Choi[1,*], and Won Sun Park[2,*]

[1]*Pharmacological Research Division, Toxicological Evaluation and Research Department, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Cheongju 28159,* [2]*Department of Physiology, Kangwon National University School of Medicine, Chuncheon 24341, Korea*

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***Correspondence**
Sun Ok Choi
E-mail: sochoi@korea.kr
Won Sun Park
E-mail: parkws@kangwon.ac.kr

Key Words
Ion channels
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Safety
Stem cell
Torsades de pointes

ABSTRACT Cardiotoxicity, particularly drug-induced Torsades de Pointes (TdP), is a concern in drug safety assessment. The recent establishment of human induced pluripotent stem cell-derived cardiomyocytes (human iPSC-CMs) has become an attractive human-based platform for predicting cardiotoxicity. Moreover, electrophysiological assessment of multiple cardiac ion channel blocks is emerging as an important parameter to recapitulate proarrhythmic cardiotoxicity. Therefore, we aimed to establish a novel *in vitro* multiple cardiac ion channel screening-based method using human iPSC-CMs to predict the drug-induced arrhythmogenic risk. To explain the cellular mechanisms underlying the cardiotoxicity of three representative TdP high- (sotalol), intermediate- (chlorpromazine), and low-risk (mexiletine) drugs, and their effects on the cardiac action potential (AP) waveform and voltage-gated ion channels were explored using human iPSC-CMs. In a proof-of-principle experiment, we investigated the effects of cardioactive channel inhibitors on the electrophysiological profile of human iPSC-CMs before evaluating the cardiotoxicity of these drugs. In human iPSC-CMs, sotalol prolonged the AP duration and reduced the total amplitude (TA) *via* selective inhibition of I_{Kr} and I_{Na} currents, which are associated with an increased risk of ventricular tachycardia TdP. In contrast, chlorpromazine did not affect the TA; however, it slightly increased AP duration *via* balanced inhibition of I_{Kr} and I_{Ca} currents. Moreover, mexiletine did not affect the TA, yet slightly reduced the AP duration *via* dominant inhibition of I_{Ca} currents, which are associated with a decreased risk of ventricular tachycardia TdP. Based on these results, we suggest that human iPSC-CMs can be extended to other preclinical protocols and can supplement drug safety assessments.

INTRODUCTION

In the 1990s to early 2000s, eight non-cardiac drugs were withdrawn from the market because of their association with Torsade de Pointes (TdP), a potentially life-threatening ventricular arrhythmia condition [1]. Up to 90% of new compounds that pass preclinical testing fail at the clinical trial phase, with cardiotoxicity accounting for the failure of 45% of the compounds [2]. Hence, the International Conference for Harmonization (ICH) presented the regulatory guidelines S7B and E14 in 2005, which focuses on two markers to assess TdP risk: *in vitro* inhibition of a single human Ether-à-go-go-Related Gene (hERG) potassium channel (representing the rapidly activating delayed rectifier potassium current, or I_{Kr}) and prolongation of the heart rate corrected QT

Author contributions: J.R.A. conceptualization, writing – original draft, data collection, data analysis. S.Y.M. and I.K.J. data analysis and interpretation. K.S.K. drafting the manuscript. C.H.K. critical revision of the paper. S.O.C. and W.S.P. conceptualization, funding acquisition, and final approval of the completed manuscript.

2. Publication: *In vitro* cardiac safety and pharmacology assessment of the 3 drugs categorized as TdP low risk, intermediate risk and high risk

Original Articles

Journal of Alternatives to Animal Experiments
16(1), December, 2022:15-24

TdP 고·중·저 위험군 3종에 대한 in vitro 심혈관계 안전성약리 평가

안진렬[1], 정인교[1], 박성혜, 김관수, 권찬혁, 최선옥*

식품의약품안전평가원 독성평가연구부 약리연구과

In vitro Cardiac Safety and Pharmacology Assessment of the 3 Drugs Categorized as TdP Low Risk, Intermediate Risk and High Risk

Jin Ryeol An[1], In Kyo Jung[1], Seong Hye Park, Kwan Soo Kim, Chan Hyeok Kwon, Sun Ok Choi*

Pharmacological Research Division, Toxicological Evaluation and Research Department, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety

ABSTRACT. The Comprehensive in vitro Proarrhythmia Assay (CiPA) project has been recently proposed to assess the proarrhythmic risk on multiple cardiac ion channels in the development of new drugs. Due to be based solely on in vitro hERG channel block and in vivo QT prolongation, the current ICH S7B nonclinical testing strategy is imperfect and used to be mis-identified for an evaluation of drug-associated torsade de pointes (TdP) risk. The inhibition of hERG channels induces QT prolongation and even generate TdP, while the balanced block of other cardiac ion channels such as Nav1.5 sodium channels and Cav1.2 calcium channels reduces QT prolongation or TdP occurrence. From this reasons, we investigated the drug effects on Nav1.5 channels and Cav1.2 channels as well as hERG channels. First, we examined the effect of the 3 CiPA training set drugs categorized as proarrhythmic low risk (mexiletine), intermediate risk (chloropromazine) and high risk (sotalol) on three cardiac ion channels. Using the manual whole-cell patch clamp technique, each cardiac ion currents were measured in cell lines expressing hERG, Nav1.5, and Cav1.2 channels, respectively. In addition, field potential duration (FPD) in human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) were recorded by performing microelectrode array (MEA) to confirm the integrative channel effect of those drugs. As a result, all of sotalol, chloropromazine and mexiletine blocked hERG channels, whereas chloropromazine and mexiletine inhibited Cav1.2 calcium currents more than sotalol, expecting the reduction of QT prolongation and TdP occurrence via balanced ion channel block by chloropromazine and mexiletine. Corresponding to these results, furthermore, sotalol induced more prolonged FPD in hiPSC-CMs as compared to chloropromazine and mexiletine. Thus, we suggest that the optimization and development of new in vitro cardiac safety and pharmacology test strategy on multiple cardiac ion channels to comprehensively predict proarrhythmic risk.

KEY WORDS: CiPA initiative, TdP, in vitro multiple ion channel assay, MEA, hiPSC-CMs

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* These authors contributed equally to this work.
** Corresponding author: Sun Ok Choi, Ph.D.
187 Osong Saengmyeong 2-ro, Heungdeok-gu,
Chungju 28159 Korea
Pharmacological Research Division, Toxicological Evaluation and Research Department, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety

Tel: 82-43-719-5210
E-mail: sochoi@korea.kr

6 Study on next-generation neurotoxicity evaluation method

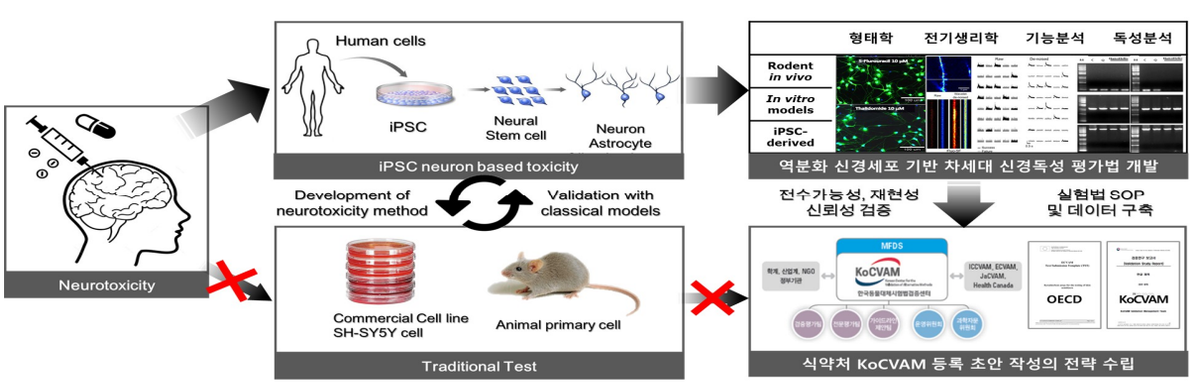
Supervising division	Pharmacology Research Divison of NIFDS
Type	Entrusted project
Title	Study on next-generation neurotixicity evaluation method (/) (19182MFDS408)

☐ Background for the study

- Recently, drug abuse cases are increasing and drug users are becoming younger and more diverse, which become a serious social issue.
  - In addition, drug abuse cases are expected to increase since new narcotics are continuously introduced and the society is becoming more complex and diverse.
- There is no neurotoxicity test method for neuroterapeutic drugs targeting adult nervous system instead of developmental stage in the world.
  - Although a safety pharmacology test of the central nervous system do exist, it remains at the level of behavioral evaluation analysis using rodents (Functional Observation Battery: FOD, Modified Irwin's)

☐ Study in brief

- Establishment of human-mimicking neuron test system* using hiPSC
  * neurons, astrocytes, and microglia
- Development and validation of neurotoxicity test method using the established neurotoxicity test system.



〈Concept mapping of the study on development of next-generation neurotoxicity test method based on iPSC-derived neurons〉

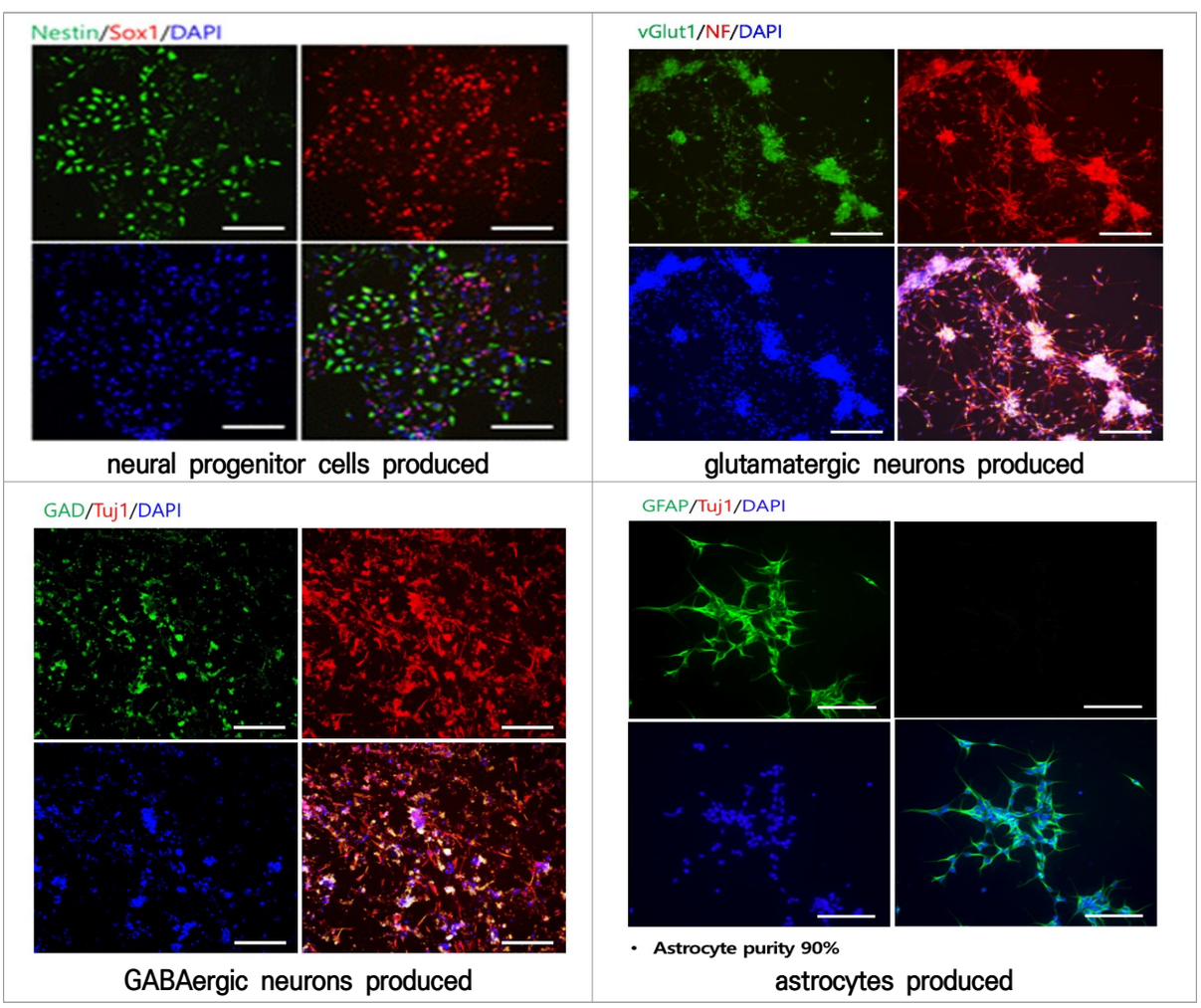
- Establishment of neurotoxicity analysis method based on morphology, electrophysiology, nerve function and apoptosis.
- Development of drug abuse test method for mid to long term exposure to drugs
  * 30 substances including reference drugs and narcotics were evaluated for toxicity

● Establishment of a platform system that can be used to encourage practical use of stem cell-based toxicity evalaution methods.

☐ Overview of the study outcome

● Establishment of hiPSCs derived from human fibroblast

- A differentiation method for stable and homogenous neuronal system has been established.
  * neural progenitor cells, glutamatergic neurons, GABAergic neurons, motor, dopaminergic neurons, and astrocytes



- Development of drug abuse evaluation method for middle to long term exposure to drugs
  * Development and validation of a cell line using for drug evaluation using a multi-electrode assay

- Establishment of neurotoxicity analysis method for toxicity evaluation based on morphology, reactive oxygen, apoptosis, and electrophysiology
  - 30 substances including reference drugs and narcotics were evaluated for toxicity
    * Preparation of SOP for toxicity evaluation on central nervous system

☐ Excellence and advantage

- Utilization of human-mimicking neuron test system established using iSPC-derived stem cells
  - The differentiation method for iSPC-derived neurons that is highly functional and efficient can be utilized as efficacy evaluation method in drug envelopment.
  - The 3D cell culture plaform can be utilized as a new cell culture technology, and in production of medical devices.

☐ Utilization of the outcome and its impact

- Publication of guidance on neurotoxicity test method using stem cells
  - Accurate prediction of human body response to drugs can reduce failure of new drug development caused by toxicity, and can be used in central nervous system safety pharmacology which is an integral part in a non-clinical study.
    * While existing methods eevaluate toxicity inducing neuronal cell apoptosis, this method evaluates electrophysiological changes and receptor abnormality of neurons that will be followed by real neurological symptom.
- Provision of scientific basis for regulation and safety management policy of substances of abuse risk including narcotic drugs
  - While there is no reliable evaluation method for drug abuse, this study proposes a standard for abuse evaluation based on human-mimicking neurons.

☐ Performance of the study

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicator
1	Publication: BAPTA, a calcium chelator, neuroprotects injured neurons *in vitro* and promotes motor recovery after spinal cord transection *in vivo*	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journal
2	Publication: Development and validation of dual-cardiotoxicity evaluation method based on analysis of field potential and contractile force of human iPSC-derived cardiomyocytes/multielectrode assay platform	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journal

☐ Major accomplishment

1. Publication: BAPTA, a calcium chelator, neuroprotects injured neurons *in vitro* and promotes motor recovery after spinal cord transection *in vivo*

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ORIGINAL ARTICLE

CNS Neuroscience & Therapeutics WILEY

BAPTA, a calcium chelator, neuroprotects injured neurons in vitro and promotes motor recovery after spinal cord transection in vivo

Kyu-ree Kang[1] | Jin Kim[2] | Bokyeong Ryu[2] | Seul-Gi Lee[1] | Min-Seok Oh[1] | Jieun Baek[1] | Xiaoping Ren[3,4] | Sergio Canavero[4,5] | C-Yoon Kim[1,4,6] | Hyung Min Chung[1]

[1]Department of Stem Cell Biology, School of Medicine, Konkuk University, Seoul, Korea

[2]Laboratory Animal Medicine, College of Veterinary Medicine, Seoul National University, Seoul, Korea

[3]Department of Orthopedics, Ruikang Hospital, Nanning, China

[4]GICUP-Global Initiative to Cure Paralysis, Columbus, Ohio, USA

[5]HEAVEN/GEMINI International Collaborative Group, Turin, Italy

[6]Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul, Korea

Correspondence
C-Yoon Kim, PhD, Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul, Korea.
Email: vivavets@gmail.com

Hyung Min Chung, PhD, Department of Stem Cell Biology, School of Medicine, Konkuk University, 120 Neungdong-Ro, Gwangjin-Gu, Seoul 143-701, Korea.
Email: hmchung@kku.ac.kr

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Abstract

Aim: Despite animal evidence of a role of calcium in the pathogenesis of spinal cord injury, several studies conducted in the past found calcium blockade ineffective. However, those studies involved oral or parenteral administration of Ca++ antagonists. We hypothesized that Ca++ blockade might be effective with local/immediate application (LIA) at the time of neural injury.

Methods: In this study, we assessed the effects of LIA of BAPTA (1,2-bis (o-aminophenoxy) ethane-N, N, N′, N′-tetraacetic acid), a cell-permeable highly selective Ca++ chelator, after spinal cord transection (SCT) in mice over 4 weeks. Effects of BAPTA were assessed behaviorally and with immunohistochemistry. Concurrently, BAPTA was submitted for the first time to multimodality assessment in an in vitro model of neural damage as a possible spinal neuroprotectant.

Results: We demonstrate that BAPTA alleviates neuronal apoptosis caused by physical damage by inhibition of neuronal apoptosis and reactive oxygen species (ROS) generation. This translates to enhanced preservation of electrophysiological function and superior behavioral recovery.

Conclusion: This study shows for the first time that local/immediate application of Ca++ chelator BAPTA is strongly neuroprotective after severe spinal cord injury.

KEYWORDS
calcium, locomotor recovery, neuronal apoptosis, oxidative stress, spinal cord injury

Kyu-ree Kang and Jin Kim contributed equally to this work as first authors, respectively.

2. Publication: Development and validation of dual-cardiotoxicity evaluation method based on analysis of field potential and contractile force of human iPSC-derived cardiomyocytes/multielectrode assay platform

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Development and validation of dual-cardiotoxicity evaluation method based on analysis of field potential and contractile force of human iPSC-derived cardiomyocytes / multielectrode assay platform

Seul-Gi Lee [a, 1], Jin Kim [b, 1], Min-Seok Oh [a], Bokyeong Ryu [b], Kyu-Ree Kang [a], Jieun Baek [a], Jin-Moo Lee [d], Sun-Ok Choi [d], C-Yoon Kim [c, *], Hyung Min Chung [a, **]

[a] Department of Stem Cell Biology, School of Medicine, Konkuk University, 120 Neungdong-Ro, Gwangjin-Gu, Seoul, 143-701, Republic of Korea
[b] Department of Laboratory Animal Medicine, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea
[c] College of Veterinary Medicine, Konkuk University, Seoul, 05029, Republic of Korea
[d] Pharmacological Research Division, Toxicological Evaluation and Research Department, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Republic of Korea

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ABSTRACT

A recent in vitro cardiovascular safety pharmacology test uses cardiomyocytes derived from human induced pluripotent stem cells (hiPSCs) to overcome the limitations of the classical test systems, such as species differences and local channel analysis. The Comprehensive in vitro Proarrhythmia Assay (CiPA) is a new proarrhythmia screening paradigm proposed by a CiPA steering expert group, which essentially requires iPSCs derived cardiomyocyte-based electrophysiological evaluation technology. Moreover, the measurement of the contractile force is also emerging as an important parameter to recapitulate non-proarrhythmic cardiotoxicity. Therefore, we constructed an multielectrode assay (MEA) evaluation method that can measure the electrophysiological changes with 6 reference drugs in hiPSC-derived cardiomyocytes. Subsequently, it was confirmed that the electrophysiological were changed in accordance with the mechanism of action of the drugs. Furthermore, based on the multi-probe impedance, we confirmed the decrease in contractile force due to treatment with drugs, and developed a platform to evaluate cardiotoxicity according to drugs along with field potential changes. Our excitation–contraction coupling cardiotoxicity assessment is considered to be more supportive in cardiac safety studies on pharmacologic sensitivity by complementing each assessment parameter.

1. Introduction

For the development of new drugs, the process of identifying adverse drug reactions (ADRs) is essential, and many potential candidate drugs fail because of their side effects. Cardiotoxicity is one of the major causes of drug withdrawal from the market and more than 2000 post-market drugs, including anticancer drugs and antibiotics on the market, were found to have cardiac ADRs [1]. 14% of the 462 drugs withdrawn from the market between 1953 and 2013 were responsible for serious cardiovascular side effects [2]. In particular, not only an acute effect but also a chronic effect with delayed onset may occur in the case of cardiotoxicity. Therefore, even after passing through preclinical screening, toxicity may be confirmed in phase 2 and phase 3 clinical trials or after being released into the market [3]. Therefore, evaluating potential cardiac toxicity is an important parameter in the drug market.

In this context, the International Conference for Harmonization (ICH) presented guidelines S7B and E14 in 2005. ICH S7B, a guideline for evaluating nonclinical cardiac toxicity, is based on ventricular repolarization (QT interval prolongation) in cell lines transfected with human Ether-à-go-go-Related Gene (hERG) or in cardiomyocytes (CMs) isolated from animals or humans. ICH E1T, a clinical trial guideline, analyzes the thorough QT/QTc study to evaluate the arrhythmia-inducing effects of non-antiarrhythmic drugs [4]. By pre-screening through the application of the above

* Corresponding author.
** Corresponding author.
E-mail addresses: vivavets@gmail.com (C.-Y. Kim), hmchung@kku.ac.kr (H.M. Chung).
[1] These authors contributed equally to this work as first and corresponding authors, respectively.

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0006-291X/

7 Study on development of a method for evaluation of (new) narcotics pharmacology and toxicity in central nervous system

Supervising division	Pharmacological Research Division of NIFDS
Type	Entrusted project
Title	Study on development of a method for evaluation of (new) narcotics pharmacology and toxicity in central nervous system (20182MFDS423)

☐ Background for the study

- As the number of new narcotics introduced into Korea is increasing, a regular system for hazard evaluation should be established in Korea. Further, the evaluation system should meet the national regulations (three years of designation period as) temporary narcotics) and the requirements by the UN and other advanced countries for designation of narcotics.
- Neurotoxicity or dependency evaluation performed on animals has a limitation of requiring a lot of samples, and thus a new assay for evaluation of toxicity and pharmacological mechanism should be developed to overcome the limitation.

☐ Study in brief

- Establishment of a new assay for evaluation of new narcotics of central nervous system actions and dependency/abuse potential
  - An electrophysiological measurement method using mouse tissues and neurons derived from human stem cells, and a method for analyzing binding force and function of mitochondria to narcotic receptors have been developed.
- Establishment of new dependency test methods by utilizing various electrical measurement methods on the existing neural cells and circuits
  - An electrical measurement method using rat-derived primitive neurons has been established.
  - An electrical measurement method using hippocampal slices and nucleus accumbens slices has been established.
- Establishment of stem cell-derived neurons for evaluation central nervous system reactions to narcotics.
  - Human stem cell-based cell lines for evaluation narcotics on central nervous system actions have been established.

- Human stem cell-based cell lines for evaluation of neurotoxicity/ dependency have been established.

- Establishment of methods for prediction of narcotic receptor binding affinity
  - Non-radioactive receptor binding assay using Surface Plasmon Resonance (SPR) has been established.
  - Narcotic receptor binding affinity is evaluated using SPR.
- Development of new test methods using analysis of mitochondria function
  - The effect of narcotics on central nervous system is evaluated by analyzing mitochondria function.
  - Neurotoixicity and (or) dependency of narcotics are evaluated by analyzing mitochondria function.

☐ Overview of the study outcome

- The following pharmacology and toxicity test method of new narcotics on central nervous system using neurons have been established: a new narcotics central neurotoxicity and dependency test method using synapse; a novel test method based on mitochondria function; and a novel SPR-based test method.
  - SPR (surface plasmon resonance) evaluation method for measurement of new narcotics receptor binding affinity
    - · The method evaluates hazard of new narcotics by assessing their binding affinity to receptors using SPR.
  - Mitochondria function test method neurons derived from human pluripotent stem cells
    - · The method evaluates safety on new narcotics on neurons by assessing their effect on the function of mitochondria in neurons derived from human pluripotent stem cells (Glycolytic ATP production and Mitochondrial ATP production).
  - Neuron activation level measurement method
    - · The method analyzes activation level of nuerons by confirming neuron excitation based on the action potential measured by changes in membrane potential of Na+ and K+ ion channels.
  - Method for measuring neurotransmitter release
    - · The method measures neurotransmitter glutamate release by analyzing membrane potential changes of neuron synapse channels (NMDAR, AMPAR, and GABAR)

- Method for measuring disruption of dopamine receptor

  · A sensor (GFAP104-GRABda2m) for measuring dopamine has been introduced to investigate changes in GFP (green fluorescent protein) that incur during interaction between dopamine and dopamin receptor.

- Method for evaluating presynapse release using paired pulsed ratio

  · The method confirms inhibition of neurotransmitter release by new narcotics by analyzing paired pulsed ratio

- *In vitro* dependency test method

  · Dependency of new narcotics is evaluated by observing LTP (long term potentiation) that regulates memory and learning. In addition, existence of dependency is determined by observing increase in synapse AMPA receptor expression, a major mechanism of LTP.

- New narcotics test method using a synapse channel

  · The method evaluates the effect of new narcotics on neurons by analyzing disruption level of synapse channel based on glutamate release measurement.

☐ Excellence and advantage

- **Academic accomplishment:** publication of papaers with 90% mrnIF

  - The study provides the scientific basis and objectivity of MFDS safety management skills and standard specifications by publishing high quality papers (2 papers with 94% and 80% mrnIF each)

    * Digital selective transformation and patterning of highly conductive hydrogel bioelectronics by laser-induced phase separation. SCIENCE ADVANCES. 2022. 8(23).

    * An Octopus-derived pepetide with antidiuretic activity in rats. Marine drugs. 2022. 20.

- **SOP:** While there is lack of research on neurotoxicity test methods for new narcotics and narcotics, this study provides research basis to research institutes or laboratories that newly perform neurotoxicity studies by establishing related test methods and preparing SOPs.

- **Action mechanism:** This study has become the first in the world to reveal the action mechanism by which four temporary narcotics (4-EA-NBOMe, LY-2183240, DCMP, AB-CHGFUPYCA) induce neurotoxicity.

- For the temporary narcotics that cannot be designated as narcotics based the the results from the existing test method (rodent behavioral test), their neurotoxicity test results

obtained in this study has been utilized to prepare technical report that provides scientific basis for revision of Narcotics Control Act.

- This study provides a neuron-based test method for temporary narcotics that are difficult to be tested by the traditional dependency test methods focused on behavioral test including 4-EA-NBOMe, and LY-2183240

☐ Utilization of the outcome and its impact

- **Utilization of the outcome**
  - The data generated in this study is utilized as scientific basis for hazard evaluation of new narcotics for designation of them as (temporary) narcotics.
  - The basis that can be used in preparation of technical report for designation of temporary narcotics (amendment to the enforcement decree of the Narcotics Control Act) has been obtained.
- **Impact of the outcome**
  - The data generated in this study are utilized as raw data for evaluation of neurotoxicity and dependency of narcotics. It is used as base data for the Narcotics Control Act and contributes to public safety management.
  - The SOPs on neuron-based toxicity test methods prepared in this study would help research institutes or laboratories that newly adopt narcotics neurotoxicity test methods.
- **Demonstration of excellence and reliability of MFDSs safety assessment methods**
  - The influx of new narcotics into Korea has emerged as a serious social problem, amplifying public anxiety. Against this backdrop, this study provides preemptive measures to safely and scientifically evaluate the new narcotics, which would respond to the needs of the people and increase the public trust to the government agency.
  - Since the 2000s, stem cells have been constantly attracting public attention with new science and technology. Announcement of practical research using stem cells is expected to contribute to increasing public trust to the MFDS while promoting the excellence of the Ministry's research capabilities.

☐ Performance of the study

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
1	Digital selective transformation and patterning of highly conductive hydrogel bioelectronics by laser-induced phase separation.	Building on expertise through academic accomplishment	Number of papered published in SCI-grade journal
2	Establishment of Neurotoxicity Assessment Using Microelectrode Array (MEA) with hiPSC-Derived Neurons and Evaluation of New Psychoactive Substances (NPS).	Building on expertise through academic accomplishment	Number of papered published in SCI-grade journal
3	Acute effects of 4-EA-NBOMe, 3, 4-DCMP, LY-2183240 and AB-CHFUPYCA on the excitability of rat cortical primary cultured neurons.	Building on expertise through academic accomplishment	Number of papered published in SCI-grade journal
4	An Octopus-derived pepetide with antidiuretic activity in rats	Building on expertise through academic accomplishment	Number of papered published in SCI-grade journal
5	Anti-stress effects of octopus cephalotocin in rats.	Building on expertise through academic accomplishment	Number of papered published in SCI-grade journal
6	Classifcation of advanced methods for evaluating neurotoxicity	Building on expertise through academic accomplishment	Number of papered published in SCI-grade journal
7	BAPTA, a calcium chelator, neuroprotects injured neurons *in vitro* and promotes motor recovery after spinal cord transection *in vivo*	Building on expertise through academic accomplishment	Number of papered published in SCI-grade journal
8	Development and validation of dual-cardiotoxicity evaluation method based on analysis of field potential and contractile force of human iPSC-derived cardiomyocytes / multielectrode assay platform	Building on expertise through academic accomplishment	Number of papered published in SCI-grade journal
9	4-EA-NBOMe, an amphetamine derivative, alters glutamatergic synaptic transmission through 5-HT receptors on both C57BL/6 mouse and Sprague-Dawley (SD) rats (The Korea Society of Toxicogenomics and Toxicoproteomics)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
10	*In vitro* evaluation of new psychoactive substances (NPS) by using synaptic channels (Korea Society of Toxicogenomics and Toxicoproteomics)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
11	*In vitro* evaluation of new psychoactive substances (NPS) by using synaptic channels (Korean Society of Toxicology)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
12	Establishment of Advanced Multi-organoid Model Based on Functional Characteristics (Korea Tissue Engineering and Regenerative Medicine Society)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
13	Establishment of advanced multi-organoid model based on functional characteristics (Korean Society for Stem Cell Research)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
14	Establishment of next-generation pharmacotocixity using iPS- derived neuron and cardiomyocyte (Korean Society of Toxicologic Pathology)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
15	Establishment of next-generation pharmacotocixity using iPS- derived neuron and cardiomyocyte (The Organoid Society)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
16	Evaluation of neurotoxicity and efficacy using in vitro alternative model: hiPSC-derived mini brain with multi-electrode array(Korean Society for Alternative to Animal Experiments)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
17	Generation of Human iPSCs Derived Heart Organoids Structurally and Functionally Similar to Heart (The Organoid Society)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
18	Novel reversible effect of N-(ortho-methoxybenzyl)-4-Ethylamphetamine (4-EA-NBOMe) on the frequency of excitatory postsynaptic currents from rat primary cortical neurons and mouse medial prefrontal cortex(Korean Society of Toxicology/Korean Environmental Mutagen Society)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
19	Reversible effect of N-(ortho-methoxybenzyl)-4-Ethylamphetamine (4-EA-NBOMe) on the frequency of excitatory postsynaptic currents *in vitro* and *ex vivo* (Korea Society of Toxicogenomics and Toxicoproteomics)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
20	Special features of N-(ortho-methoxybenzyl)-4-Ethylamphetamine (4-EA-NBOMe) on excitatory postsynaptic current from rat primary cortical neurons (Korean Society for Brain and Neural Sciences)	Building on expertise through academic accomplishment	Number of publications or presentations in national workshops
21	SPR (surface plasmon resonance) evaluation method for measuring new narcotics receptor binding affinity	Improving testing, investigation and research capabilities	Number of test method development
22	iPSC-based method for measuring mitochondria function	Improving testing, investigation and research capabilities	Number of test method development
23	Method for measuring neuron activation	Improving testing, investigation and research capabilities	Number of test method development

	Title	MFDS Performance Evaluation Standard	
		Type	Performance Indicators
24	Method for measuring Neurotransmitter release	Improving testing, investigation and research capabilities	Number of test method development
25	Dopamin receptor disruption level measurement method	Improving testing, investigation and research capabilities	Number of test method development
26	Pre-synapse release evaluation method using paired pulsed ratio	Improving testing, investigation and research capabilities	Number of test method development
27	*In vitro* dependency test method	Improving testing, investigation and research capabilities	Number of test method development
28	New narcotics test method using synapse channel	Improving testing, investigation and research capabilities	Number of test method development

☐ Major accomplishment

1. Publication: Digital selective transformation and patterning of highly conductive hydrogel bioelectronics by laser–induced phase separation

SCIENCE ADVANCES | RESEARCH ARTICLE

APPLIED SCIENCES AND ENGINEERING

Digital selective transformation and patterning of highly conductive hydrogel bioelectronics by laser-induced phase separation

Daeyeon Won[1,2]†, Jin Kim[3,4]†, Joonhwa Choi[1,2], HyeongJun Kim[5], Seonggeun Han[1,2], Inho Ha[1,2], Junhyuk Bang[1,2], Kyun Kyu Kim[1,2], Youngseok Lee[1,2], Taek-Soo Kim[5], Jae-Hak Park[3], C-Yoon Kim[4]*, Seung Hwan Ko[1,2,6]*

The patterning of poly(3,4-ethylenedioxythiophene):poly(styrene sulfonate) (PEDOT:PSS) hydrogels with excellent electrical property and spatial resolution is a challenge for bioelectronic applications. However, most PEDOT:PSS hydrogels are fabricated by conventional manufacturing processes such as photolithography, inkjet printing, and screen printing with complex fabrication steps or low spatial resolution. Moreover, the additives used for fabricating PEDOT:PSS hydrogels are mostly cytotoxic, thus requiring days of detoxification. Here, we developed a previously unexplored ultrafast and biocompatible digital patterning process for PEDOT:PSS hydrogel via phase separation induced by a laser. We enhanced the electrical properties and aqueous stability of PEDOT:PSS by selective laser scanning, which allowed the transformation of PEDOT:PSS into water-stable hydrogels. PEDOT:PSS hydrogels showed high electrical conductivity of 670 S/cm with 6-μm resolution in water. Furthermore, electrochemical properties were maintained even after 6 months in a physiological environment. We further demonstrated stable neural signal recording and stimulation with hydrogel electrodes fabricated by laser.

INTRODUCTION

Engineering conductive hydrogels with excellent electrical properties and aqueous stability is important for developing electrode materials that are of broad interest to research fields such as bioelectronics (*1*), e-skin (*2*), and energy devices (*3*). In bioelectronics, achieving tissue-like mechanical properties and retaining their electrical conductivity under strain in physiological environments are crucial for electrode materials to interface with elastic soft tissue in a long-term operation (*4*, *5*).

Hydrogels fabricated by conducting polymers [e.g., polypyrrole, polyaniline, and poly(3,4-ethylenedioxythiophene):polystyrene sulfonate (PEDOT:PSS)] are promising electrode materials (*1*) owing to superior softness, stretchability, and electrochemical stability to metal (Au and Pt) (*6*, *7*) and oxide materials (IrOx and RuO) (*6*) in physiological environments. In particular, conductive hydrogels consisting of PEDOT:PSS have attracted the most attention due to their unique electrical and ionic dual conductivity and excellent biocompatibility. However, PEDOT:PSS is disadvantageous for long-term operation in contact with biological tissues as it suffers from a relatively high Young's modulus (1 to 2 GPa), low stretchability (~2% strain) (*8*) due to the brittle PEDOT-rich domain, and water instability due to the hydrophilic PSS-rich domain. To transform PEDOT:PSS into water-stable soft hydrogels, phase separation methods that properly redistribute the networks between the conductive and hydrophobic PEDOT-rich domain and the soft and hydrophilic PSS-rich domain have been developed (*4*, *9*–*17*). For phase separation, sulfuric acid or ionic liquids with strong ionic strength were usually introduced to break the ionic bond between positively charged PEDOT ($PEDOT^+$) and negatively charged PSS (PSS^-) and rearrange the networks between them (*4*, *9*–*14*). Subsequent dry annealing was able to physically interconnect the PEDOT-rich domain that made the PEDOT:PSS hydrogel stable in aqueous environments. However, the additives were harmful to living tissues, thus requiring posttreatment of washing with water for several days before usage (*4*, *9*, *11*–*14*). For phase separation, a strong electric field was also applied with several hours of thermal annealing in a state where the ionic bond between $PEDOT^+$ and PSS^- was weakened by additives (*15*–*17*). Even after tedious and lengthy postprocesses, the hydrogels in previous studies had a low electrical conductivity of less than 200 S/cm when fully swollen in the electrolyte.

Despite the promising properties of PEDOT:PSS hydrogels, patterning them with a high spatial resolution is another major challenge for bioelectronic applications. Various printing methods [e.g., inkjet printing, screen printing, and three-dimensional (3D) printing] (*13*, *14*, *18*, *19*) were developed by mixing additives into PEDOT:PSS aqueous solution that can induce phase separation for transformation into hydrogels. However, most of the studies showed low spatial resolutions of over 100 μm and required a long detoxification process to remove cytotoxic additives. Processes to change the physical properties of PEDOT:PSS by irradiating a light source have also been attempted (*4*, *20*, *21*). The incorporation of a photo–cross-linkable monomer has been tried, but a nonconductive monomer could reduce the conductivity of PEDOT:PSS hydrogel (*4*, *20*). Supplying infrared photons to selectively remove PSS shell for conductivity enhancement has been introduced (*21*); however, it could not induce physical aggregation of PEDOT-rich domain, which cannot assure aqueous stability in physiological environments.

[1]Soft Robotics Research Center, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea. [2]Applied Nano and Thermal Science Lab, Department of Mechanical Engineering, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea. [3]Laboratory Animal Medicine, College of Veterinary Medicine, Seoul National University, 1, Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea. [4]College of Veterinary Medicine, Konkuk University, 120, Neungdong-ro, Gwangjin-gu, Seoul 05029, Republic of Korea. [5]Department of Mechanical Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 34141, Republic of Korea. [6]Institute of Advanced Machines and Design/Institute of Engineering Research, Seoul National University, Seoul 08826, Republic of Korea. *Corresponding author. Email: maxko@snu.ac.kr (S.H.K.); vivavet@konkuk.ac.kr (C.-Y.K.) †These authors contributed equally to this work.

2. Publication: Establishment of Neurotoxicity Assessment Using Microelectrode Array (MEA) with hiPSC-Derived Neurons and Evaluation of New Psychoactive Substances (NPS).

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ORIGINAL ARTICLE

Establishment of Neurotoxicity Assessment Using Microelectrode Array (MEA) with hiPSC-Derived Neurons and Evaluation of New Psychoactive Substances (NPS)

Kyu-ree Kang[1,*], C-Yoon Kim[1,2,*], Jin Kim[3], Bokyeong Ryu[5], Seul-Gi Lee[1], Jieun Baek[1], Ye-Ji Kim[4], Jin-Moo Lee[5], Yootmo Lee[5], Sun-Ok Choi[5], Dong Ho Woo[4], Il Hwan Park[6], Hyung Min Chung[1]

[1]*Department of Stem Cell Biology, School of Medicine, Konkuk University, Seoul, Korea*
[2]*Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul, Korea*
[3]*Department of Laboratory Animal Medicine, College of Veterinary Medicine, Seoul National University, Seoul, Korea*
[4]*Drug Abuse Research Group, Research Center of Convergence Toxicology, Korea Institute of Toxicology, Daejeon, Korea*
[5]*Pharmacological Research Division, Toxicological Evaluation and Research Department, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Cheongju, Korea*
[6]*Departments of Thoracis and Cardiovascular Surgery, Yonsei University Wonju College of Medicine, Wonju, Korea*

Background and Objectives: Currently, safety pharmacological tests for the central nervous system depend on animal behavioral analysis. However, due to the subjectivity of behavioral analysis and differences between species, there is a limit to appropriate nervous system toxicity assessment, therefore a new neurotoxicity assessment that can simulate the human central nervous system is required.

Methods and Results: In our study, we developed an *in vitro* neurotoxicity assessment focusing on neuronal function. To minimize the differences between species and fast screening, hiPSC-derived neurons and a microelectrode array (MEA) that could simultaneously measure the action potentials of the neuronal networks were used. After analyzing the molecular and electrophysiological characters of our neuronal network, we conducted a neurotoxicity assessment on neurotransmitters, neurotoxicants, illicit drugs, and new psychoactive substances (NPS). We found that most substances used in our experiments responded more sensitively to our MEA-based neurotoxicity assessment than to the conventional neurotoxicity assessment. Also, this is the first paper that evaluates various illicit drugs and NPS using MEA-based neurotoxicity assessment using hiPSC-derived neurons.

Conclusions: Our study expanded the scope of application of neurotoxicity assessment using hiPSC-derived neurons to NPS, and accumulated evaluation data of various toxic substances for hiPSC-derived neurons.

Keywords: Neurotoxicity assessment, Microelectrode array, iPSC-derived neuron application, Illicit drugs, New psychoactive substance

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Correspondence to **Il Hwan Park**
Departments of Thoracis and Cardiovascular Surgery, Yonsei University Wonju College of Medicine, Wonju 26426, Korea
Tel: +82-2-450-4254, E-mail: nicecs@yonsei.ac.kr
Co-Correspondence to **Hyung Min Chung**
Department of Stem Cell Biology, School of Medicine, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 05029, Korea
Tel: +82-2-2049-6028, E-mail: hmchung@kku.ac.kr
*These authors contributed equally to this work.

3. Publication: Acute effects of 4-EA-NBOMe, 3, 4-DCMP, LY-2183240 and AB-CHFUPYCA on the excitability of rat cortical primary cultured neurons

Molecular & Cellular Toxicology
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ORIGINAL ARTICLE

Acute effects of 4-EA-NBOMe, 3, 4-DCMP, LY-2183240 and AB-CHFUPYCA on the excitability of rat cortical primary cultured neurons

Hyun-A Oh[1] · Jong-Hwa Lee[1] · Dong Ho Woo[1]

Accepted: 26 January 2022

Abstract

Background Various methods to evaluate the effect of new psychoactive substances (NPSs) on the central nervous system (CNS) are available, but they are not sufficient. There is an urgent need for a method to measure and analyze the effect of NPS on the CNS for scientific research.

Objectives We investigated whether seven psychoactive substances (PSs) affect the excitability of rat cortical culture neurons by counting the number of action potentials (APs) in current clamp modes.

Results Incubation with Meth, THC, LY2183240 and AB-CHFUPYCA for 2 min increased the number of APs; in contrast, incubation with 4-EA-NBOMe and CMP reduced the number of APs. Cocaine did not show any effects on the number of APs.

Conclusion Acute treatment with PSs changed neural excitability as evidenced by the number of APs. Our data provide scientific evidence that acute PS exposure can affect the CNS.

Keywords New psychoactive substance · Meth · 4-EA-NBOMe · 3, 4-DCMP · THC · LY-2183240 · AB-CHFUPYCA · Cocaine · Rat primary cortical neurons

Abbreviations

Meth (Methamphetamine)	(RS)-*N*-methyl-1-phenylpropan-2-amine
4-EA-NBOMe (*N*-(ortho-methoxybenzyl)-4-ethylamphetamine)	1-(4-Ethylphenyl)-*N*-[(2-methoxyphenyl)methyl] propan-2-amine
3, 4-DCMP (3, 4-dichloromethylphenidate)	Methyl (2R)-2-(3,4-dichlorophenyl)-2-[(2R)-piperidin-2-yl]acetate
THC (Δ1-tetrahydrocannabinol)	(6AR,10aR)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol
LY-2183240 (5-(4-biphenylylmethyl)-*N*,*N*-dimethyl-1H-tetrazole-1-carboxamide)	*N*,*N*-Dimethyl-5-[(4-biphenyl)methyl] tetrazole-1-carboxamide
AB-CHFUPYCA	*N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1H-pyrazole-5-carboxamide
Cocaine	Methyl (1R,2R,3S,5S)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1] octane-2-carboxylate

✉ Dong Ho Woo
donghowoo24@gmail.com; dongho.woo@kitox.re.kr

[1] Department of Advanced Toxicology, Research Center for Convergence Toxicology, Korea Institute of Toxicology, Daejeon, South Korea

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4. Publication: An Octopus-derived pepetide with antidiuretic activity in rats

marine drugs

MDPI

Article

An Octopus-Derived Peptide with Antidiuretic Activity in Rats

Ye-Ji Kim [1,2,†], Jei Ha Lee [3,†], Seung-Hyun Jung [3], Ki Hyun Kim [3], Chang-Hoon Choi [1], Seonmi Jo [3,*] and Dong Ho Woo [1,2,*]

[1] Research Center for Convergence Toxicology, Korea Institute of Toxicology, Daejeon 34114, Korea; yeji.kim@kitox.re.kr (Y.-J.K.); cchcom@kitox.re.kr (C.-H.C.)
[2] Human and Environmental Toxicology, University of Science and Technology, Daejeon 34114, Korea
[3] Department of Genetic Resources, National Marine Biodiversity Institute of Korea, Seocheon 33662, Korea; jeiha@mabik.re.kr (J.H.L.); zebrajung@mabik.re.kr (S.-H.J.); tjckkh@naver.com (K.H.K.)
* Correspondence: joseonmi@mabik.re.kr (S.J.); dongho.woo@kitox.re.kr (D.H.W.); Tel.: +82-41-950-0920 (S.J.); +82-42-610-8243 (D.H.W.)
† These authors contributed equally to this work.

Abstract: Discovering new drug candidates with high efficacy and few side effects is a major challenge in new drug development. The two evolutionarily related peptides oxytocin (OXT) and arginine vasopressin (AVP) are known to be associated with a variety of physiological and psychological processes via the association of OXT with three types of AVP receptors. Over decades, many synthetic analogs of these peptides have been designed and tested for therapeutic applications; however, only a few studies of their natural analogs have been performed. In this study, we investigated the bioactivity and usefulness of two natural OXT/AVP analogs that originate from the marine invertebrate *Octopus vulgaris*, named octopressin (OTP) and cephalotocin (CPT). By measuring the intracellular Ca^{2+} or cyclic AMP increase in each OXT/AVP receptor subtype–overexpressing cell, we found that CPT, but not OTP, acts as a selective agonist of human AVP type 1b and 2 receptors. This behavior is reminiscent of desmopressin, the most widely prescribed antidiuretic drug in the world. Similar to the case for desmopressin, a single intravenous tail injection of CPT into Sprague–Dawley rats reduced urine output and increased urinary osmolality. In conclusion, we suggest that CPT has a significant antidiuretic effect and that CPT might be beneficial for treating urological conditions such as nocturia, enuresis, and diabetes insipidus.

Keywords: cephalotocin; octopressin; antidiuretic; octopus; vasopressin

check for updates

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1. Introduction

Oxytocin (OXT) and arginine vasopressin (AVP) are nonapeptides that are well known as a uterine-contracting hormone and an antidiuretic hormone, respectively. The genes encoding these two peptides are usually located close to each other on the same chromosome and are thought to have arisen through the duplication of an ancestral gene, named vasotocin [1]. Consequently, the two active nonapeptides are very similar in structure in terms of internal disulfide bonds and amidated C-termini [2,3]; only two amino acids, at positions 3 and 8, distinguish OXT and AVP. Once secreted from the posterior pituitary into the bloodstream, these peptide hormones exert their actions via one oxytocin receptor (OXTR) and three vasopressin receptors (V1aR, V1bR, and V2R), which are differentially expressed in various tissues [4]. In addition to their emerging roles in sociality and emotion [5], these hormones have physiological consequences, including uterine contraction and breast milk ejection via OXTR [6], vasoconstriction and platelet aggregation via V1aR (also called V1R), pituitary adrenocorticotropic hormone secretion in response to stress via V1bR (also called V3R), and antidiuretic water reabsorption in the kidney via V2R [7]. Inappropriate receptor activation leads to diseases and medical conditions associated with the various physiological processes described above [6,7].

5. Publication: Anti-stress effects of octopus cephalotocin in rats.

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Original Article

en
Experimental Neurobiology

Anti-stress Effect of Octopus Cephalotocin in Rats

Ye-Ji Kim[1,2], Seonmi Jo[3], Seung-Hyun Jung[3] and Dong Ho Woo[1,2*]

[1]*Research Center for Convergence Toxicology, Korea Institute of Toxicology, Daejeon 34114,*
[2]*Department of Human and Environmental Toxicology, University of Science and Technology, Daejeon 34114,*
[3]*Department of Genetic Resources, National Marine Biodiversity Institute of Korea, Seocheon 33662, Korea*

Cephalotocin is a bioactivity-regulating peptide expressed in octopus (*Octopus vulgaris*). The peptide sequence of cephalotocin is very similar to the peptide sequence of mammalian vasopressin, and cephalotocin has been proposed to mainly activate arginine vasopressin 1b receptor (Avpr1b) in the brain. However, the effects of cephalotocin on mammalian behavior have not been studied. In the current study, cephalotocin significantly reduced both the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) recorded from not only cultured neuronal cells from postnatal Sprague–Dawley (SD) rats but also hippocampal slices from 4-week-old male C57BL/6 mice. Intraperitoneal (IP) injection did not affect the open field behaviors of C57BL/6 mice. Cephalotocin was directly infused into the hippocampus because the normalized Avpr1b staining intensity divided by the DAPI staining intensity indicated that Avpr1b expression tended to be high in the hippocampus. A hippocampal infusion of 1 mg/kg cephalotocin via an implanted cannula exerted an anti-stress effect, significantly reducing the immobility time in the tail suspension test (TST). The present results provide evidence that the effects of cephalotocin on the activity of hippocampal neurons are related to ameliorating stress, suggesting that cephalotocin may be developed as an anti-stress biomodulator that functions by affecting the brain.

Key words: Cephalotocin, SD rat, Tail suspension test, sEPSCs, Intrahippocampal infusion

INTRODUCTION

Arginine vasopressin (Avp) is an important regulator of stress. Vasopressin-deficient Brattleboro rats have been shown to exhibit lower stress levels than wild-type rats in a 5 min forced swim test but not after a 15 min test, suggesting that Avp is strongly correlated with stress regulation [1]. The Avp 1a receptor (Avpr1a) is related to vascular modulation [2, 3], the Avp 2 receptor (Avpr2) is related to renal duct water reabsorption [4], and the Avp 1b receptor (Avpr1b or V3R) is related to stress regulation. Avpr1b plays key roles in social memory, aggressive behavior and stress responses [5]. An acute 38℃ treatment increases the blood Avp levels and decreases Avpr1b protein expression, suggesting that Avpr1b is strongly related to stress regulation [6]. *In situ* hybridization histochemistry (ISHH) and RT–PCR have provided evidence suggesting that Avpr1b transcripts are expressed in the hippocampal CA3, CA2 and CA1 regions [7].

The sequence of an octopus-originated peptide, cephalotocin (CPT), is similar to that of Avp and oxytocin. CPT has the potential to interact with the human oxytocin receptor and the vasopressin receptor. The 397 amino acid sequence of the receptor of CPT shares close homology with the receptor of vasopressin and is a potential Gq protein-coupled receptor that activates Ca^{2+}-activated Cl^- currents in the *Xenopus* oocyte expression system [8]. Thus, CPT is a potential agonist of oxytocin and vasopressin receptors. CPT has the potential to interact with the vasopressin receptor for two reasons. First, CPT varies from Avp only in the 4th and 9th amino acids and exhibits 78% sequence homology with oxytocin and vasopressin [9]. Second, Avp treatment increases the number of action potentials from CA1 pyramidal neurons in hippocampal slices by blocking the G protein-gated inwardly rectifying potassium (GIRK) channel and increases glutamate release in

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*To whom correspondence should be addressed.
TEL: 82-42-610-8243, FAX: 82-42-610-8252
e-mail: dongho.woo@kitox.re.kr

6. Publication: Classifcation of advanced methods for evaluating neurotoxicity

Molecular & Cellular Toxicology
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REVIEW

Classification of advanced methods for evaluating neurotoxicity

Kyung-Seok Han[1] · Dong Ho Woo[2]

Accepted: 5 August 2021

Abstract

Purpose of review As fields such as neurotoxicity evaluation and neuro-related drug research are increasing in popularity, there is a demand for the expansion of neurotoxicity research. Currently, neurotoxicity is assessed by measuring changes in weight and behavior. However, measurement of such changes does not allow the detection of subtle and inconspicuous neurotoxicity. In this review, methods for advancing neurotoxicity research are divided into molecule-, cell-, circuit-, and animal model-based methods, and the results of previous studies assessing neurotoxicity are provided and discussed.

Recent findings In coming decades, cooperation between universities, national research institutes, industrial research institutes, governments, and the private sector will become necessary when identifying alternative methods for neurotoxicity evaluation, which is a current goal related to improving neurotoxicity assessment and an appropriate approach to neurotoxicity prediction. Many methods for measuring neurotoxicity in the field of neuroscience have recently been reported. This paper classifies the supplementary and complementary experimental measures for evaluating neurotoxicity.

Keywords Neurotoxicity · Synapse · Ion channel · Synaptic plasticity · Electrophysiology · Imaging

Introduction

According to a global burden disease study, from 1990 to 2016, there was a 29.9% increase in years lived with disability (YLDs) for subjects with bipolar disorder (from 376.4 to 489.1 YLDs in thousands), a 65.6% increase for dementia (from 360.8 to 597.6 YLDs in thousands), a 13.6% increase for alcohol-related disorders (from 558.2 to 633.9 YLDs in thousands), a 36% increase for schizophrenia (from 503.3 to 685.2 YLDs in thousands), a 30.5% increase for opioid use disorders (from 1256.2 to 1638.9 YLDs in thousands), a 30.8% increase for anxiety disorders (from 1341.7 to 1755.0 YLDs in thousands), a 27.2% increase for migraine (from 1580.3 to 2010.1 YLDs in thousands), and a 27.0% increase for major depressive disorder (from 1726.2 to 2193.0 YLDs in thousands) (Collaborators, U.S.B.o.D. et al. 2018).

✉ Kyung-Seok Han
kshan@ddongguk.ac.kr

✉ Dong Ho Woo
dongho.woo@kitox.re.kr

[1] Department of Medical Biotechnology, Dongguk University-Gyeongju, 123 Dongdae-ro, Gyeongju, South Korea

[2] Research Center for Convergence Toxicology, Korea Institute of Toxicology, Daejeon, South Korea

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Neuromodulation technologies are required for the assessment of treatments and drugs administered for various diseases. Such technologies are used to improve cognition in stroke or dementia patients, aid recovery from mental illness, and rapidly assess the effects of drugs.

Current methods used to measure neurotoxicity involve assessing the effects of drugs on the central nervous system (CNS) by evaluating behaviors. However, behaviors represent many physiological functions and therefore cannot be used to assess specific neurotoxic effects. As many studies in neuroscience are being performed, various methods for measuring neurotoxicity are being developed. Systems biology, bioinformatics, and rapid assay technologies may be alternatives to the existing behavioral tests (Gibb 2008). Methods for assessing the risk of neurotoxicity are shifting from traditional animal toxicity tests to various mechanistic studies designed to clarify the toxicity mechanism and identify techniques for coping with the adverse effects of toxic substances (Krewski et al. 2009). In this paper, we present a classification system for various methods that may be used to assess neurotoxicity.

In neuroscience, the mechanisms underlying the connections between various circuits were established through the study of synapses. For example, the memory and learning processes can be explained by long-term and short-term

7. Publication: BAPTA, a calcium chelator, neuroprotects injured neurons *in vitro* and promotes motor recovery after spinal cord transection *in vivo*

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ORIGINAL ARTICLE

CNS Neuroscience & Therapeutics WILEY

BAPTA, a calcium chelator, neuroprotects injured neurons in vitro and promotes motor recovery after spinal cord transection in vivo

Kyu-ree Kang[1] | Jin Kim[2] | Bokyeong Ryu[2] | Seul-Gi Lee[1] | Min-Seok Oh[1] | Jieun Baek[1] | Xiaoping Ren[3,4] | Sergio Canavero[4,5] | C-Yoon Kim[1,4,6] | Hyung Min Chung[1]

[1]Department of Stem Cell Biology, School of Medicine, Konkuk University, Seoul, Korea

[2]Laboratory Animal Medicine, College of Veterinary Medicine, Seoul National University, Seoul, Korea

[3]Department of Orthopedics, Ruikang Hospital, Nanning, China

[4]GICUP-Global Initiative to Cure Paralysis, Columbus, Ohio, USA

[5]HEAVEN/GEMINI International Collaborative Group, Turin, Italy

[6]Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul, Korea

Correspondence
C-Yoon Kim, PhD, Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul, Korea.
Email: vivavets@gmail.com

Hyung Min Chung, PhD, Department of Stem Cell Biology, School of Medicine, Konkuk University, 120 Neungdong-Ro, Gwangjin-Gu, Seoul 143-701, Korea.
Email: hmchung@kku.ac.kr

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Abstract

Aim: Despite animal evidence of a role of calcium in the pathogenesis of spinal cord injury, several studies conducted in the past found calcium blockade ineffective. However, those studies involved oral or parenteral administration of Ca++ antagonists. We hypothesized that Ca++ blockade might be effective with local/immediate application (LIA) at the time of neural injury.

Methods: In this study, we assessed the effects of LIA of BAPTA (1,2-bis (o-aminophenoxy) ethane-N, N, N′, N′-tetraacetic acid), a cell-permeable highly selective Ca++ chelator, after spinal cord transection (SCT) in mice over 4 weeks. Effects of BAPTA were assessed behaviorally and with immunohistochemistry. Concurrently, BAPTA was submitted for the first time to multimodality assessment in an in vitro model of neural damage as a possible spinal neuroprotectant.

Results: We demonstrate that BAPTA alleviates neuronal apoptosis caused by physical damage by inhibition of neuronal apoptosis and reactive oxygen species (ROS) generation. This translates to enhanced preservation of electrophysiological function and superior behavioral recovery.

Conclusion: This study shows for the first time that local/immediate application of Ca++ chelator BAPTA is strongly neuroprotective after severe spinal cord injury.

KEYWORDS
calcium, locomotor recovery, neuronal apoptosis, oxidative stress, spinal cord injury

Kyu-ree Kang and Jin Kim contributed equally to this work as first authors, respectively.

8. Publication: Development and validation of dual-cardiotoxicity evaluation method based on analysis of field potential and contractile force of human iPSC-derived cardiomyocytes / multielectrode assay platform

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Development and validation of dual-cardiotoxicity evaluation method based on analysis of field potential and contractile force of human iPSC-derived cardiomyocytes / multielectrode assay platform

Seul-Gi Lee [a, 1], Jin Kim [b, 1], Min-Seok Oh [a], Bokyeong Ryu [b], Kyu-Ree Kang [a], Jieun Baek [a], Jin-Moo Lee [d], Sun-Ok Choi [d], C-Yoon Kim [c, *], Hyung Min Chung [a, **]

[a] Department of Stem Cell Biology, School of Medicine, Konkuk University, 120 Neungdong-Ro, Gwangjin-Gu, Seoul, 143-701, Republic of Korea
[b] Department of Laboratory Animal Medicine, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea
[c] College of Veterinary Medicine, Konkuk University, Seoul, 05029, Republic of Korea
[d] Pharmacological Research Division, Toxicological Evaluation and Research Department, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Republic of Korea

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ABSTRACT

A recent *in vitro* cardiovascular safety pharmacology test uses cardiomyocytes derived from human induced pluripotent stem cells (hiPSCs) to overcome the limitations of the classical test systems, such as species differences and local channel analysis. The Comprehensive *in vitro* Proarrhythmia Assay (CiPA) is a new proarrhythmia screening paradigm proposed by a CiPA steering expert group, which essentially requires iPSCs derived cardiomyocyte-based electrophysiological evaluation technology. Moreover, the measurement of the contractile force is also emerging as an important parameter to recapitulate non-proarrhythmic cardiotoxicity. Therefore, we constructed an multielectrode assay (MEA) evaluation method that can measure the electrophysiological changes with 6 reference drugs in hiPSC-derived cardiomyocytes. Subsequently, it was confirmed that the electrophysiological were changed in accordance with the mechanism of action of the drugs. Furthermore, based on the multi-probe impedance, we confirmed the decrease in contractile force due to treatment with drugs, and developed a platform to evaluate cardiotoxicity according to drugs along with field potential changes. Our excitation–contraction coupling cardiotoxicity assessment is considered to be more supportive in cardiac safety studies on pharmacologic sensitivity by complementing each assessment parameter.

1. Introduction

For the development of new drugs, the process of identifying adverse drug reactions (ADRs) is essential, and many potential candidate drugs fail because of their side effects. Cardiotoxicity is one of the major causes of drug withdrawal from the market and more than 2000 post-market drugs, including anticancer drugs and antibiotics on the market, were found to have cardiac ADRs [1]. 14% of the 462 drugs withdrawn from the market between 1953 and 2013 were responsible for serious cardiovascular side effects [2]. In particular, not only an acute effect but also a chronic effect with delayed onset may occur in the case of cardiotoxicity. Therefore, even after passing through preclinical screening, toxicity may be confirmed in phase 2 and phase 3 clinical trials or after being released into the market [3]. Therefore, evaluating potential cardiac toxicity is an important parameter in the drug market.

In this context, the International Conference for Harmonization (ICH) presented guidelines S7B and E14 in 2005. ICH S7B, a guideline for evaluating nonclinical cardiac toxicity, is based on ventricular repolarization (QT interval prolongation) in cell lines transfected with human Ether-à-go-go-Related Gene (hERG) or in cardiomyocytes (CMs) isolated from animals or humans. ICH E1T, a clinical trial guideline, analyzes the thorough QT/QTc study to evaluate the arrhythmia-inducing effects of non-antiarrhythmic drugs [4]. By pre-screening through the application of the above

* Corresponding author.
** Corresponding author.
E-mail addresses: vivavets@gmail.com (C.-Y. Kim), hmchung@kku.ac.kr (H.M. Chung).
[1] These authors contributed equally to this work as first and corresponding authors, respectively.

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Ⅳ Latest studies on NAMs and their regulatory acceptance

1. Study on development of OECD skin sensitization test method using nanomaterial
2. Development of mitochondria-targeted toxicity evaluation method
3. Development of alternative developmental neurotoxicity evaluation technology using stem cells
4. Study on evaluation of drug interaction using big data and machine learning
5. Study on establishment of big data-based model for prediction of drug cardiovascular safety

Ⅳ Latest studies on NAMs and their regulatory acceptance

- The Ministry of Food and Drug Safety (MFDS) has put effort into regulatory acceptance of NAMs by participating in the development of OECD Test Guidelines and working groups including OECD Expert Groups, Working Party on Manufactured Nanomaterials (WPMN) and Advisory Group on Emerging Science in Chemical Assessment (AG ESCA).
- In particular, as the use of nanomaterials with advancement of nanotechnology as well as the used of chemicals including cosmetic ingredients and medical devices have been increasing, MFDS has been working with overseas organizations to perform joint study on nanomaterials and to revise related test guidelines.
- MFDS has developed NAMs applicable to medical devices as well as conducted studies on new methods for regulatory implantation.
- MFDS has been committed to reducing the use of laboratory animals and increasing predictivity by, for example, building a system for prediction of the results of drug interaction using bid data in order to reduce adverse effect caused by pharmaceuticals.

1 Study on development of OECD skin sensitization test method using nanomaterials

Supervising division	Toxicological Research Division of NIFDS
Type	Internal Project
Title	Study on enhancing international cooperation and infrastructure for development of toxicity test methods

☐ Background for the study

- The OECD constantly publishes and revises Test Guidelines and Guidance Documents that reflect advanced technologies and animal welfare to support the mutual acceptance of data (MAD). Korea has participated in the meetings of OECD expert groups and WNT to provide inputs for publication and revision of the documents.
- As the use of nanomaterials is increasing with the development of nanotechnology, a study that informs the revision of toxicity test methods for evaluation of nanomaterials is necessary.
- MFDS has performed research on safety assessment of nanomaterials since the late 1990s and produced sufficient toxicity data on nanomaterials by performing target organ toxicity tests. The paradigm of nanotoxicity research has shifted to development of evaluation method using nanomaterials since the mid 2010s, and research keeping pace with the change is necessary.
- MFDS has conducted a joint study with the Swiss Federal Office of Public Health (FOPH) on the properties of nanomaterials in order to revise Test guideline 442D (*in vitro* skin sensitization test). The SPSF (Standard Project Submission Form) on the revision was adopted by the OECD in April, 2019.
- International joint study on incorporation of nanomaterials properties to skin sensitization test methods was conducted and the draft updated Test Guideline was prepared to include considerations for toxicity test methods using nanomaterials based on the study outcome.

☐ Study in brief

- Demonstration of scientific basis to update OECD Test Guideline to incorporate testing of nanomaterials.

- The draft OECD Test Report (project number 4.133 led by Switzerland) was accepted by the OECD WNT in April 2023.
- The results we presented was accepted as the scientific basis for the OECD Study Report.

☐ Overview of the study outcome

- **Major accomplishment:** Presenting scientific basis to incorporate testing of manufactured nanomaterials into OECD Test Guideline and enhancing Korea's profile in the world by carrying out international joint study.
- **Main outcome**
  - **OECD acceptance:** OECD WNT acceptance of the OECD Test Report in April 2023.
  - **Scientific outcome:** Publication of 7 papers in SCI-grade journals and presentations of 5 posters (awarded as outstanding poster)
  - **Social outcome:** Participation in various international conference including OECD WNT, WPMN, etc.
  - **Technocal outcome:** Monitoring of the skin sensitization tests (OECD TG 442) using nanomaterials

☐ Excellence and Advantage

- **Exellcence:** Establishment of skin sensitization test method for nanomateiral skin exposure
  - Risk assessment tests have been conducted using the clinical route of nanomaterial exposure, but few research has addressed toxicity caused by nanomaterial skin exposure.
  - The international joint study with Swiss FOPH helped establish scientific basis for the revision of OECD TG to incorporate nanomaterials properties to the skin sensitization test methods in the TG and risk assessment methods for nanomaterial skin exposure.
- **Advantage:** Establishment of scientific basis for acceptance of OECD Test Report
  - Since the mid 2010s, global paradigm of nanotoxicity research has moved towards development of nanomaterial assessment methods and many related studies have been performed.

- In particular, various studies to develop or revise test methods for nanomaterials that can by accepted in globally harmonized test guidelines have performed. We carries out an international joint study with Swiss FOPH to update OECD TG to extend the applicability of the skin sensitization test methods to nanomaterials.
- The data generated in this study has provided the scientific basis for the approval of OECD Test Report.

☐ Utilization of the outcome and its impact

- Establishment of the major scientific basis for preparation of the Test Report for OECD skin sensitization test methods incorporating nanomaterials properties.
  - The international joint study produced scientific and professional data to support nanomaterials toxicity (skin sensitization) assessment. Based on the data, Test Report of OECD skin sensitization test methods extending the applicability to nanomaterials has been prepared. The study outcome is expected to enhance practical use of the methods and have influence related national and global industry and academia.
- Improvement of the national profile through international cooperation and reinforcement of the infrastructure for toxicity tests
  - Exchange of regulations and research trend related to safety of nanomaterials, etc. at international meetings including OECD WNT and WPMN strengthened national competitiveness in development of nanomaterials safety evaluation methods and international standards and improved national profile.

☐ Performance of the study

	Title	MFDS Performance Evaluation Standards	
		Type	Performance Indicators
1	Draft updated TG442D-KeratinoSensTM	Encouragement of practical use by policy improvement, etc.	TG proposal
2	Draft updated TG442Eh-cLAT	Encouragement of practical use by policy improvement, etc.	TG proposal
3	Draft updated TG442B-LLNA:BrdU-FCM (skin sensitisation testing of nanomaterials)	Encouragement of practical use by policy improvement, etc.	TG proposal
4	35th Annual Meeting of KSOP/KEMS best workshop poster	Promotion and dissemination of research outcome	Education and promotion of research outcome
5	2020 MFDS best R&D project-1st prize winner	Promotion and dissemination of research outcome	Education and promotion of research outcome
6	KSOP best workshop poster	Promotion and dissemination of research outcome	Education and promotion of research outcome
7	KSTP best workshop poster-The research of toxicity and sensitization potential of PEGylated Silver and Gold Nanomaterials	Promotion and dissemination of research outcome	Education and promotion of research outcome
8	KSTP best workshop poster	Promotion and dissemination of research outcome	Education and promotion of research outcome
9	2021 MFDS best R&D project-1st prize winner	Promotion and dissemination of research outcome	Education and promotion of research outcome
10	Skin sensitisation testing of metal nanoparticles (3 test areas, 7 items, and 21 cases)	Building on expertise in testing, research and investigation	Monitoring
11	Skin sensitisation testing of nanomaterials (2 test areas, 6 items and 19 cases)	Building on expertise in testing, research and investigation	Monitoring
12	Skin sensitisation testing of nanomaterials (OECD TG442D)(1 test areas, 13 items and 13 cases)	Building on expertise in testing, research and investigation	Monitoring
13	Skin sensitisation testing of nanomaterials (OECD TG442E)(1 test areas, 5 items and 5 cases)	Building on expertise in testing, research and investigation	Monitoring
14	Skin sensitisation testing of nanomaterials (OECD TG442B)(1 test areas, 10 items and 10 cases)	Building on expertise in testing, research and investigation	Monitoring

	Title	MFDS Performance Evaluation Standards	
		Type	Performance Indicators
15	Participation in the 11th OECD EAGMST Meeting	Improvement of international competitiveness	International exchange and cooperation (e.g. signing of MOU, etc.)
16	Participation in the 18th OECD WPMN Meeting and Seminar	Improvement of international competitiveness	International exchange and cooperation (e.g. signing of MOU, etc.)
17	Participation in the OECD WNT30 and review of draft TGs	Improvement of international competitiveness	International exchange and cooperation (e.g. signing of MOU, etc.)
18	Participation in the 14th OECD EAGMST Meeting	Improvement of international competitiveness	International exchange and cooperation (e.g. signing of MOU, etc.)
19	Participation in the 21st OECD WPMN	Improvement of international competitiveness	International exchange and cooperation (e.g. signing of MOU, etc.)
20	Participation in the OECD WNT33 and review of draft TGs	Improvement of international competitiveness	International exchange and cooperation (e.g. signing of MOU, etc.)
21	Copper and Cobalt Ions Released from Metal Oxide Nanoparticles Trigger Skin Sensitization	Building on expertise through academic accomplishment	Publication of papers in SCO-grade journals
22	Effect of Pulmonary Inflammation by Surface Functionalization of Zinc Oxide Nanoparticles	Building on expertise through academic accomplishment	Publication of papers in SCO-grade journals
23	Evaluation of the Skin Sensitization Potential of Carbon Nanotubes Using Alternative *In Vitro* and *In Vivo* Assays	Building on expertise through academic accomplishment	Publication of papers in SCO-grade journals
24	Evaluation of the skin sensitization potential of metal oxide nanoparticles using the ARE-Nrf2 Luciferase KeratinoSensTM assay	Building on expertise through academic accomplishment	Publication of papers in SCO-grade journals
25	Flow cytometric evaluation of the potential of metal oxide nanoparticles for skin sensitization using 5-Bromo-2-deoxyuridine	Building on expertise through academic accomplishment	Publication of papers in SCO-grade journals
26	Colony-Forming Efficiency Assay to Assess Nanotoxicity of Graphene Nanomaterials	Building on expertise through academic accomplishment	Publication of papers in SCO-grade journals
27	Skin Sensitization Evaluation of Carbon - Based Graphene Nanoplatelets	Building on expertise through academic accomplishment	Publication of papers in SCO-grade journals
28	Six-well plate-based colony-forming efficacy assay and Co-Culture application to assess toxicity of metal oxide nanoparticles	Building on expertise through academic accomplishment	Publication of papers in SCO-grade journals

	Title	MFDS Performance Evaluation Standards	
		Type	Performance Indicators
29	Skin Sensitization Potential and Cellular ROS-Induced Cytotoxicity of Silica Nanoparticles	Building on expertise through academic accomplishment	Publication of papers in SCO-grade journals
30	The Research of Toxicity and Sensitization Potential of PEGylated Silver and Gold Nanomaterials	Building on expertise through academic accomplishment	Publication of papers in SCO-grade journals
31	Evaluation of metal nanoparticles on skin sensitization using OECD TG442B	Building on expertise through academic accomplishment	Publication or presentation in national workshops
32	Evaluation of the skin sensitisation potential of TiO2 and ZnO nanoparticles using the OECD skin sensitization test guideline	Building on expertise through academic accomplishment	Publication or presentation in national workshops
33	Evaluation of nanomaterials by shape difference on skin sensitization using OECD TG 442B	Building on expertise through academic accomplishment	Publication or presentation in national workshops
34	Evaluation of the skin sensitization & histopathological analysis by titanium oxide nanoparticles	Building on expertise through academic accomplishment	Publication or presentation in national workshops
35	Evaluation of the skin sensitization potential of Carbon Nanotubes using alternative methods	Building on expertise through academic accomplishment	Publication or presentation in national workshops
36	Overview of skin sensitization AOP for titanium oxide (TiO2) nanoparticles	Building on expertise through academic accomplishment	Publication or presentation in national workshops
37	Comparison of skin sensitization potential of metal nanoparticles and metal ions using OECD TG442B (LLNA: BrdU-FCM)	Building on expertise through academic accomplishment	Publication or presentation in national workshops
38	Copper and Cobalt Ions Released from Metal Oxide Nanoparticles Trigger Skin Sensitization	Building on expertise through academic accomplishment	Publication or presentation in national workshops
39	Evaluation of the Skin Sensitization Potential of Silica nanoparticles using *in vitro* and *in vivo* assay	Building on expertise through academic accomplishment	Publication or presentation in international workshops
40	Skin Sensitization Potential and Cellular ROS-Induced Cytotoxicity of Silica Nanoparticles	Building on expertise through academic accomplishment	Publication or presentation in national workshops

☐ Major accomplishment

21. Publication: Copper and Cobalt Ions Released from Metal Oxide Nanoparticles Trigger Skin Sensitization

frontiers in Pharmacology

ORIGINAL RESEARCH
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Copper and Cobalt Ions Released from Metal Oxide Nanoparticles Trigger Skin Sensitization

Sung-Hyun Kim, Jin Hee Lee, Kikyung Jung, Jun-Young Yang, Hyo-Sook Shin, Jeong Pyo Lee, Jayoung Jeong, Jae-Ho Oh and Jong Kwon Lee**

Division of Toxicological Research, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong, South Korea

Human skins are exposed to nanomaterials in everyday life from various sources such as nanomaterial-containing cosmetics, air pollutions, and industrial nanomaterials. Nanomaterials comprising metal haptens raises concerns about the skin sensitization to nanomaterials. In this study, we evaluated the skin sensitization of nanomaterials comparing metal haptens *in vivo* and *in vitro*. We selected five metal oxide NPs, containing copper oxide, cobalt monoxide, cobalt oxide, nickel oxide, or titanium oxide, and two types of metal chlorides ($CoCl_2$ and $CuCl_2$), to compare the skin sensitization abilities between NPs and the constituent metals. The materials were applied to KeratinoSens™ cells for imitated skin-environment setting, and luciferase induction and cytotoxicity were evaluated at 48 h post-incubation. In addition, the response of metal oxide NPs was confirmed in lymph node of BALB/C mice via an *in vivo* method. The results showed that CuO and CoO NPs induce a similar pattern of positive luciferase induction and cytotoxicity compared to the respective metal chlorides; Co_3O_4, NiO, and TiO_2 induced no such response. Collectively, the results implied fast-dissolving metal oxide (CuO and CoO) NPs release their metal ion, inducing skin sensitization. However, further investigations are required to elucidate the mechanism underlying NP-induced skin sensitization. Based on ion chelation data, metal ion release was confirmed as the major "factor" for skin sensitization.

Keywords: skin sensitization, alternative test, KeratinoSens™, LLNA, dissolving nanoparticles, nanoparticles, copper, cobalt

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Tian Xia, UCLA David Geffen School of Medicine, United States
Masanori Horie, Adina Institute of Science and Technology (AIST), India

****Correspondence:***
Jae-Ho Oh
mfdsnc@korea.kr
Jong Kwon Lee
jklees@korea.kr

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INTRODUCTION

Metal oxide nanoparticles (NPs) constitute one of the major types of nanomaterials (NMs) that are used in industrial, biomedical, and cosmetic applications. With an increase in the number and production volume of NPs, concerns about their toxicity have increased exponentially in the recent years. While the major NP-exposure pathways include inhalation, ingestion, and absorption into the skin, the latter can cause lesions such as local inflammation, contact allergy, and skin sensitization (Oberdörster et al., 2005; Maynard and Kuempel, 2005). With an exponential increase in the commercialization of NPs in cosmetics and relevant safety concerns, evaluation of NP safety has become important (Katz et al., 2015). In recent cosmetic tests, the importance of alternative test methods considering animal welfare and the 3R principles has been emphasized (Rusche, 2003; Kaluzhny et al., 2011). However, since these guidelines are based on chemical substances,

22. Publication: Effect of Pulmonary Inflammation by Surface Functionalization of Zinc Oxide Nanoparticles

toxics

MDPI

Article

Effect of Pulmonary Inflammation by Surface Functionalization of Zinc Oxide Nanoparticles

Ayoung Jung †, Sung-Hyun Kim †, Jun-Young Yang, Jayoung Jeong, Jong Kwon Lee, Jae-Ho Oh and Jin Hee Lee *

Division of Toxicological Research, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong, Cheongju 28159, Korea; kntp2002@korea.kr (A.J.); tjdgus32@korea.kr (S.-H.K.); yangjy6@korea.kr (J.-Y.Y.); 0jjy@korea.kr (J.J.); jkleest@korea.kr (J.K.L.); chopin68@korea.kr (J.-H.O.)
* Correspondence: tod988@korea.kr; Tel.: +82-43-719-5106; Fax: +82-43-719-5100
† These authors contributed equally to this work.

Abstract: Zinc oxide nanoparticles (ZnO NPs) are used in various industries such as food additives, cosmetics, and biomedical applications. In this study, we evaluated lung damage over time by three types of ZnO NPs (L-serine, citrate, and pristine) following the regulation of functional groups after a single intratracheal instillation to rats. The three types of ZnO NPs showed an acute inflammatory reaction with increased LDH and inflammatory cell infiltration in the alveoli 24 h after administration. Especially in treatment with L-serine, citrate ZnO NPs showed higher acute granulocytic inflammation and total protein induction than the pristine ZnO NPs at 24 h. The acute inflammatory reaction of the lungs recovered on day 30 with bronchoalveolar fibrosis. The concentrations of IL-4, 6, TNF-α, and eotaxin in the bronchoalveolar lavage fluid (BALF) decreased over time, and the levels of these inflammation indicators are consistent with the following inflammatory cell data and acute lung inflammation by ZnO NP. This study suggests that single inhalation exposure to functionalized ZnO NPs may cause acute lung injury with granulocytic inflammation. Although it can recover 30 days after exposure, acute pulmonary inflammation in surface functionalization means that additional studies of exposure limits are needed to protect the workers that produce it.

Keywords: nanoparticles; zinc oxide; intratracheal instillation; bronchoalveolar lavage; acute inflammation

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1. Introduction

In recent years, as the number of cases of applying nanomaterials in various industrial fields has increased, it has become a critical issue to identify the toxicity of nanomaterials accurately. Primarily, zinc oxide nanoparticles (ZnO NPs) are used in many commercial products, including food additives, cosmetics, textiles, paints, and personal hygiene products [1]. In addition, ZnO NPs are widely used as an ingredient of paints and coating and finishing materials in products and buildings because they provide long-term protection from ultraviolet light [2,3].

The increasing use of ZnO NPs has raised concerns about their potential toxicity to humans and the environment. ZnO NPs might enter the human body by various routes, including oral ingestion, nasal inhalation, intravenous injection, and transdermal delivery [4–8]. Therefore, the toxicological properties of ZnO NPs have been studied according to the different routes of exposure. Nanoparticles can translocate into the blood and various organs from the respiratory tract and further induce lesions [9]. Many studies have evaluated the toxicity of ZnO NPs in cell lines and animal models [10–13]. The inhalation of low levels of ZnO NPs causes marked changes and damage to pulmonary function in guinea pigs [14], and ZnO inhalation causes pulmonary impairment and systemic effects such as metal fume fever in humans [15].

Recently, due to the development of biomedical applications, studies for the application of nanomaterials are in progress, and surface modification of nanomaterials is

23. Publication: Evaluation of the Skin Sensitization Potential of Carbon Nanotubes Using Alternative *In Vitro* and *In Vivo* Assays

toxics

MDPI

Article

Evaluation of the Skin Sensitization Potential of Carbon Nanotubes Using Alternative In Vitro and In Vivo Assays

Sung-Hyun Kim *, Dong Han Lee, Jin Hee Lee, Jun-Young Yang, Hyo-Sook Shin, JeongPyo Lee, Kikyung Jung, Jayoung Jeong, Jae-Ho Oh and Jong Kwon Lee *

Division of Toxicological Research, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong, Cheongju 28159, Chungcheongbuk-do, Korea; donghan04@korea.kr (D.H.L.); tod98@korea.kr (J.H.L.); yangjy@korea.kr (J.-Y.Y.); aqua978@korea.kr (H.-S.S.); origene@korea.kr (J.L.); kikyung@korea.kr (K.J.); 0jjy@korea.kr (J.J.); chopin68@korea.kr (J.-H.O.)

* Correspondence: tjdgus32@korea.kr (S.-H.K.); jkleest@korea.kr (J.K.L.); Tel.: +82-43-719-5110 (S.-H.K.); +82-43-719-4351 (J.K.L.)

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Abstract: Carbon nanotubes (CNTs) are one of the major types of nanomaterials that have various industrial and biomedical applications. However, there is a risk of accidental exposure to CNTs in individuals involved in their large-scale production and in individuals who use products containing CNTs. This study aimed to evaluate the skin sensitization induced by CNTs using two alternative tests. We selected single-wall carbon nanotubes and multi-walled carbon nanotubes for this study. First, the physiochemical properties of the CNTs were measured, including the morphology, size, and zeta potential, under various conditions. Thereafter, we assessed the sensitization potential of the CNTs using the ARE-Nrf2 Luciferase KeratinoSens™ assay, an in vitro alternative test method. In addition, the CNTs were evaluated for their skin sensitization potential using the LLNA: BrdU-FCM in vivo alternative test method. In this study, we report for the first time the sensitization results of CNTs using the KeratinoSens™ and LLNA: BrdU-FCM test methods in this study. This study found that both CNTs do not induce skin sensitization. These results suggest that the KeratinoSens™ and LLNA: BrdU-FCM assay may be useful as alternative assays for evaluating the potential of some nanomaterials that can induce skin sensitization.

Keywords: skin sensitization; alternative to animal testing; KeratinoSens™; LLNA; nanomaterial; CNT

1. Introduction

Carbon nanotubes (CNTs) are a major type of nanomaterial that is used for various industrial and biomedical applications [1,2]. In recent years, with the growing number and production volume of CNTs, concerns about their toxicity have also increased exponentially. Generally, nanomaterials are defined as particles less than 100 nm in at least one dimension [3], which exhibit various physicochemical properties associated with a nanostructure [4].

The various physicochemical characteristics of a nanomaterial are the major determinants of its toxic potential [5,6]. In normal environmental conditions, nanomaterials are mostly poorly soluble; however, some nanomaterials have shown to be soluble in lysosomal fluid or gastric fluid [7,8]. Dissolution of nanomaterials can cause toxicity due to the release of ions [9].

The major exposure pathways of nanomaterials are inhalation, ingestion, and absorption into the skin. Absorption pathways within the skin can cause lesions, such as local inflammation, contact allergy, and skin sensitization [10,11]. Recently, with an exponential increase in the cosmetic commercialization

24. Publication: Evaluation of the skin sensitization potential of metal oxide nanoparticles using the ARE-Nrf2 Luciferase KeratinoSensTM assay

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ORIGINAL ARTICLE

Evaluation of the skin sensitization potential of metal oxide nanoparticles using the ARE-Nrf2 Luciferase KeratinoSens™ assay

Sung-Hyun Kim[1] · DongHan Lee[1] · JinHee Lee[1] · Jun-Young Yang[1] · JiHyun Seok[1] · Kikyung Jung[1] · JongKwon Lee[1]

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Abstract
Numerous studies have reported the potential of chemicals for inducing skin sensitization; however, few studies have examined skin sensitization induced by nanomaterials. This study aimed to evaluate skin sensitization induced by metal oxide nanoparticles (NPs) using the ARE-Nrf2 Luciferase KeratinoSens™ assay. Seven different metal oxide NPs, including copper oxide, cobalt oxide, nickel oxide, titanium oxide, cerium oxide, iron oxide, and zinc oxide, were assessed on KeratinoSens™ cells. We selected an appropriate vehicle among three vehicles (DMSO, DW, and culture medium) by assessing the hydrodynamic size at vehicle selection process. Seven metal oxide NPs were analyzed, and their physicochemical properties, including hydrodynamic size, polydispersity, and zeta potential, were determined in the selected vehicle. Thereafter, we assessed the sensitization potential of the NPs using the ARE-Nrf2 Luciferase KeratinoSens™ assay. Copper oxide NPs induced a positive response, whereas cobalt oxide, nickel oxide, titanium oxide, cerium oxide, iron oxide, and zinc oxide NPs induced no response. These results suggest that the ARE-Nrf2 Luciferase KeratinoSens™ assay may be useful for evaluating the potential for skin sensitization induced by metal oxide NPs.

Keywords Skin sensitization · Alternative to animal testing · KeratinoSens™ · Nanomaterial · Nanoparticle

Introduction

There are numerous reports regarding the potential of inducing skin sensitization using chemicals [1]; however, whether skin can be sensitized using nanoparticles (NPs) remains unclear.

It is well-known that NPs released into the environment can enter the human body and potentially cause damage to organs [2, 3]. Metal oxide NPs have been used in various applications including the industrial, electrical, pharmaceutical, and biomedical fields because of their unique physicochemical properties compared to bulk chemicals [4]. These physicochemical properties include a small size, easy dissolution, and a surface charge. While desirable, these properties can also lead to various adverse effects in humans. Their very small size, which is characteristic of NPs, increases the surface area to volume ratio, showing higher toxicity than larger particles of the same composition with a lower surface area to volume ratio [5]. Thus, it is important to analyze the physicochemical properties of NPs because they can persist in vivo [6–8].

The current knowledge on the chemical and biological mechanisms associated with skin sensitization has been summarized in the form of an adverse outcome pathway, starting with the molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis [9]. The first key event is that electrophilic chemicals in the irritant initially covalently react with nucleophilic thiol and primary amines in skin proteins. The second key event occurs within the keratinocytes and includes inflammatory responses, as well as changes in gene expression associated with specific cell signaling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways [10]. The ARE-Nrf2 Luciferase KeratinoSens™ test, representing the second key event, can be used to discriminate between skin sensitizers and non-sensitizers in accordance with the United Nations Globally Harmonized System of Classification and Labelling of Chemicals [9].

Park et al. [11] reported that titanium oxide NPs do not induce skin sensitization in mice according to a local

✉ JongKwon Lee
jklee81@korea.kr

[1] Division of Toxicological Research, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong, Korea

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25. Publication: Flow cytometric evaluation of the potential of metal oxide nanoparticles for skin sensitization using 5-Bromo-2-deoxyuridine

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ORIGINAL ARTICLE

Flow cytometric evaluation of the potential of metal oxide nanoparticles for skin sensitization using 5-Bromo-2-deoxyuridine

Dong Han Lee[1] · Sung-Hyun Kim[1] · Jin Hee Lee[1] · Jun-Young Yang[1] · Ji-Hyun Seok[1] · Kikyung Jung[1] · Jong Kwon Lee[1]

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Abstract

Although skin sensitization potential of various chemicals has been extensively studied, there are only a few reports on nanoparticles induced skin sensitization. Aiming to fill this lacuna, in this study we evaluated the potential of metal oxide nanoparticles (NPs) to induce skin sensitization with flow cytometry. Seven different metal oxide NPs, including copper oxide, cobalt oxide, nickel oxide, titanium oxide, cerium oxide, iron oxide, and zinc oxide were applied to Balb/c mice. After selecting the proper vehicle, the NPs were applied, and the skin sensitization potential were assessed using 5-bromo-2-deoxyuridine with flow cytometry. Physiochemical properties such as hydrodynamic size, polydispersity, and zeta potential were measured for the NPs prior to the tests. All the seven metal oxide NPs studied showed negative responses for skin sensitization potential. These results suggest that the OECD TG 442B using 5-bromo-2-deoxyuridine with flow cytometry can be applied to evaluate the potential of NPs for skin sensitization.

Keywords Nanomaterials · Nanoparticles · Skin sensitization · Local lymph node assay · 5-bromo-2-deoxyuridine

Introduction

The Guinea pig Maximisation Test (GPMT) has been used worldwide to evaluate the skin sensitization potential of substances [1]. The LLNA, an alternative to the conventional method that uses guinea pigs, was adopted as OECD TG 429 (Skin sensitization; Local Lymph Node Assay) in 2002 [2]. The LLNA is a skin sensitization test in which the proliferation of murine local auricular lymph node cells (LNCs) is measured after exposure of mice to test substances. However, the extensive use of LLNA has been restricted because it involves the use of radioisotope-labeled ^{3}H-methyl thymidine, mainly due to difficulties in the disposal of radioactive waste in some countries. A flow cytometry based LLNA using 5-bromo-2-deoxyuridine (LLNA: BrdU-FCM) was developed as a novel non-radioactive model of the LLNA with a performance similar to that of the conventional LLNA method. This variant of LLNA was adopted as OECD TG442B in 2018 [3].

While there are many reports of skin sensitization potential induced by chemicals [4], only few studies that investigate the skin sensitization potential of nanoparticles (NPs) are available. Since nanoparticles released into environments may enter the human body and can cause potential damage to organs [5, 6], it is pertinent to study the skin sensitization potential of different NPs to assess the risks they pose to public health.

Because of their unique physicochemical properties compared to bulk chemicals, metal oxide NPs have been used for various applications in several fields, including electrical, pharmaceuticals, and biomedical fields [7]. While their physicochemical properties such as small size, altered solubility, and higher surface charge, these properties can lead to various adverse effects on human body. Very small size, which is characteristic of nanoparticles, results in an increase in the surface area and consequently, high toxicity. Thus, small NPs are generally more toxic than large particles of the same composition [8]. Since NPs tend to remain in the body under physiological conditions, it is essential to analyze their physicochemical properties that influence their in vivo persistence [9–11].

✉ Jong Kwon Lee
jklee1@korea.kr

[1] Division of Toxicological Research, Ministry of Food and Drug Safety, National Institute of Food and Drug Safety Evaluation, 187, Osongsaengmyeong 2-ro, Osong-eup, Cheongju-si, Chungcheongbuk-do 28159, Korea

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26. Publication: Colony-Forming Efficiency Assay to Assess Nanotoxicity of Graphene Nanomaterials

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Article

Colony-Forming Efficiency Assay to Assess Nanotoxicity of Graphene Nanomaterials

Hansol Won †, Sung-Hyun Kim †, Jun-Young Yang, Kikyung Jung, Jayoung Jeong, Jae-Ho Oh and Jin-Hee Lee *

Division of Toxicological Research, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, 187, Osongsaengmyeong 2-Ro, Cheongju 28159, Korea; hanssol2@korea.kr (H.W.); tjdgus32@kakao.com (S.-H.K.); yangjy@korea.kr (J.-Y.Y.); kikyung@korea.kr (K.J.); 0jjy@korea.kr (J.J.); chopin68@korea.kr (J.-H.O.)

* Correspondence: tod98@korea.kr; Tel.: +82-43-719-5106; Fax.: +82-43-719-5100

† These authors contributed equally to this work.

Abstract: The nano-market has grown rapidly over the past decades and a wide variety of products are now being manufactured, including those for biomedical applications. Despite the widespread use of nanomaterials in various industries, safety and health effects on humans are still controversial, and testing methods for nanotoxicity have not yet been clearly established. Nanomaterials have been reported to interfere with conventional cytotoxicity tests due to their unique properties, such as light absorption or light scattering. In this regard, the colony-forming efficacy (CFE) assay has been suggested as a suitable test method for testing some nanomaterials without these color-interferences. In this study, we selected two types of GNPs (Graphene nanoplatelets) as test nanomaterials and evaluated CFE assay to assess the cytotoxicity of GNPs. Moreover, for further investigation, including expansion into other cell types, GNPs were evaluated by the conventional cytotoxicity tests including the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), Cell Counting Kit-8 (CCK-8), and Neutral red uptake (NRU) assay using MDCK, A549 and HepG2 cells. The results of CFE assay suggest that this test method for three cell lines can be applied for GNPs. In addition, the CFE assay was able to evaluate cytotoxicity regardless more accurately of color interference caused by residual nanomaterials.

Keywords: graphene; cytotoxicity; CFE; interference

Citation: Won, H.; Kim, S.-H.; Yang, J.-Y.; Jung, K.; Jeong, J.; Oh, J.-H.; Lee, J.-H. Colony-Forming Efficiency Assay to Assess Nanotoxicity of Graphene Nanomaterials. *Toxics* **2022**, *10*, 236. https://doi.org/10.3390/toxics10050236

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1. Introduction

In recent years, due to the rapid growth of nanotechnology, various manufacturing nanomaterials are being produced, and nanomaterials are used in various industries such as batteries, electrodes, cosmetics, displays and biomedical engineering [1–3]. The growth of the nano-industry affects our lives in a more prosperous manner and contributes to it by providing various benefits, however like a 'double-edged sword' it has the potential to induce human toxicity, both large and small when exposed to the body. Therefore, it is crucial to develop an accurate nanotoxicity evaluation method to understand the toxicity of these nanomaterials.

The Organization for Economic Cooperation and Development (OECD), European Union (EU) and other organizations stipulate the following for manufactured nanomaterials: 'Materials with a size less than 100 nm made for this purpose' [4,5]. As such, nanomaterials are nanoscopic in size, and the risk of nanomaterial products stem from its small size and the unique physicochemical properties of the nanomaterial. Taking the physical 'shape' as an example, carbon nanotube (CNT) nanomaterials with acicular structures such as asbestos or glass fibers have risks such as cancer-causing potential [6,7]. In addition, high surface reaction power and surface charge, due to their very small size, can

27. Publication: Skin Sensitization Evaluation of Carbon-Based Graphene Nanoplatelets

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Article

Skin Sensitization Evaluation of Carbon-Based Graphene Nanoplatelets

Sung-Hyun Kim, So-Hye Hong, Jin Hee Lee, Dong Han Lee, Kikyung Jung, Jun-Young Yang, Hyo-Sook Shin, JeongPyo Lee, Jayoung Jeong and Jae-Ho Oh *

Division of Toxicological Research, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong, Cheongju 28159, Korea; tjdgus32@korea.kr (S.-H.K.); shhong99@korea.kr (S.-H.H.); tod98@korea.kr (J.H.L.); donghan04@korea.kr (D.H.L.); kikyung@korea.kr (K.J.); yangjy@korea.kr (J.-Y.Y.); aqua978@korea.kr (H.-S.S.); origene@korea.kr (J.L.); 0jjy@korea.kr (J.J.)
* Correspondence: mfdsnc@korea.kr; Tel.: +82-43-719-5102

Abstract: Graphene nanoplatelets (GNPs) are one of the major types of carbon based nanomaterials that have different industrial and biomedical applications. There is a risk of exposure to GNP material in individuals involved in their large-scale production and in individuals who use products containing GNPs. Determining the exact toxicity of GNP nanomaterials is a very important agenda. This research aimed to evaluate the skin sensitization potentials induced by GNPs using two types of alternative to animal testing. We analyzed the physicochemical characteristics of the test material by selecting a graphene nanomaterial with a nano-size on one side. Thereafter, we evaluated the skin sensitization effect using an in vitro and an in vivo alternative test method, respectively. As a result, we found that GNPs do not induce skin sensitization. In addition, it was observed that the administration of GNPs did not induce cytotoxicity and skin toxicity. This is the first report of skin sensitization as a result of GNPs obtained using alternative test methods. These results suggest that GNP materials do not cause skin sensitization, and these assays may be useful in evaluating the skin sensitization of some nanomaterials.

Keywords: skin sensitization; alternative to animal testing; KeratinoSens™; local lymph node assay (LLNA); nanomaterial; graphene

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1. Introduction

Graphene nanoplatelets (GNPs), a two-dimensional monocrystalline layer form of carbon, are a major type of carbon-based nanomaterial which are used for various industrial and biomedical applications. Graphene has drawn attention across a vast field, such as in diverse devices or applied in batteries [1,2]. In recent years, as the types and production of GNPs have increased, concerns about toxicity caused by human exposure have increased exponentially. The major routes of exposure for nanomaterials are ingestion, inhalation and skin penetration in the workplace. Skin penetration of nanomaterials can induce lesions, contact allergy, local inflammation, and skin sensitization [3,4]. However, there are no studies on the toxicological database for GNP in skin sensitization which is the most easy exposure route in the workplace.

In addition, with the recent exponential increase in commercialization of nanomaterials for use in the cosmetic industry, etc., and the safety concerns associated with these, Nano safety evaluation has gained importance [5]. The focus on alternative methods in cosmetic testing is also increasing due to concerns regarding animal welfare and the 3Rs principle of replacement, reduction, and refinement [6,7]. However, as these guidelines are based on chemical substances, there is a need to develop alternative test methods that reflect the properties of nanomaterials.

The Organization for Economic Cooperation and Development (OECD) has suggested an adverse outcome pathway (AOP) leading to allergic contact dermatitis (ACD) starting

28. Publication: Six-well plate-based colony-forming efficacy assay and Co-Culture application to assess toxicity of metal oxide nanoparticles

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Six-well plate-based colony-forming efficacy assay and Co-Culture application to assess toxicity of metal oxide nanoparticles

Seong Han Lee [a,1], Hansol Won [b,1], Sung-Hyun Kim [b], Soyeon Jeon [a], Jiyoung Jeong [a], Dong-Keun Lee [a], Jun-Young Yang [b], Ji-Hyeun Seok [b], Kikyung Jung [b], Jae Ho Oh [b], Jin Hee Lee [b,**], Wan-Seob Cho [a,*]

[a] Lab of Toxicology, Department of Health Sciences, The Graduate School of Dong-A University, 37, Nakdong-daero 550 Beon-gil, Saha-gu, Busan, 49315, Republic of Korea

[b] Division of Toxicological Research, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong, Cheongju, 28159, Republic of Korea

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ABSTRACT

The development of a universal, label-free, and reliable *in vitro* toxicity testing method for nanoparticles is urgent because most nanoparticles can interfere with toxicity assays. In this regard, the colony-forming efficacy (CFE) assay has been suggested as a suitable *in vitro* toxicity assay for testing nanoparticles without such interference. Recently, the Organisation for Economic Co-operation and Development (OECD) developed a 60 × 15 mm Petri dish-based CFE assay for testing nanoparticles in MDCK-1 cells. However, further investigations are needed, including testing with other cell types, at a smaller scale for greater efficiency, and the application of the co-culture technique. In this study, we selected TiO_2, CuO, CeO_2, and SiO_2 as test nanoparticles and successfully developed a 6-well plate-based CFE assay using HepG2 and A549 cells and a co-culture assay for combinations of HepG2 cells and THP-1 macrophages or A549 cells and THP-1 monocytes. The results suggest that the 6-well-plate-based CFE assay for HepG2 and A549 cells can be applied to nanoparticles, but the co-culture CFE assay has limitations in that it is not different from the single culture study, and it inhibits colony-formation by A549 cells in the presence of macrophages; this warrant further study.

1. Introduction

The development of nanotechnology has concerns about the safety of nanoparticles (NPs). However, safety assessment of NPs is difficult because their toxicity varies according to physicochemical properties such as size, shape, and surface charge (Cho et al., 2010b). Hence, the quantitative evaluation of the impact of the physicochemical properties of NPs with expansion to the structure-toxicity relationship has been suggested as a strategy for safety assessment of NPs (Lamon et al., 2018; Liu et al., 2015; Zhang et al., 2012). In this regard, large sets of *in vitro* and *in vivo* toxicity studies are needed. Since animal experiments are limited due to ethical issues, the development of a high-throughput toxicity screening method dictating the *in vivo* toxicity is needed (Cho et al., 2013).

Cytotoxicity or cell viability assays are among the most basic *in vitro* assays, and can be used to determine the toxic potential of test materials. Since cytotoxicity assays generally utilize chromophores or fluorophores in the measurement, researchers should consider the interferences (optical or chemical) by NPs in the steps of such assays (Guadagnini et al., 2015; Holder et al., 2012; Kroll et al., 2012). Optical interference can occur due to light absorbance by the NPs present in the medium, and the adsorption of chromophores or fluorophores on the surface of NPs (Stone et al., 2009). Chemical interference includes interference in the enzymatic reaction of the lactate dehydrogenase (LDH) assay by copper oxide (CuO) and zinc oxide (ZnO) NPs (Aslantürk 2018; Kroll et al., 2009).

Since it is not possible to completely avoid interference by NPs in chromophore- or fluorophore-based cytotoxicity assays, there is a need

* Corresponding author. Lab of Toxicology, Department of Health Sciences, The Graduate School of Dong-A University, 37, Nakdong-daero 550 beon-gil, Saha-gu, Busan, 49315, Republic of Korea.
** Corresponding author.
E-mail addresses: tod98@korea.kr (J.H. Lee), wcho@dau.ac.kr (W.-S. Cho).
[1] These authors contributed equally.

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29. Publication: Skin Sensitization Potential and Cellular ROS-Induced Cytotoxicity of Silica Nanoparticles

nanomaterials

MDPI

Article

Skin Sensitization Potential and Cellular ROS-Induced Cytotoxicity of Silica Nanoparticles

Sung-Hyun Kim *, Dong Han Lee, SeoYoon Choi, Jun-Young Yang, Kikyung Jung, Jayoung Jeong, Jae Ho Oh and Jin Hee Lee *

Division of Toxicological Research, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong, Cheongju 28159, Korea; donghan04@korea.kr (D.H.L.); sugar819@korea.kr (S.C.); yangjy@korea.kr (J.-Y.Y.); kikyung@korea.kr (K.J.); 0jjy@korea.kr (J.J.); chopin68@korea.kr (J.H.O.)

* Correspondence: tjdgus32@korea.kr (S.-H.K.); tod98@korea.kr (J.H.L.); Tel.: +82-43-719-5110 (S.-H.K.); +82-43-719-5106 (J.H.L.); Fax.: +82-43-719-5100 (S.-H.K. and J.H.L.)

Abstract: Nowadays, various industries using nanomaterials are growing rapidly, and in particular, as the commercialization and use of nanomaterials increase in the cosmetic field, the possibility of exposure of nanomaterials to the skin of product producers and consumers is increasing. Due to the unique properties of nanomaterials with a very small size, they can act as hapten and induce immune responses and skin sensitization, so accurate identification of toxicity is required. Therefore, we selected silica nanomaterials used in various fields such as cosmetics and biomaterials and evaluated the skin sensitization potential step-by-step according to in-vitro and in-vivo alternative test methods. KeratinoSens™ cells of modified keratinocyte and THP-1 cells mimicking dendritic-cells were treated with silica nanoparticles, and their potential for skin sensitization and cytotoxicity were evaluated, respectively. We also confirmed the sensitizing ability of silica nanoparticles in the auricle-lymph nodes of BALB/C mice by in-vivo analysis. As a result, silica nanoparticles showed high protein binding and reactive oxygen species (ROS) mediated cytotoxicity, but no significant observation of skin sensitization indicators was observed. Although more studies are needed to elucidate the mechanism of skin sensitization by nanomaterials, the results of this study showed that silica nanoparticles did not induce skin sensitization.

Keywords: silica; nanoparticles; skin sensitization; KeratinoSens™; h-CLAT; LLNA; alternative test

Citation: Kim, S.-H.; Lee, D.H.; Choi, S.; Yang, J.-Y.; Jung, K.; Jeong, J.; Oh, J.H.; Lee, J.H. Skin Sensitization Potential and Cellular ROS-Induced Cytotoxicity of Silica Nanoparticles. *Nanomaterials* **2021**, *11*, 2140. https://doi.org/10.3390/nano11082140

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1. Introduction

Manufactured nanomaterials refer to materials that are created to have at least one cross-section of a size of 100 nm or less. The unique properties of nanomaterials are increasing their use value in various industries, including food and biomedical science fields. Silica nanomaterials are one of the major types of nanomaterials which are particularly used in the industrial and cosmetics fields [1]. Therefore, it is pivotal for the manufacturers and consumers who consume these substances to identify the silica nanotoxicity due to its potential for human exposure through various routes.

In recent years, to evaluate substances used in cosmetics, animal substitution test methods have been applied by reflecting the 3R (Replacement, Reduction and Refinement) principles for animals [2,3]. Specifically, the chemical and biological mechanisms related to skin sensitization such as allergic contact dermatitis have been summarized as the adverse outcome pathway (AOP) of skin sensitization [4]. The Organization for Economic Cooperation and Development (OECD) skin sensitization guidelines classify four

30. Publication: The Research of Toxicity and Sensitization Potential of PEGylated Silver and Gold Nanomaterials

toxics

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Article

The Research of Toxicity and Sensitization Potential of PEGylated Silver and Gold Nanomaterials

Dong-Han Lee †, Seo-Yoon Choi †, Ki-Kyung Jung, Jun-Young Yang, Ja-young Jeong, Jae-Ho Oh, Sung-Hyun Kim * and Jin-Hee Lee *

Division of Toxicological Research, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Cheongju 28159, Korea; donghan04@korea.kr (D.-H.L.); sugar0819@korea.kr (S.-Y.C.); kikyung@korea.kr (K.-K.J.); yangjy@korea.kr (J.-Y.Y.); 0jjy@korea.kr (J.-y.J.); chopin68@korea.kr (J.-H.O.)
* Correspondence: tjdgus32@korea.kr (S.-H.K.); tod98@korea.kr (J.-H.L.); Tel.: +82-43-719-5110 (S.-H.K.); +82-43-719-5106 (J.-H.L.); Fax: +82-43-719-5100 (S.-H.K. & J.-H.L.)
† These authors have contributed equally to this work.

Abstract: Polyethylene glycol (PEG) is a polymer used for surface modification of important substances in the modern pharmaceutical industry and biopharmaceutical fields. Despite the many benefits of PEGylation, there is also the possibility that the application and exposure of the substance may cause adverse effects in the body, such as an immune response. Therefore, we aimed to evaluate the sensitization responses that could be induced through the intercomparison of nanomaterials of the PEG-coated group with the original group. We selected gold/silver nanomaterials (NMs) for original group and PEGylated silver/gold NMs in this study. First, we measured the physicochemical properties of the four NMs, such as size and zeta potential under various conditions. Additionally, we performed the test of the NM's sensitization potential using the KeratinoSens™ assay for in vitro test method and the LLNA: 5-bromo-2-deoxyuridine (BrdU)-FCM for in vivo test method. The results showed that PEGylated-NMs did not lead to skin sensitization according to OECD TG 442 (alternative test for skin sensitization). In addition, gold nanomaterial showed that cytotoxicity of PEGylated-AuNMs was lower than AuNMs. These results suggest the possibility that PEG coating does not induce an immune response in the skin tissue and can lower the cytotoxicity of nanomaterials.

Keywords: skin sensitization; immunogenicity; alternative test; nanomaterials; polyethylene glycol; PEGylation

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1. Introduction

Polyethylene glycol (PEG) is composed of repeating ethylene glycol units and, when it is attached to a polypeptide or another molecule, the phenomenon is referred to as "PEGylation" [1]. PEGylation technique is commonly used in pharmaceuticals, performing an important role in nanoparticle (NP) stabilization [2]. NP is used to transport genes and drugs to target cells and tissues, and PEG prevents the NPs from opsonization, aggregation, and phagocytosis by altering NP's size and solubility [2]. Therefore, PEGylation protects the content of the delivery carrier from being degraded by proteolytic enzymes and swiftly eliminated by the kidneys, and upregulates the circulation time to increase the delivery efficiency [1,2]. With regard to the abovementioned benefits which overcome the biological limitations, PEGylated NPs have been applied to treatment of various diseases such as cancer, pulmonary diseases, and hepatocellular carcinoma [3–6].

Substantially, PEGylation of NPs is utilized for messenger ribonucleic acid (mRNA) vaccines of Coronavirus disease 2019 (COVID-19) [7]. The COVID-19 vaccine manufactured by Pfizer and BioN-Tech delivers mRNA, packaged with a PEGylated lipid nanoparticle (LNP) to improve the effectiveness of a vaccine [7]. Anaphylaxis has rarely been reported after vaccination, however mRNA vaccines containing PEG are known to sometimes

2 Development of mitochondria-targeted toxicity evaluation method

Supervising division	Toxicological Screening and Testing Division of NIFDS
Type	Entrusted project (led by Prof. Kyung-cheol Choi, Chungbuk National University)
Title	Development of mitochondria-targeted toxicity evaluation method (20183MFDS525)

☐ Background for the study

- Mitochondrial damage causes damages to tissues including cardiac and (or) muscle tissues that consume a lot of energy or liver tissues that are often exposed to pharmaceuticals. In fact, several drugs have been reported to cause adverse effects on liver, heart and muscle due to mitochondrial toxicity. In this sense, it is necessary to perform mitochondrial toxicity assay as a screening method in order to assure the safety of drugs.

☐ Study in brief

- Usefulness of mitochondria in development of an alternative model to animal toxicity testing
  - Mitochondria are organelles surrounded by double membrane that contain different kinds of protein and mitochondrial DNA (mtDNA) and play critical role in life and death of cells.
- **Development of mitochondria-targeted toxicity assay using cardiac muscle cell line, mouse embryonic stem cells and kidney cell line**
  - Test substances that affect cardiac muscle cells, liver and mitochondria were selected. Then, the substances were applied on cardiac muscle cell line and kidney cell line to determine mitochondrial toxicity potential by evaluating cell proliferation, active oxygen generation rate, mitochondrial toxicity and oxygen consumption rate.
  - MitoSOX^{TM} dye that enables analysis of mitochondrial peroxide volume and oxidative stress in cardiac muscle cell line was used to develop and evaluate reproducibility of the assay.
  - Test substances were applied on mouse kidney stem cells and changes in mitochondrial DNA copy number was measured.

- Study on mitochondrial-targeted toxicity evaluation method using liver cells and animal models
  - Following application of toxic substances on liver cell line, protein expression of mitochondrial electron transport chain and oxidative stress were evaluated. The model cells for evaluation of mitochondrial reactive oxygen species were established using the cell line showing high reproducibility.
  - Toxic substances were applied to mouse and liver toxicity index, mitochondrial membrane potential, reactive oxygen species, protein expression of electron transport chain and oxidative stress were evaluated.
- **Study on mitochondrial toxicity evaluation using clinical specimen**
  - Non-invasive biomarker for determination of mitochondrial toxicity was identified using clinical specimen of 17 patients with mitochondrial disease.
  - When normal control group and non-alcoholic fatty liver disease patients were divided by simple steatosis and NASH and compared, reduction in mtDNA copy number based on occurrence of ALT and diabetes was confirmed.

☐ Overview of study outcome

- **Major accomplishment:** Five mitochondrial toxicity assays for prediction of adverse effects by chemicals or drugs have been developed, and the study results were published and (or) presented in 5 SCI-grade journals and national international workshops.
- **Main outcome**
  - **Academic outcome:** publication of 5 papers in SCI-grade journals
    * 「Toxicology *in vitro*」, 「Toxicologycal Research」, 「Life Sciences」, 「BBA-Molecular Basis of 」 및 「Frantiers in Endocrinology」

☐ Excellence and advantage

- **Excellence:** Oxygen consumption rate analysis method was established by evaluating reactive oxygen generation rate based on mitochondrial activation in target organ cells, and high reproducibility of the method was confirmed. Correlation between application of chemicals and anticancer drugs and mitochondrial toxicity reaction was identified in *in vivo* animal models and clinical models.

- There has been no other study that established a Developmental neurotoxicity study method using embryonic body and analyzed accuracy and transferability of the method with varioud neurotoxic substances.

- **Advantage:** Biomarker was identified from clinical specimen of the patients with mitochondrial diseases

☐ Utilization of the outcome and its impact

- **Technical:** The study help improve public health by evaluating mitochondrial toxicity in chronic disease related to kidney using mouse embryonic stem cell-induced kidney cells, and by establishing mitochondrial-targeted toxicity assay and its scientific basis.

- **Economic and industrial:** It is assumed that diabetes and cardiovascular diseases are induced by mitochondrial damage. Introduction of mitochondrial toxicity assay in drug development will help reduce social and economic loss.

☐ Performance of the study

	Title	MFDS Performance Evaluation Standards	
		Type	Performance Indicators
1	Publication: Peripheral blood mononuclear cell mitochondrial copy number and adenosine triphosphate inhibition test in NAFLD	Acamedic accomplisment	Number of papers published in SCI-grade journal
2	Presentation: Peripheral blood mononuclear cells mitochondrial copy number and adenosine triphosphate inhibition test in non-alcoholic fatty liver disease	Building on expertise through academic accomplishment	Publication and presentation in international workshop
3	Presentation: Peripheral blood mononuclear cells mitochondrial copy number and adenosine triphosphate inhibition test in non-alcoholic fatty liver disease	Building on expertise through academic accomplishment	Publication and presentation in national workshop
4	Mitochondrial DNA copy number measurement method	Establishment of scientific basis for regulation	Number of test method development
5	Publication: Mitoregulin controls mitochondrial function and stress-adaptation response during early phase of endoplasmic reticulum stress in breast cancer cells	Acamedic accomplisment	Number of papers published in SCI-grade journal
6	Presentation: Monocarboxylate transporter 1 targeting strategy in tamoxifen-resistant breast cancer cells	Building on expertise through academic accomplishment	Publication and presentation in national workshop
7	Mitochondrial membrane potential measurement method using human liver cancer cell line	Establishment of scientific basis for regulation	Number of test method development
8	Draft Guideline on mitochondrial-targeted toxicity assay	Improvement of regulation and utilization in policy	Number of TG proposals
9	Publication: Effects of anticancer drugs on the cardiac mitochondrial toxicity and their underlying mechanisms for novel cardiac protective strategies	Acamedic accomplisment	Number of papers published in SCI-grade journal
10	Publication: Establishment of a platform for measuring mitochondrial oxygen consumption rate for cardiac mitochondrial toxicity	Acamedic accomplisment	Number of papers published in SCI-grade journal
11	Publication: Evaluation of mitochondrial oxidative toxicity in mammalian cardiomyocytes by determining the highly reproducible and reliable increase in mitochondrial superoxides after exposure to therapeutic durgs	Acamedic accomplisment	Number of papers published in SCI-grade journal
12	Presentation: An increase in MitoSOX by therapeutic drugs was highly reproducible and reliable to measure mitochondrial toxicity in mammalian cardiomyocytes	Utilization and dissemination of the study outcome	Level of mutual information exchange for technology dissemination

	Title	MFDS Performance Evaluation Standards	
		Type	Performance Indicators
13	Presentation: Analytical method to assess the oxidative stress in mouse renal stem cell mitochondria by anticancer chemicals	Building on expertise through academic accomplishment	Publication and presentation in national workshop
14	Presentation: Establishment of evaluation platform for cardiac mitochondrial toxicity using three cardiomyocyte cell lines	Building on expertise through academic accomplishment	Publication and presentation in national workshop
15	Presentation: Establishment of oxygen consumption rate as an evaluation platform for mitochondrial toxicity in diverse cardiomyocytes	Building on expertise through academic accomplishment	Publication and presentation in national workshop
16	Presentation: *In vitro* oxidative stress measuring methods to investigate the relationship between mitochondrial toxicity and kidney injury	Building on expertise through academic accomplishment	Publication and presentation in national workshop
17	Presentation: Methods to measure the oxidative stress in mouse kidney stem cell mitochondria by toxicant	Building on expertise through academic accomplishment	Publication and presentation in national workshop
18	Presentation: Novel toxicological method to investigate the increased ROS in mouse kidney stem cell mitochondria by doxorubicin	Building on expertise through academic accomplishment	Publication and presentation in national workshop
19	Presentation: Screening for mitochondrial toxicity in mouse kidney stem cell by measuring oxidative stress	Building on expertise through academic accomplishment	Publication and presentation in national workshop
20	Mitochondrial reactive oxygen species measurement method using human cardiac muscle cell line	Establishment of scientific basis for regulation	Number of test method development
21	Mitochondrial reactive oxygen species measurement method using mouse kidney cell line	Establishment of scientific basis for regulation	Number of test method development
22	Mitochondrial oxygen consumption rate measurement method using human cardiac muscle cell line	Establishment of scientific basis for regulation	Number of test method development

☐ Major accomplishment

1. Publication: Peripheral blood mononuclear cell mitochondrial copy number and adenosine triphosphate inhibition test in NAFLD

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Shanghai Jiao Tong University, China

REVIEWED BY
Ming Yang,
University of Missouri, United States
Karim Gariani,
Hôpitaux universitaires de Genève (HUG), Switzerland

*CORRESPONDENCE
Dae Won Jun
noshin@hanyang.ac.kr
Eileen L. Yoon
mseileen80@hanyang.ac.kr

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work

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Peripheral blood mononuclear cell mitochondrial copy number and adenosine triphosphate inhibition test in NAFLD

A-Hyeon Lee[1†], Ju Hee Oh[1†], Hyun Sung Kim[2], Jeong-Hun Shin[3], Eileen L. Yoon[3*‡] and Dae Won Jun[1,3*‡]

[1]Department of Translational Medicine, Hanyang University Graduate School of Biomedical Science and Engineering, Seoul, South Korea, [2]Department of Pathology, Hanyang University School of Medicine, Seoul, South Korea, [3]Internal Medicine, Hanyang University School of Medicine, Seoul, South Korea

Background and aim: Non-alcoholic fatty liver disease (NAFLD) is associated with mitochondrial dysfunction. This study aims to develop biomarkers for assessing mitochondrial dysfunction in patients with NAFLD.

Methods: Mitochondrion-associated transcriptome analysis was performed. Peripheral blood mononuclear cells obtained from patients with NAFLD (69) and healthy controls (19) were used to determine the mitochondrial DNA (mtDNA) copy number. A mitochondrial inhibition substrate test (ATP assay) was performed in HepG2 cells using the patient serum.

Results: Hepatic mRNA transcriptome analysis showed that the gene expression related to mitochondrial functions (mitochondrial fusion, apoptotic signal, and mitochondrial envelope) increased in patients with steatohepatitis, but not in those with NAFL. Gene set enrichment analysis revealed that the upregulated expression of genes is related to the pathways of the tricarboxylic (TCA) cycle and deoxyribonucleic acid (DNA) replication in patients with steatohepatitis, but not in healthy controls. The mtDNA copy number in the peripheral blood mononuclear cells was 1.28-fold lower in patients with NAFLD than that in healthy controls (P <.0001). The mitochondrial inhibition substrate test showed that the cellular adenosine triphosphate (ATP) concentration was 1.2-fold times less in NAFLD patients than that in healthy controls (P <.0001). The mtDNA copy number and mitochondrial ATP inhibition substrate test demonstrated negative correlations with the degree of hepatic steatosis, whereas the ATP concentration showed a positive correlation with the mtDNA copy number.

Conclusion: The mitochondrial copy number of peripheral blood mononuclear cells and mitochondrial ATP inhibition substrate can be used as biomarkers for assessing the mitochondrial dysfunction in patients with NAFLD.

KEYWORDS
NAFLD, mitochondrial dysfunction, mitochondria copy number, ATP, biomarker

5. Publication: Mitoregulin controls mitochondrial function and stress-adaptation response during early phase of endoplasmic reticulum stress in breast cancer cells

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Mitoregulin controls mitochondrial function and stress-adaptation response during early phase of endoplasmic reticulum stress in breast cancer cells

Munkyung Choi, Keon Wook Kang *

College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 08826, Republic of Korea

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Mitochondria-associated ER membrane
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ABSTRACT

The proper regulation of mitochondrial function is important for cellular homeostasis. Especially, in cancer cells, dysregulation of mitochondria is associated with diverse cellular events such as metabolism, redox status, and stress responses. Mitoregulin (MTLN), a micro protein encoded by *LINC00116*, recently has been reported to control mitochondrial functions in skeletal muscle cells and adipocytes. However, the role of MTLN in cancer cells remains unclear. In the present study, we found that MTLN regulates membrane potential and reactive oxygen species (ROS) generation of mitochondria in breast cancer cells. Moreover, MTLN deficiency resulted in abnormal mitochondria-associated ER membranes (MAMs) formation, which is crucial for stress adaptation. Indeed, the MTLN-deficient breast cancer cells failed to successfully resolve ER (endoplasmic reticulum) stress, and cell vulnerability to ER-stress inducers was significantly enhanced by the downregulation of MTLN. In conclusion, MTLN controls stress-adaptation responses in breast cancer cells as a key regulator of mitochondria-ER harmonization, and thereby its expression level may serve as an indicator of the responsiveness of cancer cells to proteasome inhibitors.

1. Introduction

Long non-coding RNAs (lncRNAs) are a group of RNA transcripts longer than 200 bp which do not encode proteins. Although lncRNAs do not encode proteins, they do have pleiotropic functions in mammalian cells, as reported in recent studies [1]. Several other studies have revealed that some lncRNAs, despite their name, encode small proteins shorter than 100 amino acids encoded from short open reading frames (sORFs). Those microproteins have been spotlighted as important mediators regulating diverse cellular processes [2]. Stein et al. recently demonstrated that *LINC00116* enriched in muscle-type cells encodes a 56-amino-acid microprotein named mitoregulin (MTLN) [3]. Subsequent studies further revealed that lipid metabolism and respiration in mitochondria can be controlled by MTLN in several cell types, including adipocytes and skeletal muscle cells [4–6].

Changes in mitochondrial function have been attracting attention as an important topic in cancer biology. Metabolic shifts in cancer cells, including the Warburg effect, are well-established phenomena [7]. However, mitochondrial dysfunction affects not only the characteristics of energy metabolism but also the various intracellular stress responses. Several studies have reported that dysregulation of mitochondrial function causes an imbalance in redox status, endoplasmic reticulum (ER) stress, and autophagy [8–10]. Thus, mitochondrial dysfunction may affect overall cellular status as well as energy metabolism. The influence of mitochondrial dysfunction on cellular stress responses could be explained by the active interaction between mitochondria and other intracellular organelles. Mitochondria are not solely-acting organelles but communicate with other intracellular organelles directly or indirectly. Among them, ER is one of the major interaction partners of mitochondria. The interaction between these two organelles is mainly through what are called mitochondria-associated ER membranes (MAMs), which enable direct membrane contact without fusion [11]. MAMs are involved in several intracellular processes such as calcium transfer from ER to mitochondria and maintenance of mitochondrial membrane potential (MMP) [12,13]. Moreover, MAMs affect the morphological control mechanisms of two organelles, including

Abbreviations: ARE, antioxidant response element; ATF4, activating transcription factor 4; CRE, cAMP response element; DRP1, dynamin-related protein 1; ER, endoplasmic reticulum; ETC, electron transport chain; GRP78, glucose regulatory protein 78; LncRNAs, long non-coding RNAs; MAMs, mitochondria-associated ER membranes; MMP, mitochondrial membrane potential; MTLN, mitoregulin; OMM, outer mitochondrial membrane; sORFs, short open reading frames; ROS, reactive oxygen species; TNBC, triple negative breast cancer; OXPHOS, oxidative phosphorylation; UPR, unfolded protein response.

* Corresponding author.
E-mail address: kwkang@snu.ac.kr (K.W. Kang).

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9. Publication: Effects of anticancer drugs on the cardiac mitochondrial toxicity and their underlying mechanisms for novel cardiac protective strategies

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Effects of anticancer drugs on the cardiac mitochondrial toxicity and their underlying mechanisms for novel cardiac protective strategies

Cho-Won Kim, Kyung-Chul Choi *

Laboratory of Biochemistry and Immunology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

ARTICLE INFO

Keywords:
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ABSTRACT

Mitochondria are organelles that play a pivotal role in the production of energy in cells, and vital maintenance of cellular homeostasis due to the regulation of many biochemical processes. The heart con lot of mitochondria because those muscles require a lot of energy to keep supplying blood through the circ system, implying that the energy generated from mitochondria is highly dependent. Thus, cardiomyocy sensitive to mitochondrial dysfunction and are likely to be targeted by mitochondrial toxic drugs. It ha reported that some anticancer drugs caused unwanted toxicity to mitochondria. Mitochondrial dysfunc related to aging and the onset of many diseases, such as obesity, diabetes, cancer, cardiovascular and degenerative diseases. Mitochondrial toxic mechanisms can be mainly explained concerning reactive species (ROS)/redox status, calcium homeostasis, and endoplasmic reticulum stress (ER) stress signalin toxic mechanisms of many anticancer drugs have been revealed, but more studying and understanding mechanisms of drug-induced mitochondrial toxicity is required to develop mitochondrial toxicity scr system as well as novel cardioprotective strategies for the prevention of cardiac disorders of drugs. This focuses on the cardiac mitochondrial toxicity of commonly used anticancer drugs, i.e., doxorubicin, mi trone, cisplatin, arsenic trioxide, and cyclophosphamide, and their possible chemopreventive agents th prevent or alleviate cardiac mitochondrial toxicity.

1. Introduction

In the cell, there is a power plant that turns nutrients supplied from the outside into energy that can be used, which is the mitochondria [1,2]. Mitochondria are double membrane-bound organelles of a eukaryote cell containing mitochondrial DNA (mtDNA) [3,4], which is the circular chromosome found inside mitochondria [5], and play an important role in energy generation through a breakdown of energy sources such as pyruvate, fatty acids and amino acids and oxidative phosphorylation [6,7]. They are vital to the maintenance of cellular homeostasis due to the regulation of many biochemical processes [8]. The most important function of mitochondria is the synthesis of adenosine triphosphate (ATP), an energy source directly used by cells [9]. They produce most of the supply of ATP [10], and consume about 98% of the total O_2 we breathe [11]. In the mitochondria, the glucose metabolism is completed, then pyruvate enters the mitochondria and is oxidized by O_2 to CO_2 and H_2O. This allows 15 times more ATP to be made than that produced by glycolysis alone although glycolysis occurs at a rate approximately 100 times faster [12–14]. In addition t plying cellular energy, mitochondria are involved in a variety of processes, such as mitochondrial regulation of intracellular ca concentration and signal transduction, cell differentiation, cell cell division, and cell growth [8,10,15,16].

Cells are exposed to free radicals by respiration or from an ex environment, which causes damage to the DNA base. In part mitochondria produce various types of reactive oxygen species and reactive nitrogen species (RNS) as by-products such as super hydrogen peroxide, nitric oxide, peroxynitrite, hypochlorous singlet oxygen, and hydroxyl radical through the process of respi Mitochondrial ROS mainly is generated at the electron transport located on the inner mitochondrial membrane during the proc oxidative phosphorylation [17]. Low levels of ROS play a major metabolic adaptation [18], stimulated by signals such as lysoph tidylcholine and Toll-like receptor 4, and are involved in the regu of the inflammatory responses [19,20]. However, high levels of chondrial ROS activate apoptosis or autophagy pathways tha

* Corresponding author at: Laboratory of Biochemistry and Immunology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chu 28644, Republic of Korea.
E-mail address: kchoi@cbu.ac.kr (K.-C. Choi).

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ORIGINAL ARTICLE

Establishment of a platform for measuring mitochondrial oxygen consumption rate for cardiac mitochondrial toxicity

Cho-Won Kim[1] · Hee-Jin Lee[1] · Dohee Ahn[1] · Ryeo-Eun Go[1] · Kyung-Chul Choi[1]

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Abstract
The heart has an abundance of mitochondria since cardiac muscles require copious amounts of energy for providing continuous blood through the circulatory system, thereby implying that myocardial function is largely reliant on mitochondrial energy. Thus, cardiomyocytes are susceptible to mitochondrial dysfunction and are likely targets of mitochondrial toxic drugs. Various methods have been developed to evaluate mitochondrial toxicity by evaluating toxicological mechanisms, but an optimized and standardized assay for cardiomyocytes remains unmet. We have therefore attempted to standardize the evaluation system for determining cardiac mitochondrial toxicity, using AC16 human and H9C2 rat cardiomyocytes. Three clinically administered drugs (acetaminophen, amiodarone, and valproic acid) and two anticancer drugs (doxorubicin and tamoxifen) which are reported to have mitochondrial effects, were applied in this study. The oxygen consumption rate (OCR), which directly reflects mitochondrial function, and changes in mRNA levels of mitochondrial respiratory complex I to complex V, were analyzed. Our results reveal that exposure to all five drugs results in a concentration-dependent decrease in the basal and maximal levels of OCR in AC16 cells and H9C2 cells. In particular, marked reduction in the OCR was observed after treatment with doxorubicin. The reduction in OCR after exposure to mitochondrial toxic drugs was found to be associated with reduced mRNA expression in the mitochondrial respiratory complexes, suggesting that the cardiac mitochondrial toxicity of drugs is majorly due to dysfunction of mitochondrial respiration. Based on the results of this study, we established and standardized a protocol to measure OCR in cardiomyocytes. We expect that this standardized evaluation system for mitochondrial toxicity can be applied as basic data for establishing a screening platform to evaluate cardiac mitochondrial toxicity of drugs, during the developmental stage of new drug discovery.

Keywords Mitochondrial toxicity · Mitochondrial dysfunction · Oxygen consumption rate · Cardiomyocytes

Introduction

Mitochondria are multifunctional organelles in eukaryotic cells that provide energy in the form of adenosine triphosphate (ATP), largely via the process of oxidative phosphorylation (OXPHOS) in which electrons generated by the citric acid cycle translocate to the mitochondrial respiratory complexes [1–3]. The heart is constantly pumping blood to supply oxygen and nutrients to organs in the body, and the energy required for this is mainly provided by mitochondria [4]. Similar to other muscle cells, particularly the cardiomyocytes are mainly powered by mitochondria. However, increased mitochondrial density at times results in skyrocketing of energy output [5, 6]. Considering the above indicates that the heart is sensitive to mitochondrial-targeted drugs and is vulnerable to mitochondrial dysfunction [7].

Mitochondrial dysfunction is now widely implicated in the etiology of medication-induced toxicities, and has become the primary cause of compound attrition and post-market drug withdrawals due to safety concerns [8, 9]. However, most assays currently used for mitochondrial toxicity provide limited mechanistic information. Besides, although clinical testing and genetic analysis are being achieved through various biochemical assays to confirm the diagnosis of mitochondrial diseases, there is no

Cho-Won Kim and Hee-Jin Lee have contributed equally as the first authors.

✉ Kyung-Chul Choi
kchoi@cbu.ac.kr

[1] Laboratory of Biochemistry and Immunology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 28644, Republic of Korea

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10. Publication: Establishment of a platform for measuring mitochondrial oxygen consumption rate for cardiac mitochondrial toxicity

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Evaluation of mitochondrial oxidative toxicity in mammalian cardiomyocytes by determining the highly reproducible and reliable increase in mitochondrial superoxides after exposure to therapeutic drugs

Dohee Ahn [1], Cho-Won Kim [1], Ryeo-Eun Go, Kyung-Chul Choi [*]

Laboratory of Biochemistry and Immunology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

ARTICLE INFO

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ABSTRACT

Mitochondria are important cytoplasmic elements present in eukaryotic cells, and are involved in conv energy to ATP through oxidative phosphorylation. Mitochondria are vulnerable to reactive oxygen species thereby making it imperative to evaluate the toxicity. However, existing methods that evaluate mitocho toxicity in cardiomyocytes are limited. In the current study, we aimed to determine a mitochondrial bio that measures the toxicity of mitochondria, and subsequently suggest an efficient evaluation system for ating mitochondrial-specific oxidative toxicity. To achieve this, AC16 human cardiomyocytes, H9C2 r diomyocytes were exposed to acetaminophen (AP), amiodarone hydrochloride (AMD), doxo hydrochloride (Dox), valproic acid sodium salt (Val), and (Z)-4-hydroxytamoxifen (4-OHT). Mitocho oxidative stress was determined by staining the drug-treated cells with MitoSOX™ red fluorescence dye, fo by imaging with a fluorescence microscope. All working concentrations of Dox showed increased levels fluorescence in AC16 and H9C2 cells, whereas exposure to Val did not alter the red fluorescence level cells. Considering our results, increased MitoSOX™ subsequent to drug exposure is a highly reproducib reliable method to measure the mitochondrial-specific oxidative toxicity. These results indicate that a scr system using MitoSOX™ has the potential to be applied as a reliable biomarker for determining mitocho oxidative toxicity in new drug development.

1. Introduction

Eukaryotic mitochondria are important organelles that convert energy in the body into ATP, via the TCA cycle. Thus, organs having a high density of mitochondria require voluminous energy, such as the heart, liver, and brain. It is well validated that mitochondrial dysfunction is strongly associated with heart diseases, such as heart failure (Zhou and Tian, 2018). This is because cardiomyocyte functions are mitochondria-dependent, and the cells are extremely susceptible to mitochondrial dysfunction. Furthermore, cardiomyocytes are vulnerable to cardiotoxicities, such as cardiomyopathy and heart failure caused by adverse side effects when using anticancer drugs (Upadhyay et al., 2021). Mitochondrial dysfunction is associated with the mechanism of various diseases caused by toxicity attributed to drugs (Chistiakov et al., 2018; Forbes and Thorburn, 2018; Johnson et al., 2021). However, there are currently no standardized methods capable of assessing the toxic mitochondria. It is therefore necessary to develop a new sta method that evaluates mitochondrial toxicity in cardiomyocytes.

Oxidative stress is one of the causes of mitochondrial dysfun Mitochondria are the organelles that mainly generate reactive o species (ROS) in cells, and the abnormally high rate of ROS produ results in apoptosis and causes pathological problems. Mitocho oxidative damage may cause mitochondrial outer membrane meabilization (MOMP)-induced transmembrane space protein re accelerating mechanisms that promote cell apoptosis. In add occurrence of ischemia or reperfusion injury may progress to p ability transition pore (PTP), in which the inner membrane be permeable to small molecules (Murphy, 2009). Superoxide ($O_2^{\cdot-}$) droxyl radical (·OH), and hydrogen peroxide (H_2O_2) are various generated in the respiratory process, utilizing oxygen in live cells.

* Corresponding author at: Laboratory of Biochemistry and Immunology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chu 28644, Republic of Korea.
E-mail address: kchoi@cbu.ac.kr (K.-C. Choi).
[1] These two authors contributed equally as the first authors.

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3 Development of alternative developmental neurotoxicity evaluation technology using stem cells

Supervising division	Toxicological Testing and Screening Division of NIFDS
Type	Entrusted Project (led by Prof. Ui-Bae Jung, Chungbuk National University)
Title	Development of alternative developmental neurotoxicity evaluation technology using stem cells (20183MFDS522)

☐ Background for the study

- With increasing global interest in animal welfare including OECD, Europe, the U.S., etc., test methods that are able to rapidly evaluate toxicity using stem cell gene expression based on key events of adverse outcome pathway (AOP) have been developed as alternatives to animal testing.
- In order to replace OECD TG426 that requires many laboratory animals, time and cost alternatives that are based on the AOP have been developed and it is considered necessary to establish technological basis for safety evaluation of developmental neurotoxicity in Korea.

☐ Study in brief

- In this study, an alternative method to animal testing was proposed by investigating and developing developmental neurotoxicity alternative test method using stem cells (Sox1-GFP) for safety evaluation of pharmaceuticals.
- Usefulness of stem cells in development of alternatives to animal testing for toxicity evaluation
  - Using totipotency stem cells, unlimited number of cells with the identical genetic background can be obtained, and thus toxicity comparison between similar drugs or cell reaction comparison under the identical condition and development of highly efficient screening method are possible.
- ***Sox1-GFP* stem cell:** *Sox1* is a gene that plays a critical role in development of central nerveous system. When *Sox1* is expressed, embryonic stem cells are induced to neuroectoderm. *Sox1* was used as a marker of neural precursor cell.
  - *Sox1*-GFP stem cells have been derived from *Sox1*-GFP knock-in reporter mouse

in which *Sox1* genes were replaced by enhanced green fluorescent protein (EGFP). The cells are characterized by their self-expression of GFP only in the neuroglial stage.

- **Principle of the method:** Developmental neurotoxicity potential is evaluated by measuring inhibition of differentiation to nerve cells and cell viability following application of test substances to *Sox1*-GFP stem cells.

☐ Overview of the study outcome

- **Major accomplishment:** This study produced social and scientific accomplishment. Development of developmental neurotoxicity assay using stem cells was selected as one of the best MFDSR&D projects and multiple papers based on the study were published in SCI-grade journals.

- **Main outcome**

  - **Academic outcome:** Publication of 3 papers in SCI-grade journals

    * 「International Journal of Molecular Sciences」, 「Reproductive Toxicology」, and 「Food and Chemical Toxicology」

  - **Award**

    · 2021 MFDS best R&D project

    · 2020 KSVS Autumn Workshop – best poster award

    · 2021 KALAS Winter Symposium – best poster award

  - Development of developmental neurotoxicity assay using *Sox1*-GFP stem cells

☐ Excellence and advantage

- **Excellence:** Development of a method for screening developmental neurotoxic substances using *Sox1*-GFP cells that were established by promoting differentiation of mouse embryonic stem cells into neurons

  - There has been no other study that established developmental neurotoxicity study method using embryonic body and used difference neurotoxic substances to confirm accuracy and transferability of the method.

- **Advantage:** Developmental neurotoxicity assay using Sox1-GFP can be used to simultaneously evaluate both neurotoxicity and developmental toxicity.

- **Developmental toxicity alternative method in other countries:** Embryonic Stem Cell Test of EURLECVAM and Hand1-Luc EST of JaCVAM
- **Neurotoxicity alternative methods in other countries:** Neurotoxicity assay using ReNCell CX of EPA

- **Public interest:** Reduction in use of laboratory animal leads to improvement in animal welfare
  - The existing developmental neurotoxicity assay (OECD TG 426) is a large scale animal experiment that requires use of many laboratory animals. However, developmental neurotoxicity assay using Sox1-GFP does not use laboratory animals nor involve in bioethical issues.

- **Utilization of the outcome and its impact:** Development of developmental neurotoxicity assay that can replace animal testing
  - To keep pace with the international changes that pose ban on animal testing, an alternative developmental neurotoxicity assay that uses stem cells instead of laboratory animals has been developed.
  - The developed assay can be used in safety evaluation of the pharmaceuticals that have been unclassified yet.

☐ Performance of the study

	Title	MFDS Performance Evaluation Standards	
		Type	Performance Indicators
1	Presentation: Alternative Developmental neurotoxicity test Based on Sox1-GFP mouse embryonic stem cells	Building on expertise through academic accomplishment	Number of publication and presentation in national workshop
2	Presentation: Establishment of a developmental neurotoxicant screening using Sox1-GFP mouse embyronic stem cells	Building on expertise through academic accomplishment	Number of publication and presentation in national workshop
3	Presentation: Establishment of a developmental neurotoxicity test (DNT) using Sox1-GFP mouse embryonic stem cells	Building on expertise through academic accomplishment	Number of publication and presentation in national workshop
4	Presentation: Establishment of a developmental neurotoxicity test using 46C cells	Building on expertise through academic accomplishment	Number of publication and presentation in national workshop
5	Presentation: Screening for developmental neurotoxicity test using 46C cells	Building on expertise through academic accomplishment	Number of publication and presentation in international workshop
6	urturing professional personnel with master's degree	Human resource training	Human resource training
7	Nurturing professional personnel with master's degree	Human resource training	Human resource training
8	Nurturing professional personnel with master's degree	Human resource training	Human resource training
9	Nurturing professional personnel with master's degree	Human resource training	Human resource training
10	MFDS best R&D award	social evaluation	Education and promotion material about the study outcome
11	Publication: Combined Exposure to Diazinon and Nicotine Exerts a Synergistic Adverse Effect *In Vitro* and Disrupts Brain Development and Behaviors *In Vivo*	Academic accomplishment	Number of papers published in SCI-grade journal
12	Publication: Establishment of a developmental neurotoxicity test by Sox1-GFP mouse embryonic stem cells	Academic accomplishment	Number of papers published in SCI-grade journal
13	Publication: Pre-validation of an alternative test method for prediction of developmental neurotoxicity	Academic accomplishment	Number of papers published in SCI-grade journal

	Title	MFDS Performance Evaluation Standards	
		Type	Performance Indicators
14	Presentation: Development neurotoxicant test in mouse Sox1-GFP cell	Academic accomplishment	Number of publication and presentation in national workshop
15	Presentation: Establishment of a developmental neurotoxicant screening using SOX1-GFP mouse embryonic stem cells	Academic accomplishment	Number of publication and presentation in international workshop
16	Presentation: Establishment of a developmental neurotoxicant screening using 46C cells	Academic accomplishment	Number of publication and presentation in national workshop
17	Presentation: Perinatal Octamethylcyclotetrasiloxane exposure to maternal mice disrupts brain development and behavior in offspring mice	Academic accomplishment	Number of publication and presentation in national workshop
18	Presentation: Pre-validation study of alternative developmental neurotoxicity test using Sox1-GFP cell.	Academic accomplishment	Number of publication and presentation in international workshop
19	Presentation: Study for the pre-validation of alternative development neurotoxicity test using Sox1-GFP cell	Academic accomplishment	Number of publication and presentation in national workshop
20	Presentation: Study for the pre-validation of alternative developmental neurotoxicity test using Sox1-GFP mouse stem cell	Academic accomplishment	Number of publication and presentation in national workshop
21	2020 KSVS Autumn Workshop - best workshop poster-Establishment of a developmental neurotoxicant screening using Sox1-GFP mouse embryonic stem cells	social evaluation	Education and promotion material about the study outcome
22	2021 KALAS Winter Symposium best poster award-Establishment of a developmental neurotoxicant screening using 46C cells	social evaluation	Education and promotion material about the study outcome
23	Preparation of the draft DNT test method using Sox1-GFP stem cells	Establishment of scientific basis for regulation	Number of TG development

☐ Major accomplishment

11. Publication: Combined Exposure to Diazinon and Nicotine Exerts a Synergistic Adverse Effect *In Vitro* and Disrupts Brain Development and Behaviors *In Vivo*

International Journal of *Molecular Sciences*

MDPI

Article

Combined Exposure to Diazinon and Nicotine Exerts a Synergistic Adverse Effect In Vitro and Disrupts Brain Development and Behaviors In Vivo

Bonn Lee †, Seon Mi Park †, SunHwa Jeong, KangMin Kim and Eui-Bae Jeung *

Laboratory of Veterinary Biochemistry and Molecular Biology, College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea; bonnleedvm@gmail.com (B.L.); qkrtjsal0321@naver.com (S.M.P.); tnsghk0202@naver.com (S.J.); jfun4my@gmail.com (K.K.)

* Correspondence: ebjeung@chungbuk.ac.kr; Tel.: +82-43-261-2397; Fax: +82-43-267-3150

† These authors contributed equally to this work.

Abstract: A real-life environment during pregnancy involves multiple and simultaneous exposures to toxic chemicals. Perinatal exposures to toxic chemicals have been reported to exert an inhibitory effect on mouse neural development and behaviors. However, the effect of combined exposures of organophosphate and nicotine has not been previously reported. In this study, we investigated whether a combined exposure of diazinon and nicotine can have a synergistic effect. The effects of the combined chemical exposure on cell viability and neuronal differentiation were examined using mouse Sox1-GFP cells. Additionally, mice were maternally administered 0.18 mg/kg diazinon, a no adverse effect level (NOAEL) dose, combined with 0.4, 1, and 2 mg/kg nicotine. Mice offspring underwent behavior tests to assess locomotor, depressive, cognitive, and social behaviors. Morphological change in the brain was investigated with immunolocalization. We revealed that the combined exposure to diazinon and nicotine can have a synergistic adverse effect in vitro. In addition, the chemical-treated mouse offspring showed abnormalities in motor learning, compulsive-like behaviors, spatial learning, and social interaction patterns. Moreover, 0.18 mg/kg diazinon and 2 mg/kg nicotine co-exposure resulted in an increase in tyrosine hydroxylase (TH)-positive dopaminergic neurons. Thus, the findings suggest that perinatal co-exposure to nicotine and diazinon can result in abnormal neurodevelopment and behavior, even at low-level administration.

Keywords: organophosphate; combined exposure; diazinon; nicotine; dopaminergic neuron

Citation: Lee, B.; Park, S.M.; Jeong, S.; Kim, K.; Jeung, E.-B. Combined Exposure to Diazinon and Nicotine Exerts a Synergistic Adverse Effect In Vitro and Disrupts Brain Development and Behaviors In Vivo. *Int. J. Mol. Sci.* **2021**, 22, 7742. https://doi.org/10.3390/ijms22147742

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1. Introduction

During pregnancy, maternal exposure to a specific chemical compound may result in abnormal brain development and behavior in humans and animals, and the real-life environment during pregnancy can involve multiple and simultaneous exposures to toxic chemicals, such as pesticides and cigarette smoke. In this regard, synergistic adverse effects generated by co-exposure of toxicants have attracted contemporary researchers. For example, even at an environmental-level exposure, a mixture of pesticides (varying from 0.001 to 0.004 ppm) can generate an inhibitory effect on aquatic ecosystems via oxidative stress or cholinergic inhibition [1]. Likewise, a combination of benzopyrene and lead (Pb) can produce a synergistic adverse effect on spatial learning and memory impairments by exacerbating oxidative stress [2]. In addition, co-exposure of dichlorvos and monocrotophos has resulted in more severe damage to neurons than from individual-exposures by depleting neurotransmitter levels [3]. Indeed, combined exposure to multiple substances may enhance or counterbalance the toxicity of chemicals, leading to unknown risks to animal health.

Diazinon is one of the most extensively used organophosphate pesticides for household or agriculture purposes [4]. Additionally, cigarettes represent one of the globally

12. Publication: Establishment of a developmental neurotoxicity test by Sox1-GFP mouse embryonic stem cells

Reproductive Toxicology 104 (2021) 96–105

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Establishment of a developmental neurotoxicity test by Sox1-GFP mouse embryonic stem cells

Seon Mi Park [a], Na Rae Jo [b], Bonn Lee [a], Eui-Man Jung [c], Sung Duck Lee [b], Eui-Bae Jeung [a,*]

[a] Laboratory of Veterinary Biochemistry and Molecular Biology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, 28644, Republic of Korea
[b] Department of Information and Statistics, College of Natural Sciences, Chungbuk National University, Cheongju, Chungbuk, 28644, Republic of Korea
[c] Department of Molecular Biology, College of Natural Sciences, Pusan National University, Busan, 46241, Republic of Korea

ARTICLE INFO

Handling Editor: Bal-Price Anna

Keywords:
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mESCs
46C cells
Sox1-GFP
Cell differentiation

ABSTRACT

Developmental toxicity tests have been generated by applying the embryonic stem cell tests at the European Centre for the Validation of Alternative Methods, or by using the embryoid body test in our laboratory. This study was undertaken to explore novel developmental neurotoxicity (DNT) assay, using a Sox1-GFP cell line (mouse embryonic stem cells with an endogenous Sox1-GFP reporter). The expression of Sox1, a marker for neuroepithelial cells, is detected by green fluorescence, and the fluorescence intensity is a critical factor for achieving neuronal differentiation. Sox1-GFP cells cultured for 24 h were exposed to eleven neurotoxicants and four non-neurotoxicants. CCK-8 assays were performed to determine IC_{50} values after 48 h of chemical treatment. The fluorescence intensity of GFP was measured 4 days after treating the cells, and it was observed to decrease after exposure to neurotoxicants at higher concentrations, thereby indicating that the neuronal differentiation of Sox1-GFP cells is inhibited by the chemicals. Taken together, the results obtained in this study provide a model for DNT using embryonic stem cells, which may be applied to evaluate the toxicity of new chemicals or new drug candidates.

1. Introduction

The nervous system is stimulated and give responds to the surrounding environment. The nervous system originates from the ectoderm of the three germ layers of the developing embryo [1]. In the early stages of gestation, neuroepithelial cells divide symmetrically or unsymmetrically when the neuroepithelial cells produce apical radial glial cells [2]. Any anomalies during this process result in neurodevelopmental disorders. About 10–15% newborn child has this disease [3]. Moreover, neurodevelopmental disorders such as attention deficit hyperactive disorder and autism are rising sharply [4]. This alarming number of newborn children with neurodevelopmental diseases is now currently drawing attention. In this paper, we suggest the test method for developmental neurotoxicity (DNT). Even though this topic has drawn much attention, however, the cause of these global neurodevelopmental disorders is only partially understood. It is well documented in the paper written by Grandjean and Landrigan [5]. Needham et al. suggested that 30~40 % of all neurodevelopmental disorder cases are due to genetic factors. In some cases, non-genetic environmental exposure is caused by anomalies interacting with genetically inherited tendencies. Fetuses are implanted on placenta, however, the placenta is unable to block all trespassing of varieties of environmentally harmful substances, which will consequently be transferred to the baby. Also, after born, the harmful chemicals can be transferred from mother to baby during lactation [6]. The developing human brain is also vulnerable to harmful chemicals during fetal and early childhood [7]. The brain of the baby is very vulnerable, and the amount of harmful chemicals affecting the fetus is a significantly smaller amount than that for an adult; this means that negligible amount of chemical could cause permanent brain damages to the baby and children during the developmental stage. Laboratory studies have reported more than 1000 substances to be neurotoxic to animals [5]. In the United States, Canada and Europe, more than 30,000 of the commonly used industrial chemicals have been exhibit developmental neurotoxicity [8].

Growing concerns on neurotoxicants lead to an increase in identifying developmental neurotoxicants, new guidelines have been

* Corresponding author at: Laboratory of Veterinary Biochemistry and Molecular Biology, Veterinary Medical Center and College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, 28644, Republic of Korea.
E-mail address: ebjeung@chungbuk.ac.kr (E.-B. Jeung).

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13. Publication: Pre-validation of an alternative test method for prediction of developmental neurotoxicity

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Pre-validation of an alternative test method for prediction of developmental neurotoxicity

SunHwa Jeong [a], Seon-Mi Park [a], Na Rea Jo [b], Jin-Sook Kwon [a], Jimin Lee [a], KangMin Kim [a], Seon Myeong Go [a], Lian Cai [c], Dohee Ahn [d], Sung Duck Lee [b], Sang-Hwan Hyun [c], Kyung-Chul Choi [d], Eui-Bae Jeung [a,*]

[a] Laboratory of Veterinary Biochemistry and Molecular Biology (LVBMB), College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, 28644, Republic of Korea
[b] Department of Information and Statistics (DIS), College of Natural Sciences, Chungbuk National University, Cheongju, Chungbuk, 28644, Republic of Korea
[c] Laboratory of Veterinary Embryology and Biotechnology (LVEB), College of Natural Sciences, Chungbuk National University, Cheongju, Chungbuk, 28644, Republic of Korea
[d] Laboratory of Biochemistry and Immunology (LBI), College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, 28644, Republic of Korea

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Validation
Developmental neurotoxicity
46C
Embryonic stem cell

ABSTRACT

Exposure to neurodevelopmental toxicants can cause permanent brain injury. Hance, determining the neurotoxicity of unknown substances is essential for the safety of substance. As an alternative method to animal studies, developmental neurotoxicity test (DNT) and the first discriminant function (DF) were established in previous study. This study aimed to increase the predictability of the DNT method and perform a mobility test. Two endpoints of 29 newly investigated substances were used to establish a second-generation DF (2nd GDF). As two endpoints, the half-inhibitory concentration of the cell viability (IC_{50}) was determined using a cell counting kit-8 assay. The half-inhibitory concentration of differentiation (ID_{50}) was determined by measuring the green fluorescent protein (GFP) intensity in 46C cells. The substances were treated dose-dependently to measure IC_{50} and ID_{50}. The 2nd GDF classified 29 chemicals accurately as toxic and non-toxic. Four participants of three independent laboratories were enrolled to test the mobility. The results of the test set were highly accurate in reproducibility (100% of accuracy, sensitivity, and specificity) and mobility (accuracy 93.33%, sensitivity 90.91%, and specificity 100%). In conclusion, the protocol is transferable, reproducible, and accurate. Therefore, this could be a standardizing method for determining a neurotoxicant as an alternative for animal experiments.

1. Introduction

Developmental neurotoxicity is one of the most important endpoints for the toxicity of substances (Li et al., 2019). Neurodevelopmental toxicants impair brain development, and their sequelae usually do not recover and persist for life. This study referred to Organization for Economic Co-operation and Development (OECD) test guideline 'TG 426', the US Environmental Protection Agency (EPA) Office of Chemical Safety and Pollution Prevention (OCSPP) guideline (CFR Title 40, Chapter I, Subchapter R), and 'Toxic Substances Control Act' to establish the developmental neurotoxicity test. These protocols were approved using a standardized approach to determine developmental neurotoxicity, but this method requires the sacrifice of large number of lab animals. Moreover, the use of animals is a time-consuming process that is restricted because of ethical issues (Fritsche et al., 2018). Several alternative methods for a laboratory animal study have been suggested to settle these issues. In humans and animals, such as rodents, embryonic stem cells (de Leeuw et al., 2020), inducible progenitor stem cells (Hofrichter et al., 2017a), neural progenitor (Masjosthusmann et al., 2019), and primary neural cells (Hofrichter et al., 2017a) neurotoxicities, were investigated by measuring the cell viability (Masjosthusmann et al., 2019) and neural cell differentiation (de Leeuw et al., 2020; Hofrichter et al., 2017a). In addition, the use of mouse embryonic stem cells genetically modified with the *Sox1* (SRY-box transcription factor 1) gene reporting system marked green fluorescent protein (GFP) gene was also recommended (Park et al., 2021). These were visualized as green

* Corresponding author. Laboratory of Veterinary Biochemistry and Molecular Biology, Veterinary Medical Center and College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, 28644, Republic of Korea.
E-mail address: ebjeung@chungbuk.ac.kr (E.-B. Jeung).

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4 Study on evaluation of drug interaction using big data and machine learning

Supervising Division	Pharmacological Research Division of NIFDS
Type	Entrusted project
Title	Study on evaluation of drug interaction using big data and machine learning (19182MFDS407)

☐ Background for the study

- Drug interaction can be the cause of negative clinical outcome including increase in occurrence of adverse effects or reduction in drug efficacy.
  - It was reported that, of all combination of drugs on the market, about 10% of drug interaction has yet been identified.
- When approving new drugs, MFDS requires to label as much drug interaction information as possible by performing as many drug interaction clinical trials as possible.
  - Clinical trials demand a lot of time and cost, and a system that is able to predict drug interaction results using algorithm is needed.

☐ Study in brief

- Development of algorithm for prediction of drug interaction using information on drugs and authorization
  - Prediction of interaction between drugs, food ingredients and drug metabolizing enzyme (CYP450, etc.)
  - Prediction of Korean-specific drug interaction and adverse effects based on gene information of Koreans
- Establishment of a platform for prediction of drug interaction based on machine learning
  - Prediction model for predicting effects of drug interaction on absorption, distribution, metabolism and excretion
  - Prediction model for predicting drug-induced toxicity and adverse effects caused by drug interaction
  - Prediction model for predicting CYP 450 reactions at a molecular level and drug metabolism derived from genetic differences between Koreans and Westerners

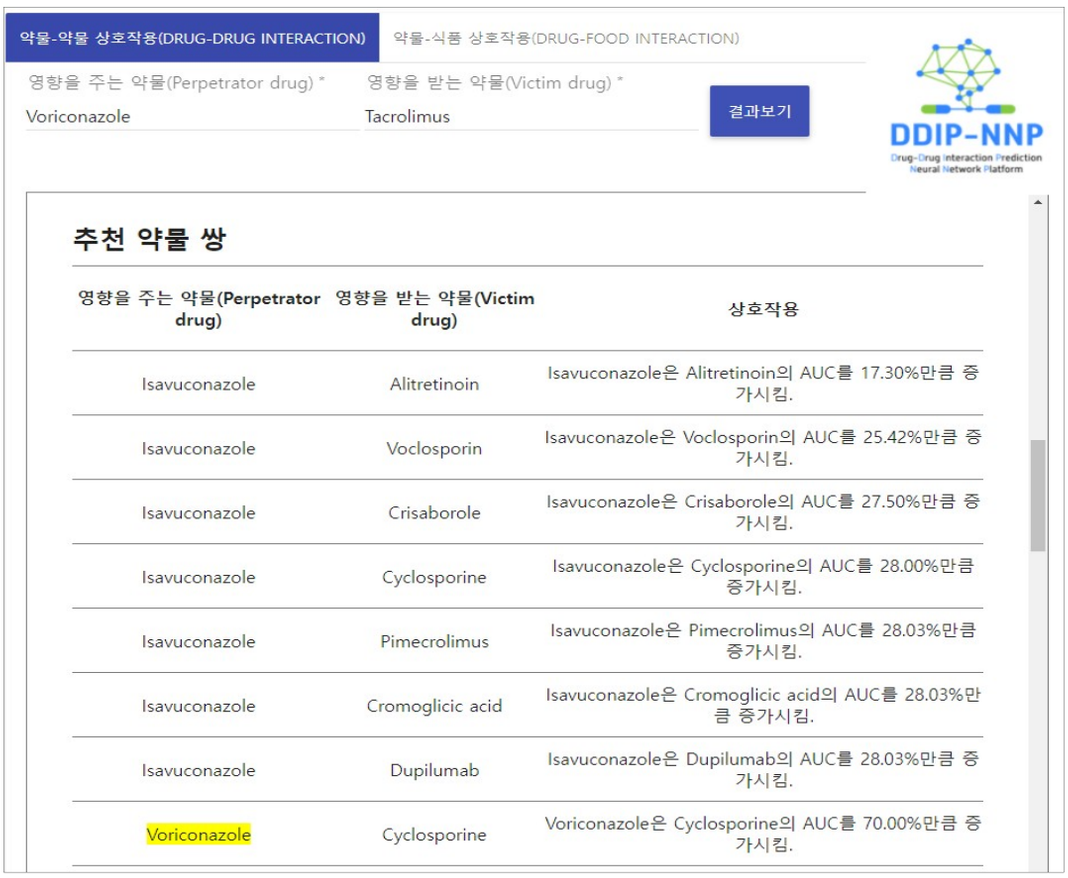
- Establishment of big data-based platform for evaluation of drug interaction
  - Establishment of DM model for prediction of interaction between drugs and foods
  - Suggestion of combination and alternative drugs that would reduce adverse drug reactions
  - Establishment of prediction model for drug metabolism and active metabolic pathways at a network level based on Korean-specific genetic information
  - Evaluation of the platform using CDW dig data of medical records of hospitals

☐ Overview of the study outcome

- Establishment of prediction model of interaction between drugs and between food ingredients
  - DDIP – NNP (Drug to drug interaction prediction neural network platform) system has been established.
    * Target: regulatory body, pharmaceutical companies developing drugs, clinical experts (e.g. physicians and pharmacists), etc.
    * Where to use
    - To predict interaction of new drugs being developed
    - To identify and (or) predict interaction between drugs taken by patients in clinical practice
    - To predict interaction of drugs that have been developed, but rarely have known interaction due to lack of clinical evidence

DDIP-NNP
Drug-Drug Interaction Prediction
Neural Network Platform

- Prediction of appropriate pharmaceutical and adequate dose volume in Korean based on drug interaction
- Suggestion of combination and alternative drugs that reduce drug adverse effects



영향을 주는 약물(Perpetrator drug)	영향을 받는 약물(Victim drug)	상호작용
Isavuconazole	Alitretinoin	Isavuconazole은 Alitretinoin의 AUC를 17.30%만큼 증가시킴.
Isavuconazole	Voclosporin	Isavuconazole은 Voclosporin의 AUC를 25.42%만큼 증가시킴.
Isavuconazole	Crisaborole	Isavuconazole은 Crisaborole의 AUC를 27.50%만큼 증가시킴.
Isavuconazole	Cyclosporine	Isavuconazole은 Cyclosporine의 AUC를 28.00%만큼 증가시킴.
Isavuconazole	Pimecrolimus	Isavuconazole은 Pimecrolimus의 AUC를 28.03%만큼 증가시킴.
Isavuconazole	Cromoglicic acid	Isavuconazole은 Cromoglicic acid의 AUC를 28.03%만큼 증가시킴.
Isavuconazole	Dupilumab	Isavuconazole은 Dupilumab의 AUC를 28.03%만큼 증가시킴.
Voriconazole	Cyclosporine	Voriconazole은 Cyclosporine의 AUC를 70.00%만큼 증가시킴.

〈Screenshot of suggested drug pairs when drug to drug interaction is searched〉

- Establishment of prediction model for predicting drug metabolism and active metabolic pathways at a network level based on Korean-specific genetic information
- Evaluation of platform using CDW big data of medical records of hospitals

〈Providing information on proteins involving in interaction 〉

☐ Excellence and advantage

- Development of a system for prediction of drug interaction based on accumulated medical big data
  - Development of machine learning algorithm can be used as an independent technology specialized in pharmaceutical sector, and can also be utilized in other drug related projects.
    * A new technology to generate valuable data by integrating and editing information related to drug interaction has been obtained.
  - The drug interaction prediction system has higher accuracy and predictivity as it is built based on medical-related big data accumulated for the past.
    * In particular, it can be used as a major part of the AI drug administration proposal system in conjunction with other algorithms that help precise customization of drug administration when building prediction and evaluation models that reflect characteristics of Koreans.

☐ Utilization of the outcome and its impact

- Providing personalized medication that minimizes adverse effects
  - The quality of medical services and for the general public has enhanced. This contributes to improving public health and quality of life, and thereby satisfaction of the people.
- Medical professionals are able to provide medication-related services more accurately.
  - Medical professionals would be constantly exposed to expert knowledge by using the platform and this can hel reduce the time and cost required for repeated education and training.
- Prioritization of drug interaction information considering the clinical situation can reduce unnecessary notification.
  - The platform can become more trustworthy by the medical professionals. Also, it can reduce unnecessary fear of adverse effects among the public and correct the understanding of drugs in the long term.

☐ Performance of the study

	Title	MFDS Performance Evaluation Standards	
		Type	Performance Indicators
1	Patent: 10-2022-0119792 Pharmacokinetic change prediction device and method	Intellectual property	Number of patent applications
2	Publication: An annotated corpus from biomedical articles to construct a drug-food interaction database	Building on Expertise through academic accomplishment	Number of papers published in SCI-grade journals
3	Publication: Machine learning-based quantitative prediction of drug exposure in drug-drug interactions using drug label information	Building on Expertise through academic accomplishment	Number of papers published in SCI-grade journals

☐ Major accomplishment

2. Publication: An annotated corpus from biomedical articles to construct a drug–food interaction database

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An annotated corpus from biomedical articles to construct a drug-food interaction database

Siun Kim [a,b], Yoona Choi [a,b], Jung-Hyun Won [b,c], Jung Mi Oh [d,1,*], Howard Lee [a,b,c,e,f,1,*]

[a] Department of Applied Biomedical Engineering, Graduate School of Convergence Science and Technology, Seoul National University, Seoul, Korea
[b] Center for Convergence Approaches in Drug Development, Graduate School of Convergence Science and Technology, Seoul National University, Seoul, Korea
[c] Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul, Korea
[d] College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Korea
[e] Department of Clinical Pharmacology and Therapeutics, Seoul National University College of Medicine and Hospital, Seoul, Korea
[f] Advanced Institute of Convergence Technology, Suwon, Korea

ARTICLE INFO

Keywords:
Biomedical corpora
Drug interaction
Drug-food interaction
Natural language processing
Information extraction

ABSTRACT

Motivation: While drug-food interaction (DFI) may undermine the efficacy and safety of drugs, DFI detection has been difficult because a well-organized database for DFI did not exist. To construct a DFI database and build a natural language processing system extracting DFI from biomedical articles, we formulated the DFI extraction tasks and manually annotated texts that could have contained DFI information. In this article, we introduced a new annotated corpus for extracting DFI, *the DFI corpus*.

Results: The DFI corpus contains 2270 abstracts of biomedical articles accessible through PubMed and 2498 sentences that contain DFI and/or drug-drug information (DDI), a substantial amount of information about drug/food entities, evidence-levels of abstracts and relations between named entities. BERT models pre-trained on the biomedical domain achieved a F1 score 55.0% in extracting DFI key-sentences. To the best of our knowledge, the DFI corpus is the largest public corpus for drug-food interaction.

Availability and implementation: Our corpus is available at https://github.com/ccadd-snu/corpus-for-DFI-extraction.

1. Introduction

Drug interaction occurs when the exposure to a drug (or *victim*) or its efficacy and/or safety is affected by another substance (or *perpetrator*) consumed together with the drug. Perpetrators include, but are not limited to, another drug, food, beverage, or a chemical. Drug interaction may increase or decrease the activity of the victim drug. For example, grapefruit can increase the blood pressure-lowering effect of some antihypertensive when ingested together [1]. In contrast, omeprazole can reduce the plasma levels of co-administered nelfinavir, an antiviral agent to treat human immunodeficiency virus, leading to the development of viral resistance [2,3].

Of various types of drug interactions, those with another drug (drug-drug interaction, DDI) or food (drug-food interaction, DFI) are clinically important because DDIs and DFIs can often be avoided if they were recognized beforehand. Drug interactions can be identified at various stages of drug development, post-marketing pharmacovigilance, and routine clinical use through prospective systematic investigations or by accident. No matter how drug interactions are identified, a drug interaction database can assist clinicians to choose a set of medications, which can be safely co-administered to avoid harmful drug interactions.

A natural language processing (NLP) system that automatically extracts drug interaction information from biomedical texts can facilitate the construction of a drug interaction database. For example, several DDI extraction NLP models developed on the DDIExtraction 2013 corpus have shown good performance in extracting and classifying DDIs [4–6]. A similar NLP model to extract DFIs from scientific literature was trained on the DDIExtraction 2013 corpus on the assumption that the structure of DDI-describing sentences is close to that of DFI-describing ones [7]. This assumption, however, may not be substantiated given the

* Corresponding authors at: Department of Clinical Pharmacology and Therapeutics, Seoul National University College of Medicine and Hospital, 103 Daehak-ro, Jongno-gu, Seoul 110-799, Korea (H. Lee) College of Pharmacy, Seoul National University, 1, Gwanak-ro, Gwanak-gu, Seoul 08826, Korea (J.M. Oh).
E-mail addresses: jmoh@snu.ac.kr (J. Mi Oh), howardlee@snu.ac.kr, shiuhn95@snu.ac.kr (H. Lee).
[1] These authors are co-corresponding authors.

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2. Publication: An annotated corpus from biomedical articles to construct a drug–food interaction database

npj | digital medicine

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ARTICLE OPEN

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Machine learning-based quantitative prediction of drug exposure in drug-drug interactions using drug label information

Ha Young Jang[1,5], Jihyeon Song[2,5], Jae Hyun Kim[3], Howard Lee[4], In-Wha Kim[1], Bongki Moon[2✉] and Jung Mi Oh[1✉]

Many machine learning techniques provide a simple prediction for drug-drug interactions (DDIs). However, a systematically constructed database with pharmacokinetic (PK) DDI information does not exist, nor is there a machine learning model that numerically predicts PK fold change (FC) with it. Therefore, we propose a PK DDI prediction (PK-DDIP) model for quantitative DDI prediction with high accuracy, while constructing a highly reliable PK-DDI database. Reliable information of 3,627 PK DDIs was constructed from 3,587 drugs using 38,711 Food and Drug Administration (FDA) drug labels. This PK-DDIP model predicted the FC of the area under the time-concentration curve (AUC) within ± 0.5959. The prediction proportions within 0.8–1.25-fold, 0.67–1.5-fold, and 0.5–2-fold of the AUC were 75.77, 86.68, and 94.76%, respectively. Two external validations confirmed good prediction performance for newly updated FDA labels and FC from patients'. This model enables potential DDI evaluation before clinical trials, which will save time and cost.

npj Digital Medicine (2022)5:88 ; https://doi.org/10.1038/s41746-022-00639-0

INTRODUCTION

A drug-drug interaction (DDI) occurs when the pharmacokinetics (PK) or pharmacodynamics (PD) of the victim drug is changed by a perpetrator drug previously taken or administered in combination. DDIs may lead to products' withdrawal from the market. For instance, astemizole, a drug for the treatment of allergic symptoms, was withdrawn from the market due to the possibility of prolongation of the QT interval and arrhythmias when combined with cytochrome P450 3A4 (CYP3A4) inhibitors, including grapefruit juice and erythromycin[1]. Mibefradil, a treatment for hypertension and chronic angina, was withdrawn from the market due to bradycardia and rhabdomyolysis when combined with various cardiovascular drugs, such as beta-blockers or statins[2]. Likewise, DDIs have been studied as one of the causes of severe adverse reactions occurring in clinical settings[3,4]. Furthermore, the increasing trend of multi-drug prescriptions increases the possibility of side effects due to DDIs[5].

However, despite this importance, numerous DDIs exist, but have not been identified. What is worse, approximately 10% of DDI pairs may have adverse reactions due to DDIs among all combinations of commercially available drugs[6]. This is because, first, the Food and Drug Administration (FDA) recommends that a clinical trial for DDIs be conducted when drugs affect only, or are affected by, a specific enzyme in an in-vitro study[7]. High costs and time-consuming clinical trials may be part of the reason for the limited number of known DDIs. Second, the mechanisms by which DDIs occur are very diverse, and each mechanism may be complex, so not all potential DDIs may be detected.

Various machine learning techniques have been developed to predict DDIs to overcome the lack of known DDI pairs. In previous studies[8–42], many models have been developed to predict the presence or absence of DDIs, discovering DDI pairs that cause side effects, or classifying the types of DDIs using open source databases (DBs). However, there are clear limitations. First, most models have only provided a simple prediction for the existence or classification of DDIs. These models do not aid in complex clinical decisions, such as precise dose adjustment or alternative drug selection. Predictions about fold change of PK parameters are needed to help physicians and pharmacists, but, to date, there are no models that have been successful in predicting this. Second, a systematically constructed true-negative dataset does not exist. The DDI DB, such as DrugBank, widely used for DDIs prediction, contains information that 'there is a DDI between drug A and B', but does not contain information that 'there is no DDI'. As a result, researchers inevitably have selected random sets of drug pairs thinking there were no interactions[9,11,19,20,28–32,34,37,42,43]. Certainly, absence of evidence is not evidence of absence. Using a model without good input makes it difficult to obtain reliable output. If the negative set is random, it is difficult to identify the exact cause when unexpected problematic output occurs.

Therefore, a sufficient amount of DDI information containing fold change of PK parameters was collected by hand search from FDA drug labels for high model performance and a reliable PK-DDI DB was constructed (Fig. 1). Using this data, a PK DDI prediction model (PK-DDIP model) is proposed that quantitatively predicts the fold change of drug PK parameters in DDIs. In addition, a standalone application, which provides predicted fold changes and reported fold changes of PK parameters, anatomical therapeutic chemical (ATC) code-based alternative drug choices, and single nucleotide polymorphism (SNP) action information was distributed.

[1]College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Republic of Korea. [2]Department of Computer Science and Engineering, Seoul National University, Seoul, Republic of Korea. [3]School of Pharmacy, Jeonbuk National University, Jeonju, Republic of Korea. [4]Department of Clinical Pharmacology and Therapeutics, Seoul National University College of Medicine and Hospital, Seoul, Korea. [5]These authors contributed equally: Ha Young Jang, Jihyeon Song.
✉email: bkmoon@snu.ac.kr; jmoh@snu.ac.kr

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npj

5 Study on establishment of big data-based model for prediction of drug cardiovascular safety

Supervising Division	Pharmacological Research Division of NIFDS
Type	Entrusted project
Title	Study on establishment of big data-based model for prediction of drug cardiovascular safety (19182MFDS406)

☐ Background for the study

- Drug adverse reactions in cardiovascular system are integral part in hazard evaluation, and many drugs have been withdrawn from the market due to their potential to cause deep vein thrombosis

  * New drugs applied for approval are evaluated using safety pharmacology test methods

- New drugs have been commercialized after non-clinical and clinical studies in accordance with international guidelines, but issues related to false negatives or false positives are persisting.

  - Cardiotoxicity evaluation using animal models have limitations including variability caused by experimental environment and interspecies differences.

☐ Study in brief

- Selection of arrhythmia drugs based on big data and development of prediction algorithm

  - Establishment of clinical big data for prediction of drug safety in cardiovascular system

  - Development of clinical big data-based algorithm for prediction of cardiovascular safety

- Preparation of non-clinical test method for verification of cardiovascular safety prediction platform

  - Establishment and validation of *in vitro* cardiovascular hazard evaluation model: Multi electrode array using hiPS-CMs

  - Validation of *in vivo* cardiovascular hazard evaluation model: Telemetry using beagle dogs

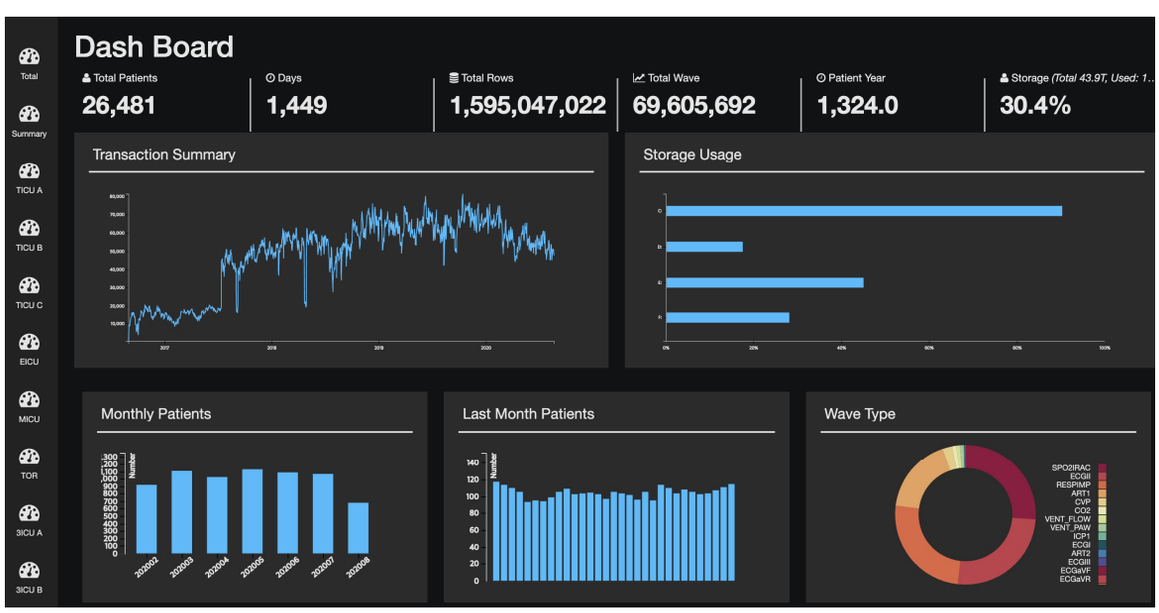
- Validation of big data-based model for prediction of cardiovascular safety

  - Validation of prediction rate using non-clincal model for evaluation of cardiovascular hazard

- Establishment of a single ion channel measuring assay for major channels related to cardiac action potential
- Obtention of data on evaluation of single channel effect induced by drugs
- Obtention of non-clinical data-based prediction model for deciding QT extension in clinical situation

☐ Overview of the study outcome

- Establishment of a platform for prediction of effects on clinical ECG using *in vitro* single ion channel assay
- Establishment of clinical biological signal big data for prediction of cardiovascular safety
  - Clinical electrocardiogram data and electronic medical records have been collected to create database.
    * As for standard 12-lead ECG, 1,040,752 ECG data from 447,632 people have been collected.



〈Dashboard of Ajou University Hospital in October 2020 showing statistics of patient biological signal collection〉

- Establishment of an active cardiovascular risk monitoring system and dashboard based on clinical big data
  - A total opf six tabs* are configured to display overall data collection and provide guidance and contextual monitoring systems
    * Dashboard, Tutorial, Standard 12-lead ECG, ICU continuous ECG, Patient specific, and pre-clinical risk evaluation

〈Dashboard〉

☐ Excellence and advantage

- Establishment of clinical biological signal big data for prediction of cardiovascular safety
  - By utilizing the data of single cardiac ion channel experiment in non-clinical stage, risk of extending QT interval in clinical stage can be predicted during new drug development.
    * This is expected to reduce cost and complexity of clinical trials by helping set the direction and scale of the trials
- An active cardiovascular risk monitoring system and dashboard based on clinical big data
  - Safe medical environment would be created thanks to early detection of drugs with deep vein thrombosis risk and timely countermeasures.
    * Through commercialization, it can be utilized as a decision support system when physicians prescribe drugs with cardiac toxicity risks

☐ Utilization of the outcome and its impact

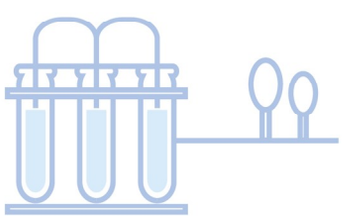
- Improvement of national health care by enabling monitoring and early screening of potential cardiovascular threats caused by drugs prescribed frequently as well as analysis of the causes
- Utilization of a big data-based cardiovascular safety prediction platform during new drug development that requires a lot of cost

〈Example of the use of a clinical data-driven cardiovascular safety prediction model 〉

- Minimizing trial errors caused by interspecies differences, reducing cost for new drug development and improving the competitiveness of national pharmaceutical industry.

☐ Performance of the study

	Title	MFDS Performance Evaluation Standards	
		Type	Performance Indicators
1	Publication: Data-driven drug-induced QT prolongation surveillance using adverse reaction signals derived from 12-lead and continuous electrocardiogram data	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journals
2	Publication: Risk of QT prolongation through drug interactions between hydroxychloroquine and concomitant drugs prescribed in real world practice	Building on expertise through academic accomplishment	Number of papers published in SCI-grade journals
3	Publication: Development of a Risk Score for QT Prolongation in the Intensive Care Unit Using Time-Series Electrocardiogram Data and Electronic Medical Records	Building on expertise through academic accomplishment	Number of papers published in non-SCI-grade journals



☐ Major accomplishment

1. Publication: Data-driven drug-induced QT prolongation surveillance using adverse reaction signals derived from 12-lead and continuous electrocardiogram data

PLOS ONE

RESEARCH ARTICLE

Data-driven drug-induced QT prolongation surveillance using adverse reaction signals derived from 12-lead and continuous electrocardiogram data

Byung Jin Choi[1☯], Yeryung Koo[2☯], Tae Young Kim[2], Hong-Seok Lim[3], Dukyong Yoon[2,4,5*]

1 Department of Biomedical Informatics, Ajou University School of Medicine, Suwon, Gyeonggi-do, Republic of Korea, **2** BUD.on Inc, Jeonju, Jeollabuk-do, Republic of Korea, **3** Department of Cardiology, Ajou University School of Medicine, Suwon, Gyeonggi-do, Republic of Korea, **4** Department of Biomedical Systems Informatics, Yonsei University College of Medicine, Yongin, Gyeonggi-do, Republic of Korea, **5** Center for Digital Health, Yongin Severance Hospital, Yonsei University Health System, Yongin, Gyeonggi-do, Republic of Korea

☯ These authors contributed equally to this work.
* dukyong.yoon@yonsei.ac.kr

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Data Availability Statement: Data for drugs known to prolong QT interval can be downloaded at CredibleMeds (https://www.crediblemeds.org/). Data for analysis on the 12-lead ECGs is based on

Abstract

Drug-induced QT prolongation is one of the most common side effects of drug use and can cause fatal outcomes such as sudden cardiac arrest. This study adopts the data-driven approach to assess the QT prolongation risk of all the frequently used drugs in a tertiary teaching hospital using both standard 12-lead ECGs and intensive care unit (ICU) continuous ECGs. We used the standard 12-lead ECG results (n = 1,040,752) measured in the hospital during 1994–2019 and the continuous ECG results (n = 4,835) extracted from the ICU's patient-monitoring devices during 2016–2019. Based on the drug prescription frequency, 167 drugs were analyzed using 12-lead ECG data under the case-control study design and 60 using continuous ECG data under the retrospective cohort study design. Whereas the case-control study yielded the odds ratio, the cohort study generated the hazard ratio for each candidate drug. Further, we observed the possibility of inducing QT prolongation in 38 drugs in the 12-lead ECG analysis and 7 drugs in the continuous ECG analysis. The seven drugs (vasopressin, vecuronium, midazolam, levetiracetam, ipratropium bromide, nifedipine, and chlorpheniramine) that showed a significantly higher risk of QT prolongation in the continuous ECG analysis were also identified in the 12-lead ECG data analysis. The use of two different ECG sources enabled us to confidently assess drug-induced QT prolongation risk in clinical practice. In this study, seven drugs showed QT prolongation risk in both study designs.

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Translator	Minhee Cha (Toxicological Testing and Screening Division)
Cantact us	Toxicological Testing and Screening Division of NIFDS Tel: 043-719-5155 Fax: 043-719-5150
Address	National Insititute of Food and Drug Safety at Ministry of Food and Drug Safety
	187, Osongsaengmyeong 2-ro, Osong-eup, Heungdeik-gu, Cheongju-si, Cheungcheongbuk-do, Republic of Korea