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ORIGINAL PAPER

In vitro estimation of metal-induced disturbance in chicken gut-oviduct chemokine circuit

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Abstract

Backgrounds: Heavy metals affect various processes in the embryonic development. Embryonic fibroblasts (EFs) play key roles in the innate recognition and wound healing in reproductive tissues.
Methods: Based on the relative toxicities of different inorganic metals and inorganic nonmetallic compounds against murine and chicken EF cells, mechanistic estimations were performed based on transcriptomic analyses.
Results: Lead (II) acetate induced preferential injuries in the chicken EF and mechanistic analyses using transcriptome revealed that chemokine receptor-associated events are potently involved in metal-induced adverse actions. As an early sentinel of metal exposure, the precision-cut intestine slices (PCIS) induced the expression of chemokines including CXCLi1 or CXCLi2, which were potent gut-derived factors that activate chemokine receptors in reproductive organs after circulation.
Conclusion: EF-selective metals can be estimated to trigger the chemokine circuit in the gut-reproductive axis of chickens. This *in vitro* methodology using PCIS-EF culture could be used as a promising alternate platform for the reproductive immunotoxicological assessment.

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Introduction

Inorganic heavy metals flow into soil and aquatic environments, where they cause long-term eco-toxicological effects[1]. Non-essential heavy metals such as lead (Pb) that are ingested via drinking water and food can accumulate and pose chronic toxic risks to the liver, kidney, brain, blood and reproductive system[2,3]. Some heavy metals that are widely distributed in aqueous and soil environments, such as inorganic arsenic (iAs), are potent human carcinogens. The gastrointestinal tract is the first line of exposure to metals in food and drinking water. Lead potently suppresses feed efficiency and growth performance in chickens via downregulation of major nutrient transporter genes during oxidative stress responses in the small intestine[4]. Moreover, barrier injuries such as epithelial cell death or leakage as a result of junctional disruption allow more exposure to luminal hazardous xenobiotics, including metals, in the intestine and subsequent translocation to other target organs[5-8]. In particular, adverse impacts of non-essential heavy metals including lead on reproductive organs have been extensively addressed in mammals and fish[9,10]. In addition to the toxic effects of lead on gametes and suppressed spermatocyte production, ovarian development is retarded, resulting in a decreased number of oviposited eggs, increased interspawn periods, and suppressed embryo development[10-12]. Moreover, egg production is decreased in hens receiving lead in feed from the day of hatch and after they have reached sexual maturity[13].

The embryo as an alternative approach to pregnant mammalian exposure is a well-addressed animal model for developmental, pharmacological, and toxicological

studies[14-17]. In particular, embryonic fibroblasts (EFs) provide sentinels to the shift of niche in reproductive organs during embryonic development[18]. Therefore, EFs are often used as feeders of embryonic stem cells. Moreover, induced pluripotency in chicken EF can generate germ cells and facilitate their optimal survival and migration to embryonic gonads via the chemokine receptor-linked pathway[19,20]. In addition to impacts on egg development, EF can play key roles in the innate immunological recognition and tissue repair following infection and inflammatory insults to the reproductive organs[21-23]. In the present study, EFs were used to compare the relative toxicities of inorganic heavy metals, which are known to contaminate mammalian and avian diets as well as drinking water. Changes in gene profiles in chicken embryonic fibroblasts exposed to metals was potently associated with early gastrointestinal exposure. The approach described herein will provide new insight into alternative models for estimation of coupling adverse events between early exposure and target organ toxicity using the *in vitro* transcriptome-based platform.

Materials & Methods

Cell culture and chemicals

The chicken embryonic fibroblast line, DF-1 (also called UMNSAH or ATCC CRL-12203^{TM}) was kindly provided by Jae Yong Han (Seoul national university, Seoul, Korea). The mouse embryonic fibroblast-like cell line, 3T3-L1, was purchased from the American Type Culture Collection (ATCC CL-173^{TM}, Rockville, MD, USA). Cells were maintained in DMEM medium [supplemented with 10% [v/v] heat-inactivated FBS, 50 U/mL penicillin, and 50 mg/mL streptomycin, all of which were purchased from Welgene (Daegu, Korea)] in a 5% CO_2 humidified incubator at 37°C. Mercury (II) chloride, cadmium acetate, cadmium chloride, copper (II) sulfate, lead (II) acetate trihydrate, sodium fluoride, sodium selenite, and arsenic trioxide were acquired from Sigma-Aldrich.

MTT assay

Viability of 3T3L1 cells and DF-1 cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay after various treatments. Cells were seeded at densities of 5×10^3 cells/well in 96-well plates until 70% to 80% confluence. Subsequently, cells were exposed to various concentrations of heavy metals for 24 h. Next, MTT (0.5 mg/mL) in phosphate buffered saline was added and cells were incubated for an additional 4 h at 37°C. At the end of the incubation period, the medium containing MTT was removed, and the formazan crystals obtained were lysed in 200 μL dimethyl sulfoxide (DMSO). Finally, the optical density was measured using a microplate reader at 490 nm (VersaMax, Molecular Devices, San Jose, CA, USA) and the cell viability was expressed as a percentage of the control culture (100%).

DNA microarray

Total RNA was extracted using RiboEX (GeneAll Biotechnology) according to the manufacturer's instructions. 2 μg of total RNA were primed with 2 μL of 100 μM random primer at 70°C for 10 min, then reversed transcribed at 42°C for 1 h in the presence of 400 U SuperScript II RTase (Invitrogen, Carlsbad, CA, USA), and 100 μM each dATP, dTTP, dGTP, with 25 μM dCTP, 25 μM Cy3 (Cy5)-labeled dCTP. Oligoarray control targets and hybridization buffer (In Situ Hybridization Kit Plus, Agilent Thechnologies, Santa Clara, CA, USA) were added, and samples were applied to microarrays enclosed in Agilent SureHyb-enabled hybridization. After hybridization, the microarray was washed using the Affymetrix Fluidics Station according to the manufacturer's protocol. The chips were scanned using an Agilent G2565AA scanner. Functional grouping of the identified genes was done by manual editing of gene ontology categories obtained through the DAVID annotation tool (https://david.ncifcrf.gov).

Real-time reverse transcription PCR

RNA was extracted using RiboEX (GeneAll Biotechnology, Seoul, South Korea) according to the manufacturer's instructions. RNA (100 ng) from each sample was then transcribed to cDNA using Prime Moloney murine leukemia virus reverse transcriptase (Genetbio, Nonsan, South Korea), after which samples were amplified using N-Taq DNA polymerase (Enzynomics, Seoul, South Korea) in a MyCycler thermal cycler (BioRad). The amplification parameters were as follows: denaturation at 95°C for 2 min, followed by varying numbers of cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s. The following primers were used for PCR: chicken GAPDH, 5′-AGT CAA CGG ATT TGG CCG TA-3′ and 5′-ACC TGC ATC TGC CCA TTT GA-3′; chicken CXCLi1, 5′-AGC GAT TGA ACT CCG ATG CC-3′ and 5′-GCC TTG TCC AGA ATT GCC TT-3′; chicken CXCLi2, 5′-CTC TGT CGC AAG GTA GGA CG-3′ and 5′-GCT GAG CCT TGG CCA TAA GT-3′. Aliquots of each PCR product were subjected to 1% (w/v) agarose gel electrophoresis and visualized by ethidium bromide (ETBR) staining. For real-time PCR, FAM was conjugated to the 5′ ends of the probes as the fluorescent reporter dye to

detect amplified cDNA. Analyses were conducted using an iCycler thermal cycler (BioRad, Hercules, CA, USA) to subject the samples to the following conditions: denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 98°C for 10 s, annealing at 59°C for 30 s, and elongation at 72°C for 45 s. All analyses were conducted in triplicate. Relative quantification of gene expression was performed using the comparative threshold cycle (CT) method, in which the CT value is defined as the point at which a statistically significant increase in fluorescence has occurred. The number of PCR cycles (CT) required for FAM intensity to exceed a threshold immediately above the background was calculated for test and reference reactions. In all experiments, GAPDH was used as the endogenous control.

Ethics statement

Experimental protocols performed in this study were reviewed and approved by the Animal Ethics Committee of Pusan National University (PNU)-IACUC (PNU-2017-1555). The animal study was performed in accordance with the guidelines for animal experiments issued by PNU-IACUC.

PCIS (precision-cut intestinal slices)

Three to four week old chicken intestines were cut into pieces about 1 inch in length. After having closed one end of the intestine using a thread, the intestine was filled gently with low-melting-point agarose (Sigma-Aldrich) to stabilize the intestinal tissue. For solidification, the sample was put on ice. Samples were then arranged in a straight position about 1 cm in length, after which the thread was removed and five pieces of intestine were put into the cool tissue holding adapter of a Krumdieck tissue slicer (TSE systems, Bad Homburg, Germany). The space between the intestine and the walls of the adapter were then filled with low-melting-point agarose. After solidification, the intestine was cut with the Krumdieck tissue slicer into slices approximately 400 μm thick with a cycle speed of 60 slices/min. The precision-cut intestinal slices were then incubated in 1 mL of Williams's Medium E containing L-glutamine (Life Technologies, Carlsbad, CA, USA), D-glucose (14 mM), gentamycin (50 μg/mL) and amphotericin B (2.5 μg/mL) per slice in a 24-well plate at 37°C and 5% CO_2.

Computational analysis plus cytoscape

Gene ontology (GO) analysis, which is commonly used for functional studies of large-scale genomic or transcriptomic data, classifies functions with respect to molecular function (MF), cellular components (CC), and biological processes (BP). The Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.kegg.jp/) pathway database is widely used for systematic analysis of gene functions and linking genomic data with higher order functional data. To extract biological relevance from the genes that were differentially expressed in lead, arsenic or DON-treated DF-1 cells, we used the DAVID (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov) bioinformatics tool, which provides functional interpretation of genes based on gene ontology enrichment analysis, among other criteria. Gene ontology analysis was applied to genes that were up- or down-regulated by 2× in lead, arsenic or DON-treated DF-2 cells. A network graph was then constructed and visualized using Cytoscape v3.6.1 (http://cytoscape.org/).

Statistical analysis

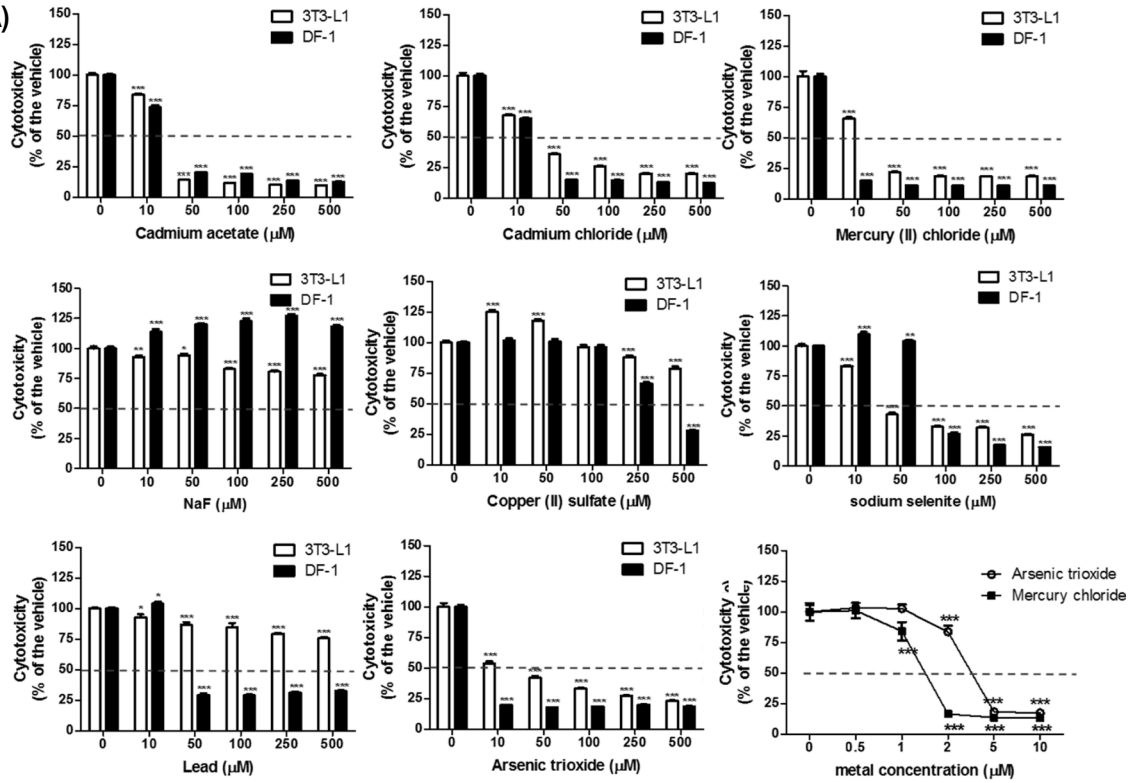
Statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). A Student's *t*-test was used for comparative analysis of the two groups of data. To compare multiple groups, data were subjected to ANOVA, and pairwise comparisons were made using the Student-Newman-Keuls method.

Results

Toxicity of heavy metals in mouse embryo fibroblast-like 3T3-L1 or chicken fibroblast DF-1 cells

To compare between mammalian and avian responses, two EF cells (the mouse cell line 3T3-L1 and the chicken cell line DF-1) were exposed to metals. The cytotoxicity of inorganic metals (mercury (II) chloride, cadmium acetate, cadmium chloride, copper (II) sulfate, lead (II) acetate, arsenic trioxide) and inorganic non-metallic compounds (sodium fluoride, sodium selenite) was quantified in EF cells after exposure for 24 h using a MTT assay. Chicken EF showed more resistance to sodium fluoride and sodium selenite than murine cells (Figure 1A). In contrast, 3T3-L1 murine EF showed more resistance to lead acetate than chicken cells. The half maximal inhibitory concentration (IC_{50}) was also compared based on the cytotoxicity assay (Figure 1B). Among the tested inorganic compounds, arsenite trioxide and mercury chloride were most toxic to chicken EF cells. In particular, chicken EF cells showed more susceptibility to arsenite trioxide, mercury chloride and lead acetate than murine cells. When the relative toxicity of each chemical was compared by calculating IC_{50} in mouse cells per IC_{50} in chicken cells, lead was found to be the most selectively toxic to chicken EF, followed by mercury and arsenic trioxide.

(A)



(B)

IC_{50} (μM)	NIH 3T3-L1 (A)	DF-1 (B)	Chicken selectivity (A/B)
Arsenic trioxide	36.84	3.45	10.68
Cadmium acetate	29.33	29.64	0.99
Cadmium chloride	51.18	26.37	1.94
Copper(II) sulfate	845.58	364.34	2.32
Lead(II) acetate	1087.45	38.09	28.54
Mercury(II) chloride	28.71	1.45	19.80
Sodium fluoride	1146.49	3176.12	0.36
Sodium selenite	42.99	87.07	0.49

Figure 1. Effects of inorganic compounds on cell viability of mouse and chicken embryo fibroblast cells. (A) Mouse 3T3-L1 and chicken DF1 cells were treated with various concentrations of each compound and the cell viability was determined by MTT assay. The asterisks represent significant differences from each vehicle control (*$P<0.05$, **$P<0.01$, and ***$P<0.001$) using 2-tailed, unpaired Student's *t* test. (B) Based on the MTT assay, the half maximal inhibitory concentration (IC_{50}) was calculated for each cell line.

Chemokine-linked pathways are altered in chicken EFs that are susceptible to metal toxicity

Based on the MTT results, we selected lead (II) acetate as a representative metal posing preferential risk to chicken EFs. Arsenic trioxide was also chosen as a representative mutagenic metal with high selectivity to chicken cells. Cellular gene expression was assessed in DF-1 cells after exposure to IC_{20} doses of lead or arsenite for 24 h. After integrating both lead- and arsenite-induced gene expression profiles, functional categorization was evaluated in DF-1 cells. Among the various type of gene-linked functions, the category of chemokine-chemokine receptor interaction showed the highest value of probability of involvement (Figure 2A). In particular, analysis of the gene revealed that the CC subfamily or CXC subfamily genes were involved in the cytokine-cytokine receptor interaction pathway through the KEGG pathway (Figure 2B).

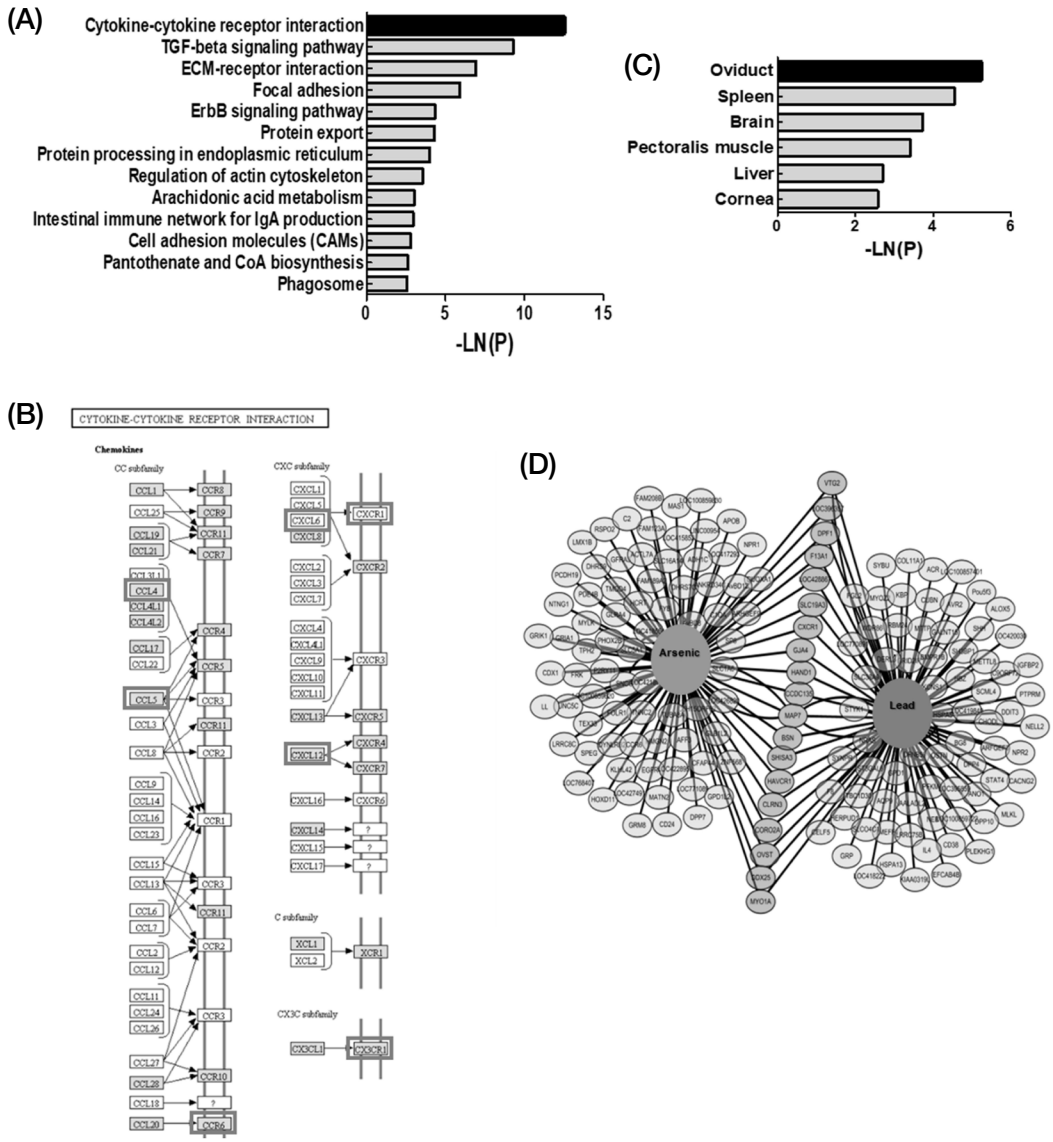


Figure 2. Metal-induced transcriptomic profiles in chicken EF cells. (A) DF-1 cells were treated with the 20% inhibitory concentration (IC_{20}) of lead (18.59 μM) or arsenite (1.79 μM) for 24 h. Cells were analyzed using cDNA microarray, and functional gene ontology of 2-fold increased and decreased genes was categorized based on their enrichment scores (-Ln (*P*) values). (B) Cytokine-cytokine receptor interaction using the KEGG database in 2-fold increased genes in lead and arsenic-treated DF-1 cells. (C) Tissue localization of 2-fold increased and decreased genes following treatment with lead and arsenite was profiled using DAVID and BioGPS analyses. (D) Genes commonly upregulated by both lead and arsenite treatment were visualized by Cytoscape 3.3.0.

CXCR1, CX3CR1, and CCR6 were upregulated by the chicken EF-toxic metals. Along with induced CXCR1, coupling ligands such as CXCL6 were also elevated by the metals. Moreover, geometric distribution analysis of the integrated gene profile indicated that oviduct-located genes are most frequently altered by treatment with lead and arsenite, followed by spleen- and brain-associated genes (Figure 2C). Based on a cutoff of a 2-fold increase, nineteen genes were commonly upregulated by both lead and arsenite treatment in DF-1 cells (Figure 2D). Taken together, analyses of gene profiles revealed that chemokine receptor-associated events are potently involved in metal-induced adverse actions in reproductive organs, including the ovary, embryo and oviduct.

Gut-derived chemokines may contribute to activation of CXCR1-linked outcomes in reproductive tissues

Dietary exposure would trigger the gut lining and its

mucosal immune-related tissues as the frontline against metals in feed or drinking water. The impact of lead on gut tissues was assessed using precision-cut intestine slices (PCIS) since the tissue culture model may simulate the relatively intact tissue contexture in response to luminal xenobiotics. Chicken PCIS produced a highly reproducible and regular morphology of chicken intestine (Figure 3A); therefore, it represents a mini gut model that was well suited for studying luminal exposure in toxicological research because it contains all of the cells of the gut, including mucosal immune cells, while maintaining intracellular and cell-matrix interactions[24]. Chicken PCIS-based chemokine gene expression was assessed based on the assumption that luminal xenobiotics may trigger chemokine release from the early exposure site (gut). As a positive xenobiotic agent, the effects of *Fusarium*-producing deoxynivalenol (DON) were also evaluated because this fungal toxin is a representative gut barrier disruptor capable of strong pro-inflammatory insult that is found in feed and foodstuff worldwide[25,26]. In the chicken small intestine, lead induced production of chemokine CXCLi1 or CXCLi2 in a time-dependent manner, and this occurred only slightly later than the induction by DON (Figure 3B). When compared with the chemokine induction in the small intestine, lead and DON had more influential effects on CXCLi2 than CXCLi2 (Figure 3C). The induction of CXCLi2 (corresponding to human interleukin 8, CXCL8) was initiated early and maintained for 12 h in the large intestine following exposure to lead or DON. In response to chemokine induction in the exposure site (gut), DON also enhanced CXCR1 as the receptor for CXCLi1/2 in chicken EF cells (Figure 3D). Gastrointestinal exposure to metals, including lead, causes reproductive disorders such as reduced egg production and infertility. The organ-to-organ interaction was mechanistically estimated by the culture-based gene analysis. Therefore, the present culture-based estimation of the chemokine-chemokine receptor circuit during xenobiotic exposure provides a potent alternate platform for assessment of the effects of exposure and target events in chickens.

Discussion

It is well known that developmental exposure to lead is associated with embryonic toxicity or subsequent teratogenic outcomes[27,28]. Male exposure to lead increases infertility, resulting in in alterations in embryonic gene integrity in the murine model[29]. Female offspring rats that undergo neonatal exposure to lead suffer detrimental suppression of follicular development in the ovary[30]. In the primate model, luteal function was suppressed by chronic treatment with levels of lead that had marginal effects on ovulation[31]. In line with extensive evidence of the ability of metals to cause reproductive organ injuries, we assessed its effects on EFs, which serve as the sentinel to environmental changes in the embryo and oviduct. In the present study, chicken EFs were more sensitive to lead and arsenite than the murine EF. Additionally, EF gene expression profiling showed that chemokine receptor-linked events were particularly disturbed by lead exposure, indicating that they may be potently triggered by gut-derived chemokines in response to luminal lead. Moreover, altered gene profiles were closely associated with oviduct localization, which may account for the gut-to-oviduct axis in the reproductive toxicity of lead (Figure 4A).

Chemokine-chemokine receptor crosstalk is crucial in the fate of gamete cells in the oviduct and ovary. Germ cells first appear outside of the embryonic gonads, then move toward the developing genital ridges during early embryonic development. For this migratory behavior of germ cells and guidance in the oviduct, chemokines and their receptor-linked messengers must regulate and facilitate the process of reproduction. Chemotactic molecules guide directional migration toward gonadal somatic cells, whereas chemorepellent-expressing somatic cells forcibly repel migrating germ cells or indirectly induce disruption of chemoattractant signals in the surrounding somatic cells. Chicken germ cells passively migrate from the central to the anterior region, then actively move toward the germinal crescent[32]. In the second migratory phase, chicken germs cells were shown to penetrate developing blood vessels and circulate until they finally settled into the genital ridges[33]. In particular, G-protein-coupled chemokine receptors (CXCR4 and CXCR6) were found to facilitate germ cell migration[34]. The receptor CXCR4, which is known as fusion or cluster of differentiation 184 (CD184), specifically binds to chemotactic ligands including CXCL12, which facilitate the chemoattraction between germ cells and target sites[35]. Therefore, CXCLi1/2-activated CXCR1 in the embryonic fibroblast would contribute to the reproductive disturbance caused by lead exposure in the chicken model, indicating that it warrants further mechanistic investigations *in vivo*.

In cases of the mammalian exposure, implantation, placentation, and the first and early second trimester of pregnancy resemble the wound healing process that occurs through reproductive inflammatory responses. For instance, the blastocyst has to break through the epithelial lining of the uterus to implant and damage endometrial tissues to invade, which is followed by trophoblast replacement of the endothelium and vascular smooth muscle of blood vessels to secure an adequate placental-fetal blood supply[36], all of which require sub-

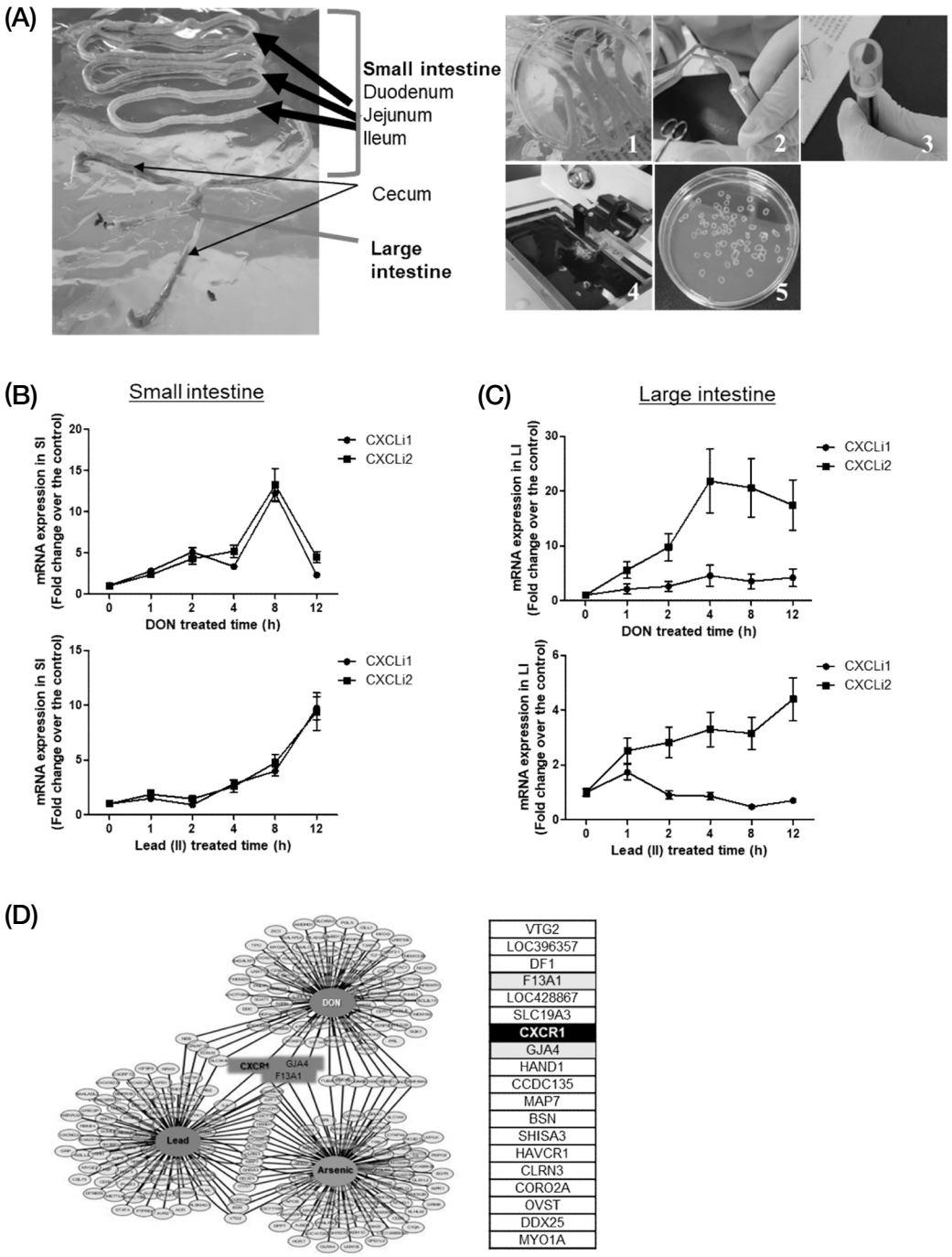


Figure 3. PCIS-based gene expression analysis. (1) Preparation and incubation of chicken intestinal slices. After the fecal components were removed from the intestinal segment, one side was tied and the lumen was filled with agarose (1), the segment was anchored in side of precooled cylindrical mold plunger (2), the outside of the segment was filled with agarose in the mold plunger (3), after which the segment was cut with a Krumdieck tissue slicer (4). Slices of 2-4 mg (wet weight) were cut and incubated in 12-well plates (5). (B and C) Small intestine (B) or large intestine (C) slices were treated with 20% inhibitory concentration (IC_{20}) of lead (18.59 μM) or DON (500 ng/mL) for the indicated time. Each mRNA was then measured using RT real-time PCR (mean + SEM, n = 3). (D) Genes commonly upregulated in DF-1 cells treated with lead, arsenite and DON were visualized using Cytoscape 3.3.0.

sequent repair of the uterine epithelium with the clearance of cellular debris in an adequate time sequence[37,38]. However, altered inflammatory insults by metals would interfere with the physiological inflammatory process in

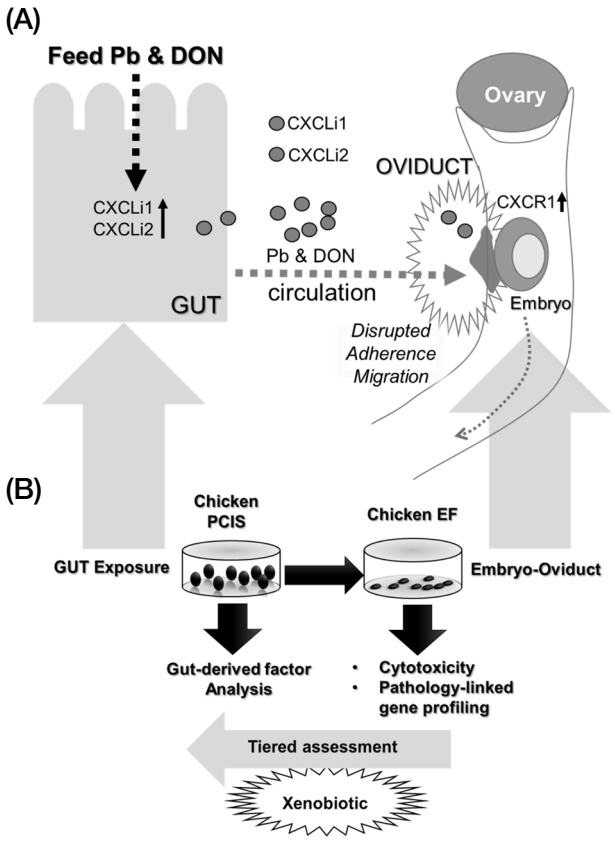


Figure 4. (A) A putative mechanism for the roles of lead-induced chemokines in chicken embryonic development to eggs. (B) Chicken EF-PCIS-based platform for assessment of reproductive immunotoxicity consisting of the cytotoxicity, pathology-linked gene profiling in the chicken EF cell line (Tier1), and analysis of gut-derived factors in chicken PCIS (Tier 2).

the reproductive cycle. Mechanistically, metal-disrupted chemokine homeostasis would be detrimental to embryonic programming via (i) activation of the inflammatory cell stress response, which alters optimal gene profiling with lasting impacts on developmental trajectory, (ii) epigenetic modification-induced permanent changes in phenotypes and (iii) inflammation-induced cytotoxicity (apoptosis, necrosis, and pyroptosis) and loss of tissue components. Even small perturbations in blastomere number and inner cell mass/trophectoderm allocation in the blastocyst could influence later growth trajectory of the fetus and offspring[39]. Lead-induced chemokine and chemokine receptor pathways would account for disorganization of the wound healing processes in reproductive organs[40-42].

In terms of risk assessment, PCIS-based monitoring provides an easy methodology for screening of gut-derived factors in the chicken exposure model. PCIS is a culture system for differentiated intestinal epithelial linings surrounded by submucosa, muscularis, and serosa that is similar to the gut luminal exposure system. Originally, PCIS was used for physiological measurements of the mucosal barrier similar to an Ussing chamber. PCIS has also been used to evaluate drug metabolism and transport in mammalian systems[43-45]. In terms of the mucosal immune system, this chicken PCIS system can be used as an alternate avian infection model since it includes the intestinal lymphoid tissues for defense[46]. In the present study, both the chicken small intestine and colon were sliced to assess early events following gastrointestinal exposure to metals and DON under the assumption that these xenobiotics frequently contaminate the diet and drinking water. This monitoring using PCIS can support alternate immunotoxicological assessments for detection of early gut-derived factors, which can account for chemokine-linked immunological or developmental distress in reproductive organs (Figure 4B).

Conclusion

The susceptibility of the chicken reproductive system to various metals was evaluated using chicken EF cells and transcriptomic profiling in the present study. Lead (II) acetate induced preferential adverse actions in the chicken EFs, potently via chemokine receptor-associated pathways. Moreover, as a readout of early exposure, gut-derived chemokines including CXCLi1 or CXCLi2 were induced in the intoxicated gut using PCIS. Metal-induced gut chemokines are promising triggers of target-specific disorders in the reproductive organs via interactions with the chemokine receptors. This *in vitro* methodology using a PCIS-EF-based tiered platform in the gut-to-oviduct/embryo axis could be incorporated as a new simplified alternate platform of animal reproductive immunotoxicological assessment.

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Conflict of Interest Ki Hyung Kim, Juil Kim, Jae Yong Han and Yuseok Moon declare that they have no conflicts of interest associated with the contents of this article.

Human and animal rights The article does not contain any studies with human. Animal experimental protocols performed in this study were reviewed and approved by the Animal Ethics Committee of Pusan National University (PNU)-IACUC (PNU-2017-1555). The animal study was performed in accordance with the guidelines for animal experiments issued by PNU-IACUC.

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