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Effects of indium chloride exposure on sperm morphology and DNA integrity in rats

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ABSTRACT

Indium, a Group IIIA element of the periodic chart and a rare earth metal characterized by high plasticity, corrosion resistance, and a low melting point, is widely used in the electronics industry where released streams can contaminate the environment. Consequently, indium can reach humans mainly by natural ways, which could result in a health hazard. Although reproductive toxicities have been surveyed in some studies in animal models, the infertility effects of sperm function induced by indium compounds have not been greatly investigated. We designed a study to investigate the toxicities of subacute exposure to indium compounds on male sperm function and the process of spermatogenesis in a rodent model. Fourteen Sprague-Dawley rats on postnatal Day (PND) 84 were randomly divided into exposure and control groups, and weekly received intraperitoneal injections of indium chloride (1.5 mg/kg body weight) and normal saline, respectively, for 8 weeks. Cauda epididymal sperm count, motility, morphology, chromatin DNA integrity, mitochondrial membrane potential (MMP), reactive oxygen species (ROS) generation, and testis DNA content were investigated. The indium chloride exposed group showed significant toxicity to sperm function, as well as an increased percentage of sperm morphological abnormality and chromatin DNA damage. Furthermore, positive correlations between abnormal sperm morphology, chromatin DNA damage, and superoxide anion generation were also noted. The results of this study demonstrated the toxic effect of subacute low dose indium exposure during sexual

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maturation on sperm function, resulting in sperm chromatin DNA damage through an increase in sperm ROS generation in a rodent model.

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1. Introduction

Indium, a metallic rare earth element belonging to Group IIIA, is a soft, silvery, white metal with superior ductility, malleability, and plasticity that is recovered from fumes, dusts, slags, residues, and alloys from zinc and lead–zinc smelting. Indium has many uses, but the principal one, which accounts for about 65% of the indium consumed in industry, is as thin films of indium-tin oxide for liquid crystal displays. Indium is also used in semiconductors, in the form of indium phosphide (InP). Other indium compounds, such as indium chloride, are used to produce transparent, conductive indium-tin oxide films by the dip-coating and vacuum-sputtering method. Indium compounds have also been used for medical diagnostic and clinical investigations in humans. Prior to the beginning of the 1990s, because of a lack of information on the adverse effects of exposure to indium compounds on the health of humans or animals, indium compounds were not considered harmful. However, occupational exposure to indium compounds has attracted increased attention, resulting from the pulmonary toxicities which have been demonstrated clinically in Japan [1,2]. In a study of workplace exposure to unspecified indium compounds at a factory in Japan conducted by Miyaki et al [3], the concentration of indium in blood were reported as 10-fold in increment in geometric mean to those workers exposed to water-insoluble, partially-respirable indium-containing particles in workplace air, than those who were not exposed.

Taiwan has become a major flat panel display (FPD) and semiconductor manufacturer in Asia, with flat panel display and semiconductor-related industries also covering a wide range of other areas. In response to Taiwan's industrial development and technical advancements, the consumption of indium and its compounds continues to grow annually to provide the raw materials for various commercial products. Chen [4] estimated that >30,000 people were employed in 2007 among 350 semiconductor manufacturing firms in Taiwan. Although a large number of studies have been performed on pulmonary toxicities and carcinogenesis, little attention has been given to male reproductive toxicities [5]. For example, it has been reported that testicular toxicity could result from repetitive intratracheal instillation of indium arsenide (InAs) and InP in hamsters for 2 years [6], whereas indium-induced testicular toxicity in rats is controversial [7]. Additionally, the mechanisms of male reproductive toxicities induced by indium compounds have not been fully investigated.

In accordance with the ever-increasing consumption of indium and its compounds, assessment of the related health hazards would provide an important reference for environmental and occupational health agencies when implementing the necessary precautionary measures and legislation governing its industrial use. This paper is intended as an investigation of the adverse effects of subacute indium chloride exposure on the sperm functions, and the mechanisms involved, using a rodent model.

2. Materials and methods

2.1. Treatment of animals

The experiment is designed as a subacute exposure. Thirteen male Sprague-Dawley rats were purchased on postnatal Day (PND) 42 from the National Laboratory Animal Center (Taipei, Taiwan). They were housed in a rodent vivarium of the Laboratory of Industrial Hygiene, National Kaohsiung First University of Science and Technology (Kaohsiung City, Taiwan), where the temperature was maintained at 21–25°C, with a relative humidity of 40–60%. Animals were kept in a 12 hour light-dark cycle and provided a Laboratory Rodent Diet 5001 (LabDiet, Richmond, IN, USA) and water *ad libitum*. After they had been acclimated to PND 84, 14 animals were divided into two groups. Seven rats were in the indium chloride exposed group and seven rats were in the unexposed control group. Each rat in the indium chloride group was given a total dose of 1.5 mg/kg of body weight of indium chloride, for 8 weeks, as eight intraperitoneal injections. Indium chloride was obtained from Sigma-Aldrich Corporation, (St. Louis, MO, USA), had a purity of >99.999%, was powdery in character, and soluble in water. An amount of 30 mg indium chloride totally dissolved in 20 mL normal saline to form indium chloride solution.

All of the injections were carried out on PND 84, PND 91, PND 98, PND 105, PND 112, PND 119, PND 126, and PND 133. Each rat in the group exposed to indium chloride received 1 mL/kg body weight of indium chloride solution. By contrast, the unexposed control group received the same amount of physiological saline (1 mL/kg body weight) in the same experimental conditions. During the experimental periods, the two groups of rats were daily weighed in the light phase (from 8:00 AM to 12:00 AM). The animals were sacrificed by carbon dioxide on PND 150.

2.2. Body and reproductive organ weights

The paired testis, paired epididymis, and seminal vesicles were removed and weighed on the day the rats were sacrificed (PND 150). These organs were immediately freed of adipose tissues. Relative organ weight was estimated by calculating the ratio between organ weight and body weight measured on PND 150. The left testis was used for DNA content analysis by FCM (flow cytometry) and for histopathological observation.

The left cauda epididymis was used to perform sperm chromatin structure assay (SCSA) analysis. The sample of sperm suspension was measured for sperm count, motility, morphology, mitochondrial membrane potential (MMP), and reactive oxygen species (ROS) generation.

2.3. Serum indium level analysis

The serum indium (S-In) level analysis was modified from methods of Hamaguchi et al [8]. Each rat provided 10 mL of blood drawn by needle aspiration from the heart into two vacuum blood-drawing tubes without any anticoagulant and centrifuged at 3000 × *g* for 15 minutes to separate blood cells and serum on PND 150. Serum was stored at −85°C until the analysis of S-In. For pretreatment, 0.25 mL of serum was diluted by 2.5 mL of 68–70% ultra-pure nitric acid (HNO_3, Guarantee Reagent (GR) for analysis, Merck, Taipei, Taiwan) and 0.25 mL of 30% ultra-pure hydrogen peroxide (H_2O_2, TAMAPURE-AA-100). After digestion by a 100 W/vessel microwave oven (Mars, microwave digestion system, CEM Corporation, U.S.A) [9], 0.1 mL rhodium at 10 μg/L was added to each sample for external calibration. Finally, inductively-coupled plasma mass spectroscopy (ICP-MS, Perkin Elmer Sciex ELAN DRC II, San Jose, CA, USA) was used to analyze the indium concentration.

2.4. Sperm count and motility analysis

The left cauda epididymis of each control and exposed male rat was removed and placed in a medium of 1 mL human tubule fluid maintained at 34°C in an environment saturated with 5% carbon dioxide. After 5 minutes, the cauda epididymis was minced with curved scissors and the sperm was dispersed. After 20 minutes, the sperm was collected and transferred to a fresh tube. Aliquots of sperm suspension (10 μL) were placed on a prewarmed Makler chamber (10 μm depth; Sefi-Medical Instruments, Haifa, Israel) and an epididymal sperm count was performed under a phase-contrast microscope (Olympus CH2, Olympus Corporation, Tokyo, Japan). Sperm motility was expressed as the ratio between the number of motile sperm and the total number of sperm.

2.5. Sperm morphology analysis

The sperm suspension was diluted with phosphate-buffered saline then mixed with 1% aqueous eosin Y (10:1). The resulting mixture was set aside and left still for 30 minutes. After that, a drop of the mixture was placed on a clear slide and a uniform smear was made. Two samples were made for each rat. After air drying, the slides were briefly rinsed in methanol to remove excess stain. They were air dried again, and cover-slipped with mounting medium. Two hundred cells from each rat were examined for morphological abnormalities under a light microscope (400×; Zeiss, Axioskop 2, Zeiss, Jena, Germany). Abnormalities were classified as abnormal head, abnormal neck, abnormal tail, and multiple abnormalities. Abnormal head included headless sperm, pin head, and flattened head. Abnormal neck was defined as kinked or coiled neck or midpiece and abnormal tail was defined as kinked or coiled tail. It should be noted that it is possible for multiple abnormalities or tailless sperm to be seen from a single sperm that was classified into multiple abnormalities. Total morphologically abnormal sperm indicated the sum of abnormal head, abnormal neck, abnormal tail, and multiple abnormalities [10,11].

2.6. SCSA

The SCSA was applied following the procedure described by Evenson and Jost [12], with minor modifications. Aliquots of samples from the left cauda epididymis of each rat were placed in TNE (tris soldium chloride EDTA) buffer (0.15M NaCl, 0.01M Tris-HCl, 1mM disodium EDTA, pH 7.4) in a Petri dish and minced. After allowing the tissue fragments to settle, the sperm suspension was filtered through a 0.2 mm nylon mesh into 2 mL cryogenic vials and mixed with glycerol to form a final solution at a concentration of 10% (v/v). All aliquots were properly diluted in TNE buffer in order to work with comparable concentrations of 1–2 × 10^6 sperm/mL. A 50 μL aliquot was subjected to acidic denaturation in100 μL of a low pH detergent solution containing 0.1% Triton X-100, 0.15M NaCl, and 0.08 N HCl, pH 1.2. After 30 seconds, 0.3 mL of a staining solution containing 6 mg/L acridine orange (AO) (chromatographically purified; Molecular Probes, Eugene, OR, USA) in 0.2M Na_2HPO_4, 1mM disodium EDTA, 0.15M NaCl, and 0.1M citric acid monohydrate (pH 6.0) was added for staining over 3 minutes. AO is a metachromatic fluorochrome used to stain differentially double-stranded/single-stranded nucleic acids. When excited by blue light at 488 nm, AO intercalates into double-stranded DNA and fluoresces green (FL1, native DNA). If, however, DNA is damaged and exposed single-stranded DNA, AO will attach to single-stranded nucleic acids and exhibit a red fluorescence. The stained sample was placed in the flow cytometer FACScan FCM (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) for analysis. A total of 10,000 spermatozoa were collected and analyzed at a flow rate of 100–200 cells/s. Scatter gram analysis on raw data, with each point representing the coordinates of red and green fluorescence intensity values for every individual sperm, was carried out using Becton Dickinson standard software and FCS Express software version 2 (De Novo Software, Thornhill, ON, Canada). Events which accumulated in the lower left corner corresponded to sample debris and were excluded from the analysis. The bivariate data can be conveniently expressed by the DNA fragmentation index (DFI), which is the ratio of red to total (red + green) fluorescence intensity [13], thus representing the amount of denatured, single-stranded DNA over the total cellular DNA. The DFI is calculated for each sperm cell in a sample and the results are expressed as the mean DFI, the standard deviation of the DFI distribution (SD DFI), and as the frequency of cells with high DFI values, usually called cells outside the main population (% DFI).

2.7. Sperm MMP

The lipophilic, cationic compound, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) has been used to differentially label mitochondria with high and low membrane potential [14–16]. JC-1 possesses the ability to form multimers known as J-aggregates after

accumulation in mitochondria with high membrane potential. In addition to J-aggregates, JC-1 can form monomers in mitochondria with low membrane potential. Thus, JC-1 has the distinct advantage of differentiating cells of high and low MMP. The excitation maximum for JC-1 is 488 nm, with the emission spectra of the monomer in the green range (530 nm) and the emission of J-aggregates in the high orange wavelength (590 nm). Stock solutions of 1.53mM JC-1 (Molecular Probes) were prepared in DMSO. The sperm suspension was diluted to 1–2 × 10^6 sperm/mL and stained with JC-1 to a final stain concentration of 3.0 μM. The samples were set aside for 10 minutes at 34°C, and immediately assessed for orange and green staining by flow cytometry (FACScan FCM). The percentage of orange stained (sperm of active mitochondrial potential) cells were recorded as MMP (%).

2.8. Sperm ROS generation

Dihydroethidine (DHE; Molecular Probes, Inc.) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Inc.) detected superoxide anion ($O_2^{-\bullet}$) and H_2O_2, respectively. DCFH-DA is a stable compound that passively diffuses into cells and is hydrolyzed by intracellular esterase to yield DCHF, which is trapped inside cells. H_2O_2 produced by cells oxidizes DCFH to the highly fluorescent compound, 2,7-dichlorofluorescein (DCF) which is fluorescent at 530 nm. The DHE can be directly oxidized into ethidium bromide by $O_2^{-\bullet}$ produced by sperm. We modified the method used by Fisher et al [17] to evaluate H_2O_2 levels in sperm; sperm $O_2^{-\bullet}$ levels were measured using a modification of a previously described method [18]. DCFH-DA (2.5 mM) and DHE (0.33 mM), were added to the sperm suspension at a concentration of 1–2 × 10^6 sperm/mL and incubated at 34°C for 30 minutes for both DCFH-DA and DHE. The final concentrations of DCHF-DA and DHE were 12.5 μM and 1.65 μM, respectively. Each aliquot was analyzed using FACScan FCM (Becton Dickinson FACScan). Intracellular H_2O_2 and $O_2^{-\bullet}$ levels were measured with excitation and emission settings at 488 nm and 530 nm, respectively. Data were expressed as the percentage of fluorescent spermatozoa which indicated positive ROS cells [19].

2.9. FCM DNA content analysis of testis cells

The left testis was minced and treated with 1 mL 0.1% pepsin-HCl solution gently mixed by magnetic stirring at room temperature for 10 minutes. These monocellular suspensions were then filtered through a 37 mm nylon mesh to separate the cells from tissue. The resulting monocellular suspensions were stabilized with 4 mL of 95% ethanol and stored at −20°C for several weeks. Fixed samples were added to 0.5% pepsin-HCl solution to a 1:1 volume at the time point for FCM analysis. Testicular cells were then immediately brought to a concentration of 2 × 10^6 cells/mL in separation medium and diluted 1:1 with propidium iodide solution (0.1M Tris-HCl pH 7.5, 0.04 mg/mL DNAse-free RNAse, and 0.05 mg/mL propidium iodide) after 10 minutes of gentle magnetic stirring. The fluorescence intensity of DNA content of the testis cells was measured using a FACScan FCM. Based on the DNA content, main germ cell peaks can be classified into four categories: (1) mature haploid (elongated spermatids); (2) immature haploid (round and elongating spermatids); (3) diploid (spermatogonia, secondary spermatocytes, tissue somatic cells); and (4) tetraploid (mostly primary spermatocytes). The region between the diploid and tetraploid peaks, called the S-phase, is comprised of cells that actively synthesize DNA. The relative proportions of haploid, diploid, S-phase, and tetraploid cell types were calculated.

2.10. Sample treatment for testicular histopathological studies

The left testis was fixed in Bouin's solution for at least 48 hours. To prepare them for histological studies, the testes were embedded in paraffin and sections (3–4 μm) were stained with hematoxylin and eosin. The damage to seminiferous tubules was evaluated in cross sections. Sections were observed under 400 × magnification with an Olympus microscope (Milton Keynes, Buckinghamshire, UK) connected to a computer on which the digital images were saved.

2.11. Statistical analysis

Results are expressed as mean ± SD of the mean. All statistical comparisons were performed using the JMP statistical package (SAS Institute Inc., Gary, NC, USA) for the unexposed control and exposed groups. The body weights of the group exposed to indium chloride and the control group were compared with the Student t test and $p < 0.05$ was considered statistically significant. Welch's test was used instead of the Student t test if the variances of the two groups were not equal. The reproductive organ weights, sperm count, motility, morphology, SCSA, MMP, generation of H_2O_2 and $O_2^{-\bullet}$, and DNA content of testis cells of the group exposed to indium chloride and the control group were compared using the nonparametric Wilcoxon rank sums test, and $p < 0.05$ was considered statistically significant. The relationships among sperm morphology, DFI, and $O_2^{-\bullet}$ were measured using general linear regression analysis.

3. Results

3.1. Body and reproductive organ weights

The trend of weight gain was significantly decreased in the exposure group when compared to the control group. In addition, there was a significant reduction in mean body weight between the indium chloride exposed and control groups during PND 134–139 (Fig. 1). There were no significant differences in absolute and relative weights of body, paired testis, paired epididymis, paired cauda epididymis, and seminal vesicle between the group exposed to indium chloride and the unexposed control group (Table 1).

3.2. Serum indium level, sperm count, motility, and morphology

There were also no significant differences in sperm count and motility between the two groups, as shown in Table 2. There

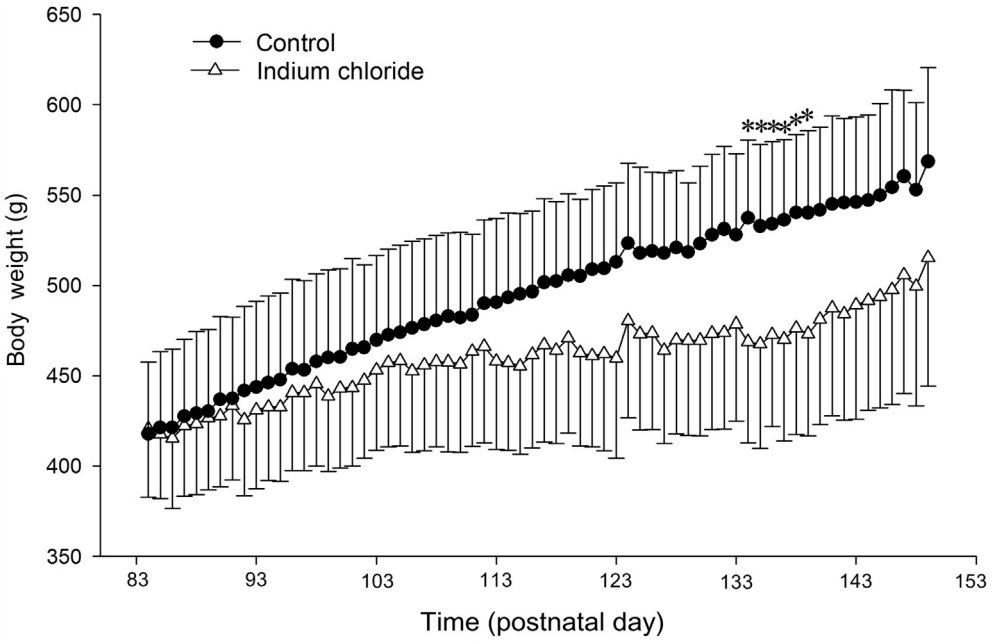


Fig. 1 – Body weight of each rat recorded daily and presented as mean ± standard deviation between the indium chloride and unexposed control group. * Significant differences as compared with the control group ($p < 0.05$).

was a significant increase in percentage of abnormal head, abnormal neck, abnormal tail, multiple abnormalities, and total morphologically abnormal sperm in the exposed group as compared with the control group (Fig. 2). The mean S-In level displayed 138.7 μg/L in the exposed group and 0.26 μg/L in the control group (Table 2).

3.3. *FCM analysis of SCSA, MMP, and ROS generation*

The sample of cauda epididymal sperm was analyzed by FCM SCSA to investigate whether there was any indium chloride-induced damage in sperm chromatin DNA integrity. There was a significant increase of % DFI (percentage out of the main populations) and mean of DFI in the indium chloride exposed group, while there was no significant difference in SD DFI between the indium chloride and the control groups (Table 2). There was no significant difference in MMP between the group exposed to indium chloride and the control group. To evaluate sperm ROS generation, we measured H_2O_2 and $O_2^{-\bullet}$ in the groups treated with indium chloride. Furthermore, we did not find such a difference in the generation of H_2O_2 and $O_2^{-\bullet}$ between the two groups (Table 2).

Table 1 – Body and tissue weight of rats exposed to indium chloride and the unexposed controls at postnatal Day 150.

Parameters	Treatment		*p* Wilcoxon
	Control ($n = 7$)	Indium chloride ($n = 7$)	
Absolute			
Body weight (kg)	0.50 ± 0.08	0.51 ± 0.05	0.7015
Testis (g)	3.34 ± 0.37	3.55 ± 0.30	0.3067
Epididymis (g)	1.14 ± 0.12	1.14 ± 0.08	0.5229
Cauda epididymis (g)	0.50 ± 0.07	0.51 ± 0.05	0.7015
Seminal vesicles (g)	2.03 ± 0.20	1.80 ± 0.37	0.2013
Relative			
Testis (g/kg BW)	6.00 ± 0.9	7.00 ± 1.11	0.0967
Epididymis (g/kg BW)	2.05 ± 0.33	2.26 ± 0.35	0.3067
Cauda epididymis (g/kg BW)	0.90 ± 0.20	1.00 ± 0.16	0.5229
Seminal vesicles (g/kg BW)	3.64 ± 0.50	3.41 ± 0.44	0.4433

Data are presented as mean ± standard deviation.
BW = body weight.

Table 2 – Sperm quality, sperm chromatin structure assay (SCSA), mitochondrial membrane potential (MMP), and reactive oxygen species (ROS) generation in rats exposed to indium chloride and the unexposed control group.

Parameters	Treatment		*p* Wilcoxon
	Control ($n = 7$)	Indium chloride ($n = 7$)	
Sperm count (10^6/mL)	16.3 ± 4.5	16.9 ± 5.7	0.9484
Sperm motility (%)	51.0 ± 8.4	43.5 ± 8.5	0.1768
S-In (μg/L)	138.7 ± 34.7	0.3 ± 0.2	0.0022**
Mean DFI (AU)	206.9 ± 6.4	234.6 ± 15.6	0.0033**
% DFI	2.3 ± 1.3	23.4 ± 16.3	0.0022**
MMP (%)	83.0 ± 16.4	86.4 ± 16.2	0.7983
DHE (%, $O_2^{-\bullet}$)	0.5 ± 0.6	1.2 ± 0.5	0.0553
DCFH-DA (%, H_2O_2)	0.7 ± 0.5	0.4 ± 0.2	0.2243

Data are presented as mean ± standard deviation.
*$p < 0.05$, **$p < 0.01$ as compared with control group.
AU = stain intensity; DFI = DNA fragmentation index; MMP = mitochondrial membrane potential.

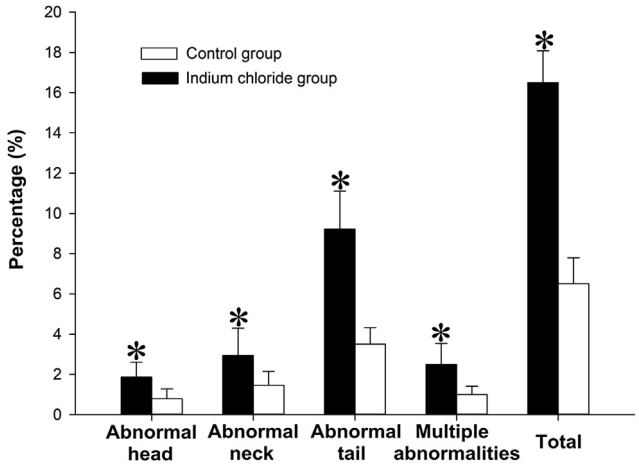


Fig. 2 – Percentage occurrence of different sperm abnormalities observed between rats exposed to indium chloride and the unexposed control group. * Significant differences as compared with control group ($p < 0.05$).

3.4. *DNA content analysis of testis cells*

The frequencies of cells belonging to various testis subpopulations were expressed as relative fractions (figure not shown). There were no significant differences between the two groups in the testis DNA content among the four cell types (mature haploid, immature haploid, diploid, and tetraploid) and that of the S-phase cell (data not shown).

3.5. *Relationships among abnormal sperm morphology, mean DFI, % DFI, and ROS generation*

We performed a regression analysis of the incidence of total morphologically abnormal sperm, mean DFI, % DFI, and percentage of positive DHE cells (Figs. 3 and 4). The incidence of morphologically total abnormal sperm was positively and significantly associated with % DFI ($r = 0.64$, $p = 0.0132$) and mean DFI ($r = 0.73$, $p = 0.0026$). Furthermore, the percentage of positive DHE cells (positive superoxide anion sperm) was positively associated with mean DFI ($r = 0.62$, $p = 0.0169$).

3.6. *Histopathology of the testis*

We found severe vacuolar degenerative change in both interstitial and seminiferous tubules in the exposed group. There was some interstitial vacuolar degeneration in the control group but it was less severe than in the exposed group (Fig. 5).

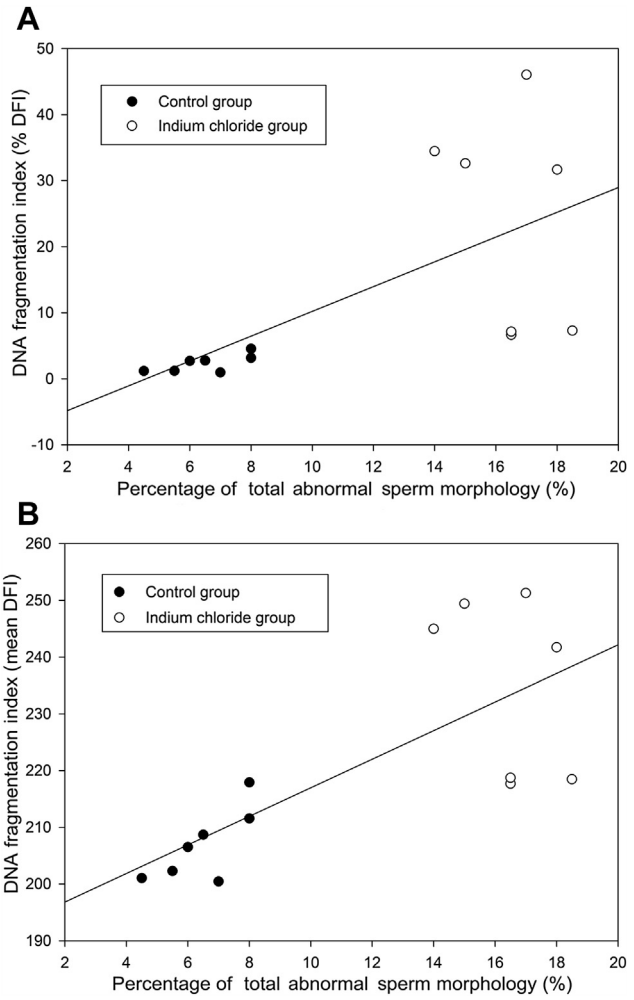


Fig. 3 – Relationships among percentages of total sperm abnormalities, % DNA fragmentation index (DFI), and mean of DFI (fluorescence intensity) by general linear model. (A) Increased percentage of total sperm abnormalities in relation to % DFI. % DFI = −8.57 + 1.88 × (% total abnormal sperm morphology) ($r = 0.64$, $p = 0.0132$); (B) increased percentage of total sperm abnormalities in relation to mean of DFI. Mean DFI = 191.69 + 2.53 × (% total abnormal sperm morphology) ($r = 0.73$, $p = 0.0026$).

4. Discussion

We sought to demonstrate male rat reproductive toxicities following subacute exposure to indium chloride, which can help to provide understanding of the effects of indium exposure to male reproductive function. To put it differently, the results of this study showed that subacute exposure to indium chloride in male rats might reduce body weight, increase the percentage of abnormal sperm morphology, and enhance sperm chromatin integrity damage. Although we found that the generation of $O_2^{-\bullet}$ was increased more in the exposed group than the control group, it did not reach a significant difference. Moreover, there were no significant differences in sperm counts and MMP between the group exposed to indium chloride and the control group, possibly due to the small size of the groups. However, the result of continued loss of body weight gain in the exposed rats from the initiation of this experiment, reaching a significant difference from PND 134 to PND 139, was similar to the results of the study conducted by Samira et al [20], which proved to be the effect of both body weight reduction and hypophagia induced by indium compounds. Other studies showed some similar findings of male reproductive toxicities induced by indium compounds. In

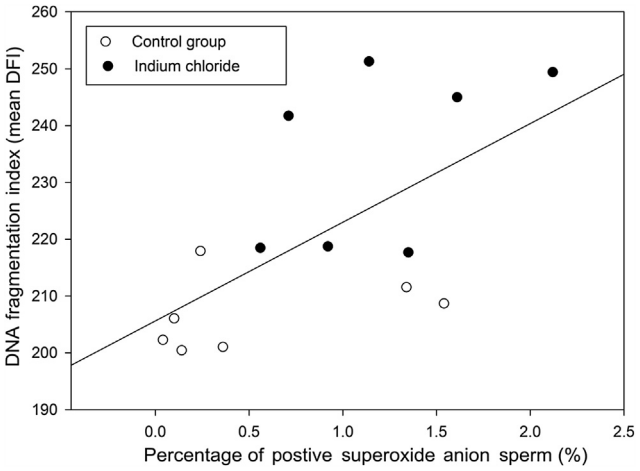


Fig. 4 – Relationship between mean of DNA fragmentation index (DFI) and percentage of positive superoxide anion cell by linear regression. Mean of DFI (fluorescence intensity) = 205.63 + 17.37 × (percentage of positive superoxide anion sperm) ($r = 0.62$, $p = 0.0169$).

research to determine testicular toxicities in rats exposed to gallium arsenide and InAs by repetitive intratracheal instillation twice/week for a total of 16 times, it was discovered that the incidence of morphologically abnormal sperm was slightly increased in the InAs group, but did not reach a significant difference [7]. Omura et al [6] further conducted another investigation in hamsters exposed to either InAs or InP repetitive intratracheal instillation twice weekly for eight times, which proved definite testicular toxicity during 2 years examination, as well as decreased hamster body weight, testis weight, epididymis weight, and caudal sperm count, and caused severe histopathologic changes in the testis. These histopathologic changes were similar to our findings in the exposed group.

Based on our results, significant damage of rat sperm chromatin integrity induced by indium chloride exposure was noted by the SCSA test. The ultrastructure in the testis of rats contained indium particles which were deposited in the lysosomes of Leydig and Sertoli cells and were demonstrated in the study of Samira et al [20]. This result also indicated that indium compounds could be transferred from blood to the testis, which may affect sperm chromatin structure and genome. The structure and composition of the sperm chromatin is remarkably different from that of the chromatin in somatic cells. During spermiogenesis, histones are replaced by protamines, which condense and protect sperm DNA generated highly compacted sperm chromatin [21–23]. Furthermore, chemical interactions with chromatin protamines, included heavy metal, which prevents normal sperm chromatin condensation which may induce changes in the sperm genome and thus might affect male fertility [24–27].

The SCSA test is used to measure DNA susceptibility of DNA denaturation after exposure to mild acid. DNA of sperm with a normal chromatin structure does not denature, whereas if the DNA is somewhat damaged and contains breaks in the DNA strands, it can reach different degrees of denaturation. To determine the degree of sperm DNA denaturation after mild acid treatment, the sperm cells are stained with AO. Because AO is a metachromatic fluorochrome, it fluoresces green when intercalated between intact double-stranded DNA and fluoresces red in single-stranded DNA. Measurement of fluorescence at both of the wavelengths after denaturation assesses the percentage of fragmented DNA named as DFI. This assay can be assessed by determining its level of compaction and has been extensively applied to studies of human infertility [28]. Previous studies indicated that a DFI value >27% was associated with pregnancy failure in assisted reproductive technology [29,30].

Our data even demonstrated positive correlations among abnormal sperm morphology, chromatin integrity damage, and $O_2^{-\bullet}$ generation. Given that spermatogenesis and sperm maturation is a highly dynamic process in terms of sperm DNA replication and packaging, it is probable that any heritable or environmental perturbation to this process will be reflected in the production of morphologically abnormal sperm. There have been several studies supporting the

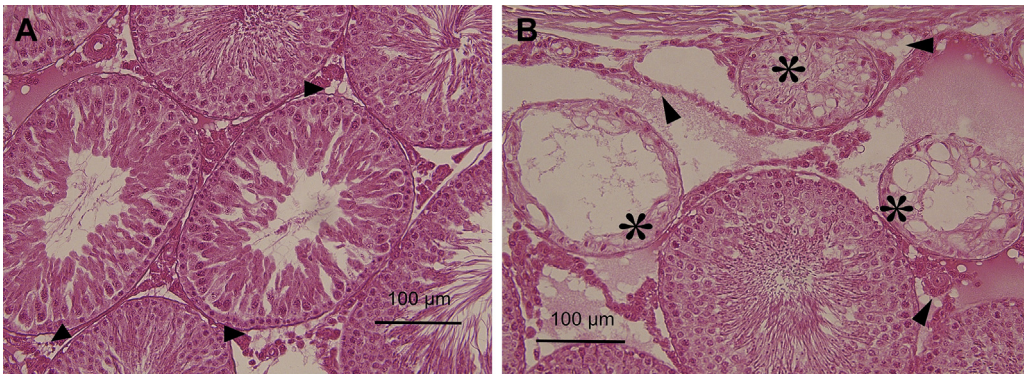


Fig. 5 – Tissue sections of testis stained with hematoxylin and eosin stain were obtained from the control group and the indium chloride treated group. (A) The interstitial cells of the testis in the control group show mild vacuolar degeneration. (B) In the indium chloride-treated rats, the seminiferous tubules and interstitial cells show severe vacuolization. The seminiferous tubules were not only associated with complete losses of spermatozoa and spermatids, but still retained both basement membrane and a few spermatogonia. * Vacuolization of seminiferous tubules; arrowhead, vacuolization of interstitial cells; scale bar = 100 μm.

hypothesis that abnormal sperm morphology is statistically associated with an increase in the incidence of chromosomal abnormalities and chromatin integrity damage [31–34]. Correspondingly, there is a great deal of evidence indicating that most of the sperm chromatin integrity damage we observed was associated with ROS generation, especially in the spermiogenesis process [35].

Although the human toxicity of indium compounds through normal inhalation and ingestion is not clearly understood, the intraperitoneal injection route of exposure was used in the subacute rodent model to obtain a more stable internal dose of indium in this work. The findings herein may be used in assessing human toxicity associated with its production, use, and disposal.

In conclusion, the main specific findings of this study were the enhancement of the percentage of abnormal sperm morphology, and the damage of chromatin DNA integrity induced by indium chloride exposure. In addition, positive correlations among abnormal sperm morphology, chromatin DNA damage, and ROS generation were demonstrated. These findings may lead to a hypothesis that male reproductive toxicities may result from oxidative DNA damage after indium chloride exposure and further influence the sperm chromatin remodeling associated with abnormal sperm morphology, resulting in male infertility. Given the current wide utility of these indium compounds in industries and medicine, it is noteworthy that the implications for indium toxicity and male reproductive health could be considerable.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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