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RESEARCH ARTICLE

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Effect of cadmium and nickel on expression of CatSper 1 and 2 genes in mice

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ABSTRACT

This study was designed to investigate the effect of the administration of cadmium and nickel on the histology of the testes, sperm parameters, and the expression of CatSper 1 and CatSper 2 genes in adult male mice. Despite the vital role of CatSper genes in male fertility, very little is known about the factors that regulate their expression. Thirty-two adult male mice were randomly allocated into four groups. The control group received no treatment. The sham group was injected with normal saline. The cadmium and nickel groups were injected with 2 mg/kg/day of cadmium chloride or 5 mg/kg/day of nickel chloride for 2-weeks as models for testicular injury. Histological study and the analysis of their sperm were performed according to WHO's guidelines for the examination of human sperm. In addition, a prooxidant antioxidant balance assay and real-time PCR were performed 35 days after the treatment, as the duration of spermatogenesis cycle in mice is 35 days. The data were analyzed using SPSS software using ANOVA. Both nickel and cadmium injections caused a reduction in sperm parameters as well as a decrease in the thickness of the germinal epithelium. The administration of cadmium caused down-regulation in the expression of both CatSper1 and CatSper2 genes. Only cadmium increased the PAB values.

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CatSper; cadmium; nickel; gene; expression

Introduction

Heavy metals, such as lead, cadmium, and nickel, are known to produce adverse effects on health (Permenter *et al.* 2011). Cadmium and nickel are used extensively in manufacturing batteries, paints, plastics, alloys, metal plating, and many other products. Exposure to these metals may occur through diet, smoking, and environmental pollutants (Thompson and Bannigan 2008, Permenter *et al.* 2011, Shariati-Rad *et al.* 2015). Cadmium and nickel exposure at toxic levels may cause damage to the liver, the testes, and the kidneys (Das *et al.* 2008, Siu *et al.* 2009). These heavy metals accumulate gradually in various tissues, including the reproductive organs, and they can cause detrimental effects on the production of sperm. Cadmium and nickel decrease sperm count and sperm motility, and can cause irreversible damage to the germinal epithelium (Das and Dasgupta 2002, Gupta *et al.* 2007, Thompson and Bannigan 2008). Severe hemorrhage, edema, and necrosis, as well as the destruction of tubules, have been reported after exposure to cadmium (Siu *et al.* 2009). The half-life of cadmium in blood in man is 2–3 months, but, after absorption, it can

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remain in the tissues of the body for decades (WHO, Cadmium 2000, Siu *et al.* 2009). Human studies have shown that men who have occupational exposure to cadmium have higher levels of cadmium in their plasma than normal, and the quality of the sperm in their semen, especially the motility of the sperm, is decreased significantly (Benoff *et al.* 2000). Testicular damage, including an increase in the volume of the interstitial cells and a decrease in the size of the germinal epithelium, was reported in male rats that were exposed to 20 mg/kg of $NiCl_2$ (Massányi *et al.* 2007). Exposure to nickel also decreased sperm motility, the number of sperm, and the normal sperm morphology in male rats (Das and Dasgupta 2002). Gupta *et al.* (2007) reported an increase in lipid peroxidation and a reduction in antioxidant enzyme in the semen of welders exposed to nickel. Shrinkage of the seminiferous tubules, congestion, and necrosis were observed in the rats that were treated with nickel (Jargar *et al.* 2012). Several studies have reported that cadmium has an adverse effect on gene expression that is involved in apoptosis, cell cycles, and hormone regulators (Benoff *et al.* 2000, Oliveira *et al.* 2009, Siu et al 2009). The genotoxic effects of nickel, such as breaks in DNA strands, gene mutations, and cell transformation also have been reported (Doreswamy *et al.* 2004). Also, a higher incidence of chromosomal aberrations was found in workers exposed to nickel compared to the healthy control group (Danadevi *et al.* 2003).

There are unique calcium channels in sperm, that include the CatSper 1 to CatSper 4 proteins, that have a key role in controlling the motility of sperm and the uptake of calcium by the sperm (Mohammadi *et al.* 2009). These channels are present exclusively in the testes and are encoded by CatSper genes. The knocking out of CatSper genes in mice showed that the presence of these channels is essential for fertility in the male mice, normal motility of the sperm, the sperm's penetration into the egg, and for the entry of calcium ions (Ren *et al.* 2001).

Studies have shown that cadmium, due to its similarity to calcium ions, can substitute for calcium ions and block the calcium channels, including L-Type in infertile people (Benoff *et al.* 2000). It is yet to be determined whether the exposure to environmental factors, such as heavy metals, e.g. cadmium, nickel, lead, and mercury, affect the expression of the CatSper gene or the calcium flow in these unique channels. Therefore, the aim of this study was to evaluate the effects of treatment with cadmium and nickel in male mice NMRI on the histology of their testes, the quality of their sperm, PAB values, and the expression of CatSper 1, 2 genes.

Materials and methods

Animals and ethics

This study was performed after being approved by the Ethics Committee of Gonabad University of Medical Sciences NMRI male mice were purchased from the Razi Institute (Mashhad, Iran), and they were kept for two weeks in an animal home at Gonabad University of Medical Sciences. The animals were kept under standard conditions of environment (12:12 h light–dark cycles at 22 °C) with free access to drinking water and standard pellets. Thirty-two NMRI male mice were divided randomly into four groups (control, sham, cadmium, nickel, and groups). The eight mice in the control group did not receive any injections. The sham group was injected with normal saline. The eight mice in the cadmium group were injected intraperitoneally with 2 mg/kg of cadmium chloride (Oliveira *et al.* 2009). The mice in the nickel group were injected with 5 mg/kg of nickel chloride (Murawska-Ciałowicz *et al.* 2012). The injections were performed intraperitoneally once daily for two weeks.

RNA extraction

After completion of the injection, the mice were sacrificed rapidly by cervical dislocation, and, after opening the abdominal cavity, right testis was extracted and kept in the freezer at −80 °C until the extraction process was performed. Total RNA was extracted using the RNA plus solution (CinnaGene, Iran) and according to the manufacturer's protocol. The primers used for CatSper 1, 2, and βActin genes have been taken from Mohammadi *et al.* (2009)

Sperm analysis

To evaluate the quality of the sperm 14 days after the final injection, the tail of the left epididymis was isolated and completely cut by scissors and was put on PBS (phosphate buffer saline) and was incubated for 20 min at 37 °C. The motility, count, and morphology of the mice's sperm were assessed according to WHO's guidelines using a neuobar hemocytometer.

Prooxidant antioxidant balance (PAB assay)

Twenty milli liter of sodium acetate buffer were added to 16 mg of powdered TMB (tetramethylbenzidine dissolved in dimethyl sulfoxide (DMSO) (solution 1). Seventy milli liter of Chloramine T solution were incubated with this solution for two hours. The first solution was added to the U25 peroxidase enzyme

solution, 10 ml of sodium acetate buffer, 200 ml of TMB/DMSO, and 10 ml of TMB. This solution with 10 ml of blood serum and the standard was added to the ELISA reader. After 10 min, 100 mM HCl was added and the absorbance was read at the wave length of 450 nm (Alamdari *et al.* 2008).

Reverse transcription reaction

Oligo dT was added to 1 mg of extracted RNA, and its volume was increased to 12 ml by adding water. A microtube was incubated at 70 °C for 5 min in the thermal cycler, and then 4 ml of replication buffer 5×, 1 ml of RNase, and 2 ml of mixed dNTP were added to the denatured RNA. The reaction mixture was incubated for 5 min at37 °C. After adding the MMLV enzyme, the microtube was placed for 60 min at 37 °C and then 10 min at 70 °C. The reaction product was kept at −20 °C until they were used in the PCR reaction.

Real time PCR

The relative expression of CatSper1 and CatSper2 genes was measured using SYBR and β-Actin housekeeping gene. In a microtube, 2 ml Master Mix, 0.6 ml primer Reverse [Fermentas], 0.6 ml primer Forward [Fermentas], 2 ml cDNA, 9.3 ml deionized water were added and the microtubes were placed in the Stratagene Max 3000p and was established with the following protocol:

Initiation step: 95 °C for 10 min, 1 cycle; Denaturation step: 95 °C for 25 s, 40 cycles; Annealing step: 60 °C for 30 s, 40 cycles; Extension step: 72 °C for 30 s, 1 cycle.

Histological evaluation

Left testis was placed on Bouin's fixative for histological examination (Merck, Germany). The slides were gradually dehydrated through graded ethanol, immersed in xylene and mounted with Entellan (Merck, Germany). Then, the tissues were molded with paraffin (Merck, Germany) and were cut using a microtome (Leica, Germany) into sections with thicknesses of 5μ and were stained using the H&E method (Sigma, England); then, the stained slides were studied with light microscope.

Statistical analysis

The normality of data was assessed using the Kolmogorov–Smirnov test. Data analysis was performed using SPSS software (version 20), the ANOVA and *post hoc* Tukey. $p < .05$ was considered significant.

Results

Histological examination of the cadmium- and nickel-treated groups

The histology of seminiferous tubules was normal in the control group as well as sham group. The morphological findings in the cadmium-treated group were similar to those of the nickel group except that all mentioned changes (especially maturation arrest and leydig cell hyperplasia) were more pronounced in the cadmium group (Figure 1). Histological examination of the tissue slides in the nickel-treated group showed congestion of blood vessels, disintegration of germ cells from their basement membrane, and distorted intra tubular architecture. In addition, there were some degrees of leydig cell hyperplasia, maturation arrest in some tubules, and scattered apoptotic cells.

Effects of cadmium or nickel on sperm parameters in the adult male mice

Data about the sperm parameters from the control, cadmium, and the nickel groups are presented in Table 1.

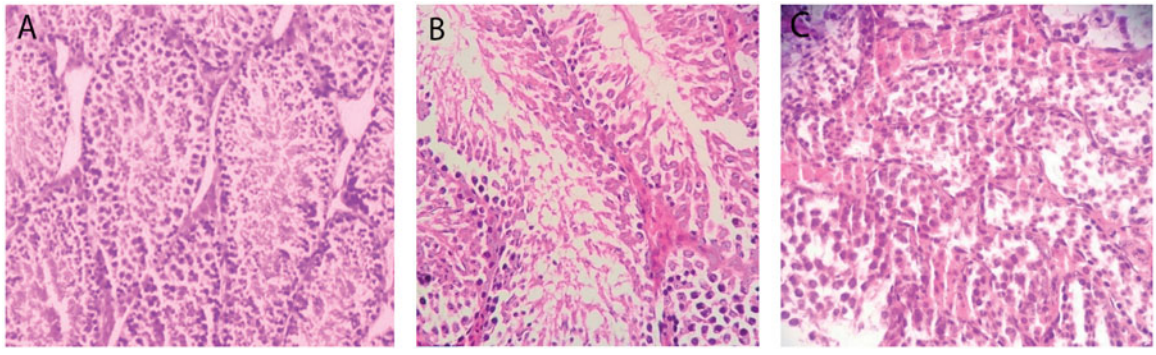

Figure 1. Images of seminiferous tubules in the control (A), nickel treated (B) and cadmium treated (C) rats (H&E staining, 400 × magnification). B: The sections show cellular disintegration and scattered apoptotic cells in nickel treated rats. C: Seminiferous tubules of a cadmium treated subject. Cellular disintegration, maturation arrest and leydig cell hyperplasia is evident ($n = 8$ mice in each group).

Table 1. Effect of cadmium and nickel treatment on sperm count, sperm motility, and normal sperm morphology in mice.

Sperm parameters	Control group	Sham group	Cadmium group	Nickel group
Sperm Count (million/ml)	4.87 ± 0.10	4.66 ± 0.30	3.92 ± 0.22*	3.16 ± 0.21*
Sperm motility (%)	58.87 ± 4.67	60.00 ± 3.62	26.5 ± 2.66*	38.62 ± 2.56*
Normal morphology rate (%)	84 ± 4.14	86 ± 3.20	68.25 ± 2.25*	69.75 ± 2.12*

Values are presented as Mean ± SD of eight mice in each group. Data were analyzed by ANOVA test. ($n = 8$ mice in each group).
*$p < .05$ compared to the control group.

Table 2. Effect of cadmium and nickel treatment on PAB values in mice.

	Control group	Sham group	Cadmium group	Nickel group
PAB value	0.51 ± 0.1	0.61 ± 0.30	1.62 ± 0.64*	0.66 ± 0.21

Values are presented as Mean ± SD of eight mice in each group. Data were analyzed by ANOVA test. ($n = 8$ mice in each group).
*$p < .05$ compared to the control group.

The sperm parameters were affected by the cadmium administration. Administration with 2 mg/kg cadmium caused significant decrease in sperm count and motility ($p < .05$; Table 1). There was a significant difference in the sperm parameters between the nickel and control groups ($p < .05$). The percentage of normal sperm morphology was affected by the cadmium or nickel group compared to the control group. The most common types of morphologic abnormalities in cadmium group was bent neck (28.75%) and coiled tail (30.25%), while it was head deformity (49.75%) in the nickel group and (8%) in the control group.

Effects of cadmium and nickel on prooxidant antioxidant balance in the adult male mice

As shown in Table 2, PAB values were significantly higher in mice treated with cadmium compared to the control group (1.62 ± 0.64 vs. 0.51 ± 0.1; $p < .05$). There was no significant difference between the PAB values in nickel group compared to the control group (0.66 ± 0.21 vs. 0.51 ± 0.11; $p = .13$).

Effects of cadmium on CatSper 1, 2 genes expression in the adult male mice

As shown in Figure 2, the relative expression of CatSper 1 was lower in the cadmium group compared to the control group (−0.26 ± 1.87 vs. 1 ± 0.46, $p = .97$). Besides, the relative expression of CatSper 2 was significantly lower in the cadmium group compared to the control group (−2.20 ± 0.95 vs. 1 ± 0.46, $p < .05$).

Effects of nickel on CatSper 1, 2 gene expression in the adult male mice

The relative expression of CatSper genes in the nickel group is presented in Figure 2. The relative expression of the CatSper 1 in the nickel group was not statistically higher than that of the control group (−0.82 ± 2.40 vs. 1 ± 0.46, $p = .33$). The relative expression of CatSper 2 in the nickel group was significantly lower than that in the control group (−0.76 ± 1.45 vs. 1 ± 0.46, $p < .05$). The negative values mean a reduction in gene expression.

Discussion

Our findings showed that the administration of a dose of 2 mg/kg cadmium caused a decrease in the thickness of germinal epithelium as well as sperm parameters. Besides, a down-regulation of CatSper 1 and CatSper 2 genes was observed in the cadmium group. In the nickel group, spermatic arrest was not observed in the seminiferous tubules but the expression of CatSper gene 2 decreased compared to the control group. There was no significant difference between the PAB values in the nickel group compared to the control group, while these values were significantly higher in the cadmium group compared to the control group. There has been considerable interest in CatSper gene functions in the past decade due to their critical role in sperm motility (Jin *et al.* 2005). The focus on heavy metals makes sense for two reasons, one that their cationic state would have a biological interaction with the CatSper channels, and two that cadmium has been implicated as a weak endocrine disrupter that could impact CatSper expression.

Testicular damage, assessed as an increase in interstitial cell volume and a decrease of germinal epithelium, was found in male rats exposed to 20 mg/kg of $NiCl_2$ (Massányi *et al.* 2007). Inconsistent with this study, the thickness of epithelium decreased in nickel-treated mice but it was not statistically significant. Zhou *et al.* (2004) showed that subcutaneous injection of 5 μmol/kg $CdCl_2$ induced necrosis and large residual bodies in testis tissue. Less than 1% of the seminiferous tubules were damaged, and no obvious change was observed in interstitial tissue. A dose of 10 μmol/kg of cadmium caused excessive necrosis and hemorrhage in the testes. Similarly, our findings

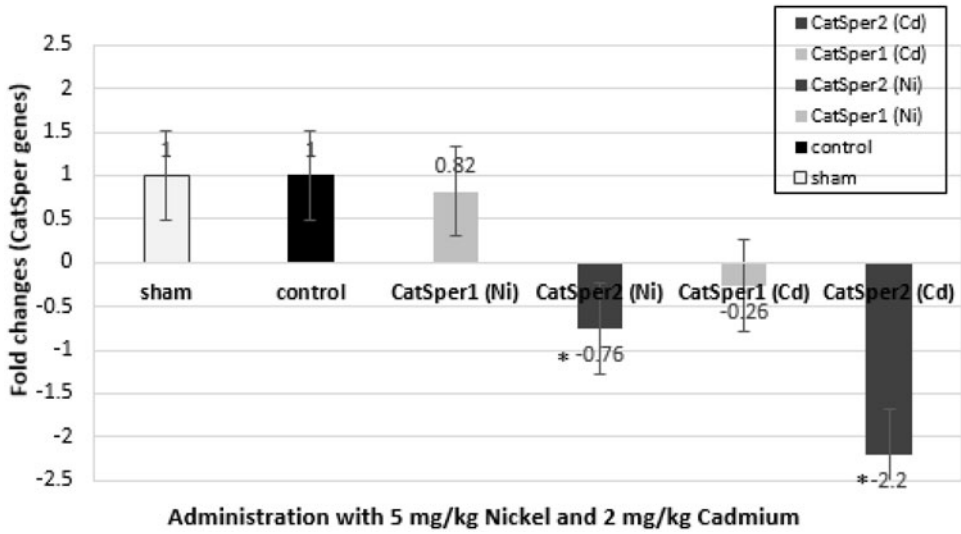

Figure 2. Comparing the relative\ expression of CatSper 1, 2 gene to ß-actin gene expression in control, cadmium and nickel groups: ($n = 8$ mice in each group). Statistical analysis showed the down-regulation of CatSper 2 gene expression compared to control group; $^{*}p < .05$. The negative values mean a reduction in gene expression.

showed spermatic arrest as well as decrease in thickness of germinal epithelium in the cadmium group.

Administration with 10 mg/kg nickel sulfate caused a significant reduction in sperm count, sperm motility, body weight, and testicular somatic index as compared to the control group (Jargar *et al.* 2012). Helena Oliveria *et al.* (2009) studied the effects of cadmium chloride on sperm function parameters in 8-week-old male mice by subcutaneous injections of 1, 2, and 3 mg $CdCl_2$/kg bw. The short-term effects in 24 h and long-term effects in 35 days were evaluated. The short terms effects of cadmium chloride increased sperm with abnormal forms, premature acrosome reaction, and reduced motility. Long-term effects included a drastic reduction of sperm cell number and sperm motility and also DNA damages. In agreement with these reports, in the present study sperm parameters decreased in mice treated with either cadmium or nickel.

Gupta *et al.* (2007) reported increase in lipid peroxidation and decrease in antioxidant enzymes in the semen of welders exposed to nickel. Acharya *et al.* (2004) also showed cadmium cytotoxic effects on various organs of animals. In this study, intraperitoneal injection of $CdCl_2$ (1 mg/kg body weight) into Swiss mice increased lipid peroxidation. The activity of enzymes belonging to superoxide dismutase, catalase, and peroxidase significantly decreased. In line with this study, we found that treatment with nickel or cadmium, especially cadmium, can increase PAB values compared to the control mice.

Rossman (2000) reported that heavy metals influence gene expression using the microarray method. Also, up-regulation of genes involved in cell growth and apoptosis was observed in nickel-exposed workers compared to the healthy controls (Bonin *et al.* 2011). Das *et al.* also reported that intraperitorally administration nickel (20 mg/kg) significantly decreased the expression of nucleic acids concentration in the testes of male Wistar rats fed protein-restricted and normal protein diets (Benoff *et al.* 2000). Su *et al.* (2011) reported that after 30 days, the expression of Bax and c-kit increased in rats treated with 2.5 and 5 mg/kg of nickel sulfate. Similar to this result, our findings showed that the expressions of CatSper 1, 2 genes were affected by 5 mg/kg nickel treatment.

Zhou *et al.* (1999) investigated the effect of 0, 5, 10, and 20 μmol/kg $CdCl_2$ which was subcutaneously injected into male rats after on apoptosis and necrosis genes. MT1 gene was expressed in the testes and C-jun gene was observed in the first of 96 h, but after 12 and 48 h was not obvious every three hours. P53 gene expression decreased with increasing doses of cadmium. In another study, subcutaneous injection of 10 μmol/kg cadmium into male rats caused a 1.6 increase in the expression of the glyceraldehyde-3-phosphate gehydrogenase gene, but it caused no effect on α1A-and β2-androgenic receptors gene expression (Gunnarsson *et al.* 2007). These results are in agreement with our study that the expressions of CatSper 1, 2 genes were affected by 2 mg/kg cadmium treatment.

Several mechanisms have been reported to explain the effects of cadmium in testis damages. Siu *et al.* (2009) reported that one of the most destructive effects of cadmium is the testicular dysfunction of blood-testis barrier and also disturbance the balance of calcium and magnesium ions resulting in creation of toxic effects in

the testes. Benoff *et al.* (2000) showed that calcium and potassium channels are influential on acrosomal reaction. Cadmium, due to its similarity to calcium ions, substitutes for calcium and blocks calcium channels including L-TYPE in infertile people. The calcium channels, when exposed to lead and cadmium, show different reactions. A high level of cadmium was observed in patient with varicocele and also, a high level of lead was found in semen of infertile patient (Benoff *et al.* 2000). Therefore, it seems that heavy metals such as cadmium and nickel, may substitute with calcium ions and block calcium channels, such as CatSper. Sivakumar *et al.* (2017) reported that CatSper gene expression in cattle with low sperm motility did not significantly difference from cattle with high sperm motility. Wang *et al.* (2016) reported that the administration of bisphenol reduced the sperm motility, CatSper gene and protein expression. It seems that sperm motility and expression of CatSper gene is affected by exposure to metals such as cadmium, nickel, and bisphenol.

In this study, a single dose of cadmium and nickel was used that was a limitation in this study. It was better that different doses were examined at different times, but due to limitation in financial support it was not possible. In this study, the i.p. route of exposure selected as a model for testicular injury. It was better that mice exposed with cadmium instead of intraperitoneally injection with cadmium. This study shows effects of cadmium and nickel on CatSper 1, 2 gene expressions. It recommends the effects of other heavy metals such as lead and mercury faced with CatSper genes expression. The present study showed adverse effects of administration with nickel or cadmium on sperm parameters. Besides, administrations with these heavy metals decrease CatSper 2 gene expression in the mice testis. Hence, it may cause adverse effect on spermatogenesis by affecting the CatSper genes expression in men exposed to nickel or cadmium.

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Disclosure statement

The authors declare that they have no declarations of interest.

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