Original article

Protective role of silibinin over nickel sulfate-induced reproductive toxicity in male rats

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Abstract

Nickel damages the male reproductive system. We investigated the beneficial effects of silibinin which has metal-chelating and antioxidant properties over nickel toxicity. Both anti-oxidative effects in testes and overall effects related to sperm motility, membrane and acrosome integrity of orally administered Silibinin were evaluated against the harmful effects of 30 day of intraperitoneal nickel sulfate (5 mg/kg/day) administration in rats. Male rats were randomized into control (Group1; n=6) and three experimental groups (n=6, each): Group2 Nickel sulfate (5 mg/kg/day), Group3 Silibinin (150 mg/kg/day), and Group 4 Nickel sulfate (5 mg/kg/day) + Silibinin (150 mg/kg/day). We found higher sperm motility, viable sperm and total sperm count in Groups 3 and 4 than the Group 2 treatment groups and the percentage of abnormal spermatozoa was similar in both groups (Groups 2 and 4). Increased apoptosis, activation of caspase3, 8, 9 and TUNEL were detected in Group 2. However, activation of caspase3, 8, 9 and TUNEL was reduced in Group 4. The protective effects of silibinin were demonstrated on histopathologic findings and some sperm parameters (sperm motility percentage, viable spermatozoa, sperm count, and abnormal spermatozoa percentage) in rats exposed to nickel.

Key words: nickel, silibinin, male, rats, reproductive toxicity

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Introduction

Nickel is a trace element extensively used in industrial branches (Ijomone et al. 2018). It is also an environmental pollutant with genotoxic, immunotoxic and teratogenic effects (Adjroud 2013), and nickel is classified as carcinogenic (IARC 2012). Nickel sulfate damages the liver (Adjroud 2013), testes, sperm, and prostate and seminal glands (Doreswamy et al. 2004, Das et al. 2008, Lukac et al. 2014) by inducing oxidative stress. Many studies have been conducted to determine the protective effects of various substances (bromelain, nano selenium, zinc, sesamin, L ascorbic acid and grape seed proanthocyanidin extract) against the harmful effects of nickel sulfate (Sidhu et al. 2006, Das and Buchner 2007, Su et al. 2011, Liu et al. 2013, Cebi Sen et al. 2019, Zhang et al. 2019). Silibinin (SB) is the largest flavonolignan of silymarin, which is found in milk thistle (*Silybum marianum*) (Bosch-Barrera et al. 2017) and has metal-chelating and antioxidant properties (Muthumani and Prabu 2012). SB strongly protects the testes (Oufi et al. 2012, Oufi and AlShawi 2014), as a result of the sequelae of antioxidative processes and modulation of the expression of effector proapoptotic caspase3 activity (Ligeret et al. 2008). SB was also found effective against arsenic toxicity (Muthumani and Prabu 2012). However SB efficacy in nickel toxicity has not previously been investigated. This study was aimed at investigating the protective effect of SB against the toxic effects of nickel sulfate on the testes in rats.

Materials and Methods

Experimental animals

Twenty four healthy adult male rats (Sprague Dawley, 6 weeks old weighing 200±20 g) were obtained from an experimental research unit of Fırat University. In the standard laboratory conditions (12h light:12h dark and 23±2°C, 60-65% humidity) the animals were fed with a diet consisting of 22.21% protein, 3.32% fat, 3.12% fibre, 68% carbohydrate, vitamins, and minerals. Food and water were provided ad libitum. The Local Ethics Committee approved the experiment protocol.

Experimental design

The rats were divided into four groups for thirty-day administration. As the control group the rats were injected with 1 ml isotonic saline solution, intraperitoneally (Group1, n=6). Nickel sulfate (Acros Organics) was given alone at doses of 5 mg/kg/day (Su et al. 2011) to Group 2 (n=6), respectively. In the SB (Sigma Chemical) group (Group 3, n=6) the rats received only daily SB in 150 mg/kg/day dose (Oufi and AlShawi 2014). SB, 150 mg/kg/day was added to nickel sulfate 5 mg/kg/day in Group 4 (n=6). Nickel sulfate was always diluted with 1 ml isotonic saline solution and administered intraperitoneally and SB was suspended in a 0.3% carboxymethylcellulose (CMC) (Sigma Chemical) solution and administered orally (Lu et al. 2009). The rats were sacrificed by cervical dislocation on the thirty-first day. Following collection of testes and sperm samples, the serum was prepared by centrifugation (1500xg, 15 min, 4°C), frozen and stored at 20°C; the sperm samples obtained from fresh cauda epididymis were held in 0.9% NaCl and were processed according to the method described previously (Muralidhara and Narasimhamurthy 1991) to determine the sperm counts. Motility, membrane, and acrosome integrity of the sperms were checked using the method described previously (Sen et al. 2017).

Histological procedures

For histopathology, the tissue samples were fixed in buffered neutral 10% formaldehyde and embedded in paraffin blocks. 4mm thick sections were then cut and stained with Hematoxylin-Eosin (HE) (Histostain Plus Kit; Zymed). Tissue analysis was performed using an Olympus BX53 microscope equipped with Olympus Cell B software for image acquisition and analysis. Histopathological changes were evaluated semiquantitatively as 0: none, 1: mild, 2: moderate, 3: severe. The paraffin-embedded tissue blocks were cut into 4 μm thick sections on polylysine coated glass slides for the immunohistochemical studies. The Streptavidin-Biotin-Peroxidase Complex method was used to investigate in all tissues. They were stained with the streptavidin-biotin-peroxidase complex (ABC) technique after routine deparaffinization and rehydration procedures. Immunohistochemical staining was performed according to the specified method indicated by Yumusak et al. (2018). All tissues were marked with caspase3 (1:200, Invitrogen), caspase8 (1:50, Abcam) and caspase9 (1:100, Abcam) primer antibodies. Diaminobenzidine (DAB, Dako) was used as the chromogen. Cytoplasmic staining was scored according to brown staining presence as 0-negative, 1-weak, 2-intermediate, or 3-strong (Detre et al. 1995). The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay method was used according to the catalog procedure (In Situ Cell Death Detection Kit, POD; Roche, Germany) to determine apoptosis-related DNA breaks in testes cells. The method indicated by Yumusak et al. (2018) was applied to all procedures. In the final evaluation, on the basis of the percentages of the stained nuclei, staining indexes were scored as 0-negative, <1% positive; 1-weak, 1-25%; 2-intermediate, >25-75%; or 3-strong, >75%.

Table 1. Post-treatment sperm motility, viable spermatozoa rate, sperm count and abnormal spermatozoa of four groups of rats (data are mean ± SEM).

Groups	Sperm motility (%)	Viable spermatozoa (%)	Sperm Count (millons/ml)	Abnormal spermatozoa rate (%)
1 (Control)	74.00±0.0a	83.40±1.0a	83.25±3.0b	12.80±1.1b
2	28.00±5.7c	38.20±2.9c	55.25±2.5c	21.20±0.7a
3	78.00±2.7a	80.60±2.2a	93.75±1.6a	12.40±0.9b
4	44.00±2.2b	48.60±2.5b	68.70±2.3b	21.20±1.0a
p	0.00	0.00	0.00	0.00

a,b,c are used to refer to and distinguish the values of the different groups.

Table 2. TUNEL results in testis histological damage and scoring in all experimental groups of rats.

Variables	Group1	Group 2	Group 3	Group 4
Testes degeneration	-	+	-	+
Germinal cell detachment	-	++	-	+
Vesiculation in germinal cells	-	++	-	+
Testes dystrophy	-	++	-	++

() – no lesion, (+) – mild lesions, (++) moderate lesions, (+++) –severe lesions. *The intensity of staining was scored as – (< 1% positive), + (1 to 25 % positive), ++ (>25 % to %75 positive), +++ (>75 % positive) according to the percentage of positive staining cells (Sen et al. 2017) .

Statistics

Statistical analyses were carried out using SPSS, Version 16.0 (SPSS Inc., Chicago, USA). Quantitative data analyses related to tissue pathology Chi square was used. The group causing the difference was determined using the Duncan test if variance analysis showed a difference between groups. In quantitative data, peak value as a central tendency measure of variance, if there were more than one peak value, was used for groups.

Results

Spermatological results

Sperm motility was similar in Groups 1 and 3 (p>0.05), Groups 1 and 3 had a higher sperm motility rate than the others (p<0.05). Group 2, with the lowest sperm motility rate, differed significantly from Group 4 (p<0.05). The viable spermatozoa rates of Groups 1 and 3 were similar (p>0.05) and higher than those of others (p<0.05). The lowest sperm viability rate was in Group 2 (p<0.05). The lowest sperm count and sperm viability rate was in Group 2 (p<0.05). The sperm count of Group 4 was significantly higher than that of Group 2, the percentage of abnormal spermatozoa was similar in both groups (Groups 2 and 4) and higher than the other Groups (p<0.05). There was no significant difference between the other Groups (1 and 3) for the percentage

of abnormal spermatozoa (p>0.05). Better spermatological parameters were seen in Group 4, and Group 3 (SB only) had higher sperm counts (Table 1).

Microscopic results

In the histopathological examination of testicular tissue sections Groups 1 and 3 demonstrated the absence of microscopic lesions. Toxicity signs of lesions in the interstititium and seminiferous tubules (germinal cell loss and detachment, seminiferous tubule degeneration, cytoplasmic vacuolisation, and testes dystrophy) were significantly less severe in Groups 2 and 4 (Fig. 1). In immunohistochemical staining of Activated Caspases in rat testes (Fig. 2); all antibody and TUNEL stainings were shown to have no detectable immunostaining for Group 1 (a1, b1, c1, d1) and Group 4 (a4, b4, c4, d4). Severe immunopositive reactions were seen in Group 2 (a2, b2, c2, d2) and Group 5 (a5, b5, c5, d5). However, in Group 3 (a3, b3, c3, d3) and Group 6 (a6, b6, c6, d6) mild focal reactions were shown in the testes. In Group 2 in tubules and the interstitium mild cellular positiveness, showing local immunopositive apoptotic reactions, was observed. Similar changes, such as local tubular reactions, were seen in Group 4. The immunohistochemical and TUNEL assay of the testicular tissues (Table 2) showed apoptosis of varying severity in all groups except Groups 1 and 3 (p<0.05). Group 2 in particular showed significantly higher levels of TUNEL assay than the other groups (p<0.05).
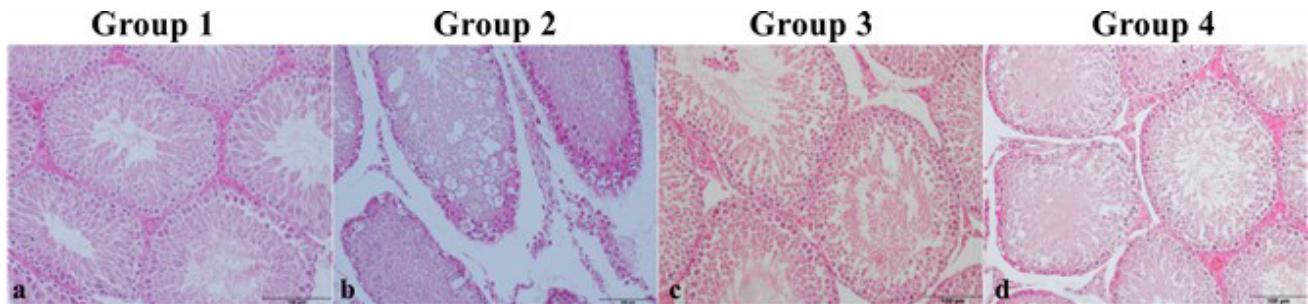
Fig. 1. Histological examination of hematoxylin and eosin-stained (HE) sections of rat testes tissues (HE, x100).
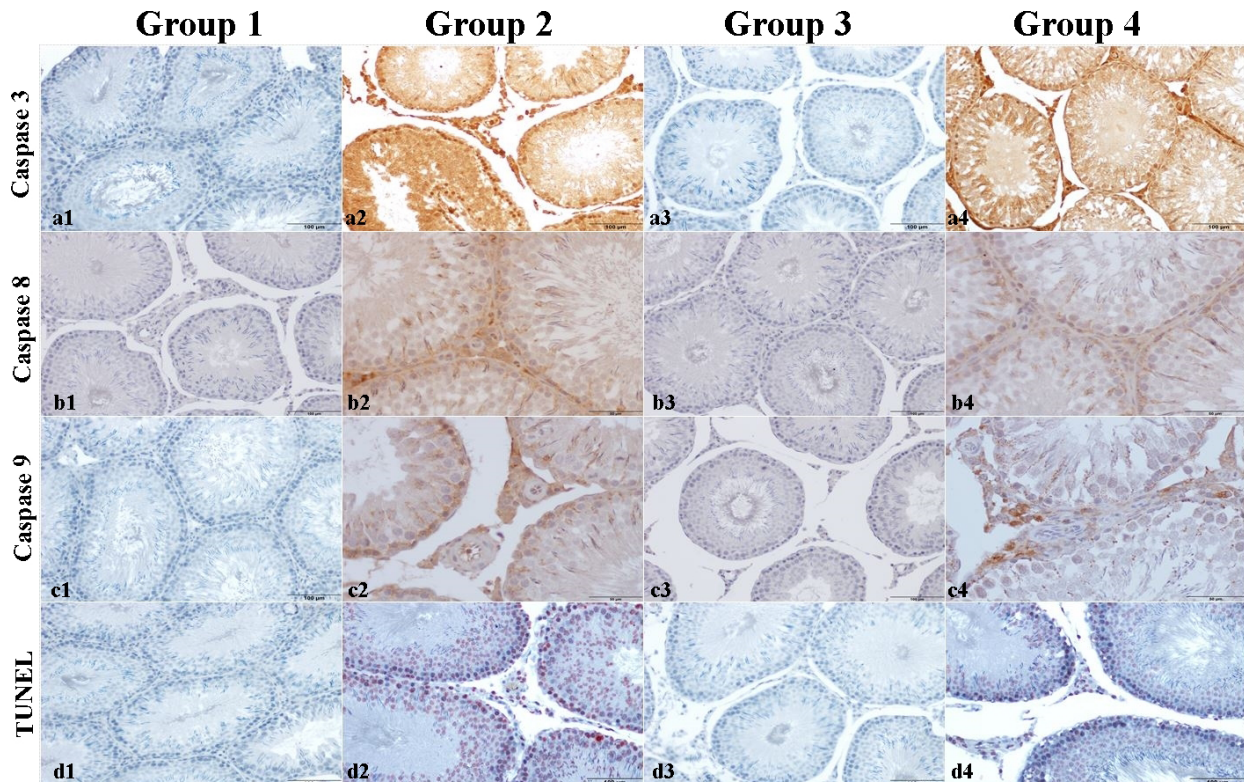

Fig. 2. Immunohistochemical staining of rats testes for Caspases and TUNEL (x100).

Discussion

In the present study, it was determined that SB showed a protective effect on the reproductive system of male rats that were exposed to nickel sulfate. It is well known that nickel has various carcinogenic effects on internal organs such as the liver, kidneys and lungs, and a toxic effect on the immune system, genes, blood, nervous system, and reproductive system (Das et al. 2008). Several studies have shown the harmful effects of nickel on testicular tissue (Doreswamy et al. 2004, Oufi et al. 2012). On the basis of the results obtained with the histopathological and immunohistochemical staining of the testicular tissues of Group 4 it was concluded that SB had a protective effect against the adverse effects of nickel sulfate. There are several studies that have demonstrated the antioxidant effect

of SB (Oufi and AlShawi 2014, Baeeri et al. 2018). Sozen et al. (2015), reported that, when used against the adverse effects of itraconazole in rats, SB shows a cytoprotective effect owing to its antioxidant property. Furthermore, Prabu and Muthumani (2012) indicated that, when used for prophylactic purposes in rats that were administered arsenic, SB showed an antioxidant effect, provided protection in particular against free oxygen groups, regulated the glutathione level, and, as understood from the expression level of caspase3 in the renal tissue, protected the kidneys against apoptosis. Similarly, in the present study, it was determined that the level of apoptosis in the testicular tissues was lower in Group 4 compared to Group 2.

Nickel damages nuclear proteins by increasing the level of endogenous cellular hydrogen peroxide, which in return reduces the enzymatic activity required

for DNA replication, transcription, recombination and repair (Das et al. 2008). Research has shown that SB prevents the adverse effects of the genotoxic effects of methotrexate on sperm motility, and reduces testicular cell damage in mice (Oufi and AlShawi 2014). In the present study, Group 2 presented with lower sperm motility, viability spermatozoa rates and sperm counts than those of the other groups, and also suffered from the most severe histopathological adverse impact on the testicular tissue. Furthermore, when compared to the groups that received nickel alone (Group 2), those groups which were administered SB together with nickel (Group 4), were determined to have significantly better sperm motility and viability spermatozoa rates. In Group 4 the sperm motility, viability spermatozoa rates and sperm counts were higher than those detected in Group 2, suggesting that SB had a protective effect. In the present study, the best sperm parameters were detected in Groups 1 and 3. In fact, the sperm counts of Group 3 were found to be significantly higher than those of Group 1. Furthermore, the histopathological parameters of the testes were observed to be unaffected in Groups 1 and 3, while SB was determined to have significantly reduced histopathological damage in Group 4. Pandey (1999) determined that, in adult male mice, nickel sulfate decreased the epididymal sperm count and sperm motility rate, and increased the percentage of abnormal spermatozoa. Upon administering different doses of nickel sulfate and nickel chloride to mice, Pandey and Srivastava (2000) observed that with higher doses the percentage of abnormal sperm increased, whilst the epididymal sperm count and sperm motility rate decreased. Lukac et al. (2014), reported that a single intraperitoneal dose of nickel chloride reduced sperm motility and concentration in adult male mice. Oufi et al. (2012) determined that, when administered to mice at several different doses, SB reduced the number of nonviable sperm and improved sperm motility, and also observed that the percentage of abnormal spermatozoa decreased. Similarly, in the present study, in Group 4 it was ascertained that SB had a protective effect against the adverse effects of nickel on sperm. As is well known, nickel has the ability to directly pass the testis blood barrier (Cavicchiaet al. 1996). Research has shown that nickel accumulates in the testes and causes the degeneration of germ cells (Pandey et al. 1999). In the present study, in Group 2, testicular degeneration and the percentage of abnormal sperm were determined to have increased, whilst the sperm viability rate was detected to have decreased. These results are likely to have been caused by the accumulation of nickel in the testicular tissue. Massányi et al. (2007) who administered nickel chloride intraperitoneally to male mice, determined that the functional semi-

niferous epithelium, where spermatozoa are produced, is adversely affected, and the TUNEL assay showed a high level of apoptosis in the interstitium. In the present study, the immunohistochemical and TUNEL assay of the testicular tissues demonstrated the presence of apoptosis at varying levels of severity in all of the groups, excluding Groups 1 and 3 ($p<0.05$). Particularly in Group 2, the results obtained for the Caspase3, 8, 9 and TUNEL assays were observed to be significantly more severe than those obtained in the other groups ($p<0.05$). Additionally, caspase3, 8, 9 and TUNEL activities were observed to be lower in Group 4 The low caspase3, 8, 9 and TUNEL activities observed in the groups that were given SB were attributed to the antioxidant property of this substance. The present study demonstrates that SB has a protective effect on the testicular tissues and certain spermatological parameters in rats exposed to nickel sulfate. Further detailed research is needed to be able to make use of this protective effect of SB in clinical practice.

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