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Protective effects of selenium nanoparticles against doxorubicin-induced testicular apoptosis in rats

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ABSTRACT

The chemotherapeutic drug doxorubicin (Dox) is prescribed for cancer treatment. In addition to cancer cells, its cytotoxic effect affects healthy tissues with a high proliferation index, such as the testes. This study examined the potential of selenium nanoparticles (SeNPs) to attenuate Dox-induced testicular apoptosis. Thirty-two male albino rats were divided into four experimental groups (n=8): Control (group I), Dox (group II), SeNPs (group III) and Dox + SeNPs (group IV). For four weeks, Dox (3 mg/kg body weight) was administered intraperitoneally weekly, while SeNPs (0.5 mg/kg) were administered orally daily. After experimental treatments, testicular tissues were harvested for histological, immunohistochemical and molecular analyses. SeNPs treatment with Dox (group IV) markedly decreased testicular histological lesions induced by Dox (group II) and up-regulated (p<0.05) the defensive antioxidant nuclear factor erythroid 2–related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) genes. SeNPs also decreased (p<0.05) the protein levels of pro-apoptotic P53, Bax, caspase-3 and increased (p<0.05) anti-apoptotic Bcl-2 genes in group IV compared to group II. To conclude, SeNPs alleviate Dox-induced testicular damage and apoptosis by improving the antioxidant capacity of spermatogenic cells and by inhibiting apoptosis.

Keywords: Doxorubicin, selenium nanoparticles, Nrf2, Apoptosis, testicular injury

1. INTRODUCTION

Chemotherapy is one of the most effective strategies for treating patients with cancer. However, despite the beneficial effects of chemotherapeutic drugs, they lack specificity, resulting in cytotoxicity not only to malignant cells, but also to healthy tissues with rapid cellular cycles (Kluwe, 2016). One side effect of chemotherapy is a testicular injury which can lead to male infertility (Delessard et al., 2020). Therefore, it is crucial to understand the reproductive toxicity mechanisms of various chemotherapy drugs and to explore protective measures for patients treated with chemotherapy.

Dox (also known as Adriamycin) is an anthracycline antibiotic group of

chemotherapeutic drugs commonly used in the treatment of different types of cancer (D'Angelo et al., 2022). In the male reproductive system, Dox causes injury to the testicular germinal epithelium and disrupts spermatogenesis, which can result in azoospermia (Abdrabou et al., 2021). The underlying mechanism of Dox-induced testicular damage has been attributed to increased oxidative stress (Belhan et al., 2020) and germ cell apoptosis (Shinoda et al., 1999). Therefore, antioxidants may play a pivotal role in protecting against Dox-induced testicular toxicity.

Nrf2 is a key modulator of cellular defence mechanisms in reaction to oxidative stress. Nrf2 activates the transcription of antioxidant response elements (ARE) of cytoprotective and antioxidant enzyme genes, such HO-1, ultimately reducing oxidative stress and protecting against apoptosis (Egbujor et al., 2021). Activation of Nrf2 has been shown to protect against Dox-mediated toxicity in various tissues, including the heart, liver and kidneys (Mirzaei et al., 2021). Nrf2 has also been reported to be effective in alleviating cisplatin-induced testicular damage (Abdel-Wahab et al., 2020), suggesting a potential role in reducing Dox-mediated testicular toxicity.

Selenium (Se) is a micronutrient with known antioxidant properties that is necessary for animal and human health (Radomska et al., 2021). Concerning male reproduction, it was found to be crucial for testosterone biosynthesis, healthy testicular development, spermatogenesis and sperm quality and function (Qazi et al., 2019). Se deficiency has been implicated in numerous male reproductive defects, including seminiferous tubule degeneration, spermatogenesis abnormalities and poor sperm maturation and quality (Abdel-Halim et al., 2016). Moreover, a study by Boussada et al., (2017) demonstrated that Se ameliorates Dox-induced gonadotoxicity in male rats by attenuating stress conditions and associated apoptosis. These findings support the importance of Se in male reproductive functions and its potential role in overcoming gonadotoxicity. However, the therapeutic use of Se is limited because of its low safety margin (Bhattacharjee et al., 2019).

As arising nanomedicine, SeNPs have received considerable interests due to their superior antioxidant effects and lower toxicity compared to the other selenocompounds (Ferro et al., 2021). Previous reports have shown that, in comparison to Se, SeNPs increased testicular antioxidant activity and improved sperm concentration, motility and viability in rats (Abd-Allah and Hashem, 2015). Furthermore, several studies have shown that SeNPs are effective in ameliorating testicular damage caused by the anticancer drug cisplatin (Rezvanfar et al., 2013) and other environmental agents (Rashad et al., 2018). This study was conducted to investigate the possible protective effects of SeNPs and evaluate the role of Nrf2 and key apoptotic proteins (P53, Bcl-2, Bax, Caspase-3) as possible mechanisms for alleviating Dox-induced testicular injury.

2. MATERIALS AND METHODS

Experimental animals

Thirty-two male albino rats weighing 120–150 g was used in this study. They were housed in separate cages under standard experimental conditions (12 h day/night cycle and 22 °C) with ad libitum access to food and water. The animals were acclimatised to their environment for one week before the initiation of the experiment. All experimental protocols involving animals in this study were performed according to the guidelines of the Ethical Committee, Department of Pharmacology and Toxicology, Faculty of Medicine, Al-Azhar University, Egypt. This study was conducted from July 2021 to November 2022.

Study design

All rats were randomly divided into four groups (n = 8rats/group) as follows: Group I acted as a control and was injected with 0.5 ml of a single intraperitoneal (IP) injection of normal saline daily for four weeks. Group II was used as the Dox-treated group and was administered a single IP injection of Dox (Shaanxi Xinheng Biotech, China) for four weeks at a dose of 3 mg/kg/week prepared in normal saline (Kumar et al., 2016). Group III served as the SeNP-treated group and was administered 0.5 mg/kg SeNPs (Nano-Tech, Cairo, Egypt) by oral gavage daily for four weeks (Asadpour et al., 2020). Group IV served as the Dox + SeNP-treated group and received 3 mg/kg/week IP of Dox and 0.5 mg/kg/day of SeNPs by oral gavage for four weeks

Samples collections

Twenty-four hours after the last experimental treatment, the rats were anaesthetised with ether and euthanised by cervical dislocation. One testis sample from each experimental animal group was fixed using 10% neutral buffered formalin (Sigma–Aldrich, USA) for histological and immunohistochemical analysis and the others were immediately frozen in liquid nitrogen and stored at −80 ◦C for gene/protein expression analysis.

Histological staining

Formalin-fixed testes samples from all groups were dehydrated through an ascending gradient of ethanol, cleared in xylene, embedded in paraffin wax and sectioned at 4 μm. For histological analysis, tissue sections were stained with haematoxylin and eosin (H & E), observed under a light microscope and scored according to (Johnsen, 1970).

Immunohistochemical staining for caspase-3

Assessment of apoptotic markers was carried out using a caspase-3 antibody and an HRP/DAB (ABC) immunohistochemical staining detection kit (Abcam, UK) according to the manufacturer's instructions. The sections were counterstained with Mayer's haematoxylin and examined under a light microscope. To quantify immunostaining, caspase-3 positive cells were analysed and scored according to (Khalil et al., 2020).

Elisa assay for quantitative determination of protein expression of apoptotic regulators P53, Bax and Bcl-2

Teste tissue homogenates from all experimental groups were prepared and protein expression of apoptotic regulators P53, Bax and Bcl-2 were analysed according to the manufacturer's instructions using a quantitative sandwich CUSABIO ELISA kit (Wuhan, China).

Real-time quantitative PCR assay

To measure the expression of Narf2 and the antioxidant enzyme HO-1 genes, total RNA was extracted from all samples using Direct-zol RNA Miniprep Plus (Zymo Research Corp., USA). Additionally, quantity and quality were assessed using a Beckman dual spectrophotometer (USA). Extracted RNA was reverse transcribed using the Super Script IV One-Step RT-PCR kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. SYBR Green dye PCR Master Mix, along with the primers listed in Table 1 was used for real-time PCR analysis. Furthermore, fold changes in genes of interest in each experimental group were determined using the comparative threshold cycle (Ct) method after normalising gene expression to the endogenous control GAPDH (Pfaffl, 2001).

Table 1 Prime sequences

	Forward sequence	Reverse sequence
Nrf2	AGGACATGGAGCAAGTTTGG	TTGCCCTAAGCTCATCTCGT
Ho-1	TCAGGTGTCCAGAGAAGGCTTT	CTCTTCCAGGGCCGTGTAGA
GAPDH	CACCCTGTTGCTGTAGCCATATTC	GACATCAAGAAGGTGGTGAAGCAG

Statistical analysis

All data were analysed using the statistical software program SPSS for Windows, version 20, USA. The data were examined for normality using the Shapiro-Wilk test. The homogeneity of variance was checked using Levene's test. One-way ANOVA followed by Tukey's post-hoc multiple comparisons for parametric data and Kruskal-Wallis followed by Dunn's test for multiple comparisons for non-parametric data were applied $p < 0.05$.

3. RESULTS

Ameliorative effect of SeNPs on Dox-induced testicular histological lesions

Alterations in the morphology and structure of rat testes were examined using H & E-stained tissue sections from different treatment groups. Tissue sections from Dox-treated group II exhibited testicular damage in the form of tubular vacuolation with germ cell sloughing and irregular and compressed seminiferous tubules with an increase in intertubular spaces (Figure 1B, C, D) compared to control group I, which showed normal testicular histology (Figure 1A). SeNP-treated group III showed testicular tissue architecture comparable to that of control group I (Figure 1E). However, tissue sections from group IV displayed rejuvenated testicular morphology with markedly decreased testicular lesions in the form of focal sloughed spermatogenic cells in comparison to group II (Figure 1F). Evaluation of the histopathological effect of each treatment on spermatogenesis activity (Figure 1G) revealed a significant decrease in testicular injury score in group II compared to the normal control group I. Group III showed comparable results to group I. However, Group IV showed significant improvement in spermatogenesis compared to group II.

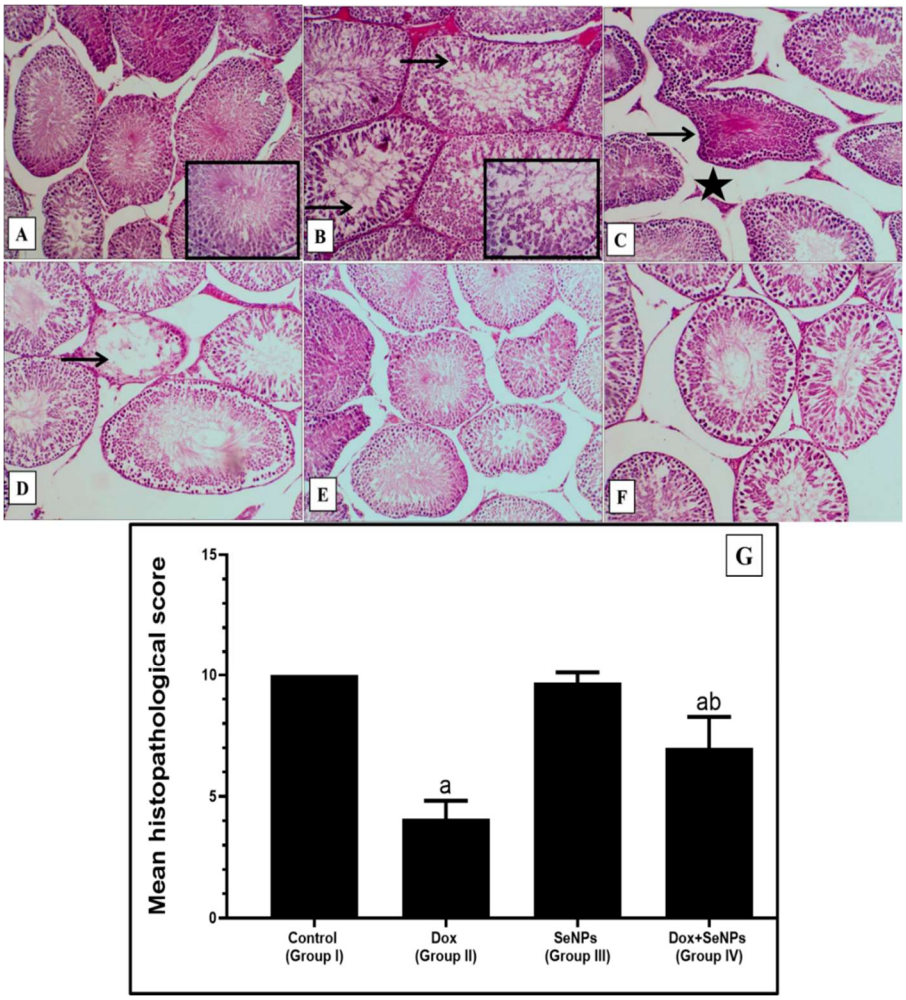

Figure 1 SeNPs ameliorate Dox-induced testicular histopathological changes. (A-F) Photomicrographs of testis sections from different treatment groups (H & E, 100x, 400x inserts). (A) Control group I shows normal testicular histology with normal seminiferous tubules (insert). (B-D) Dox-treated group II shows testicular tubular vacuolation with germ cell sloughing (arrows) (B), irregular and compressed seminiferous tubules (arrow) with an increase in intertubular space (star) (C) and complete loss of germ cells in some seminiferous tubules (arrow) (D). (E) SeNPs treated group III shows testicular histological structure comparable to those of control group I. (F) Dox + SeNPs treated group IV shows focal sloughed spermatogenic cells. (G) Quantification of the effect of each treatment on spermatogenesis. Thirty random tubules from each experimental group were graded. Data represent mean ± SE (n=4). The letter (a) indicates significance versus control group I; letter (b) indicates significance versus Dox group II when $p < 0.05$

Anti-apoptotic effect of SeNPs against Dox-induced testicular apoptosis

To establish the effect of SeNP treatment on Dox-induced testicular apoptosis, the expression of the apoptotic marker caspase-3 was investigated in testicular tissues from each experimental group by immunohistochemistry (Figure 2). Tissue sections from control group I showed negative immunostaining for caspase-3 (Figure 2A). Compared to the normal control Group I, Dox-treated group II revealed strong caspase-3 positively stained spermatogenic cells (Figure 2B). SeNP-treated group III (Figure 2C) showed comparable staining results as control group I. However, Dox + SeNP-treated group IV (Figure 2D) showed weak caspase-3 positively stained cells compared to group II. Quantification of the immunostaining expression of caspase-3 (Figure 2E) revealed a significant increase in caspase-3 expression ($p<0.05$) in Dox-treated group II compared to that in control group I. The increase in caspase-3 expression observed in group II was significantly reduced in group IV compared with that in group II (Figure 2E).

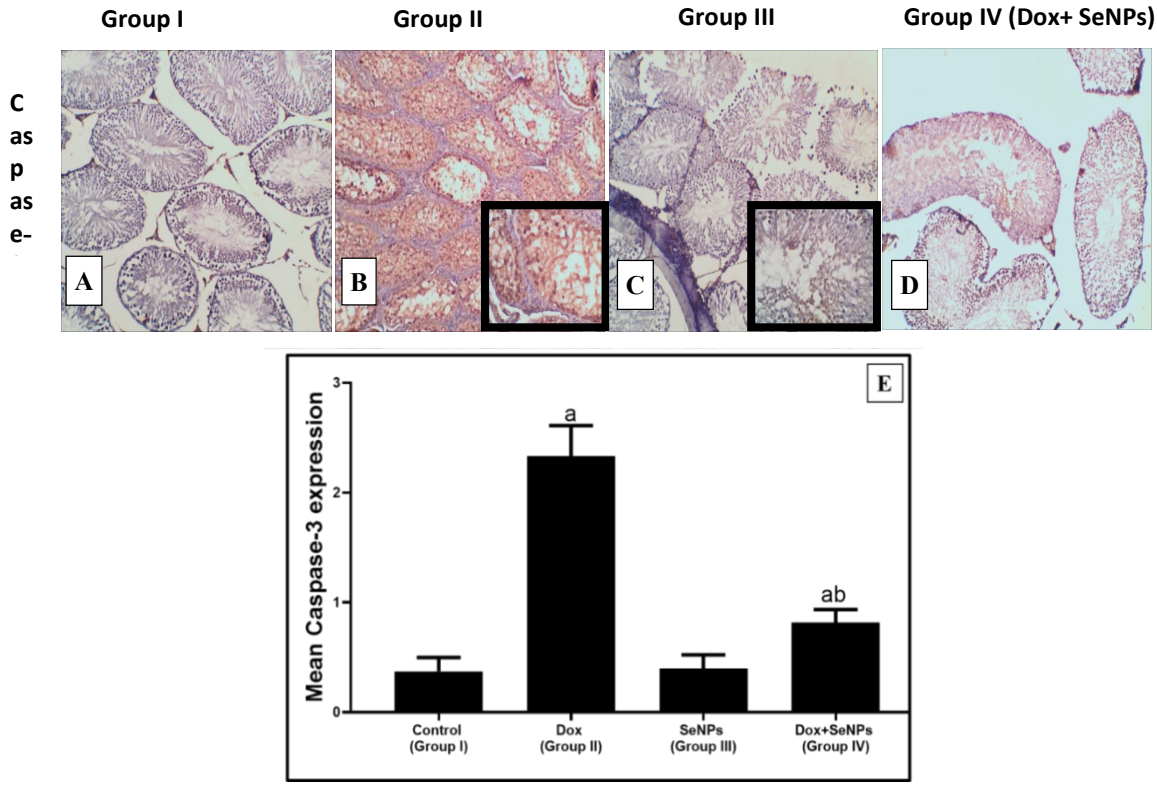

Figure 2 SeNPs reduce Dox-induced testicular apoptosis. (A-D) Photomicrographs of immunohistochemistry assays on testicular sections from all treatment groups for the apoptotic marker caspase-3 (H & E, 100x, 400x inserts). (A) Group I show negative staining. (B) Group II staining is evident in spermatogenic cells. (C) Group III shows comparable results to control group I. (D) Group IV shows weak staining compared to group II. (E) Quantification of the impact of each treatment on caspase-3 expression. Data represent mean ± SE (n=4). The letter (a) indicates significance versus control group I; letter (b) indicates significance versus Dox group II when $p < 0.05$.

The effect of SeNPs on the protein levels of P53, Bax and Bcl-2

Dox-treated group II showed an increase ($p<0.05$) in the protein levels of P53 (Figure 3A) and Bax (Figure 3B) and a decrease ($p<0.05$) in Bcl-2 as compared to control group I. SeNP-treated group III showed no significant change in the protein levels compared to group I. However, Dox + SeNP treated in group IV showed a decrease ($p<0.05$) in P53 and Bax and an increase ($p<0.05$) in Bcl-2 compared to group II (Figure 3A, B, C).

Up-regulatory effect of SeNPs on Nrf2/HO-1 gene expression

Dox-treated group II showed down regulation ($p<0.05$) in the gene expression of the Nrf2 transcription factor and antioxidant enzyme HO-1 compared to the control group I (Figure 4A, B). However, the Dox + SeNPs group IV showed up regulation ($p<0.05$) in Nrf2 and HO-1 gene expression compared to group II (Figure 4A, B).

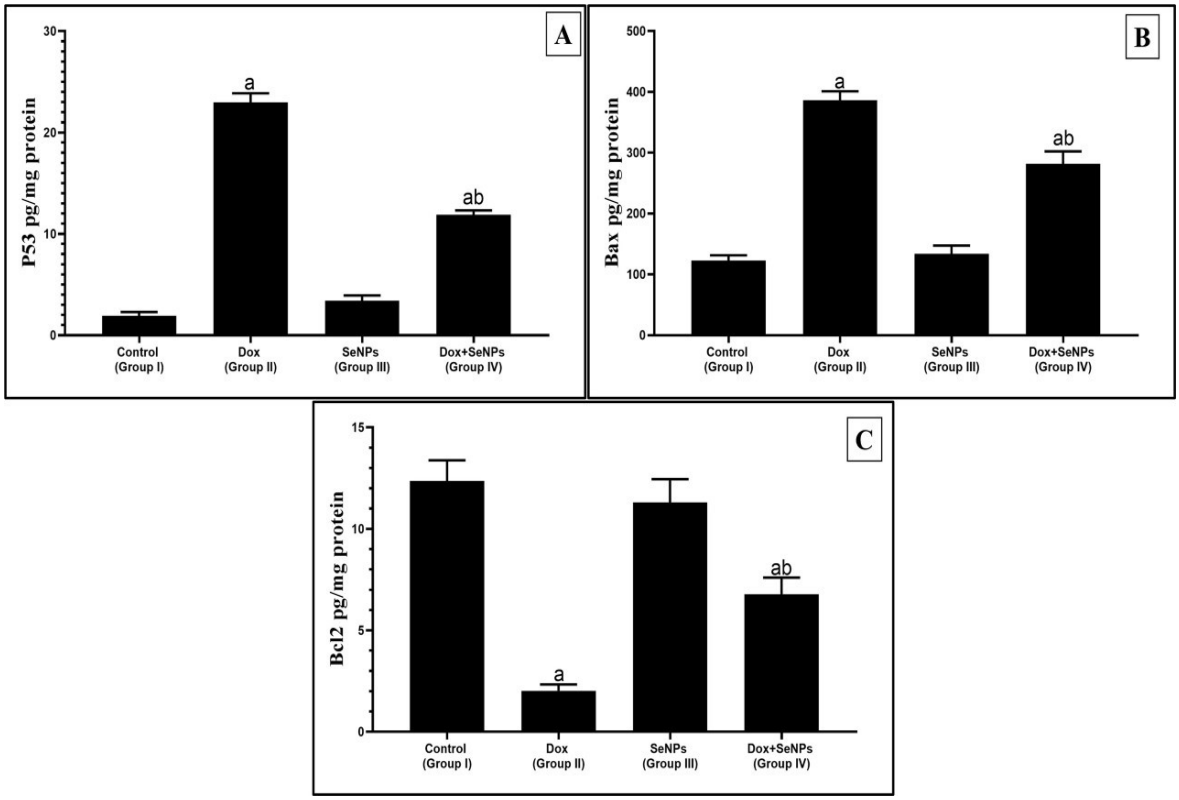

Figure 3 Quantification of protein levels of pro-apoptotic P53, BAX and anti-apoptotic Bcl-2 regulator genes in different experimental groups as analysed by Elisa. Data represent mean ± SE (n=4). The letter (a) indicates significance versus control group I; letter (b) indicates significance versus Dox group II when $p < 0.05$.

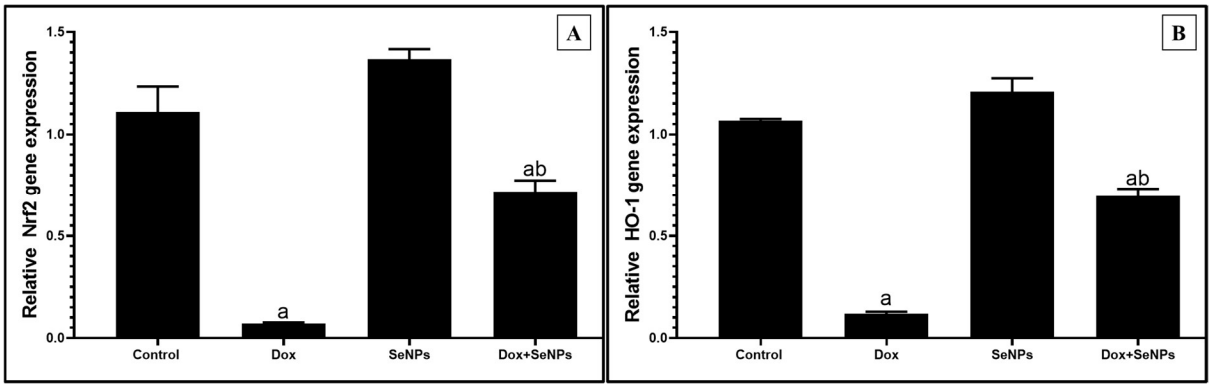

Figure 4 Gene expression of Nrf2 (A) and HO-1 (B) in different experimental groups. Data represent mean ± SE (n=4). The letter (a) indicates significance versus control group I; letter (b) indicates significance versus Dox group II when $p < 0.05$.

4. DISCUSSION

Treatment with chemotherapeutic drugs has several adverse effects, including testicular degeneration (Delessard et al., 2020). Dox is an anthracycline-derived antibiotic that plays a significant role in the treatment of several cancers (D'Angelo et al., 2022). However, its clinical use is limited by its destructive side effects on the kidneys, liver and testicular tissues (Pereverzeva et al., 2007).

In this study, the therapeutic potential of SeNPs against Dox-induced testicular damage and apoptosis were investigated. The current study found that the intraperitoneal injection of Dox induced testicular histological alterations. Furthermore, it causes down regulation of the antioxidant defence pathway Nrf2/HO-1, resulting in testicular germinal epithelial apoptosis. These testicular alterations were ameliorated by the co-treatment with SeNPs.

Kato et al., (2001) attributed testicular damage to Dox due to its histological changes either in the stroma or parenchyma. The present study found that intraperitoneal injection of Dox induced testicular vacuolation with germinal epithelium cell sloughing and significantly decreased the testicular injury score. These results are in agreement with those reported by Kumar et al., (2016)

and Divya et al., (2017). In contrast, treatment with SeNPs significantly improved the testicular histological alterations and injury scores induced by Dox. This finding is in line with the protective effect of SeNPs against testicular structural alterations caused by various agents, including the anticancer drug cisplatin (Rezvanfar et al., 2013), the antibiotic gentamicin (Hamoud, 2019), the heavy metal lead (El-Fakharany et al., 2022) and environmental agents (Rashad et al., 2018).

A previous study reported that germ cell apoptosis is one of the main mechanisms underlying Dox-induced testicular damage (Shinoda et al., 1999). Anti-apoptotic Bcl-2 and pro-apoptotic Bax regulator proteins, which belong to the Bcl-2 family, together with the tumor suppressor p53, are key components involved in apoptosis (Harada and Grant, 2003; Bunz et al., 1998). Moreover, caspase-3 activation is considered the end stage of the apoptotic process and is a marker of the apoptotic process (Salvesen, 2002). In the present study, Dox administration markedly elevated the protein expression of caspase-3 in spermatogenic cells. This represents an increase in apoptosis (Ujah et al., 2021) supporting the findings of a previous study by Türedi et al., (2015), who reported that Dox administration induces apoptosis of spermatogonia and all other spermatogenic cells. Furthermore, in the current study, Dox was found to upregulate ($p<0.05$) the testicular protein levels of the pro-apoptotic P53, Bax and downregulated ($p<0.05$) the protein levels of the anti-apoptotic Bcl-2 regulator genes. All of these events eventually result in the activation of caspase-3, the primary executor of apoptosis (Salvesen, 2002). These findings were in accordance with previous studies reporting that apoptosis induced by Dox in the testis is manifested by elevated expression of pro-apoptotic genes, such as P53, Bax and caspase-3 and decreased expression of anti-apoptotic genes, such as Bcl-2 (Yeh et al., 2008; Ujah et al., 2021; Safaei-Pourzamani et al., 2022). In contrast, administration of SeNPs significantly reduced testicular germ cell apoptosis induced by Dox, as evidenced by the increased ($p<0.05$) expression of anti-apoptotic Bcl-2 and decreased ($p<0.05$) expression of P53, Bax and caspase-3 protein. This anti-apoptotic effect of SeNPs is consistent with a previous study by Zhang et al., (2019), who reported that SeNPs attenuated NiSO4-induced testicular injury and apoptosis by down regulating and up regulating the expression of anti-apoptotic and pro-apoptotic regulatory genes, respectively. Furthermore, the anti-apoptotic effect of SeNPs against cadmium-induced apoptosis in the brain is associated with decreased P53, Bax expression and increased Bcl-2 expression (Al-Kahtani, 2020), supporting the anti-apoptotic role of SeNPs observed in this study.

Another key mechanism underlying Dox-mediated testicular damage is oxidative stress (Belhan et al., 2020), which can be mitigated by Nrf2. Nrf2 is a transcription factor that controls the expression of cytoprotective and antioxidant enzyme genes, such as HO-1, through interaction with the ARE (Egbujor et al., 2021). Its activation is considered one of the compensating endogenous antioxidant protective systems as a result of reactive oxygen species (ROS) accumulation (Hu et al., 2016). Nrf2 is also one of the main modulators of apoptosis, causing a reduction in apoptosis by activating the expression of Bcl-2, down regulating Bax and reducing caspases-3 activity (Niture and Jaiswal, 2012; Niture and Jaiswal, 2013; Renu and Valsala-Gopalakrishnan, 2019). In this study, Dox significantly down regulated the mRNA expression of Nrf2 and HO-1, in agreement with the studies conducted by Ujah et al., (2021) and Khodir et al., (2021), which reported that Dox-induced apoptosis in the testis is associated with decreased expression of Narf2/OH-1. In contrast, co treatment with SeNPs and Dox significantly up regulated the testicular Nrf2/HO-1 pathway. This finding concurs with a previous study by Yuan et al., (2022), who stated that SeNP administration provides neuroprotection against pentylenetetrazole-induced hippocampal oxidative stress by up regulating the Nrf2/HO-1 pathway, thereby inhibiting neuronal apoptosis. The protective effect of SeNPs against Dox-induced testicular apoptosis observed in this study can be explained by their antioxidant and anti-apoptotic effects.

5. CONCLUSION

Dox administration induces testicular histological alteration, apoptosis and down regulation of the Nrf2/HO-1 pathway, whereas SeNPs provide testicular protection by improving the testicular architecture, decreasing testicular apoptosis and up regulating the Nrf2/HO-1 pathway.

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Ethical approval

The study was approved by the Ethical Committee, Department of Pharmacology and Toxicology, Faculty of Medicine, Al-Azhar University, Egypt (Code: Pha. 3Med.Research._0000003).

Informed consent

Not applicable.

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Conflict of interest

The authors declare that there is no conflict of interests.

Data and materials availability

All data sets collected during this study are available upon reasonable request from the corresponding author.

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