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RESEARCH ARTICLE Indian Journal of Animal Research

Therapeutic Potency of Prodigiosin Conjugated with Silver-nanoparticles in Male Rats Exposed to Cadmium Chloride-induced Testicular Toxicity

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ABSTRACT

Background: Cadmium (Cd) is a frequent environmental toxin that causes harm to humans, animals and plants. In the current Study, the therapeutic effects of Prodigiosin conjugated silver nanoparticles (PG-AgNP2) (a red pigment generated by Serratia marcescens strain) on cadmium chloride ($CdCl_2$)-induced testicular toxicity in rats was assessed.

Methods: $CdCl_2$ (6.5 mg/kg) was injected intraperitoneally (i.p.) into male albino rats followed by an i.p. injection of PG-AgNP2 (3 mg/kg) for seven days.

Result: As a result of Cd exposure, a considerable decline in the amount of serotonin (5-HT) neurotransmitters in the hypothalamus was observed. In addition, injection with $CdCl_2$ caused an increase in residual Cd levels in the testis and a decrease in serum levels of FSH, LH and testosterone. Testicular tissue suffered from considerable oxidative damage due to $CdCl_2$ exposure. MDA and NO levels were increased by $CdCl_2$ and natural antioxidant proteins were less abundant. In addition, the inflammatory mediators were markedly increased as a result of Cd exposure. Testicular cells exposed to $CdCl_2$ underwent apoptosis, as shown by increased Bax and caspase 3 expression and decreased Bcl_2 expression. Our research indicates that PG-AgNP2 may function as a natural remedy to ameliorate harm in the hypothalamic pituitary testicular axis associated with $CdCl_2$ exposure.

Key words: Apoptosis, Inflammation, Nanoparticles, Prodigiosin, Testosterone, Testis.

INTRODUCTION

The origin of male infertility has been linked to a wide range of variables, including varicocele, accessory gland infection, immunological issues, malignancies, genetic abnormalities, endocrine disorders and exposure to environmental contaminants including heavy metals such as cadmium (Cd) (Sharma *et al*., 2021).

Cadmium is one of the most pervasive environmental toxins that can have harmful effects on living things at low or high concentrations (Genchi *et al*., 2020). Serotonin and Gonadotropin-releasing hormone (GnRH) dysregulation in the hypothalamus of the male rat are accelerated.

by exposure to Cd (Zakharova *et al*., 2020). The hypothalamus secretes GnRH, which stimulates the pituitary gland to create LH and FSH, which in turn stimulates the testes to produce testosterone. GnRH modulates the hypothalamic-pituitary-testicular (HPT) axis. Through negative feedback, testosterone inhibits the synthesis of GnRH, FSH and LH from the hypothalamus and pituitary gland.

Numerous bacteria produce natural red pigment named prodigiosin (PG) which shares a pyrrolyl pyrromethene structure. It was first characterized in *Serratia marcescens*. The antifungal, immunosuppressive and antiproliferative properties of this promising pigment are demonstrated.

For potential use in the food business, prodigiosin might take the place of synthetic colorants. PG was demonstrated to be a potent proapoptotic agent against several cancer cell lines, including ones that were resistant to numerous medications, while having little to no effect on healthy cell lines (Sudhakar *et al*., 2021). Moreover, PG has antibacterial, antiparasitic, insecticidal and immunomodulatory activities (Suryawanshi *et al*., 2017). Many studies have been conducted on natural pigments like PG during the past 10 years since they seem to be a desirable bioactive alternative.

The effective properties of PG enable its use in models in which oxidative stress might play a role; therefore, in the present study, we investigated the effect of PG-conjugated AgNP2 on hypothalamic pituitary testicular axis in male rats intoxicated with $CdCl_2$.

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How to cite this article: El-khadragy, M.F., Salem, F.E.H., Yehia, H.M. and Al-Megrin, W.A. (2023). Therapeutic Potency of Prodigiosin Conjugated with Silver-nanoparticles in Male Rats Exposed to Cadmium Chloride-induced Testicular Toxicity. Indian Journal of Animal Research. doi:10.18805/IJAR.BF-1663.

Submitted: 05-05-2023 **Accepted:** 04-07-2023 **Online:** 22-.07-2023

MATERIALS AND METHODS

Bacterial isolation, preparation, extraction, purification and quantification of prodigiosin, as well as the formation of PG-conjugated AgNP2 and its characterization, were carried out according to Farag *et al*. (2017) and El-batal *et al.* (2017). Additionally, the anhydrous cadmium chloride ($CdCl_2$) was provided by Sigma Chem Co. (St. Louis, MO, U.S.A.).

Forty adults male Wister rats (weighing 130-160g) were used to study the therapeutic impact of PG-AgNP2 on $CdCl_2$ reproductive toxicity. The rats were obtained and housed in Department of Biology, College of Science, Princess Nourah bint Abdulrahman University. For the in vivo experiment. The animals were housed in wire-bottomed cages under standard conditions of illumination with a 12-h light-dark cycle and at a temperature of 25±1°C for 1 week until the beginning of treatment. The animals were provided with tap water and a balanced diet ad libitum.

The animal care procedures agreed with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, eighth edition and were approved by the Institutional Animal Ethics Committee for Laboratory Animal Care at Princess Nourah bint Abdulrahman University, (Approval number: 23-0435/13/4/2023).

After the acclimation phase, the animals were divided into four groups at random (n=10 rats/group).

1. Control group: rats were intraperitoneally (i.p.) injected with 0.1 ml of 0.9% NaCl.
2. Prodigiosin-conjugated nano silver (PG-AgNP2) group: animals were i.p. treated with PG-AgNP2 (3 mg/kg) according to (El-batal *et al*., 2017).
3. Cadmium (Cd) group: rats were i.p. injected with $CdCl_2$ (6.5 mg/kg) according to (Elkhadragy *et al*., 2018).
4. Cd+PG-AgNP2 group: animals were i.p. injected with PG-AgNP2 (3 mg/kg) after 2 h of Cd (6.5 mg/kg) exposure.

The treatment was given for seven days. The animals were killed by sudden decapitation 24 h after the last treatment, serum was collected and stored at -80°C for measurement of the male sex hormonal profile and brains were rapidly excised from skulls, blotted and chilled. The brain tissue was rapidly wiped dry with filter paper then the hypothalamus was isolated and kept at (-80°C) for the measurement of serotonin (5-HT). Testes were immediately dissected, weighed and homogenized in ice-cold 50 mM of Tris-HCl buffer (pH 7.4), centrifuged for 10 min at 3,000×g and the supernatants were stored at "80°C for assessment of oxidative status and antioxidants biomarkers as well as pro-inflammatory cytokines and apoptotic markers. Serotonin was assayed using HPLC as described by Pagel *et al*. (2000). The relative testis weight was calculated according to the following formula:

$$\text{Relative testicular weight} = \frac{\text{Testis weight}}{\text{Body weight}} \times 100$$

Cadmium concentration in the testicular tissues was determined by using atomic absorption spectrophotometer at 228.8 nm according to the method described by Kubaszewski *et al*. (2014). Determination of blood Testosterone, FSH and LH was done by enzyme-linked immunosorbent assay (ELISA) kits according to Chen *et al*. (1991), Rose, (1998) and Rebar *et al*. (1982) respectively. Using the technique outlined by Ohkawa *et al*. (1979) the concentration of malondialdehyde (MDA), a lipid peroxidation (LPO) biomarker, was measured in the testicular tissue. Additionally, using the Lodovici *et al*. (1997) approach, testicular DNA was isolated and hydrolyzed to estimate 8-hydroxy-2-deoxyguanosine (8-OHdG).

By reducing Elman's reagent with GSH to yield a yellow molecule, Glutathione (GSH) was measured (Giustarini *et al*., 2014). According to Aebi, (1984) catalase (CAT) activity was estimated. The Nishikimi *et al.* (1972) method was used to measure the superoxide dismutase (SOD) activity. The Paglia and Valentine (1967) method was used to test GRx activity. GPx activity was calculated using a reaction combined with glutathione reductase as the reduction in NADH per minute (GR).

TNF-α (tumor necrosis factor-α) and interleukin-6 (IL-6) concentrations were measured using commercial ELISA kits (RandD System, Minneapolis, MN, USA) in accordance with the manufacturer's instructions.

In addition, according to the manufacturer's instructions, a colorimetric caspase-3 assay kit (Sigma-Aldrich Co. USA) was used to examine testicular tissue homogenates prepared in lysis buffer. By using ELISA kits, B cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax) levels in the tissue homogenate were determined (LifeSpanBioSciences, Inc., Seattle, WA, USA).

Finally,Data analysis was done using the Statistical Package (SPSS) for the Social Sciences. The results were presented as the mean ± standard error of the mean (SEM). To acertain significance, Duncan's test was used after a one-way analysis of variance (ANOVA). The acceptable level of significance was 5% ($p<0.05$).

RESULTS AND DISCUSSION

Heavy metal exposure in the environment, particularly exposure to Cd, has been strongly linked to the development of a number of pathological failures by causing oxidative damage. One of the organs that are most adversely affected by cadmium poisoning is the testes (Zhu *et al*., 2020). Here, we investigated the possible therapeutic efficacy of PG-AgNB2 in treating rats against $CdCl_2$-induced hypothalamic-pituitary-testicular dysfunction in rats. Rats exposed to $CdCl_2$possessed lower concentrations of 5-HT in their hypothalamus, which indicated a disruption in serotonergic neurotransmission and this was clear from the reduction in the content of 5-HT as compared to the control. Interestingly, the levels of this neurotransmitter were considerably restored ($p<0.05$) by the injection of PG-AgNPs (Fig 1).

According to our findings and earlier research, rats exposed to Cd exhibit neurotoxic effects (Salem *et al*., 2022). In fact, Cd has the ability to accelerate the initiation and progression of serious neurological problems. The blood

brain barrier (BBB's) permeability has been observed to increase in response to Cd, focusing mostly on the brain tissue that has been identified as a target for Cd-mediated toxicity. Our findings showed that Cd can lower levels of 5-HT in the hypothalamus. Following Cd toxicity, serotonergic disruption has been documented in animal models (Lawes *et al.,* 2020). After exposure to Cd, 5-HT neurotransmitter levels may have decreased as a result of the production of ROS, which inhibits the enzymes involved in monoamine biosynthesis, disturbs monoamine metabolism by encouraging monoamine breakdown, elimination and blocks monoamine uptake (Lizarraga *et al.,* 2015).

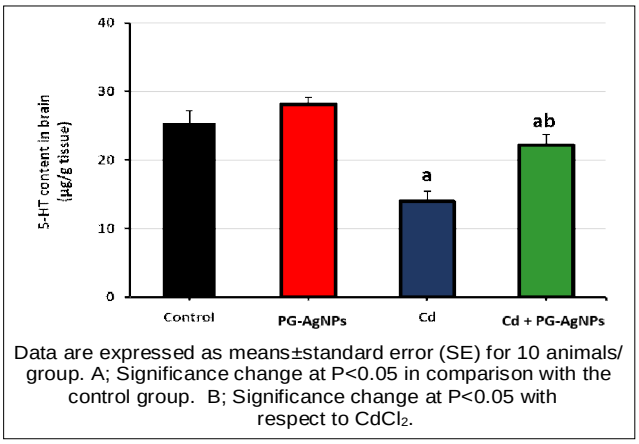
Additionally, it was recently shown by Salem *et al.* (2022) that Cd intoxication stimulated monoamine oxidase (MAO), an enzyme that catalyzes the oxidative deamination of monoamines, which causes an increase in hydroxyl radicals in the brain producing a change in the amount of 5-HT. It is important to note that the gonadotropin-releasing hormone (GnRH) is controlled by serotonin production from the hypothalamus. The maintenance of gonadotropin secretion and regular reproductive function depends on the release of the gonadotropin-releasing hormone (GnRH). A similar final route controls LH and FSH and the neurons that generate GnRH are primarily located in the hypothalamus. The observed decrease in 5-HT concentration in the hypothalamus following exposure to Cd may affect indirectly pituitary gonadotropins (FSH and LH) secretion.

On the other hand, it has been hypothesized that prodigiosin can treat a variety of diseases linked to environmental pollutants. The use of metal-based nanoparticles has become a promising trend in the pharmaceutical business due to the enhanced bioavailability, delivery progression and drug inflow to the target tissues supplied by these treatment formulations compared to normal medication formulations. Several investigations showed that exposure to metal-based nanoparticles in high doses over an extended period of time causes a metal build-up in the cells. According to Patlolla *et al*. (2015), a low dose of AgNP2 administered for 7 days did not significantly increase accumulation or toxicity in animal cells; instead, it improved the delivery of the intended treatment to the cells.

On the other hand, the neuroprotective effects of PG have received little attention, our results recorded amelioration in the content of Hypothalamic 5-HT. These findings are consistent with those of Salem *et al*. (2022), who observed that PGs have the ability to control neurotransmission in brain tissues, particularly monoamines, by reducing the level of MAO-caused reduction in the oxidative deamination of monoamines and elevating their concentration. Increased levels of 5-HT in the hypothalamus may lead to amelioration in pituitary gonadotropin production (FSH and LH).

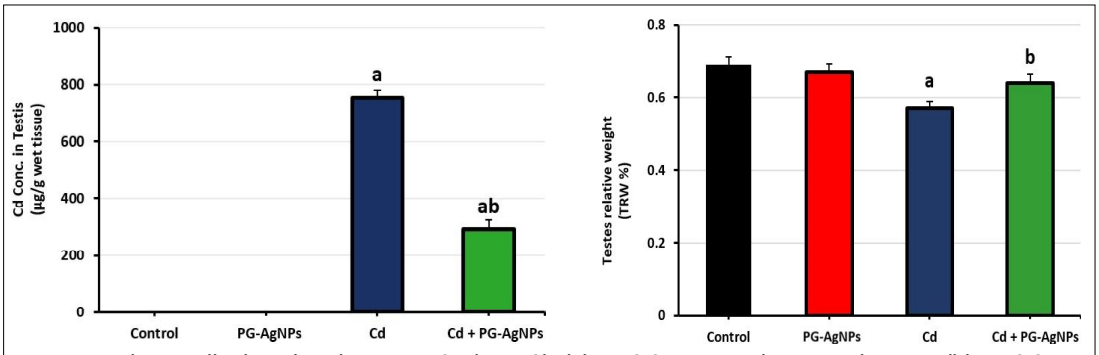
Moreover, the use of organ weight as a significant toxicological marker has been widespread. After receiving $CdCl_2$ treatment for seven days, our findings showed a reduction in the relative weight of the testicles (Fig 2). This weight loss may be to reduce the number of germ cells that lead to testicular failure (Fan *et al*., 2018).

The results in Fig 3 showed that the serological levels of testosterone, LH and FSH reduced in response to Cd exposure, which was also associated with a decrease in



Data are expressed as means±standard error (SE) for 10 animals/ group. A; Significance change at P<0.05 in comparison with the control group. B; Significance change at P<0.05 with respect to $CdCl_2$.

Fig 1: The effect of treatment with PG-AgNP2 (3mg/kg) on the content of serotonin (5-HT) in the hypothalamus in rats intoxicated with $CdCl_2$ for 7 days.

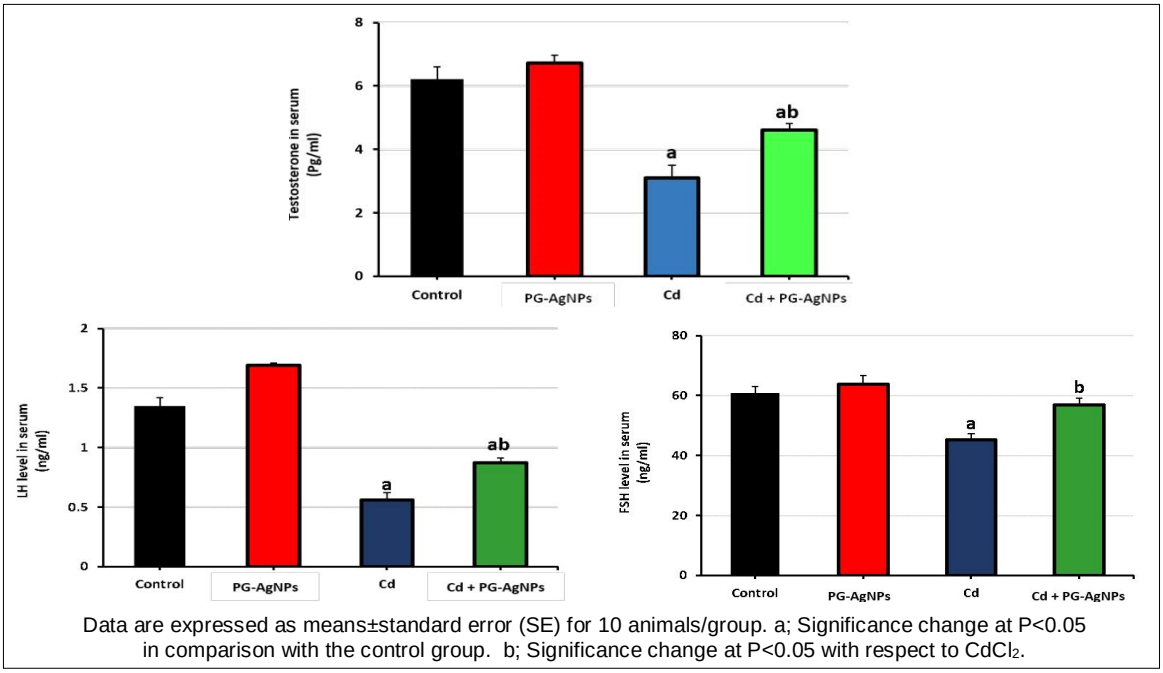


Results are displayed as the mean±SE (n = 10). (a); p<0.05 versus the control group; (b); p<0.05 versus the Cd-treated group.

Fig 2: Bioaccumulation of Cd in testicular tissue and the relative testis weight in response to PG-AgNPs and/or Cd treatment.

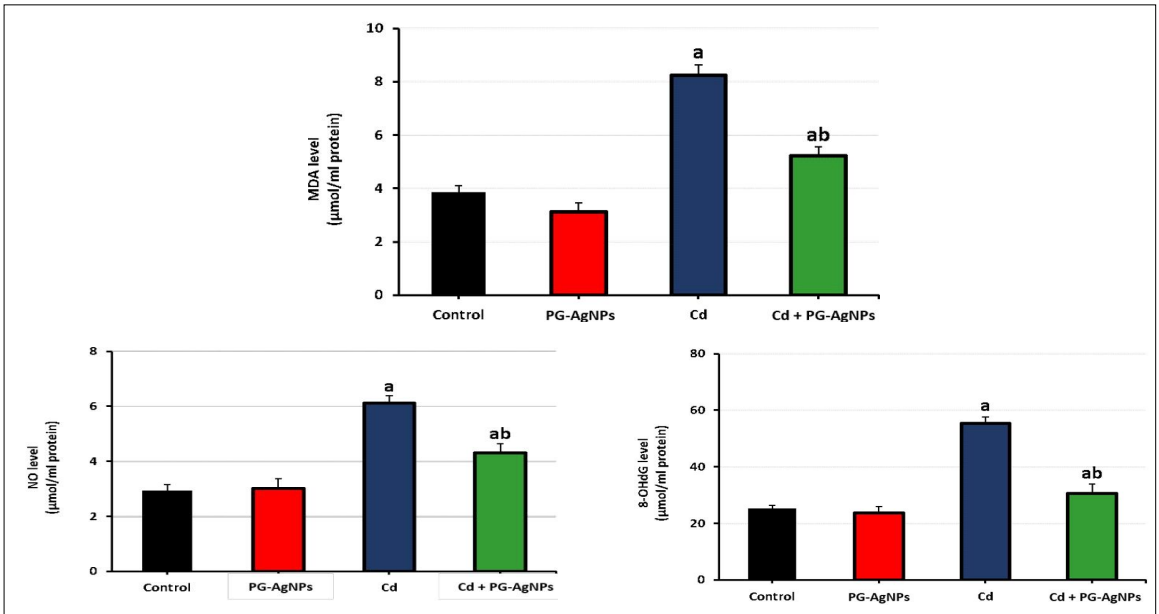
testicular weight. The inactivation of steroidogenic enzymes, such as 3β- and 17β-hydroxysteroid dehydrogenase, which disrupts androgen synthesis and inhibits testosterone production, is thought to be the cause of the decline in the evaluated sex hormones (Hachfi and Sakly 2010). Meanwhile, PG-AgNP2 treatment was able to ameliorate and minimize Cd-induced testicular weight loss; this may be due to its high and rich nutraceutical constituents. PG-AgNP2 treatment against Cd toxicity is extended to restore the levels of testosterone, LH and FSH to be close to the normal values.

Oxidative stress is produced by the generation of ROS, which causes Cd testicular toxicity. The ability of Cd in the



Data are expressed as means±standard error (SE) for 10 animals/group. a; Significance change at P<0.05 in comparison with the control group. b; Significance change at P<0.05 with respect to $CdCl_2$.

Fig 3: The effect of treatment with PG-AgNP2 (3 mg/kg) on the content of serum testosterone, luteinizing hormone (LH) and follicular stimulating hormone (FSH) in rats intoxicated with $CdCl_2$ for 7 days.



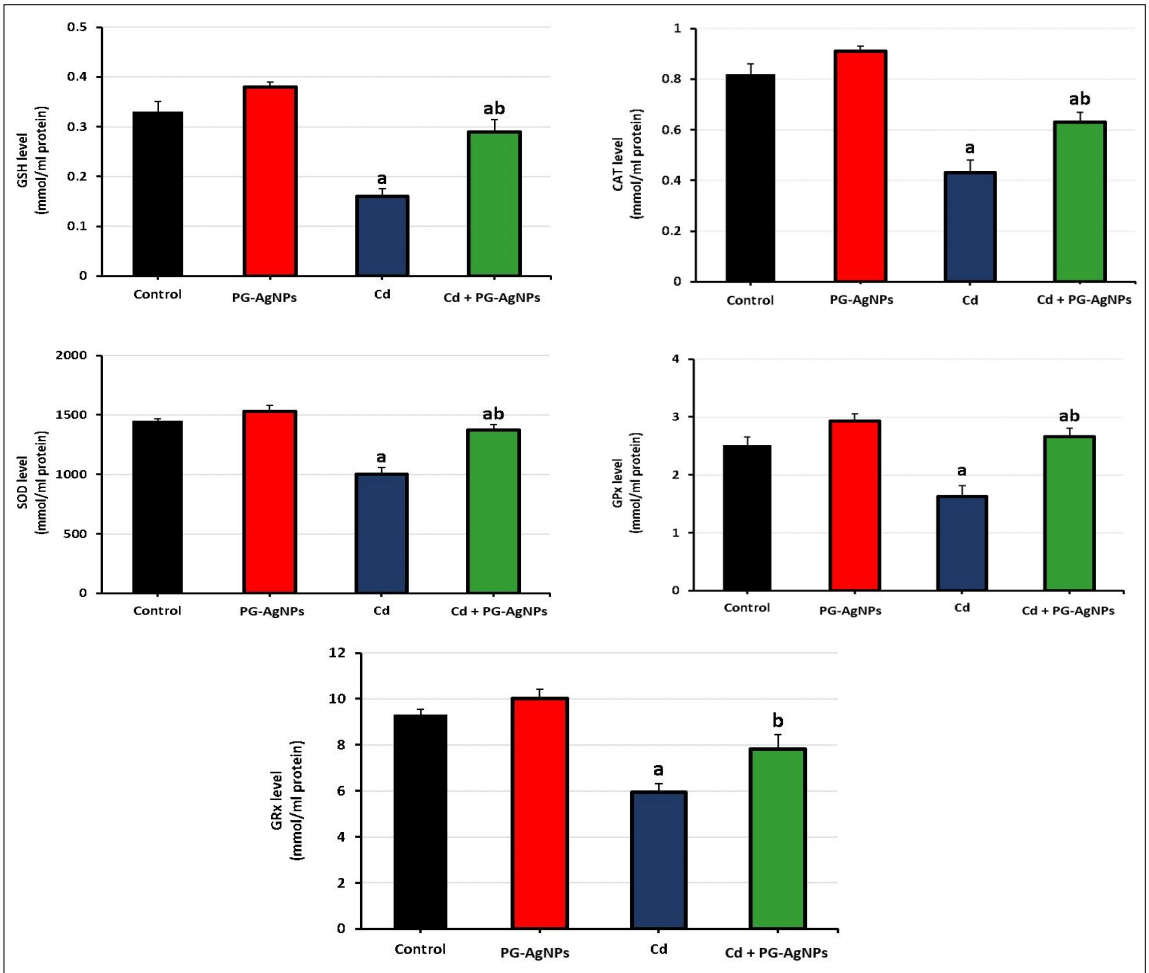
Data are expressed as means±standard error (SE) for 10 animals/group. a; Significance change at P<0.05 in comparison with the control group. b; Significance change at P<0.05 with respect to $CdCl_2$.

Fig 4: The effect of treatment with PG-AgNP2 (3 mg/kg) on Testicular levels of oxidative stress indicators in rats intoxicated with $CdCl_2$ for 7 days. Data are expressed as means±standard error (SE) for 10 animals/group.

production of ROS was confirmed by measuring the level of NO, MDA and 8-OHdG in addition to determining the activity of antioxidant enzymes (GSH, GPx, GR, CAT and SOD) in the testicular tissue homogenate of rats. Our findings (Fig 4 and 5) showed that a seven-day continuous exposure to Cd (6.5 mg/kg body weight) resulted in a testicular injury because the antioxidant defense mechanisms were depleted, which disrupted cellular redox and led to oxidative stress. In the current investigation, a rise in NO levels was also seen in response to Cd exposure, indicating the presence of nitrosative stress in the testicular tissue. The damage is caused by the coupling of NO with singlet oxygen, which creates peroxynitrite anion (ONOO), which may be more toxic than its precursors in terms of generating tissue toxicity (Lee *et al.* 2019). This conclusion was supported by a fall in GSH levels as well as the activity of SOD, CAT, GR and GPx.

The reduction in antioxidant enzyme levels may be triggered by the accumulation of Cd in testicular tissue, which reduces the GSH pool. The fact that GSH, SOD, CAT, GPx and GR levels and activities decreased in response to Cd intoxication shows that all of these abnormalities contribute to Cd-mediated testicular damage. The ability of Cd to react with -SH group of enzymes, which in turn suppresses the GSH pool enzymes, glutathione consumption during free radical elimination (Winiarska-Mieczan, 2018), increased lipid peroxidation levels and excessive production of superoxide anions are just a few possible mechanisms that could be responsible for the suppression of endogenous antioxidants.

According to our findings, the administration of PG-AgNPs prevented Cd induces alterations in the redox status of brain tissue. This was shown by the reduction of ROS production, the formation of MDA, 8-OHdG and NO, as well as the stimulation of the antioxidant system. These results confirm promising testicular protection and antioxidant characteristics of PG-AgNPs. PG inhibit NADPH oxidase2 activity and ROS production to protect tissues from oxidative and nitrative damages brought on by hypoxia and ischemia.



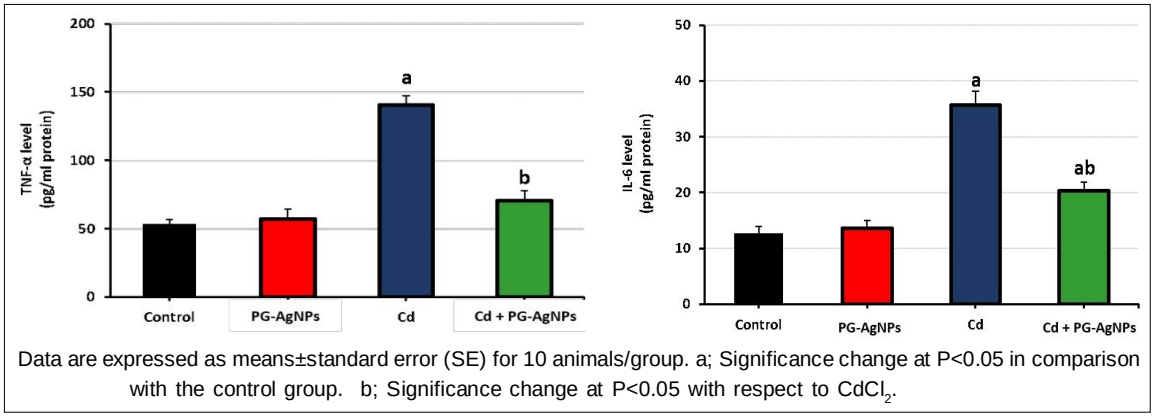
a; Significance change at P<0.05 in comparison with the control group. b; Significance change at P < 0.05 with respect to $CdCl_2$.

Fig 5: The effect of treatment with PG-AgNP2 (3mg/kg) on Testicular antioxidant enzyme activities with $CdCl_2$ for 7 days.

Additionally, PG reduced ROS generation and stimulated 8-OHdG to reduce microcystin LR-mediated oxidative stress in HepG2 cells (Chen *et al*., 2019).

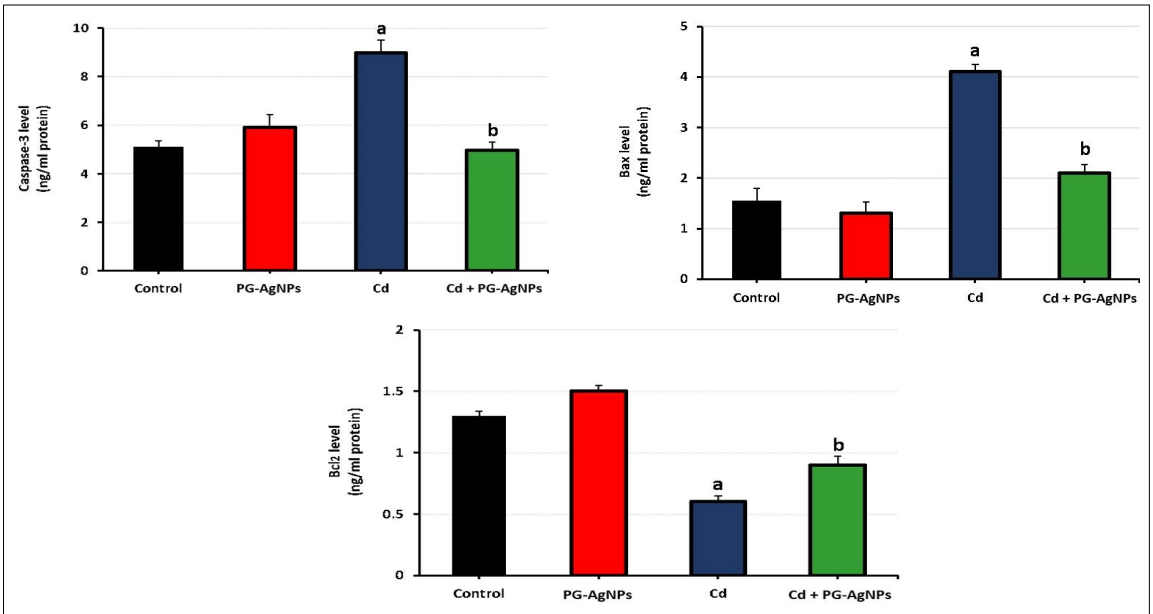
In addition to the increase in the oxidative state of the testicular tissue, the injection with $CdCl_2$-induced Toxicity led to Testicular inflammation, which was detected by significantly higher tissue levels of pro-inflammatory cytokines (TNF-α and IL-6) compared to those found in the control group ($p<0.05$). These inflammatory responses were dramatically reduced in PG-AgNPs-treated rats compared to the $CdCl_2$ group, demonstrating the anti-inflammatory effect of PG-AgNPs in the $CdCl_2$-induced Testicular toxicity model (Fig 6). Previous studies have reported that exposure to Cd not only damages the antioxidant defence system but also triggers the body to initiate an inflammatory reaction. Pro-inflammatory cytokines, particularly TNF-α and IL-6, cause and exacerbate testicular injury and inflammation (Kassab *et al.* 2020). The inflammatory cytokines stimulate the accumulation of neutrophils, which in turn increases tissue damage and inflammation. The treatment with PG-AgNP2 in the current study decreased the elevation of inflammatory cytokines (TNF-α and IL-6) in testicular tissues. The powerful anti-inflammatory action of PG-AgNP2 may be the mechanism by which it restores the damage caused by Cd and ameliorates the studied cytokines (Lin *et al*., 2019).

To explore apoptotic events in the $CdCl_2$-induced testicular toxicity model rats and the potential anti-apoptotic



Data are expressed as means±standard error (SE) for 10 animals/group. a; Significance change at P<0.05 in comparison with the control group. b; Significance change at P<0.05 with respect to $CdCl_2$.

Fig 6: The effect of treatment with PG-AgNP2 (3 mg/kg) on testis inflammatory markers in rats intoxicated with $CdCl_2$ for 7 days. Data are expressed as means±standard error (SE) for 10 animals/group.



Data are expressed as means±standard error (SE) for 10 animals/group. a; Significance change at P<0.05 in comparison with the control group. b; Significance change at P<0.05 with respect to $CdCl_2$.

Fig 7: The effect of treatment with PG-AgNP2 (3 mg/kg) on testicular level of apoptosis markers in rats intoxicated with $CdCl_2$ for 7 days. Data are expressed as means±standard error (SE) for 10 animals/group.

role of PG-AgNPs treatment, the levels of Bcl-2 and Bax and caspase-3 activity were examined in testicular tissue. Compared with the control group, rats exposed to $CdCl_2$ exhibited significant elevations ($p<0.05$) in the levels of apoptogenic proteins (Bax and caspase-3), whereas a significant reduction ($p<0.05$) in the Bcl-2 level (anti-apoptotic protein) was observed. However, PG-AgNPs injection prevented the apoptotic cascade and reversed the $CdCl_2$-exposure-induced changes in apoptotic proteins compared with the untreated $CdCl_2$ levels, indicating the effective role played by, PG-AgNPs against neuronal loss following $CdCl_2$ exposure (Fig 7). These results are in line with those of Amanpour *et al.* (2019), who showed that mitochondria play a key role in Cd-induced apoptosis. According to previous reports, Cd produced mitochondrial malfunction that led to the opening of the mitochondrial permeability transition pore, which allowed the release of the apoptosis-inducing factor (AIF) and cytochrome c, which then activated the caspase cascade and caused apoptosis. Apoptosis of germ cells was also thought to be induced by Cd *via* oxidative damage. Oxidative stress caused dysregulation of Ca^{2+} channels, altering the permeability of the mitochondrial membrane, releasing cytochrome c and ultimately stimulating the caspase cascade and DNA destruction.

Apoptosis was inhibited in the testicular tissue of rats given therapy with PG-AgNP2. A decrease in the production of pro-apoptotic proteins (Bax and caspase-3) and an increase in the expression of the anti-apoptotic protein Bcl-2, however, indicated that treatment with PG decreased the Cd-induced loss of testicular cells. Many previous studies have proved thatPG prevented apoptosis in rats suffering from different malformations. Lapenda *et al.* (2020), studied the anti-apoptotic action of PG and discovered that it prevented the apoptotic cascade associated with stomach lesions caused by injections of acidified ethanol.

CONCLUSION

According to the study's findings, PG-AgNP2 significantly reduces Cd-induced testicular toxicity in rats by regulating hypothalamic serotonergic transmission, controlling the release of reproductive hormones (testosterone, FSH and LH), suppressing pro-oxidative insults (8-OHdG, NO and MDA) and strengthening antioxidant defense mechanisms (GSH, GPx, GR, SOD and CAT), reducing inflammation (TNF-á and IL-6), preventing apoptosis by lowering pro-apoptotic factors and increasing the anti-apoptotic protein in rat testicular tissue. Our research recommends the use of PG-AgNP2 as a natural therapy to ameliorate toxicity in the hypothalamic pituitary testicular axis associated with $CdCl_2$ exposure.

ACKNOWLEDGEMENT

The authors extend their appreciation to the Deputyship for Research and Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number RI-44-0333.

Conflict of interest: None.

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