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Therapeutic Effects of Biogenic Silver Nanoparticles against Diethyl Nitrosamine-Induced Gonadal Toxicity in Male Rats

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Declaration
Authors' Contribution
Research design: Nayab Aslam and Gul Nabi Khan; experimental work: Nayab Aslam; article writing: Syed Zawar Ali Shah, Wali Ullah, Gul Nabi Khan, manuscript review: Gul Nabi Khan.

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ABSTRACT

Background: The testicular cancer is one of the most common types of malignancies in young adult men, and cause infertility and hormonal disproportions which remains a major concern in the male reproductive health. **Aim:** The current research aimed to assess the carcinogenic effect of DEN (Diethylnitrosamine) and to determine the protective effect of biogenic silver nanoparticles (AgNPs) against DEN-induced damage in gonadal tissues of male rats. **Materials & Methods:** Twenty male albino rats were divided randomly into four experimental groups, including Control, DEN, DEN+AgNPs, and AgNPs. Animals were sacrificed after five weeks of treatment and the alterations in the body weight, hormonal profile, histopathology, genomic DNA and apoptosis related molecules were observed. **Results:** A significant reduction in the body weight and a marked increase in follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were observed in DEN-treated rats. Furthermore, degeneration of germinal epithelium, necrosis and fibrotic seminiferous tubules were found in the DEN group. AgNPs co-treatment showed recovery in body weight, attenuation of the histopathological damage and improved the gonadal architecture. A significant decline in the expression level of p53, DR5 and caspase-9 was noted while AgNPs co-administration reversed the pro-apoptotic gene levels. **Conclusion:** Altogether, AgNPs have a protective effects against DEN-induced toxicity in the gonadal tissues in male rats. Furthermore, these results showed that AgNPs can be considered as a therapeutic agent against testicular carcinogenesis.

INTRODUCTION

Testes are the primary male reproductive organs responsible for spermatogenesis and the production of hormones such as testosterone, which are essential for male fertility and reproductive health [1, 2]. Testes comprise three main cell types i.e. germ cells, Sertoli cells, and Leyding cells, where germ cells develop into spermatozoa, Sertoli cells provide structural and nutritional support, and regulate the testicular microenvironment while Leydig cells produce Testosterone [3]. The coordinated interaction between germ cells, Sertoli cells, and Leydig cells play a crucial role in maintaining normal testicular function, and disruption in this balance can lead to impaired spermatogenesis and pathological conditions [4, 5]. Testicular cancer is a pathological conditions and the most common malignancy in males between 15 and 40 years of age and accounts for approximately 1% of all cancers in men worldwide, with a high survival rate when diagnosed early [6]. Moreover, it is primarily classified into germ cell and non-germ cell tumors, with testicular germ cell tumors (TGCTs) representing nearly 95% of cases [7]. TGCTs are further divided into seminomas and non-seminomas, which differ in their biological behavior and treatment response [8]. Several risk factors, including infertility, genetic predisposition, and environmental exposures, have been associated with the development of testicular cancer [9].

Environmental carcinogens, particularly N-nitrosamines, play a significant role in the initiation of testicular toxicity and carcinogenesis [10]. Diethylnitrosamine (DEN) is a potent nitrosamine widely used in experimental models due to its strong carcinogenic and mutagenic properties [11]. Furthermore, DEN generates reactive intermediates upon metabolic activation by cytochrome P450 enzymes that

induce DNA damage and oxidative stress [12]. In testicular tissue, DEN exposure leads to histopathological alterations such as degeneration of germinal layers, disruption of spermatogenesis, and reduced sperm production [13].

Apoptosis, or programmed cell death, is a fundamental process involved in maintaining cellular homeostasis and eliminating damaged cells [14, 15]. This process is regulated by a balance between pro- and anti-apoptotic proteins. Key regulators include p53, which induces apoptosis in response to DNA damage, and members of the Bcl-2 family such as Bax and Bcl-2, which control mitochondrial integrity [16]. Activation of caspases, mainly caspase-9 and caspase-3, leads to the execution phase of apoptosis, resulting in cellular breakdown and death. Disruption of these apoptotic pathways contributes to carcinogenesis and disease progression [17].

Recent advances in nanotechnology have provided new opportunities for cancer treatment through targeted drug delivery and reduced systemic toxicity [18]. In this context, Silver nanoparticles (AgNPs) have gained attention due to their unique physicochemical properties and biological activities, including antibacterial, anti-inflammatory, and anticancer effects [19]. AgNPs exert their therapeutic effects by inducing oxidative stress and activating apoptotic pathways in cancer cells. Their effectiveness is influenced by factors such as size, shape, and surface characteristics [20].

The present study was designed to investigate the protective effects of biogenic AgNPs against DEN-induced testicular toxicity in male rats, with a focus on the modulation of apoptotic biomarkers and tissue histology.

MATERIALS AND METHODS

This research was conducted in the Main Research Laboratory, Department of Zoology, Islamia College Peshawar, Pakistan.

Animals Maintenance

Adult male Sprague-Dawley rats (180-200g) were procured from the Veterinary Research Institute, Peshawar, Pakistan. The rats were housed in ventilated cages and were provided with regular food and water. Animals were kept in a room with natural, automatic light cycles (12 hours light/dark) at 25°C and standard humidity levels.

Ethical Approval

The Ethical Institutional Review Board (EIRB), Islamia College University, Peshawar, Pakistan, approved the study protocols (August 22, 2024/47-AS&RB/ICP). Health of the rats was monitored twice daily. In order to reduce the suffering of animals during dissection, every procedure was carried out with high care.

Chemical Reagents and Dosage Preparation

Diethyl nitrosamine (DEN) was procured from Sigma (Aldrich, N0258- 1G), and a stock solution (5%) was made in physiological saline. This prepared stock was aliquoted and stored at -20°C. Furthermore, 200 mg of silver nanoparticles (AgNPs) were suspended in 3 ml of distilled water and these solutions were kept at -20°C pending usage.

Experimental Groups

The test animals were divided into four groups with each group containing five rats.

Group I (Normal Control): In this group, the rats were given normal saline through Intraperitoneal (I.P.) injections for five weeks.

Group II (DEN-treated): These rats received one dosage of DEN (90mg/kg; I.P.) every week.

Group III (DEN + AgNPs): In this group, the rats were first administered DEN, followed by an oral gavage of AgNPs (100mg/Kg/week) via an infant feeding tube for five weeks.

Group IV (AgNPs): Rats were administered AgNPs (100mg/kg/week) orally for a period of five weeks.

Plant Extract and Silver Nanoparticles

The plant extract was prepared, and the biogenic silver nanoparticles were synthesized as described previously [21]. The nanoparticles were characterized by Fourier Transform Infrared spectroscopy (FTIR), Energy-Dispersive X-ray Spectroscopy (EDX), X-ray diffraction (XRD), and Scanning Electron Microscopy (SEM) in the Central Resources laboratory, University of Peshawar, Pakistan.

Dissection

After five weeks of the experimental period, rats were anesthetized with chloroform and sacrificed. Blood samples were collected via intracardiac puncture using 3 mL heparin syringes and transferred into EDTA tubes and serum gel tubes for biochemical analysis.

Serum Hormonal Profile

Testicular and other related reproductive hormone levels, such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (TS) were measured using commercially available, relatively cheap enzyme-linked immunoassay (ELISA) kits as per the manufacturer.

Histopathology

Tissues were fixed in formalin solution (10%, pH.7.2) and sections of 3-5µm thickness were made using standard microtomy procedures followed by staining with eosin and hematoxylin. A camera fixed light microscope (Nikon, Japan) was used to study the slides, and images were recorded for further analysis.

DNA Extraction and Ladder Assay

DNA extraction was carried out using the phenol-chloroform method. Tissues (0.1 mg) were homogenized in the lysis buffer (0.5% SDS, 10 mM Tris-HCl and 1 mM EDTA) incubated at 25°C, and centrifuged. The pellet was re-treated with lysis buffer, proteinase K, and SDS at 37°C for 12 h. DNA was isolated from supernatant using phenol-chloroform-isoamyl alcohol extraction, *via* precipitation with sodium acetate and chilled isopropanol. DNA pellet was rinsed with 70% ethanol, air-dried, dissolved in double distilled water, and stored at 4°C. DNA was loaded on 1% agarose gel followed by electrophoresis. DNA visualization was conducted via UV transilluminator using a BioDoc analyzer (Cat No: 97-0170-01).

RNA Isolation and Gene Expression Analysis

Total RNA was isolated from tissue samples using the TRIzol method under RNase-free conditions [22]. All equipment and working surfaces were sterilized with 70% ethanol, and samples were processed on ice with centrifugation performed at 4 °C to prevent RNA degradation. Tissues were homogenized in 1 mL TRIzol reagent following grinding in liquid nitrogen and incubation at room temperature for 10 minutes. Furthermore, 400 µL chloroform was added, mixed, and incubated for 5 mins, followed by centrifugation at 13,000 rpm for 10 min at 4 °C to achieve phase separation. Moreover, the aqueous phase was transferred to a fresh tube, and RNA was precipitated with an equal volume of cold isopropanol, incubated at −20 °C for 10 min, and centrifuged at 13,000 rpm for 10 min. The resulting RNA pellet was washed twice with 95% ethanol, air-dried, and dissolved in 40 µL RNase-free water, then stored at −80 °C. RNA concentration and purity were determined via a NanoDrop spectrophotometer, and samples with a 260/280 ratio of 1.7–2.0 were considered acceptable. cDNA synthesis was performed using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit, where RNA and oligo(dT) primers were first incubated at 65 °C for 5 min and chilled on ice, followed by addition of reverse transcription master mix. The reaction was incubated at 42 °C for 60 min and terminated at 85 °C for 5 min. The synthesized cDNA was either used immediately or stored at −20 °C. Polymerase chain reaction (PCR) was carried out using gene-specific primers (Table 1) in a reaction mixture containing cDNA template, primers, dNTPs, buffer, MgCl2, Taq-DNA polymerase, and nuclease-free water. Amplification was performed under optimized cycling conditions, including initial denaturation, followed by repeated cycles of denaturation, primer-specific annealing, and extension, with a final extension step to ensure complete amplification.

Table 1

Forward (F) and reverse (R) primer sequences used for amplification of target genes.

S.No	Primers	Tm	Sequence	Amplicon
1.	ß-Actin-F	65°C	AAGATCCTGACCGAGCGTGG	127 bp
	ß-Actin-R	62.5°C	CAGCACTGTGTTGGCATAGAGG	
2.	Caspase 3-F	60°C	GTGGAACTGACGATGATATGGC	135 bp
	Caspase 3-R	61.3°C	CGCAAAGTGACTGGATGAACC	
3.	Bax-F	55°C	ATCAGCAAACATGTCAGCT	145 bp
	Bax-R	58.4°C	GTCCACCAAGAAGCTGAG	
4.	TRP53-F	56.3°C	AACTTACCAGGGCAACTATG	141 bp
	TRP53-R	58.9°C	CTGACCCACAACTGCAC	
5.	DR5-F	55.4°C	CAAATACGGTGTGTCGATG	140 bp
	DR5-R	60.4°C	GGAGACACACTTCCGGT	
6.	mTOR-F	61.6°C	CCGCTACTGTGTCTTGGCAT	118 bp
	mTOR-R	61.9°C	CAGCTCGCGGATCTCAAAGA	
7.	Caspase 9-F	63.1°C	TGCCCTTGCCTCTGAGTAGT	163 bp
	Caspase 9-R	58.4°C	AACAAAGAAACGCCCACAAC	

Statistical Analysis

The statistical analysis was done using the SPSS software (version 16.0). One-way ANOVA was then used to analyze data and compare it using post hoc tests as a means to establish differences between groups (Tukey and Duncan tests). All the results of the experiments were deemed to be statistically significant if $p<0.05$.

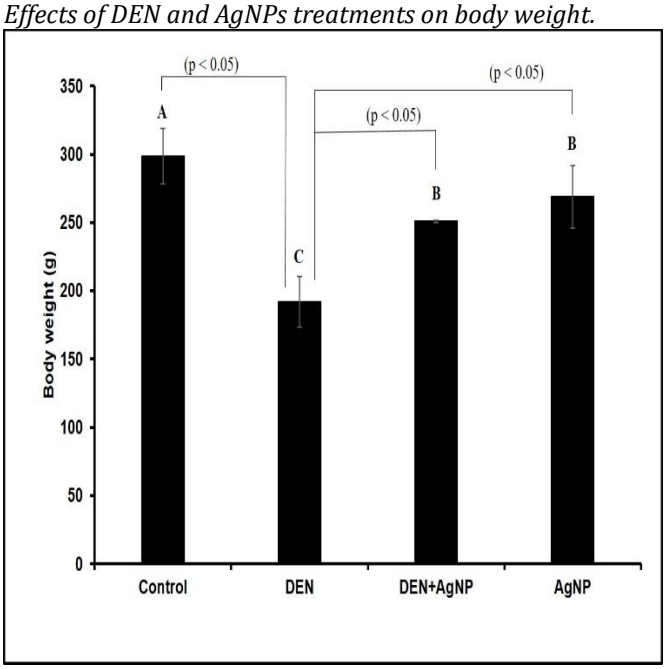
RESULTS

Body Weight

Variations in body weight were found among the different experimental groups. A significant decrease in the body weight of DEN-treated rats was observed as compared to the control group ($p\leq0.05$). However, recovery was observed in body weight with DEN+AgNPs treatment compared to DEN (Figure 1).

Figure 1

Effects of DEN and AgNPs treatments on body weight.



A significant decrease in body weight of the rats was observed in DEN-treated group compared to the control ($p\leq 0.05$). However, recovery in body weight was found with AgNPs treatment. Data is presented as mean ± standard deviation (n = 5).

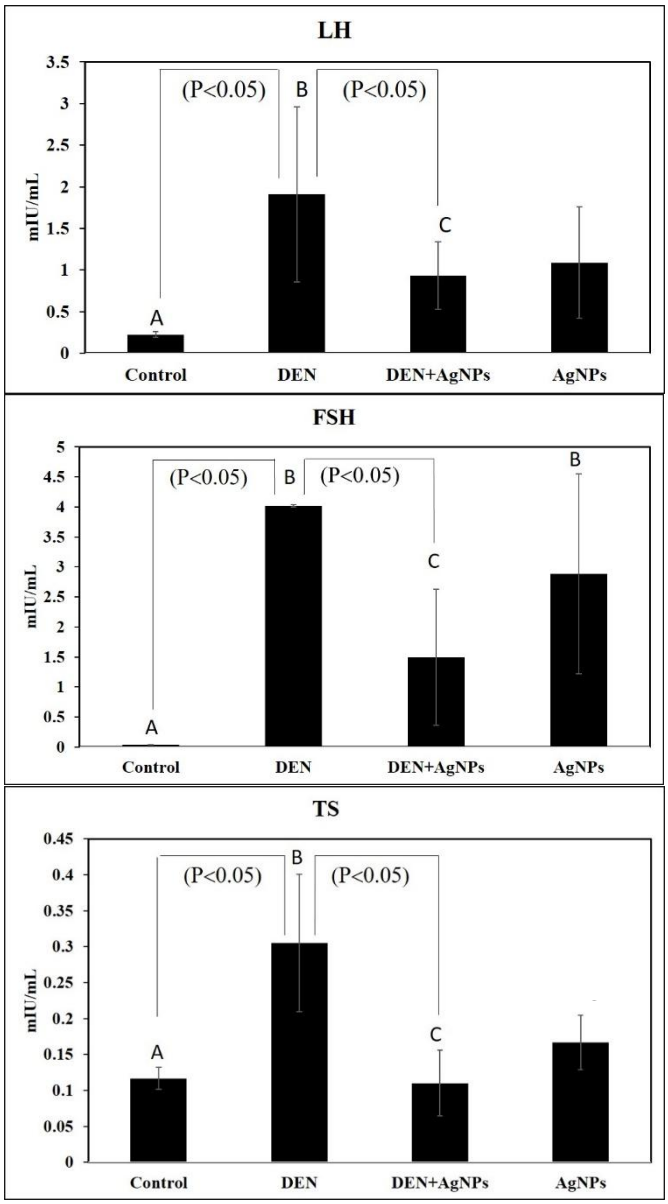
Evaluation of Reproductive Hormones

Significant differences were observed in levels of reproductive hormone among the experimental groups ($p< 0.05$). Luteinizing hormone (LH), follicle-stimulating

hormone (FSH), and testosterone (TS) were significantly higher in the DEN-treated group as compared to the control group. Furthermore, in DEN+AgNPs-treated, LH, FSH and TS showed significant decrease as compared to DEN group. However, AgNPs-treated group demonstrated the same hormone levels as that of the control group (Figure 2).

Figure 2

Effects of DEN and AgNPs treatments on Reproductive Hormone Levels.



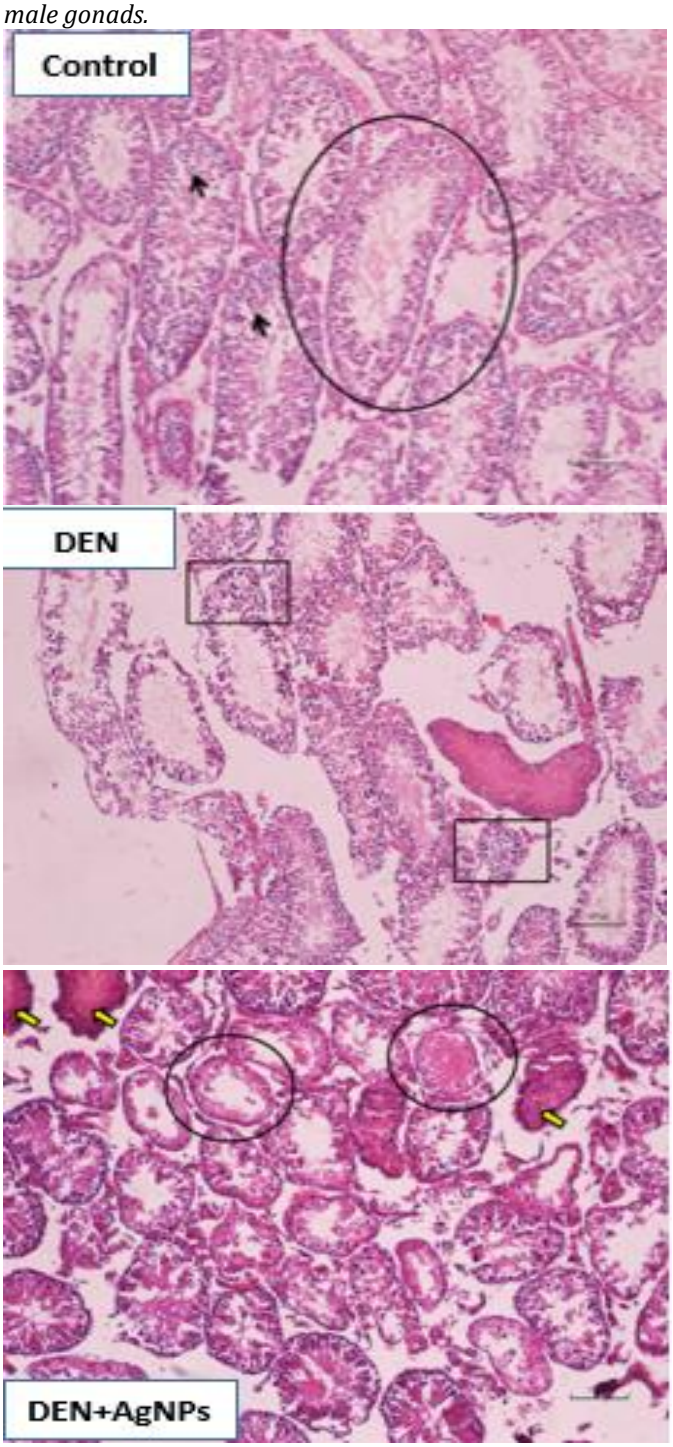
Serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone (TS) were measured in Control, DEN, DEN+AgNPs, and AgNPs groups. Data is presented as mean ± SD, statistical significance among groups was determined at $p < 0.05$, with different letters (A, B, and C) indicating significant differences.

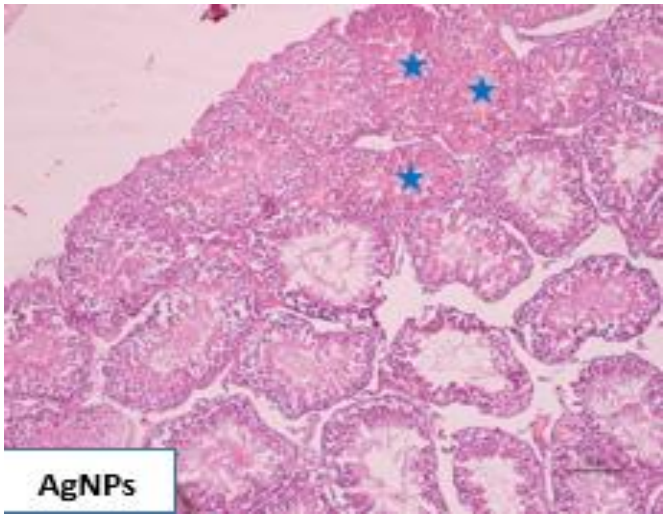
Evaluation of Histopathology

In the control group, seminiferous tubules with intact basement membrane (Black circle) and normal spermatogenesis (black arrows) were observed. In DEN group, seminiferous tubules with intact basement membrane and enlarged, hyperchromatic spermatocytes (Black Square) were found. Furthermore, in DEN + AgNPs groups, mild necrosis (Yellow arrows) and fibrosis (Black circle) were observed in the seminiferous tubules along with normal tubules. However, in the AgNPs-treated group, only mild necrosis in seminiferous tubules (Blue stars) was observed along with normal seminiferous tubules (Figure 3).

Figure 3

Effects of DEN and AgNPs treatments on Histopathology of male gonads.





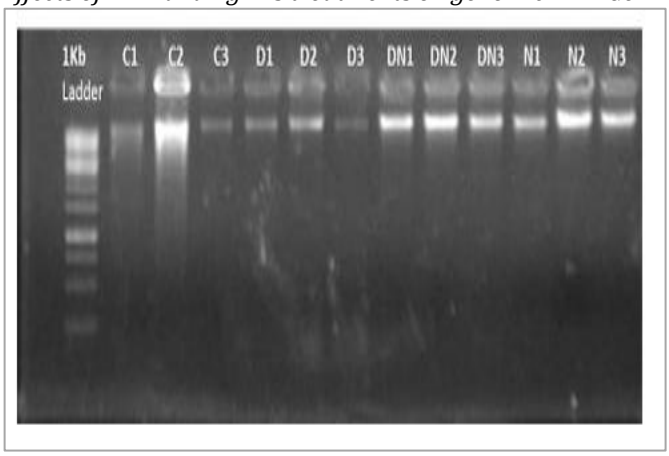
H&E-stained testis sections from control, DEN+AgNPs, and AgNPs groups, evaluated for histological changes associated with DEN treatment with 20x magnification , and a 100 µm scale bar.

DNA damage and Ladder Assay

DNA was found intact in the control group, however, a smear in genomic DNA was observed in DEN-treated group. Meanwhile, light smear of DNA was observed in DEN+AgNPs and AgNPs-treated groups (Figure 4).

Figure 4

Effects of DEN and AgNPs treatments on genomic DNA Gel



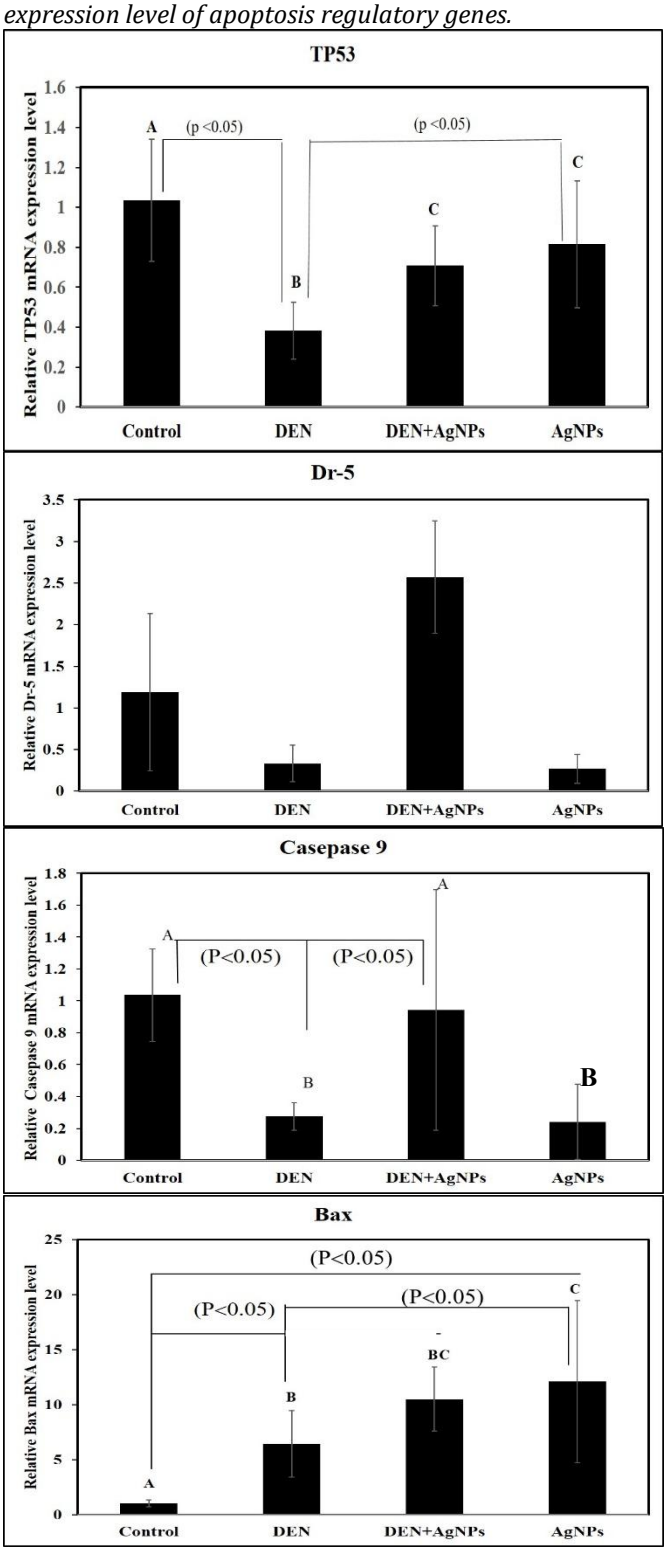
electrophoresis showed genomic DNA bands parallel run with 1KB standard commercial ladder. C1-C3, Control group; D1-D3, DEN group; DN1-DN3, DEN+AgNPS; N1-N3, AgNPs group.

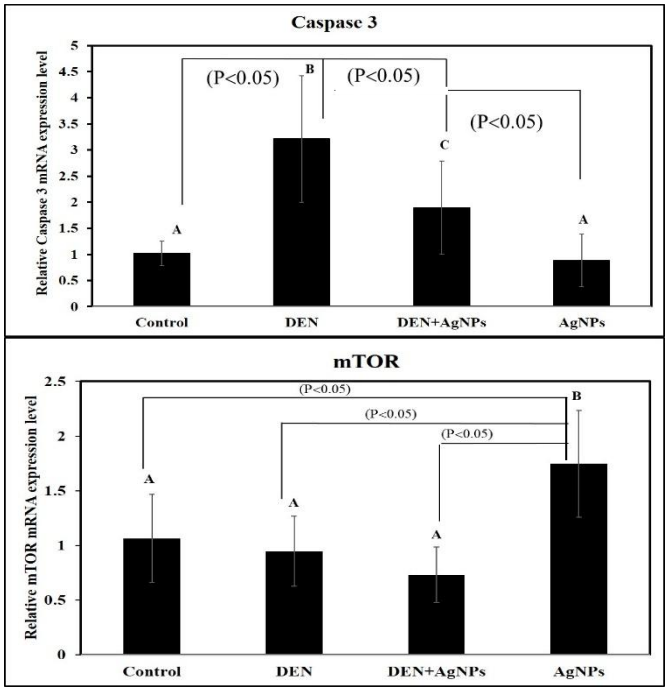
Analysis of Apoptosis Marker Genes

The expression regulations of apoptosis related genes were examined among the experimental groups. p53, DR5, and Caspase-9 were significantly downregulated in DEN-treated group as compared to the control; however, Bax, and Caspase-3 were significantly upregulated compared to control. In the DEN+AgNPs-treated group, p53, Caspase-9, Bax, and DR5 were significantly upregulated and Caspase-3 was significantly downregulated in comparison to the DEN group. Meanwhile, m-TOR showed no significant changes when comparing DEN group to control or DEN+AgNPs group. Moreover, AgNPs-alone group showed no significant differences among all the gene expressions, as compared to the control group (Figure 5).

Figure 5

Effects of DEN and AgNPs treatments on relative mRNA expression level of apoptosis regulatory genes.





Bar graph showing relative mRNA expression levels of selected marker genes, in Control, DEN, DEN+AgNPs, and AgNPs groups. Data is presented as mean ± SEM. Statistical differences among groups were indicated by different alphabetic letters (A–C), groups with different letters are significantly different ($p < 0.05$).

DISSCUSSION

The versatility of the applications of Diethyl nitrosamines (DEN) creates a question of its possible implication on human health and the environment [23]. The use of biogenic silver nanoparticles (AgNPs) has gained considerable attention in cancer treatment due to their potential cytotoxic effects against various cancer cells [24]. Hence, the present study was designed to assess the toxic effects of DEN on gonadal functions while analyzing the male sex hormone profile, histopathological alterations in gonadal tissues, DNA damage, and dysregulation in apoptosis-related gene expression of male rat. In addition, this study aimed to assess the therapeutic efficacy of green synthesized AgNPs, prepared using the root extract of *Operculina turpethum* as a reducing agent, in ameliorating DEN-induced gonadal toxicity. Understanding these effects is crucial for elucidating the impact of DEN toxicity and the protective role of AgNPs on gonadal structure, function, and overall reproductive integrity in male rats.

In this study, DEN caused significant reduction in body weight in comparison to normal group. The body weight of rats in the previous study was reduced in DEN-treated group [18, 25]. Therefore, this research suggests that the reduction in the body weight is a result of DEN administration that induces toxicity, DNA damage and metabolic disturbances, which was attenuated with AgNPs treatment.

In the present study, serum levels of FSH, LH, and testosterone (TS) were significantly increased in the DEN-treated group compared to the control. This elevation may reflect a compensatory endocrine response where DEN-induced testicular toxicity and oxidative stress reduce testosterone secretion, which leads to increased pituitary release of FSH and LH due to disrupted negative feedback [26]. Moreover, systemic stress and hypothalamic–pituitary–thyroid axis activation could contribute to the rise in TS. Such alterations in hormone regulation have been observed in endocrine-toxicant models where pituitary and hormonal homeostasis are disturbed by carcinogenic compounds [27].

Histopathological evaluation shows that male gonads in DEN-treated group exhibited seminiferous tubules with intact basement membranes but contained enlarged, hyperchromatic spermatocytes, indicating early degenerative changes. However, in DEN+AgNPs normal tubules with necrotic seminiferous tubules and fibrosed seminiferous tubules treated group shows a slight recovery against DEN. Previous study of DEN–treated rats showed moderate to severe degenerative changes in tubules along with multi-nucleated cells and moderate degeneration of spermatogonial cells in seminiferous tubules along with multi focal necrosis and inflammation were observed [28]. Therefore, our study suggests that the AgNPs worked effectively against DEN-induced carcinogenesis.

DEN induces ceramide; a bioactive sphingolipid, that functions as a key signaling molecule at the cellular level, which is responsible for promoting tissue fibrosis, inflammation, DNA damage, and apoptosis. In the present study, a smear of genomic DNA in DEN-treated rats reflected a major sign of genotoxicity. However, the DEN-induced genotoxicity was attenuated with biogenic AgNPs. Previous studies have shown that DEN-induced DNA damage in cells occurs via the formation of DNA adducts [29]. Furthermore, DNA damage repair is vital for the maintenance of genomic stability. Compromised DNA repair mechanism and damaged signaling, trigger the tumorigenesis [30]. In the present study, a significant DNA repair with AgNPs treatment against DEN in testis rat model was observed. Our results showed that AgNPs also have marked therapeutic potential against DEN at the genomic level.

Apoptosis is responsible for the removal of damaged or unnecessary cells; however, dysregulation of apoptosis can lead to abnormal cell deaths or uncontrolled cell proliferation leading to cancer [31]. In the present study, the expression of p53, caspase-9 and DR5 was downregulated as compared to control group. However, AgNPs attenuated the effects of DEN by an increase in the expression of these genes. p53 is a widely known tumor suppressor gene which serves as the genome guardian to ensure the genomic and cellular stability [32, 33]. p53 is activated by numerous cellular stresses, especially the DNA damage, leading to cell cycle arrest, apoptosis, or senescence, thus inhibiting tumorigenesis by removing the damaged or possibly precancerous cells. In addition, Caspase-9 is known for its role in the regulation of physiological cell death and degeneration of pathological tissue through intrinsic pathway [34]. Moreover, DR5 is a receptor for tumor necrosis factor–related apoptosis-inducing ligand (TRAIL). DR5 is highly expressed in many

types of cancer [35]. Our study is supported by previous literature in which DEN downregulated the expression of TP53, Caspase-9 and DR5 [36, 37]. Altogether these results show that AgNPs restored the carcinogenic effect of DEN by upregulation of these genes which underlines its therapeutic role. In contrast, the expression of BAX is upregulated with DEN-treatment in comparison to control group. Furthermore, Caspase-3 is a crucial cysteine protease in the process of apoptosis and is essential for normal development and tissue homeostatis [38]. In this study, the expression of caspase-3 was upregulated in DEN-treated group while it was downregulated in the co-treated group. This study is supported by a previous study in which DEN was administered for short time, which lead the cells towards apoptosis by causing the dysregulation of BAX and Caspase-3 [39]. The PI3K-Akt-mechanistic target of the rapamycin (mTOR) signaling pathway is important in a variety of biological activities, including cellular proliferation, survival, metabolism, autophagy, and immunity [40]. In the current study, no significant difference in the expression of mTOR among the control and other experimental groups was observed. However, a study conducted previously had found that there were elevated mTOR levels in DEN-treated rats [41]. Thus, the current result does not support the earlier findings. Altogether, DEN administration reduced body weight as compared to control group. Furthermore, DEN exposure caused histopathological degeneration with necrotic, and fibrosed seminiferous tubules. Moreover, molecular analysis via RT-PCR showed that there was a significant decrease in TP53, Caspase-9, and DR5. These detrimental effects were efficiently overcome by co-administration with. AgNPs treatment restored body weight, tissue architecture and expression profile of the apoptosis modulators. Hence, AgNPs can be an effective Nano therapeutic approach to the prevention and treatment of chemically induced testicular cancer and other reproductive disorders. Further studies are needed to confirm the therapeutic efficacy of AgNPs against DEN in testicular carcinoma.

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