

Research Article

Ginseng Nanoparticles Protect Against Methotrexate-Induced Testicular Toxicity in Rats

Madonna E. F. Kamel, Hala M. F. Mohammad, Cherine Maurice, and Magda M. Hagra

Department of Clinical Pharmacology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

Abstract. Testicular toxicity of methotrexate (MTX) is a clinically important adverse effect. Ginseng has been demonstrated to stimulate spermatogenesis, prevent chemotherapy-induced testicular injury and to possess antiapoptotic and antioxidant actions. Owing to its low bioavailability, ginseng was formulated to nanoform in the current study. As there is no available data about the protective effects of ginseng or ginseng nanoparticles against MTX-induced testicular toxicity, this study was initiated. Seventy-two male rats were enrolled. Rats were given either ginseng (500 mg/kg/day), or ginseng nanoparticles (125 and 250 mg/kg/day) orally for 28 consecutive days. Rats received a single dose of MTX (20 mg/kg) intraperitoneally on day 25. Ginseng and ginseng nanoparticles pre-treatment in rats significantly alleviated the testicular histopathological effects induced by MTX. Also, they significantly restored the impaired spermatogenesis induced by MTX via significantly increasing the Johnsen's tubular biopsy score (JTBS). Ginseng and ginseng nanoparticles treatment prior to MTX administration in rats significantly ameliorated MTX-induced testicular apoptosis by significantly decreasing the percentage of caspase-3-immunostained testicular area. Ginseng and ginseng nanoparticles pretreatment caused nonsignificant increase in serum testosterone levels that were significantly decreased by MTX. The results indicate that ginseng and ginseng nanoparticles protect against MTX-induced testicular toxicity in rats, which is suggested to be through inhibition of MTX-induced testicular apoptosis. The protective effect of ginseng nanoparticles was supposed to be better than ginseng in the given doses.

Keywords: Chemotherapy; Nanoginseng; Testicular toxicity; Caspase-3; JTBS

Corresponding Author

Hala M. F. Mohammad
hala_mohamed@med.suez.edu.eg
halafathy_2@yahoo.com

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1. Introduction

Methotrexate (MTX) is a cytotoxic chemotherapeutic drug used to treat a wide variety of malignancies and non-neoplastic diseases including rheumatoid arthritis [1]. It has a narrow therapeutic window and is known for its toxic effects in various organ systems [2]. Testicular toxicity of MTX is an important adverse effect due to possibility of subsequent infertility [3]. Several human studies and case reports have documented cases of oligospermia, sexual dysfunction and reversible sterility in men receiving MTX [4]. Several previous studies have reported damage in the seminiferous tubules of testis, decrease in sperm number [5] and sperm DNA damage following MTX administration [2] in addition to a decrease in testosterone concentration [6].



Testicular toxicity of MTX is suggested to be due to its direct toxic effect [7] via dihydrofolate reductase enzyme inhibition preventing DNA synthesis with cell cycle arrest [2] and causing DNA damage. This is aggravated by MTX-induced oxidative stress due to generation of free oxygen radicals [5, 8]. Several studies have reported the important role of apoptotic cell death in the pathogenesis of MTX-induced testicular damage [9, 10]. Caspases are cysteine proteases that are activated in response to proapoptotic signals and trigger a cascade of proteolytic cleavage of cellular substrates that lead eventually to cell death. Caspase-3 is a key caspase involved in apoptosis, the execution phase of apoptosis requires proteolytic cleavage of caspase-3 through both extrinsic and intrinsic signaling pathways [11]. Methotrexate administration was associated with increased levels of caspase-3 in testes of rats [12].

Ginseng is a medicinal herbal plant that has been used for thousands of years as an adaptogen to increase physical energy and enhance fertility [13]. It has been reported to have positive effects on sexual dysfunction and sperm abnormalities [14]. Ginseng was reported to have immunoenhancing action preventing MTX-induced macrophage cell regression [15]. Most of ginseng pharmacological actions are attributed to steroidal saponins called ginsenosides, the major bioactive constituents of ginseng [16]. Akram et al. reported that treatment with ginseng protected sperms against cyclophosphamide insult in rats [17]. Ginseng also has beneficial effects against testicular toxicity induced by doxorubicin [18] and busulfan in male rats [19]. Therefore, ginseng therapy could have an application in the recovery of male fertility under chemotherapy.

Nanotechnology has been recently used in medicine for therapeutic purposes [20]. The reduction of particle size of plant extracts increases the surface area, thus improving absorption, bioavailability and release of functional ingredients [21]. Ginseng oral bioavailability is low due to low absorption rate of ginseng saponins [22]. It has been reported that using ginseng nanoparticles was more effective than ginseng extract in enhancing male rat's fertility by decreasing sperm abnormalities through inhibition of DNA damage, increasing secretion of male reproductive hormones (testosterone, FSH and LH) and increasing expression levels of reproductive genes in the testis and pituitary gland. The formulation of the ginseng particles to nanoform increased the ability of its active ingredients to reach the target cells of the hypothalamus-pituitary-testis axis and enhance the fertility of male rats [23].

To the best of our knowledge there is no data describing the protective effect of ginseng and ginseng nanoparticles against MTX-induced testicular injury in rats. So, this study aimed to evaluate the possible protective effect of ginseng and ginseng nanoparticles against MTX-induced testicular injury in rats.

2. Materials and Methods

2.1. Animals

A total of 72 adult male albino Wistar rats, weighting 200-250 gm each, were used in this study. Animals were purchased from the National Research Institute, Cairo, Egypt. Animals were housed in polyethylene cages and kept under controlled temperature, humidity and day/night cycle with free access to standard rodent chow diet.

2.2. Drugs

2.2.1. Methotrexate

Methotrexate (MTX) powder with yellow color was purchased [Sigma chemical co, St. Louis, MO, USA]. It was freely dissolved in normal saline.

2.2.2. Ginseng

Ginseng was supplied as yellow brownish powder color from [MEPACO-MEDIFOOD (Arab company for Pharmaceuticals and Medicinal plants), Sharkeya, Egypt]. It was extracted from the roots of *Panax ginseng* C.A.Meyer. The total ginsenosides content of the ginseng extract powder was 10% as was detected by HPLC. The powder was dissolved in distilled water.

2.2.3. Ginseng nanoparticles

Ginseng nanoparticles were prepared by [NanoTech Egypt for Photo-Electronics company, City of 6 October, Al Giza, Egypt]. Ginseng nanoparticles were supplied in the form of nanoemulsion of the *Panax ginseng* extract. Size and shape of ginseng nanoparticles were tested by using high resolution transmission electron microscope (TEM). Nanoemulsion of *Panax ginseng* extract was prepared by using tween 80 surfactant as an emulsifying agent. Tween was added to ethanolic solution of ginseng, mixed together and sonicated for 30 min using an ultrasonic processor (according to Linjawi [23], Shahavi et al. [24] and Akhter et al. [25] with modifications).

Properties of ginseng nanoparticles emulsion (Figure 1 in Supplementary Material available online at <http://www.kenzpub.com/journals/ejbcp/2019/101397/>) as shown by TEM as follows:

Appearance (color): yellow

Appearance (form): liquid (suspended solution)

Solubility: dispersed in H₂O

Average size (TEM): 300 nm ± 50

Shape (TEM): spherical shape

2.3. Chemicals and kits

Rabbit caspase-3 polyclonal antibody kit was purchased from [GeneTex, Irvine, California, USA], and Elecsys® Testosterone hormone II kit was purchased from [Roche Diagnostics Ltd., CH-6343 Rotkreuz, Switzerland].

2.4. Study design

The current study was carried out on seventy-two rats. They were divided randomly into 8 groups (9 animals each). Group 1 (control group): rats received distilled water from day 1 to day 24, then on day 25 they received intraperitoneal saline. Group 2 (MTX-induced testicular injury group): rats received no treatment from day 1 to day 24, then on day 25 they received

intraperitoneal MTX as a single dose of 20 mg/kg intraperitoneally on day 25 of the experiment (modified from Yulug et al., Vardi et al. and Oufi and Al-Shawi [5, 12, 29] and confirmed by a pilot study). Groups 3 (ginseng control group 500 mg/kg/day): rats received ginseng orally daily for 28 days [17, 23, 36]. Groups 4 and 5 (ginseng nanoparticles control groups: 250 mg/kg/day and 125 mg/kg/day): rats received ginseng nanoparticles orally daily for 28 days modified from Linjawi, Gu et al., Kitts and Hu and Cho et al. [23, 27, 36]. Group 6 (ginseng 500 mg/kg/day + MTX group): rats received ginseng orally daily for 28 days, and on day 25 of the experiment MTX as a single dose of 20 mg/kg intraperitoneally. Groups 7 and 8 (ginseng nanoparticles 250 mg/kg/day and 125 mg/kg/day + MTX group): rats received ginseng nanoparticles orally daily for 28 days, and MTX was administered as a single dose of 20 mg/kg intraperitoneally on day 25 of the experiment. By the end of the experiment, rats were anesthetized before sacrifice using pentobarbital (75 mg/kg) intraperitoneally [9] and blood samples were withdrawn from retro-orbital venous plexus for serum testosterone analysis [28]. Rats were sacrificed after 28 days from the onset of the experiment (three days after MTX administration) [12] by decapitation [17]. Both testes were dissected from each rat after sacrifice for histopathological and immunohistochemical analysis [9].

2.5. Methods

2.5.1. Testicular histopathological examination

Tissue samples from both testes [9] were fixed in Bouin's fluid, dehydrated and blocked in paraffin for light microscopic histopathological examination [12, 29]. Slides of four different serial sections were prepared out of each block [30]. Structural changes of each rat testis were evaluated [7].

- Semi quantitative evaluation of spermatogenesis was done using Johnsen's tubular biopsy score (JTBS) in 20 seminiferous tubules from each testicular section [5]. Testicular tubule sections in each group were evaluated and were given a score from 1-10 according to specific criteria described by Johnsen [26]. JTBS was calculated by dividing the sum of all scores by the total number of seminiferous tubules examined [5].
- Semi quantitative evaluation of histopathological testicular changes was done. Four slides from rat's testis were examined and scored for the histopathological changes [0 (no injury), 1 (mild injury), 2 (moderate injury), 3 (severe injury)]. Thirty seminiferous tubules from each rat were examined randomly, each tubule took a score of (0, 1, 2 or 3). The histopathologic changes were categorized into 7 parameters; disruption of seminiferous tubules, detachment of spermatogenic cells, inflammation, edema of interstitium, congestion of vessels, degeneration of Sertoli cells and degeneration of Leydig cells. For each experimental group, the average scores for slides were taken and assessed statistically (according to Padmanabhan et al. [32] and Yuncu et al. [30] with some modifications).

2.5.2. Assessment of apoptosis by testicular caspase-3 immunohistochemical analysis

Thick testicular sections were mounted on slides and the caspase 3 kit was used [12] according to the instructions of the manufacturer, then examined by light microscope and photo analyzed by image J program.

2.5.3. Serum testosterone hormonal assay

Serum samples were separated from blood samples obtained from each rat. Then quantitative estimation of total testosterone hormone was carried out in the samples of rat's serum [23, 29] by electrochemiluminescence immunoassay (ECLISA) using cobas e411 automated immunoassay analyzer, Roche Diagnostics.

2.6. Statistical analysis

All the grouped data were statistically evaluated using statistical package for social sciences (SPSS) program. Descriptive statistics were done using means \pm SD for each group. Data derived from three groups or more were evaluated by One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. Data were considered statistically significant with a P value < 0.05 . Confidence interval (CI) was considered to be 95%.

3. Results

3.1. Assessment of spermatogenesis by Johnsen's tubular biopsy score (JTBS) and Serum testosterone hormonal assay

Table 1 shows that administration of MTX in a dose of 20 mg/kg caused spermatogenesis impairment as evident by significant decrease in JTBS when compared to the control group. Oral ginseng (500 mg/kg/day) and ginseng nanoparticles (125 and 250 mg/kg/day) pre-treatment significantly restored the impairment of spermatogenesis induced by MTX in rats.

MTX caused a significant decrease in serum testosterone levels compared to control group rats. Pre-treatment of male rats with ginseng and ginseng nanoparticles increased the levels of testosterone that were decreased in MTX-treated rats, but this increase was found to be statistically insignificant. However, ginseng pre-treatment was shown to normalize the levels of serum testosterone in rats treated with MTX (Table 1 and Figure 2 and in Supplementary Material available online at <http://www.kenzpub.com/journals/ejbcp/2019/101397/>).

Table 1: The effect of ginseng and ginseng nanoparticles on the serum levels of total testosterone ng/ml in methotrexate-induced testicular toxicity in rats, and on methotrexate-induced impairment of spermatogenesis assessed by Johnsen's tubular biopsy score (JTBS).

Group No.	Group name	Serum total testosterone (ng/ml)	Johnsen's tubular biopsy score (JTBS)
1	Control	1.56 ± 0.62 ^{b,d}	9.33 ± 0.5 ^b
2	MTX	0.08 ± 0.07 ^{a,c,d}	3.78 ± 0.97 ^a
3	G 500	1.77 ± 1.03 ^{b,d}	8.89 ± 0.33 ^b
4	GN 250	3.25 ± 1.41 ^{a,b,c}	8.78 ± 0.44 ^b
5	GN 125	1.84 ± 1.04 ^{b,d}	8.79 ± 0.44 ^b
6	G 500/MTX	1.02 ± 0.93 ^d	7.89 ± 0.78 ^{a,b}
7	GN 250/MTX	0.13 ± 0.07 ^{a,c,d}	8.22 ± 0.83 ^{a,b}
8	GN 125/MTX	0.2 ± 0.15 ^{a,c,d}	8.56 ± 0.53 ^b

n (9 rats/group), MTX: methotrexate (20 mg/kg), G 500: ginseng (500 mg/kg/day), GN 250: ginseng nanoparticles (250 mg/kg/day), GN 125: ginseng nanoparticles (125 mg/kg/day), G 500/MTX: ginseng (500 mg/kg/day) + methotrexate (20 mg/kg), GN 250/MTX: ginseng nanoparticles (250 mg/kg/day) + methotrexate (20 mg/kg), GN 125/MTX: ginseng nanoparticles (125 mg/kg/day) + methotrexate (20 mg/kg). Data are expressed as mean ± S.D, and the difference between groups was evaluated using one-way analysis of variance (ANOVA) test followed by Bonferroni multiple comparison test.

^asignificant difference compared to control group at P-value < 0.05

^bsignificant difference compared to MTX group at P-value < 0.05

^csignificant difference compared to G 500 group at P-value < 0.05

^dsignificant difference compared to GN 250 group at P-value < 0.05

CI = 95%

3.2. Testicular histopathological changes

Methotrexate caused significant moderate-to-severe disruption of seminiferous tubules when compared to the control group (Table 2). Ginseng and ginseng nanoparticles treatment prior to MTX administration, significantly reduced the disruption of seminiferous tubules caused by MTX (Figure 1). In the MTX group, spermatogenic cells were detached from the wall of seminiferous tubules and shed into the lumen. Degeneration affected all germ cell lines with cell shrinkage. Sperms disappeared from some tubules with accumulation of immature germinal cells in the tubule's lumen (Figure 1). The score of injury was significantly moderate-to-severe in the MTX group compared to the control group (Table 2). Ginseng and ginseng nanoparticles pretreatment decreased the score of detachment of germ cells caused by MTX significantly (Table 2). In the MTX group, there was significant mild-to-moderate inflammatory reaction in some testicular sections compared to the control group (Figure 1), which was decreased significantly by ginseng and ginseng nanoparticles pretreatment (Table 2). Significant mild-to-moderate interstitial edema was caused by MTX increasing the inter-tubular spaces compared to the control group (Figure 1). This edema was reduced significantly by ginseng and ginseng nanoparticles pretreatment (Table 2). There was significant moderate-to-severe congestion of vessels in the MTX group compared to the control group (Table 2). Pre-treatment with ginseng and ginseng nanoparticles decreased the severity of congestion significantly in rats that received MTX (Figure 1). Significant moderate-to-severe degeneration of Sertoli cells with vacuolization

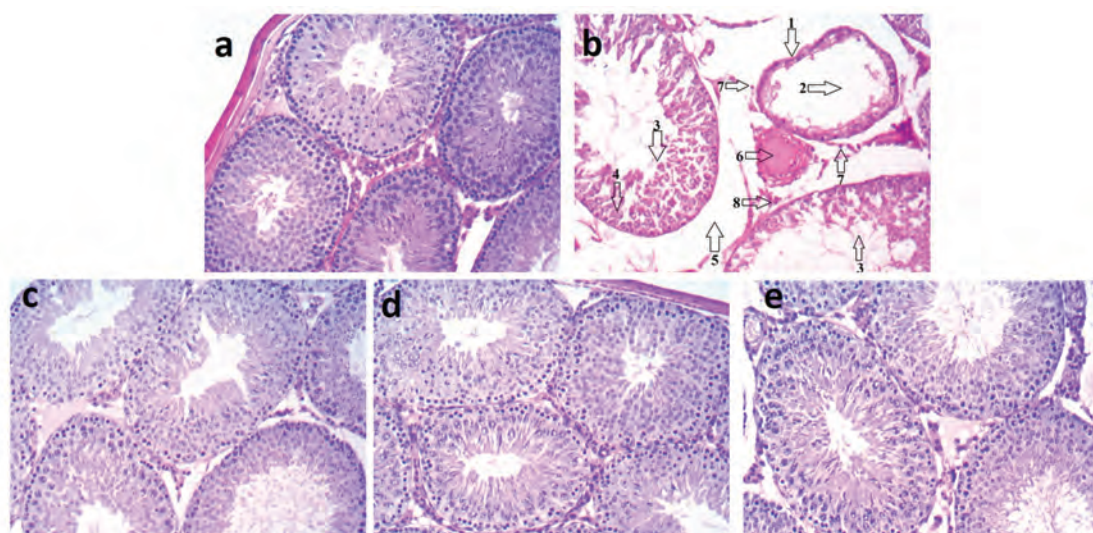


Figure 1: The effect of ginseng and ginseng nanoparticles on testicular histopathological changes induced by methotrexate in rats. (a) Control group, (b) Methotrexate group (20 mg/kg), (c) Ginseng (500 mg/kg/day) + Methotrexate (20 mg/kg) group, (d) Ginseng nanoparticles (250 mg/kg/day) + Methotrexate (20 mg/kg) group, (e) Ginseng nanoparticles (125 mg/kg/day) + Methotrexate (20 mg/kg) group. 1-Disruption of seminiferous tubules with irregularity in outline and shrinkage, 2-Severe disruption of seminiferous tubules with complete loss of spermatogenic cell lines and Sertoli cells, 3-Shedding of the spermatogenic cells into the lumen giving vacuolated appearance in some seminiferous tubules, 4-Degeneration of Sertoli cells, 5-Interstitial edema, 6-Congested blood vessels, 7-Inflammatory cells, 8-Degeneration and loss of Leydig cells.

and shrinkage of cytoplasm was caused by MTX compared to the control group (Table 2). Ginseng and ginseng nanoparticles pretreatment significantly decreased the injurious effects of MTX on Sertoli cells (Figure 1). Moderate degeneration and loss of Leydig cells were detected in the MTX group compared to the control group (Figure 1). Ginseng and ginseng nanoparticles reduced significantly the effects of MTX on Leydig cells (Table 2).

3.3. Assessment of apoptosis by testicular caspase-3 immunostaining

Methotrexate was found to increase apoptosis of testicular cells as evident by causing a significant increase in the percentage of testicular area immunostained with caspase-3 compared to the control group (Table 3). Brown immunostaining with caspase-3 was observed in basal and luminal spermatogenic cells and was also observed in Sertoli cells and interstitial Leydig cells in the MTX group as shown in figure (2). Pre-treatment with oral ginseng and ginseng nanoparticles in rats significantly ameliorated MTX-induced increase in testicular apoptosis (Figure 2). The results indicated that ginseng nanoparticles pre-treatment in the dose of 125 mg/kg/day was significantly better than the double-dose ginseng nanoparticles and the quadruple-dose ginseng. Also, ginseng nanoparticles pre-treatment in the of dose of 250 mg/kg/day was significantly better than ginseng (Table 3).

Table 2: The effect of ginseng and ginseng nanoparticles on the testicular histopathological changes induced by methotrexate in rats.

Group	Histopathological parameter						
	Disruption of seminiferous tubules	Detachment of spermatogenic cells	Inflammation	Edema of interstitium	Congestion of vessels	Sertoli cells degeneration	Leydig cells degeneration
1. Control	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b
2. MTX	2.78 ± 0.44 ^a	2.67 ± 0.5 ^a	1.44 ± 0.88 ^a	2.56 ± 0.53 ^a	2.33 ± 0.5 ^a	2.11 ± 0.78 ^a	2 ± 0.71 ^a
3. G 500	0.22 ± 0.44 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b
4. GN 250	0.44 ± 0.53 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b
5. GN 125	0.22 ± 0.44 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b
6. G 500/MTX	0.56 ± 0.53 ^b	0.44 ± 0.53 ^b	0 ± 0 ^b	0.33 ± 0.5 ^b	0.56 ± 0.53 ^b	0.56 ± 0.53 ^b	0.33 ± 0.5 ^b
7. GN 250/MTX	0.44 ± 0.53 ^b	0.67 ± 0.5 ^b	0 ± 0 ^b	0.33 ± 0.5 ^b	0.44 ± 0.53 ^b	0.56 ± 0.53 ^b	0.33 ± 0.5 ^b
8. GN 125/MTX	0.44 ± 0.53 ^b	0.22 ± 0.44 ^b	0 ± 0 ^b	0.11 ± 0.33 ^b	0.44 ± 0.53 ^b	0.33 ± 0.5 ^b	0.22 ± 0.44 ^b

n (9 rats/group), MTX: methotrexate (20 mg/kg), G 500: ginseng (500 mg/kg/day), GN 250: ginseng nanoparticles (250 mg/kg/day), GN 125: ginseng nanoparticles (125 mg/kg/day), G 500/MTX: ginseng (500 mg/kg/day) + methotrexate (20 mg/kg), GN 250/MTX: ginseng nanoparticles (250 mg/kg/day + methotrexate (20 mg/kg), GN 125/MTX: ginseng nanoparticles (125 mg/kg/day) + methotrexate (20 mg/kg). Data are expressed as mean ± S.D, and the difference between groups was evaluated using one-way analysis of variance (ANOVA) test followed by Bonferroni multiple comparison test.

^asignificant difference compared to control group at P-value < 0.05

^bsignificant difference compared to MTX group at P-value < 0.05

CI = 95%

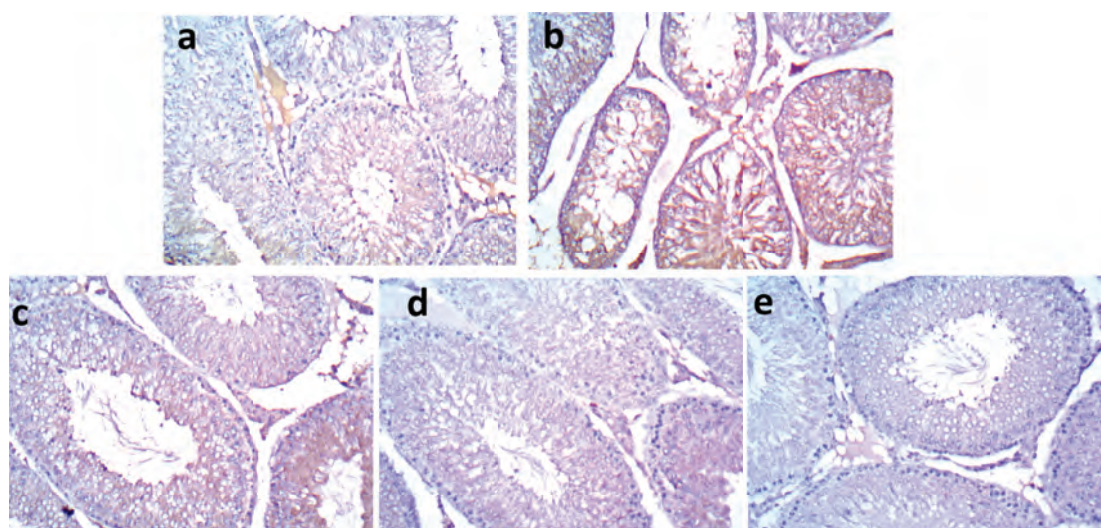


Figure 2: Apoptosis in testicular tissue assessed by the percentage of testicular area immunostained with caspase-3 (brown color). (a) Control group, (b) Methotrexate group (20 mg/kg), (c) Ginseng (500 mg/kg/day) + Methotrexate (20 mg/kg) group, (d) Ginseng nanoparticles (250 mg/kg/day) + Methotrexate (20 mg/kg) group, (e) Ginseng nanoparticles (125 mg/kg/day) + Methotrexate (20 mg/kg) group.

Table 3: The effect of ginseng and ginseng nanoparticles on methotrexate-induced testicular apoptotic changes rats (the percentage of area of testicular tissue stained with caspase-3).

Group No.	Group name	Percentage of testicular area stained with caspase-3
1	Control	5.76 ± 2.49 ^{b,c,d}
2	MTX	28.71 ± 2.83 ^{a,c,d}
3	G 500	8.59 ± 1.82 ^{b,c,d}
4	GN 250	11.39 ± 0.77 ^{a,b,c}
5	GN 125	9.98 ± 1.26 ^{a,b,c}
6	G 500/MTX	16.91 ± 3.33 ^{a,b,d}
7	GN 250/MTX	12.85 ± 3.15 ^{a,b,c}
8	GN 125/MTX	5.73 ± 2.63 ^{b,c,d}

n (9 rats/group), MTX: methotrexate (20 mg/kg), G 500: ginseng (500 mg/kg/day), GN 250: ginseng nanoparticles (250 mg/kg/day), GN 125: ginseng nanoparticles (125 mg/kg/day), G 500/MTX: ginseng (500 mg/kg/day) + methotrexate (20 mg/kg), GN 250/MTX: ginseng nanoparticles (250 mg/kg/day + methotrexate (20 mg/kg), GN 125/MTX: ginseng nanoparticles (125 mg/kg/day) + methotrexate (20 mg/kg). Data are expressed as mean ± S.D, and the difference between groups was evaluated using one-way analysis of variance (ANOVA) test followed by Bonferroni multiple comparison test.

^asignificant difference compared to control group at P-value < 0.05

^bsignificant difference compared to MTX group at P-value < 0.05

^csignificant difference compared to G 500/MTX group at P-value < 0.05

^dsignificant difference compared to GN 250/MTX group at P-value < 0.05

CI = 95%

4. Discussion

Methotrexate has been documented to cause testicular toxicity in human and animal studies [4]. It is considered as the most widely used antimetabolite agent in the young age, [9] which raises the concern for alleviating its potential risk on fertility of males in the reproductive age [33, 34]. So, this study was designed to elucidate the possible protective effect of ginseng against MTX-induced testicular histopathological changes and apoptosis via measuring testicular caspase-3 levels in rats and whether using ginseng nanoparticles has better protective effect than ginseng. Ginseng is a medicinal plant used widely to treat sexual dysfunction, it has been reported in several human and animal studies to have positive improving effects on sexual dysfunction and sperm abnormalities [14].

In the current study, ginseng and ginseng nanoparticles treatment of rats prior to MTX administration was found to significantly ameliorate the injurious effects of MTX on testicular tissue restoring the normal histological architecture. Pre-treatment with ginseng and ginseng nanoparticles restored the impaired spermatogenesis induced by MTX in rats (as assessed by JTBS). In agreement with the study results, several previous studies have proved that ginseng could restore spermatogenesis after testicular injury against various toxic insults as anticancer chemotherapy including; doxorubicin, [18] busulfan [19] and cyclophosphamide [17]. Thus, ginseng could be used to protect against male infertility during cancer treatments.

In the present study, the mechanism underlying the effect of ginseng and ginseng nanoparticles pre-treatment in ameliorating MTX-induced testicular toxicity in rats could be partially due to antiapoptotic action on testicular tissue. Oral ginseng and ginseng nanoparticles administration to rats significantly ameliorated MTX-induced increase in testicular apoptosis (as assessed by caspase-3 immunostaining of testicular tissue). The effect was dose-dependent in case of ginseng nanoparticles in the current study. Ginseng nanoparticles pre-treatment in the dose of 125 mg/kg/day caused significant decrease in the MTX-induced increase in the percentage of testicular area immunostained with caspase-3 that was significantly more than that achieved by ginseng nanoparticles pre-treatment in the dose of 250 mg/kg/day. Ginseng pre-treatment in dose of 500 mg/kg/day to rats that received MTX caused significant decrease in the percentage of testicular area immunostained with caspase-3 caused by MTX, that was significantly less than the two doses of ginseng nanoparticles.

Consistent with the study results, the testicular protective action of ginseng against toxicity has been attributed to inhibition of germ cell apoptosis. Ginseng prevented severe testicular toxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats through inhibiting apoptotic DNA damage via its antioxidant activity [35]. Pre- and co-treatment with ginseng was reported by Cho et al. to ameliorate zearalenone-induced testicular degenerative changes and impairment of spermatogenesis by decreasing apoptotic DNA damage of germ cells via decreasing the expression of death receptor Fas and Fas ligand [36]. Wang et al. demonstrated that ginsenosides were found to protect Sertoli cells in vitro against the cytotoxic damage of bisphenol A [16]. Ginsenosides significantly inhibited bisphenol A-induced decrease in Sertoli cell viability and increase in apoptosis via inhibiting extracellular signal-regulated kinase (ERK 1/2) phosphorylation, modulation of Bcl-2 and Bax protein expression in Sertoli cells. Also, ginsenosides antiapoptotic action was mediated through their potent antioxidant free radical scavenging activity and enhancement of cellular antioxidant enzymes. Linjawi reported that ginseng and ginseng nanoparticles prevented nicotine-induced sperm abnormalities by inhibiting testicular apoptotic DNA damage caused by nicotine in rats [23]. Ginseng reduced DNA damage of sperms in vitro [13]. Ginseng in the present study was used to ameliorate MTX-induced testicular toxicity in rats based on the previous reports of the anti-apoptotic action of ginseng against testicular toxicity. Ginseng was reported by Jang and Shin to protect against MTX-induced immunotoxicity in vitro by preventing MTX-induced macrophage cell regression and enhancing the immune responses by macrophages [15]. Therefore, ginseng can be used as an adjuvant immunomodulating agent under MTX chemotherapy.

Although ginseng and ginseng nanoparticles pretreatment ameliorated MTX-induced testicular histopathological effects and increased apoptosis in the present study, it didn't restore serum testosterone hormone level disrupted by MTX toxicity completely. The pretreatment increased the levels of the hormone compared to MTX group but wasn't statistically significant. In agreement with this observation, Jang et al. demonstrated that ginseng protected mice against ethanol-induced toxicity on male fertility and insignificantly increased serum testosterone hormone levels [37]. In contrast to this observation, Linjawi demonstrated that ginseng and ginseng nanoparticles protected rat testis against nicotine-induced toxicity via indirect action through increasing the secretion of male reproductive hormones (testosterone, FSH and LH) [23]. Kopalli et al. stated also that ginseng treatment ameliorated spermatogenesis impairment in aged rat testes via significantly improving the decrease in the expression of sex hormone receptors [38].

Kim et al. proved also that ginseng was effective in ameliorating heat stress-induced damage in rats' testes through significantly ameliorating the temperature-induced reduction in the expression of sex hormone receptors (androgen receptor, LH receptor and FSH receptor) [39]. The cause of the current observation may be attributed to differences in the mechanisms of action of MTX and ginseng on the hypothalamus-pituitary-gonadal axis. Ginseng was reported to act directly on androgen receptors [38, 39]. Another explanation for such observation can be attributed to the differences in ginseng type and treatment duration used between the various studies and the current study. These can result in differences in ginseng abilities to enhance sexual function and testosterone production [14]. Interestingly, treatment with 5% ginseng in rats significantly increased blood testosterone levels, while no effect was observed with 1% ginseng treatment [40]. Ginseng administered to rats in the current study was 10% *Panax ginseng* root extract i.e the amount of contained ginsenosides was 10% of the extract. As well known, the potency of the ginseng extract is often measured by the percentage of contained ginsenosides. Total ginsenosides content of the ginseng extract may vary by species of ginseng, cultivation conditions, [22] method of processing and the part of the plant from which the extract is prepared [41].

It is evident from the current study that ginseng nanoparticles in the small dose was better than ginseng nanoparticles in the large dose and ginseng as a pre-treatment in ameliorating MTX-induced increase in testicular apoptosis. This can be explained by the increased efficacy and enhanced kinetics of ginseng nanoparticles. This observation agrees with several previous studies conducted on the nanoformulation of ginseng that was proved to markedly increase its absorption, bioavailability and efficacy as demonstrated by Song et al., [42] Linjawi [23] and Voruganti et al. [43]. Lipid-based ginsenosides nanoparticles (ginsomes) were proved to be more effective than free ginsenosides in enhancing the immune response in mice when co-administered as an adjuvant with a vaccine [42]. Similar to the current study, ginseng nanoparticles were proved to be more effective in ameliorating nicotine-induced testicular toxicity, sperm abnormalities and apoptosis than ginseng [23]. Voruganti et al. used nanoparticles of ginsenoside Gs25 encapsulated by polylactic-co-glycolic acid and coated by polyethylene glycol nanoparticles (Gs25NP). Gs25NP was proved to have dramatically better oral bioavailability and anticancer efficacy in mice than free unencapsulated Gs25 [43].

The rationale of using nanoformulation of ginseng extract in the current study was to improve its pharmacokinetic properties since ginseng oral bioavailability is known to be very poor [22, 41]. Oral bioavailability of ginseng can be enhanced by increasing the dose to saturate its metabolism or by changing its pharmaceutical formulation [22] such as nanoparticles formulation [23, 43]. Ginseng nanoparticles in our study were prepared in the form of nanoemulsion. Nanoemulsion was chosen in this research as it is characterized by stability of the suspension in several environmental circumstances such as thermal changes, dilution and this stability may extend for months [44]. The differences in the efficacy on male reproductive system of ginseng nanoparticles between Linjawi and our study in enhancing testosterone hormone levels may be also attributed to the preparation method of nanoparticles from *Panax ginseng* root extract. Linjawi used ginseng nanoparticles that were encapsulated with polylactic-co-glycolic acid (PLGA) [23]. Established methods of nanoformulation in previous studies that proved to enhance ginseng bioavailability and efficacy included encapsulation with PLGA, [23] polyethylene glycol (PEG) [43] or liposomes [42]. One of the limitations of the present study was

confined to the preparation of nanoemulsion of ginseng with no carrier material or encapsulation used. Secondly, the total ginsenosides content of the ginseng extract powder was 10 % only. Therefore, further studies are needed to evaluate the best method for preparation of ginseng nanoparticles.

5. Conclusion

In conclusion, it is evident from the current study that ginseng was able to protect against the testicular toxicity induced by MTX administration to rats. The suggested mechanisms included direct antiapoptotic action of ginseng on testicular tissue via inhibition of MTX-induced increase in testicular caspase-3 immunostaining. The protective effect of ginseng nanoparticles was demonstrated to be better than ginseng in decreasing MTX-induced increase in testicular apoptosis.

5.1. Participation of the researchers

- MK:** Research idea and planning, research methods, carrying out the practical work, preparation and writing of study results and discussion. Writing and revision of the research paper.
- HM:** Research idea and planning, research methods, participation and supervision of the practical work, preparation and writing of study results and discussion. Writing and revision of the research paper. Corresponding author.
- CM:** Research idea and planning, research methods, participation and supervision of the practical work the practical work, preparation and writing of study results and discussion. Writing and revision of the research paper.
- MH:** Research idea and planning, research methods, revision of the research paper. Supervision of all research process.

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Ethical Approval

All applicable international, national and faculty of medicine ethics committee guidelines for care and use of the animals were followed. The approval of Faculty of Medicine's (Suez Canal University) Ethics Committee was taken before starting the research.

Competing Interests

The authors declare no competing interests.

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