Canadian Journal of Physiology and Pharmacology
Revue canadienne de physiologie et pharmacologie

Oxidative stress burden inhibits spermatogenesis in adult male rats: testosterone protective effect

Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2017-0459.R1
Manuscript Type:	Article
Date Submitted by the Author:	29-Sep-2017
Complete List of Authors:	Makary, Samy; Suez Canal University Faculty of Medicine, Physiology Abdo, Mohamed; faculty of Medicine, Suez Canal University, Physiology Fekry, Ereny; Suez Canal University Faculty of Medicine, Histology
Is the invited manuscript for consideration in a Special Issue?:	N/A
Keyword:	spermatogenesis, aromatase inhibitor, testosterone, methotrexate, oxidative stress

SCHOLARONE™
Manuscripts

Oxidative stress burden inhibits spermatogenesis in adult male rats: testosterone protective effect

Samy Makary^{a*}, Mohamed Abdo^a, Ereny Fekry^b

^aDepartment of Physiology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

^bDepartment of Histology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

***Corresponding author:** Samy Makary

Address: Department of Physiology, Faculty of Medicine, Suez Canal University, Ismailia
41522, Egypt

Tel.: +20 127-241-6076

E-mail address: samy.makary@med.suez.edu.eg

Abstract

In this study, we aimed to investigate the protective effects of androgens, using letrozole (LET; an aromatase inhibitor), grape seed extract (GSE; a naturally occurring aromatase inhibitor and antioxidant), and testosterone propionate (Tp), against methotrexate (MTX)-induced testicular toxicity in adult male rats. MTX has been shown to induce oxidative stress and exhibit antiproliferative effects in the testes. Adult male rats received oral saline gavage (control group with no treatment), the potential protective agents (LET, GSE, or Tp) alone, MTX alone, or a combination of one of the potential protective agents and MTX. The testicular levels of oxidative stress markers and cytokines (tumor necrosis factor- α and interleukin-1 β) were measured. Spermatogenesis and sperm viability were microscopically evaluated. Administration of LET and GSE 7 days before MTX improved spermatogenesis and sperm viability, as well as reduced the levels of oxidative stress markers and cellular cytokines. Exogenous testosterone exhibited anti-inflammatory and antioxidant activities, similar to GSE and LET. We also showed that enhancing the endogenous androgenic activity by LET and GSE protected spermatogenesis against MTX-induced testicular toxicity *via* reduction of inflammation and oxidative stress in the testes. Our data suggest that testosterone protected spermatogenesis owing to its antioxidant and anti-inflammatory properties.

Keywords

Testes, spermatogenesis, aromatase inhibitor, testosterone, estradiol, methotrexate, oxidative stress

Introduction

Spermatogenesis involves multiple processes of meiosis and mitosis that take place in the testicular seminiferous tubules (SNT) (Fok et al. 2014). The balance between cell proliferation and apoptosis controls the total cell number (Russell et al. 2002). Testicular Leydig cells secrete testosterone (T), which regulates spermatogenesis. Testosterone deficiency has an adverse impact on the spermatogenic processes (Smith and Walker 2014).

Methotrexate (MTX) is an alkylating agent with various clinical applications for treatment of malignant tumors and autoimmune diseases (Chan and Cronstein 2013). MTX inhibits spermatogenesis owing to its antiproliferative effects, damage of germ cell DNA (Padmanabhan et al. 2008), and enhancement of hydrogen peroxide and oxidative stress-induced cellular toxicity (Chan and Cronstein 2013). Previous studies have shown that MTX exhibits inhibitory effects on both T production and spermatogenesis (Badri et al. 2000; Padmanabhan et al. 2008).

Decrease in androgen concentration with disturbed estrogen/T ratio in some diseases, such as obesity and diabetes, (Vodo et al. 2013) was linked to male infertility (Cabler et al. 2010). In diabetes, low T concentrations were associated with an increase in reactive oxygen species (ROS) levels in the local testicular milieu (Vodo et al. 2013). In addition, low levels of T have been associated with an increase in the levels of proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). In aged men, androgen replacement with testosterone propionate (Tp) improved spermatogenesis (de and de Jong 2011; Jackson and Jackson 1984); besides, it exerted anti-inflammatory effects (Maggio et al. 2005; Maggio et al. 2006; Malkin et al. 2004; Norata et al. 2006).

Aromatase is crucial for estrogen synthesis since it converts the testicular and adrenal androgens to estradiol (E2). Aromatase has both gonadal and extra-gonadal activities (de and de Jong 2011). Extra-gonadal aromatase is present in the adipose tissues, liver, muscles, and bones (de and de Jong 2011; Gooren and Toorians 2003). Aromatase inhibitors (AI) were shown to normalize T and E2 levels, as well as the disturbed follicular stimulating hormone (FSH) and luteinizing hormone (LH) levels (Raman and Schlegel 2002). In aged men with low T levels, letrozole (LET), a third-generation AI, increased T levels (T'Sjoen et al. 2005) and decreased plasma E2 levels, within the male physiological range (de and de Jong 2011); in addition, it increased FSH (Raven et al. 2006) and LH levels (Pitteloud et al. 2008). Grape seed extract (GSE), a natural extract isolated from *Vitis vinifera* seeds, contains polyphenols, which are mainly flavonoids and proanthocyanidins, with potent antioxidant properties (El-Ashmawy et al. 2007; Shi et al. 2003). Procyanidin, a proanthocyanidin, exhibited a potent AI activity (Kijima et al. 2006).

MTX rat model has dual inhibition on T production and spermatogenesis. It is still debatable whether improvement of the endogenous T levels by LET or GSE could improve the semen parameters owing to their antioxidant and anti-inflammatory activities. In this study, we focused on the association between T levels and oxidative stress, and their effects on spermatogenesis.

Materials and methods

Animals

This study was conducted at Department of Physiology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt. Rats were supplied by the center for experimental animals, Faculty of Veterinary Medicine, Suez Canal University. Animal experiments were carried out in accordance with the ethical guide for the care and use of laboratory animals (2003),

Canadian Council on Animal Care (CCAC), as previously described (Beca et al. 2013; Samah et al. 2012). The study protocol was approved by the institutional animal care ethics committee at Faculty of Medicine, Suez Canal University (Research #3170). Rats were housed in plastic cages with free access to standard animal pellet diet and water, and allowed to acclimatize to the laboratory conditions for one week. They were maintained at controlled temperature (22-24 °C), with 12/12 h light/dark cycles.

Animals Model

Sixty-five adult albino Sprague-Dawley rats (200-250 g) were included in the study. The total MTX dose was adjusted at 30 mg/kg (Yulug et al. 2013), and modified as previously described (Badri et al. 2000) to reduce the mortality rate (Supplementary, Table S1). Briefly, MTX was administered as a single dose (2 mg/kg, intraperitoneal) on three consecutive days for a total period of five weeks. The control group (CTL) received no treatment (NT; normal saline by oral gavage), or one of the potential protective agents, LET (Sigma, Egypt; 0.5 mg/kg/day, oral gavage; $n = 7$) (Buzdar et al. 2002; de and de Jong 2011; Kafali et al. 2004), GSE (commercially available; 50 mg/ kg/day, oral gavage; $n = 7$) (Cetin et al. 2008; Hassan et al. 2014), or Tp (Sigma, Egypt; 100 µg/kg/day, subcutaneous; $n = 6$) (Verjans et al. 1975)) without MTX. The MTX-model groups received MTX alone ($n = 6$), a combination of MTX + LET ($n = 7$; experiment 1), MTX + GSE ($n = 6$; experiment 1), or MTX + Tp ($n = 6$; experiment 2; Supplementary Table S2). Experiments 1 and 2 were conducted to test the endogenous and exogenous testosterone effects, respectively. MTX was administrated starting from day 7 after administration of the protective agents (day 0) till day 35.

Blood and tissue sampling

On day 35, rats were tranquilized and anesthetized with isoflurane (Beca et al. 2013). Blood samples were collected by cardiac puncture, using a 5-gauge syringe to determine the

concentrations of serum FSH, LH, T, and E2. Next, the rats were sacrificed by exsanguination (aortic dissection) at the end of the procedure. The right and left testes from each animal were removed for histological and chemical assessments. The body and testicular weights of each animal were measured on day 35 (Table 1). Testicular tissue samples were collected from several regions of the left testes (except the mediastinal region), and stored at -40 °C (Castro et al. 2002) for measurement of the testicular levels of oxidative stress markers, superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GPx), as well as inflammatory markers, TNF- α and IL-1 β .

For histological assessment of SNT spermatogenesis and interstitial tissue, the right testes were stored in Bouin fixative, embedded in paraffin blocks, and sectioned at 3 μ m. The sections were examined using a light microscope.

Assessment of sperm count and motility

We studied the sperm motility of rats according to the WHO standard method for manual examination of sperm motility (Catanzariti et al. 2013). Briefly, epididymal sperm samples were collected by cutting the caudal epididymis into small pieces. They were placed in 2.9 % sodium citrate solution and then diluted with the same solution (1:10) to examine sperm motility and count. A drop of the sperm suspension was smeared on a glass slide and stained by eosin-nigrosin stain. The stained smears were examined to determine the percentage of sperm viability (alive/dead). For sperm count determination, 20 μ L of sperm sample was placed in Neubauer counting chamber and examined at 200 $\times$ magnification. For sperm motility evaluation, only the motile sperms were counted within ten boxes. The average of four times of counting was calculated. Motility was evaluated under the microscope, according to the following equation: motility (%) = (motile sperms/total motile and non-motile sperms) $\times$ 100 (Khosravanian et al. 2014).

Serum FSH, LH, T, and E2

All blood samples were centrifuged ($1600 \times g$, 20 min, 4 °C), and serum was separated and kept at -20 °C until assayed. The concentrations of FSH, LH, T, and E2 were determined using commercial kits (Vidas, Biomerieux, France). For interassay variation reduction, all collected samples for one experiment were assayed at the same time. The corresponding intra-assay and interassay coefficients of variation (CVs) were as follows: FSH, 5.9 and 4.7 %; LH, 5.7 and 6.3 %; T, 5.2 and 5.1 %; and E2, 3.2 and 4.6 %, respectively.

Testicular oxidative stress markers

Samples of the left testes were weighed and homogenized in 10 % (w/v) Tris buffer (0.32 M sucrose, 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid [EDTA], pH = 7.8) using a Teflon homogenizer (Glas-Col homogenizer system, Vernon Hills, USA). The homogenate was then sonicated and centrifuged at $20,000 \times g$ for 15 min, and the supernatant was used for determination of SOD, MDA, and GPx levels spectrophotometrically using specific kits (Biodiagnostics, Egypt). Absorbance values were measured by a UV-visible spectrophotometer (UV-1601PC, Shimadzu, Japan). MDA, SOD, and GPx levels were measured as previously described (Ohkawa et al. 1979) (Nishikimi et al. 1972; Paglia and Valentine 1967).

Serum and testicular inflammatory markers

The testicular samples were processed for assaying the inflammatory markers, TNF- α and IL-1 β . TNF- α and IL-1 β concentrations were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Morphological spermatogenesis assessment

Hematoxylin and eosin (H&E)-stained sections of SNTs were examined using the light microscope. Johnsen's criteria were used to assess spermatogenesis by scoring 'cross-sectional' profiles of the SNTs. A score from 1 to 10 was assigned, according to the presence or absence of germ cells (Johnsen 1970).

SNT diameter was measured at 100× magnification; besides, the epithelium height and Leydig cell count were assessed at 400× magnification using the light microscope. Images were analyzed using ImageJ software (version 1.50b, USA) by a blinded histologist. Sertoli/germ cell ratio was assessed using SNTs with score 7 of the Johnsen's criteria. Leydig cells were counted in five fields, and the average count was calculated, as described previously (Bartlett et al. 1986; Castro et al. 2002; Wing and Christensen 1982).

Statistical analysis

Comparisons among groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test, whereas for comparisons between two groups, the *t*-test was used. Data are expressed as means ± standard error of the mean. *P* values < 0.05 indicated statistical significance. Data were analyzed using Origin Pro software (version 8.0724, OriginLab Corporation, Northampton, MA, USA). N refers to the number of rats.

Results

Effects of LET and GSE on MTX-induced changes in body and testicular weights

In this study, the LET and GSE protective effects on MTX-induced changes in body and testicular weights were assessed. As summarized in Table 1, a significant decrease in mean testicular weights was found in the MTX-treated group compared to that of the NT group (0.97 ± 0.09 g, *n* = 6; vs 1.41 ± 0.01 g, *n* = 7, *P* < 0.001, respectively), LET, and GSE-treated groups. MTX + LET (1.23 ± 0.03 g, *n* = 7, *P* < 0.05) and MTX + GSE (1.24 ± 0.04 g, *n* = 6, *P* < 0.05) combinations protected the rats against MTX-induced reduction of testicular weight. In addition, the body weights of MTX-treated rats (164.8 ± 5.59 g, *n* = 6) were significantly (*P* < 0.001; Table 1) lower than that of the NT group (249.0 ± 5.36 g, *n* = 7), and rats of the other groups. However, there was no significant difference in body weight between

rats in the MTX-treated and rats in the MTX + GSE (186.7 ± 4.43 g, $n = 6$, $P = 0.26$) groups. In contrast, the MTX + LET combination (204.0 ± 9.83 g, $n = 7$, $P < 0.01$) protected the rats against MTX-induced reduction of body weight.

Sperm count and motility

To explore the effect of MTX on functional sperm production, rats epididymal sperm samples were collected to evaluate this capacity in Table 2. A significant decrease in sperm count ($\times 10^6$) was found in the MTX-treated group compared to that of the NT group (16.46 ± 2.09 , $n = 6$; vs 65.89 ± 1.97 , $n = 7$, $P < 0.001$, respectively), as well as the protected groups MTX + LET (41.76 ± 3.03 , $n = 7$, $P < 0.01$) and MTX + GSE (51.99 ± 2.42 , $n = 6$, $P < 0.001$). Regarding sperm motility, there was a significant decrease in the mean percentage of spermatozoa with progressive motility in the MTX group compared to that of the NT, LET, GSE and protected groups. Moreover, rats receiving MTX + GSE exhibited greater sperm motility than those receiving MTX + LET (74.27 ± 2.35 %, $n = 6$; vs 54.59 ± 2.69 %, $n = 7$, $P < 0.001$; vs, respectively; Table 2).

Serum FSH, LH, T, and E2 concentrations

We next examined pituitary-testicular hormonal axis, and the peripheral T and E2 levels in the protected and unprotected MTX-treated rats (Fig. 1). MTX had no significant effect on the serum concentrations of FSH and LH (Fig. 1A and 1B). However, it resulted in a significant reduction of serum T (MTX, 0.98 ± 0.32 , $n = 6$; vs NT, 3.39 ± 0.11 ng/mL, $n = 7$; $P < 0.05$) and E2 concentrations (MTX, 4.86 ± 0.56 pg/mL, vs $n = 6$; NT, 11.81 ± 0.67 , $n = 7$; $P < 0.001$; Fig. 1C and 1D). Co-administration of MTX + LET and MTX + GSE ameliorated the decrease in serum T (Fig. 1C) and E2 concentrations (Fig. 1D) compared to that of the LET- and GSE-treated groups. In addition, serum T, but not E2, concentrations, increased (Fig. 1C) in the MTX + LET and MTX + GSE groups compared to those of the MTX group (5.31 ± 0.81 , $n = 7$; 5.04 ± 0.59 , $n = 6$; 0.98 ± 0.32 , $n = 6$; $P < 0.001$,

respectively, Fig. 1C). Regarding, LET group alone, but not GSE (4.29 ± 0.32 , $n = 7$; $P = 0.77$) group alone, was also able to increase serum T concentrations, compared to that of the NT (LET, 6.81 ± 0.47 , $n = 7$; NT, 3.39 ± 0.11 , $n = 7$; $P < 0.001$, Fig. 1C). Opposite results were observed for E2 concentrations, when compared to that of the NT group (Fig. 1D).

SNT cellular assessment

Histopathological examination of the testicular tissues revealed normal spermatogenesis in NT, LET, and GSE groups (Fig. 2 and Fig. 3). SNTs were populated by normal Sertoli cells (Fig. 2 and Fig. 3-A); in addition, it showed normal height and diameter of the epithelium (Fig. 2, 3C, and 3D), various stages of germ cell maturation till the mature spermatozoa, and normal interstitial Leydig cells (Fig. 2A, 2C, 2E and 3B). Testicular sections from rats in the MTX group showed marked hypospermatogenesis, disorganization, vacuolation, maturation arrest with absence of the mature sperms, and disturbed interstitial space (Fig. 2B). These deleterious effects associated with MTX administration were significantly ameliorated by co-administration of MTX + LET and MTX + GSE (Fig. 2D, 2F, and 3A-3E).

Effects of Tp injection on spermatogenesis and sex hormones

To explore the importance of T on spermatogenesis, we examined its effect by administering exogenous T in the form of Tp (Table 3). Subcutaneous Tp administration increased testosterone levels above the normal level (Tp, 18.89 ± 0.98 ng/mL, $n = 6$; NT, 3.84 ± 0.12 ng/mL, $n = 7$; $P < 0.001$; Table 3). Serum E2 levels showed no reduction ($P = 0.70$) in the Tp and MTX + Tp groups (11.72 ± 0.43 pg/mL, $n = 6$; 10.91 ± 0.57 pg/mL, $n = 6$, respectively) compared to the NT (11.24 ± 0.60 pg/mL, $n = 7$) group; however, they were higher ($P < 0.001$) than that in the MTX group (4.19 ± 0.39 pg/mL, $n = 6$; Table 3). Rats in the Tp and MTX + Tp groups exhibited lower serum FSH and LH levels compared to those of the NT group. The Tp-treated rats showed significant reduction in testicular weight

compared to NT-treated rats (Table 3). Rats cotreated with MTX + Tp showed preservation of sperm count and motility compared to rats the treated with MTX (Table 3); however, they were lower than that in the NT group.

LET, GSE, and Tp reduced testicular oxidative stress markers

Furthermore, we also investigated the effects of LET, GSE, and Tp on testicular oxidative stress (Fig. 4). MTX induced oxidative stress in rat testes, as evidenced by the significant increase in MDA concentrations and decrease in SOD and GPx concentrations compared to those of the control NT group (Fig. 4). To investigate the antioxidant effects of exogenous T, rats were treated with Tp only or MTX + Tp. Exogenous T significantly improved all oxidative stress markers (Fig. 4). Similarly, MTX + LET and MTX + GSE ameliorated MTX-induced oxidative stress (Fig. 4).

Top, LET, and GSE reduced testicular inflammatory markers

We used cytokines analysis to investigate changes in MTX-treated rats with or without protective effect of Tp, LET, and GSE (Fig. 5). TNF- α and IL-1 β levels significantly increased in the MTX group; in addition, the proinflammatory cytokine levels correlated with Johnsen's score ($R^2 = 0.82, 0.94$, respectively; Fig. 5A and 5E). Combination of MTX with LET, GSE, or Tp resulted in TNF- α and IL-1 β reduction and improvement in Johnsen's score (Fig. 5B, 5C, 5F, and 5G). In particular, Johnsen's score improved significantly in the groups receiving combinations of MTX + LET (7.00 ± 0.98 , $n = 7$; $P < 0.01$), MTX + GSE (7.17 ± 0.88 , $n = 6$; $P < 0.01$), or MTX + Tp (6.50 ± 0.76 , $n = 6$; $P < 0.05$) compared to that of the MTX (2.33 ± 0.49 , $n = 6$) group (Fig. 5). TNF- α and IL-1 β levels significantly decreased in the protected groups compared to the MTX group (Fig. 5). There was no significant difference in TNF- α and IL-1 β levels among MTX + LET, MTX + GSE, and MTX + Tp groups.

Discussion

Defects in spermatogenesis are among the causes of male infertility. Hormonal treatment and AI therapy are used to treat spermatogenic defects; however, they have limited clinical benefits (Anawalt 2013; de and de Jong 2011). Vitamin E and T ameliorated varicocele-induced damage to rat Leydig cells, enhanced T levels, and improved spermatogenesis *via* improvement of the testicular antioxidant status and endocrine activities (Khosravanian et al. 2014). In the present study, we investigated the effects of increasing endogenous T and antioxidant defenses by LET, GSE, and exogenous T (Kijima et al. 2006) on MTX-induced spermatogenesis impairment in rats.

Spermatogenesis is dependent on Sertoli and Leydig cells, which are the primary source of local androgen (Mruk and Cheng 2004; Wang et al. 2009). LH regulates the secretion of T, which binds to the androgen receptors in Sertoli cells to initiate spermatogenesis (Wang et al. 2009). Deficiency of T can arrest spermatogenesis during meiosis (Chang et al. 2004; De et al. 2004; Yeh et al. 2002). Furthermore, T-suppressed rats were unable to maintain the attachment of Sertoli cells to the spermatids, and germ cells were released prematurely (Holdcraft and Braun 2004; O'Donnell et al. 1996). In line with our findings, MTX caused a significant decrease in serum T levels owing to its inhibitory effects on steroidogenesis (Badri et al. 2000). This effect was reflected by the reduction in testicular weight, sperm count, and motility. Microscopically, we observed atrophied interstitial cells, interstitial space edema, reduced spermatogenic cells, and atrophied seminiferous tubules. These findings are in agreement with the results of many previous studies (Badri et al. 2000; Chan and Cronstein 2013; Padmanabhan et al. 2008). Treatment with LET and GSE

significantly ameliorated MTX-induced damage and protected the interstitial cells. These findings suggested that LET and GSE exhibited protective effects on spermatogenesis.

AI reversibly inhibits aromatase enzyme activity (Geisler 2011). However, it does not result in complete depletion of plasma E2 levels (Raven et al. 2006; T'Sjoen et al. 2005). This incomplete inhibition is attributed to the high plasma concentrations of T, the main precursor of E2. Similarly, we observed incomplete inhibition of serum E2 synthesis in rats treated with LET alone, which was accompanied by an increase in serum T levels. A study on young and aged men showed that inhibition of E2 synthesis by LET resulted in increased basal LH and T levels (T'Sjoen et al. 2005). This effect is mainly attributed to the decrease in peripheral E2 levels (an inhibitor of LH release), and thus increasing LH release (Raven et al. 2006). Therefore, modulation of peripheral T, E2, FSH, and LH levels might provide a good treatment strategy for male infertility (Patry et al. 2009). However, in the current study, we did not observe any significant difference in FSH or LH levels in rats treated with LET, compared to that of rats in the control (NT) group. This could be because of the difference in species, treatment duration, or the factors affecting gonadotrophin release in the two studies (T'Sjoen et al. 2005; Turner et al. 2000). Chronic administration of AI did not affect spermatogenesis but can result in sporadic disturbed germ/Sertoli cell ratio, as observed in the current study (Turner et al. 2000).

In rats with Leydig cell abolition, exogenous T replacement was able to maintain the spermatogenic epithelium (Delic et al. 1987; Jackson and Jackson 1984). However, our results showed that exogenous T exhibited an inhibitory effect on spermatogenesis, which could be attributable to its negative effect on the pituitary-hypothalamic axis (Crosnoe et al. 2013; Kolettis et al. 2015) or the difference in type and method of T administration (Delic et al. 1987).

GSE has been shown to have AI (Conway et al. 2015) and antioxidant effects (Abdel-Kawi et al. 2016; Alkhedaide et al. 2016; Bayatli et al. 2013; El-Ashmawy et al. 2007; Hassan et al. 2014; Li et al. 2015; Su et al. 2011; Zhao et al. 2014). In this study, we did not observe the AI effects of GSE like LET. However, it protected spermatogenesis in MTX-treated rats and prevented MTX-induced decrease in sperm count and motility, which were confirmed microscopically. These results indicated that GSE and LET supported spermatogenesis by enhancing endogenous T production and decreasing oxidative stress, particularly GSE.

Oxidative stress is one of the main causes of male infertility since it can enhance the apoptosis of germ cells and reduce the synthesis of T (Aprioku 2013). Exogenous T was able to decrease oxidative stress in rats suffered testicular physical injury and enhanced oxidative stress markers (MDA, SOD, and GPx) (Khosravanian et al. 2014). MTX treatment was shown to induce marked oxidative stress and inflammatory responses (increase in ROS, MDA, IL-1 β , and TNF- α levels) in the sciatic nerve of rats (Celik et al. 2013). MTX has independent toxicity that involves modifying the cellular metabolic processes through altering the anti-inflammatory and antioxidant pathways (Neradil et al. 2012). The antioxidant, resveratrol was able to ameliorate MTX-induced oxidative stress and testicular damage (Yulug et al. 2013). Our results showed that MTX increased MDA and reduced SOD and GPx levels (Fig. 4); besides, it elevated TNF- α and IL-1 β concentrations (Fig. 5) in rat, which were reduced by MTX + LET and MTX + GSE co-administration. The antioxidant and anti-inflammatory effects of Tp were similar to those of LET and GSE, which indicated that T exerted protective effects in the testes. It is known to modify immune responses by reducing the proinflammatory markers (Furman et al. 2014), wherein T therapy reduced inflammatory cytokines, TNF- α and IL-1 β in hypogonadal men (Malkin et al. 2004; Soljancic

et al. 2013; Vodo et al. 2013; Xu et al. 2008). Therefore, the protective effects of androgens on spermatogenesis may be attributed to their anti-inflammatory and antioxidant effects.

Potential future significance

In the present study, we showed the GSE antioxidant and anti-inflammatory effects. However, rats treated with GSE exhibited small but significant body weight reduction compared to CTL group (Table 1). Polyphenols in GSE may reduce food intake, digestibility and delay absorption. Moreover, polyphenols stimulate lipolysis, which supports sustained satiety, and increases hepatic fat oxidation (Ohyama et al. 2011; Tebib et al. 1994) causing degrading fat stores (Ardevol et al. 2000). This effect should be considered if patients receive GSE as a supplement alone or with other medications.

MTX is an important treatment for chronic rheumatic and non-rheumatic inflammatory diseases (Chan and Cronstein 2013). Patients who suffer from these chronic inflammatory conditions could exhibit the oxidative stress impairment effect on spermatogenesis. GSE and LET with their antioxidant, and anti-inflammatory effects would benefit these patients, particularly if there is hypoandrogenic state.

Conclusion

Based on the results of this study, we suggest that chemical or physical insults to the testis can decrease T levels, which, in turn, affects spermatogenesis and decreases anti-inflammatory and antioxidant protection of the testis. Therefore, increasing the endogenous androgen levels by AI and enhancing the antioxidant and anti-inflammatory effects by GSE and AI could support spermatogenesis and protect the germinal and non-germinal cells from chemical or physical insults because of medical, occupational, or environmental exposures.

Additional physiological mechanistic studies are required to elucidate the significance of peripheral testosterone regulatory mechanisms in health and disease.

Conflict of interest

The authors declare that there are no conflicts of interest associated with this study.

Acknowledgements

There is no conflict of interest. This study was not supported by any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

Draft

References

- Abdel-Kawi, S.H., Hashem, K.S., and Abd-Allah, S. 2016. Mechanism of diethylhexylphthalate (DEHP) induced testicular damage and of grape seed extract-induced protection in the rat. *Food Chem. Toxicol* **90**: 64-75. doi: S0278-6915(16)30026-6 [pii];10.1016/j.fct.2016.02.003 [doi].
- Alkhedaide, A., Alshehri, Z.S., Sabry, A., Abdel-Ghaffar, T., Soliman, M.M., and Attia, H. 2016. Protective effect of grape seed extract against cadmium-induced testicular dysfunction. *Mol. Med. Rep* **13**(4): 3101-3109. doi: 10.3892/mmr.2016.4928 [doi].
- Anawalt, B.D. 2013. Approach to male infertility and induction of spermatogenesis. *J. Clin. Endocrinol. Metab* **98**(9): 3532-3542. doi: 98/9/3532 [pii];10.1210/jc.2012-2400 [doi].
- Aprioku, J.S. 2013. Pharmacology of free radicals and the impact of reactive oxygen species on the testis. *J. Reprod. Infertil* **14**(4): 158-172.
- Ardevol, A., Blade, C., Salvado, M.J., and Arola, L. 2000. Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes. *Int J Obes Relat Metab Disord* **24**(3): 319-324.

Badri, S.N., Vanithakumari, G., and Malini, T. 2000. Studies on methotrexate effects on testicular steroidogenesis in rats. *Endocr. Res* **26**(2): 247-262.

Bartlett, J.M., Kerr, J.B., and Sharpe, R.M. 1986. The effect of selective destruction and regeneration of rat Leydig cells on the intratesticular distribution of testosterone and morphology of the seminiferous epithelium. *J. Androl* **7**(4): 240-253.

Bayatli, F., Akkus, D., Kilic, E., Saraymen, R., and Sonmez, M.F. 2013. The protective effects of grape seed extract on MDA, AOPP, apoptosis and eNOS expression in testicular torsion: an experimental study. *World J. Urol* **31**(3): 615-622. doi: 10.1007/s00345-013-1049-8 [doi].

Beca, S., Ahmad, F., Shen, W., Liu, J., Makary, S., Polidovitch, N., Sun, J., Hockman, S., Chung, Y.W., Movsesian, M., Murphy, E., Manganiello, V., and Backx, P.H. 2013. Phosphodiesterase type 3A regulates basal myocardial contractility through interacting with sarcoplasmic reticulum calcium ATPase type 2a signaling complexes in mouse heart. *Circ. Res* **112**(2): 289-297. doi: CIRCRESAHA.111.300003 [pii];10.1161/CIRCRESAHA.111.300003 [doi].

Buzdar, A.U., Robertson, J.F., Eiermann, W., and Nabholz, J.M. 2002. An overview of the pharmacology and pharmacokinetics of the newer generation aromatase inhibitors anastrozole, letrozole, and exemestane. *Cancer* **95**(9): 2006-2016. doi: 10.1002/cncr.10908 [doi].

Cabler, S., Agarwal, A., Flint, M., and du Plessis, S.S. 2010. Obesity: modern man's fertility nemesis. *Asian J. Androl* **12**(4): 480-489. doi: aja201038 [pii];10.1038/aja.2010.38 [doi].

Castro, A.C., Berndtson, W.E., and Cardoso, F.M. 2002. Plasma and testicular testosterone levels, volume density and number of Leydig cells and spermatogenic efficiency of rabbits. *Braz. J. Med. Biol. Res* **35**(4): 493-498. doi: S0100-879X2002000400014 [pii].

Catanzariti, F., Cantoro, U., Lacetera, V., Muzzonigro, G., and Polito, M. 2013. Comparison between WHO (World Health Organization) 2010 and WHO 1999 parameters for semen analysis - interpretation of 529 consecutive samples. *Arch. Ital. Urol. Androl* **85**(3): 125-129. doi: aiua.2013.3.125 [pii];10.4081/aiua.2013.3.125 [doi].

Celik, F., Gocmez, C., Bozkurt, M., Kaplan, I., Kamasak, K., Akil, E., Dogan, E., Guzel, A., and Uzar, E. 2013. Neuroprotective effects of carvacrol and pomegranate against methotrexate-induced toxicity in rats. *Eur. Rev. Med. Pharmacol. Sci* **17**(22): 2988-2993.

Cetin, A., Kaynar, L., Kocyigit, I., Hacıoglu, S.K., Saraymen, R., Ozturk, A., Sari, I., and Sagdic, O. 2008. Role of grape seed extract on methotrexate induced oxidative stress in rat liver. *Am. J. Chin Med* **36**(5): 861-872. doi: S0192415X08006302 [pii];10.1142/S0192415X08006302 [doi].

Chan, E.S., and Cronstein, B.N. 2013. Mechanisms of action of methotrexate. *Bull. Hosp. Jt. Dis.* (2013.) **71 Suppl 1**: S5-S8.

Chang, C., Chen, Y.T., Yeh, S.D., Xu, Q., Wang, R.S., Guillou, F., Lardy, H., and Yeh, S. 2004. Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. *Proc. Natl. Acad. Sci. U. S. A* **101**(18): 6876-6881. doi: 10.1073/pnas.0307306101 [doi];0307306101 [pii].

Conway, R., Low, C., Coughlan, R.J., O'Donnell, M.J., and Carey, J.J. 2015. Methotrexate use and risk of lung disease in psoriasis, psoriatic arthritis, and inflammatory bowel disease: systematic literature review and meta-analysis of randomised controlled trials. *BMJ* **350**: h1269.

Crosnoe, L.E., Grober, E., Ohl, D., and Kim, E.D. 2013. Exogenous testosterone: a preventable cause of male infertility. *Transl. Androl Urol* **2**(2): 106-113. doi: 10.3978/j.issn.2223-4683.2013.06.01 [doi];tau-02-02-106 [pii].

De, G.K., Swinnen, J.V., Saunders, P.T., Schoonjans, L., Dewerchin, M., Devos, A., Tan, K., Atanassova, N., Claessens, F., Lecureuil, C., Heyns, W., Carmeliet, P., Guillou, F., Sharpe, R.M., and Verhoeven, G. 2004. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proc. Natl. Acad. Sci. U. S. A* **101**(5): 1327-1332. doi: 10.1073/pnas.0308114100 [doi];0308114100 [pii].

de, R.W., and de Jong, F.H. 2011. Aromatase inhibitors in men: effects and therapeutic options. *Reprod. Biol. Endocrinol* **9**: 93. doi: 1477-7827-9-93 [pii];10.1186/1477-7827-9-93 [doi].

Delic, J.I., Harwood, J.R., and Stanley, J.A. 1987. Time dependence for the protective effect of androgen from procarbazine-induced damage to rat spermatogenesis. *Cancer Res* **47**(5): 1344-1347.

El-Ashmawy, I.M., Saleh, A., and Salama, O.M. 2007. Effects of marjoram volatile oil and grape seed extract on ethanol toxicity in male rats. *Basic Clin. Pharmacol. Toxicol* **101**(5): 320-327. doi: PTO125 [pii];10.1111/j.1742-7835.2007.00125.x [doi].

Fok, K.L., Chen, H., Ruan, Y.C., and Chan, H.C. 2014. Novel regulators of spermatogenesis. *Semin. Cell Dev. Biol* **29**: 31-42. doi: S1084-9521(14)00020-2 [pii];10.1016/j.semcdb.2014.02.008 [doi].

Furman, D., Hejblum, B.P., Simon, N., Jojic, V., Dekker, C.L., Thiebaut, R., Tibshirani, R.J., and Davis, M.M. 2014. Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination. *Proc. Natl. Acad. Sci. U. S. A* **111**(2): 869-874. doi: 1321060111 [pii];10.1073/pnas.1321060111 [doi].

Geisler, J. 2011. Differences between the non-steroidal aromatase inhibitors anastrozole and letrozole--of clinical importance? *Br. J. Cancer* **104**(7): 1059-1066. doi: bjc201158 [pii];10.1038/bjc.2011.58 [doi].

Gooren, L.J., and Toorians, A.W. 2003. Significance of oestrogens in male (patho)physiology. *Ann. Endocrinol. (Paris)* **64**(2): 126-135. doi: MDOI-AE-04-2003-64-2-0003-4266-101019-ART15 [pii].

Hassan, H.A., El-Kholy, W.M., and Nour, S.E. 2014. Proanthocyanidin as a cytogenetic protective agent against adverse effects of plant growth regulators supplementation in rats. *Cytotechnology* **66**(4): 585-596. doi: 10.1007/s10616-013-9607-x [doi].

Holdcraft, R.W., and Braun, R.E. 2004. Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids. *Development* **131**(2): 459-467. doi: 10.1242/dev.00957 [doi];131/2/459 [pii].

Jackson, C.M., and Jackson, H. 1984. Comparative protective actions of gonadotrophins and testosterone against the antispermatogenic action of ethane dimethanesulphonate. *J. Reprod. Fertil* **71**(2): 393-401.

Johnsen, S.G. 1970. Testicular biopsy score count--a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Hormones* **1**(1): 2-25.

Kafali, H., Iriadam, M., Ozardali, I., and Demir, N. 2004. Letrozole-induced polycystic ovaries in the rat: a new model for cystic ovarian disease. *Arch. Med. Res* **35**(2): 103-108. doi: 10.1016/j.arcmed.2003.10.005 [doi];S0188-4409(03)00156-5 [pii].

Khosravanian, N., Razi, M., Farokhi, F., and Khosravanian, H. 2014. Testosterone and vitamin E administration up-regulated varicocele-reduced Hsp70-2 protein expression and ameliorated biochemical alterations. *J. Assist. Reprod. Genet* **31**(3): 341-354. doi: 10.1007/s10815-013-0165-0 [doi].

Kijima, I., Phung, S., Hur, G., Kwok, S.L., and Chen, S. 2006. Grape seed extract is an aromatase inhibitor and a suppressor of aromatase expression. *Cancer Res* **66**(11): 5960-5967. doi: 66/11/5960 [pii];10.1158/0008-5472.CAN-06-0053 [doi].

Kolettis, P.N., Purcell, M.L., Parker, W., Poston, T., and Nangia, A.K. 2015. Medical testosterone: an iatrogenic cause of male infertility and a growing problem. *Urology* **85**(5): 1068-1072. doi: S0090-4295(15)00105-3 [pii];10.1016/j.urology.2014.12.052 [doi].

Li, S.G., Ding, Y.S., Niu, Q., Xu, S.Z., Pang, L.J., Ma, R.L., Jing, M.X., Feng, G.L., Liu, J.M., and Guo, S.X. 2015. Grape Seed Proanthocyanidin Extract Alleviates Arsenic-induced

Oxidative Reproductive Toxicity in Male Mice. *Biomed. Environ. Sci* **28**(4): 272-280. doi: 10.3967/bes2015.038 [doi].

Maggio, M., Basaria, S., Ceda, G.P., Ble, A., Ling, S.M., Bandinelli, S., Valenti, G., and Ferrucci, L. 2005. The relationship between testosterone and molecular markers of inflammation in older men. *J. Endocrinol. Invest* **28**(11 Suppl Proceedings): 116-119.

Maggio, M., Blackford, A., Taub, D., Carducci, M., Ble, A., Metter, E.J., Braga-Basaria, M., Dobs, A., and Basaria, S. 2006. Circulating inflammatory cytokine expression in men with prostate cancer undergoing androgen deprivation therapy. *J. Androl* **27**(6): 725-728. doi: jandrol.106.000141 [pii];10.2164/jandrol.106.000141 [doi].

Malkin, C.J., Pugh, P.J., Jones, R.D., Kapoor, D., Channer, K.S., and Jones, T.H. 2004. The effect of testosterone replacement on endogenous inflammatory cytokines and lipid profiles in hypogonadal men. *J. Clin. Endocrinol. Metab* **89**(7): 3313-3318. doi: 10.1210/jc.2003-031069 [doi];89/7/3313 [pii].

Mruk, D.D., and Cheng, C.Y. 2004. Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocr. Rev* **25**(5): 747-806. doi: 25/5/747 [pii];10.1210/er.2003-0022 [doi].

Neradil, J., Pavlasova, G., and Veselska, R. 2012. New mechanisms for an old drug;. *Klin. Onkol* **25 Suppl 2**: 2S87-82S92.

Nishikimi, M., Appaji, N., and Yagi, K. 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem. Biophys. Res. Commun* **46**(2): 849-854.

Norata, G.D., Tibolla, G., Seccomandi, P.M., Poletti, A., and Catapano, A.L. 2006. Dihydrotestosterone decreases tumor necrosis factor-alpha and lipopolysaccharide-induced inflammatory response in human endothelial cells. *J. Clin. Endocrinol. Metab* **91**(2): 546-554. doi: jc.2005-1664 [pii];10.1210/jc.2005-1664 [doi].

O'Donnell, L., McLachlan, R.I., Wreford, N.G., De Kretser, D.M., and Robertson, D.M. 1996. Testosterone withdrawal promotes stage-specific detachment of round spermatids from the rat seminiferous epithelium. *Biol. Reprod* **55**(4): 895-901.

Ohkawa, H., Ohishi, N., and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem* **95**(2): 351-358.

Ohyama, K., Furuta, C., Nogusa, Y., Nomura, K., Miwa, T., and Suzuki, K. 2011. Catechin-rich grape seed extract supplementation attenuates diet-induced obesity in C57BL/6J mice. *Ann Nutr Metab* **58**(3): 250-258. doi: 10.1159/000330444.

Padmanabhan, S., Tripathi, D.N., Vikram, A., Ramarao, P., and Jena, G.B. 2008. Cytotoxic and genotoxic effects of methotrexate in germ cells of male Swiss mice. *Mutat. Res* **655**(1-2): 59-67. doi: S1383-5718(08)00200-3 [pii];10.1016/j.mrgentox.2008.07.003 [doi].

Paglia, D.E., and Valentine, W.N. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab Clin. Med* **70**(1): 158-169. doi: 0022-2143(67)90076-5 [pii].

Patry, G., Jarvi, K., Grober, E.D., and Lo, K.C. 2009. Use of the aromatase inhibitor letrozole to treat male infertility. *Fertil. Steril* **92**(2): 829-822. doi: S0015-0282(09)01090-5 [pii];10.1016/j.fertnstert.2009.05.014 [doi].

Pitteloud, N., Dwyer, A.A., DeCruz, S., Lee, H., Boepple, P.A., Crowley, W.F., Jr., and Hayes, F.J. 2008. Inhibition of luteinizing hormone secretion by testosterone in men requires aromatization for its pituitary but not its hypothalamic effects: evidence from the tandem study of normal and gonadotropin-releasing hormone-deficient men. *J. Clin. Endocrinol. Metab* **93**(3): 784-791. doi: jc.2007-2156 [pii];10.1210/jc.2007-2156 [doi].

Raman, J.D., and Schlegel, P.N. 2002. Aromatase inhibitors for male infertility. *J. Urol* **167**(2 Pt 1): 624-629.

Raven, G., de Jong, F.H., Kaufman, J.M., and de, R.W. 2006. In men, peripheral estradiol levels directly reflect the action of estrogens at the hypothalamo-pituitary level to inhibit gonadotropin secretion. *J. Clin. Endocrinol. Metab* **91**(9): 3324-3328. doi: jc.2006-0462 [pii];10.1210/jc.2006-0462 [doi].

Russell, L.D., Chiarini-Garcia, H., Korsmeyer, S.J., and Knudson, C.M. 2002. Bax-dependent spermatogonia apoptosis is required for testicular development and spermatogenesis. *Biol. Reprod* **66**(4): 950-958.

Samah, M., El-Aidy, A., Tawfik, M.K., and Ewais, M.M. 2012. Evaluation of the antifibrotic effect of fenofibrate and rosiglitazone on bleomycin-induced pulmonary fibrosis in rats. *Eur. J. Pharmacol* **689**(1-3): 186-193. doi: S0014-2999(12)00469-4 [pii];10.1016/j.ejphar.2012.05.026 [doi].

Shi, J., Yu, J., Pohorly, J.E., and Kakuda, Y. 2003. Polyphenolics in grape seeds-biochemistry and functionality. *J. Med. Food* **6**(4): 291-299. doi: 10.1089/109662003772519831 [doi].

Smith, L.B., and Walker, W.H. 2014. The regulation of spermatogenesis by androgens. *Semin. Cell Dev. Biol* **30**: 2-13. doi: S1084-9521(14)00024-X [pii];10.1016/j.semcdb.2014.02.012 [doi].

Soljancic, A., Ruiz, A.L., Chandrashekar, K., Maranon, R., Liu, R., Reckelhoff, J.F., and Juncos, L.A. 2013. Protective role of testosterone in ischemia-reperfusion-induced acute kidney injury. *Am. J. Physiol Regul. Integr. Comp Physiol* **304**(11): R951-R958. doi: ajpregu.00360.2012 [pii];10.1152/ajpregu.00360.2012 [doi].

Su, L., Deng, Y., Zhang, Y., Li, C., Zhang, R., Sun, Y., Zhang, K., Li, J., and Yao, S. 2011. Protective effects of grape seed procyanidin extract against nickel sulfate-induced apoptosis and oxidative stress in rat testes. *Toxicol. Mech. Methods* **21**(6): 487-494. doi: 10.3109/15376516.2011.556156 [doi].

T'Sjoen, G.G., Giagulli, V.A., Delva, H., Crabbe, P., De, B.D., and Kaufman, J.M. 2005. Comparative assessment in young and elderly men of the gonadotropin response to aromatase inhibition. *J. Clin. Endocrinol. Metab* **90**(10): 5717-5722. doi: jc.2005-0982 [pii];10.1210/jc.2005-0982 [doi].

Tebib, K., Rouanet, J.M., and Besancon, P. 1994. Effect of grape seed tannins on the activity of some rat intestinal enzyme activities. *Enzyme Protein* **48**(1): 51-60.

Turner, K.J., Morley, M., Atanassova, N., Swanston, I.D., and Sharpe, R.M. 2000. Effect of chronic administration of an aromatase inhibitor to adult male rats on pituitary and testicular function and fertility. *J. Endocrinol* **164**(2): 225-238. doi: JOE03541 [pii].

Verjans, H.L., van der Molen, H.J., and Eik-Nes, K.B. 1975. Relation between circulating levels of testosterone lh and fsh in intact and castrated, adult, male rats after testosterone administration. *Acta Endocrinol. (Copenh)* **79**(2): 380-386.

Vodo, S., Bechi, N., Petroni, A., Muscoli, C., and Aloisi, A.M. 2013. Testosterone-induced effects on lipids and inflammation. *Mediators. Inflamm* **2013**: 183041. doi: 10.1155/2013/183041 [doi].

Wang, R.S., Yeh, S., Tzeng, C.R., and Chang, C. 2009. Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. *Endocr. Rev* **30**(2): 119-132. doi: er.2008-0025 [pii];10.1210/er.2008-0025 [doi].

Wing, T.Y., and Christensen, A.K. 1982. Morphometric studies on rat seminiferous tubules. *Am. J. Anat* **165**(1): 13-25. doi: 10.1002/aja.1001650103 [doi].

Xu, Q., Wells, C.C., Garman, J.H., Asico, L., Escano, C.S., and Maric, C. 2008. Imbalance in sex hormone levels exacerbates diabetic renal disease. *Hypertension* **51**(4): 1218-1224. doi: HYPERTENSIONAHA.107.100594 [pii];10.1161/HYPERTENSIONAHA.107.100594 [doi].

Yeh, S., Tsai, M.Y., Xu, Q., Mu, X.M., Lardy, H., Huang, K.E., Lin, H., Yeh, S.D., Altuwaijri, S., Zhou, X., Xing, L., Boyce, B.F., Hung, M.C., Zhang, S., Gan, L., and Chang,

C. 2002. Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. *Proc. Natl. Acad. Sci. U. S. A* **99**(21): 13498-13503. doi: 10.1073/pnas.212474399 [doi];212474399 [pii].

Yulug, E., Turedi, S., Alver, A., Turedi, S., and Kahraman, C. 2013. Effects of resveratrol on methotrexate-induced testicular damage in rats. *ScientificWorldJournal* **2013**: 489659. doi: 10.1155/2013/489659 [doi].

Zhao, Y.M., Gao, L.P., Zhang, H.L., Guo, J.X., and Guo, P.P. 2014. Grape seed proanthocyanidin extract prevents DDP-induced testicular toxicity in rats. *Food Funct* **5**(3): 605-611. doi: 10.1039/c3fo60486a [doi].

Draft

Tables

Table 1 Body and testicular weights of rats in different study groups

Groups	Body weight (g)	Testicular weight (g)
	Day (35)	Day (35)
	Mean ± SEM	Mean ± SEM
NT (n=7)	249.0 ± 5.359	1.41 ± 0.01
LET (n=7)	241.0 ± 6.495###	1.26 ± 0.07#
GSE (n=7)	213.3 ± 5.58*###	1.31 ± 0.06##
MTX/LET (n=7)	204.0 ± 9.83***##	1.23 ± 0.03#
MTX/GSE (n=6)	186.7 ± 4.43***	1.24 ± 0.04#
MTX (n=6)	164.8 ± 5.59***	0.97 ± 0.09***

One-way ANOVA and post hoc Tukey’s tests were done between groups on scarification day. NT, no treatment; LET, letrozole; GSE, grape seed extract; MTX, methotrexate. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs NT; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs MTX. n, indicates number of rats.

Table 2 Effect of LET, GSE and MTX on sperm parameters

Groups	Count ($\times 10^6$)	Motility (%)
	Mean $\pm$ SEM	Mean $\pm$ SEM
NT (n=7)	65.89 $\pm$ 1.97	91.30 $\pm$ 2.40
LET (n=7)	61.45 $\pm$ 4.59 ^{###}	82.39 $\pm$ 2.05 ^{###}
GSE (n=7)	60.32 $\pm$ 7.29 ^{###}	91.15 $\pm$ 2.16 ^{###}
MTX/LET (n=7)	41.76 $\pm$ 3.03 ^{**##}	54.59 $\pm$ 2.69 ^{***###}
MTX/GSE (n=6)	51.99 $\pm$ 2.42 ^{###}	74.27 $\pm$ 2.35 ^{***###§}
MTX (n=6)	16.46 $\pm$ 2.09 ^{***}	22.22 $\pm$ 2.52 ^{***}

One-way ANOVA and post hoc Tukey's tests between groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs NT; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs MTX. Motility % parameter, § $P < 0.001$ MTX/LET vs MTX/GSE. n, indicates number of rats.

Table 3 Effect of testosterone injection on intact male rat reproductive parameters

	Testicular weight	Count ($\times 10^6$)	Motility (%)	FSH (mIU/ml)	LH (mIU/ml)	T (ng/ml)	E2 (pg/ml)
NT	1.36 $\pm$	67.32 $\pm$	85.73 $\pm$	5.20 $\pm$	3.09 $\pm$	3.84 $\pm$	11.24 $\pm$
(n=7)	0.06	2.74	3.23	0.63	0.36	0.12	0.60
MTX	0.88 $\pm$	13.79 $\pm$	20.56 $\pm$	4.15 $\pm$	2.12 $\pm$	0.94 $\pm$	4.19 $\pm$
(n=6)	0.089**	1.91***	1.51***	1.01	0.44	0.25*	0.39***
Tp	0.97 $\pm$	50.33 $\pm$	59.55 $\pm$	1.55 $\pm$	0.73 $\pm$	18.89 $\pm$	11.72 $\pm$
(n=6)	0.09*	4.47*####	1.68***####	0.32** [#]	0.09*** [#]	0.98***####	0.43####
MTX/Tp	0.79 $\pm$	44.67 $\pm$	54.58 $\pm$	1.95 $\pm$	0.60 $\pm$	17.18 $\pm$	10.91 $\pm$
(n=6)	0.08***	4.33*####	1.92***####	0.26*	0.09*** [#]	1.12***####	0.57####

One way ANOVA were done between rats injected with testosterone propionate (Tp) in the absence and presence of MTX. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs NT; [#] $P < 0.05$, ^{##} $P < 0.01$, ^{###} $P < 0.001$ vs MTX, respectively. n, number of rats per group.

Figure captions

Fig.1. Serum FSH, LH, T, and E2 concentrations in adult male rats. Protective effect of letrozole (LET, 0.5 mg/Kg), and grape seed extract (GSE, 50 mg/Kg) on pituitary and sex hormones in methotrexate (MTX, 30 mg/Kg) treated adult male rats. (A) Serum level of follicular stimulating hormone (FSH), (B) luteinizing hormone (LH), (C) testosterone, and (D) estradiol are presented as mean $\pm$ SEM. Measurement were done on day 35 before scarification in control (CTL-NT, No-treatment), LET, GSE alone or in combination with MTX. Values are the mean $\pm$ SE. Statistical analysis was done using ANOVA and post hoc Tukey's test to compare means between groups. $*P < 0.05$, $***P < 0.001$ LET compared with CTL; $***P < 0.001$ MTX + LET compared with MTX; $^{\#}P < 0.05$, $###P < 0.001$ CTL compared with MTX. Non-significant (ns), $P > 0.05$. N indicates number of rats per group.

Fig. 2. Testicular seminiferous tubules (SNTs) microscopic graphs. Photomicrographs of hematoxylin and eosin (H&E) stained histological slides of the testes on day 35 of experiment. SNTs are shown at (200 $\times$) magnification; (A) CTL-NT, (B) MTX, (C) LET, (D) MTX + LET, (E) GSE, and (F) MTX + GSE. (A) Normal CTL group shows the SNT and interstitial tissue structures are well-maintained, (B) MTX group shows disorganization and vacuolization (black arrows) with widening of interstitial space (black triangles) are seen. (C, E) LET and GSE groups shows preservation of SNT height, layers and interstitial space. (D, F) MTX + LET or MTX + GSE groups, there is mild loss of epithelium height, vacuolar changes and preservation of interstitial tissue. Bar = 50 μ m

Fig. 3. Effect of LET, GSE and MTX on different testicular functional structures. Effect of LET and GSE alone, or in combination with MTX as MTX + LET and MTX + GSE compared with CTL (NT) and MTX on SNTs structure were estimated at 400× magnification. The measurements were done on (A) germ/sertoli cell ratio, (B) Leydig cell number, (C) epithelium height, and (D) SNTs diameter. The levels are expressed as the mean $\pm$ SEM. One-way ANOVA test with post hoc Tukey's test for between groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ MTX + LET or MTX + GSE compared with MTX; #### $P < 0.001$ CTL compared with MTX. Non-significant (ns), $P > 0.05$. N indicates number of rats per group.

Fig. 4. Effect of LET, GSE and testosterone propionate on oxidative stress in testicular homogenates. (A) Assessment of oxidant marker malondialdehyde (MDA), (B) superoxide dismutase (SOD), and (C) glutathione peroxidase (GPx) reaction to MTX in testicular tissue. LET, GSE and testosterone propionate (Tp) showed protecting effect when combined with MTX. Statistical analysis of data were carried out using one-way ANOVA and Tukey's post hoc test for mean comparison between groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ MTX + LET, MTX + GSE or compared MTX + Tp compared with MTX; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ CTL or MTX + LET compared with MTX. Non-significant (ns), $P > 0.05$. N indicates number of rats per group.

Fig. 5. Correlation between Johansen score and testicular inflammatory cytokines. Correlation between the Johansen score (J Score, 1-10 assessed histologically), inflammatory markers TNF α (A–D), and IL1 β (E–H) levels. Data were fitted with a Pearson correlation (P and R^2 values are shown). one-way ANOVA and Tukey's post hoc test for mean comparison

between MTX and other groups regarding J Score and inflammatory markers are shown. Significance was defined as $P < 0.05$. *J SCORE, P value, MTX + LET (< 0.01), MTX + GSE (< 0.01), MTX + TP (< 0.05) compared with MTX; *TNF α : P value, MTX/LET (< 0.05), MTX/GSE (< 0.001), MTX/TP (< 0.01) compared with MTX; *IL1 β : P value, MTX vs MTX/LET (< 0.01), MTX/GSE (< 0.001), MTX/TP (< 0.01).

Draft

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Supplementary material

Table S1 Methotrexate doses and mortality

MTX doses	N	Week 1 1 st dose	Week 2 2 nd dose	Week 3 3 rd dose	Week 4 4 th dose	Week 5 5 th dose
(mg/kg) x doses numbers						
(2) x 3 days/week	35	0/35	0/35	1/35	1/35	2/35
(10) x single dose	3	2/3	3/3	NA	NA	NA
(20) x single dose	3	1/3	3/3	NA	NA	NA
(30) x single dose	3	3/3	NA	NA	NA	NA

Number of rats used in MTX experiments: MTX, MTX/LET, MTX/GSE, MTX/Tp.

MTX doses; 2 mg/kg (intraperitoneal, ip), for three consecutive days, for five weeks. N, number of rats. 10, 20, and 30 mg/kg (ip) doses were pilot experiments.

Table S2 Change in rats body weight of different study groups

Groups	Start	Scarification (day 35)	
	Mean $\pm$ SEM	Mean $\pm$ SEM	<i>t-test</i>
Experiment 1			
NT (n=7)	233.60 $\pm$ 3.89	249.0 $\pm$ 5.36	P<0.05
LET (n=7)	227.1 $\pm$ 6.53	241.0 $\pm$ 6.49 [§]	P<0.001
GSE (n=7)	227.9 $\pm$ 4.06	213.3 $\pm$ 5.58* [§]	P>0.05
MTX/LET (n=7)	229.3 $\pm$ 2.54	204.0 $\pm$ 9.83* [§]	P<0.05
MTX/GSE (n=6)	231.0 $\pm$ 3.47	186.7 $\pm$ 4.43*	P<0.001
MTX (n=6)	234.2 $\pm$ 4.55	164.8 $\pm$ 5.59*	P<0.001
Experiment 2			
NT (n=7)	228.6 $\pm$ 4.46	242.1 $\pm$ 4.06	P< 0.01
Tp (n=6)	230.8 $\pm$ 2.51	250.8 $\pm$ 3.96 [#]	P< 0.01
MTX/Tp (n=6)	235.8 $\pm$ 4.32	200.0 $\pm$ 4.08 ^{§#}	P< 0.01
MTX (n=6)	233.2 $\pm$ 3.66	165.3 $\pm$ 6.68 [§]	P< 0.001

Paired t-test between weight at the start and scarification on day 35. One way ANOVA were done between groups at the same category. *P* value < 0.05 is considered significant. n, number of rats per group. Experiment 1, * and [§] *P* < 0.05 compared to NT and MTX, respectively. Experiment 2, [§] and [#] *P* < 0.05 compared to NT and MTX, respectively.