

ORIGINAL ARTICLE

Ameliorating effect of kisspeptin-10 on methotrexate-induced sperm damages and testicular oxidative stress in rats

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Abstract

The purpose of this study was to determine the kisspeptin-10 (Kiss) administration on the damages in testicular oxidant-antioxidant system, reproductive organ weights and some spermatological characteristics resulted from methotrexate (MTX) exposure. Group 1 (n:6) received saline only; group 2 (n:6) received 50 nmol/kg kisspeptin-10 for 10 days; group 3 (n:10) received single-dose methotrexate 20 mg/kg; and group 4 (n:10) received MTX 20 mg/kg single dose and, after 3 days, received kisspeptin-10, 50 nmol/kg, lasted for 10 days by intraperitoneal injection. At the end of the study, malondialdehyde levels were found to have increased following the application of MTX while showing a significant reduction in group 4 with Kiss administration. With respect to the spermatological parameters, administering MTX decreased motility and increased the rates of abnormal spermatozoa in group 2, while improvements were observed in group 4 in the form of increased motility in the spermatozoa and fewer abnormal spermatozoa. In addition, Kiss treatment provided statistically significant increases in the absolute weight of the seminal vesicles and the relative weights of the right cauda epididymis and seminal vesicles resulting from MTX administration. MTX administration damaged some spermatological parameters and increased oxidative stress when compared to the control group. However, Kiss treatment was observed to mitigate these adverse effects as demonstrated by the improvements in coadministration of Kiss and MTX when compared to the MTX group. It is concluded that Kiss treatment may reduce MTX-induced reproductive toxicity as a potential antioxidant compound.

KEYWORDS

kisspeptin, methotrexate, oxidative stress, sperm quality

1 | INTRODUCTION

Many chemotherapy agents, radiotherapy applications and some surgical procedures cause impairments in the reproductive organs including hypogonadism as well as permanent infertility and menopause. Although it is stated that the age for cancer is 60 years or over, about 4% of all cancers occur between the ages of 15 and 40. As many of these patients do not already have

children, the unwanted effects of treatment agents on fertility can cause a variety of problems (Şen & Aydinler, 2011).

Methotrexate (MTX) is a folic acid (FA) antagonist with anti-inflammatory, antiproliferative, immunosuppressive and antipsychotic effects in addition to possessing antimetabolic features. It is used to treat many disorders and various types of cancer including but not limited to rheumatoid arthritis, lymphoma, leukaemia, osteosarcoma, tumours in the head and neck region, lung and breast

cancer (Feagan & Alfadhli, 2004). The cytotoxic effects of anticancer medicine including MTX are not selective for cancer cells. Therefore, MTX is highly toxic to healthy tissues with high cellular proliferation rates such as bone marrow, bowel mucosa and hair follicles. Several studies have shown that MTX impairs the antioxidant system rendering the cells susceptible to ROS (reactive oxygen species) and leading to injuries. As for its effects on the reproductive system, the oxidative stress damages the seminiferous tubules of the testes and causes a reduction in the germ cells (Babiak, Campello, Carnieri, & Oliveira, 1998; Gao, Tomitori, Igarashi, & Horie, 2002). Antioxidants are thought help protect testicular tissue against oxidative stress (Vernet, Aitken, & Drevet, 2004).

Kisspeptins are neuropeptides transcribed by the *KISS1* gene (1q32). The Kiss-1 protein consists of 145 amino acids while the "ss" tag in Kiss refers to the suppressor sequence (Lee & Welch, 1997; Lee et al., 1996, 1999). Kisspeptin is synthesised as a result of gonadotrophs secreted from neurons in the hypothalamus and the anteroventral periventricular region. Kisspeptin has many roles in the regulation of homeostatic and neuroendocrine functions, but these roles have not been precisely defined (Mikkelsen, Bentsen, Ansel, Simonneaux, & Juul, 2009).

Kisspeptin's known derivatives are kisspeptin-10, 13, 14, 52 and 54 (Thompson et al., 2004). One of these, kisspeptin-10, is released from cultured human trophoblast cells and includes C-terminal decapeptide-10 (Bilban et al., 2004). The receptor is GPR54, which defines a G protein-linked Kiss-1/GPR54 system (Shahed & Young, 2009).

Kisspeptin is an effective peptide that stimulates the secretion of gonadotropin releasing hormone (GnRH) and the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) (Mikkelsen et al., 2009). The application of kisspeptin using different methods was seen to stimulate the HPG axis in various experimental models (Dhillon et al., 2005). In addition, peripheral kisspeptin-10 administration caused LH release in male monkeys and increased LH and FSH release in adult rats (Matsui, Takatsu, Kumano, Matsumoto, & Ohtaki, 2004; Navarro et al., 2005; Shahab et al., 2005; Thompson et al., 2004).

The relationship between oxidative stress and gonadotropins has been demonstrated in various studies. The decreased release of LH and FSH in hypothalamic dysfunction results in oxidative stress (Hurtado de Catalfo, Ranieri-Casilla, Marra, De Alaniz, & Marra, 2007; Muthuvel et al., 2006). Recent studies have shown that kisspeptin protects against oxidative damage (Akkaya, Kilic, Dinc, & Yilmaz, 2014; Aslan, Erkanli Senturk, Akkaya, Sahin, & Yilmaz, 2017; Aydin, Oktar, Yonden, Ozturk, & Yilmaz, 2010; Milton, Chilumuri, Rocha-Ferreira, Nercessian, & Ashioti, 2012).

Defects in spermatogenesis and imperfections in sperm quality, disorders in the hypogonadal (HPG) system and sexual dysfunction are the undesirable effects of chemotherapeutic agents on the reproductive system (Demirci, Benekli, Buyukberber, & Coskun, 2010; Ragheb & Sabanegh, 2010; Schrader, Müller, Straub, & Miller, 2001). Even though anticancer medicine causes toxication in tissues with high proliferation rates such as gonadal tissues with gonadal

damage being common in these patients, this side effect has been studied less than others (Kim, Kim, & Chung, 1999; Saxena, Dhungel, Bhattacharya, Jha, & Srivastava, 2004). Furthermore, there are no studies investigating the effects of kisspeptin on MTX-induced oxidative stress and reproductive dysfunction.

Therefore, this study was conducted to investigate whether Kiss has any preventive effect on the adverse MTX-induced changes in sperm quality and the testicular lipid peroxidation associated with oxidative stress.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental protocol

This study was conducted with the permission of the Local Ethics Committee of Experimental Investigations of Firat University (Elazig, Turkey). The study protocol and the features of animal care complied with the NIH Guide for the Care and Use of Laboratory Animals. Thirty-two male Wistar-Albino rats, of 4–5 months old, were obtained from Firat University Experimental Animal Research Centre to be used in the study. The experimental part of the study was conducted at Firat University Animal Application and Research Center (Elazig, Turkey). Rats were maintained at $25 \pm 2^\circ\text{C}$ constant temperature in 60%–65% humidity and in ventilated rooms under standard conditions. They were exposed to 12 hr of daylight and 12 hr of darkness, and they were fed ad libitum with standard rat diet. Their cages were cleaned daily.

The rats were randomly divided into four groups. The rats in group 1 ($n:6$) were administered saline only (control group); the rats in group 2 ($n:6$) were injected kisspeptin 50 nmol/kg for 10 days (Kiss group); the rats in group 3 ($n:10$) were administered MTX injected as a single dose of 20 mg/kg (MTX group); and finally, the rats in group 4 ($n:10$) were injected a single dose of MTX followed by the administration of 50 nmol/kg kisspeptin for 10 days (Kiss + MTX group). All administrations were performed by intraperitoneal injection. 50 nmol/kg of kisspeptin-10 administered daily to the rats that were chosen to be studied as it contains the shortest molecular structure for receptor binding and activation (Kotani et al., 2001; Ohtaki et al., 2001).

2.2 | Sample collection and homogenate preparation

At the end of the treatment period, the rats were sacrificed under xylazine/ketamine anaesthesia. Testes, epididymis, seminal vesicles and ventral prostate were removed from the body and weighed. The ventral prostate was weighed being separated from the accessory glands and semilunar vesicles. Absolute and relative weights of the reproductive organs were recorded. The relative organ weight was calculated using the following formula: relative organ weight = absolute organ weight (g)/body weight (g) $\times$ 100. Testis samples were stored at -20°C for biochemical analyses. At the time of analyses, they were taken from the -20°C freezer and

immediately transferred to the cold glass tubes. Then, the testis tissues were diluted with a ninefold volume of PBS (pH 7.4). For the enzymatic analyses, testes tissues were minced in a glass and homogenised by a tissue homogenisator for 5 min in cold physiological saline on ice.

2.3 | Determination of testicular oxidative stress, antioxidant enzyme activities

All analyses were performed with the aid of a spectrophotometer (Shimadzu 2R/UV-visible, Tokyo, Japan). Oxidative stress and lipid peroxidation (LPO) levels were measured referencing the concentration of thiobarbituric acid reactive substances. The amount of malondialdehyde (MDA) produced was used as the indicator of LPO levels. MDA level at 532 nm was expressed as nmol/g protein (Placer, Cushman, & Johnson, 1966). Reduced glutathione (GSH) levels were measured using the method described by Sedlak and Lindsay (1968). The level of GSH at 412 nm was expressed as nmol/g protein. Glutathione peroxidase (GSH.Px, EC 1.11.1.9) activity was determined according to the method described by Lawrence and Burk (1976). The GSH.Px activity at 340 nm was expressed as IU/g protein. Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm and was expressed as k/g protein, where k is the first-order rate constant (Aebi, 1984). Protein concentration was determined using the method of Lowry, Rosebrough, Farr, and Randall (1951).

2.4 | Analyses of spermatozoa

All spermatozoal analyses were made using the methods reported in the study of Türk et al. (2008). The spermatozoa concentration in the right caudal epididymal tissue was determined with a haemocytometer. Freshly isolated left caudal epididymal tissue was used for the analysis of spermatozoal motility. The percentage of spermatozoal motility was evaluated using a light microscope with a heated stage. To determine the percentage of morphologically abnormal spermatozoa, the slides stained with eosin-nigrosin (1.67% eosin, 10% nigrosin and 0.1 M of sodium citrate) were prepared. The slides were then viewed under a light microscope at 400× magnification. A

total of 300 spermatozoa were examined on each slide (2,400 cells in each group) with their heads and tails, and the total abnormality rates of spermatozoa were expressed as percentages.

2.5 | Statistical analysis

The descriptive data of our study were presented as the means with standard error ($X \pm Sx$) values. A value of $p < 0.05$ was considered significant. The Shapiro–Wilk normality test was applied to determine whether the raw values of each group were normally distributed in all measured parameters.

One-way analysis of variance (ANOVA) was used to compare group averages, and Duncan test was used to determine differences between groups.

All analyses were carried out using the SPSS/PC software programme (version 22.0; SPSS, Chicago, IL, USA).

3 | RESULTS

3.1 | Changes in spermatological parameters

When the data on spermatological parameters are examined in Table 1, the results showed that MTX caused a decrease in sperm motility ($p < 0.01$). Numerical increases were observed with Kiss treatment, but the differences were not significant compared to the control group. MTX caused significant decreases in sperm motility, increases in abnormalities in the sperm tails and, finally, total sperm abnormalities when compared to control group. On the other hand, the Kiss treatment had ameliorative effects on the sperm motility and spermatozoon head/tail abnormality to the MTX only group. Regarding the sperm concentrations, there are numerical decreases in MTX but increases in Kiss groups. However, no statistical difference was found.

3.2 | Changes in reproductive organ weights

When the data on the reproductive organ weights among the groups in Tables 2 and 3 are examined, a statistically significant increase was observed in absolute and relative weights of seminal vesicles of only

TABLE 1 Mean $\pm$ SEM values of sperm parameters

Groups	Sperm parameters				
	Motility (%)	Concentration (million/right cauda epididymis)	Abnormal sperm rate (%)		
			Head	Tail	Total
Control	82.00 $\pm$ 2.00 ^{ab}	140.67 $\pm$ 2.91	3.40 $\pm$ 0.93	4.40 $\pm$ 1.17 ^a	7.80 $\pm$ 2.08 ^a
Kiss-10	88.66 $\pm$ 2.26 ^b	150.67 $\pm$ 14.14	5.33 $\pm$ 1.99	3.67 $\pm$ 0.67 ^a	9.00 $\pm$ 1.69 ^a
MTX	72.22 $\pm$ 2.78 ^c	108.33 $\pm$ 10.85	5.83 $\pm$ 0.79	8.83 $\pm$ 1.40 ^b	14.66 $\pm$ 1.54 ^b
Kiss-10 + MTX	82.00 $\pm$ 3.74 ^{ab}	149.75 $\pm$ 18.12	4.75 $\pm$ 1.11	4.38 $\pm$ 0.80 ^a	9.13 $\pm$ 1.11 ^a
Significance	$p < 0.01$	$p > 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.05$

Note. The mean values having different superscripts within the same column significantly differ from each other.

TABLE 2 Mean $\pm$ SEM values of body and absolute reproductive organ weights of rats

Groups	Absolute organ weights (g)					
	Body weight(g)	Testis (right + left/2)	Whole epididymis (right + left)/2	Right cauda epididymis	Seminal vesicles	Ventral prostate
Control	448.21 ± 5.053	1.775 ± 0.086	0.673 ± 0.026	0.254 ± 0.017	1.426 ± 0.127 ^a	0.683 ± 0.088 ^{ab}
Kiss-10	439.75 ± 10.783	1.750 ± 0.061	0.688 ± 0.016	0.268 ± 0.007	1.878 ± 0.104 ^b	0.840 ± 0.069 ^{bc}
MTX	404.40 ± 9.636	1.618 ± 0.042	0.624 ± 0.009	0.223 ± 0.012	1.030 ± 0.081 ^c	0.623 ± 0.047 ^a
Kiss-10 + MTX	406.33 ± 9.759	1.794 ± 0.072	0.679 ± 0.027	0.279 ± 0.015	1.589 ± 0.077 ^a	0.756 ± 0.039 ^{abc}
Significance	<i>p</i> > 0.05	<i>p</i> > 0.05	<i>p</i> > 0.05	<i>p</i> > 0.05	<i>p</i> < 0.001	<i>p</i> < 0.05

Note. The mean values having different superscripts within the same column significantly differ from each other.

TABLE 3 Mean $\pm$ SEM values of relative reproductive organ weights of rats

Groups	Relative organ weights [absolute organ weight (g)/final body weight (g) $\times$ 100]				
	Testis (right + left)/2	Whole epididymis (right + left)/2	Right cauda epididymis	Seminal vesicles	Ventral prostate
Control	0.385 $\pm$ 0.019	0.149 $\pm$ 0.005 ^{ab}	0.058 $\pm$ 0.002 ^a	0.331 $\pm$ 0.027 ^a	0.163 $\pm$ 0.023 ^{ab}
Kiss-10	0.413 $\pm$ 0.022	0.162 $\pm$ 0.008 ^a	0.063 $\pm$ 0.004 ^a	0.441 $\pm$ 0.025 ^c	0.197 $\pm$ 0.016 ^b
MTX	0.364 $\pm$ 0.008	0.136 $\pm$ 0.005 ^b	0.049 $\pm$ 0.002 ^b	0.230 $\pm$ 0.012 ^b	0.139 $\pm$ 0.009 ^a
Kiss-10 + MTX	0.424 $\pm$ 0.014	0.160 $\pm$ 0.004 ^a	0.065 $\pm$ 0.002 ^a	0.376 $\pm$ 0.019 ^a	0.178 $\pm$ 0.008 ^{ab}
Significance	$p > 0.05$	$p < 0.05$	$p < 0.01$	$p < 0.001$	$p < 0.01$

Note. The mean values having different superscripts within the same column significantly differ from each other.

Groups	Biochemical parameters			
	MDA (nmol/g protein)	rGSH (nmol/g protein)	GSH.Px (IU/g protein)	CAT (k/g protein)
Control	14.27 $\pm$ 0.45 ^a	2.28 $\pm$ 0.12 ^a	13.85 $\pm$ 0.99 ^a	3.12 $\pm$ 0.06 ^a
Kiss-10	15.19 $\pm$ 0.31 ^a	2.56 $\pm$ 0.17 ^a	14.88 $\pm$ 2.69 ^a	6.77 $\pm$ 0.41 ^b
MTX	16.93 $\pm$ 0.43 ^b	2.19 $\pm$ 0.09 ^a	8.08 $\pm$ 0.71 ^c	5.57 $\pm$ 0.71 ^b
Kiss-10 + MTX	15.08 $\pm$ 0.62 ^c	2.64 $\pm$ 0.05 ^b	11.24 $\pm$ 0.38 ^{ab}	4.08 $\pm$ 0.38 ^b
Significance	$p < 0.05$	$p < 0.05$	$p < 0.01$	$p < 0.001$

Note. The mean values having different superscripts within the same column significantly differ from each other.

TABLE 4 Mean $\pm$ SEM values of malondialdehyde, reduced glutathione levels and glutathione peroxidase and catalase activities

Kiss-treated group when compared with the control group. However, MTX treatment caused statistically significant diminutions in the weight of absolute seminal vesicles ($p < 0.01$) as well as relative weights of right cauda epididymis ($p < 0.01$) and seminal vesicles ($p < 0.001$) in comparison with the control group. Cotreatment with Kiss and MTX provided statistically significant increases in decreased values of absolute weight of seminal vesicles and relative weights of right cauda epididymis and seminal vesicles caused by MTX administration ($p < 0.05$).

3.3 | LPO levels and antioxidant activities in the testicular tissue

The levels of MDA, GSH and GSH.Px, CAT activities are given in Table 4. Kiss administration caused significant increases in the

activity levels of CAT ($p < 0.05$) compared to the control group. MTX administration caused significant increases in MDA ($p < 0.05$) levels and significant decreases in the activity levels of GSH.Px compared to the control group. Beside this, in the MTX + Kiss treatment group, MDA levels decreased significantly in the kisspeptin group ($p < 0.05$) and GSH.Px levels increased significantly in the MTX group ($p < 0.05$).

4 | DISCUSSION

In this study, we examined in detail the possible adverse effects of MTX on the organs and cells of the male reproductive system and the curative effect of the protective agent kisspeptin. To our

knowledge, this is the first study to show that kisspeptin therapy alleviates testicular lipid peroxidation, demonstrating that it inhibits the negative effects of MTX-induced oxidative stress and improves sperm parameters.

The detrimental effects of MTX, an anticarcinogenic drug, can lead to a large number of undesirable consequences in patients given treatment regimens containing this molecule. It is known that chemotherapeutic agents also affect healthy tissues and cells resulting in undesirable consequences, and their effects on the male reproductive system can manifest in the form of infertility and sterility (Feagan & Alfadhli, 2004; Gao et al., 2002; Sabanegh & Ragheb, 2009).

Lipids are an important component of cell structure. Increase in the production of free radicals leads to lipid peroxidation. MDA is the best indicator of oxidative stress as it is the end product of multi-chain unsaturated fatty acids. Several studies have shown that MTX increases the levels of MDA in the testis, liver and kidneys at doses of 20 mg/kg and higher (Armagan et al., 2008; Jahovic et al., 2004). Our study also showed an increase in the MDA levels in the testes following MTX application.

Methotrexate administration resulted in a significant decrease in the activity levels of GSH.Px and CAT compared to the control group ($p < 0.05$). The increase in lipid peroxidation-induced metabolites may lead to an increase in free radicals, which in turn can lead to significant problems in the in vitro protective mechanisms against oxidative stress, including reduced intracellular antioxidant enzymes and GSH levels. These protective mechanisms are therefore of great importance for gonads with the enzymes involved including SOD, GSH.Px, glutathione reductase and CAT, all of which render the products of the oxidative stress harmless. SOD and GSH.Px are considered to be the most important enzymes involved in clearing ROS and free radicals in the male reproductive system. (Kaneko et al., 2002). On the other hand, MTX reduces the effectiveness of the antioxidant enzyme system causing the cells to become susceptible to ROS and eventual cellular injuries.

Studies have been conducted on the effects of kisspeptin on the HPG axis and reproductive system in recent years (Dhillon et al., 2007; George et al., 2011; Kuohung & Kaiser, 2006). In recent years, studies have been carried out to determine whether this peptide has an antioxidant effect (Akkaya et al., 2014; Aslan et al., 2017; Aydin et al., 2010; Milton et al., 2012). A study by Akkaya, Eyuboglu, Erkanlı Senturk, and Yilmaz (2017) on the testicular tissue has suggested that kisspeptin may reduce lipid peroxidation and may have antioxidant effects. Our study results demonstrated that Kiss has antioxidative effect by reducing MTX-induced increment in MDA level and increasing MTX-induced diminution in GSH and GSH.Px activities in testis tissue. Our data showed that the levels of GSH and activity of GSH.Px in the testis were significantly increased, indicating the protective effects of kisspeptin on testicular damage.

The study results demonstrated that MTX administration led to significant decreases in the number of seminal vesicles ($p < 0.001$), in prostate weight ($p < 0.05$) and in sperm motility ($p < 0.01$). It also led to abnormalities in the sperm tails ($p < 0.05$)

and total abnormalities in the sperms ($p < 0.05$). However, while a numerical reduction was observed, there was no statistical decrease in sperm concentration compared to the control group. The results of our study are similar to those reported by previous studies (Choudhury, Ghosh, & Palo, 2001; Padmanabhan, Tripathi, Vikram, Ramarao, & Jena, 2009).

A study by Ramzan and Qureshi (2011) reported that the application of kisspeptin-10 did not lead to differences in testicular weights. Administering Kiss administration did not lead to significant differences in the testicular weights in this study, either. However, the MTX + Kiss-10 group did show significant increases in the absolute and relative weights of the vesicula seminalis compared to the MTX group. These increases in the absolute and relative weights of the seminal vesicles were also observed in the Kiss-10 group compared to the control group ($p < 0.001$).

Oxidative stress leads to damage in the seminiferous tubules and a reduction in the number of germ cells (Wathes, Abayasekara, & Aitken, 2007). The consequences of oxidative stress include impairments in the sperm cell membrane, death of spermatozoa, abnormalities in the spermatozoa and loss of motility. These parameters, such as spermatozoal morphology, sperm motility and sperm concentration, are among the spermatological quality criteria, and they are closely related to each other. Central or peripheral administration of kisspeptin-10 or kisspeptin-54 stimulates HPG axillary (Dhillon et al., 2005; George et al., 2011). However, studies investigating the effects of Kiss-1 and GPR54 on sperms and spermatogenesis are limited. Our study results demonstrate that sperm motility in the Kiss-10 group was significantly higher than the control group ($p < 0.01$), whereas there were no differences in the sperm density and abnormal spermatozoa counts. Kiss treatment had a healing effect on some of the specified spermatological parameters such as total sperm abnormalities, sperm tail abnormalities, sperm motility and dead sperm rate. In addition, the sperm tail abnormality in the MTX + Kiss group was statistically lower than that detected in the MTX only group. These results demonstrate that Kiss has healing effects on sperm damage.

As a result, our findings show that kisspeptin-10 as an endocrine hormone can have antioxidant effects when administered exogenously. In our study, the administering of Kiss resulted in a decrease in MDA levels and an increase in GSH levels and in GSH.Px activities, which are reduced by MTX, and also demonstrated healing effects on sperm parameters. These results suggest that this peptide may help improve antioxidant activity and male infertility. However, further elaboration of the antioxidant activity of the different forms and analogs of kisspeptin will provide more specific data on the spermatogenesis of these peptides, thereby allowing for more data to be obtained.

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