

Ameliorating Effect of *Ziziphora tenuior* Hydro-Alcoholic Extract against Reprotoxic Effects of Formaldehyde in Male Mice

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ABSTRACT

Background: *Ziziphora tenuior* extract has anti-oxidant, anti-inflammatory and anti-tumorigenic properties. In the present study the protective effect of *Ziziphora tenuior* extract (ZTE) against formaldehyde (FA) sperm toxicity was evaluated.

Methods: Thirty male mice (6-8 weeks old) were allotted to five random groups (n=6) including vehicle (distilled water) and control which received intraperitoneal (IP) injection of FA (10 mg/kg). The other experimental groups were received IP injection of FA plus 50, 100 and 150 mg/kg of ZTE, respectively. This trial was continued for 35 days. Then sperm evaluation parameters (count, viability and motility), leydig cell number and serum testosterone level were assessed. The statistical analyses were done using SPSS, version 17, and the data are presented as means \pm SEMs. Statistical difference was evaluated using the one-way analysis of variance (ANOVA), followed by the post hoc LSD test.

Results: ZTE with dose of 50 and 100 mg/kg increased leydig cell number and testosterone level in comparison with control group which had received FA ($P < 0.001$). Also, sperm count, motility, viability and histological structure were improved.

Conclusion: ZTE may provide protective effects against detrimental effects of FA in male mice.

Key words: *Ziziphora*, Formaldehyde, Spermatozoa, Mice

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ATP: Adenosine Triphosphate; ROS: Reactive Oxygen Species

ABBREVIATIONS

ZTE: *Ziziphora tenuior* Extract; FA: Formaldehyde; IP: Intraperitoneal; NMRI: Naval Medical Research Institute; HTF: Human Tubal Fluid; KMU: Kerman University of Medical Sciences; ANOVA: One-way Analysis of Variance;

INTRODUCTION

The human spermatozoa are highly vulnerable to oxidative stress. This process induces oxidative injury in the sperm plasmalemma and DNA fragmentation in its genome [1]. Herbal medicine is as a valuable source of remedies and numerous plants have been

suggested for the treatment of diseases that affect the reproductive system [2]. The *Ziziphora tenuior* L. (ZT) is an herbaceous and annual plant with a height of 5-15 cm. It is named as Kakoti in Persian [3]. ZT is known as a medicinal herb, because its extract has antibacterial and antifungal properties. It has potent disinfection effects and its vapor is used to treat upper respiratory tract infection. Besides, it has a traditional medicinal role in gastrointestinal disorders. ZT is carminative and is a tonic for the stomach. Talebi claim to have found aphrodisiac properties in ZT [4]. Some of well-known antioxidants of ZT are pulegone, limonene and cineol. Some types of pollutants such as formaldehyde (FA), xylene, and toluene have harmful effects on human health [5].

FA (CH₂O) is a cheap, volatile organic compound widely used in laboratories (histology and pathology), hospitals, and chemical industry [6]. Increasing evidence has demonstrated that the adverse effects of FA on human health in general [7,8]. In addition, there is growing number of evidences for the adverse effects of FA on male reproduction system [9,10]. Some studies revealed that FA exposure can inhibit spermatogenesis and induced cell death of spermatogenic cells in rodents [11,12]. DNA mutation can be induced by FA in the paternal germ line in mice [9]. Oxidative stress is one of key mechanisms of reproductive injuries of FA [13-15]. Since ZTE has potent antioxidant properties, the study was designed to investigate the ZTE protective effects against FA-induced reproductive toxicity in adult male mice.

MATERIALS AND METHOD

Extract preparation

The ZT was obtained from herbal market and authenticated by a botanist. A Voucher specimen was preserved at the herbarium of Pharmacy faculty of Kerman Medical University (KF1246). A portion of leaves dried plant material (100 g) was extracted by maceration with methanol for 48 h at 22°C. Then the extracted powder was dried, which is to note that it is made up of 62.4% concentrated liquid herb, after that, it is collected, and the resulting powder was stored at 5°C until use.

Experimental groups

The study was registered in Ethics Committee of Kerman University of Medical Sciences as KMUS. 2016. 95/415. Thirty the naval medical research institute (NMRI) male mice (6-8 weeks old) were allotted to five random groups (n=6) including vehicle (distilled water) and control which received IP injection of and FA (10 mg/kg), respectively [16]. The other experimental groups were received IP injection of FA plus simultaneously administration of 50, 100 and 150 mg/kg of ZTE (gavage), respectively. This trial was continued for 35 days. The animals were kept in a controlled environment

(22°C ± 1.0°C) and automatically day and night cycle (12 h) and free access to food and water.

Sperm parameters

The caudal part of the left epididymis was taken out and placed in 1 mL human tubal fluid (HTF) supplemented with 4 mg/mL BSA and cut into several fragments to allow the spermatozoa to come out from the reproductive ducts. Samples were incubated for 15 min at 37°C and the following parameters (e.g., sperm number, motility, viability and morphology) were studied in control and treated group of animals. Sperm number was assessed by an improved Neubauer hemocytometer technique (CNMEDIATECH, Jiangsu, China); sperm suspensions were diluted 1:20 in a diluting solution (50 g/L Na₂HCO₃ and 1% FA; Merck, Kenilworth, NJ) in distilled water. The diluted samples were put into the counting chamber and a number of sperms were counted under a light microscope (Nikon Ts100, Tokyo, Japan) and expressed as sperm number/mL. Sperm motility was analyzed by counting 200 motile (sperm with slight movement without forward progression were also considered motile) and immotile sperm in at least five microscopic fields (400X).

Sperm viability was determined by the eosin (EO) dye exclusion test. For the EO test, the sperm suspension was mixed thoroughly and 10 mL of the suspension was mixed with 10 mL of EO-nigrosin dye. A thin smear was prepared after 1 min and the number of viable sperms was determined out of 200 sperms in at least 10 microscopic fields (400X).

The live spermatozoa remained unstained and the dead ones were stained red. For sperm morphology assessment, a smear of sperm sample was fixed with formalin, dehydrated by graded alcohols in ascending order and stained with EO. After preparation, the slides were examined under a light microscope at 400X magnification. For each sample, 300 sperm cells were examined on each slide and the number of morphologically normal sperm was assessed. To assay sperm abnormality, smears were prepared from the sperm suspension (10 L) and stained with the Papanicolaou's method [16].

Histological study

The right-side testes after washing with saline were dissected, weighed and fixed in buffered formalin 10% solution for 48 h. Thereafter, specimen dehydrated and embedded in paraffin and 7-μ sections were prepared, stained with hematoxylin-eosin (H&E) method and examined under light microscope (Nikon i50) equipped by camera. In each testis, seminiferous tubules diameter and their epithelium thickness were assessed in 50 randomly selected tubules using software Image J.

Testosterone assay

Blood samples were collected from all animals in the

vehicle and treated groups, and the serum testosterone level was then determined by radioimmunoassay using Coat-A-Count total testosterone direct kit (Diagnostic Products Corporation, USA). The serum hormone concentrations were presented in ng/ml.

Statistical analysis

The obtained data were analyzed by one way analysis of variance (ANOVA) and Tukey tests using SPSS18 statistical software. p values less than 0.05 were considered significant. All results presented as mean ± standard error (SE).

RESULTS

Testis parameters

FA treatment markedly (p<0.001) decreased testes parameters including weight, length, volume and width, compared to vehicle group in male mice. While treatment of the mice with different doses of ZTE (50, 100 and 150 mg/kg) significantly ameliorated the adverse effects of FA (p<0.001) (Table 1).

Table 1: Testis indices in adult mice exposed to FA

Groups	Testis weight (mg)	Testis length (mm)	Testis width (mm)	Testis volume (mm ³)
Vehicle	111.40 ± 1.96	7.87 ± 0.30	5.57 ± 0.17	105 ± 2.91
FA	102.20 ± 2.22*	7.08 ± 0.20*	5.07 ± 0.15*	95.60 ± 1.50*
ZTE (50 mg/kg)	108.80 ± 3.05	7.69 ± 0.16#	5.48 ± 0.11#	103.4 ± 3.76#
ZTE (100 mg/kg)	107.80 ± 2.43	7.77 ± 0.13#	5.38 ± 0.15	104.60 ± 2.01#
ZTE (150 mg/kg)	106.60 ± 2.65	7.29 ± 0.11	5.20 ± 0.14	100 ± 1.70

Values are the mean ± SD
 *Significant difference versus vehicle group (P<0.05).
 #Significant difference versus FA group (P<0.05).
 FA: Formaldehyde; ZTE: *Ziziphora tenuior* extract.

Histological assessment

Histological assessments of the testes from vehicle treated mice showed normal architecture showing all germ cell types. However, FA caused destructive changes in the seminiferous tubules exhibiting the germ and Leydig cells degeneration, significantly diminish the diameter of seminiferous tubules, epithelial thickness and Leydig cell number as compared to vehicle group (p<0.001). ZTE treatment at the doses of 50, 100 and 150 mg/kg resulted in increasing the germ and Leydig cells number, seminiferous tubular diameter and thickness comparing to FA-treated animals (p<0.001) (Table 2) (Figure 1(A-E) and Figure 2).

Table 2: Testis structure and testosterone levels in adult mice exposed to FA

Groups	Seminiferous tubule diameter (µm)	Seminiferous epithelium thickness (µm)	Testosterone level (ng/ml)
Vehicle	131.36 ± 0.92	35.44 ± 0.31	8.98 ± 0.12
FA	111.81 ± 1.45*	30.74 ± 0.43*	8.17 ± 0.11*
ZTE (50 mg/kg)	128.35 ± 1.24#	31.6 ± 0.43#	8.51 ± 0.08#
ZTE (100 mg/kg)	129.41 ± 0.86#	32.61 ± 0.62#	8.59 ± 0.12#
ZTE (150 mg/kg)	124.41 ± 3.07#	32.04 ± 0.49#	8.21 ± 0.09#

Values are the mean ± SD.
 *Significant difference versus vehicle group (P<0.05).
 #Significant difference versus FA group (P<0.05).
 FA: Formaldehyde; ZTE: *Ziziphora tenuior* extract.

Testosterone concentration

The mean values of the testosterone levels differ significantly among different groups. The data revealed that FA treatment significantly decreased (p<0.001) testosterone level as compared to vehicle. ZTE treatment at the doses of 50, 100 and 150 mg/kg significantly

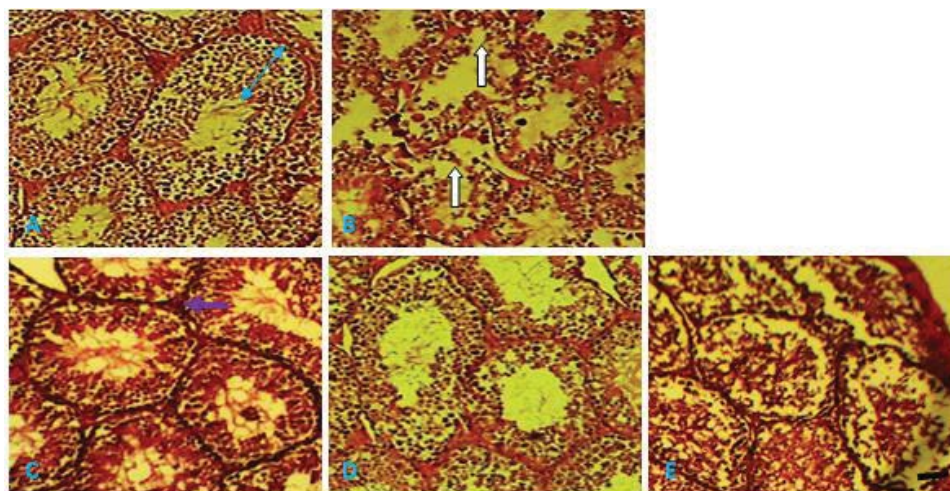


Figure 1: Morphology of tubules in the testis of (A) vehicle, (B) FA and (C-E) ZTE treated (50, 100 and 150 mg/kg respectively) groups were studied by H&E staining (Disorganized seminiferous tubules were observed in FA group (white arrow); ZTE administration (especially 50 mg/kg) resulted in reduction of adverse effects of FA (c); Violet arrow shows the leydig cell in the interstitial tissue of mouse testis and blue two headed arrow demonstrates the seminiferous epithelium thickness) (Staining: Hematoxylin and eosin (H&E). Scale bar: 100 µm)

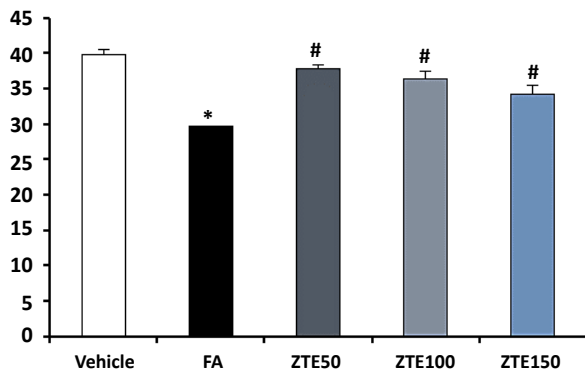


Figure 2: ZTE treatment resulted in increasing of leydig cell number in testis of mice (*Significant difference versus vehicle group ($P<0.001$); #Significant difference versus FA ($P<0.001$)).

increased the testosterone level as compared to FA group ($p<0.001$) (Table 2).

Sperm parameters

FA exposure significantly diminished the sperm parameters as compared to vehicle ($p<0.001$) in the treated animals and ZTE administration at 50, 100 and 150 mg/kg doses for 35 days significantly increased these sperm parameters ($p<0.001$) (Table 3). The sperms in the FA group demonstrated abnormality in the head, neck and tail parts as compared to vehicle group.

Table 3: Sperm profile and morphology in adult mice exposed to FA

Groups	Sperm viability (%)	Sperm motility (%)	Sperm count $\times 10^6$	Normal Sperm morphology (%)
Vehicle	79.80 \pm 1.59	82.40 \pm 1.07	241.19 \pm 22.39	79.20 \pm 1.28
FA	58.40 \pm 1.86*	47.60 \pm 1.66*	90.41 \pm 5.00*	63.20 \pm 1.59*
ZTE (50 mg/kg)	74.60 \pm 1.69#	79.00 \pm 1.22#	204.67 \pm 20.68#	76.00 \pm 1.78#
ZTE (100 mg/kg)	73.80 \pm 2.22#	79.86 \pm 1.24#	198.41 \pm 17.77#	74.80 \pm 2.31#
ZTE (150 mg/kg)	69.40 \pm 1.32#	75.60 \pm 1.63#	156.69 \pm 17.83#	70.60 \pm 1.43#

Values are the mean \pm SD.

*Significant difference versus vehicle group ($P<0.05$).

#Significant difference versus FA group ($P<0.05$).

FA: Formaldehyde; ZTE: *Ziziphora tenuior* extract.

DISCUSSION

The current research confirms that testosterone level, testis indices and sperm parameters were decreased in formalin-treated mice and administration of ZTE resulted in ameliorating in testosterone level, sperm parameters and testicular changes.

Previous *in vivo* and *in vitro* studies showed that administration of FA decreased the viability, motility and number of spermatozoid cells [17,18]. Diverse scientific works demonstrated that FA increases the production

of Reactive oxygen species (ROS) in different organs [12,19] such as testis [20]. Extreme free radicals in testis increase germ cell death and reduce the activity of sperms [21,22]. The mechanism of FA on sperm profile has been revealed clearly. Lipid peroxidation of the sperm outer membrane, that in turn leads to loss of motility [23], and enhanced chromatin injury [24]. Disruption in processes of membrane ion exchange and its enzymes diminish sperm motility [25,26]. ROS also hinders intracellular enzyme; consequently, adenosine triphosphate (ATP) cannot be available for sperm motility [27]. On the other hand, peroxidation of sperm membrane constituents result in reduced enzymatic activity of Na/K-ATPase and finally declined sperm motility [26].

In the present study, the sperm morphology assay confirmed that sperm abnormality in general increased in FA-treated mice and abnormal sperm rate diminished following the ZTE treatment. The sperm abnormality rate may show FA general toxicity on germ cells and their [18,19]. Tramer et al. demonstrated that ROS cause lipid peroxidation of sperm cell membranes that finally leads to infertility [28]. In this work, ZTE had more likely protective properties on sperm against ROS injury induced by FA. This finding is agreement with previous work [29]. It has also been confirmed that FA decreased diameter of seminiferous tubules and epithelium height in the testis of mouse and ZTE could prevent these testicular injuries.

According to Feldman, FA can inhibit nucleic acid and proteins synthesis [30]. In addition, Tang et al., showed that FA administration resulted in some anatomical disorders in the testes [18]. These abnormal changes consist of degeneration of sperms and Leydig cells as well as disorganization of the seminiferous tubules. These data are supported by our findings.

It is reported that FA directly deteriorates the testis antioxidant system [12], so we can conclude that ZTE treatment may scavenge ROS and protect the spermatogenic cells through its antioxidant activities. Moreover, FA administration significantly reduced testosterone level and leydig cell numbers in treated mice and improvement was found in ZTE treated groups. These effects of ZTE may be due to its flavonoids components because it has been shown that flavonoids of herbs have androgenic effects [31].

Phytochemical tests showed that main components of ZT are pulegone, isomenthone, thymol, and piperitone [32] which are suggested to be responsible for the medicinal properties. ZTE can inhibit inflammation by reducing myeloperoxidase activity and cellular lipid peroxidation [33]. In addition, the anti-inflammatory effects of pulegone, as the main active components of ZTE was confirmed [34], which may be partially responsible for the beneficial effects of the used extract.

CONCLUSION

According to this data, it may be concluded that ZTE administration ameliorates the adverse effects of FA-induced toxicity due to its antioxidant and anti-inflammatory effects in male mice. However, further studies are necessary to reveal the exact mechanisms of FA-induced reprotoxicity, and protective effects of ZTE.

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CONFLICT OF INTERESTS

Authors declare there is no conflict.

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