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Effect of etodolac hydrazone, a new compound synthesised from etodolac, on spermatozoon quality, testicular lipid peroxidation, apoptosis and spermatozoon DNA integrity

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Summary

The aim of this study was to investigate the effect of etodolac hydrazone (EH), a new compound synthesised from etodolac, on spermatozoon quality, testicular lipid peroxidation, apoptosis and spermatozoon DNA integrity in rats. Group 1 ($n = 8$) received 1 ml dimethyl sulfoxide (DMSO) daily (Control); group 2 ($n = 8$) was treated with 5 mg kg^{-1} day^{-1} EH, dissolved in 1 ml DMSO (EH-5); and group 3 ($n = 8$) was treated with 10 mg kg^{-1} day^{-1} EH, dissolved in 1 ml DMSO (EH-10). All administrations were performed by gavage and maintained for 8 weeks. Both doses of EH administration caused significant decreases in absolute and relative weights of testis, whole epididymis, right cauda epididymis, and spermatozoon motility, spermatozoon count in comparison with the control group. Only 10 mg kg^{-1} day^{-1} EH administration caused significant decreases in absolute and relative weights of seminal vesicles and serum testosterone level, and significant increases in testicular lipid peroxidation level, and numbers of TUNEL+ apoptotic germ cells and spermatozoa with damaged DNA along with some histopathological damages when compared to the control group. However, body and ventral prostate weight, and testicular antioxidant markers (glutathione, glutathione-peroxidase and catalase), were unaffected significantly by both doses of EH administration. In conclusion, two different doses of EH, in particular its high dose, damage to testicular spermatogenic cells and spermatozoon DNA and, it decreases spermatozoon motility, count and testosterone level in healthy rats.

Introduction

Etodolac *(R,S)* 2-[1,8-diethyl-1,3,4-tetrahydrapyrano(3,4-b)indole-1-yl]acetic acid is a nonsteroidal anti-inflammatory agent with analgesic and antipyretic properties. Etodolac and other nonsteroidal anti-inflammatory drugs (NSAIDs) have inhibitory effect on cyclooxygenase-2 (COX-2) activation. Its mechanism of action is inhibition of COX with reduction in the synthesis of prostaglandins from arachidonic acid, which is an unsaturated fatty acid liberated from phospholipids of cell membranes (Bennett & Tavares, 2001). Prostaglandin E and F series have been shown to exist endogenously in the male reproductive organs including testis, epididymis, vas deferens, accessory glands as well as seminal fluid, with prostaglandin E predominance (Cosentino *et al.*, 1984). Prostaglandins are important regulators of epididymis contractions (Cosentino *et al.*, 1984) and stimulate spermatozoon motility (Gottlieb *et al.*, 1988).

In contrast to therapeutic effects of NSAIDs, they have also detrimental effects on the body, in particular gastric mucosa, by increasing the levels of reactive oxygen species (ROS) and causing lipid peroxidation (Yoshikawa *et al.*, 1993; Maity *et al.*, 2009). Lipid peroxidation mediated by

oxygen free radicals is an important cause of destruction and damage to cell membranes, because polyunsaturated fatty acids (PUFAs) of the cellular membranes are degraded by the lipid peroxidation with consequent disruption of membrane integrity. Spermatozoa require a high PUFA content to provide the plasma membrane with the fluidity essential at fertilisation. However, this makes spermatozoa particularly vulnerable to attack by ROS (Wathes *et al.*, 2007). However, it has been reported that NSAIDs including etodolac have free radical scavenging activity (Fernandes *et al.*, 2004; Costa *et al.*, 2005).

Hydrazones containing an azometine -NHN = CH- proton are synthesised by heating the appropriate substituted hydrazines/hydrazides with aldehydes and ketones in solvents such as ethanol, methanol, tetrahydrofuran, butanol, glacial acetic acid and ethanol-glacial acetic acid. Hydrazide–hydrazones are very effective organic derivatives and an important class of compounds for new drug development. Therefore, many researchers have synthesised these compounds as target structures and evaluated their biological activities (Rollas & Küçükgüzel, 2007). In addition, the formation of hydrazone is one of the useful methods for pro-drug synthesis due to conversion of active drug by hydrolysis. Based on these observations, a new compound etodolac hydrazone (EH, 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(4-chlorophenyl)methylene] hydrazide) was synthesised from etodolac to determine its anticancer activity in cancer line (Çıkla *et al.*, 2013) because NSAIDs have also anticancer activity (Shigemura *et al.*, 2005). The researchers (Çıkla *et al.*, 2013), who developed EH, have reported that EH exhibited anticancer activity against prostate cancer cell line (PC-3) and did not display cytotoxicity towards L-929 rat prostatic fibroblast cell compared to etodolac. However, the *in vivo* effect of EH has not been studied so far. Therefore, this study was conducted to investigate whether EH, a new compound synthesised from etodolac, has beneficial or adverse effects on spermatozoon quality, testicular lipid peroxidation, apoptosis and spermatozoon DNA integrity in rats.

Materials and methods

All chemicals were purchased from Merck, Sigma-Aldrich or Fluka (Turkey distributors, İstanbul, Turkey). Etodolac was supplied by Bilim Pharmaceutical Industry Inc. Methyl (1,8-diethyl-1,3,4,9-tetrahydropyrano [3,4-*b*] indole-1-yl) acetate (Compound 1), 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano [3,4-*b*]indole-1-yl) acetohydrazide (Compound 2) and EH (2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetic acid[(4–chlorophenyl) methylene]hydrazide) synthesised according literature method (Çıkla *et al.*, 2013). Chemical route for synthesis of compounds 1, 2 and EH is shown in Fig. 1.

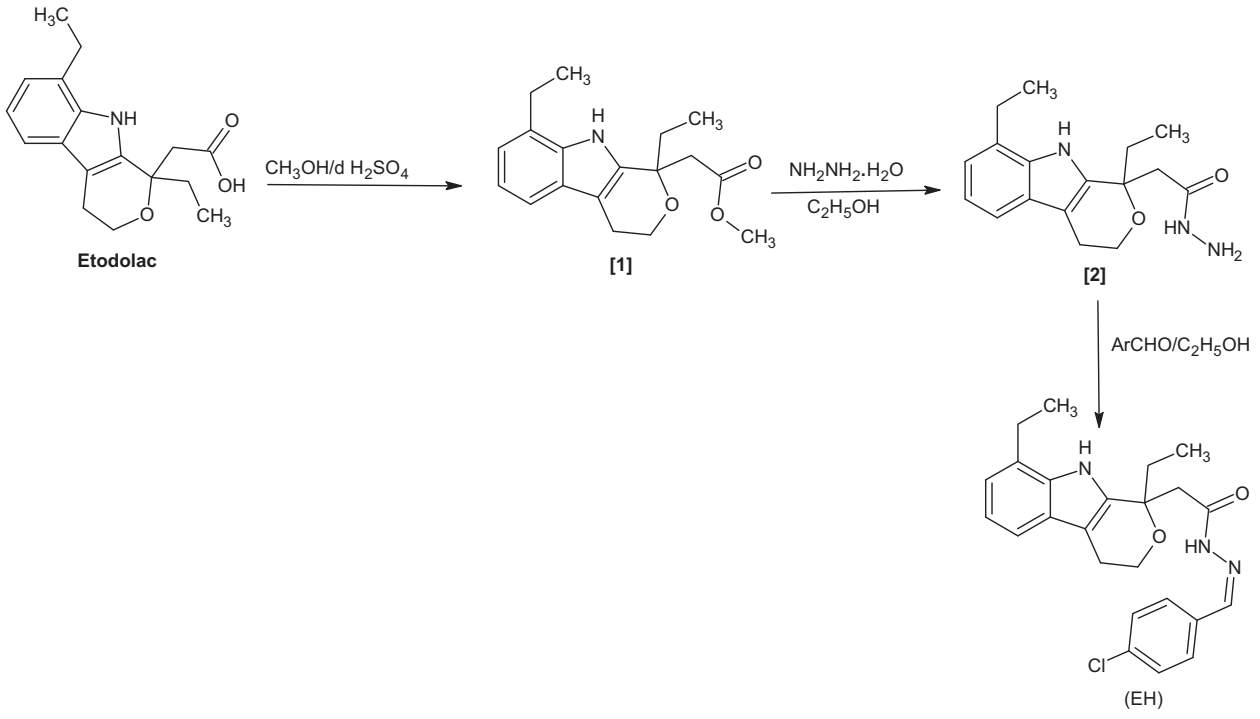

Fig. 1 Synthesis of etodolac hydrazone (EH, 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(4-chlorophenyl)methylene] hydrazide) from etodolac.

Animals and experimental protocol

The experimental protocol was approved by the Fırat University Animal Experimentations Local Ethics Committee (Elazığ, Turkey). Animal care and experimental protocol complied with the NIH Guide for the Care and Use of Laboratory Animals. Twenty-four healthy adult male Wistar albino rats, aged 3 months, were obtained from Fırat University Experimental Research Centre (Elazig, Turkey) and maintained therein during the study. The animals were housed in polycarbonate cages in a room with a 12-h day–night cycle, at a temperature of 24 ± 3 °C and humidity of 45% to 65%. During the whole experimental period, animals were fed with a balanced commercial diet (Elazığ Food Company, Elazığ, Turkey) and fresh drinking water was given *ad libitum*.

Dimethyl sulfoxide (DMSO) was used as vehicle because EH is hardly dissolved in natural conditions. The rats were randomly divided into three groups; each containing eight rats. Group 1 received 1 ml DMSO daily (Control group); group 2 was treated with 5 mg kg^{-1} day^{-1} EH, dissolved in 1 ml DMSO (EH-5 group); group 3 was treated with 10 mg kg^{-1} day^{-1} EH, dissolved in 1 ml DMSO (EH-10 group). All administrations were performed by gavage and maintained for 8 weeks. The doses of EH given to rats in this study were selected based on the doses used in previous studies for etodolac (Kishimoto *et al.*, 2000; Okamoto *et al.*, 2008). Since the spermatogenic cycle, including spermatocytogenesis, meiosis and spermiogenesis is 48–52 days (Bennett & Vickery, 1970), and epididymal transit of spermatozoon is approximately 1 week (Kempinas *et al.*, 1998) in rats, the treatment period used herein was set at 8 weeks to achieve a maximum effect. Each rat was weighed weekly and the dose level of EH within the DMSO solution was adjusted for the changes in body weights during the experimental period.

Sample collection and homogenate preparation

The rats were sacrificed using xylazine/ketamine anaesthesia at the end of 8th week. The blood samples were taken by sterile injector from heart. Testes, epididymides, seminal vesicles and ventral prostate were removed, cleared from adhering connective tissue and weighed. Absolute and relative [organ weight (g)/final body weight (g) × 100] reproductive organ weights were recorded. Collected blood samples were centrifuged at 3000 ***g*** for 10 min to obtain serum. One of the testis samples was fixed in Bouin's solution for histopathological examination. The other testis samples and blood sera were stored at −20 °C for biochemical analyses. Testes were taken from a −20 °C freezer and immediately transferred to the cold glass tubes. Then, the testes were diluted with a 9-fold volume of PBS (pH 7.4). For the enzymatic analyses, testes were minced in a glass and homogenised by a Teflon-glass homogenisator for 3 min in cold physiological saline on ice.

Serum testosterone assay

The serum testosterone level was measured using electrochemiluminescence immunoassay (ECLIA) method and commercial testosterone kit (Elecsys® Testosterone II, Roche Diagnostics Ltd, Rotkreuz, Switzerland) in the device of Cobas e 602 module. The testosterone level was expressed as ng dl^{-1}.

Determination of testicular tissue lipid peroxidation level and antioxidant enzyme activities

All analyses were performed with the aid of a spectrophotometer (Shimadzu 2R/UV-visible, Tokyo, Japan). Lipid peroxidation level was measured according to the concentration of thiobarbituric acid reactive substances, and the amount of malondialdehyde (MDA) produced was used as an index of lipid peroxidation. The MDA level at 532 nm was expressed as nmol g^{-1} protein (Placer *et al.*, 1966).

Reduced glutathione (GSH) level was measured using the method described by Sedlak & Lindsay (1968). The level of GSH at 412 nm was expressed as nmol g^{-1} protein. Glutathione-peroxidase (GSH-Px, EC 1.11.1.9) activity was determined according to the method described by Lawrence & Burk (1976). The GSH-Px activity at 340 nm was expressed as IU g^{-1} 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^{-1} protein, where k is the first-order rate constant (Aebi, 1983). Protein concentration was determined using the method of Lowry *et al.* (1951).

Spermatozoon analyses

All spermatozoon analyses were made using the methods reported in the study of Türk *et al.* (2008). The spermatozoon count in the right cauda epididymal tissue was determined with a haemocytometer. Freshly isolated left cauda epididymal tissue was used for the analysis of spermatozoon motility. The percentage of spermatozoon 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 (2400 cells in each group), and the head, tail and total abnormality rates of spermatozoa were expressed as percentage.

Histopathological examination

Testis tissues were fixed in Bouin's solution for 48 h, they were dehydrated through graded concentrations of ethanol, embedded in paraffin wax, sectioned at 5 μm thicknesses and stained with Mayer's haematoxylin & eosin. Twenty seminiferous tubules were randomly examined per section, and the lesions were photographed.

Determination of apoptotic germ cell number

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay with the ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Chemicon, Temecula, CA, USA) was used to detect apoptotic germ cell number according to the manufacturer's instructions. The fixed testicular tissues in Bouin's solution were embedded in paraffin and sectioned at 4 μm thickness. The paraffin sections were deparaffinised in xylene, dehydrated through graded alcohol and washed in PBS. The sections were treated with 20 mg ml^{-1} proteinase K for 5 min, followed by treatment with 3% H_2O_2 for 5 min to inhibit endogenous peroxidase. After re-washing with PBS, sections were then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) enzyme and digoxigenin-11-dUTP at 37 °C for 1 h in humidified chamber, and then, stop-wash buffer was applied for 30 min at 37 °C. Sections were visualised with 3-amino-9-ethylcarbazole (AEC) substrate. Negative controls were performed using distilled water in the place of the TdT enzyme. Finally, sections were counterstained with Mayer's haematoxylin, rinsed in tap water and mounted with glycerol. To detect TUNEL+ apoptotic germ cell number, 20 seminiferous tubules of each section were randomly selected and examined at original magnification ×200. TUNEL+ apoptotic germ cells were counted in the defined areas with the aid of Image J software programme for quantitative histomorphometric analysis and photographed.

Determination of spermatozoa with damaged DNA by comet assay

Diluted sperm samples extracted from epididymis were centrifuged at 300 ***g*** for 10 min at 4 °C. Supernatant was removed, and the remaining spermatozoa were washed with Ca^{2+} and Mg^{2+} free PBS (Arabi, 2005). Spermatozoa with damaged DNA were determined using the single-cell gel electrophoresis (comet) assay that was generally performed at high alkaline conditions. Firstly, diluted spermatozoon samples were embedded in agarose gel. Each microscope slide was pre-coated with a layer of 1% normal melting point agarose in PBS and dried thoroughly at room temperature. Next, 100 μl of 0.7% low melting point agarose at 37 °C was mixed with 10 μl of the cell suspension and dripped onto the first layer. Slides were allowed to solidify for 5 min at 4 °C in a moist box. The coverslips were removed, and the slides were immersed in freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na_2–EDTA, 10 mM Tris, 1% Triton X-100 and 40 mM dithiothereitol (pH 10) for 1 h at 4 °C. Then, the slides were incubated overnight at 37 °C in 100 μg ml^{-1} proteinase K added to the lysis buffer. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at room temperature at 25 V and was adjusted to 300 mA. Subsequently, the slides were washed with a neutralising solution of 0.4 M Tris, pH 7.5, to remove the alkali ions and detergents. After neutralisation, the slides were stained with 50 μl of ethidium bromide (1 μg ml^{-1}) and covered with a coverslip. All steps were performed under dim light to prevent further DNA damage (Haines *et al.*, 1998). The images of 100 randomly chosen nuclei from spermatozoon sample of each animal were visually analysed, and spermatozoa with damaged DNA were counted. Observations were made at a magnification of 400× using a fluorescent microscope (Olympus, Tokyo, Japan). Damage was detected by a tail of fragmented DNA that migrated from the spermatozoon head, causing a 'comet' pattern, whereas whole spermatozoon heads, without a comet, were not considered to be damaged (Verit *et al.*, 2006).

Statistical analysis

Data are presented as the mean ± SEM. The degree of significance was set at $P < 0.05$. Nonparametric Kruskal–Wallis analysis of variance test was used to determine the differences between the groups, and nonparametric Mann–Whitney *U*-test was used for multiple comparisons with respect to all parameters studied. All the analyses were carried out using the SPSS/PC software programme (Version 22.0; SPSS, Chicago, IL, USA).

Results

Changes in body and reproductive organ weights

The mean data related to body, absolute and relative reproductive organ weights are presented in Tables 1 and 2. No statistically significant differences were found between the groups in terms of final body weight as well as absolute and relative ventral prostate weight. However, both doses of EH administration caused significant reductions in absolute and relative weights of testis ($P < 0.001$), whole

Table 1 Changes in body and absolute reproductive organ weights in response to different dose EH treatment

Variables	Groups			
	Control	EH-5	EH-10	Significance
Body weight (g)	284.88 ± 10.13	283.00 ± 10.44	274.13 ± 8.76	NS
Absolute reproductive organ weights (g)				
Testis (Right+left/2)	1.337 ± 0.050[a]	1.166 ± 0.031[b]	0.917 ± 0.015[c]	$P < 0.001$
Whole epididymis (Right+left/2)	0.539 ± 0.028[a]	0.433 ± 0.014[b]	0.318 ± 0.013[c]	$P < 0.001$
Right cauda epididymis	0.216 ± 0.016[a]	0.141 ± 0.005[b]	0.128 ± 0.005[b]	$P < 0.01$
Seminal vesicles	1.563 ± 0.066[a]	1.520 ± 0.062[a]	1.160 ± 0.030[b]	$P < 0.01$
Ventral prostate	0.543 ± 0.026	0.504 ± 0.049	0.443 ± 0.031	NS

EH-5, etodolac hydrazone (5 mg kg^{-1}); EH-10, etodolac hydrazone (10 mg kg^{-1}); NS, nonsignificant.
Data are expressed as mean ± SEM.
Different superscript letters (a, b, c) within the same line show statistically significant differences between the groups.

Table 2 Changes in relative reproductive organ weights in response to different dose EH treatment

Relative reproductive organ weights [organ weight (g)/final body weight (g) × 100]	Groups			
	Control	EH-5	EH-10	Significance
Testis (Right+left/2)	0.471 ± 0.016[a]	0.414 ± 0.014[b]	0.337 ± 0.013[c]	$P < 0.001$
Whole epididymis (Right+left/2)	0.189 ± 0.005[a]	0.154 ± 0.008[b]	0.117 ± 0.007[c]	$P < 0.001$
Right cauda epididymis	0.076 ± 0.005[a]	0.050 ± 0.003[b]	0.047 ± 0.002[b]	$P < 0.01$
Seminal vesicles	0.553 ± 0.028[a]	0.539 ± 0.021[a]	0.427 ± 0.021[b]	$P < 0.05$
Ventral prostate	0.193 ± 0.013	0.179 ± 0.017	0.164 ± 0.014	NS

EH-5, etodolac hydrazone (5 mg kg^{-1}); EH-10, etodolac hydrazone (10 mg kg^{-1}); NS, nonsignificant.
Data are expressed as mean ± SEM.
Different superscript letters (a, b, c) within the same line show statistically significant differences between the groups.

epididymis ($P < 0.001$) and right cauda epididymis ($P < 0.01$) as compared to the control group. A total of 10 mg kg^{-1} dose of EH significantly reduced the absolute ($P < 0.01$) and relative ($P < 0.05$) weights of seminal vesicles when compared to control group. In addition, the absolute and relative weights of testis, whole epididymis and seminal vesicles were significantly lower in EH-10 group than that of the EH-5 group.

Changes in serum testosterone level and oxidative stress markers

Serum testosterone level, testicular tissue lipid peroxidation, demonstrated as MDA, and GSH level, GSH-Px and CAT activities of all the groups are given in Table 3. EH administration at the dose of 10 mg kg^{-1} significantly reduced the testosterone level ($P < 0.05$) and significantly increased the MDA level ($P < 0.05$) when compared to control group. With respect to data related to antioxidant markers (GSH, GSH-Px and CAT), no statistically significant differences were observed between the groups.

Changes in spermatozoon parameters

Epididymal spermatozoon motility, spermatozoon count and abnormal spermatozoon rate in all groups are presented in Table 4. Significant decreases in spermatozoon motility ($P < 0.001$) and spermatozoon count ($P < 0.001$) were observed between EH-5 and control, between EH-10 and control, as well as between EH-5 and EH-10 groups. However, no significant change was observed between the groups with respect to head, tail and total abnormal spermatozoon rates.

Changes in testicular histological structure

Figure 2 demonstrates the changes observed in the testicular histological structure of each group. The sections of

Table 3 Changes in testosterone levels and oxidative stress markers in response to different dose EH treatment

	Groups			
Variables	Control	EH-5	EH-10	Significance
Testosterone (ng dl^{-1})	256.50 ± 28.96[a]	203.81 ± 26.49[ab]	167.83 ± 13.27[b]	$P < 0.05$
MDA (nmol g^{-1} protein)	3.66 ± 0.38[a]	4.53 ± 0.10[ab]	4.87 ± 0.18[b]	$P < 0.05$
GSH (nmol g^{-1} protein)	8.30 ± 0.84	8.84 ± 0.79	10.04 ± 0.62	NS
GSH-Px (IU g^{-1} protein)	0.63 ± 0.14	0.55 ± 0.12	0.62 ± 0.13	NS
CAT (k g^{-1} protein)	49.52 ± 12.01	51.96 ± 8.54	42.84 ± 10.18	NS

EH-5, etodolac hydrazone (5 mg kg^{-1}); EH-10, etodolac hydrazone (10 mg kg^{-1}); MDA, malondialdehyde; GSH, reduced glutathione; GSH-Px, glutathione-peroxidase; CAT, catalase; NS, nonsignificant.
Data are expressed as mean ± SEM.
Different superscript letters (a, b) within the same line show statistically significant differences between the groups.

Table 4 Changes in spermatozoon characteristics in response to different dose EH treatment

	Groups			
Variables	Control	EH-5	EH-10	Significance
Spermatozoon motility (%)	74.88 ± 1.26[a]	47.14 ± 1.73[b]	30.42 ± 0.98[c]	$P < 0.001$
Spermatozoon count (million/right cauda epididymis)	108.76 ± 1.97[a]	73.72 ± 1.95[b]	54.00 ± 1.04[c]	$P < 0.001$
Abnormal spermatozoon rate (%)				
Head	8.14 ± 2.03	11.71 ± 1.82	12.40 ± 1.44	NS
Tail	4.29 ± 0.75	5.14 ± 1.18	6.20 ± 1.39	NS
Total	12.43 ± 2.40	16.85 ± 2.03	18.60 ± 1.86	NS

EH-5, etodolac hydrazone (5 mg kg^{-1}); EH-10, etodolac hydrazone (10 mg kg^{-1}); NS, nonsignificant.
Data are expressed as mean ± SEM.
Different superscript letters (a, b, c) within the same line show statistically significant differences between the groups.

control group showed normal testicular architecture with normal germ cell polarity and regular seminiferous tubular morphology. The Sertoli cells between the germ cells were observed to be normal in control group (Fig. 2a). The testis tissue section from 5 mg kg^{-1} EH-treated group showed a normal testicular architecture, although there were a few degenerated seminiferous tubules and capillary congestion (Fig. 2b). However, many histopathological changes such as degeneration, disorganisation in germinal cells, capillary congestion and also necrotic and atrophied tubules were observed in the sections of EH-10 group when compared to control group. Microscopic examination of the testis tissue showed degenerated seminiferous tubules (Fig. 2c).

Changes in the numbers of TUNEL+ apoptotic germ cells and spermatozoa with damaged DNA

The microphotographic view of apoptotic germ cells and their numbers in all groups are presented in Figs 3 and 4 respectively. Although no increase in TUNEL+ apoptotic germ cell number was detected in the testis tissue of control group (Fig. 3a), gradual increase was observed in EH-5 (Fig. 3b) and EH-10 (Fig. 3c) groups. When the apoptotic germ cell numbers in 20 seminiferous tubules of each group were statistically compared, a significant ($P < 0.01$) increase was observed in only EH-10 group, but not EH-5 group, versus control group (Fig. 4).

The microphotographic view of spermatozoa with damaged DNA and their percentage values in all groups are presented in Figs 5 and 6 respectively. Although no comet pattern, which is an indicator of DNA damage, was observed in control group (Fig. 5a), the prominent and the best prominent comet patterns were detected in EH-5 (Fig. 5b) and EH-10 (Fig. 5c) groups respectively. When the percentage values of spermatozoa with damaged DNA were statistically compared, a significant ($P < 0.001$) increase was observed in EH-5 and EH-10 groups versus control group. In addition, the percentage value of spermatozoa with damaged DNA was found to be higher significantly ($P < 0.001$) than that of the EH-5 group (Fig. 6).

Discussion

The detrimental effects of NSAIDs on male reproductive system (Kumar & Chinoy, 1988; Tanyıldızı & Bozkurt,

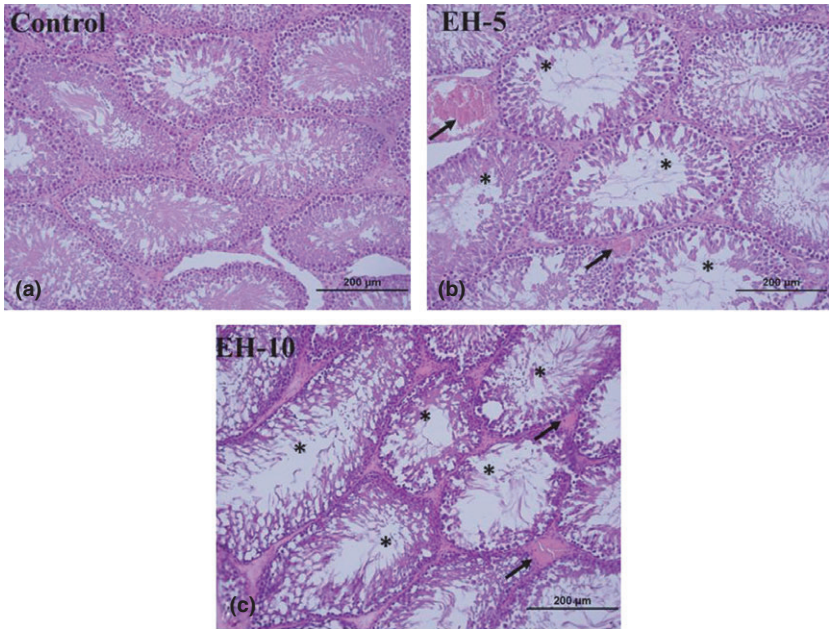

Fig. 2 Representative photomicrographs of the testis in each experimental groups. (a) Control group had normal morphological appearance. (b) Degenerative changes as a few degenerated seminiferous tubules and capillary congestion in EH-5 group. (c) The structure of the seminiferous tubules severely damaged in EH-10 group. Star, degenerative tubules, arrow, capillary congestion (H&E, 200×).

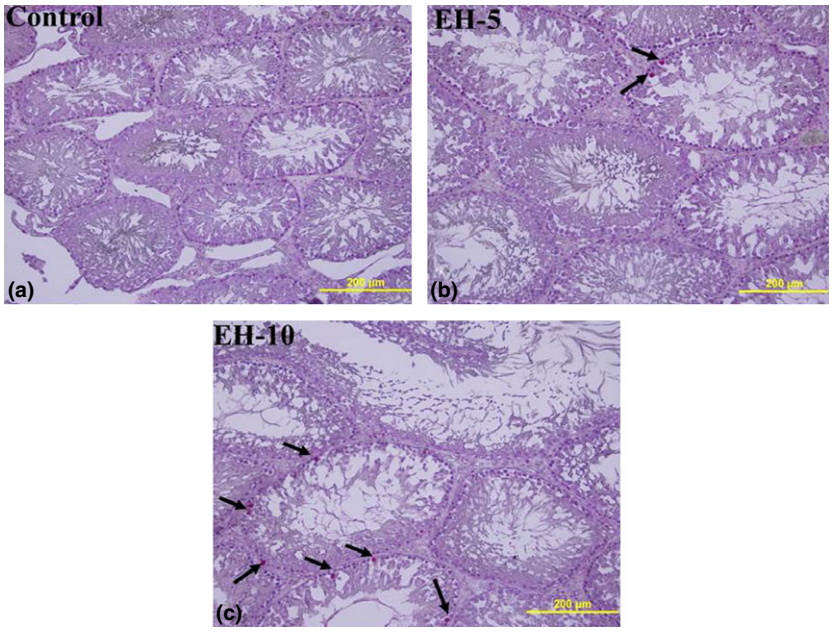

Fig. 3 Effects of different dose of EH on testicular germ cell apoptosis. Apoptotic cells were labelled with terminal deoxynucleotidyl transferase-mediated digoxigenin-dNTP nick end-labelling (TUNEL) method. (a) Control group showed a few TUNEL+ apoptotic germ cells. (b) An increase in TUNEL+ apoptotic germ cells in the testis section of EH-5 group compared to control group. (c) After 10 mg kg^{-1} EH treatment, TUNEL+ apoptotic germ cells markedly increased in the seminiferous tubules. Arrow: TUNEL+ apoptotic germ cell (Mayer's haematoxylin, 200×).

2003; Karahan *et al.*, 2006; Oyedeji *et al.*, 2013) have been reported in contrast to their therapeutic effects against inflammation, analgesia (Bennett & Tavares, 2001) and carcinogenesis (Shigemura *et al.*, 2005; Okamoto *et al.*, 2008). Hydrazide–hydrazones are very effective organic derivatives and an important class of compounds for new drug development. Therefore, many researchers have synthesised these compounds as target structures and evaluated their biological activities (Rollas & Küçükgüzel, 2007). For this purpose, EH was synthesised from etodolac as a new compound and its effect on prostate cancer was studied *in vitro* (Çıkla *et al.*, 2013). However, *in vivo* animal experimentations of this compound have not been studied so far. Therefore, we investigated the changes in male reproductive organ weights, spermatozoon quality parameters, testicular histopathology, germ cell apoptosis and spermatozoon DNA fragmentation to see the *in vivo* effects of EH in male rats. In addition, the obtained findings from this study are the first results related to *in vivo* effect of EH on male reproductive system.

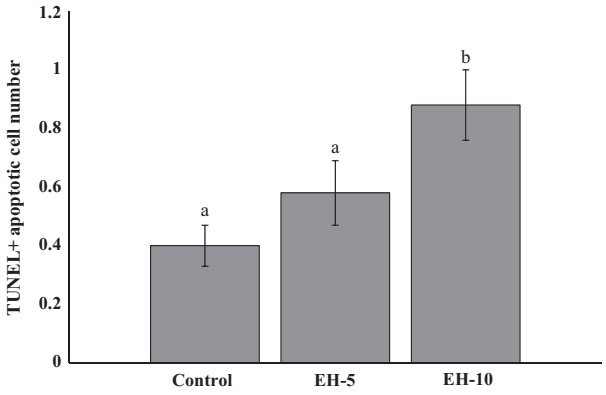

Fig. 4 Changes in TUNEL+ apoptotic germ cell number in response to different dose EH treatment. EH-5, etodolac hydrazone (5 mg kg^{-1}), EH-10: etodolac hydrazone (10 mg kg^{-1}); Data are expressed as mean ± SEM. Different superscript letters (a, b) show statistically significant differences between the groups ($P < 0.01$).

Prostaglandins modulate the hypothalamus–pituitary–gonadal axis pathway (Di Luigi *et al.*, 2007), and testicular prostaglandin system is highly sensitive to NSAIDs (Albert *et al.*, 2013). In addition, increased ROS-induced lipid peroxidation acts directly on Leydig cells to diminish testosterone production by inhibiting cytochrome P450 side chain cleavage enzyme and steroidogenic acute regulatory protein (Tsai *et al.*, 2003). Permanent androgenic stimulation is necessary for normal growth and functions of testes, epididymides and accessory sex organs (Klinefelter & Hess, 1998). Therefore, disturbances in the synthesis of androgens can cause negative changes in reproductive organ weights (Fernandes *et al.*, 2007). It has been reported that acetylsalicylic acid has a marked effect in decreasing the testicular weight in immature rats (Didolkar *et al.*, 1980) and mature mice (Chaloob *et al.*, 2010; Mohan & Sharma, 2011), and in altering the metabolism of testis, cauda epididymis, seminal vesicles and vas deferens in adult rats (Kumar & Chinoy, 1988). Some experimental studies have suggested that NSAIDs (aspirin, celecoxib, indomethacin) decrease the testosterone concentration (Selmanoğlu *et al.*, 2006; Albert *et al.*, 2013; Oyedeji *et al.*, 2013). In the present study, while 10 mg kg^{-1} EH administration caused significant reductions in absolute and relative weights of all reproductive organs and serum testosterone level except ventral prostate weight, 5 mg kg^{-1} EH administration significantly decreased only the absolute and relative weights of testis, whole epididymis and right cauda epididymis as compared to the control group. These decreases in reproductive organ weights observed in the present study may possibly be explained by EH-induced decreased steroidogenic activity, as evidenced by decreased testosterone level in this study, due to the ROS-induced lipid peroxidation, as evidenced by increased MDA level in this study. Inhibition of prostaglandin synthesis and lipid peroxidation induced by EH may possibly be responsible for the decreased testosterone level observed in the present study. Decreased testosterone level and increased lipid peroxidation might result in decreased reproductive organ weights due to the structural damages such as necrosis and atrophy in testis, as evidenced by necrotic and atrophied seminiferous tubules in the present study, and epididymis rather than functional damages. However, the decrease in seminal vesicles weight observed in this study after EH

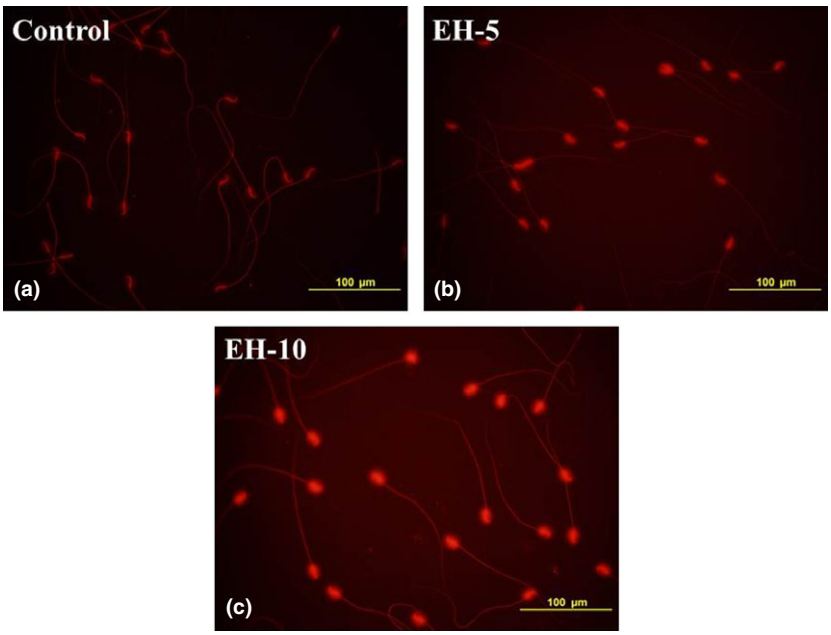

Fig. 5 No comet pattern, which shows spermatozoa with undamaged DNA, in control group (a). The prominent and the best prominent comet patterns, which show spermatozoa with damaged DNA, in EH-5 (b) and EH-10 (c) groups respectively (ethidium bromide staining, 400×).

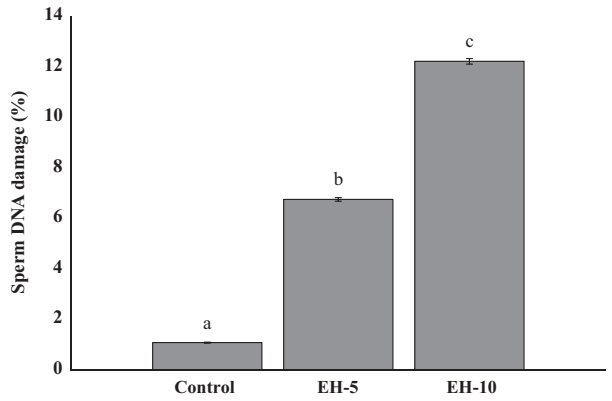

Fig. 6 Changes in percentage values of spermatozoa with damaged DNA in response to different dose EH treatment. EH-5, etodolac hydrazone (5 mg kg^{-1}); EH-10, etodolac hydrazone (10 mg kg^{-1}). Data are expressed as mean ± SEM Different superscript letters (a, b, c) show statistically significant differences between the groups ($P < 0.001$).

administration is possibly related to the reduction in secretion of seminal fluid due to the functional damage rather than structural damage of this organ because the large amount (70–80%) of seminal fluid is secreted from seminal vesicles. The reason of lack of effect of EH on ventral prostate weight may be explained that EH has no structural and functional impact on normal fibroblast cells of prostate (Çıkla *et al.*, 2013).

Reproductive cells and tissues remain stable when free radical production and the scavenging antioxidants remain in balance. When the balance was broken in favour of free radicals under pathologic conditions, ROS can attack and inactivate or alter the biological activity of molecules such as lipids, are essential for cell function, due to the lipid peroxidation (Wathes *et al.*, 2007). Although some authors suggested that NSAIDs including etodolac had free radical scavenging activity (Fernandes *et al.*, 2004; Costa *et al.*, 2005), some authors reported that NSAIDs increase ROS level and cause lipid peroxidation (Yoshikawa *et al.*, 1993; Maity *et al.*, 2009). In this study 10 mg kg^{-1}, but not 5 mg kg^{-1}, EH administration significantly increased the MDA level, by-product of lipid peroxidation, with no effect on antioxidant markers when compared to control group. In the present study, the increased lipid peroxidation in testes may be related to the increased free radicals induced by EH.

Despite the low oxygen tensions that characterise the testicular microenvironment, spermatozoa and other cells within the testis remain vulnerable to oxidative stress due to the abundance of highly PUFAs and the presence of potential ROS-generating systems. On the other hand, spermatozoa are also vulnerable to oxidative damage during the epididymal transit due to the maturational changes in spermatozoon plasma membrane (Aitken & Roman, 2008). Thus, excessive generation of free radicals in pathologic conditions can induce the lipid peroxidation by oxidative breakdown of PUFAs in the membranes of cells. Obviously, peroxidation of spermatozoon lipids destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with rapid loss of intracellular ATP leading to axonemal damage, decreased spermatozoon viability and increased mid-piece morphological defects, and even it completely inhibits spermatogenesis in extreme cases (Aitken & Roman, 2008; Turner & Lysiak, 2008). Besides, prostaglandins have been reported to be important regulators of epididymis contractions (Cosentino *et al.*, 1984) and $PGF_2\alpha$ stimulates seminiferous tubule contractions (Farr & Ellis, 1980) and spermatozoon motility (Gottlieb *et al.*, 1988). Thus, spermiation within the testis (Farr & Ellis, 1980) and spermatozoon transport through epididymis (Cosentino *et al.*, 1984) may be regulated by prostaglandins. In the present study, significant decreases in spermatozoon count and motility, and insignificant increases in head, tail and total abnormality rates were observed in both EH-5 and EH-10 groups when compared to the control group. These findings are in agreement with the earlier reports that demonstrated a reduced spermatozoon count (Tanyıldızı & Bozkurt, 2003; Oyedeji *et al.*, 2013) and spermatozoon motility (Karahan *et al.*, 2006; Oyedeji *et al.*, 2013), and also an increased spermatozoon shape abnormality (Oyedeji *et al.*, 2013) in NSAIDs-treated animals. However, there is a contradictory between our results and some author findings where NSAIDs have been reported to increase in spermatozoon count in partial obstructive (Martin-Du Pan *et al.*, 1997) and nonobstructive azoospermic men (Montag *et al.*, 1999) and also increase in spermatozoon count and motility in oligospermic men (Barkay *et al.*, 1984). This discrepancy may probably be due to the factors such as using of different species and different spermatozoon collection methods or being healthy and having pathologic conditions of the species used in the studies. Increased lipid peroxidation, as evidenced by increased MDA level in this study, may be responsible for the impaired spermatozoon quality observed in EH-treated rats. In addition, the reason of reduced spermatozoon count may also be explained by the detrimental effect of EH administration on spermiation within the testis and spermatozoon transport through caput and corpus regions of epididymis due to the decreased prostaglandin-induced inhibition of contractility of seminiferous tubules and epididymis.

It has been reported that degeneration in germ cells, shrinkage in the tubules, oedema, decrease in blood vessels, change in Sertoli cell morphology, decrease in spermatid count and increase in size of spermatocytes nuclei

in testis sections of aspirin-treated animals (Biswas *et al.*, 1978; Didolkar *et al.*, 1980; Chaloob *et al.*, 2010), and decrease in Leydig cell numbers, extraction in Sertoli cells, degeneration in seminiferous tubules, intratubular vacuolisation and necrotic debris in tubule lumen in testis sections of diclofenac sodium-treated mice (Mohan & Sharma, 2011) were observed. Similarly, prominent testicular histopathological damages such as degeneration, disorganisation in germinal cells, capillary congestion and also necrotic and atrophied tubules were observed in the EH-treated groups, in particular EH-10 group, when compared to the control group in this study. However, some researchers have claimed that aspirin (Oyedeji *et al.*, 2013) and celecoxib (Selmanoğlu *et al.*, 2006) have no significant adverse effect on testicular structure. The integrity of spermatozoon DNA has a vital importance to the spermatozoon cell. Apoptosis is known to be a programmed cell death for controlling the spermatogonial population within the testis. However, increased number of apoptotic germ cells in pathologic conditions disrupts this program leading to excessive cell death (Blanco-Rodriguez, 1998). Excessive generation of free radicals-induced DNA damage results in increased testicular apoptotic germ cells (Maheshwari *et al.*, 2009) and increased spermatozoa with damaged DNA (Rajesh *et al.*, 2002). NSAIDs such as benoxaprofen, naproxen, ketoprofen and tiaprofenic acid have been reported to induce DNA-breakage (Artuso *et al.*, 1991). However, Kristensen *et al.* (2012) have suggested that paracetamol, aspirin and indomethacin have no significant effect on the rate of apoptotic gonocytes in foetal rat testis. A significant increase was observed in the numbers of TUNEL+ apoptotic germ cells only in EH-10 group and spermatozoa with damaged DNA in EH-5 and EH-10 groups, versus control group in the present study. Increased lipid peroxidation level induced by EH administration might possibly cause the testicular histopathological damages and the increase in the numbers of TUNEL+ apoptotic germ cells and spermatozoa with damaged DNA.

In conclusion, although EH, a new compound synthesised from a NSAID etodolac, has anticarcinogenic effect on prostatic cancer cell line (Çıkla *et al.*, 2013), its consumption for a long time (8 weeks) causes significant damages on male reproductive organs and cells by inhibiting the prostaglandin synthesis like other NSAIDs and also increasing the testicular lipid peroxidation level. Besides, the results cannot be directly interpreted to humans because this study was conducted in healthy rats. Therefore, further studies are required to see the positive or negative effects of EH on reproductive system of men with healthy or having inflammation in different organs and tissues, in particular in testis and epididymis.

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