Prevention of H$_2$O$_2$ Induced Oxidative Damages of Rat Testis by *Thymus algeriensis*

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**Abstract**

**Objective** We evaluate the effects of *Thymus algeriensis* (TEO) against hydrogen peroxide (H$_2$O$_2$) toxicity on body and testis weight, testis sperm count, testis lipid peroxidation, and antioxidant enzyme activities in rats.

**Methods** Rats were treated with low (LD) and high dose (HD) of H$_2$O$_2$ (0.1 and 1 mmol/L) in the presence or absence of TEO (150 mg/kg).

**Results** The results exhibited a significant decrease in body weight and testis weight, in total sperm number decrease ($P<0.05$), sperm motility and percentage of sperm viability, leading to complete arrest, in sperm flagellar beat frequency by the gavage of 1 mmol/L H$_2$O$_2$ compared to controls. The administration of H$_2$O$_2$ resulted in a significant reduction in testis GSH, GPx, CAT, SOD, and GST activity and significant increase ($P<0.05$) in MDA concentration compared with the untreated control animals. TEO pre-treatment protected testis from the H$_2$O$_2$ generated oxidative stress. These results were confirmed by histological architecture examinations.

**Conclusion** H$_2$O$_2$ has the ability to alter the sperm function, characteristics and development of testis. However, TEO is an efficient natural agent, which can prevent the testis from H$_2$O$_2$-induced oxidative damage in rats.

**Key words:** Testis; Hydrogen peroxide; TEO; Antioxidants; Infertility

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**INTRODUCTION**

Recently, oxidative stress has become the focus of interest as a potential cause of male infertility. Oxidative stress is induced by reactive oxygen species (ROS) which plays some key roles in the development of testis toxicity. Testis toxicity is a sporadic and challenging issue for pharmaceutical drug development$^{[1]}$. Some level of oxidative stress are required for sperm capacitation, hyperactivation, and sperm-oocyte fusion. However, extreme levels of ROS can negatively impact sperm quality, motility and increase DNA damage in sperm. Subsequently, sperm-oocytes fusion efficacy decreased too much. Therefore, equilibrium is required between the ROS

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Biographical note of the first author: Fatma Guesmi, born in 1980, female, PhD, majoring in medicinal plants and molecular biology.
production and antioxidant scavenging activity in the male reproductive organs\textsuperscript{[2]}

Among a great variety of ROS, hydrogen peroxide (H$_2$O$_2$) plays a crucial role in male infertility. H$_2$O$_2$ is generated from various sources of oxidative stress and oxygen free radicals, which can diffuse freely in and out of cells and tissues\textsuperscript{[3]}. It has also been recognized as the most toxic oxidizing species for human spermatozoa\textsuperscript{[4]}. H$_2$O$_2$ exposure induces lipid peroxidation and decreases activities of antioxidant enzymes; superoxide dismutase (SOD), catalase (CAT) & cellular glutathione (GSH). These scavenger enzymes in semen are considered as the part of first line defence against ROS\textsuperscript{[5]}. Generation of oxygen metabolites induced by the direct addition of H$_2$O$_2$ to sperm was shown to cause a significant increase in DNA fragmentation\textsuperscript{[6]}. These effects are counteracted by oxidative defence enzymes and antioxidants including glutathione peroxidase isoforms (GPx1) and (GPx4), glutathione reductase (GR), and reduction in GSH\textsuperscript{[7]}. Furthermore, GST is responsible for eliminating and detoxifying xenobiotic and their metabolic products\textsuperscript{[8]}. The first defense line against the accumulation of ROS is superoxide dismutase (SOD), which converts the O$_2$− radicals to H$_2$O$_2$\textsuperscript{[9]}. Modification in protein contents as a result of ROS has emerged as a key parameter for measuring oxidative stress\textsuperscript{[10]}. Tissue levels of malondialdehyde (MDA) and the activities of SOD, GPx, and CAT are proven indicators of oxidative stress\textsuperscript{[11]}

It has been hypothesized that reduction in oxidative stress through the natural ROS scavengers may be effective to improve the sperm function and treatment of male infertility. As natural resources are the safe and best sources, natural extracts, and essential oils will be good ROS scavengers. The genus \textit{Thymus} \textit{L.} (Lamiaceae) comprises more than 250 species growing worldwide and is well known for its medicinal importance including its antioxidant properties. Extensively published literature on genus \textit{Thymus} revealed that it has many natural compounds with multiple health benefit, such as health tonic, anti-inflammatory, antitussive, antimicrobial, antiparasitic, antiulcer, and carminative properties. It also has the potential to work as an antioxidant, antimicrobial, and antiulcer\textsuperscript{[12-13]}. Essential oil of \textit{Thymus algeriensis} (Boiss. et Reut.) contains a wide range of monoterpenes among which oxygenated monoterpen hydrocarbon includes borneol, camphor, 1,8-cineole, linalool and terpinen-4-ol\textsuperscript{[12]}

An endemic aromatic herb; \textit{T. algeriensis}, found in Tunisia are largely used in folk medicine for the treatment of respiratory disorders, in digestion and pregnancy complications\textsuperscript{[14]}. However, whether the essential oil of this plant can be used as an antioxidant against oxidative stress for the male infertility has not been studied before. To the best of our knowledge, this is the first study to explain the antioxidant potential of \textit{T. algeriensis} essential oil against the oxidative stress produced by H$_2$O$_2$ in animal model.

We evaluated the effect of \textit{T. algeriensis} essential oil against the hydrogen peroxide toxicity on organs weight and histoarchitecture of testis, DNA fragmentation in sperm, alteration in sperm morphology and motility, sperm counts as well as glutathione content (or oxidative status) in testis of rats. On the basis of these findings, \textit{T. algeriensis} can be suggested to use for the restoration of H$_2$O$_2$ toxicity.

**MATERIALS AND METHODS**

**Reagents**

The reagents used for the experiments were of analytical grade. TBA, Reduced glutathione (GSH), bovine serum albumin (BSA), Ellman’s reagent, [5, 5’-dithiobis-(2-nitrobenzoic acid)] (DTNB), H$_2$O$_2$, Tris-HCl buffer purchased from Merck chemicals.

**Plant Collection and Extraction**

\textit{Thymus sp. algeriensis} was collected from the mountain of Orbata, Gafsa (Tunisia). Plants were identified and a specimen was collected by Herbarium of the National Institute of Agronomy of Tunisia (INAT) for future reference. One hundred grams of air dried aerial part of \textit{Thymus algeriensis} were used for the extraction process. Plant extract was prepared by steam distillation for 6 h, and the appropriate stock solution of the active compound (150 mg/kg) were prepared.

**In Vivo Assay of Testis Toxicity**

**Animals and Experimental Design**

Adult male Wistar rats (90 days old; 140 to 180 g body weight) obtained from the animal laboratory of Pasteur Institute of Tunis, Tunisia (Ethics No. LNSP/Pro 152012) were housed in a specific pathogen-free environment (in clean polypropylene cages) with 12 h light/dark cycle, relative humidity (55%±10%), and a temperature of 20-25 °C with \textit{ad libitum} access.
to food and water. All experimental interventions were given daily by oral gavage. The animals were acclimatized 1 week prior to the experiments, and examined on daily basis for 5 min to acclimatize them to human contact and minimize their physiological responses to handling for subsequent protocols. All ethical themes of the studies on animals were considered carefully. After the adaptive period of 1 week, the animals were randomly divided into six groups consisting of six animals each. Group 1 served as normal control receiving saline vehicle through the experimental period. Group 2 rats were administered 0.1 mmol/L H$_2$O$_2$ (LD) orally for 2 weeks without administration of TEO. Group 3 treated with 1 mmol/L H$_2$O$_2$ (HD). Group 4 rats served as drug control group and received TEO [150 mg/(kg·day)] dissolved in normal saline by oral gavage. Group 5 rats received TEO+LD H$_2$O$_2$ [150 mg/(kg·day)] and 0.1 mmol/L, respectively by oral gavage (1 h before treatment with 0.1 mmol/L H$_2$O$_2$). Group 6 rats received TEO+HD H$_2$O$_2$ [150 mg/(kg·day)] and 1 mmol/L, respectively. The rats were sacrificed on day fifteen of the experiment. The testis were carefully dissected out and quickly fixed in 10% buffered formaldehyde for routine histological study after Haematoxylin & eosin (H&E) method. Sperm count, motility and parameters of specific interest, such as GSH level and antioxidant enzyme activities (SOD, CAT, GSH-Px, and GST) were done in all the groups under investigation.

Reproductive Rgans Weights Body weights of all experimental rats were accurately recorded daily before, during and after the treatments from day 1 till day 15. All rats were euthanized at the end of the experiment. After animal dissection, the testis were excised out quickly, weighed and the index weight (IW) of this organ were calculated according to Matousek [15] IW=organ weight (g)/100×body weight (g).

Sperm Morphology For the analysis of morphological abnormalities, sperm smears were drawn on clean and grease-free slides, and allowed to air-dry overnight [16]. The slides were stained with 1% eosin-Y/5% nigrosin and examined at 400× for morphological abnormalities in each sample.

Sperm Count and Motility Assessment of sperm motility was done according to WHO laboratory manual protocol for the examination of human semen and sperm-cervical mucus interaction [17]. In brief, 10 μL of the sperm suspension was placed on semen analysis chamber. A minimum of five microscopic fields was assessed to evaluate sperm motility on at least 200 sperm for each animal.

Sperm Viability Eosin-nigrosin staining was used to assess sperm viability according to WHO protocol [11]. Briefly, eosin and nigrosin was prepared in distilled water. One volume of sperm suspension was mixed with two volume of 1% eosin. After 30 s, an equal volume of nigrosin was added to this mixture. Thin smears were then prepared and observed under a light microscope at 1000× magnification. Viable sperms remained colorless while nonviable sperms stained red.

Histopathological Studies Testis of the different experimental group was removed and quickly fixed in 10% formalin for 24 h, and processed through the conventional paraffin embedding technique, sectioned at 4 μm thick and stained with haematoxylin and eosin (H&E). Photomicrographs of the desired sections were made for further observations [18].

DNA Fragmentation Analysis The integrity of extracted genomic DNA was checked by electrophoresis in 1% agarose gels. The gel was stained with ethidium bromide and visualized under UV light.

In Vitro Assay of Oxidative Stress Marker Enzymes

Preparation of Homogenate The testis were weighed and homogenized in a buffer solution of potassium phosphate (pH 7.4) and centrifuged at 3000 rpm/15 min at 4 °C. The supernatant was used for the enzymatic, non-enzymatic and TBARS assays. All enzyme activity data were normalized by the total protein content in the testis and were expressed as units of activity per mg of protein (U/mg Pr).

Protein Estimation Protein content of the testis tissue was estimated by the method of Lowry [19] using a bovine serum albumin as a standard.

Lipid Peroxidation (LPO) Lipid peroxide content in testis tissues was determined by thiobarbituric acid reaction as described by Ohkawa [20]. The lipid peroxide concentration was expressed as nmol MDA/mg protein.

Assessment of Enzymatic and Non-enzymatic Antioxidants Catalase (CAT) was assayed as previously mentioned [21]. Superoxide dismutase (SOD) was estimated by the method of Marklund [22]. Glutathione peroxidase (GPx) was determined according to the method of Hafeman [23]. GST was estimated by the method described by Habig [24]. The reaction was initiated with 20 mmol/L 1-chloro-2,4-dinitrobenzene (CDNB) and an ethanol
solution was added for conjugation of GST with CDNB.

\[
\text{GSH + CDNB } \rightarrow \text{GS-DNB + HCl}
\]

Change in OD was read at 340 nm for 3 min at an interval of 30 s. A complete assay mixture without sample was used as control. The GST activity (U) was expressed as nmol CDNB conjugate formed min\(^{-1}\) mg\(^{-1}\) protein using a molar extinction coefficient of 9.6×10\(^3\) mol·L\(^{-1}\)·cm\(^{-1}\).

Total reduced glutathione (GSH) was assayed according to the method of Sedlak and Lindsay (1968)\(^{[25]}\). 5,5’-dithiobis-(2-nitrobenzoic acid) is reduced by thiol (SH) group to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The color developed was read at 412 nm against a reagent blank. A standard calibration curve was prepared using reduced glutathione (GSH). The amount of glutathione was expressed as µmol/mg protein.

Statistical Analysis

The values were reported as mean±S.E.M. The significance (\(P<0.05\)) of the results was assessed by one-way analysis of variance (ANOVA), followed by Bonferroni’s test for multiple comparison or Dunnett’s multiple range test.

RESULTS

Organs Weights

The effects of TEO treatment on body weights are shown in Figure 1. All TEO-treated male rats gained weight during the period of treatment. However, the weight gain in treated groups was less than that in the control group except in TEO treated rats. \(\text{H}_2\text{O}_2\) induced a decrease in body and testis weight (Figure 2). Administration of \(\text{H}_2\text{O}_2\) at only 1 mmol L\(^{-1}\) day\(^{-1}\) resulted in significant decrease in the absolute weights of testis (\(P<0.05\)). The average body weight (BW) of 1 mmol/L \(\text{H}_2\text{O}_2\) treated was 146±0.71 g. In the present study, reduction in the testis weight after \(\text{H}_2\text{O}_2\) treatment was indicative of toxicity. The average testicular weight of treated group with LD and HD of \(\text{H}_2\text{O}_2\) was 567±0.37 mg/100 g BW and 538±0.65 mg/100 g BW, respectively.

Sperm Count, Motility, Viability, and Morphology

In the present study \(\text{H}_2\text{O}_2\), one of the most toxic reactive oxygen species, has the ability to alter sperm function characteristics. Spermatozoa stained with Hematoxilin & Eosin showed that there was no significant difference between sperm count in TEO group compared to control group (Table 1). However, an opposite trend was encountered when the cells were treated with \(\text{H}_2\text{O}_2\). The total numbers of spermatozoa per testis were significantly decreased in dose dependent manner.

The sperm motility was significantly (\(P<0.05\)) preserved in TEO group compared to that of the control group (Table 1). The percentage of viable sperm was significantly (\(P<0.05\)) preserved in TEO group compared to that of the control group (Table 1).

In the present investigation, reduction in sperm number and motility was associated with an increase of sperm abnormalities in rats exposed to \(\text{H}_2\text{O}_2\). As shown in Table 1, it is evident that rat administered with high dose of \(\text{H}_2\text{O}_2\) resulted in profound altered sperm morphology. Unlike the control rat, in which 1.2% of the testis spermatozoa exhibited abnormal morphology; low and high dose \(\text{H}_2\text{O}_2\) treated rat showed 15.37% and 50.67% abnormal spermatozoa, respectively. These abnormalities included: amorphous
head, hookless head, doublet heads, compact head tail with a cytoplasmic droplet, irregular tail and coiled tail. The notable abnormality in high dose H$_2$O$_2$-treated sperms was the appearance of predominant deformities such as the detached and amorphous head with the highly folded tail. We observed significant improvement in morphological abnormalities of sperms by TEO administration.

### Tissue Total Proteins

A significant decrease in the total protein of testicular tissue was observed in H$_2$O$_2$ treated group (Table 2). While, TEO significantly increased total protein and alleviated the negative effects in H$_2$O$_2$-treated group.

### MDA Levels and Antioxidant Enzymes

Mammalian spermatozoa are sensitive to oxygen-induced damages mediated by lipid peroxidation of the cell membrane. Malonaldehydes, markers of lipid free radical peroxidation, were significantly elevated in all testis samples from infertile rats except for the control and TEO subgroups. Testicular cells demonstrated a significant (P<0.01) rise in lipid peroxidation following H$_2$O$_2$ exposure at a 1 mmol/L concentration and above, the MDA level was increased significantly in stressed rats (P<0.01) (25 %)

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**Table 1. Effects of TEO and H$_2$O$_2$ on Morphological Abnormalities of Sperm**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sperm Count ($x\times10^6$/ml)</th>
<th>Viability (%)</th>
<th>Motility (%)</th>
<th>Morphology (%)</th>
<th>Abnormality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85.65±0.54**</td>
<td>80.44±0.32**</td>
<td>83.20±1.24**</td>
<td>88.40±3.98**</td>
<td>1.20±0.04**</td>
</tr>
<tr>
<td>LD H$_2$O$_2$</td>
<td>67.14±0.47</td>
<td>65.51±2.08</td>
<td>61.95±3.90</td>
<td>64.87±2.54</td>
<td>15.37±1.38</td>
</tr>
<tr>
<td>HD H$_2$O$_2$</td>
<td>61.32±0.65**</td>
<td>52.49±0.54**</td>
<td>47.18±0.06**</td>
<td>50.10±3.87**</td>
<td>50.67±0.63**</td>
</tr>
<tr>
<td>TEO</td>
<td>94.81±3.07**</td>
<td>93.97±4.76**</td>
<td>94.63±2.70**</td>
<td>98.50±0.07**</td>
<td>2.30±0.02**</td>
</tr>
<tr>
<td>TEO + LD H$_2$O$_2$</td>
<td>87.04±4.00**++</td>
<td>88.15±0.98**</td>
<td>90.25±3.70**</td>
<td>82.65±0.91**</td>
<td>9.89±0.71**</td>
</tr>
<tr>
<td>TEO + HD H$_2$O$_2$</td>
<td>70.20±0.21</td>
<td>62.90±0.06</td>
<td>67.70±0.04</td>
<td>60.74±0.63**</td>
<td>34.21±0.82</td>
</tr>
</tbody>
</table>

**Note.** **Indicate significance from the control group at P<0.05 and P<0.01 probability level, respectively. ++Indicate significance from the H$_2$O$_2$ groups at P<0.01 probability level.**

**Table 2. Effect of H$_2$O$_2$ on Oxidative Stress Status**

<table>
<thead>
<tr>
<th>Biochemical Parameters</th>
<th>Control</th>
<th>LD H$_2$O$_2$</th>
<th>HD H$_2$O$_2$</th>
<th>TEO</th>
<th>TEO + LD H$_2$O$_2$</th>
<th>TEO + HD H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins concentration (µg/ml)</td>
<td>280.00±34.60*</td>
<td>188.00±14.80*</td>
<td>182.00±92.70*</td>
<td>265.00±32.98*</td>
<td>255.00±53.21*</td>
<td>242.00±3.98*</td>
</tr>
<tr>
<td>CAT (µmol H$_2$O$_2$ consumed min$^{-1}$ mg$^{-1}$ protein)</td>
<td>56.34±0.42*</td>
<td>8.30±0.11*</td>
<td>4.79±0.19*</td>
<td>45.18±0.36*</td>
<td>20.78±0.01*</td>
<td>14.53±0.00*</td>
</tr>
<tr>
<td>SOD U/mg of protein</td>
<td>44.52±1.45*</td>
<td>19.23±0.06*</td>
<td>13.33±4.30*</td>
<td>49.46±1.70*</td>
<td>38.65±0.26*</td>
<td>33.60±2.30*</td>
</tr>
<tr>
<td>GPx (µmol GSH/mg protein mL$^{-1}$)</td>
<td>5.70±0.04*</td>
<td>2.10±0.08*</td>
<td>1.84±0.08*</td>
<td>5.49±0.14*</td>
<td>5.33±0.32*</td>
<td>3.54±0.00*</td>
</tr>
<tr>
<td>GSH (µmol/mg protein)</td>
<td>6.42±0.00*</td>
<td>0.18±0.04*</td>
<td>0.09±0.00*</td>
<td>4.96±0.90*</td>
<td>2.97±0.05*</td>
<td>1.24±0.62*</td>
</tr>
<tr>
<td>GST (nmol of CDNB conjugate Formed min$^{-1}$ mg$^{-1}$ protein)</td>
<td>38102.00±34.60*</td>
<td>8090.00±66.72*</td>
<td>7428.00±45.80*</td>
<td>35300.00±19.06*</td>
<td>15152.00±98.80*</td>
<td>10340.00±88.90*</td>
</tr>
<tr>
<td>LPO (nmol MDA/mg protein)</td>
<td>120.23±5.98*</td>
<td>201.17±16.23*</td>
<td>204.00±36.80*</td>
<td>132.60±19.04*</td>
<td>147.00±76.42*</td>
<td>154.00±24.80*</td>
</tr>
</tbody>
</table>

**Note.** This experiment consists of the control (Group 1), the experimental groups: LD H$_2$O$_2$ (Groups 2), received 100 µmol/L of the H$_2$O$_2$; HD H$_2$O$_2$ (Groups 3), received 1 mmol/L of the H$_2$O$_2$; TEO (Groups 4), received 150 mg/kg of the TEO; TEO + LD H$_2$O$_2$ (Groups 5), received 150 mg/(kg-day) and 100 µmol/L, respectively by oral gavage (1 h before treatment with 100 µmol/L H$_2$O$_2$); TEO + HD H$_2$O$_2$ (Groups 6), received 150 mg/(kg-day) and 1 mmol/L, respectively by oral gavage (1 h before treatment with 1 mmol/L H$_2$O$_2$). Value are expressed as mean±SEM (n=6), significant difference at P<0.05 (ANOVA followed by Dunnett’s test) compared with normal control and intoxicated group (HD H$_2$O$_2$), MDA, Malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH, reduced glutathione; GPx, glutathione peroxidase; GST, glutathione transferase; *Significant when compared to the HD H$_2$O$_2$ groups; †Significant when compared to the control group.
versus the control group whereas that of recovery
group rats did not differ from controls (Table 2). However, the effect was not significant at the lowest
concentration of H$_2$O$_2$ (0.1 mmol/L). Remarkably TEO (150 mg/kg BW) treatment of rats reduced levels of
MDA and as a consequence improvement in GSH
level and enzymatic antioxidant activities in testis
increased. It should be noted that elevated malonaldehyde values obtained in the studied of
infertile rats corresponded with observed SOD activity changes.

The activities of the testicular SOD, CAT, and GST
were significantly reduced in stress group as well as in
recovery group rats compared to controls (Table 2). After being treated with H$_2$O$_2$, the testis levels of
SOD, CAT, GSH-Px, and GST were significantly
decreased ($P<0.01$) in comparison of the
respective control. Both, superoxide dismutase (SOD) and catalase from treated cells showed a
significant ($P<0.01$) decline in their activities above a
100 µmol/L concentration of H$_2$O$_2$. Interestingly, the
decline in the activity of glutathione S-transferase (GST) also revealed that TEO reverses the effect of
H$_2$O$_2$ treatment. However, the change in GST activity
is lesser than the control one. The activity of GPrx
was significantly reduced in stress group compared to
controls, while the recovery group was significantly higher than stressed rats and did not
differ from controls. Total reduced GSH was
significantly ($P<0.001$) decreased (0.093±0.00
µmol/mg protein) in response to H$_2$O$_2$ treatment as
compared to non-treated animals. The H$_2$O$_2$-induced
alterations in enzymatic activities and GSH level
were significantly prevented by TEO pretreatment
(Table 2).

To establish the relationship between MDA levels and other sperm parameters, linear
parametric coefficients were calculated and results
are shown in Table 3. Positive significant ($P<0.05$)
correlations confirmed between proteins concentration ($r$=-1.00), CAT ($r$=-0.88), SOD ($r$=-0.97),
GPrx ($r$=-0.96), GSH ($r$=-0.92), GST ($r$=-0.84), sperm
Count ($r$=-0.87), Motility ($r$=-0.86), morphology
($r$=-0.83), daily body weight gain ($r$=-0.93), testis
weight ($r$=-0.93) and MDA levels. The results
illustrated total lack of correlation between viability
($r$=-0.78), abnormality ($r$=-0.70) and MDA levels was
detected.

**Effect of TEO Treatment on Histological
Architecture of Testis**

We observed the normal structure of seminiferous
tubules (ST), each one is lined with spermatogenic
cells and sertoli cells, and swirling movement of
sperm in untreated as well as only TEO treated rats.
In H$_2$O$_2$-treated rats, many histopathological
abnormalities were observed according to treatment
dose. At LD H$_2$O$_2$-treated rats, most ST were
separated by wide intertubular spaces, few numbers of
sertoli cells and degenerated spermatogenic
epithelium with incomplete spermatogenesis, devoid
of spermatozoa (Figure 3).

At HD level, vacuolation in the intratubular (ST)
(white and black arrows), necrosis (long arrows) and
hemorrhage, tunica albugenea contains myoid cells,
dark nuclei of spermatogonia, primary spermatocytes, spermatids, spermatozoa, intratubular degeneration in spermatogenic stages;
leydig cells, wide intertubular spaces of ST; loss of
spermatogenic stages, loss of elongated spermatids,
degenerated Leydig cells. Further whether, TEO
prevent this LD H$_2$O$_2$-induced morphological
alteration was also determined. We examine the
effect of LD H$_2$O$_2$ on TEO-pretreated rats and
observed that ST, spermatogenic cells and sertoli
cells were appeared almost similar to the control.
These histological studies also justify the antioxidant
potential of TEO against H$_2$O$_2$-induced morphological
abnormalities.

**Table 3. Linear Correlation Coefficients between MDA Concentration, and Sperm Parameters Quality**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>LPO (nmol MDA/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins concentration (µg/mL)</td>
<td>-1.00*</td>
</tr>
<tr>
<td>CAT (µmol H$_2$O$_2$ consumed min$^{-1}$mg$^{-1}$ protein)</td>
<td>-0.88*</td>
</tr>
<tr>
<td>SOD U/mg of protein</td>
<td>-0.97*</td>
</tr>
<tr>
<td>GPrx (µmol GSH mg$^{-1}$ protein mL$^{-1}$)</td>
<td>-0.96*</td>
</tr>
<tr>
<td>GSH (µmol/mg protein)</td>
<td>-0.92*</td>
</tr>
<tr>
<td>GST (nmol of CDNB conjugate formed min$^{-1}$mg$^{-1}$ protein)</td>
<td>-0.84*</td>
</tr>
<tr>
<td>Sperm Count (× 10$^6$/mL)</td>
<td>-0.87*</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>-0.78</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>-0.86*</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>-0.83*</td>
</tr>
<tr>
<td>Abnormality (%)</td>
<td>0.70</td>
</tr>
<tr>
<td>Daily body weight gain</td>
<td>-0.93*</td>
</tr>
<tr>
<td>Testes weight</td>
<td>-0.93*</td>
</tr>
</tbody>
</table>

**Note.** *Significant correlation at $P<0.05$.
Prevention of H\textsubscript{2}O\textsubscript{2}-induced testicular damages by *thymus algeriensis*

**Figure 3.** Histology of testes stained with hematoxylin-eosin in control rats, rats treated with TEO and different concentrations of H\textsubscript{2}O\textsubscript{2}. a) Testis showing the normal structure of the seminiferous tubules in groups of control in male rats, swirling movement of sperm in the seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue in the testes of these rats; b) Testes of male rats intoxicated with single dose of LD H\textsubscript{2}O\textsubscript{2} showing complete testicular necrosis and germinal epithelium of all layers. hemorrhage, shrunken, buckled, disorganized seminiferous tubules, sloughing germinal epithelium (asterisks) in the lumen of the seminiferous tubules of rats who received HD of H\textsubscript{2}O\textsubscript{2}; c) Photomicrograph of testis showing degenerative and necrotic seminiferous tubules (white arrow), few spermatozoa (black arrow), vacuolation (small arrows), hemorrhage between seminiferous tubules (arrows) and some of the spermatogenic cells desquamate or vanish (H&E×200) Group 3 (HD H\textsubscript{2}O\textsubscript{2}); d) Pretreatment with TEO (Group 4) reversed damage produced by H\textsubscript{2}O\textsubscript{2} suggesting that TEO prevent testicular dysfunction induced by H\textsubscript{2}O\textsubscript{2} and showed the lesser of degenerative and atrophic changes of the tubular epithelium. The testicular histomorphology of rats in the recovery does not resemble that of the stressed rats or control. There was a vacuolization of the seminiferous epithelium (Figure 3c) and the spermatozoa were more abundant than the stressed rats. A marked improvement in spermatogenesis, as evidenced by the presence of spermatids and spermatozoa in the majority of rats treated with Group 4 (TEO) and Group 5 (TEO) + LD H\textsubscript{2}O\textsubscript{2}; e) and f) Rats treated with TEO + H\textsubscript{2}O\textsubscript{2} show a significant improvement in all of these deterioration (e), although partial (f).
**Effect of H₂O₂ and TEO on DNA Fragmentation**

DNA was extracted from sperms of different untreated and treated groups, and examined by agarose-gel electrophoresis. DNA fragmentation was observed in H₂O₂ treated testis samples. However, there was no DNA fragmentation in control and TEO treated testis (Figure 4). H₂O₂ induced DNA damage was evaluated by comparing DNA profiles using 1.5% agarose gel electrophoresis. In 1 mmol/L H₂O₂ treated rats; a clear DNA fragmentation was observed (Figure 4), where the fragments formed a typical apoptotic ladder in the gel, with most of the DNA accumulated in the lower bands. The degradation of DNA into oligonucleotide fragments confirmed the induction of apoptosis by H₂O₂ (Figure 4). Treatment with TEO led to significant protection against H₂O₂ induced DNA fragmentation in testis.

**DISCUSSION**

ROS are supposed to play one of the key roles in the development of testis toxicity, which is a sporadic and challenging issue for pharmaceutical drug development[26]. Since organ weight is a fundamental benchmark for the toxicological studies[27], we determined the weight loss of H₂O₂ treated testis, which is basically dependent on the mass of the differentiated spermatogenic cells. Weight loss of the testis can occur due to decreased number of germ cells, inhibition of spermatogenesis and steroidogenic enzyme activity[28]. Recently, EL-Kashoury (2009)[29] has shown that the weight of testis was significantly lowered in H₂O₂ treated male rats. Thus, the observed weight loss of the testis may be due to the adverse effect of H₂O₂ on the number of germ cells and elongated spermatids. In some reports, the decrease in testicular weight was also the result of reduction of tubule size, spermatogenic arrest and inhibition of steroid biosynthesis of Leydig cells[30]. Other than weight of testis, overall morphology, sperm count, motility should also be analysed to determine semen quality[31]. If the sperm motility is examined only 5% to 10%, sperm viability testing is recommended because low motility can occur due to sperm death or necrospermia[32]. Besides sperm motility and sperm counts, sperm morphology has also been considered as a good indicator of semen quality[33]. A number of morphological alterations occurs due to some abnormal process in sperm morphogenesis and subsequently reduces the fertilizing ability[34]. Oxidative stress has a direct adverse effect on sperm count and motility as excess of ROS induces germ cell apoptosis and loss of mitochondrial membrane potential[35]. Progressive sperm motility was also significantly lower in the H₂O₂ toxicated groups compared to others. The current data concur with the previous studies of Chaki and Misro, who found that 60 µmol/L H₂O₂ dose showed little effect on motility, the first parameter to be affected at higher concentrations[36]. Administration of H₂O₂ caused a significant increase in sperm abnormalities and ratio of viable to dead sperm count, and suppression of sperm motility. Our results are supported by a previous study which showed that ROS induced damage of spermatozoa involves an oxidative attack on the lipids of sperm plasma membrane causing spermatozoa to lose their motility[37]. Sperms with abnormal morphology were also shown to be less motile. Fadwa et al., found that treatment of drinking water with H₂O₂ (0.5%) caused a significant (P<0.05) decrease in the percentage of ratio of live to dead sperms with a significant (P<0.05) increase
induced impairment of superoxide dismutase activity has been previously reported. SOD stabilizes the plasmalemma of spermatozoa and so increases motility. Cell membranes are protected by GPx from ROS-mediated oxidative damage resulting from the formation of hydrogen peroxide (H₂O₂) during normal metabolism in the cells mitochondria. The reduction in the activities of CAT and GPx may reflect the inability of testicular mitochondria, microsomes and cytosol to eliminate the H₂O₂ produced by SOD. This may also be attributed to enzyme inactivation caused by excess ROS production. The testis has high concentration of GSH, which plays an important role in spermatogenesis. GSH concentrations may be very less due to oxidative stress, and the major peroxide detoxification enzyme GSH peroxidase. Interestingly, TEO pretreatment attenuated testicular dysfunction and renewed the activities of the antioxidant enzymes and GSH level by down-regulating the ROS level.

H₂O₂ induced sever testicular toxicity as shown in the histopathological results, which coupled with the marked changes of biochemical results. In the present study, our investigation demonstrated that exposure to H₂O₂ induces histopathological changes of testis in a concentration-dependent manner. The histomorphological alteration of the testis of recovery group rats did not resemble with the stressed or the control rats. The results showed the cystic degenerative deviations with some vacuolization of seminiferous epithelium, and spermatozoa were more abundant when compared to the control sections.

Rahim presumed that treatment with H₂O₂ markedly increased MDA in serum and testis homogenates, as well as testicular histopathological alterations. In addition, our findings supported by the other studies confirms that H₂O₂ at a higher concentration in the medium is toxic and affects cell survival even at physiological concentrations H₂O₂ (30-50 µmol/L) was shown to induce apoptosis in testicular Leydig cells in vitro. Histological examination revealed that the number of spermatogonia was dramatically decreased.

Our data exhibited that normal testicular architecture was observed in controls (Figure 3a) and only TEO-administered group (Figure 3d). The H₂O₂-treated rats show vacuolization in germinal epithelium, lost of germinal line and total reduction of spermatogenesis. Our findings are consistent with Creasy, who explained the presence of vacuoles within the seminiferous epithelium, which is
sensitive to a variety of toxicants.

DNA fragmentation is not determine in male infertility, but represents an extremely important parameter indicative of infertility and potential outcome of assisted reproduction treatment\(^{[50]}\). Our observations indicated that the direct effect of higher concentrations of \(H_2O_2\) causes DNA damage which can be seen in the form of a base damage (depurination or depyrimidination), single strand breaks, double strand breaks, chromosomal aberrations, and cross-linking between DNA\(^{[51]}\). Although this would appear to be good evidence for the involvement of an endonuclease, DNA fragmentation was restored in the presence of TEO (Figure 4). In this study we selected histological parameters of the adult rat testis and tested the protective effects exerted by TEO in \(H_2O_2\) treated rats.

**CONCLUSIONS**

The present study demonstrated that oxidative stress generated by \(H_2O_2\) resulted in decline of sperm motility and morphological alterations with the decreased sperm count and induction of DNA damage. The reduction in the level of GSH, disturbed sperm parameters, increased lipid peroxidation and sperm DNA fragmentation revealed an adverse effect of \(H_2O_2\) on the reproductive system of developing male rats. The eco-friendly, and nontoxic natural compounds such as TEO can prevent these adverse effects. Further preclinical studies and clinical trials in humans are needed to find out a possible place for it in therapies of fertility disturbances.

**CONFLICT OF INTEREST**

Authors have no conflict of interests.

**AUTHORS’ CONTRIBUTIONS**

GF conceived and designed the study. GF, BM, and BH performed the animal experiments and histological assessment. GF, BR, and BH participated in DNA damage assessment. GF and AKT drafted the manuscript. GF performed the statistical analysis and AKT revise the manuscript. All authors read and approved the final manuscript.

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