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## Protective effect of ethyl pyruvate on testicular histology and fertilization potential in cyclophosphamide treated mice

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Article Info	Abstract
<b>Article history:</b> Received: 20 January 2018 Accepted: 22 May 2018 Available online: 15 March 2020	In the present study, we aimed to address the use of ethyl pyruvate (EP) against the harmful effects of cyclophosphamide (CP) treatment. Thirty-nine adult male mice were divided into three groups including control group received normal saline [0.20 mL per day; intraperitoneally (IP)], CP group received CP (15.00 mg kg <sup>-1</sup> per week, IP) and CP+EP group received EP (40.00 mg kg <sup>-1</sup> per day, IP) along with CP and treated for 35 days. After preparation of paraffin sections and Hematoxylin and Eosin staining, the histomorphometric studies were performed on the testicular tissue. Additionally, the serum superoxide dismutase (SOD) and testosterone level, testis malondialdehyde (MDA) and <i>in vitro</i> fertilization rate were assessed. The results showed an increase in the tubal differentiation index, repopulation index, spermiogenesis index, thickness of testicular capsule, mean distribution of active Sertoli cells, SOD and testosterone levels of the CP+EP group in comparison with the CP group. Moreover, the MDA levels in the CP+EP group were lower than the CP group. An increase occurred in the percentage of fertilization in the CP+EP group compared to the CP group. Results of this study revealed that the EP ameliorates deleterious side effects of CP on testicular histology and <i>in vitro</i> fertility.
<b>Keywords:</b> Cyclophosphamide Ethyl pyruvate <i>In vitro</i> fertilization Mice Spermatogenesis	

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### Introduction

Cyclophosphamide (CP) with brand names of Revimmune, Procytox, Neosar, Cytoxan, and Endoxan, is an alkylating agent belonging to the oxazophorine group drugs, which has been widely used as a chemotherapy chemical. The CP links the alkyl group to the guanine of DNA in number seven atoms of nitrogen on the imidazole ring. Furthermore, due to its antitumor and immune suppressor properties, it is used for the treatment of various types of cancers and autoimmune disorders.<sup>1</sup> Extensive studies have been done in order to identify the side effects of CP, which were confirmed its toxicity in heart,<sup>2</sup> reproductive organs,<sup>3</sup> liver,<sup>4</sup> immune system<sup>5</sup> and urinary tract.<sup>6</sup> Moreover, the CP is known for its impact on genetic mutations,<sup>7</sup> secondary malignancies, particularly cancers of the urinary bladder,<sup>8</sup> suppression of hematopoietic tissue,<sup>9</sup> brain oxidative injuries,<sup>10</sup> diabetes<sup>11</sup> and embryo development as a teratogen.<sup>12,13</sup> Phosphoramidate mustard and acrolein<sup>14</sup> are known as active metabolites of CP. According to previous findings, acrolein is the main

cause of CP toxicity.<sup>15</sup> Acrolein generates oxygen free radicals, which are responsible for mutations in mammalian cells.<sup>16</sup> Reactive oxygen species (ROS) decrease sperm quality and fertility in atrazine-treated male rats.<sup>17</sup> Previous studies have confirmed the role of antioxidants in decreasing sperm damage reduction and pregnancy rate increase.<sup>18,19</sup> Half of total referral couples to infertility clinics are observed to be caused by males<sup>20</sup> and the most important factors involved in male infertility are drugs and chemical agents.<sup>21</sup> Cyclophosphamide as a current chemotherapeutic drug in addition to antimetabolic effect causes genital tract toxicity through ROS production and lipid peroxidation elevation.<sup>22</sup>

Studies have shown that the antioxidants are able to beneficially reduce the toxic effects of hydrogen peroxide on embryos.<sup>23</sup> The previous studies have confirmed the role of antioxidants in the amelioration of embryo development impairment by decreasing the ROS production of sperms.<sup>24</sup> Using the pyruvate as a drug is limited due to its unstable nature, so it used in the form of ethyl pyruvate (EP) containing pyruvic acid and ethanol.

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Pyruvate as a final metabolite of glycolysis is a substrate with three carboxylic acid cycle and acts as a mediator in metabolism.<sup>25</sup> The pyruvate is involved in ROS elimination in cell.<sup>26</sup> This study designed to evaluate the effects of EP in moderating CP side effects on the reproductive tract.

## Materials and Methods

**Chemicals.** Cyclophosphamide (Baxter Oncology GmbH, Halle, Germany) was prepared from a local drugstore and ethyl pyruvate was purchased from Sigma (St. Luis, USA).

**Animals and treatment groups.** In this experimental study, 39 adult male mice (6-8 weeks; 20-25 g) were kept in a standard condition of  $22.00 \pm 2.00$  °C and humidity of 30.00-60.00% and a period of 14 hr light and 10 hr darkness. They were randomly divided into three groups as follows: Control group receiving normal saline [0.20 mL per day, intraperitoneally (IP)], CP group receiving CP (15.00 mg kg<sup>-1</sup> per week, IP)<sup>27</sup> and CP+EP group receiving CP plus EP (40.00 mg kg<sup>-1</sup> per day, IP).<sup>28</sup> All procedures for animal care and use were carried out in accordance with the guidelines approved by the Animal Ethics Committee of Urmia University (IR-UU-AEC-1210/AD3/).

**Sampling.** At the end of the treatment period (35 days), all animals were euthanized by intraperitoneal injection of ketamine (300 mg kg<sup>-1</sup>; Alfasan, Woerden, The Netherlands) and right testes from 5 mice in each group were sampled for histomorphometric study and malondialdehyde (MDA) evaluation. The epididymides from eight mice were also sampled in each group for the evaluation of the *in vitro* fertilization (IVF) rate. Superoxide dismutase (SOD) and testosterone concentrations have been measured in the serum of animals.

**Histomorphometric study.** After opening the abdominal cavity of animals, testes were harvested. The right testes were fixed in 10% formal saline for 72 hr. The tissues were processed through paraffin embedding and cut at 6.00 to 7.00 µm thicknesses. Then, the slides were stained using Hematoxylin and Eosin method. Using optical microscopy (model BH-2; Olympus, Tokyo, Japan), the morphometrical analyses were conducted. The mean thickness of the testicular capsule was measured by a graded objective lens calibrated previously and mean distribution of active Sertoli cells (the number of accumulating locations of maturing sperms in the apical portion of Sertoli cells) was counted by the latticed objective lens in 0.25 mm<sup>2</sup> of testicular tissue. The percentage of active to resting spermatogonia with dark or pale nuclei was calculated as a repopulation index (RI), the percentage of seminiferous tubules with three or more to tubules with less than three germinal epithelial cell layers was estimated as a tubal differentiation index (TDI) and the spermiogenesis index (SI) was conducted by calculating the percentage of seminiferous tubules with or without maturing sperm.<sup>27</sup>

**Hormonal assessment.** The serum testosterone level was evaluated using immune radiometric methods using the kits of WHO/Sigma Asso-RTGC-768/98 (Abbott Laboratories, Abbott Park, USA). The intra-assay coefficients variance and inter-assay coefficient variances (for 10 times) for testosterone were 3.56 and 8.98, respectively.

**Superoxide dismutase determination.** The serum level of SOD was evaluated using specific kits (Randox Laboratories, Crumlin, UK). Serum was incubated in 1 M TRIS buffer and pirogalol (1.20 mg mL<sup>-1</sup>) and auto-oxidation inhibition was measured at 420 nm in a spectrophotometer (UV-1650 PC; Shimadzu, Tokyo, Japan) as described by Marklund and Marklund.<sup>29</sup> The results were expressed as SOD U nL<sup>-1</sup> of erythrocytes.

**Determination of MDA levels.** For determining of lipid peroxidation rate, the MDA content of the testis samples was measured using thiobarbituric acid (TBA). In brief, 0.30-0.40 g of the testis samples were homogenized in ice-cold KCL (150 mM), then the mixture was centrifuged at 3,000 *g* for 10 min; 0.50 mL of the supernatant was added to 3.00 mL phosphoric acid (1.00%; v/v), and after that followed vortex mixing, 2.00 mL of 6.70 g L<sup>-1</sup> TBA was added to the samples. The samples were boiled at 100 °C for 45 min and chilled on ice. Finally, 3.00 mL N-butanol was added and the samples were further centrifuged at 3,000 *g* for 10 min. The absorbance of the supernatant was measured with a spectrophotometer at 532 nm and the MDA concentration was calculated according to the simultaneously prepared calibration curves using MDA standards. The rate of MDA was expressed as nmol per mg protein of the samples.<sup>28</sup> The protein composition of the sample was assayed according to the Lowry method.<sup>30</sup>

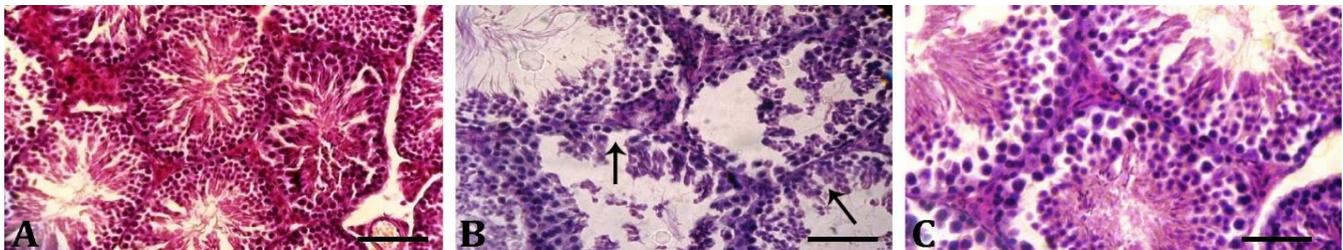
**The methodology of IVF.** For IVF and sperm DNA damage evaluation, eight animals in each group were euthanized and sperms were collected from the tail of the epididymis. Sperm DNA damage was evaluated by acridine orange staining method. For IVF method, hCG (Intervet, Boxmeer, The Netherlands) (0.10 mL, IP) was injected to 72 female adult mice, 10-12 hr later all animals were euthanized and their oviduct was dissected in human tubal fluid (HTF; Sigma) medium in 37.00 °C The ovulated oocytes were collected and after washing, they were transferred to drop under the mineral oil contained HTF medium and bovine serum albumin (4.00 mg mL<sup>-1</sup>; Sigma). Then, a suspension was prepared from motile and capacitated sperms (10<sup>6</sup> mL<sup>-1</sup>) in a medium and 3 to 5 hr after adding the sperms to the drop containing oocyte, the fertilization was evaluated by two pro-nuclei observation. After that, following washing of all prepared zygotes, they were transferred to a new medium that was previously prepared for all groups. The rate of cleavage was evaluated after 24 hr and embryo development was analyzed five days after fertilization.

**Statistical analysis.** The data were analyzed by SPSS (version 20.0; SPSS Inc., Chicago, USA) and Bonferroni post hoc test was also applied after one way ANOVA. The IVF data were analyzed using two ways ANOVA, by Minitab (version 16.0; Minitab Inc., Boston, USA). A  $p$ -value of less than 0.05 considered a significant difference.

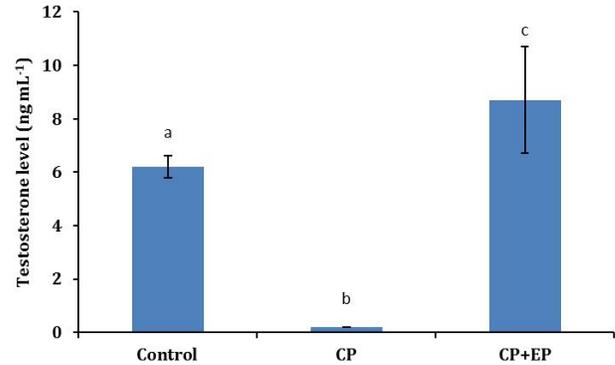
## Results

**Morphometrical findings.** The present study showed that the thickness of the testes capsule in the CP group was significantly decreased compared to the control and CP+EP groups ( $p < 0.05$ ). However, the thickness of the testes capsule in the CP+EP group was not significantly different from the control group. The RI in the CP group showed a significantly lower percentage of seminiferous tubules with active spermatogonia in comparison with the CP+EP group ( $p < 0.05$ ). The average percentage of TDI in the CP group was significantly lower than the control group. Moreover, observations showed that the TDI in CP+EP group was significantly increased compared to the CP group ( $p < 0.05$ ). The mean percentages of SI in the seminiferous tubules of CP+EP group were significantly higher than those in CP group ( $p < 0.05$ ). In all spermatogenesis indices, there were significant differences between control and CP+EP groups. An average number of active Sertoli cells in 1.00 mm<sup>2</sup> of testicular tissue was remarkably decreased in the CP group in comparison with other groups ( $p < 0.05$ ). Meanwhile, the administration of EP along with CP considerably increased the number of active Sertoli cells in comparison with the CP group. Moreover, there was a significant difference between control and CP+EP groups ( $p < 0.05$ ), (Fig. 1 and Table 1).

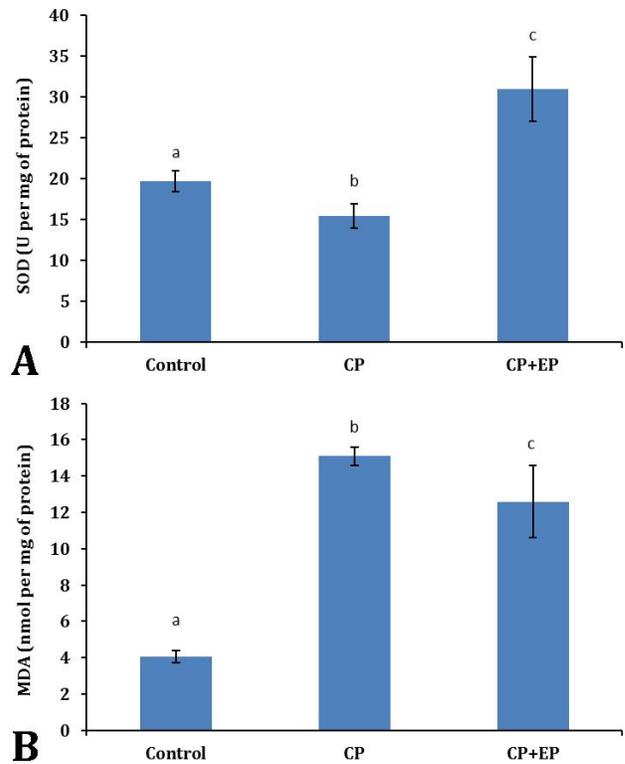
**Biochemical analyses.** Biochemical analyses showed that CP caused a severe reduction in serum level of testosterone compared to the control group ( $p < 0.05$ ). However, in CP+EP group, testosterone level significantly enhanced in comparison with the CP group ( $p < 0.05$ ; Fig. 2). In order to evaluate the serum level of SOD, observations showed that EP elevated the SOD level in comparison with the CP treated group. On the other hand, the animals in the CP+EP group exhibited a remarkably lower MDA level in comparison with CP only-treated animals, but that was not significant (Fig. 3).



**Fig. 1.** Cross-section of seminiferous tubules in **A)** Control group, **B)** CP group in which germinal epithelium shows disarrangement (arrows) and less thickness, and **C)** CP+EP group which amelioration was observed in terms of histoarchitecture approximately the same as the control group, (H&E; Scale Bar = 185  $\mu$ m). CP: Cyclophosphamide; EP: Ethyl pyruvate.



**Fig. 2.** The levels of serum testosterone in different groups (mean  $\pm$  SE). CP: Cyclophosphamide; EP: Ethyl pyruvate.  
abc Non-similar letters indicate a significant difference ( $p < 0.05$ ).



**Fig. 3. A)** The SOD level in different groups (mean  $\pm$  SE), **B)** The MDA level in testicular tissue of different groups (mean  $\pm$  SE). CP: Cyclophosphamide; EP: Ethyl pyruvate.  
abc Non-similar letters indicate a significant difference ( $p < 0.05$ ).

**Table 1.** Histomorphometric parameters in different experimental groups (mean  $\pm$  SE).

Groups	Capsule thickness ( $\mu\text{m}$ )	RI (%)	TDI (%)	SI (%)	Active Sertoli (cells per 0.25 mm <sup>2</sup> )
Control	17.91 <sup>a</sup>	79.80 <sup>a</sup>	83.00 <sup>a</sup>	79.60 <sup>a</sup>	61.72 <sup>a</sup>
CP	13.50 <sup>b</sup>	61.73 <sup>b</sup>	50.80 <sup>b</sup>	52.80 <sup>b</sup>	21.86 <sup>b</sup>
CP+EP	17.61 <sup>a</sup>	75.26 <sup>c</sup>	61.40 <sup>c</sup>	67.00 <sup>c</sup>	28.34 <sup>c</sup>

CP: Cyclophosphamide; EP: Ethyl pyruvate; RI: Repopulation index; TDI: Tubal differentiation index; SI: Spermiogenesis index.

<sup>abc</sup> Different letters in each column indicate significant differences at  $p < 0.05$ .

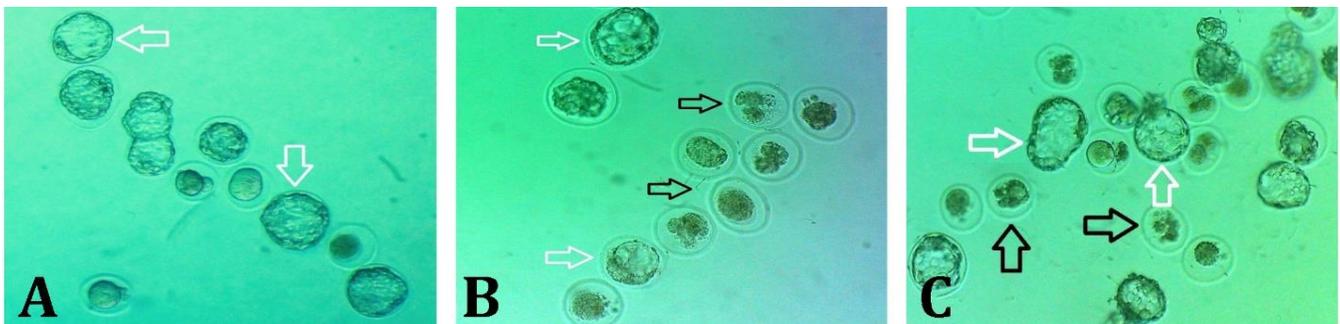
**In vitro fertilization.** The IVF results showed a notable decrease in the mean percentage of fertilization in the CP group, but the administration of EP along with CP caused a significant increase in the average percentage of fertilized oocytes in comparison with the CP group ( $p < 0.05$ ). The mean percentage of the two-cell embryos and blastocysts in CP+EP group was higher than the CP group ( $p < 0.05$ ), but they were lower than the control group ( $p < 0.05$ ). Results showed that the total number of arrested embryos in CP+EP group was significantly decreased in comparison with the CP group, but the reduction was not the same as control group ( $p < 0.05$ ). The percentage of type I, II and III arrested embryos in the CP+EP group was decreased in comparison with the CP group, but it was significant only in types I and II ( $p < 0.05$ ; Fig. 4 and Table 2).

## Discussion

Chemotherapeutic drugs exhibit antineoplastic function by an effect on a mitotic division pathway of malignant cells.<sup>31</sup> A study in this field has shown that besides the antineoplastic role, these drugs have cytotoxic side effects and cause a lot of free radicals production.<sup>32</sup> However, previous studies have revealed the protective role of antioxidants in the reduction of free radicals adverse effects in the body.<sup>33</sup> Accordingly, the high production of ROS by CP has been shown in serum.<sup>34</sup>

Several researchers have reported positive effects of EP on testicular and sperm damages leading to successful fertilization.<sup>35,36</sup> This finding confirmed in the present study by decreasing of serum SOD and increasing of testicular MDA in CP group. The EP as a substrate of mitochondria can provide the ATP for damaged cells, ameliorate the production of enzymatic antioxidants<sup>37</sup> and decrease MDA levels increased by CP, these results are in accordance with previous study.<sup>38</sup> It has been well understood that the oxidative stress results in intensive reduction of the serum antioxidant status via reducing the enzymatic potential including SOD and glutathione.<sup>39</sup> However, our biochemical analyses showed that administrating of EP along with CP resulted in remarkable up-regulation of serum SOD level. Moreover, the tissue MDA was evaluated as an oxidative stress indicator. The results of the present study revealed that EP significantly reduced the testicular MDA in CP+EP group. Therefore, we can conclude that EP diminished the CP-induced oxidative stress through enhancing the antioxidant capacity.

On the other hand, it has been reported that increased oxidative stress results in severe damages at Leydig cells level leading to a remarkable reduction at a serum level of testosterone.<sup>40</sup> Considering the critical role of testosterone in provoking the spermatogenesis, here in the present study we assessed the serum level of testosterone as a marker for endocrine status.



**Fig. 4.** Photomicrograph from pre-implantation embryos. **A)** Control group: Normal embryos that some of them are at the blastocyst stage (white arrows) **B)** CP group: Most of the embryos are in arrested and lysed conditions (black arrows). **C)** CP+EP group: A low number of embryos is arrested and lysed.

**Table 2.** In vitro fertilization parameters in different experimental groups (mean  $\pm$  SE).

Groups	Oocyte count	Fertilized oocytes (%)	Two-cell embryos (%)	Blastocysts (%)	Arrested embryos (%)	Type I arrested embryos (%)	Type II arrested embryos (%)	Type III arrested embryos (%)
Control	181	93.37	87.00	66.86	33.13	1.18	4.14	27.81
CP	148	75.67 <sup>a</sup>	75.00 <sup>a</sup>	17.86 <sup>a</sup>	82.14 <sup>a</sup>	11.60 <sup>a</sup>	14.29 <sup>a</sup>	56.25 <sup>a</sup>
CP+EP	152	87.50 <sup>b</sup>	80.45	37.59 <sup>ab</sup>	62.40 <sup>ab</sup>	0.00 <sup>b</sup>	0.00 <sup>ab</sup>	62.40 <sup>a</sup>

CP: Cyclophosphamide; EP: Ethyl pyruvate.

<sup>ab</sup> Different letters indicate the significant difference between control and the experimental groups, respectively.

This study showed that the thickness of the testicular capsule decreased in CP group. Oxidative stress may induce inflammatory mechanisms such as mast cells number increase leading to testicular capsular changes due to proteases secretion by these cells.<sup>41</sup> Observations showed that CP significantly down-regulated the serum level of testosterone and reduced the percentage of tubules with positive TDI, RI, and SI. Meanwhile, EP remarkably inhibited CP-induced derangements. Thus, it can be concluded that EP improved the testicular endocrine potential via antioxidant status up-regulation. Ultimately, it could be able to enhance the spermatogenesis through Leydig cells salvation from CP-induced oxidative stress. Higher numbers of active Sertoli cells in EP-treated animals confirm this theory. Considering the essential and key roles of Sertoli cells in structural evolution and maturation of germ cells in testis<sup>42</sup> as well as their physiological dependency for testosterone,<sup>43</sup> we can come close to this fact that CP-induced damages are partly dependent to Sertoli cells derangement. Accordingly, the spermatogenesis parameters (TDI, RI, and SI) were enhanced via Sertoli cells survival up-regulation in EP-treated animals.

The ROS production increase causes changes in all bases, removal, and uncoupling of complement bases, deformation and cross-linking of DNA strands and re-arrangement of chromosomes<sup>44</sup> and administration of CP in long-duration causes a significant breaking in DNA strands and cross-linking of DNA-DNA in the mice sperm.<sup>45</sup> It is also attendant with breaking of one or both strands of DNA.<sup>46</sup> The previous studies on human beings have confirmed the negative effect of sperm DNA damage on *in vitro* embryo development.<sup>46</sup> On the other hand, some reports have emphasized the role of antioxidants in DNA damage reduction and increasing fertility and implantation.<sup>36,47</sup> Hence, it could be concluded that the decrease in the rate of fertilization, two-cell embryos and blastocysts and generally low-level fertility in the CP group in comparison with other groups can be due to the function of CP metabolites such as phosphoramidate mustard causing cross-linking between DNA strands.<sup>18</sup> In spite of that, EP had protective effects by decreasing the percentage of sperm DNA damage and caused a significant increase in the percentage of fertility in the CP+EP group in comparison with the CP group. The major reason for the low percentage of embryo production in the IVF is the high level of ROS leading to the arrest in oocytes meiotic division<sup>48</sup> and embryo development along with apoptosis induction.<sup>16</sup> The rate of ROS production by sperm is inversely related to the sperm quality and fertilizing capacity in IVF.<sup>49</sup> In addition to all studies that showed the negative effects of CP on fertility rate, this study confirmed the protective effects of EP as a synthetic antioxidant causing notable fertility rate improvement in CP-treated mice via ROS production reduction.

This study showed that EP significantly ameliorated the CP induced impacts including testicular histological structure alterations as well as IVF and embryo developmental arrest through antioxidant status and testicular endocrine potential up-regulation. Therefore, EP increased the serum level of SOD and testosterone concentration and decreased tissue level of MDA minimizing the CP-induced derangements. Finally, EP could be considered as an appropriate chemical to be administered simultaneously by CP.

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### Conflict of interest

There are no conflicts of interest to be declared.

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