Protective Effect of Gallic Acid on Testicular Tissue, Sperm Parameters, and DNA Fragmentation against Toxicity Induced by Cyclophosphamide in Adult NMRI Mice

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**Purpose:** This study aimed to investigate the protective effect of gallic acid (GA) on the cyclophosphamide (CP) toxicity induced in the reproductive system.

**Materials and Methods:** After a pilot study for dose responses of gallic acid, forty adult male NMRI mice were divided into 5 groups (n=8): control, sham (NaCl Serum: 0.2mL per day), CP (15 mg kg⁻¹ per week; IP), GA (12.5 mg kg⁻¹ per day; IP) and GA (12.5 mg kg⁻¹ per day; IP) + CP (15 mg kg⁻¹ per week; IP). After treatment, the left testis was detached and used for histological examination and right testis used for malondialdehyde (MDA) measures. Left caudal epididymis was placed in the Ham’s F10 medium and released spermatozoa were used in order to analyze sperm parameters. Sperm DNA fragmentation was assessed by Sperm Chromatin Dispersion (SCD) method.

**Results:** In the CP group, there was a significant increase in the sperm DNA fragmentation (% 23.91 ± 57.89) compared with control group (% 10.27 ± 24.52). That was significantly improved by GA (12.5 mg kg⁻¹ per day; IP) in GA+CP group (% 8.85 ± 28.4) compared to CP group (p<.001). A significant increase was reported about MDA levels in CP group (2.59 ± 6.26) in compared with the control group (2.05 ± 4.30), But GA (1.33 ± 3.24) decreased it in GA+ CP group (p<.01). The histopathological investigation revealed marked testicular atrophy in CP group, whereas GA diminished these deviations (P<.05).

**Conclusion:** Gallic acid can modify the reproductive toxicity of cyclophosphamide in NMRI mice and increase the antioxidant capacity of testis tissue.

**Keywords:** cyclophosphamide; gallic acid; sperm; DNA fragmentation; toxicity

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**INTRODUCTION**

Infertility is the most important reproductive problem affecting about 10-15% of young couples(5). Several reasons may cause infertility, 35% of them occur because of male factors(5). In a review of the causes of male infertility, documented evidence suggests that injuries to sperm by reactive oxygen species (ROS) play an essential role on sperm motility(1). This can be caused by the use of antibiotics, toxic substances, pesticides, radiations, stress, air pollution, special medications used in the chemotherapy, and inadequate intake of vitamins(3).

Cyclophosphamide (CP) is one of chemotherapy drugs, which is widely used for treating various cancers and autoimmune diseases. With increasing the number of young people who have recovered from cancer, the risk of infertility has caused major concerns in these patients (1-3). Despite various clinical applications of this drug, it has an adverse influence on the reproductive system in humans and in the experimental animals(2,3).

Administration of CP is associated with oligospermia, azoospermia, and histological and biochemical changes in the testis and epididymis(6). Additionally, altering gonadotrophin secretion, testicular damage, and decreased plasma testosterone levels are observed in patients treated with CP, therefore fertility recovery is unpredictable in such patients and in some cases may last for years(6).

Although the defined mechanism by which CP causes testicular toxicity is unclear, in general it is known that CP interrupts with the balance of free radicals in the tissue and interferes with the antioxidant defense system(5).

The structure of DNA is very sensitive to oxidative damage(6). Oxidative damage to the DNA causes mutagenic changes, which in severe forms, disturb the quality of the germ cells and prevent fertilization. Oxidative stresses play a significant role in the germ cells’ apoptosis and DNA damage(6).

In general, damages caused by oxidative stress in human sperm is associated with reduced motility, morphological defects, increased sperm abnormality, DNA...
damage, lipid peroxidation and reduces the sperm acrosome reaction, capacitation, and fertility rates after Assisted Reproductive Technology (ART)(5,7-10). Oxidative stress can be destructive for testicular tissues, which have a high metabolism, so the antioxidant capacity of the tissue is very important(9). Thus, these studies indicate that administration of safe antioxidants might decrease CP-induced reproductive toxicity(4,11).

Gallic acid (also known as 3,4,5-trihydroxybenzoic acid), is a polyphenolic acid found in various plants such as Ceratonia Siliqua, green tea, sumac, grape seed, and apple. Gallic acid has antioxidant, anti-fungal, anti-viral, and anti-allergic activities. It also plays an important role in traditional medicine in some countries (13,14). As mentioned before, Gallic acid has antioxidant properties, which can protect the cells against oxidative damage by scavenging free radicals(12-14). Consequently, the present study was done to define whether Gallic acid has protective effects against CP-induced toxicity on sperm parameters, DNA fragmentation, and testicular tissue changes.

MATERIALS AND METHODS

In this experimental study, adult male NMRI mice (6-8 weeks old), weighing 20-25 g, were kept in wire mesh cages, under standard conditions with humidity of 30-60%, temperature of 20-25°C, and light/dark cycles of 12 hours and fed with commercial mice chow and water ad libitum and received humane care. All procedure on the animals were studied carefully and the protocol of the Ethics Committee of the Shahid Beheshti University of Medical Sciences was considered (IR.SBMU.MSP > REC > 1395.132).

Cyclophosphamide (CP) dose responses

Testicular and epididymal impairment was reached by single intraperitoneal administration of cyclophosphamide according to the report of Bakhtiari et al.(4).

Gallic acid dose responses

In various studies, the optimal amount of Gallic acid affecting the testes of the mice was variable and different(13,15,16); therefore, a pilot study was done for determining the optimal concentration of Gallic acid. To do this, 54 mice (6 mice per groups) were divided in 9 groups and were treated with different doses of Gallic acid (12.5, 25, and 50 mg kg⁻¹ per day;IP), CP (15 mg kg⁻¹ per week ;IP), CP+ Gallic acid co treatment (12.5, 25, and 50 mg kg⁻¹ per day ;IP), sham (NaCl Serum: 0.2 mL per day :IP), and then compared with the control.
group by analyzing sperm parameters (count, morphology, motility). The optimal concentration of gallic acid, which had the better results in evaluating the sperm parameters, was determined 12.5 mg kg⁻¹ per day. Samples size was estimated based on previous works on the testis and sperm parameters. After the pilot study, 40 male mice were allocated to five groups randomly and treated for 35 days: 1. The control group without any administration 2. The sham group that received NaCl solution (0.2 mL per day) intraperitoneally. 3. The CP group that received CP (15 mg kg⁻¹ per week) intraperitoneally. 4. The Gallic acid group that received only 12.5 mg kg⁻¹ per day of gallic acid intraperitoneally. 5. The Gallic acid + CP group that received gallic acid (12.5 mg kg⁻¹ per day) plus CP (15 mg kg⁻¹ per week) concurrent intraperitoneally.

**Preparation of drugs**

500 mg CP (Baxter, Germany) was prepared and dissolved in 50 mL of sterile 0.9 sodium chloride. Gallic acid powder was purchased from the SIGMA (USA-ALDRICH-G7384) and then dissolved in sterile Phosphate Buffered Saline (PBS).

**Histopathology**

Left testis was fixed with 10% formalin solution. Destruction of tissues was done in a graded series of ethanol and fixed in paraffin. Thin sections (4-5 μm) were made by microtome, after staining with hematoxylin and eosin, all slides were evaluated by light microscope.

**Lipid peroxidation level**

Malondialdehyde (MDA) levels of testis were measured by using the thiobarbituric acid (TBA) method at 532 nm, as explained by Ohkawa and colleagues in 1979. The level of MDA was defined as nmol / mg protein.

**Evaluation of sperm parameters**

At the end of the treatment period, the caudal epididymis was removed and sliced in 2 mL of Ham’s F10 medium with 1% BSA (bovine albumin serum). To swim sperm into the medium, the dishes were put in the CO₂ incubator of 37°C for 30 min. A smear of sperm suspension was made on a microscope slide then sperm counts and motility were assessed by the method defined in the WHO Manual, 2010. For the sperm morphology, 10 μm sperm was smeared then the slides were dried and stained by Diff quick kit (Avicenna, Iran). Percentage of spermatozoa with abnormal morphology was calculated.

**DNA fragmentation assessment**

Sperm DNA fragmentation was assessed by a sperm DNA fragmentation kit (Avicenna, Iran) using the sperm chromatin dispersion (SCD) method according to the manufacturer’s manual. Briefly, the sperm specimens were washed twice with the PBS buffer, and a suspension of 15-20 million sperm was prepared. Then, spermatozoa were immersed in agarose micro gel and smeared on the slide. In the order, denaturation by acid and lysis solution, dehydration and staining with Diff-quick were done.

Sperms with large halos (that were similar or larger than the diameter of sperm’s head) and sperms with medium sized halos (halo greater than 1:3 of the smallest diameter of the sperm's head and less than the smallest diameter) were defined as spermatozoa having no fragmentation.

**Stereological study**

Intestinal tissue volumes were evaluated by the Cavalieri method. The Cavalieri method was applied as a testis volume estimator. Thus, eight sections were chosen using a systematic sampling method and stereological estimations were started randomly. Each section of the samples was studied with a video-microscopy system (Nikon, E-200, Japan) which was linked to a video camera (SONY, SSC-D18P, Japan) a P4 PC computer, and a LG monitor (795 FT Plus). The stereological probe (points) was placed over the images of the tissue sections and was observed through the monitor. The following formula was used to estimate the testis volume:

\[
\text{Volume} = \frac{\text{number of points} \times \text{area of monitor}}{\text{area of tissue section}}
\]

**Table 3.** The effect of Gallic acid on the sperm parameters in the mice's treated with CP

<table>
<thead>
<tr>
<th>group</th>
<th>Sperm count(10⁶/ml)</th>
<th>Rapid Progressive sperm (%)</th>
<th>Slow progressive sperm (%)</th>
<th>No motile sperm (%)</th>
<th>Vitality (%)</th>
<th>Normal morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.23 ± 7.55</td>
<td>48.72 ± 7.26</td>
<td>23.22 ± 8.21</td>
<td>28.03 ± 5.87</td>
<td>72.04 ± 5.69</td>
<td>62.02 ± 6.65</td>
</tr>
<tr>
<td>Shau</td>
<td>33.37 ± 6.46</td>
<td>48.85 ± 10.05</td>
<td>24.56 ± 7.31</td>
<td>26.55 ± 4.40</td>
<td>74.94 ± 7.06</td>
<td>68.20 ± 8.74</td>
</tr>
<tr>
<td>GA</td>
<td>36.09 ± 5.31</td>
<td>58.91 ± 6.50</td>
<td>30.51 ± 18.32</td>
<td>48.79 ± 14.73</td>
<td>12.07 ± 34.74</td>
<td>47.68 ± 5.59</td>
</tr>
<tr>
<td>GA+CP</td>
<td>39.84 ± 6.61</td>
<td>53.76 ± 13.41</td>
<td>17.89 ± 3.04</td>
<td>23.17 ± 5.09</td>
<td>73.92 ± 5.33</td>
<td>63.37 ± 8.74</td>
</tr>
</tbody>
</table>

Statistical analyses were carried out through one way ANOVA/ Kruskal Wallis. Data are presented as means ± SD

a: p < .01 compared with control group.  
b: p < .001 compared with CP+GA group.  
c: p < .01 compared with GA group.  
d: p < .01 compared with sham group.

**Table 4.** The effect of Gallic acid on the forms of sperm morphological abnormalities in the CP-treated mice

<table>
<thead>
<tr>
<th>group</th>
<th>Abnormal head (%)</th>
<th>Abnormal mid-piece (%)</th>
<th>Abnormal tail (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.43 ± 4.01</td>
<td>22.03 ± 5.91</td>
<td>1.99 ± 1.78</td>
</tr>
<tr>
<td>Sham</td>
<td>10.12 ± 5.87</td>
<td>19.97 ± 7.73</td>
<td>1.55 ± 1.09</td>
</tr>
<tr>
<td>CP</td>
<td>14.15 ± 3.39</td>
<td>35.94 ± 6.60 ab</td>
<td>2.39 ± 0.98</td>
</tr>
<tr>
<td>GA</td>
<td>13.17 ± 5.13</td>
<td>21.79 ± 8.82</td>
<td>1.66 ± 1.68</td>
</tr>
<tr>
<td>GA+CP</td>
<td>12.82 ± 6.75</td>
<td>25.10 ± 9.19</td>
<td>1.62 ± 1.55</td>
</tr>
</tbody>
</table>

Statistical analyses were carried out through one way ANOVA/ Kruskal Wallis. Data are presented as means ± SD

a: p < .05 compared with control group.  
b: p < .05 compared with CP+GA group.
V (total) = \sum \left( \frac{A}{p \cdot d} \right)

Where the “V (total)” was the testis volume, “ΣP” was the sum of the points on the section profile, “a/p” was the zone linked with each point at the level of tissue, and “d” was the space between the samples’ sections. Each cell type was evaluated by this formula (N=number of cells counted/area of frame × number of frames × depth). The number of cells per testis was counted based upon NV (NV × testis weight). The germ cells were grouped into spermatogonia, primary spermatoocytes, round spermatids (RS), Sertoli cells, and Leydig cells.

Data analysis methods
All the data were analyzed using SPSS software. All the values were reported as mean ± SEM. All values were computed with the one way ANOVA/ Kruskal Wallis followed by post hoc Tukey test. P < .05 was considered as statistically significance.

RESULTS
As shown in Table 1, there was a significant difference between the mice’s testis weight (mg), total testis volume, and testis interstitial layer volume after 35 days treatment with CP and control group. Gallic acid administration with CP could significantly improved this reduction (P < .05). The total count of germ cells and Leydig cells was reduced by CP administration, whereas gallic acid co-treatment restored all these changes (P < .05) (Table 2).

Figure 1, shows the testes in the control, sham, and gallic acid groups with normal architecture and normal germinal epithelium (spermatogonia, primary spermatocytes, spermatids). Moreover, there were focal areas of atresia of the germinal layer. Interestingly, the testes of mice co-treated with CP and gallic acid seems normal.

The effects of Gallic acid on sperm parameters are shown in Table 3. The mean percent of progressively motile sperm was significantly reduced in CP group (20.67 ± 5.74 %) versus the control group (48.72 ± 7.26 %) (P < .001). In addition, it was noticed that it was significantly higher in gallic acid +CP group (53.76 ± 13.41 %) (P < .001). There was no significant difference in sperms with non-progressive motility between the groups (P = .17) but the immotile sperm was significantly increased in CP group (48.79 ± 14.73 %) (P < .001) and decreased in the CP+Gallic acid group (16.30 ± 10.79 %) compared with the control (28.03 ± 5.78 %) (P = .015) (Table 3).

A significant decrease was observed when comparing the sperms count in the CP group (19.71 ± 6.37) and the control group (31.23 ± 7.55) (P = .001). The count of sperms increased significantly in the CP and gallic acid group (39.84 ± 4.61) compared with the control (P = .008) and the CP group (P < .001) (Table 3).

The mean percentage of alive sperms (i.e. the viability of the sperm) decreased significantly in the CP group (34.71 ± 12.07 %) in comparison with all the other groups (P = .01). On the other hand, the vitality of sperm in the CP + gallic acid (77.19 ± 6.44 %) group was recovered (Table 3).

Considering the variety of sperm abnormalities (Figure 2), it can be seen that the mean percentage of sperms with normal morphologies decreased significantly in CP group (47.68 ± 5.59 %) compared with the control group (62.02 ± 6.65 %) (P = .001). In addition, the percentage of sperms with normal morphology revived in the CP plus gallic acid group (59.77 ± 9.93 %) (P = .008).
On the other hand, it was shown that treatment with cyclophosphamide led to abnormality in the mid-piece of sperm and in this regard, the CP group had a significant difference with control group ($P = .001$) (Table 4).

The CP-treated group ($6.26 \pm 2.59 \mu$moL/mg protein) showed a significant rise in the MDA concentration (Figure 3) compared with the control group ($4.30 \pm 2.05$). On the other hand, administration of gallic acid with CP ($3.24 \pm 1.33$) decreased the MDA concentration significantly in comparison with the CP group ($P = .002$).

As demonstrated in Figure 4, the percentage of DNA fragmentation was high in the CP group ($57.89 \pm 23.91\%$) in comparison with control group ($24.52 \pm 10.27\%$) ($P < .05$).

The concurrent administration of gallic acid and CP ($28.4 \pm 8.85\%$) significantly decreased the percentage of sperms with DNA fragmentation compared with the CP group ($P < .001$). Picture from DFI (DNA Fragmentation Index) was illustrated in Figure 5.

DISCUSSION

According to data obtained in this study, CP may cause reproductive toxicity through oxidative stress, which can be reduced by concurrent administration of gallic acid as an antioxidant.

In some studies, CP has the ability to produce free radicals, lipid peroxidation, and oxidative stress induction (20,21). Long-term use of CP in low doses may cause fertility system impairments in male mice (13,15,22) and the level of intracellular thiol has an important role in determining cell susceptibility to CP-induced damages (20).

4-hydroxycyclophosphamide can react with cellular thiols and produce a thioalkyl derivative which can induce oxidative stress (20).

Our results presented that the weight of the mice's testicles decreases significantly during the treatment in the CP group, which is in line with some other studies (24,25). The decrease in the number of seminiferous germ cells, Leydig cell lysis, and reduction in sperm production can be the possible reasons for the testicles weight loss. The testosterone level decrease along with Leydig cells lysis (21,26).

In addition to disrupting spermatogenesis, low concentration of testosterone associated with negative effects on epididymal tissue function can be associated with impairments in maturity and quality of the sperms (27). In addition, this weight loss can be attributed to a decrease in testicle size, the diameter of the seminiferous tubules, epithelial thickness, or increased interstitial space, which ultimately leads to the destruction of the testicle (24).

Our results suggest that the CP can cause reproductive toxicity on germinal layer through creating oxidative stress, although this toxicity effect could be reduced by concurrent administration of gallic acid as an antioxidant (13,16). Various studies have reported possible mechanisms of CP and its toxic metabolite, Acrolein, in producing ROS, lipid peroxidation, and oxidative stress (20,26,27). In addition, testicles are suitable places for the side effect of chemotherapeutic drugs, such as CP, that targeting highly proliferative cells as germ cells (27) since it has been confirmed the role of CP in oligospermia and azoospermia (28), as well as the destruction of spermatogenetic cycles in adult men treated with this drug (29). In present investigation, gallic acid was used as an antioxidant. (13,16). Table 3. On the other hand, it was shown that treatment with cyclophosphamide led to abnormality in the mid-piece of sperm and in this regard, the CP group had a significant difference with control group ($P = .001$) (Table 4).

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Figure 4. The effect of Gallic acid on DNA Fragmentation of sperm in mice's treated with CP.

Data are mean ± SE.

a: $p < .05$ compared with control group.

b: $p < .01$ compared with CP+GA group.

Figure 5. Sperm DNA fragmentation test using the Sperm Chromatin Dispersion (SCD) technique. When sperm classification is performed using the images provided by the SCD-Halosperm® method, normal sperm containing nonfragmented DNA are scored as the sperm population showing large or medium halos of dispersed chromatin surrounding a compact and well-defined core (1 N). Spermatozoa either containing small halos or no halos, i.e., leaving only the chromatin core visible, are considered as those containing fragmented DNA (1 S). Spermatozoa exhibiting highly degraded chromatin are characterized by the presence of small nucleoids presenting nonuniform or faintly stained chromatin core in association with the absence of a halo of dispersed chromatin after direct staining (1 D).
antioxidant to modify the side effects of oxidative stress induced by CP and we found it could prevent this destructive effects beside antioxidant capacity. Gallic acid indicated an anti-lipid peroxidation effects through decreasing Malondialdehyde (MDA) level\textsuperscript{(31)} which was higher in the CP group.  

Previous reports have revealed that increased MDA concentration is due to lipid peroxidation and reduced antioxidant enzymes levels resulted from oxidative damages\textsuperscript{(47) (48)}.  

Sperms mobility and viability are considered as the most important sperm parameters to measure the sperm membrane integrity and the fertilization ability. Spermatozoa are so sensitive to oxidative damage\textsuperscript{(25,33)}. Oxidative stress can lead to lipid peroxidation, ATP depletion, and disturbance in the axonemal protein phosphorylation process, decrease the function of the membrane and ion channel enzymes, and membrane fluidity, and consequently, loss of sperm motility\textsuperscript{(25,33)}. Cell death may occur following the ATP depletion, which can be the reason for reduced sperm viability in the present study\textsuperscript{(33)}.  

Various studies have reported the reduction of sperm motility during in vivo and in vitro studies\textsuperscript{(13,15,20,40)} and the sperm viability\textsuperscript{(25,33)} after treatment with CP. To support this idea, we have shown that gallic acid significantly modified the effects of CP on motility and viability of sperm.  

A significant reduction in epididymal sperms in CP-treated mice was observed that may be related to increase in the free radicals and oxidative stress\textsuperscript{(13,15,36)}, reduction in antioxidant defense system of testicle and sperms\textsuperscript{(37,38)}, testicular germ cells and Sertoli cells apoptosis caused by oxidative stress\textsuperscript{(39)}, changes in the concentration of sex hormones and disorders in the endocrine system\textsuperscript{(25,33)}, and destruction of the connections between Sertoli cells in testicles\textsuperscript{(40)}.

Gallic acid could modify the destructive effects of CP on the sperm count and morphology and triggered the survived germ cells to proliferate,\textsuperscript{(13,15,20,40)} by its antioxidant and anti-lipid peroxidation capacity \textsuperscript{(39,41-44)}. Surprisingly, there were two statistically significant differences between GA + CP and control groups in sperm count and no motile sperm parameters without significant difference between GA and control. Our hypothesis is that each of the substances (GA or CP) alone can have a good or bad effects on the reproductive system, but it is may be due to the synergistic effects of the two substances in condition of co-treatment that result in a destructive effects beside antioxidant capacity. Gallic acid indicated an anti-lipid peroxidation effects through decreasing Malondialdehyde (MDA) level\textsuperscript{(31)} which was higher in the CP group.

CONCLUSIONS

CP showed severe toxicity to the male reproductive system by elevating oxidative stress in the testicular and epididymal tissue, decreased spermatogenesis and morphologically mis-shaped sperm probably via stimulation of apoptosis and increased fragmentation of sperm DNA structure. This study suggests that using GA as antioxidant therapy with CP might have a protective effect against toxicity of CP on the reproductive system.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

REFERENCES


29. Shabanian S, Farahbod F, Rafieian M, Ganji...


