Impact Of Royal Jelly and L-Carnitine on Improving the Histological Changes of Cyclophosphamide-Induced Testicular Cytotoxicity in Adult Male Albino Rats

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ABSTRACT

Background: Cyclophosphamide is anticancer chemotherapeutic drug and immunosuppressive agent for organ transplantation. Wide range of adverse effects including reproductive toxicity demonstrated following its administration.

Aim of the study: Evaluation the impact of royal jelly and L-Carnitine on improving the histological and Immunohistochemical changes of experimentally Cyclophosphamide-induced testicular cytotoxicity in adult male albino rats.

Materials and Methods: forty adult male albino rats divided into two groups, the control group1 (ten rats) and treated group 2 (thirty rats) subdivided into three equal subgroups (a, b and c). Subgroup (a): Ten rats treated with cyclophosphamide. Subgroup (b): Ten rats given royal jelly with cyclophosphamide. Subgroup (c): Ten rats given L-Carnitine and cyclophosphamide. The testes excised and prepared for light microscopic examination and stained with H&E and Masson’s trichrome stains and immunohistochemically for detecting bcl-2 proteins.

Results: Seminiferous tubules distorted and shrunken with marked reduction in the thickness of the germinal epithelium that appeared detached from irreglar thick basement membrane and wide empty lumen devoid of spermatozoa, increased amount of interstitial tissue, contain congested blood vessels and covered by thick tunica albuginea. Royal jelly and L-Carnitine had a potent protective effect against the testicular toxicity and showed a notable effect.

Conclusion: Cyclophosphamide produced marked changes in the histological structure of the. Royal jelly and L-Carnitine have a potent protective effect against the toxicity.

Keywords: Cyclophosphamide; royal jelly; L-Carnitine; bcl-2.

INTRODUCTION

Survival rates from most childhood cancers have increased dramatically in recent decades, with chemotherapy a mainstay of treatment for the majority of these patients. Ideally, chemotherapeutic agents would specifically target cancerous cells with many off-target effects of cytotoxic treatments, including on the gonads. 1 Cyclophosphamide (CP) is a drug belongs to the class of oxazaphosphorines, which is essential anticancer chemotherapeutic drug, immunosuppressive for organ transplantation and many acute and chronic benign diseases as systemic lupus erythematosus, multiple sclerosis, and nephritic syndrome. Also effectively maintained remission of childhood nephrotic syndrome with frequent relapses. 2 A range of adverse effects of CP have been reported including reproductive toxicity, as diminished sperm counts and absence of spermatogenic cycles in their testicular tissue. 3

Royal jelly is one of honey products. It stimulates cell survival, cell growth and cell differentiation; it has anti-tumor and anti-metastatic effect. 4 Royal jelly and L-Carnitine (LC) found in epididymis and sperm, its concentration is about 2000 times higher than in plasma, it is important in lipid metabolism that facilitates long-chain fatty acids β-oxidation in mitochondria and is necessary for energy production. It acts as a substantial non-enzymatic antioxidant that protects the cell mitochondrial membrane, and DNA integrity against free oxygen radicals. 5 Several studies performed to evaluate the effect of carnitine on infertile men, indicating the
improvement of sperm fertility, count, and motility, so it is effective in improving fertility.

This work aimed to investigate the impact of royal jelly and L-Carnitine on improving the histological, Immunohistochemical changes of experimentally Cyclophosphamid induced testicular cytotoxicity.

MATERIAL AND METHODS

Forty rats used, divided into two groups; control group (1): ten rats injected intraperitoneal with normal saline. Second group: subdivided into three subgroups (a, b and c). (a): ten rats injected intra-peritoneal with cyclophosphamide solution at a dose of 50 mg/kg/day in every alternative day for 14 days. (b): ten rats given royal jelly orally at dose of 1000 μg/kg/day for 1 week before and with the use of cyclophosphamide at a same dose with second group. (c): ten rats given L carnitine intra-peritoneal at daily dose of 500 mg/kg/day for 1 week before and with the use of cyclophosphamide at a same dose with second group. Testes excised and put in Bouin’s fixative. Sections (5um thick) obtained and stained with H&E, Masson’s trichrome stains and immunohistochemically for localization of bcl-2 proteins.

Image analysis and morphometric study:

Stained sections were morphometrically analyzed using image analyzer computer system to detect the significance of changes in area% of collagen fibers, area% and optical density for bcl-2 proteins.

Statistical analysis:

The data analyzed by using SPSS version 23, expressed as means and standard deviation (SD) then analyzed statistically using one-way analysis of variance (ANOVA) for comparison between the different groups with p value less than 0.05 (the level of significance).

RESULTS

Group I: Testicular tissue formed of regular rounded or oval shaped sections of seminiferous tubules, surrounded by a regular thin basement membrane, and lined by seminiferous epithelium formed of spermatogenic cells at different stages of maturation with lumen full of spermatozoa (Fig. 1a). The interstitial tissue contains thin-walled blood vessels, cells of Leydig covered by a fibrous tunica albuginea (Fig. 2a). Group IIa: Seminiferous tubules markedly distorted with irregular outlines, smaller in diameters than control and show many vacuoles and the lumen is devoid of spermatozoa (Fig. 1b). The amount of interstitial areas increased and contain some thick walled congested blood vessels and increased number of cells of Leydig that arranged in small clumps and covered by a thick tunica albuginea (Fig. 2b). Group IIb: The majority of the seminiferous tubules appeared regular in their outlines and architecture with the appearance of the germinal cell populations including different types of germ cells with some vacuoles and the lumen contain spermatozoa (Fig. 1c). Interstitial tissue, similar to the control group, contains thin-walled blood vessels, cells of Leydig, covered by a thin fibrous tunica albuginea (Fig. 2c). Group IIc: Regular seminiferous tubules, normal architecture with the appearance of the germinal cell populations with lumen full of spermatozoa (Fig. 1d). Interstitial tissue similar to that of control group, thick walled blood vessels in some sections, covered by a thick tunica. (Fig. 3d).

Immunohistochemical study: (1) group I: Strong immune reactivity of bcl-2 protein in the spermatids, primary spermatocytes, however moderate reactivity in spermatogonia (fig.3a). Group IIa: Negative immune reactivity in spermatogonia with minimal immune reactivity in spermatids and primary spermatocytes (fig.3b). Group IIb: Strong expression of bcl-2 protein in the spermatids and primary spermatocytes, Spermatogonia showed moderate expression. (fig.3c). Group IIc: it was similar to (group IIb) (fig.3d).

Morphometrical and statistical results:

1. Area % of collagen fibers: Significant increase (P<0.001) in CP only treated (group IIa) in relation to control (group 1). Also, significant increase (P<0.01) in (group IIb) and (group IIc) in relation to (group 1) and a significant decrease in relation to (group IIa). However, there was a non-statistically significant difference (p > 0.05) in (group IIb) in relation to (group IIc). (Table 1& Fig. 4).

2. Area % of bcl-2 expression in the germinal epithelium: There was a significant decrease of expression in the germinal epithelium of seminiferous tubules (P<0.001) in (group IIa) in relation to (group 1). Also, there was a significant decrease in bcl-2 expression (p<0.01) in (group IIb) and (group IIc) in relation to (group 1), and a significant increase in relation to (group IIa). However, there was a non-statistically significant difference (p > 0.05) in (group IIb) in relation to (group IIc). (Table 2, Fig. 5).

DISCUSSION

Testis is considered the most important organ in male reproductive system. It is characterized by two main functions, synthesis of steroid hormones and production of spermatozoa. Various chemical factors affect spermatogenesis such as drugs and toxic elements. Spermatogenesis is the sophisticated process of sperm formation and maturation. It takes place in the seminiferous epithelium and is represented by continuous germ cell maturation towards the center, high incidence of apoptosis occurs to discard the excessive germ cells. Obeyes and Mahood, reported that the testicular dysfunction is a common long term sequel of cytotoxic drugs used in the treatment of many malignancies.
**Fig. 1:** Group (a): Seminiferous tubules (ST) lined by well-arranged epithelium have lumen (L) containing sperms with interstitial tissue (IT) showing Leydig cells (Lc) and normal blood vessel (Bv). Group (b): (ST) containing vacuoles (V), (L) devoid of sperms. (IT) showing (Lc), thick walled (Bv). Group (c): (ST) lined by well-differentiated epithelium and have (L) containing sperms and some (V). (IT) showing (Lc). Group (d): (ST) lined by well-differentiated germinal epithelium, (L) full of spermatozoa. (Hx&E, X400)

**Fig. 2:** Group (a) (ST) surrounded by thin basement membrane (yellow arrow). (IT) containing normal (Bv), covered by a fibrous tunica albuginea (T). Group (b): (ST) surrounded by thick basement membrane, (IT) showing great amount of collagen fibers (red arrow) covered by thick (T). Group (c): (ST) with regular basement membrane (arrow), covered by a thin fibrous (T). Group (d): (ST) surrounded by thin, regular basement membrane (arrow), (IT) showing fair amount of collagen fibers (arrowhead) and covered by thick (T). (Masson’s Trichrome Stain, X400).
**Fig. 3:** Group (a) Maximum expression of bcl2 in spermatids (red arrow) and primary spermatocytes (yellow arrows) with moderate immunoreactions in spermatogonia (arrow heads). Group (b) Median expression in primary spermatocytes (arrows), minimal immunoreactions in spermatogonia (arrowhead). Group (c) Positive in spermatids (red arrow), primary spermatocytes (yellow arrow) and moderate reactivity in spermatogonia (arrowhead). Group (d) Positive reactivity in spermatids (red arrow), primary spermatocytes (yellow arrow) and moderate in spermatogonia (arrowhead). (Immunoperoxidase technique for bcl-2 X400).

<table>
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<th>Collagen fibers</th>
<th>Mean &amp; SD</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>9.77 ± 2.176</td>
<td>10.30</td>
<td>6.10, 13.30</td>
<td>7.88, 11.13</td>
<td>&lt; 0.001</td>
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<td>Cyclophosphamide group</td>
<td>22.52 ± 3.024</td>
<td>22.10</td>
<td>18.70, 28.00</td>
<td>19.80, 24.92</td>
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<tr>
<td>L-Carnitine group</td>
<td>14.16 ± 2.775</td>
<td>14.80</td>
<td>8.20, 17.70</td>
<td>13.27, 15.70</td>
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<tr>
<td>Royal jelly group</td>
<td>16.52 ± 1.902</td>
<td>16.60</td>
<td>13.50, 20.30</td>
<td>15.23, 17.48</td>
<td></td>
</tr>
</tbody>
</table>

Data is expressed as mean and standard deviation, median, range and interquartile range. P is significant when < 0.05.

**Table 1:** Comparison between the area percent of collagen fibers among the studied groups.

**Fig. 4:** Variations of the area percent of collagen fibers means among the studied groups.
Histology

Table 2: Comparison between the area percent of bcl-2 expression among the studied groups.

<table>
<thead>
<tr>
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<th>Mean &amp; SD</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>5.98 ± 1.570</td>
<td>6.13</td>
<td>3.85, 8.22</td>
<td>4.50, 7.29</td>
<td>&lt; 0.001</td>
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<td>Cyclophosphamide</td>
<td>2.35 ± 1.393</td>
<td>2.49</td>
<td>0.57, 4.05</td>
<td>0.75, 3.71</td>
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<td>L-Carnitine group</td>
<td>4.05 ± 1.476</td>
<td>4.19</td>
<td>1.99, 5.88</td>
<td>2.46, 5.42</td>
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<tr>
<td>Royal jelly group</td>
<td>4.09 ± 1.146</td>
<td>4.54</td>
<td>2.36, 5.65</td>
<td>2.81, 4.94</td>
<td></td>
</tr>
</tbody>
</table>

Data is expressed as mean and standard deviation, median, range and interquartile range. P is significant when < 0.05.

Fig. 5: Variations of the area percent of bcl-2 expression means among the studied groups.

Since the period of spermatogenic cycle in rats is 12 days, the treated animals left 14 days in subgroup (a) and 21 days in subgroups (b) and (c), a period, which is more than one spermatogenic cycle, to achieve the morphological affection of cyclophosphamide treatment and protection by other agents.

Cyclophosphamide produced marked degenerative changes in the form of reduction of the diameter of the seminiferous tubules, distortion of their shapes, irregularity in outlines, thickening of their basement membrane and an arrest of the normal stages of development of their germinal epithelium with absence of mature sperms. Many vacuoles, more numerous towards the basement membrane, these findings were in agreement with those of Kamarzaman et al. Tunica albuginea was very thick as compared to control group confirmed by Masson’s trichrome stain. These findings correlated with observations of Kanth et al.

The oxidative stress caused by cyclophosphamide results in generation of reactive oxygen species (ROS) which play a critical role in the initiation and progression of fibrotic diseases. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the predominant enzyme source for ROS generation and now recognized as a key mediator of cell proliferation and matrix accumulation.

When adding royal jelly to the treatment with cyclophosphamide for two weeks showed marked degree of recovery from the testicular damage and spermatogenic cells reduced to few discrete layers, so lumen of the tubules appeared wide as compared to control. Testis of this group showed the majority of the seminiferous tubules appeared regular in their outline, germinal epithelium consisted of spermatogenic cells and Sertoli cells. The newly formed spermatogonia were next to the basement membrane, while the advanced spermatogenic stages were very close to the lumina in which the spermatozoa appeared and tubules surrounded by a thin basement membrane covered externally by a thin tunica albuginea. The interstitial tissue stroma, containing normal blood vessels, the results were in agreement with Karaca et al. The protective effect of royal jelly may be due to its components, such as antioxidants, vitamins A, E, C, D and B complex, these vitamins themselves had anticancer effect and protective effects against the genotoxicity of chemotherapy and radiotherapy.

The treatment with cyclophosphamide and LC showed also marked degree of recovery; the majority
of the seminiferous tubules appeared regular in their outline and the germinal epithelium consisted of spermatogenic cells and spermatooza appeared in the lumen. LC is a nutrient with antioxidant effects, which has pivotal roles in energy production as it facilitates the passage of fatty acids into the mitochondria matrix for oxidation. Since the main source of energy in testicular cells is fatty acid oxidation, LC role in transporting fatty acids into the sperm mitochondrial matrix for energy production is essential. 

The expression of Bcl-2 was downregulated in testicular tissues from cyclophosphamide treated rats compared to healthy controls. Similar findings reported by Hoda et al., that the germ cell apoptosis predominantly occurred in spermatogonia and to less extent in spermatocytes. Group (IIa) revealed negative reactivity in spermatogonia, minimal reactivity in spermatids and primary spermatocytes, CP induced apoptosis in the spermatogenic cells, as indicated by the highly significant decrease in the expression of bcl-2 protein. CP initiate a series of events that end in apoptosis. The ROS are key elements in this apoptosis, the amounts and activities of ROS-scavenging enzymes, including peroxidase, ascorbate, catalase and superoxide dismutase are strongly reduced with CP. These data imply that CP-exposed cells lose their ability to scavenge ROS and this loss results in oxidative damage and cell apoptosis. 

Group (IIb): Strong expression in the spermatids and primary spermatocytes, these findings were in agreement with Karaca et al, who studied the effects of (RJ) on testicular damage in streptozotocin (STZ)-induced diabetic rats. Cyclophosphamide with LC were nearly similar to group IIb this was in agreement with Roy et al., who indicated that mice fed with LC showed increased PGC1α levels in the testis, which probably regulates steroidogenesis by increasing Bcl2 mRNA abundance. In addition, LC effectively prevents apoptosis by carrying the accumulated long chain fatty acid surrounding mitochondria in testes of diabetic rats. These findings were in agreement with Vardiyen R et al, who found that an increase in bcl-2 expression and a decrease in Bax expression in mice administered both formalin and L-carnitine. Mohammadi V et al, found out that LC successfully increased expression of bcl-2 gene in rooster’s testis. Finally, oxidative stress leads to an increase in the release of cytochrome C from the mitochondria, which activates the apoptotic mechanism in the cells by activating caspases (caspase-3/7, 8 and 9) and pushing the cells to the programmed cell death. Kooppeyna, F et al, demonstrated that treatment with L-carnitine significantly reduced the gene expression of Caspase-9 and significantly increased the gene expression of Bcl-2.

CONCLUSION

the present work showed that cyclophosphamide treatment produced hazardous effects on the histological structure of the testis. These effects appeared as destruction in the germinal epithelium and sperms with marked reduction in spermatooza. These alterations may lead to infertility. Supplementation with Royal jelly or L-carnitine produces relative improvement in the testicular tissue parameters.

REFERENCES


