POSSIBLE PROTECTIVE EFFECT OF CROCIN ON CISPLATIN-INDUCED CHANGES OF TESTIS OF PREPUBERTAL ALBINO RAT: HISTOLOGICAL, IMMUNOHISTOCHEMICAL AND MOPHOMETRIC STUDY

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ABSTRACT

Background: Cisplatin is a common antineoplastic drug used to treat various solid cancers. Crocin possesses anti-inflammatory, anti-cancer, antioxidant, and memory-enhancing properties. This study aimed to study the effect of CIS on prepubertal rat testes and protective effect of Crocin on CIS-induced testicular toxicity. Material and Methods: 90 (5-days) old albino Wistar rats were divided into 3 main groups (n = 30 each): (control negative group); rats weren’t given any treatment. (Control positive 1): received 0.15 ml saline /day intraperitoneal (i.p) only for 5 days before scarification. (Control positive 2): received Crocin at a dose of (200mg/kg) for 4 consecutive days. (CIS treated group): received Cisplatin (CIS) as a single subcutaneous injection in the back of the neck one day just before scarification and subsequent laparotomy at a dose of 5μg/g. (CIS+Crocin group): received 200 mg/kg of Crocin for 4 consecutive days before treatment with CIS. The last dose of Crocin was given one hour before CIS. Each group was further divided into 3 subgroups based on rat age on the day of sacrifice (PND11, PND17, and PND30). Rats were anesthetized; their testes were extracted and subjected to histological, immunohistochemical, and morphometric examinations. Results: CIS administration significantly decreased body weights, testicular weights, and serum testosterone levels in all age’s groups. Also, it caused degeneration of testicular cells with loss of cellular organization, marked cellular atrophy, and disruption of tubular basement membranes. A significant decrease in tubular diameter and a significant increase in percentage of apoptotic cells were observed in the CIS-treated rats. Crocin administration minimally mitigated these hazardous effects of CIS in the prepubertal rat testes. Conclusion: These findings demonstrated that CIS has a cytotoxic and apoptotic effect on the testicular germinal epithelium and Crocin could minimally improve these alterations. Keywords: Cisplatin, Crocin, germinal epithelium, male prepuberty, spermatogonia.

INTRODUCTION

Cisplatin (cis-diamminedichloro platinum -II; CIS) is a platinum-based chemotherapeutic drug most commonly used as an anti-neoplastic agent for various cancers, such asovarian, cervical, bladder, and testicular malignancies. It can be used alone or in conjunction with other medications to treat cancers in children and adolescents (Kelland, 2007).

The toxicity of CIS appears to be dose-dependent owing to the buildup of this drug in the organs, which induces necrotic alterations within the tissues. Moreover, the toxicity may be attributed to the production of reactive oxygen species (ROS) or nitrogen species (NS) and the interaction with the DNA of the cells (Sheriff et al., 2017). The testes play both exocrine and endocrine roles; they are known to be involved in the production of spermatozoa in the seminiferous tubules and the generation of testosterone by leydig cells in the interstitial compartment. Spermatozoa are produced via spermatogenesis, which involves cell division and differentiation and begins in adolescence. After adolescence, this process can be differentiated into three stages: spermatogonial proliferation, spermatocyte meiosis (which produces haploid spermatids), and spermiogenesis (which entails the differentiation of spermatids into spermatozoa). The duration of spermatogenesis is determined by the species; for example, humans take 74 days to form the spermatozoa, whereas, in rats and mice, the...
duration is reported to be 52 and 35 days, respectively (Delessard et al., 2020).

The prepubertal stage is a susceptible period for reproductive development due to the occurrence of both spermatogenesis and steroidogenesis during this time; consequently, prepubertal testes are more sensitive to hazardous substances (Ing et al., 2018; Yaman and Topcu-Tarladacalisir, 2018). Several studies have evaluated the reproductive effects of CIS treatment, alone or in conjunction with other chemotherapeutic agents in adults. However, the consequences of this drug during the various phases of reproductive development remain unknown (Johnson et al., 2010). Although few reports have demonstrated the effect of CIS on prepubertal rat testes, the different stages of development, especially during the infantile and juvenile stages, have not been examined (Allen et al., 2020; Hamam et al., 2022).

However, Smart et al. (2018) reported a decrease in the spermatogonia pool with lesion in the spermatogonia stem cells (SSCs) and spermatogonia and also reduce the sertoli cell number on postnatal day (PND) 7 in mice exposed to chemotherapeutic drugs. Likewise, Stumpp et al. (2004) reported decreased spermatocyte counts in rats at PND30 after chemotherapeutic exposure.

Restoring fertility mainly depends on the status of the germ cell population in the testes, which is primarily determined by the presence of a sufficient number of SSCs. Chemotherapeutic medicines primarily affect the survival of these stem cells (Allen et al., 2020).

Saffron, or Crocus sativus, is a rare and valuable Chinese herbal medicine that improves blood circulation and eliminates blood stasis (Abdullaev and Espinosa-Aguirre, 2004). It is a natural product made from the dried stigma of the Crocus sativus flower. Crocin, crocetin, carotene, and safranal are some of the biologically active chemicals in the saffron extract. Crocinahas antioxidant, anti-cancer, anti-inflammatory, and memory-improving qualities (Das et al., 2010). Additionally, it is reported to have anti-hyperlipidemia, anti-atherosclerotic, cardioprotective, hepatoprotective, and neuroprotective properties (Goyal et al., 2010; Jnaneshwari et al., 2013).

Crocin has a substantial anti-tumor impact, an important trait for a fetoprotective substance; additionally, it can reduce the damaging effects of cyclophosphamide, an anti-cancer medicine with potent pro-oxidant properties, on the ovary (Di Emidio et al., 2017).

THE AIM OF THE WORK
The aim of this study was to evaluate the effect of Crocin on Cisplatin-induced damage in the testis of prepubertal rats.

MATERIAL AND METHODS

I. Chemicals
- Cisplatin (CIS): it was derived from EIMC (Egyptian international medical center) United Pharmaceuticals, Badr Industrial City, Egypt in vials. Each vial (50mg/ 50ml) was dissolved in normal saline (0.9% sodium chloride).
- Crocin (C_{44}H_{66}O_{24}): it was derived from Sigma-Aldrich, American fork, USA, as a red powdered form. 2 g of Cronin powder was diluted with 200 ml of distilled water as a stock solution to create the therapeutic dose.

II. Experimental animals:
The study used 90 newborn albino wistar rats all of which were (5-10 gm) and their age was 5 days. The animals were collected from the animal house at Zagazig University's Faculty of Medicine. The newborn rats were housed with their mothers in plastic cages with free access to commercial food pellets and water at room temperature (20-26°C) and normal relative humidity before the beginning of the experiment for acclimatization. All through the experiment, the rats were kept in 12-hour light/dark cycles. All experimental procedures were carried out in compliance with Zagazig University Institutional Review Board's guidelines for Institutional Animal Care (IRB) (ZU-IACUC/3/F/164/2021)

III. Experimental design:
The rats were divided into three main groups, each with 30 rats:

GroupI: (Control group) was subdivided into (control negative group); rats weren’t given any treatment all through the experiment, (control positive1) that received 0.15 ml saline /day intraperitoneal (i.p) only for 5 days before scarification (Naghizadeh et
Possible protective effect of Crocin...

al., 2008) and (control positive 2) where rats were given Crocin at a dose of (200mg/kg) for 4 consecutive days (Naghizadeh et al., 2008).

Group II (CIS treated group): received Cisplatin (CIS) as a single subcutaneous injection in the back of the neck one day just before scarification and subsequent laparotomy at a dose of 5 μg/g equivalent to the recommended therapeutic dosage (Bodenner et al., 1986). The subcutaneous route was chosen since it provides a longer period of absorption (Gad, 2019).

Group III (CIS + Crocin group): the rats in this group received both Crocin and Cisplatin. Crocin was given at a dose of (200mg/kg) for 4 consecutive days before beginning treatment with Cisplatin. The last Crocin dose was one hour before the single dose of Cisplatin (Naghizadeh et al., 2008).

Each main group is subdivided into three subgroups (11, 17 and 30) with 10 rats in each subgroup based on the rat’s age at the day of scarification i.e., all rats of the same age subgroup were sacrificed at the mentioned day: PND 11 was the day for extracting testes from subgroup 1 rats, PND 17 from subgroup 2 rats, and PND 30 from subgroup 3 rats. At the specified days, scarification followed by laparotomy was performed for each animal after anesthesia via intraperitoneal injection of sodium thiopental (75 mg/kg) (IACUC, 2013). The animal weight was recorded before laparotomy. After laparotomy, the testes were retrieved and their weights were reported.

IV. Hormonal analysis
Under anesthesia, blood samples were collected from the left ventricle using an injector. Whole blood centrifugation (3000 g, 20 min, and 4°C) was performed to obtain the serum. The amount of testosterone in the serum was tested using an enzyme-linked immunosorbent assay kit from the Cayman Chemical Company (Ann Arbor, MI, USA), according to the manufacturer’s recommendations (Rao et al., 1990).

V. Histological analysis
The extracted testes were preserved in 10% formalin, bathed in distilled water, and dried in a graded sequence of ethanol (70%–100%) before embedding in paraffin (Merck Millipore, Darmstadt, Germany). Subsequently, the samples of 5 μm thickness were stained with hematoxylin and eosin (H & E) for general architecture assessment (Bancroft and Gamble, 2008) or Periodic Acid Schiff (PAS) for detecting carbohydrate molecules found in the basal lamina and connective tissue (Bancroft and Gamble, 2008).

VI. Immunohistochemical analysis:
1. For proliferative cellular nuclear antigen (PCNA) (Yaman and Topcu-Tarladacalisir, 2018): 5 um formalin-fixed, paraffin-embedded sections were utilized. They were rehydrated and deparaffinized and then exposed to Heat-mediated antigen retrieval. After that, the sections were treated to 3 % H2o2 for 10 min to stop endogenous peroxidase activity. An HRP biotinylated secondary antibody solution (Invitrogen, Carlsbad, CA) was used after 1 h of merging with PCNA primary antibody (Novus Biologicals, Littleton, CO) diluted 1: 2,000 in phosphate buffer solution at room temperature. Diaminobenzidine was used to see the immunoreactivity (DAB, Vector Laboratories, and Burlingame, CA). Hematoxylin was used to counterstain the sections.

2. For Caspase3 (Saral et al., 2016): Polylsine-coated slides were used to mount 5um thick sections. Following rehydration, samples were placed in citrate buffer (pH 7.6) and processed in an oven (1200) for 10 min then left for cooling at room temperature, after that, the sections were washed in phosphate-buffered saline (PBS). Then, the fragments were placed in 0.3 % H2o2 for 7 min until they are washed with PBS. Primary rabbit-polyclonal Caspase-3 antibody (Ab4051, Abcam Ltd, Cambridge, UK) was incubated for 2 hours on sections (Picut et al., 2015). They were soaked with PBS and incubated with biotinylated goat antipolyvalent for 10 min at room temperature. After 15 min of chromogen positive substrate base, the slides were mixed for 1 min with Mayer's haematoxylin then rinsed with water and dehydrated.

VII. Morphometric analysis:
At a magnification of 400, the diameters of seminiferous tubules from at least 30 rounded
tubular profiles that were chosen randomly from H&E slides and measured for each animal, basement membrane thickness from PAS stained slides and the area percentage of the PCNA and Caspase 3 immunolabeled cells from the immuno-stained slides were all morphometrically assessed. The PCNA cell count was calculated by counting the number of PCNA positive cells stained with brown color. A double-blinded investigator methodology was used to score the slides at a magnification of 400 xs. Data were estimated using image analyzer software (Leica Qwin 500 Image Analyzer, England) at the Image Analyzing unit in the Human Anatomy and Embryology Department, Faculty of Medicine, Zagazig University, Egypt. The measurement units (Pixels) were automatically changed to micrometers by calibration. Ten non-overlapping fields were randomly picked from five serial sections of each specimen in the different groups and evaluated (Alsemeh et al., 2022).

STATISTICAL ANALYSIS:
The statistical analyses were performed using Statistical Package of Social Sciences version 22 (SPSS), and the results were presented in tables and graphs. Quantitative variables, such as weight, were expressed as the mean±standard deviation. After checking for normality, appropriate statistical tests of significance were performed. P-value of <0.05 was considered significant, and that of < 0.001 was considered highly significant (Emsley et al., 2010).

RESULTS

Body weights and testicular weights

The mean body weights of the rats were highly significantly different between the control group and the CIS-treated or CIS+Crocin groups at all ages (P<0.001). Furthermore, a significant difference in body weight was observed between the CIS-treated and CIS+Crocin groups at PND17 (P=0.04) but not at PND11, (P=0.06) and PND 30 (P=0.14) (Table 1).

As regard testicular weight, a highly significant (P<0.001) difference was observed between the control and CIS-treated groups at all ages. Furthermore, a significant difference was noted between the CIS-treated and CIS+Crocin groups at PND11 and 17 (P=0.003), but not PND 30 (P=0.56). No significant difference was seen between the control and CIS+Crocin groups at PND11 (P=0.17); however, a significant and highly significant differences were noted at PND17 (P=0.002) and PND 30 (P<0.001), respectively (Table 2).

Plasma testosterone level

A highly significant (P<0.001) difference in the serum testosterone level was seen between the control and CIS-treated groups at all ages. Highly significant differences were observed between the control and CIS+Crocin groups at PND11 and 17 (P<0.001) while at PND30, the difference was a significant between them (P=0.002). Likewise, a significant difference in the testosterone level was noted between the CIS-treated and CIS+Crocin groups at PND11 and 30 (P=0.002), but not at PND 17 (P=0.09) (Table 3).

Histological and morphometrical results

H&E results

Examination of all the control subgroups (control negative, control positive 1, control positive 2) revealed nearly the same results; hence, the results of one group were reported in this study.

H& E staining examination results revealed that in control group of PND11 the testis was covered with thin normal tunica albuginea and it is composed of multiple rounded or oval solid seminiferous tubules without a lumen. The tubules were separated by interstitial tissue containing few blood vessels and clusters of acidophilic spindle-shaped cells, called the interstitial cells of Leydig. The tubules were lined with multiple spermatids and spermatogonia, constituting the pseudo stratified layer. The spermatids were identified as pyramidal cells with elongated to polygonal nuclei (Fig. 1A, 1B).

In CIS-treated group of PND11, the tubules appeared smaller in diameter and distorted in shape. Some of their lining cells were sloughed in the inside the tubules. The tunica albuginea was thick and fibrotic and the tunica vasculosa consisted of dilated and congested blood vessels. The tubules were separated by wider spaces with areas of congestion at some sites (Fig. 1C, 1D).

In CIS+Crocin group of PND11, the sizes and shapes of the tubules were enhanced,
to some extent. Leydig cells were seen in the narrower interstitial spaces compared to those seen in the CIS group (Figs. 1E, 1F). The lining cells (spermatogonia, sertoli cells) were distinguishable.

Multiple seminiferous tubules were visible in the testis when H&E stained sections from the PND17 control group. These tubules are surrounded by thin normal tunica albuginea. The diameters of the tubules and the number of the lining cells increased with age. On the 17th day, most tubules appeared having a lumen, while others did not have a lumen (Figs. 2A, 2B). The tubules were surrounded by a thin basement membrane upon which the spermatogonia and pyramidal sertoli cells rested. Near to the tubular lumen, the spermatocytes appeared as large cells with rounded nuclei; minimal interstitial tissue consisting of leydig cells was observed between the tubules (Fig. 2B).

In CIS Treated group of PND17, the tunica albuginea was thicker, and the tubules were disorganized and smaller in diameter compared to those in control groups. The tubules were separated by wide interstitial spaces. There were areas of hemorrhage in between the tubules (Figs. 2C, 2D). Loss of normal tubular cellular stratification with cellular disorganization was observed. The lining cells showed vacuolation with darkly-stained pyknotic nuclei. Some cells were sloughed within the tubular lumens (Fig. 2D).

Improvement in the tubular pattern with some distortion was seen in CIS+Crocin group of PND17 (Figs. 2E, 2F). The lining epithelium showed some signs of organization with distinct cellular layers within normal tubules. Some cells demonstrated sloughing, and some presented with loss of the regular arrangement (Figs. 2E, 2F).

In control group ofPND30, the diameters of the seminiferous tubules were increased, and the lumens were located at the center and the surrounding tunica albuginea was thin (Figs. 3A, 3B). The lining epithelium became more stratified with the progress in the phases of spermatogenesis. The lining cells from the tubular basal membrane to their lumens were arranged as, the spermatogonia presented as rounded cells with spherical nuclei on the basement membrane, separated by pyramidal sertoli cells, the spermatocytes on the inner side of the tubules appeared larger, with large spherical nuclei grouped in single or double layers. In addition to the rounded spermatids, which was located near the lumen of the tubules. The tubules were lined by thin basement membranes and separated by interstitial spaces containing leydig cells (Fig. 3B).

The tunica albuginea was found to be thicker in CIS Treated PND30; the seminiferous tubules appeared irregular and shrunken. The tubules showed loss of stratification and disarray of the lining epithelium. Some germ cells were detached from the basement membrane and exfoliated toward the lumen with the disappearance of the early rounded spermatids (Figs. 3C, 3D), whereas other cells presented with vacuolation. Some spermatogenic cells appeared dark with pyknotic nuclei. Branching was seen in some tubules, whereas others were filled with acidophilic material (Fig. 3D).

**Tubular diameter and thickness of tunica albuginea**

A highly significant (P<0.001) difference in tubular diameter was observed between the control of PND11 (49.9±2.1), PND17 (55.1±1.8), PND30 (162.4±16.7) and CIS-treated groups of PND11 (34.7±2.9), PND17 (45.7±4.3), PND30 (110.1±6.5), (P<0.001). No significant difference was noted between the control and CIS+Crocin groups at PND11 (45.8±2.3), PND17 (51.2±0.6) (P>0.05) but at PND 30; still a significant difference exhibited between the control and CIS+Crocin group (139.4±4.2), (P<0.05). A highly significant difference was noticed between CIS treated group and CIS+Crocin group at PND11 and PND30 P<0.001but a significant difference in tubular diameter was observed at PND17 (P<0.05) (Fig. 4A).

Regarding thickness of tunica albuginea, there was a high significant difference (P<0.001) between control of PND11 (3.8±0.4), PND17...
Results of PCNA immunostaining
Proliferating cell nuclear antigen (PCNA) immunohistochemical staining among the different studied groups in different stages of development was shown in Figure 6. In control group, the seminiferous tubules revealed abundant positive PCNA immunostaining in the nuclei of the germ cells in different stages of development (Figs. 6A, 6D, 6G). In contrary, CIS treated group exhibited decreased in PCNA immunopositive cells and increase in PCNA unlabeled cells during the different stages of the developing germ cells (Figs. 6B, 6E, 6H). Interestingly, PCNA immuno-positive cells were increased inside the tubular germ cells in the CIS+Crocin group; however, few unlabeled cells were still seen in this group (Figs. 6C, 6F, 6I). Morphometrical and statistical analysis were carried out to demonstrate the changes in the area % of PCNA and PCNA cell count among the different studied groups respectively at the different ages. As regard area % of PCNA, there was a high significant difference (P<0.001) among the three studied groups at all different ages (PND11, PND17, and PND30). In relation to PCNA cell count; there was a high significant difference (P<0.001) between the control and CIS-treated and between the CIS-treated and CIS+Crocin groups at all different ages. Also the difference between control and CIS+Crocin groups was highly significant (P<0.001) at PND11 and PND30 while it was only significant (P<0.05) at PND17 (Fig. 6J).

Results of Caspase 3 immunostaining
Caspase 3 was used to determine the degree of apoptosis in the testicular cells among the different studied groups in different stages of development as shown in Figure 7. In the control groups, no Caspase 3 reactivity was observed in the germ cells and interstitial cells of Leydig at all the different ages (Figs.7A, 7D, 7G). Alternatively, strong positive cellular immunoreactivity was detected for Caspase 3 in the germ cells and interstitial cells in the CIS-treated group at all the different ages (Figs. 7B, 7E, 7H). In the CIS+Crocin group, a few negatively-stained germinal and interstitial cells were detected, along with some positively-stained germ cells (Figs.7C, 7F, 7I). Morphometrical and statistical analysis were carried out which revealed a significant difference (P<0.05) in the area percentages of labeled cells for Caspase 3 between the control and CIS-treated groups and CIS-treated and CIS+Crocin groups for all ages. No significant differences were noted between the control and CIS+Crocin groups at PND11 (P>0.21), PND17 (P>0.06), and PND30 (P>0.05) (Fig. 7J).
Table (1): Comparative table between Control groups and treated group with cisplatin and Group III (CIS and Crocin) at different ages regarding rat weight.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Control group</th>
<th>CIS Treated group</th>
<th>CIS + Crocin group</th>
<th>F</th>
<th>P</th>
<th>Post hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>PND11</td>
<td>18.51± 0.91</td>
<td>15.15± 1.18</td>
<td>16.03±0.75</td>
<td>32.72</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001<strong>¹ &lt;0.001</strong>² 0.06 NS³</td>
</tr>
<tr>
<td>PND17</td>
<td>26.02± 1.67</td>
<td>19.70± 1.24</td>
<td>21.25±1.85</td>
<td>42</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001<strong>¹ &lt;0.001</strong>² 0.04³</td>
</tr>
<tr>
<td>PND30</td>
<td>68.54± 3.51</td>
<td>52.03± 5.38</td>
<td>55.18±3.65</td>
<td>42.22</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001<strong>¹ &lt;0.001</strong>² 0.14 NS³</td>
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</table>

F: ANOVA test     Post hoc: Tukey’s test
**: Highly significant (P<0.001), *significant P-value <0.05
P1: Control versus CIS treated group,
P2: Control versus CIS + Crocin group,
P3: CIS treated versus CIS + Crocin group

Table (2): Comparative tables between Control groups and treated group with cisplatin and Group III (CIS and Crocin) at different ages regarding Testicular weight.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Control group</th>
<th>CIS Treated group</th>
<th>CIS + Crocin group</th>
<th>F</th>
<th>P</th>
<th>Post hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>PND 11</td>
<td>15.02± 1.85</td>
<td>12.06±0.70</td>
<td>13.92±1.59</td>
<td>10.43</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001**¹ 0.17 NS² 0.003*³</td>
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<tr>
<td>PND 17</td>
<td>47.50± 3.52</td>
<td>41.16±1.24</td>
<td>42.84±0.97</td>
<td>21.77</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001**¹ 0.002<em>² 0.03</em>³</td>
</tr>
<tr>
<td>PND 30</td>
<td>301.8± 11.08</td>
<td>278.5±10.63</td>
<td>281±8.13</td>
<td>16.26</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001<strong>¹ &lt;0.001</strong>² 0.56 NS³</td>
</tr>
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F: ANOVA test     Post hoc: Tukey’s test
**: Highly significant (P<0.001), *significant P-value <0.05
P1: Control versus CIS treated group,
P2: Control versus CIS + Crocin group,
P3: CIS treated versus CIS + Crocin group

Table (3): Comparative tables between Control groups and treated group with cisplatin and Group III (CIS and Crocin) at different ages regarding Testosterone hormone level.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Control group</th>
<th>CIS Treated group</th>
<th>CIS + Crocin group</th>
<th>F</th>
<th>P</th>
<th>Post hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>PND 11</td>
<td>0.83± 0.03</td>
<td>0.58± 0.06</td>
<td>0.65±0.01</td>
<td>108.48</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001<strong>¹ &lt;0.001</strong>² 0.002*³</td>
</tr>
<tr>
<td>PND 17</td>
<td>0.89± 0.04</td>
<td>0.66±0.07</td>
<td>0.72±0.08</td>
<td>33.1</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001<strong>¹ &lt;0.001</strong>² 0.09 NS³</td>
</tr>
<tr>
<td>PND 30</td>
<td>0.91± 0.09</td>
<td>0.71±0.03</td>
<td>0.79±0.05</td>
<td>26.43</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001**¹ &lt;0.002<em>² 0.03</em>³</td>
</tr>
</tbody>
</table>

F: ANOVA test     Post hoc: Tukey’s test
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P1: Control versus CIS treated group,
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P3: CIS treated versus CIS + Crocin group

F: ANOVA test     Post hoc: Tukey’s test
**: Highly significant (P<0.001), *significant P-value <0.05
P1: Control versus CIS treated group,
P2: Control versus CIS + Crocin group,
P3: CIS treated versus CIS + Crocin group

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P1: Control versus CIS treated group,
P2: Control versus CIS + Crocin group,
P3: CIS treated versus CIS + Crocin group
Possible protective effect of Crocin

**Fig. (1):** Photomicrographs of sections of testis of albino rat (PND 11) among the different studied groups: Control group (A, B) shows many rounded to oval solid seminiferous tubules (ST) with Leydig cells (LC) and blood vessels (BV) in interstitial tissue between tubules and covered with normal thin tunica albuginea (TA) as seen in the inset of figure 1A. The tubules are lined with rounded spermatogonia (Sg), pyramidal Sertoli cells (Se.C). CIS treated group (C, D) shows smaller disorganized tubules (STt) separated by wide interstitial space (green star). The blood vessels (red BV) are thickened and congested in tunica vasculosa and in between tubules. The germinal cells are sloughed (green wavy arrow). The tubules are covered with thick tunica albuginea (TA) and some germ cells are pyknotic with dark stained nuclei (blue wavy arrow) as seen in the inset of figure 1C. Testis of CIS + Crocin group (E, F) shows both normal seminiferous tubules (ST) and still some few distorted ones (STt) with Leydig cells (LC) in between. Wide interstitial tissue (green star) can be seen in between tubules. The lining cells are obvious spermatogonia (Sg), Sertoli cells (Se.C). The tubules are covered with slightly thin tunica albuginea (TA) as seen in the inset of figure 1E. (H&E A, C, E X 100, Scale bar=200 μm; B, D, F X 400, Scale bar=50 μm).

**Fig. (2):** Photomicrographs of sections of testis of albino rat (PND 17) among the different studied groups: Control group (A, B) shows many rounded to oval seminiferous tubules (ST) with their lumens (L). The tubular basement membrane is thin and well defined (thin black arrow). Germinal cells are arranged as spermatogonia (Sg), spermatocytes (Sc) and separated by some Sertoli cells (Se.C). Tubules are separated by scanty interstitial tissue containing Leydig cells (LC) and covered with normal thin tunica albuginea (TA) as seen in the inset of figure 2A. Testis of CIS treated group (C, D) is covered with thick tunica albuginea (TA) as seen in the inset of figure 2C. Testicular tubules are disorganized (STt) and separated with wide interstitial spaces (green star) and surrounded by thick basement membrane (thick black arrow). The germinal cells are either atrophied or sloughed in center of tubules (green wavy arrow) or vacuolated (black curved arrow). Areas of Congestion (brown wavy arrow) and hemorrhage (Hg) in between the tubules are also noticed. Testis of CIS + Crocin group (E, F) shows recovered normal tubules (ST) with clear lumen (L) and covered with slightly thin tunica albuginea (TA) as seen in the inset of figure 2E. There are normal lining cells (spermatogonia (Sg), spermatocytes (Sc) and Sertoli cells (Se.C) surrounded with thin basement membrane (thin black arrow). Other few tubules are still distorted (STt) with thick basement membranes (thick black arrow). (H&E A, C, E X 100, Scale bar=200 μm; B, D, F X 400, Scale bar=50 μm).
Possible protective effect of Crocin

Fig. (3): Photomicrographs of sections of testis of albino rat (PND 30) among the different studied groups; Control group (A, B) shows seminiferous tubules (ST) with central lumens (L) surrounded by thin well defined basement membranes (thin black arrow) and covered with normal thin tunica albuginea (TA) as seen in the inset of figure 3A. The tubules are lined with many layers of cells arranged as spermatogonia (Sg) near the basal membrane with Sertoli cells (Se.C) in between, spermatocytes (Sc) and rounded spermatids (Sp). Leydig cells (LC) are seen in between tubules. Testis of CIS treated group (C, D) is covered with thick tunica albuginea (TA) as seen in the inset of figure 3C. Testicular tubules show disorganizing (STt) or branching (black star). Some tubular epithelium shows areas of shedding (black wavy arrow). The lining germinal cells show vacuolation (black curved arrow), pyknosis (black arrow heads) or sloughing in center of tubules (green wavy arrow). The basement membrane was thick (black thick arrow). Acidophilic material (black right angled arrow) appears in the center of some tubules. Testis of CIS + Crocin group (E, F) shows some recovered normal tubules (ST) with recovered normal lining cells; spermatogonia (Sg), Sertoli cells (Se.C), spermatocytes (Sc) and rounded spermatids (Sp) and covered with slightly thin tunica albuginea (TA) as seen in the inset of figure 3E. Other tubules are still distorted (STt) with shedding of the lining cells (black wavy arrow). There are areas with thin basement membrane (thin black arrow) and others with thick one (thick black arrow).

(H&E A, C, E X 100, Scale bar=200μm; B, D, F X 400, Scale bar=50μm)

Fig. (4): Bar charts demonstrate the changes in the diameter of tubules (A) and thickness of tunica albuginea (B) at different ages among the different studied groups. Values are mean of 10 rats per group ± S.D. Highly significant P-value <0.001, significant P-value <0.05, non significant P-value >0.05, * Control versus CIS treated group.

Control versus CIS+Crocin group. * CIS treated versus CIS+Crocin group. Duplication of the symbols (aa, bb, cc) means that the difference is highly significant.
Fig. (5): Photomicrographs of PAS-stained sections of testis of albino rat among the different studied groups at different ages (PND 11, 17, 30): Tubules of control group (A, D, G) are lined with thin well defined intact basement membrane (double thin black arrows). While the tubules of CIS treated group (B, E, H) shows either disruption or degeneration (empty black arrow heads) or thickening at some sites (double red arrows). In CIS + Crocin group (C, F, I): the basement membrane shows either thinning and regularity (double thin black arrows) or thickening (double red arrows). (J) Bar chart demonstrates the changes in the thickness of the basement membrane at the different ages from the different studied groups. Values are mean of 10 rats per group ± S.D. Highly significant P-value <0.001, significant P-value <0.05, non significant P-value >0.05. a Control versus CIS treated group. b Control versus CIS+Crocin group. c CIS treated versus CIS+Crocin group. Duplication of the symbols (aa, bb, cc) means that the difference is highly significant. (PAS ×400, Scale bar=50μm).
**Fig. (6):** Photomicrographs of PCNA stained sections of testis of albino rat among the different studied groups at different ages (PND 11, 17, 30). Control group (A, D, G), CIS treated group (B, E, H) & CIS + Crocin group (C, F, I). (Black zigzag arrow) indicating dark brown staining of PCNA immuno-positive cells.

(J, K) Bar charts demonstrate the changes in the area % of PCNA and PCNA cell count among the different studied groups respectively at the different ages. Values are mean of 10 rats per group ± S.D. Highly significant P-value <0.001, significant P-value <0.05, non significant P-value >0.05. * Control versus CIS treated group. **Control versus CIS+Crocin group. ***CIS treated versus CIS+Crocin group. Duplication of the symbols (aa, bb, cc) means that the difference is highly significant.

*(PCNA immunoperoxidase stain counter stained with H.; X400, Scale bar= 50 μm)*
**DISCUSSION**

Cisplatin (CIS) is the commonest antineoplastic chemotherapeutic agent for various cancers; however, its use is restricted due to its negative effects, particularly on the kidney and nervous system. Testicular dysfunction is the most common complication of CIS poisoning due to the high rate of testicular growth (Amin et al., 2012). The effects induced by chemotherapy treatment on the prepubertal gonads are much less well understood than those on the adult gonads. The lack of prepubertal researches might be attributed to a lack of animal experimental sets to study prepubertal testis development, or it could be attributable to the reality that the prepubertal testis was originally assumed to be largely dormant (Rey, 1999).

Crocin is a dicarboxylic acid diester created from the disaccharide, gentiobiose, and the dicarboxylic acid, crocetin. It is a water-soluble carotenoid known for its antioxidant properties (Jnaneshwari et al., 2013).

Regarding (In this study) the body weights of rats in the CIS-treated group were lower than those in the control group at all ages. A similar finding was reported in a previous study (Almeer and Abdel Moneim, 2018) comprising mature male Wistar albino rats treated with a single dosage of CIS for 5 days. In another study (Yucel et al., 2019),
a decrease in the weights of adult rats treated with a single intraperitoneal dose of CIS was reported; the authors attributed this to body starvation linked to the harmful effects of CIS. Similar to the study by Ilbey et al. (2009), found that a significant decrease in testicular weight was observed between the CIS-treated and control groups in the current study. Likewise, similar findings were reported in another study (Adejuwon et al., 2015a), which observed substantial testicular parenchymal atrophy in rats exposed to CIS treatment that could probably be linked to CIS-induced oxidative damage in the rat testes. On the contrary, Kaya et al. (2015), found that CIS did not influence the testicular weights of rats, despite being treated with the same doses; this discrepancy could be due to variations in the sensitivity to the medication (Yucel et al., 2019).

In the present study, Crocin did not significantly restore the decrease in testicular weight in rats treated with CIS at PND30. In a recent study, which investigated the effects of different doses of Crocin (6.25, 25, and 100 mg/kg) on rats injected with CIS, the highest dose (100mg/kg) considerably prevented the loss of both body weight and testicular weight in the CIS-treated rats (Mesbahzadeh et al., 2021). The results of the CIS+Crocin group in the current study were significantly different from those in the control group. This was coped with (Vafaei et al., 2020) in an experiment to explore effect of Crocin on testes of male Balb/c mice exposed to electromagnetic field, which may be due to the short duration of exposure.

Tousson et al. (2014) reported a substantial drop in serum testosterone concentrations in 9-week-old rats treated with CIS; this finding was corroborated in the current study and could be explained by the CIS-induced destruction of the Leydig and Sertoli cells (Maines et al., 1990). Moreover, long-term CIS administration can cause hypophysitis-testis depression and a decrease in testosterone levels (Tousson et al., 2014). However, treatment with Crocin improved this effect in the current study. In the study by Mesbahzadeh et al. (2021), 6.25 and 25 mg/kg of Crocin did not influence the decreased levels of testosterone in animals treated with CIS; however, 100 mg/kg of the drug resulted in a dramatic improvement in the testosterone level.

Several mechanisms may be involved in the drop in the testosterone level following CIS treatment. According to Beytur et al. (2012), CIS may interfere with LH receptor expression, impede cholesterol mobilization to mitochondrial cytochrome P450scc (or lower the activity of this enzyme), and interact with the earliest stages in testosterone generation. In (2016), Soniet al., found that CIS harms the testes by affecting the amount of the StAR protein (Soni et al., 2016). StAR protein is required for cholesterol transport from the external to the internal mitochondrial membrane to produce testosterone. StAR protein was considerably reduced in the CIS-treated groups compared to those in the control group, indicating that testosterone release was hindered in the CIS-treated rats (Soni et al., 2016).

In the current study, the testes of rats in the CIS-treated group presented with a thickened tunica albuginea and an irregularly thickened basement membrane. A similar finding was reported in another study (Mohammadnejad et al., 2012), which employed an electron microscope to observe the thickening of the basement membrane in CIS-treated rats; according to the authors, this might have been caused by the breakdown of actin filaments in the basement membrane of myoid cells, or by an increase in actin production by Sertoli or myoid cells, or by a decrease in proteolysis. PAS staining revealed a thickening of the tubular basement membrane in the CIS-treated group. Similar findings have been reported in irradiated rats and after efferent ligation, which could be attributed to myoid cell contraction caused by the secretion of oxytocin and prostaglandins from the damaged epithelial cells (Sawada and Esaki, 2003).

Widening of interstitial spaces was detected in the testes of animals in the CIS-treated group. Lirdi et al. (2008), reported a similar finding after exposing prepubertal rats to one dose of CIS. The volume of the testicular interstitial space can increase for various reasons, including changes in testosterone
levels (Maddocks and Sharpe, 1989) and cytotoxic poisoning (Lirdi et al., 2008). Several pathological changes were observed in the germ cells of animals from the CIS-treated group; for example, vacuolation of the germinial epithelium has been reported in previous studies (Favareto et al., 2011) and (Brilhante et al., 2012). The first authors explained this as a result of the presence of immature germ cells following the disruption of the sertoli cell by CIS. Smart et al. (2018), observed the same outcome when using cytotoxic medicines, such as cyclophosphamide, CIS, or doxorubicin, in an in vitro model of prepubertal mouse testicular tissue. Furthermore, cellular detachment and sloughing observed in the current study were also reported in a previous study comprising adult rats exposed to CIS (Mohammadnejad et al., 2012); separation of the spermatogonial cells from each other and the basal lamina was considered as a pre-apoptotic sign. In the present study, Crocin improved the histological effects of CIS to some extent. Khorasani et al. (2021), reported considerable improvements in diabetes-induced testis alterations in rats after treatment with Crocin.

The degenerative testicular changes observed in the current study have been reported in previous studies. In (2014), Harman and Richburg utilized an undifferentiated spermatogonial stem cell pool and niche from adult mice (C57/BL/6J) to determine the effect of CIS on the testis (Harman and Richburg, 2014). They reported significant cell loss, disorder of the seminiferous epithelium, and apical sloughing with shedding of cellular debris and stated that the predominant types of cells observed in the seminiferous tubules were spermatogonia and Sertoli, indicating complete disruption of normal spermatogenesis (Harman and Richburg, 2014). Agu et al. (2020), discovered degenerative changes in the testes of adult rats treated with CIS via intraperitoneal injection in the form of germ cell degeneration, disruption in the arrangement of the germ cell layers, and necrotic germ cells.

In the present study, the blood vessels in the interstitial tissue of the CIS-treated rats were dilated and congested. Abd-Elhafiz et al. (2021), reported similar findings in CIS-treated rats compared to the control group, which could be attributed to a significant drop in the serum testosterone level in the treated rats; the use of coenzyme Q10, improved this issue. Interstitial congestion has been reported in rat kidneys treated with CIS (Alhoshani et al., 2017). Likewise, the tubular branching observed in the current study has been previously described as an anomaly in patients born with testicular and respiratory anomalies and was reported with oligospermia and infertility (Averback, 1980). Many theories exist about the mechanics of CIS cytotoxicity. It is thought that CIS forms covalent adducts with cytosine and guanine-rich DNA regions, impairs basic cellular activities such as replication, transcription, and DNA repair, and results in programmed cell death (apoptosis) (Jung and Lippard, 2007). According to Fallahzadeh et al. (2017), CIS causes oxidative stress by creating ROS and NS, which cause cellular damage and necrosis via lipid peroxidation of triglycerides. Furthermore, it may cause cellular inflammation or DNA fragmentation and death (Eid et al., 2016).

In the present study, the findings of a decreased PCNA-stained area (in %) in the CIS-treated rats compared to those in the control group were consistent with the results of a previous study (Yaman and Topcu-Tarladasilasir, 2018). Lopes et al. (2021) examined the histological abnormalities in prepubertal mouse testis exposed to CIS and Doxorubicin in vitro and found that both medications caused loss of SSCs. This effect was mostly observed with increased dosages of CIS, which inhibited the development of the germinative epithelium over time. It was suggested that germ cell injury could deleteriously impact the supporting somatic sertoli cells. Treatment with Crocin improved this effect.

A recent study (Rossi et al., 2021) showed that crocetin exposure could reduce the gonadotoxic effects of ionic radiation in pubertal rats. Although, according to earlier research, this improvement was much closer to the control outcomes. According to the findings of this research, there is
a considerable difference between the control and CIS+Crocin groups only for the earliest age. This could be due to a brief time of exposure.

In a recent study (Allen et al., 2020), the exposure of PND5 testes to CIS resulted in a decrease in the number of proliferative germ cells in the prepubertal testis, which was attributed to the medication targets and destruction of proliferating cells or the prevention of subsequent germ cell multiplication.

A significant increase in the area percentage of tissues positively-stained with Caspase 3 was observed in the CIS-treated group. Similar findings have been reported previously (Mohammadnejad et al., 2012; Coşkun et al., 2013), wherein increased percentages of apoptotic cells were observed in CIS-treated adult rats. Coşkun et al. used Caspase3,8, and 9 to demonstrate the apoptotic impact of CIS on the testes and proposed that CIS injection triggered apoptosis by activating procaspase-3; according to them, apoptosis could occur via both intrinsic and extrinsic pathways. The intrinsic pathway was more effective. This was also emphasized in another study (Adejuwon et al., 2015b), which demonstrated apoptosis and germ cell death after CIS therapy using TUNEL labeling. Apoptosis plays an important role in removing damaged spermatogonial cells and preventing aberrant sperm production.

Crocin therapy improved CIS-induced apoptosis in the present study. Fani et al. (2018), demonstrated the ameliorating effect of Crocin on testicular apoptosis in the male pups of Atrazine-treated female dogs. Similarly, another study (Potnuri et al., 2018), reported decreased apoptosis in the testes of rats administered with Crocin and cyclophosphamide.

A significant decrease in the mean tubular diameter was noted in the CIS-treated group compared to the control group in this study. Similar findings were reported in previous studies, which theorized as epithelial thinning and sloughing (Aldemir et al., 2014; Saral et al., 2016). In another study, 7-week-old male rats exposed to CIS showed a substantial decrease in the mean seminiferous tubular diameter (Kohsaka et al., 2020). However, Yaman and Topcu-Tarladacalisir (2018), reported a slight difference in the adult group and no significant difference in the mean tubular diameter of seminiferous tubules in the prepubertal phase. This discrepancy in findings may be due to differences in the methods of CIS administration. Yaman and Topcu-Tarladacalisir used a single intraperitoneal dose of 5 mg/kg of CIS, and the observed changes were partially improved following Crocin treatment at some PND ages. Similar findings were reported in another study (Vafaei et al., 2020), which examined the combined effect of Crocin with electromagnetic fields emitted from mobile phones on Balb/c mice testis; improvements in both tubular diameter and epithelial thickness were seen, statistical significance notwithstanding.

Crocin has garnered scientific attention and has earned a significant place in pharmaceutical research owing to its anti-inflammatory, antioxidant, anti-spasm, anti-depression, anti-cancer, and neuroprotective characteristics. In one study, Crocin promoted the levels of antioxidant enzymes and testosterone, and minimized testicular damage in cyclophosphamide-treated mice (Bakhtiyari et al., 2014). In accordance with these findings, improvements in the histology and the testosterone level and a decrease in Caspase 3 were observed in the current study.

CONCLUSION

In summary, CIS decreased the number of proliferating germ cells in the testes of prepubertal rats and caused extensive apoptosis along with disruption of the germinal epithelium; Crocin improved these alterations to some extent. However, it can be recommended that additional studies focusing on CIS-targeted germ cell types are required to determine the mechanisms of injury to prepubertal testis and how Crocin can preserve the testes exposed to Cisplatin.

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