The protective Role Of Honey-Be products Against The Genotoxic Effects Of Cyclophosphamide in Male Mice


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ABSTRACT

The protective effects of honey-bee products (HP) (1 gm/kg.b.w) against genotoxicity induced by Cyclophosphamide (CP) were investigated in mice. 20 mg/kg.w of cyclophosphamide were dissolved in 150 ul (high concentration dose, CP1) or in 300 ul (low concentration dose, CP2) of sterilized distilled water. Then, high dose (CP1)/animal or low dose (CP2)/animal were used and injected intraperitoneally. The HP treatments were assessed at the same time of CP injection or two weeks before using CP treatment. CP treatment induced significant increases of chromosome aberrations, DNA fragmentation and sperm-shape abnormalities as well as a significant depression of mitotic activity as compared to control group. Also, CP injection revealed a marked damaging effect on liver tissues. CP in a high dose caused severe damages of genetic, sperm and histological parameters as compared to low dose. The treatment with HP at the same time of injection of CP (CP1 HP or CP2 HP groups) or two weeks before using CP (HP CP1 HP or HP CP2 groups) significantly ameliorated the genetic, sperm and histological parameters as compared to the treatment with CP alone. However, the best protective effect was observed by using the HP treatments at the same time with CP injection, where, more improvements of genetic materials and sperm morphology as well as more normalization of liver tissues occurred in CP1 HP or CP2 HP groups than those observed in HPCP1 or HPCP2 groups. The results of most genetic and sperm examinations, except some histological measurements showed that there were no significant differences between the protective effect of HP against high dose (CP1) of CP and the protective role of HP against low dose (CP2) of CP, suggesting the potent antimutagenic activity of the HP. In conclusion, the present study demonstrated that side dangerous effect of anti-cancer drug CP could be avoided by using the treatment of honey-bee products especially at the same time with CP injection. This strategy is necessary for diminishing the deleterious side effects of anti-cancer drug with preservation of its chemotherapeutic efficacy.

Key words: Cyclophosphamide, Honey-bee products, Mice, Chromosome aberrations, DNA damage, Sperm morphology, Histopathology.

Introduction

Cyclophosphamide (CP) is extensively used as an antineoplastic agent for treatment of various cancers as well as an immunosuppressive agent (Dollery, 1999). This antineoplastic agent undergoes bioactivation by hepatic microsomal cytochrome P450 mixed function oxidase system to active metabolites that enter the circulatory system. Phosphoramide mustard and acrolein are the two active metabolites of cyclophosphamide (Luderman, 1999). The antineoplastic effects of cyclophosphamide are associated with phosphoramide mustard, whereas, acrolein is linked to toxic side effects like cell death, apoptosis, oncrosis and necrosis (Kern and Kehrer, 2002). In spite of its therapeutic importance, a wide range of adverse effects including genetic and reproductive toxicity as well as histological alterations have been demonstrated following cyclophosphamide treatment in humans and experimental animals (Anderson et al., 1995; Selvakumar et al., 2006; Rezvanfar et al., 2008; Jalali et al., 2011). The generation of free radicals and other reactive oxygen species (ROS) as well as Lipid peroxidation (LPO) have been reported to be major mechanisms in CP toxicity (Anderson et al., 1995; Murata et al., 2004; Vernet and Aitken, 2004; Tripathi et al., 2011; Jalali et al., 2011). Wherein CP and its metabolite acrolein in liver cause inactivation of microsomal enzymes and results in increase ROS and LPO (Lear et al., 1992; Anderson et al., 1995; Jalali et al., 2011). Subsequently single and double strand scissor of DNA are produced (Vijayalaxmi and D’Souza, 2004; Asita and Molise, 2011; Amador et al., 2012) leading to chromosome aberrations and depression of mitotic index as a result of disturbance of DNA replication (Gimmilerluz et al., 1999; Sumanth et al., 2011). On the other hand, adult male patients treated with CP have demonstrated diminished sperm counts and an absence of spermatogenic cycles in their testicular tissue (Kern and Kehrer, 2002). Also oligospermia, azospermia and histological alterations in the testis and epididymis have

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been confirmed in CP-treated experimental animals (Meistrich et al., 1995; Kaur et al., 1997; Jalali et al., 2011; Ramos et al., 2013). Moreover, the decrease in weight of reproductive organs, impaired fertility, growth and development of next generation were also observed in CP-treated male rats (Trasler et al., 1986; Jalali et al., 2011; Abrikwu et al., 2012). Spermatozoa are more susceptible to peroxidative damage because of high concentration of polyunsaturated fatty acids and low antioxidant capacity (Vernet and Aitken, 2004; Jalali et al., 2011; Ramos et al., 2013). Consequently, from above aforementioned studies, combination of the CP drug together with potent and safe antioxidant may be the appropriate approach to reduce its mutagenicity and reproductive toxicity. The natural honey-bee products such as pure honey, royal jelly and pollen grains have been found to have antioxidant and antimutagenic factors. Several studies have shown that these products are rich in flavonoids, phenolic acids, some enzymes (e.g. glucose oxidase, catalase), ascorbic acid, carotenoid-like substances, organic acids, amino acids and proteins (Beretta et al., 2005; Vela et al., 2007; Estevinho et al., 2008; Alvarez-Suarez et al., 2010). Many of these compounds have been shown to be cytoprotective by scavenging superoxide anion, hydroxyl radical, hydrogen peroxides, other reactive oxygen species (ROS) and reducing lipid peroxidation (Facino, 2001; Mckibben and Engeseth, 2002; Meda et al., 2005; Lopez-Lazaro, 2006; Baltrusaite et al., 2007; Beretta et al., 2007; Alvarez-Suarez et al., 2010). So, the present study was designed to evaluate the protective role of honey-bee products (honey, royal jelly and pollen grains) against CP-induced genotoxicity in mice. Chromosome analysis, mitotic index calculation, DNA fragmentation test, sperm morphology examination and histopathological assay have been investigated.

Materials and Methods

Experimental Animals:

Male Swiss Albino mice (Mus musculus) three months old weighting 25-30 grams were obtained from the Animal House Lab at National Research Centre, Giza, Egypt. The animals were maintained on standard casein diet and water ad libitum at the Animal House Laboratory and housed in a temperature-controlled 25±3.2°C on light/dark cycle of 12/12 hours and artificially illuminated room, free from any source of chemical contamination.

Cyclophosphamide (CP) or Endoxan:

Cyclophosphamide (CP) was purchased from Sigma chemical Company (st. Louis, Mo, USA).

Honey-bee products:

Honeys or Honey-bee products (pure honey, Royal Jelly and Pollen Grains) were obtained from national pharmacy in Cairo, Egypt. These products were collected from bee-house.

Drugs Preparation:

Cyclophosphamide (CP) drug:

20 mg/kg.b.w.of CP were dissolved in 150 ul (high concentration dose, CP1) or in 300 ul (low concentration dose, CP2) of sterilized distilled water. The high dose (CP1)/animal or low dose (CP2)/animal were used and injected intraperitoneally, according to method of Walker and Bole, (1973) and Premkumar et al. (2004).

Honey-bee products (HP) drug:

1 gm/kg.bw of HP (500 mg of honey+ 250 mg of Royal jelly+ 250 mg of Pollen grains) was dissolved in 10 ml of sterilized distilled water. Then 0.3 ml of this solution was used and injected intraperitoneally/animal, according to method of Wang et al. (2002); Al-Waili (2003) and Schramm et al., (2003).

Experimental Design:

Mice were randomly divided into eight groups (10 mice for each) as follows:

- Negative control group; animals were fed only on normal basal diet and water.
- The second group was injected with 0.3 ml of prepared honey-bee products drug /animal/day for two weeks. These animals were also fed on basal diet and water.
- The third group was injected with drug of Cyclophosphamide (CP1 dose) for only one time. Then animals were fed on basal diet and water for two weeks.
- The fourth group was injected with honey-bee products (0.3 ml /animal) at the same time with injection of CP1 dose (only one time). Then the animals treated with honey-bee products for two weeks. The animals were also fed on basal diet and water.
- The fifth group was injected with drug of Cyclophosphamide (CP1), and then these animals were injected with drug of Cyclophosphamide (CP1 dose) for only one time. After that, the animals were injected with drug of honey- bee products for two weeks. Through these periods, the mice fed on basal diet and water.
- The sixth group was injected with drug of Cyclophosphamide (CP2 dose) for only one time. Then animals were fed on basal diet and water for two weeks.
- The seventh group was injected with honey-bee products at the same time with injection of CP2 dose (only one time). Then the animals treated with honey-bee products for two weeks. The animals were also fed on basal diet and water.
- The eighth group was injected with drug of honey- bee products for two weeks before injection with Cyclophosphamide (CP2 dose), then these animals were injected with drug of Cyclophosphamide (CP2 dose) for only one time. After that, these animals were injected with drug of honey- bee products for two weeks. Through these periods, the animals fed on basal diet and water.

**Sampling Schedule:**

After the above treatments of the last dose for each group, mice were tested for genetic and histopathological analyses. Animals were injected intraperitoneally with 0.5 ml of colchicine (0.05 %) solution for two hours before sacrifice. Then, animals were sacrificed by cervical dislocation. Immediately, bone marrow, testis and liver samples were collected.

Bone marrow samples were used to study the chromosome aberrations and mitotic index. Testis cells were examined for sperm morphology analysis. The liver tissues were analyzed for DNA fragmentation and histopathological changes.

**Chromosome analysis:**

Metaphases for analysis of chromosomal aberrations in bone marrow cells were prepared according to the method of Preston et al. (1987). At least 50 Metaphases were scored per animal.

For structural chromosomal aberrations analyses, breaks, gaps, deletions, centromeric attenuations (CA) and endomitosis were considered. For numerical chromosome aberrations analyses, aneuploidies, polyploidy were considered. For mitotic activity of cells, the number of dividing cells were recorded and the mitotic index was calculated as the following formula: Mitotic index % (M.I)= the number of dividing cells/Total number of bone marrow cells counted/ per 1000 cells.

**DNA fragmentation:**

Liver samples were collected immediately after sacrificing the animals. The tissues were lysed in 0.5 ml lysis buffer containing 10 mM tris-HCl (PH 0.8), 1 mM EDTA, 0.2 % triton X-100, centrifuged at 10000 r.p.m. (Eppendorf) for 20 minutes at 4ºC. The pellets were resuspended in 0.5 ml of lysis buffer. To the pellets (P) and supernatants (S), 1.5 ml of 10% trichloroacetic acid (TCA) was added and incubated at 4ºC for 10 minutes. The samples were centrifuged for 20 minutes at 10000 r.p.m. (Eppendorf) at 4ºC and the pellets were suspended in 750 µl of 5% TCA, followed by incubation at 100ºC for 20 minutes. Subsequently, to each sample 2 ml of DPA solution (200 mg DPA in 10 ml glacial acetic acid, 150 µl of sulfuric acid and 60 µl acetaldehyde) was added and incubated at room temperature for 24 hours (Gibb et al., 1997). The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula:

\[
\text{DNA fragmentation} = \frac{\text{OD of fragmented DNA (S)}}{\text{OD of fragmented DNA(S) + OD of intact DNA (P)}} \times 100
\]

**Sperm morphology analysis:**

For sperm-shape analysis, the epididymis excised and minced in about 8 ml of physiological saline, disappeared and filtered to exclude large tissue fragments. Smears were prepared after staining the sperms with Eosin Y (aqueous), according to the methods of Wyrobek and Bruce (1975; 1978) and Narayana (2008). At least
1000 sperms from each mouse were examined for morphological abnormalities under a light microscope (400 x).

**Histopathological Examination:**

Specimens of liver were dissected from all animals immediately after killing, washed thoroughly with formal saline and then fixed in 10% neutral-buffered formal saline for 72 hours at least. All the specimens were washed in tap water for half an hour, dehydrated in ascending grades of alcohol (70-90-95 % absolute), cleared in xylene and then embedded in paraffin wax. Serial sections of 6 um thick were cut and stained with Haematoxylin and eosin (Drury and Wallington, 1980) for histopathological investigation. Images were captured and processed using Adobe photoshop version 8.0.

**Statistical Analysis:**

Statistical analysis was performed with SPSS software. Data were analyzed using one-way analysis of variance (ANOVA) followed by Durcan’s post hoc test for comparison between different treatments in same sex. The values were expressed as mean ± S.E. and difference were considered as significant when P< 0.05.

**Results:**

**Chromosomal analysis:**

Chromosomal examination in the present study (Table.1) showed structural and numerical aberrations. Structural chromosomal aberrations were chromatid breaks, chromatid gaps, deletions, centromeric attenuations (CA) and endomitosis. Numerical aberrations included aneoploidy and polyploidy.

The present results showed that the mice group treated only with honey- bee products (HP group) had few of chromosomal aberrations as compared to control group. Statistical analysis showed that there were no significant differences between the two groups. The only exception to this the frequencies of total numerical aberrations were significantly decreased in HP groups than those of control group.

On the other hand, the groups of mice injected with CP1 or CP2 doses had more frequencies of structural chromosome aberrations (especially chromatid breaks) than control group. Statistical analysis showed significant differences (p<0.05 or p<0.01) for the frequencies of total structural aberrations (especially chromatid breaks) between control group and CP1 or CP2 groups. The CP1 group had the high frequencies of total structural aberrations than CP2 group, and there were significant differences (p<0.05) between the two groups. Also, CP1 group had significant increases of aneoploidy, polyploidy and total numerical aberrations as compared to control group or CP2 group. However, the frequencies of numerical aberrations in CP2 group were similar with those found in control group, and there were no significant differences between the two groups.

The mice group that treated with honey- bee products at the same time with injection of CP1 (CP1HP group) showed significant (p<0.05) decreases of the frequencies of each of chromatid breaks and chromatid gaps as well as high significant (p<0.01) decreases of the frequencies of total structural aberrations as compared to mice group injected only with CP1 alone.

Also, the mice group that treated with honey- bee products at the same time with injection of CP2 (CP2 HP group) had high significant (p<0.01) decreases of each of chromatid breaks and chromatid gaps and significant (p<0.05) decreases of each of C.A and total structural chromosome aberrations as compared to mice group injected only with CP2 alone. Concerning the numerical aberrations, the CP1HP or CP2 HP groups had significant reduction (p<0.01 or p<0.05) for the frequencies of aneoploidy, polyploidy and total numerical aberrations as compared to mice group injected only with CP1 or CP2 alone.

Statistical analysis showed that there were no significant differences for the frequencies of structural and numerical chromosome aberrations between CP1HP and CP2HP groups. The only exception to this the CP2HP group had significant reduction (p<0.05) of the frequency of chromatid breaks than CP1HP group.

On the other hand, the mice group treated with honey- bee products before injection of CP1 or CP2 (HPCP1 or HPCP2 groups) had significant decreases (p<0.05 or p<0.01) of the frequencies of chromatid breaks, chromatid gaps and total structural chromosome aberrations as compared to mice group treated with CP1 or CP2 alone.

Moreover, the mice group treated with honey- bee products before injection of CP2 had high significant decreases of deletions, C.A and total structural aberrations as compared to mice group treated only with CP2 alone. On the other hand, HPCP1 or HPCP2 groups had significant decreases (p<0.01 or p<0.05) of the frequencies of aneoploidy, polyploidy and total numerical aberrations as compared to CP1 or CP2 groups.
The results showed that there were no significant differences for the frequencies of chromosome aberrations between HPCP1 and HPCP2 groups, except for chromatin breaks which significantly decreased in HPCP2 and deletions which significantly decreased in HPCP1.

The present results showed that there were no significant differences of the frequencies of structural and numerical chromosomal aberrations between mice groups treated with honey- bee products at the same time with injection with CP1 or CP2 and mice groups treated with honey- bee products two weeks before injection of CP1 or CP2. The only exception to this the frequencies of deletions significantly decreased in CP2HP than HPCP2.

### Table 1: Effect of HP on CP-induced chromosomal aberrations in bone marrow cells of male mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Structural Chromosomal Aberrations</th>
<th>Numerical Chromosomal Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breaks</td>
<td>Gaps</td>
</tr>
<tr>
<td>Control</td>
<td>0.90±0.02</td>
<td>0.00±0</td>
</tr>
<tr>
<td>HP Group</td>
<td>0.35±0.01</td>
<td>0.00±0</td>
</tr>
<tr>
<td>CP1 Group</td>
<td>5.00±0.4</td>
<td>0.10±0.05</td>
</tr>
<tr>
<td>CP1 HP Group</td>
<td>1.5±0.15</td>
<td>0.25±0.05</td>
</tr>
<tr>
<td>HP CP1 Group</td>
<td>5.00±0.02</td>
<td>0.00±0</td>
</tr>
<tr>
<td>CP2 Group</td>
<td>3.6±0±0.05</td>
<td>1.5±0±0.05</td>
</tr>
<tr>
<td>CP2 HP Group</td>
<td>0.5±0±0.02</td>
<td>0.00±0</td>
</tr>
<tr>
<td>HP CP2 Group</td>
<td>0.00±0</td>
<td>0.00±0</td>
</tr>
</tbody>
</table>

Data were expressed as mean ±SE. Means with different superscript letters (a,b,c) are significant different (p<0.05).

### Table 2: Effect of HP on CP-induced mitotic index depression in bone marrow cells of male mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of Cells (mean)</td>
</tr>
<tr>
<td>Control</td>
<td>1000</td>
</tr>
<tr>
<td>HP Group</td>
<td>1000</td>
</tr>
<tr>
<td>CP1 Group</td>
<td>1000</td>
</tr>
<tr>
<td>CP1 HP Group</td>
<td>1000</td>
</tr>
<tr>
<td>HPCP1 Group</td>
<td>1000</td>
</tr>
<tr>
<td>CP2 Group</td>
<td>1000</td>
</tr>
<tr>
<td>CP2 HP Group</td>
<td>1000</td>
</tr>
<tr>
<td>HPCP2 Group</td>
<td>1000</td>
</tr>
</tbody>
</table>

Data were expressed as mean ±SE. Means with different superscript letters (a,b,c,d) are significant different (p<0.05).

### Mitotic index:

The present results (Table 2) showed a significant increase of mitotic activity in mice group treated only with honey- bee products as compared to control group. On the other hand, the groups of mice injected with CP1 or CP2 had high significant depression of mitotic activity as compared to control group. The mitotic activity of CP1 group slightly raised than those of CP2 group and there were no significant differences between the two groups. However, the mice groups that treated with honey- bee products at the same time with injection of CP1 or CP2 (CP1HP or CP2HP groups) had significant increases (p<0.05 or p<0.01) of mitotic index as compared to mice groups treated only with CP1 or CP2. The CP2HP group had high mitotic activity than CP1 HP group and there were significant differences (p<0.05) between the two groups.

Also, the mice groups treated with honey- bee products before injection of CP1 or CP2 (HPCP1 or HPCP2 groups) had significant or highly significant of mitotic activity as compared to mice groups injected only with CP1 or CP2 alone. HPCP2 group had the higher percentage of mitotic activity as compared to HPCP1 group and there were high significant (p<0.01) differences between the two groups.

Moreover, the results showed that the treatment with honey- bee products before the injection with CP1 or CP2 by two weeks led to enhancing of mitotic activity as compared to treatment with honey- bee products at the same time with injection with CP1 or CP2, this enhancing was only significant (p<0.05) in HPCP2 group than CP2HP group.

### DNA fragmentation:

The present results (Table 3) showed that the rates of DNA fragmentation were few in group of mice treated with only honey- bee products as compared to those found in control group; however, statistical analysis showed...
that there were no significant differences between the two groups. On the other hand, the groups of mice injected with low (CP1 group) or high (CP2 group) doses of Cyclophosphamide (Endoxan) had high significant increases of DNA fragmentation as compared to control group. However, the mice groups that treated with honey-bee products at the same time with injection of CP1 or CP2 (CP1HP or CP2HP groups) had significant (p<0.001 or p<0.01) reduction of rate of DNA fragmentation in comparison with groups of mice injected only with CP1 or CP2 alone. CP2HP group had the lower rate of DNA fragmentation as compared to CP1HP group; however, there were no significant differences between the two groups.

Also, the groups of mice treated with honey-bee products before injection of CP1 or CP2 (HPCP1 or HPCP2 groups) had significant decreases of DNA fragmentation as compared to groups of mice injected only with CP1 or CP2 alone. HPCP2 group had the lower percentage of DNA fragmentation as compared to HPCP1 group, however, there were no significant differences between the two groups.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>DNA fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>2.2 ± 0.45*</td>
</tr>
<tr>
<td>HP Group</td>
<td>1.2 ± 3.15*</td>
</tr>
<tr>
<td>CP1 Group</td>
<td>23.0 ± 2.1*</td>
</tr>
<tr>
<td>CP1HPGroup</td>
<td>3.5 ± 1.3*</td>
</tr>
<tr>
<td>HPCP1Group</td>
<td>5.5 ± 2.5*</td>
</tr>
<tr>
<td>CP2 Group</td>
<td>15.5 ± 3.1*</td>
</tr>
<tr>
<td>CP2HPGroup</td>
<td>2.5 ± 1.2*</td>
</tr>
<tr>
<td>HPCP2Group</td>
<td>4.5 ± 2.4*</td>
</tr>
</tbody>
</table>

Table 3: Effect of HP on CP-induced DNA fragmentation in liver cells of male mice.

Data were expressed as mean ±SE. Means with different superscript letters (a,b,c,d) are significant different (p<0.05).

Moreover, the results showed that the treatment with honey-bee products at the same time with injection with CP1 or CP2 led to significant decreases of DNA fragmentation as compared to treatment with honey-bee products two weeks before the injection of CP1 or CP2.

**Sperm examination:**

Sperm examination in the present study (Table 4) showed head and tail abnormalities. The mice group treated only with honey-bee products had few of head and tail abnormalities as compared to control group, statistical analysis showed significant (p<0.01) decrease of total sperm head abnormalities than control group. Also, statistical analysis showed non significant reduction of all tail abnormalities and total sperm abnormalities in HP group as compared to control group.

On the other hand, the mice groups injected only with CP1 or CP2 doses had more frequent of sperm head and tail abnormalities as compared to control group. Statistical analysis showed that CP1 or CP2 groups had significant (P<0.05 or P<0.01 or P<0.001) increases of sperm head and tail abnormalities as compared to control group. Moreover, CP1 group had significant increases of all sperm shape abnormalities except big head as compared to CP2 group.

The mice group treated with honey-bee products (CP1HP or CP2HP groups) at the same time with injection with CP1 dose had significant or high or very high significant reduction of all sperm-shape abnormalities as compared to mice group injected only with CP1 or CP2 alone. However, there were no significant differences for the frequencies of coiled tail abnormalities between CP2HP and CP2 groups.

Statistical analysis showed that there were no significant differences for the frequencies of sperm-shape abnormalities between CP1HP and CP2HP groups. The exception to this, CP2HP group had significant (p<0.01) reduction of total sperm head abnormalities as compared to CP1HP, while CP1HP group had significant (p<0.05 or p<0.01) reduction of coiled tail abnormalities and total sperm tail abnormalities as compared CP2HP.

Also, the mice group treated with honey-bee products before injection of CP1 or CP2 (HPCP1 or HPCP2 groups) had significant (p<0.05 or p<0.01) reduction of all sperm-shape abnormalities as compared to mice group injected with only CP1 or CP2 alone.

However, the frequency of coiled tail abnormalities non significantly decreased in HPCP2 than those found in CP2 group. Also, the results showed that there were no significant differences between HPCP1 and HPCP2 groups. For sperm abnormality, except for the frequency of big head which significantly decrease in HPCP2 group than those of HPCP1 group.
The present results showed that there were no significant differences of most frequencies of sperm-shape abnormalities between mice groups treated with honey- bee products at the same time with injection with CP1 or CP2 and mice groups treated with honey- bee products two weeks before injection of CP1 or CP2. The exception to this, the frequencies of big head and total sperm tail abnormalities significantly decreased in CP1HP group than HPCP1 group. Also, the frequency of total sperm head abnormalities significantly decreased in CP2 HP group than HPCP2, however, the frequencies of coiled tail abnormalities and total sperm tail abnormalities significantly decreased in HPCP2 group than CP2HP group.

**Table 4: Effect of HP on CP-induced sperm morphological abnormalities in male mice.**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Head Abnormalities</th>
<th>Tail Abnormalities</th>
<th>Total Sperm Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Head</td>
<td>No. Head</td>
<td>Big Head</td>
</tr>
<tr>
<td>Control Group</td>
<td>0.3± 0.05*</td>
<td>0.2± 0.05*</td>
<td>0.5± 0.05*</td>
</tr>
<tr>
<td>HP Group</td>
<td>0.1± 0.05*</td>
<td>0.1± 0.07*</td>
<td>0.1± 0.05*</td>
</tr>
<tr>
<td>HPCP1</td>
<td>5± 0.2</td>
<td>6± 0.1</td>
<td>5± 0.7</td>
</tr>
<tr>
<td>CP1 HP Group</td>
<td>2.5± 0.5*</td>
<td>1.3± 0.24*</td>
<td>3.8± 0.18*</td>
</tr>
<tr>
<td>CP2 HP Group</td>
<td>2.9± 0.15</td>
<td>4.6± 0.2</td>
<td>4.6± 0.3</td>
</tr>
<tr>
<td>HPCP2</td>
<td>1.3± 0.05*</td>
<td>1.2± 0.05*</td>
<td>0.3± 0.05</td>
</tr>
</tbody>
</table>

Data were expressed as mean ±SE

**Histological examination:**

Results of the present study revealed the normal structure of the liver tissue in control group (Fig.1). Receiving honey-bee products made the normal architecture of liver tissue more regular (Fig.2). It was also revealed that Cyclophosphamide had a marked damaging effect on liver tissue that was dose-dependent, as using Cyclophosphamide in a high dose caused severe vacuolar degeneration in most of hepatocytes in addition to marked dilatation ad congestion of blood vessels (Fig. 3). This effect was greatly alleviated by using honey-bee products with high dose of Cyclophosphamide at the same time, where reduction of vacuolar degeneration and disappearance of blood vessels congestion were observed (Fig. 4). A less positive result was obtained by using honey-bee products 2 weeks before using Cyclophosphamide in a high dose, as marked dilatation with congestion of blood vessels was seen. The hepatocytes showed nuclear apoptotic figures (Fig.5). The low dose of Cyclophosphamide had a less destructive effect than that seen infig.3, only some hepatocytes showed variable degrees of vacuolar degeneration, also mild dilatation with no congestion was observed (Fig. 6). The best protective effect was observed by using honey-bee products with low dose of Cyclophosphamide at the same time, where normalization of liver tissue occurred except for very slight dilatation of blood sinusoids and mild increase in kupffer cells number (Fig. 7). Using honey-bee products 2 weeks before Cyclophosphamide gave less positive results than that seen in the previous group. Some hepatocytes appeared with dark nuclei and others with fragmented DNA, also, blood sinusoids showed dilatation and congestion (Fig. 8).

**Histopathological report:**

![Fig. 1: A photomicrograph of a section of liver tissue from a control mouse shows the normal structure of this tissue. The hepatic cells are arranged in cords radiating from the central vein (CV) and separated from each other by blood sinusoids (arrow).](image-url)
Fig. 2: A photomicrograph of a section of liver tissue from a mouse received honey-bee products...shows a quite normal structure and even more regular than the previous section.

Fig. 3: A photomicrograph of a section of liver tissue from a mouse received Cyclophosphamide (high dose) shows a severe damage in the form of vacuolar degeneration of most of hepatocytes (arrow), dilatation of blood sinusoids (arrowhead) and dilatation with congestion of central vein (CV). The normal architecture of the tissue is completely distorted.

Fig. 4: A photomicrograph of a section of liver tissue from a mouse received Cyclophosphamide (high dose) with honey-bee products at the same time shows a very fine vacuolar degeneration in some hepatocytes (arrow), while the rest of cells appear normal. Central vein shows no congestion or dilatation (CV), although the blood sinusoids are still mildly dilated (arrowhead).
**Fig. 5:** A photomicrograph of a section of liver tissue from a mouse received honey-bee products... for 2 weeks and then Cyclophosphamide (high dose) shows some hepatocytes appear with deep dark nuclei (arrow) or with apoptotic figure (arrowhead) and some are normal. The central vein is still congested and dilated (CV).

**Fig. 6:** A photomicrograph of a section of liver tissue from a mouse received Cyclophosphamide (low dose) shows vacuolar degeneration of variable degrees in some cells (arrow), apoptotic figures in others (arrowhead) and mild dilatation of most of blood sinusoids. The normal architecture of the tissue is somehow preserved.

**Fig. 7:** A photomicrograph of a section of liver tissue from a mouse received Cyclophosphamide (low dose) with honey-bee products... at the same time shows a quite normal liver tissue except for slight dilatation of blood sinusoids (arrow) and increased number of Kupffer cells (arrowhead).
Discussion:

In the present study, the treatment with CP in mice induced significant increases of chromosome aberrations and DNA fragmentation as well as a significant depression of mitotic activity as compared to those found in negative control. The inducing of genetic alterations in the present study, were similar with that reported in other studies, where CP induced significantly higher frequencies of chromosome aberrations (Gimmler-Luz et al., 1999; Sumanth et al., 2011; Tripathi et al., 2011) and significant depression of mitotic activity (Tripathi et al., 2011) in adult mouse bone marrow cells as compared to negative control. Also, several studies determined the mutagenic effect of CP by employing mouse bone marrow micronucleus test (DNA damage)(Vijayalaxmi and D'Souza, 2004; Premkumar et al., 2004; Asita and Molise, 2011; Naghshvar et al., 2012), the findings of these studies demonstrated significant increases of micronucleated polychromatic erythrocytes (MnPCEs) in CP groups as compared to those found in negative controls.

Moreover, Amador et al. (2012) revealed the CP mutagenicity by using comet assay of DNA in cells of Chinese hamster (ovary and CHO-k1 cells) in vitro study (6 ug/ml of 25 cm² tissue culture flasks). After 24 hrs of treatment, the results showed higher frequencies of DNA damage in cells of treated flasks as compared to those observed in cells of untreated flasks. So, the treatment with cp is known to be a main cause for the genotoxicity (Makhani et al., 2009). Reactive oxygen species (ROS) and Lipid peroxidation (LPO) have been reported to be major mechanisms in CP toxicity (Anderson et al., 1995; Selvakumar et al., 2006; Rezvanfar et al., 2008; Tripathi et al., 2011; Jalali et al., 2011). Wherein CP and its metabolite acrolein in liver cause inactivation of microsomal enzymes and results in increase ROS and LPO (Lear et al., 1992; Anderson et al., 1995; Tripathi et al., 2011; Jalali et al., 2011). Subsequently single and double strand scissor of DNA are produced (Vijayalaxmi and D'Souza, 2004; Asita and Molise, 2011; Amador et al., 2012). Also, CP and its metabolite phosphoramido mustard forms DNA adduct at guanine N-7 position (Young et al., 2006; Shanafelt et al., 2007; Makhani et al., 2009). The induction of DNA mutation may lead to abnormalities in the chromosomes as a result of disturbance of DNA replication (Gimmler-Luz et al., 1999; Sumanth et al., 2011).

Also, the present results showed that the sperm-shape abnormalities significantly elevated in mice injected with CP, suggesting that CP might have induced generalization toxicity in mice including testes. The present findings were supported by reports of other studies: Auroux et al., (1990) revealed that CP had a damaging effect on spermatogonia in rats causing significant decreases of the mean number of spermatocytes and spermatids. Also, Rezvanfar et al., (2008) indicated that in CP-treated rats, a decrease of sperm quality had been found and associated with increase of DNA damage and decrease of chromatin quality. Moreover, Jalali et al. (2011) and Abrikwu et al. (2012) demonstrated the toxic effect of CP on reproductive tissues in male rats, and their results showed that CP treatment led to significant decreases in sperm count and motility with an increase of sperm-shape abnormalities. The increasing generation of free radicals is one of the possible mechanisms involved in CP-induced sperm abnormalities (Anderson et al., 1995; Sikka, 2004). Spermatozoa are more susceptible to oxidative damage because of high concentrations of polyunsaturated fatty acids and low antioxidant capacity (Vernet and Aitken, 2004). Therefore, oxidative stress could play a critical role in the induction of sperm abnormalities through DNA denaturation and fragmentation (Agarwal and Saleh, 2002).

Histological study in liver might reflect and explain the above genetic alterations. Microscopic examination of the liver section of the CP-treated mice in the present study showed severe vacuolar generation in most of...
hepatocytes, in addition to marked dilatation and congestion of blood vessels. The noxious influence of CP and its metabolites may cause acute toxicity and histopathological changes (Nicolini et al., 2004; Young et al., 2006; Shanafelt et al., 2007; Makhani et al., 2009). These effects may be due to the accumulation of CP or its metabolites in liver, leading to pathological changes (Nelius and Klatte, 2009; Makhani et al., 2009).

On the other hand, it has been pointed out that oxidative or mutagenic damages of CP exposure could be inhibited by intake of antioxidants and/or free radical scavengers (Premkumar et al., 2004; Jalali et al., 2011). At this context honey-bee products (HP) have been found to have antioxidant and antimutagenic factors (Nada et al., 2005; Mahesh et al., 2009; Alvarez-Suarez et al., 2010). To our best knowledge, the protective role of honey-bee products against CP-induced genotoxicity in mice has not so far been studied, therefore, the present results used HP for this purpose. The present results showed that the treatment with HP at the same time of injection of CP (E1HP or E2HP) or two weeks before using CP (HPE1 or HPE2) significantly ameliorated the genetic, sperm and histological parameters as compared to the treatment with CP alone. These improvements may be due to that HP are composed of several compounds previously demonstrated to display varying degrees of antimutagenic activity (Wang et al., 2002; Nada et al., 2005) and antioxidants (Nakjima et al., 2009; Alvarez-Suarez et al., 2010). These compounds included phenolic acids, flavonoids, sugars, organic acids, vitamins (ascorbic acid) ad enzymes (glucose oxidase and catalase) (White, 1975; Gheldof et al., 2002). Certain phenolic antioxidant compounds have been found to be antimutageic against common dietary mutagens (Alldrick et al., 1986; Karekar et al., 2000). Also, flavonoids have been reported to display antimutagenic effects in the Ames assay using various Salmonella strains (Bjeldanes and chang, 1977; Macgregor and Jurd, 1987). Moreover, the treatment with flavonoids significantly reduced the chromosome abnormalities and delayed tumorigenesis in adult mice exposed to foetal irradiation (Devi and Satyamitra, 2004).

Sugar has been reported to display complex behavior toward the enhance and/or inhibition of mutagenic activity in model and cooked food systems (Skog, 1993). It was of interest to determine that honeys from different floral sources (acacia, buckwheat, fireweed, soybean, tupelo and Christmas berry) were capable of antimutagenic action against commonly encountered food mutagen (Trp-p-1) (Wang et al., 2002). Furthermore, the antimutagenic effects of HP (honey, royal jelly, propolis, caffeic acid and bee venom) significantly inhibited tumor growth and formation of metastasis in murine tumor models (Nada et al., 2005).

In previous study, ethanolic extract of propolis had inhibitory effect on the mutagenicity power of daunomycin (TA 102), benzo (a) pyrene (TA 100) and aflatoxin B1 (TA98), also, the venom acted against the mutagenicity of 4-nitro-o-phenylenediamine (TA98) and daunomycin (TA102) (Varanda et al., 1999). The increasing of genotoxic effect of AFB1 in human lymphocytes in vitro which was assessed by micronucleus test (DNA damage), was diminished by addition of propolis (Turkez and Youssif, 2009).

On the other hand, flavonoids and phenolic acids were found to improve semen quality and quantity, as well as decrease the sperm –shape abnormalities and histological damage in reproductive tissues of different animals exposed to several toxicants (Purdy et al., 2004; Turk et al., 2008, Khattab et al., 2010). Moreover, honey significantly increased the sexual behavior and fertility of male rats previously exposed to toxic effect of cigarette smoke (Mohamed et al., 2012). The protective effect of honey against oxidative stress of Acetaminophen (APAP) on liver tissues of rats was evaluated by histological examination (Mahesh et al., 2009), and the results revealed that honey reduced the incidence of liver lesions induced by APAP.

The present results observed that the injection of honey-bee products together with CP at the same time led to increases antigenotoxic effects, comparing with that observed when these agents (CP and Hp) have been injected separately. These observations indicate the possible interaction of honey-bee products with CP.

Other studies on the antigenotoxicity of honey-bee products against other mutagens in different mammalian tissues have shown that the dose required to obtain a significant effect is about 1 gm/kg body weight (Wang et al., 2002; Al-Waili, 2003; Schramm et al., 2003). Hence, in this study a similar dose was used. Several explanations have been offered for anti-mutagenic activity of honey-bee products, one of which related to their have a large number of potent antioxidants (Alvarez-Suarez et al., 2010). So, the mechanisms of the protective role of HP against CP genotoxicity may be due to one or more of the following: antioxidant action, trapping of free radicals, formation of complex with mutagen, modulation of mutagen metabolism or by adsorbing the xenobiotic. This is feasible because many naturally occurring compounds are known to exhibit discrete mechanisms of protection (Wattenberg, 1985; Morse and Stoner, 1993; Alvarez-Suarez et al., 2010).

In conclusion, the present study demonstrated that dangerous effect of anticancer drug CP could be avoid by using the treatment of HP especially at the same time with CP injection. This strategy is necessary for diminishing the deleterious side effects of anticancer drug with preservation of its chemotherapeutic efficacy.

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References


