Investigation of the Protective Effect of Echinacea Extract on Cisplatin-Induced DNA Damage, Chromosomal Aberrations and Micronuclei Formation in Mice

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Abstract: This work aimed to reduce the cisplatin-induced genotoxicity and cytotoxicity by using the natural echinacea extract for more success in cancer chemotherapy. This study was conducted on 96 adult male mice that were divided into 16 equal groups. Mice were received either separately or in combination aqueous echinacea extract (0 or 100 mg/kg) once daily through oral route for 10 consecutive days and cisplatin (0, 2, 4, or 8 mg/kg) as a single intraperitoneal injection concurrently with the last dose of echinacea. Mice were killed after 24 h post last treatment. Obtained results revealed that, cisplatin treatment for 24 h induced significant increases \( (P < 0.001) \) in the averages of chromosomal aberrations, micronucleated polychromatic erythrocytes (Mn-PCEs) and tail moment of comet cells as well as significant decreases \( (P < 0.001) \) of percentages of polychromatic erythrocytes (PCEs) and mitotic indices in bone marrow cells. Echinacea did not induce genotoxicity however; it significantly \( (P < 0.001) \) enhances the percentages of PCEs, which indicates that echinacea extract, had a proliferative activity. By comparative analysis, echinacea extract induced a less marked reduction in averages of chromosomal aberrations, Mn-PCEs and tail moment of comet cells induced by cisplatin, except a significant \( (P < 0.5) \) reduction in tail moment of comet cells derived from animals treated with the low dose of cisplatin \( (2 \text{ mg/kg}) \). Mice treated with combined doses of echinacea extract and cisplatin showed pronounced high significant \( (P < 0.001) \) increases in percentages of PCEs and mitotic indices in comparison to those treated with cisplatin alone. In conclusion, echinacea was not considered genotoxic or cytotoxic but it has cytotoxic stimulant effect on the proliferative bone marrow cells against myelosuppression induced by cisplatin. In addition, echinacea may be act as promising agent to inhibit the secondary malignancies. The inhibition of secondary malignancies by echinacea needs further experimentation to provide more successful chemotherapy.

1. Introduction

Cisplatin is the first inorganic antitumor drug and it is one of the most widely used active anticancer drugs in clinics at the present time \( \text{(Arnesano and Natile, 2008)} \). Cisplatin was first discovered by \textit{Roseberry et al.} \( \text{(1969)} \) which revealed strong antitumor properties. Cisplatin has been widely used for treatment of malignancies such as malignant melanomas, testicular tumors, osteogenic sarcoma, and carcinoma of bladder, lung, uterine cervix and ovary \( \text{(Arnesano and Natile, 2008)} \). Cisplatin has bifunctional alkylating action, producing DNA- interstrand and intrastrand cross- links \( \text{(Cherry et al., 2004)} \) and DNA – protein cross-linking as the major cause of cytotoxicity \( \text{(Basu and Krishnamurthy, 2010)} \). Low concentrations of cisplatin induced severe and prolonged inhibition of DNA synthesis \( \text{(Barabas et al., 2008)} \). Cisplatin inhibits DNA synthesis \( \text{(Todd and Lippard, 2009)} \). Cisplatin induced positive results in several genotoxicity (DNA damage) assays, such as chromosomal aberration \( \text{(Attia, 2010)} \), micronucleus formation \( \text{(Serpeloni et al., 2010)} \), single cell gel electrophoresis \( \text{(Serpeloni et al., 2011)} \). Regardless the positive genotoxic and cytotoxic effects of platinum compounds, they are poisons and have side effects which include nausea and vomiting, decreased blood cell and platelet production in bone marrow (myelosuppresion), immunosuppression, nephrotoxicity, neurotoxicity and hearing loss \( \text{(Florea and Büsselberg, 2006; Tsang \textit{et al.}, 2009; Olszewski and Hamilton, 2010)} \).

Many compounds are extracted from Echinacea plant such as polysaccharides, alkalamides, polyphenols and glycoproteins which exert immunomodulatory, antioxidative and anti-inflammatory properties \( \text{(Santos \textit{et al.}, 2006; Masteikova, 2007)} \). The oxidative stress are enhanced by cancer, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases \( \text{(Masteikova, 2007)} \). Stimulation by Echinacea extracts of various parameters of cellular, humoral immunity and on tumor amelioration in different experimental models \( \text{(Miller, 2005)} \).

The aim of the present work is to explore the antimutagenicity of echinacea extract as a natural food additive. For this exploration, metaphase chromosomal aberrations and micronucleated polychromatic erythrocytes from bone marrow were investigated in mice treated with single dose of cisplatin and echinacea extract either separately or in combination for one week.
2- Materials and Methods
2-1 Chemicals:
Cisplatin was purchased from local pharmacy under the trade name Cytoplatin-10<sup>®</sup> manufactured by CIPLA LTD, India. Cisplatin is dissolved in saline solution and administered as intraperitoneal injections within 1 h next preparation at dose levels 2, 4 and 8 mg/kg b.w. (Attia, 2010). Echinacea extract was purchased under the trade name Immulant<sup>®</sup> produced by Arab Co. for Pharmaceuticals and Medicinal Plants MEPACO- EGYPT. The extract is delivered as dry material in capsules. The capsule content is dissolved in dis. water. The animals received the soluble extract via oral gavage in a dose equivalent to 100 mg/kg b.w. for 10 consecutive days (Raso et al., 2002). Fetal bovine serum was provided from Gibco BRL (Grand Island, NY, U.S.A.). All other chemicals were of the analytical grade.

2-2 Animals:
The experiments were carried out on 96 adult male albino mice 8-9weeks and 30-35 g in weight. The animals were purchased from The Holding Company for Biological Products and Vaccines (VACSERA), Giza, Egypt. During the experiment the animals were fed with commercial food pellets and water ad libitum. The animals were divided randomly into 16 groups each with 6 animals per cage. The animals received care according to the standard humane animal care protocols.

2-3 Treatments:
A total of 96 mice were grouped into 4 groups and each Group one included 24 animals treated with single intraperitoneal (IP) injection of cisplatin equivalent to 0, 2, 4 and 8 mg/kg b.w. for 24 h for chromosomal study. Group two included 24 animals treated with single intraperitoneal (IP) injection of cisplatin equivalent to 0, 2, 4 and 8 mg/kg b.w. for 24 h for micronucleus and comet assays. Group three included 24 mice treated by gavage with 100 mg/kg/day echinacea for 10 consecutive days and the animals received single dose of 0, 2, 4, and 8 mg/kg cisplatin 24 h prior to animal scarification for chromosomal study. Group four included 24 animals treated by gavage with100 mg/kg/day echinacea for 10 consecutive days and the animals received single dose of 0, 2, 4, and 8 mg/kg cisplatin 24 h prior to animal scarification for micronucleus and comet assays.

2-4 Micronucleus test:
Animals were killed by cervical dislocation and bone marrow of femur was aspirated in 2 cc fetal bovine serum. The bone marrow cells were centrifuged at 500 r.p.m. for 3 min. The cell pellet was suspended in 0.5 cc fetal bovine serum and bone marrow smears were made on clean dry glass slides. Bone marrow smears were stained with May-Grünwald Giemsa protocol (Albanese and Middleton, 1987). 2000 polychromatic erythrocytes (PCEs) per animal were scored for micronuclei induction and 1000 erythrocytes per animal were scored to establish the percentage of polychromatic erythrocytes among erythrocytes [PCEs /100 (PCEs+NCEs)]. Micronuclei were observed directly under microscope.

2-5 Chromosomal aberrations assay:
Chromosomes were prepared from bone marrow cells of mice according to the method previously postulated by Hliscs et al. (1997). The slides were stained with 5% Giemsa stain. 100 well spread metaphases were examined per animal with oil immersion of Meiji microscope. The chromosomal aberrations were classified according to Savage (1976). Mitotic indices were determined from scoring 1000 cells for each animal. The counts were carried out with the hand tally counter.

2-6 Comet assay:
The marrow was collected from the femur bones and suspended in 1 ml of chilled homogenizing buffer. The cell suspensions were diluted in chilled homogenizing buffer appropriately and subjected to the alkaline comet assay immediately (Tice et al., 2000). The alkaline comet assay was preformed basically as described by Miyamae et al. (1998) the slides were stained with 50 µEtBr (20 µl/ml) and covered with a cover slip. To prevent drying, the slides were stored in a humidified container until microscopic examination. The slides were examined at 200x magnification using Olympus fluorescent microscope. A total of 1000 randomly selected cells from two replicate slides (500 cells per slide) were examined per sample. Ten comet cells were chosen for calculation of the tail length and tail DNA percentage to deduce the averages of tail momentum (Olive et al., 1999).

2-7 Statistical analysis
Results of the different treatment groups were compared using Students’ one-tailed t-test (Fowler et al., 1998). Significance was indicated by t<sub>values</sub> <0.05.

4- Results and Discussion
The crucial role of this work is to reduce the cisplatin-induced genotoxicity and cytotoxicity by using the natural echinacea extract for more success in cancer chemotherapy as well as to prevent the induction of secondary malignancies induced by anticarcinogenic agents. For these purposes, mice were treated with echinacea extract and received a concurrent intraperitoneal injection of cisplatin and animals were killed after 24 h. Bone marrow cells were extracted for preparation of metaphase chromosomal aberrations assay, micronucleus test in polychromatic erythrocytes and comet assay for calculation of tail momentum of the comet cells (Figure 1).
As shown in Table 1, mice treated with cisplatin alone showed appreciably significant ($P < 0.001$) increases in averages of chromosomal aberrations and damage cells with increasing the dose level. Chromosomal aberrations induced by cisplatin are of structural chromatid-type and showed up in the form of chromatid break, chromatid gap, acentric chromatid fragment, and chromatid deletion. The damage cells are cells having one or more than one chromosomal aberrations. Cisplatin induced significant ($P < 0.001$) production of Mn-PCEs and damage of DNA in the form of comet cells as well as, it induced cytotoxicity through reduction of the percentages of PCEs (as shown in table 2). Mn-PCEs appeared in the form of PCEs containing small rounded bodies (Albanese and Middleton, 1987). In the present work, cisplatin induced chromosomal aberrations and micronuclei formation which previously proved by earlier investigators (Adler and el-Tarras, 1989; Edelweiss et al., 1995; Choudhury et al., 2000; Attia, 2010; Al-Zubairi et al., 2011). On the DNA damage level, cisplatin induced comet in many test systems (Brozovic et al., 2011; Serpeloni et al., 2011). Cisplatin is one of the potent alkylating agents. The alkylating agents are classified as monofunctional, bifunctional alkylating agents and some others are topoisomerase inhibitors and some function as free radical generating agents. It is supposed that, cisplatin induced its genotoxicity and cytotoxicity via interaction with DNA (Basiak and Kowalik, 2001; Yilmaz et al., 2010; Rassouli et al., 2011). Animals treated with cisplatin developed malignancies (Hisamoto et al., 2007). Furthermore, humans treated with the anticarcinogenic agent, cisplatin can develop secondary neoplasms (Meadows et al., 2009). One of the most acceptable explanations for the genotoxicity of cisplatin is related to induction of free radicals (Manda et al., 2009; Florea and Busselberg, 2011; Kovacic and Somanathan, 2011). Cisplatin proved to be cytotoxic where; it induced myelotoxic on haemopoietic progenitor cells of mice bone marrow. The cytotoxicity of cisplatin appeared in the form of highly significant decreases ($P < 0.001$) in the percentages of both mitotic indices and PCE as shown in tables (1) and (2). The decreases in the percentages of both mitotic indices and PCE were dose dependent. Previous observations postulated by Pannacciulli et al. (1989), Mazur and Czyzewskia (2001), Mazur et al. (2002) and Molyneux et al. (2011) showed that, cisplatin is cytotoxic in bone marrow cells. Cisplatin as well as many of the anticancer drugs act as mitotic inhibitors by directly acting on the microtubules (Peterson and Mitchison, 2002; Jordan and Wilson, 2004; Altmann and Gertsch, 2007).

Mice treated for 10 consecutive days with echinacea alone did not induce significant increases in the averages of chromosomal aberrations, Mn-PCEs and comet cells. However, echinacea could induce a significant ($P < 0.001$) enhancement in the percentages of PCEs (as shown in table 2). The results indicated that echinacea is neither cytotoxic nor clastogenic when compared with the negative control. Previous reports showed that echinacea have a proliferative activity on bone marrow cells due to the presence of the alkyl-amides such as echinacin, isobutylamides such as penta-decadienes and hexadecadienes, polyacetylene in the echinacea extract (Chow et al., 2006).

Simultaneous treatment with both echinacea and cisplatin showed reduction in the averages of chromosomal aberrations, Mn-PCEs and comet cells induced by cisplatin in animals treated with 2 mg/kg cisplatin (as shown in tables 1 and 2). Apart from that, the tail momentum of comets in mice treated with a combined of echinacea and 2 mg/kg cisplatin, the reduction of comet tail momentum was significant ($P < 0.05$, Figure 2). These results may indicate that, echinacea is weak antigenotoxic because it exerted its protective effect on low dose (2 mg/kg) of cisplatin while it is failed to protect against genotoxicity in mice treated with high doses. However, it is clearly shown that echinacea exerts a pronounced anticytotoxic effect on cisplatin-induced myelotoxicity in the form of increased mitotic indices and PCEs in comparison to the results derived from mice treated with cisplatin alone (as shown in table 1).

Figure (1): Cell showing comet tail (Arrow) from bone marrow cells of mouse treated with 8 mg/kg cisplatin.

Echinacea is an antioxidant that can scavenge free radicals and protect cellular macromolecules, including proteins, from oxidative damage induced by various agents (Raso et al., 2002; Huntimer et al., 2006; Sullivan et al., 2008). Many of its ingredients are powerful immune system stimulators. Its contents
include high molecular weight polysaccharides, essential oils, alkyl-amides such as echinacein, isobutylamides such as penta-decadienes and hexadecadienes, polyacetylene, tannins, inulin, heteroxylan, flavonoids and vitamin C. (Block and Mead, 2003; Miller, 2005; Ezz, 2011).

It is concluded from this study that, echinacea was not genotoxic or cytotoxic. Also, it has cytotoxic stimulant effect on the proliferative bone marrow cells. Echinacea could exert a weak antigenotoxic action on cisplatin induced DNA damage. Echinacea is a powerful anticytotoxic agent where it is significantly protects against cisplatin-induced myelocytotoxicity. In addition, echinacea could be used as an adjuvant therapy with chemotherapeutic agents to reduce the cytotoxic impact of such drugs as well as echinacea may be act as promising agent to inhibit the secondary malignancies. The inhibition of secondary malignancies by echinacea needs further investigations to provide more successful chemotherapy.

Figure (2): Averages tail momentum of comet cells in bone marrow of mice treated intraperitoneally with 0, 2, 4 and 8 mg/kg cisplatin for 24 h and/or oral doses of 0 and 100 mg/kg echinacea for 10 days. Note that, cisplatin alone induced highly significant ($P<0.001$) increases in averages of tail momentum in comparison to the tail momentum of control untreated mice. While, combined treatment of echinacea and cisplatin induced a low reduction in the averages of tail momentum in comparison to those treated with cisplatin alone except, samples of mice treated with the low dose of cisplatin (2 mg/kg) the average was significantly ($P<0.5$) reduced (Column with starlet).

Table (1): Averages of chromosomal aberrations, damage cells and the percentages of mitotic index in bone marrow cells of mice treated intraperitoneally with 0, 2, 4 and 8 mg/kg cisplatin for 24 h and/or oral doses of 0 and 100 mg/kg echinacea for 10 days.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Average of chromosomal damage / 100 metaphase spreads ± S.D.</th>
<th>Damage cells / 100 metaphases ± S.D.</th>
<th>% of mitotic index ±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin Echinacea</td>
<td>0 0 1.33 ± 0.82</td>
<td>1.33 ± 0.82</td>
<td>3.65 ± 0.62</td>
</tr>
<tr>
<td>2 0</td>
<td>3.67 ± 1.03 **</td>
<td>2.83 ± 0.75 **</td>
<td>2.25 ± 0.34 ***</td>
</tr>
<tr>
<td>4 0</td>
<td>9.67 ± 1.97 ***</td>
<td>6.50 ± 1.05 ***</td>
<td>1.47 ± 0.33 ***</td>
</tr>
<tr>
<td>8 0</td>
<td>13.17 ± 0.75 ***</td>
<td>8.33 ± 1.37 ***</td>
<td>1.02 ± 0.15 ***</td>
</tr>
<tr>
<td>0 100</td>
<td>1.00 ± 0.63</td>
<td>1.00 ± 0.63</td>
<td>4.22 ± 0.50</td>
</tr>
<tr>
<td>2 100</td>
<td>1.83 ± 0.75 **</td>
<td>1.50 ± 0.84 *</td>
<td>3.88 ± 0.28 ***</td>
</tr>
<tr>
<td>4 100</td>
<td>6.83 ± 1.47 #</td>
<td>5.00 ± 0.89 #</td>
<td>3.45 ± 0.52 ***</td>
</tr>
<tr>
<td>8 100</td>
<td>11.83 ± 1.47</td>
<td>9.67 ± 1.21</td>
<td>2.27 ± 0.44 ***</td>
</tr>
</tbody>
</table>

Note: *= $P<0.5$, ** = $P<0.01$, *** = $P<0.001$ (in comparison with results of untreated control mice).

# = $P<0.5$, ## = $P<0.01$, ### = $P<0.001$ (in comparison with results of corresponding cisplatin-treated mice).

Table (2): Averages of Mn-PCEs percentages of PCEs and tail momentum of comet cells in bone marrow cells of mice treated intraperitoneally with 0, 2, 4 and 8 mg/kg cisplatin for 24 h and/or oral doses of 0 and 100 mg/kg echinacea for 10 days.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Average of Mn-PCEs / 2000 PCEs ± S.D.</th>
<th>% PCEs ± S.D.</th>
<th>Tail momentum for comet cells ±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin Echinacea</td>
<td>0 0 3.83 ± 0.75</td>
<td>53.33 ± 1.63</td>
<td>2.37 ± 0.60</td>
</tr>
<tr>
<td>2 0</td>
<td>6.83 ± 1.47 **</td>
<td>36.67 ± 3.93 ***</td>
<td>7.43 ± 1.69 ***</td>
</tr>
<tr>
<td>4 0</td>
<td>13.67 ± 2.66 ***</td>
<td>18.67 ± 1.63 ***</td>
<td>20.19 ± 3.20 ***</td>
</tr>
<tr>
<td>8 0</td>
<td>20.00 ± 1.55 ***</td>
<td>14.33 ± 2.50 ***</td>
<td>37.95 ± 2.91 ***</td>
</tr>
<tr>
<td>0 100</td>
<td>4.17 ± 0.75</td>
<td>58.50 ± 1.64 ***</td>
<td>2.15 ± 0.71</td>
</tr>
<tr>
<td>2 100</td>
<td>5.00 ± 1.26 *</td>
<td>51.83 ± 2.32 ***</td>
<td>5.07 ± 0.96</td>
</tr>
<tr>
<td>4 100</td>
<td>10.67 ± 1.21</td>
<td>31.67 ± 4.27 ***</td>
<td>18.13 ± 2.89</td>
</tr>
<tr>
<td>8 100</td>
<td>17.83 ± 1.72</td>
<td>29.83 ± 2.40 ***</td>
<td>36.57 ± 1.92</td>
</tr>
</tbody>
</table>

Note: *= $P<0.5$, ** = $P<0.01$, *** = $P<0.001$ (in comparison with results of untreated control mice).

# = $P<0.5$, ## = $P<0.01$, ### = $P<0.001$ (in comparison with results of corresponding cisplatin-treated mice).
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References


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