ORIGINAL ARTICLE
EVALUATION OF CISPLATIN INDUCED GENOTOXICITY IN MALE SPRAGUE DAWLEY RATS

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Background: Cisplatin is an inorganic, divalent platinum-based antineoplastic drug that is used alone or in combination chemotherapy regimens for the treatment of wide range of human cancers. Objective was to study cisplatin induced genotoxicity in reticulocytes and colonic epithelial cells of male Sprague Dawley rats. Methods: Randomized controlled trial study was conducted at Department of Physiology, Army Medical College, Rawalpindi from Oct 2014 to Apr 2016. Trial was conducted on sixty male Sprague Dawley rats having average age 80–90 days and weight 250±50 grams. Rats were randomly allocated into two groups with 30 rats each. Rats in group I were administered intraperitoneal normal saline 10 ml/Kg body weight twice a week for 4 weeks whereas rats in group II received intraperitoneal cisplatin 2 mg/Kg body weight twice a week for 4 weeks. After 4 weeks, animals were sacrificed and blood sample was obtained for evaluation of serum malondialdehyde and micronucleated reticulocytes. After collection of blood sample, rats were dissected and small part of colon was excised and studied for micronucleated colonic epithelial cells. Results: In group I, mean micronucleated reticulocytes were (0.09±0.07%) micr0nucleated colonic epithelial cells were (0.24±0.11%) and serum malondialdehyde was (3.3±0.35 µmol/L) whereas in Group-II, mean micronucleated reticulocytes were (0.33±0.12%), micronucleated colonic epithelial cells were (2.97±0.47%) and serum malondialdehyde was (7.9±0.46 µmol/L). Results of all three variables were significantly raised (p<0.001) in group II as compared to Group-I. Conclusion: Cisplatin administration produced oxidative stress by increased generation of reactive oxygen species which consequently resulted in genotoxicity in male Sprague Dawley rats.

Keywords: Cisplatin, Genotoxicity, Micronucleus, Oxidative stress.

INTRODUCTION
Cisplatin is an inorganic, divalent platinum-based antineoplastic drug that is used alone or in combination chemotherapy regimens for the treatment of wide range of human cancers such as male and female gonadal tumors, adrenocortical and head and neck tumors. DNA is the main cellular target of cisplatin and rapidly dividing cancerous cells are particularly sensitive because they actively synthesize new DNA. Although cisplatin is one of the most effective anti-cancer drug, its clinical use has been associated with proven toxicity to the normal tissue in different animals. Precise mechanism of cisplatin toxicity is not known, however, plausible mechanism involves development of oxidative stress through excess formation of reactive oxygen species (ROS). Superoxide anions (O2•-), hydrogen peroxide (H2O2) and hydroxyl radicals (OH•) interact with cellular DNA, lipids and proteins. Reactive oxygen species causes lipid peroxidation (LP) of membranes that is considered fundamental mechanism by which cisplatin causes severe impairment of normal cellular functioning and toxic damage to the organs. Genotoxicity is the damage to the genetic information of the cell by either exogenous or endogenous insults. A great variety of factors are involved in the damage to the chromatin material and DNA including exposure to irradiations (ultraviolet, X-rays and gamma-rays), heavy metals and metal containing drugs, environmental pollutants like diesel and gasoline emissions and ROS. Chromosomal aberration, micronucleus test and sister chromatid exchange in hematopoietic stem cells, peripheral blood, colonic epithelial cells and spermatogonia have been proven markers of oxidative stress induced genotoxicity in different animals. Micronucleus test has been one of the most sensitive marker used to detect genomic damage. Micronuclei are round chromatin-containing bodies which arises from lagging chromosome fragments or whole chromosomes that are not integrated into daughter nuclei following mitosis. In Red blood cells lineage, erythroblasts extrude their nucleus and chromatin material by the process of pyknosis, karyorrhexis and karyolysis and become reticulocytes (RET). Fragments of nuclear material or condensed nucleus as a result of toxic injury remains inside the RET after nucleus extrusion which gives them appearance of micronucleated reticulocytes (MnRETs). Cut off value for MnRETs is <0.08% of total RETs in the peripheral
blood. Presence of increased number of MnRETs in blood and micronucleated colonic epithelial cells in gut tissue (cut-off value <0.2%) have been documented as sensitive indicator of genomic damage. The goal of the current study was to determine development of oxidative stress produced by cisplatin followed by evaluation of oxidative stress induced genotoxicity in rats.

MATERIAL AND METHODS

The design of current study was randomized controlled trial. It was conducted at the Department of Physiology, Army Medical College, Rawalpindi, in collaboration with National Institute of Health (NIH) Islamabad. Duration of study was 18 months from Oct 2014 to Apr 2016. A total number of 60 healthy male Sprague Dawley rats aged 80–90 days, weighing 250±50 grams, were acquired from NIH, Islamabad. Animals were kept in separate room where ventilation, daily photoperiod of 12 hours light and dark cycle and temperature (22±3 °C) were maintained. Rats were given standard pallet feed and water ad libitum. Pallet feed was prepared as per guidelines laid down by the Universities Federation for Animals Welfare. Rats were randomly divided into two groups with 30 rats each. Group I was control and group II was cisplatin group. Rats in Group-I did not receive any medication, however, to maintain stress level with other group, they were administered an intraperitoneal injection of normal saline 10 mL/Kg body weight two times a week for 4 weeks. Rats in Group-II were administered an intraperitoneal injection of cisplatin 2 mg/Kg body weight two times a week for 4 weeks.

The animals were euthanized after 4 weeks. Terminal blood sample of 4–5 mL was obtained by intracardiac puncture. Three mL of blood was centrifuged in cold centrifuge machine (Model 5810R; Eppendorf, Germany) at 4 °C and 4,000 rpm for 15 minutes. Obtained serum was used for quantitative measurements of malondialdehyde (MDA) by ELISA using TBARS essay kit on Stat Fax® 2100 Microplate Reader. Twenty μL of blood (without anticoagulant) was mixed with equal amount of foetal bovine serum. Two smears of blood film were prepared from 5 μL each of this sample on acridine orange supravital stain coated glass slides. Slides were examined under the florescence microscope for scoring of MnRETs per 1,000 RETs. After collection of blood sample, rats were dissected and small part of colon was excised and washed immediately with phosphate-buffered saline. The sample was everted on a glass rod and immersed in trypsin-EDTA solution at 30 °C for 15 min. The crypts were isolated and dispersed into single cell by pipetting with Pasteur pipettes. The isolated cells were suspended in methanol acetic acid (Carnoy’s fixative) and slides were prepared. Slides were stained with May-Grünwald and Giemsa stain and examined under the microscope using high magnification power objective lens (100×) for the evaluation of micronuclei.

Data was analyzed using SPSS-23. Numerical variables were stated as Mean±SD. As per normality distribution of data, Independent-Samples Mann-Whitney U test was applied for MnRETs and micronucleated colonic epithelial cells. Independent-Samples t-test was applied for MDA to determine the difference among two groups and p<0.05 was considered statistically significant.

RESULTS

The mean serum MDA level in the control group was 3.3±0.35 μmol/L whereas in cisplatin group it was 7.9±0.46 μmol/L. The mean serum MDA level in cisplatin group was significantly raised (p<0.001) with comparison to the control (Table-1). The mean frequency of MnRETs in control group was 0.09±0.07% of total RETs whereas in cisplatin group it was 0.33±0.12% of total RETs (Figure-1). The values were significantly raised (p<0.001) in cisplatin group with comparison to the control (Table-1). The mean frequency of micronucleated colonic epithelial cells (Figure-2) in control group was 0.24±0.11% whereas in cisplatin group it was 2.97±0.47%. The values were increased in cisplatin group was as compared to the control group and this increase was found to be statistically significant (p<0.001).

Table-1: Comparison of MnRETs, micronucleated colonic epithelial cells and MDA in group I and II

<table>
<thead>
<tr>
<th>Variables</th>
<th>Study Groups</th>
<th></th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Cisplatin</td>
<td></td>
</tr>
<tr>
<td>MnRETS (% of total RETs)</td>
<td>0.09±0.07</td>
<td>0.33±0.12</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Micronuclei in Colonic Epithelial cells (%)</td>
<td>0.24±0.11</td>
<td>2.97±0.47</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Serum MDA (μmol / L)</td>
<td>3.3±0.35</td>
<td>7.9±0.46</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*pSignificant; MnRETs: micronucleated reticulocytes; RET: reticulocytes; MDA: malondialdehyde

Figure-1: Micronucleated Reticulocyte
DISCUSSION

The current study was an effort to analyze the potential genotoxicity in RETs and colonic epithelial cells caused by cisplatin administration. Apart from its potent anticancer action, cisplatin induced genotoxicity to normal tissue has detrimental consequences among survivors of cancer chemotherapy. Results of the current study reflected that the administration of cisplatin developed oxidative stress which significantly increased (p<0.001) mean frequency of MnRETs and micronucleated colonic epithelial cells. Evidence suggests that the underlying mechanism of oxidative stress and consequent genotoxicity by cisplatin chemotherapy regimen is associated with increased production of ROS. It is postulated that cisplatin accumulates in the mitochondria and forms adduct with mitochondrial DNA (mDNA). Persistent binding with mDNA interferes with mDNA transcription. Reduced expression of mDNA encoded components of electron transport chain leads to impaired cellular respiration and consequently increased ROS generation. In our study, the mean serum MDA levels were found to be significantly raised (p<0.001) in cisplatin group with comparison to the control. ROS mediated lipid peroxidation in the membranes of cell and subcellular organelle is the basis of high serum MDA levels. The results of our study showed cisplatin induced lipid peroxidation of cellular membrane phospholipids and ensuing MDA formation. Serum MDA has been widely used as a convenient biomarker to determine oxidative stress in clinical situations. In 2015, Akman et al. carried out a study to compare the effect of cisplatin administration on various parameters of oxidative stress in Sprague Dawley rats. Their results revealed significantly raised (p<0.001) serum MDA levels in cisplatin treated group with comparison to the control which are comparable to our study.

In the current study, intraperitoneal administration of cisplatin led to significant increase (p<0.001) in the number of MnRETs in cisplatin group as compared to the control group. This showed that cisplatin treatment led to DNA damage of haematopoietic stem cells and therefore increased number of MnRETs in the blood. It has been documented that the administration of cisplatin increases production of MnRETs by both directly binding and damaging the DNA and by excess production of ROS in the erythropoietic stem cells of the bone marrow. Basu et al. (2015) carried out a study to evaluate the effect of cisplatin induced oxidative stress on bone marrow cells. Their study revealed significantly increased (p<0.001) micronucleus formation in haematopoietic stem cells in cisplatin group as compared to the control group. The authors also reported significant myelosuppression in cisplatin group. These results are comparable to our study whereby we also found significantly increased (p<0.001) micronucleus formation in cisplatin group as compared to the control group.

In the present study, the mean frequency of micronuclei in colonic epithelial cells was significantly raised (p<0.001) in the cisplatin treated group as compared to the control group. Our results showed ROS mediated damage to the genome of colonic epithelial cells. The underlying mechanism of cisplatin induced DNA damage in the colonic epithelial cells is similar as seen in the reticulocytes. It has been documented that administration of cisplatin damages the DNA by both directly binding and forming DNA adducts and by excess production of ROS. The evidence of cisplatin induced micronuclei formation in the colonic epithelial cells is lacking in the literature however, plausible mechanism of micronuclei formation in the colonic epithelial cells is the ROS mediated activation of endonuclease enzyme which causes enzymatic excision of chromatin loops. In 1996, Zhurkov et al. carried out a study to evaluate the oxidative stress induced micronuclei formation in the colonic epithelial cells, hepatocytes and bone marrow. The researchers induced oxidative stress by administration of 1,2-dimethylhydrazine which increased production of ROS, an effect similar to the toxic effect of cisplatin. They documented significantly increased (p<0.001) number of micronucleated colonic epithelial cells in drug treated group as compared to the control group in a dose dependent manner. In 2012, Khan et al. confirmed the cisplatin induced genotoxicity in the colonic epithelial cells by evaluating the levels of activated p38 mitogen-activated protein kinase (MAPK), an indicator of ROS mediated DNA damage. They documented increased activation of p38 MAPK in the cisplatin treated group as compared to the control group. These results are analogous to our study whereby we also found significantly increased oxidative stress induced damage in colonic epithelial cells in cisplatin group as compared to the control group.
CONCLUSION
Cisplatin administration produced oxidative stress by increased generation of reactive oxygen species which consequently resulted in genotoxicity in male Sprague Dawley rats.

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