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Effect of varying doses of Cisplatin on rat intestinal cell apoptosis

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Abstract

The purpose of this study was to (a) determine the dosage of cisplatin required to induce intestinal epithelial cell apoptosis, without causing lethality or necrosis and (b) to assess the biochemical changes in intestinal mucosa with different doses of cisplatin administration. A total of 9 WNIN weanling male rats were divided into three groups (n=3). Groups I and II rats were administered low and high doses of cisplatin intraperitoneally (3 and 12 mg/kg body weight) for three weeks, based on computation from human dose while control rats were administered saline intraperitoneally. Morphometric apoptotic counts increased significantly in both villus and crypt regions of the jejunum in animals of group I but not in animals of group II as compared to control. These changes in apoptotic index correlated with enhanced caspase-3 activity and DNA ladder pattern studies. Increased cell death also resulted in loss of functional integrity of the jejunal mucosa and these events were linked to increased oxidative stress and altered antioxidant enzyme activities. A weekly dose of 3 mg cisplatin/kg body weight appeared to induce intestinal epithelial cell apoptosis in WNIN strain, without causing mortality and could be used as a model for determining the therapeutic and cytoprotective potential of novel test candidates.

Key words: **apoptosis, cisplatin, epithelial cells, oxidative stress**

Introduction

Injury to normal but rapidly proliferating cells in the bone marrow and intestine often complicates the treatment of patients with neoplastic disease (Van Hayen *et al*. 1998; Wadler *et al.* 1998). Systemic chemotherapy exerts cytoablative actions via several different mechanisms, ultimately leading to cell cycle arrest and/or apoptosis and produces changes in the structure of the intestinal mucosa (Baskerville and Bater-Hatton, 1997; Levin, 1968; Slavin *et al.* 1978) that are associated with increased permeability of the intestine (Siber *et al*. 1980).

Cisplatin (cis-diamminodichloroplatinum, II) is one of the most frequently used anticancer drugs. The therapeutic efficacy of cisplatin derives from its ability to form complexes with DNA (Cohen and Lippard, 2001), which exert their cytotoxicity by directly inhibiting DNA and RNA synthesis and inducing apoptosis (Meyn *et al*. 1995; Sorenson *et al.* 1990). In addition, cisplatin has been shown to induce production of reactive oxygen species (ROS) that are important mediators of stress response in many cell types (Adler *et al.* 1999; Benhar *et al*. 2001). Also, it has been shown that mitochondrial density within a cell could also determine its response to

cisplatin exposure (Miyajima *et al*. 1999). In fact, increased intracellular reduced glutathione (GSH) concentrations are found in cells resistant to cisplatin (Godwin *et al.* 1992). However, cisplatin-induced apoptosis also involves events that are not ROS-dependent (Miyajima *et al.* 1997).

Despite the extensive use of cisplatin in cancer chemotherapy, there are few animal models currently available to assess the effects, if any, of micronutrient deficiencies on cisplatin induced apoptosis and the probable biochemical/molecular mechanisms underlying such an effect.

The present study was conducted to arrive at the optimum dose of cisplatin in WNIN rats that would induce apoptosis in intestinal mucosal cell, without causing mortality in the rat. This study helps in looking into cisplatin induced apoptosis which could be used to study the effects of compounds including micronutrients, their deficiencies and intervention on intestinal mucosal cell apoptosis.

Materials and Methods

Animals and study design

The animal study protocols were approved by the Scientific Advisory Committee as well as Institutional Animal Ethics Committee. Nine weanling male, WNIN rats were obtained from and acclimatized in National Center for Laboratory Animal Sciences for 1 week and maintained at 24 ± 2 °C, 50-60% relative humidity, with a 12 h light-dark cycle. They were housed in polypropylene cages with stainless steel top grill with food and water spouts and closed bottom. Autoclaved paddy husk was used for bedding. They were fed for 17 weeks on a casein based (20 % protein) control diet, till achieving adulthood.

After 17 weeks, the animals were divided into three groups (n=3, each group). Group I and II were administered cisplatin intraperitoneally, once weekly, at a dose of 3 and 12 mg/kg body weight for three weeks and the control group was administered the vehicle (saline). This dosage of cisplatin was computed taking into consideration the human therapeutic dosage of 50-120 mg/m^2 body surface. Cisplatin (1 mg/ml) was diluted in 0.9% saline to obtain appropriate dosage before administration. The intraperitoneal dose of cisplatin gives blood levels equivalent to a routine intravenous administration of the drug (Reed and Kohn, 1990).

The rats had free access to feed and water. Their food intake (daily) and body weights (weekly) were recorded during the course of the experiment. The animals were observed for survival, body weights, hemoglobin, RBC, WBC and platelet counts after 7 days of last dose.

At the end of feeding and treatment regimen, venous blood was collected from all rats after a 17 h fasting, through orbital sinus vein puncture (Riley, 1960) into heparin containing vials. Rats from each group were euthanized in a CO_2 chamber. A 20-cm segment of the jejunum, beginning 12-cm distal to the ligament of Treitz, was immediately excised via a midline abdominal incision and freed from mesentery and fat. It was then processed for evaluating the changes in apoptotic rates.

Chemicals and Reagents

Cisplatin was obtained from Dabur Pharmaceuticals, India. The substrate for caspase-3 (Ac-DEVD-pNA) was procured from Calbiochem, San Diego, CA, USA. RNase, proteinase K, Nonidet NP-40, agarose, lys, ala- 7-amido-4-methyl coumarin were procured from Sigma chemical company, St Louis, MO, USA. All other chemicals were of the highest analytical grade procured from local sources.

Processing of the jejunum

A 20-cm segment of jejunum was gently washed with ice-cold phosphate buffered saline (PBS), divided randomly into three segments of 5, 5 and 10 cm each and processed by standard procedures (Bodiga *et al.* 2005). They were used respectively for light microscopic observations, isolation of epithelial cells and extraction of DNA and determination of enzyme activities, parameters of oxidative stress and antioxidant status (Bodiga *et al.* 2005; Vijayalakshmi *et al*. 2005).

Detection of Apoptosis

Samples of rat jejunum fixed in buffered formalin for 24 h were dehydrated, embedded in paraffin and the number of apoptotic cells per 1000 cells were scored in 4-mm thick serial sections using a light microscope, after staining with hematoxylin and eosin. Apoptotic bodies were identified by the presence of shrunken/pyknotic nuclei with surrounding rim of irregular cytoplasm and vacuolation all around (Kerr *et al.* 1972). Also, the DNA isolated from the jejunal epithelial cells was resolved on an agarose gel and the DNA fragments visualized under a UV trans-illuminator after ethidium bromide staining (BioRad) (Gong *et al.* 1994). Caspase-3 activity was also determined according to previously reported method (Henkels and Turchi 1999).

Oxidative stress, antioxidant status

The frozen samples of the mucosal scrapings were processed and the activities of caspase-3, Cu, Zn-SOD, glutathione peroxidase, catalase were determined as described by us earlier (Bodiga *et al.* 2005). Tissue oxidative stress was quantified by the estimation of TBARS and protein carbonyls (Bodiga *et al.* 2005; Vijayalakshmi *et al*. 2005).

Functional integrity of villus

Activities of alkaline phosphatase and lys, ala dipeptidyl amino peptidase were measured in 12000g supernatant, as markers of functional integrity of the mucosal membrane (Vijayalakshmi *et al*. 2005).

Statistical analysis

All the results were expressed as mean ± SE. Data was analyzed statistically by one way analysis of variance (ANOVA) followed by Post Hoc multiple comparison tests of significance using the SPSS package (Version 10.0, Chicago, USA). Since no heterogeneity of variance was observed with any of the parameters tested. Differences among the groups were tested by the parametric, least significant difference (LSD) test. The differences were considered significant only if $P \leq 0.05$.

Animals treated with cisplatin at dose of 3 mg/kg body weight

tolerated the dose well and survived till the end of 3 weeks, while those treated with 12mg/kg body weight did not tolerate the dose as seen in the form of weakness, lethargy and gross morbidity Therefore, group II rats were sacrificed after 2 weeks, before mortality occurred.

Results

Jejunal mucosal apoptosis

Apoptotic index measured in jejunal mucosa showed a significant increase in the percentage of apoptotic cells in both villus and crypt regions (Figs.1 and 2) in group I while a single dose of 12 mg/kg body weight of cisplatin in group II did not show any increase in the apoptotic index in either villus or crypt regions (Table 1). Caspase-3 activity was found to correlate with the induction of apoptosis. Enhanced caspase activity was observed in group I rat intestine, while group II has not shown increased activation, compared to controls (Table1). The electrophoretic separation of DNA isolated from the intestinal mucosal cells showed shearing of DNA and not the ladder pattern characteristic of apoptosis (data not given).

Table 1 : Cisplatin effect on the apoptotic rates of epithelial cells

Group (n=3)	Villus Apoptotic Index	Crypt Apoptotic Index	Caspase-3 activity (mmoles of p-NA/mg protein)
	MEAN ± SE	MEAN ± SE	MEAN ± SE
Control	3.37 ^{a} ± 0.35	0.19 ^{a} ± 0.01	0.045 ^{a} ± 0.003
Group I	6.43 ^{b} ± 0.15	2.30 ^{b} ± 0.26	0.177 ^{b} ± 0.015
Group II	2.63 ^{a} ± 0.52	0.20 ^{a} ± 0.00	0.057 ^{a} ± 0.012

Values with different superscripts are significantly different ($P \leq 0.05$)

Tissue oxidative stress

Enhanced protein oxidation was observed significantly in group I and group II as compared to control, but the extent of oxidation seems to be relatively less in group II compared to group I. The thio barbituric acid reacting substance (TBARS) were observed significantly in group I compared to other groups while glutathione was significantly reduced in experimental groups compared to controls (Fig. 1).

Tissue antioxidant status

Accompanying changes in antioxidant enzyme showed that cytosolic SOD activity was significantly increased in group I and II (but lesser in group II) as compared to controls. Catalase and glutathione peroxidase (GPX) showed a reverse trend, where the activity was found to be significantly reduced in both experimental groups in comparison to control group (Fig. 2).

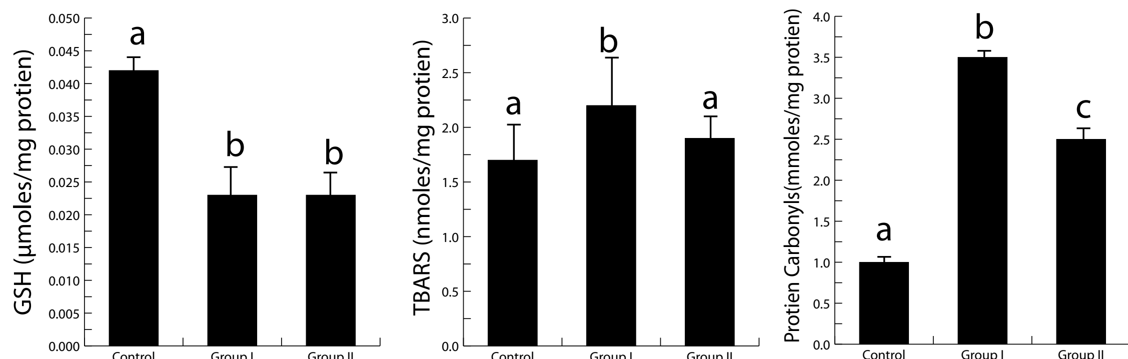
Functional integrity of the jejunal villi

Lys-ala-dipeptidyl aminopeptidase activity was significantly less in group I but comparable in group II to controls. Significantly decreased activity of alkaline phosphatase (maximal towards to the upper half of the villus) was seen in both experimental groups as compared to controls (Fig. 3).

Discussion

Essentially, group I rats received a cumulative dose of 9 mg cisplatin/kg body weight during the 3-week period and the tissues were available for all the analysis. Although group II rats were sacrificed within 2 weeks to get the tissues for analysis, the data may not be comparable to group I, because they were not time-matched. In line with earlier reports that chemotherapy causes intestinal damage with apoptosis in intestinal crypts, and hypoproliferation resulting in crypt hypoplasia (Keefe *et al.* 2000). Our present study also revealed that the crypt region showed greater sensitivity to cisplatin. We observed that cisplatin could induce apoptosis in both differentiated (villi) and proliferative (crypt) regions of the intestinal epithelium indicating that its action is not cell cycle specific.

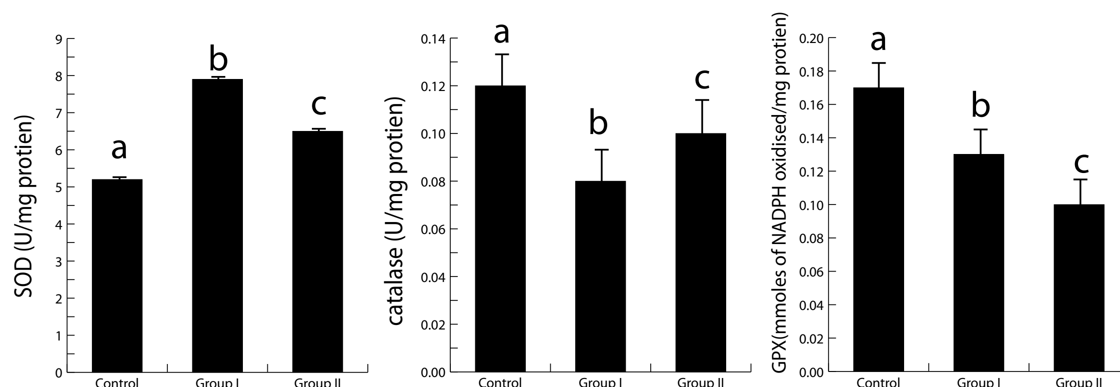
Fig 1. Effect of cisplatin on oxidative stress in IECs.



Values with different superscripts are significantly different ($P \leq 0.05$)

Vertical bars are means with standard error (n=3 per group). Levels of lipid peroxides (TBARS) do not show significant difference while glutathione (GSH) and protein carbonyls are significantly different.

Fig 2. Antioxidant enzymes status in control and cisplatin treated groups



Values with different superscripts are significantly different (P ≤0.05)

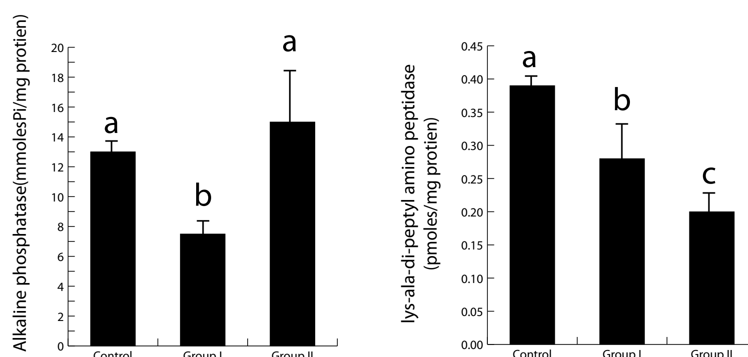
Vertical bars are means with standard error (n=3 per group) Superoxide dismutase (SOD), catalase and GPX levels are significantly different between groups.

Caspase 3 is an effector caspase activated by many apoptotic stimuli. The present study also showed increased effector caspase activation in rats treated with cisplatin confirming that intestinal epithelial cells (IEC) death in cisplatin treated rats is truly apoptotic and not necrotic, the sensitivity of IECs to cisplatin induced apoptosis was further assessed by DNA fragmentation in these cells. Despite a constitutive low rate of apoptosis in IECs of control rats, no DNA fragmentation was detectable. Cisplatin treatment *per se* significantly increased DNA fragmentation. This could be due to DNA breakage as a result of drug interactions with DNA.

There is a balance maintained between oxidative stress and antioxidant enzymes for normal functioning of the cells. To cope with increased oxidative stress, antioxidant enzymes are generally up-regulated. It is well known that cisplatin is a redox cycler that generates toxic reactive oxygen species and causes oxidative injury to various cells and tissues (Chu, 1994; Masuda *et al.* 2003). The significantly higher levels of TBARS and protein carbonyls (oxidative products of lipids and proteins) in the IECs of cisplatin treated rats indicates increased oxidative stress and the data suggests that cisplatin treatment substantially increased the ROS formation in the intestinal mucosa (Benhar *et al*. 2001). Enhanced protein oxidation was observed in group I and II as compared to controls. However, it was lesser in group II than group I because of the short-term exposure to the drug.

Endogenous ‘antioxidant’ systems are of singular importance in limiting oxidative cellular damage. Elevated intracellular levels of GSH, the cellular antioxidant involved in free radical scavenging activity, is associated with resistance to chemotherapeutic agents (Zhang *et al*. 1998). The GSH levels in the IECs treated with cisplatin in our study were decreased

Fig 3. Effect of cisplatin on IEC marker enzyme activities



Values with different superscripts are significantly different (P ≤ 0.05)

Vertical bars are means with standard error (n=3 per group). No change in lys-ala-di-peptyl amino peptidase but significant differences seen with alkaline phosphatase between the groups.

markedly. Cisplatin treatment increased SOD and decreased catalase and GPX levels than control rats. These changes in the antioxidant enzymes observed in this study are in general agreement with earlier reports where up-regulation/over expression of SOD and catalase has been shown to decrease the toxicity of cisplatin (Davis *et al.* 2001) and down-regulation of GPX was associated with mitochondrial dysfunction in the kidney epithelial cells (Sugiyama *et al.* 1989). The increased SOD activity in cisplatin treated animals, could be in response to increased generation of superoxide (O_2^-) radicals known to occur during the interaction between cisplatin and DNA and are involved in the toxic effects of this drug (Masuda *et al.* 2003). These toxic effects can be decreased by overexpression of Mn-SOD and catalase enzymes (Davis *et al.* 2001). However, in the current study the increased SOD activity could not protect the cells probably because there was a decrease in catalase and GPX activities. These changes in antioxidant enzyme activities may enhance the accumulation of hydroxyl radicals causing damage and apoptosis.

It appears that the possible increase in the accumulation of H_2O_2 or R_2O_2 as a consequence of the imbalance in the antioxidant enzyme activities could enhance the intrinsic sensitivity of the intestinal mucosa to cisplatin-induced apoptosis. It is known that along with O_2^- radicals, ·OH radicals also play role in cisplatin toxicity (Masuda *et al.* 2003). Thus increased oxidative stress seems important in cisplatin induced apoptosis/cytotoxicity.

We intended to look into the integrity of the mucosal cells, which is compromised upon exposure to chemotherapeutic agents due to increased oxidative stress. Cisplatin administration significantly altered mucosal marker enzyme activities in IECs of control rats indicating altered structural and functional integrity of mucosa. These results indicate that cisplatin *per se* increases oxidative stress in general and lipid peroxidation in particular, which damage the membranes involving membrane fluidification (Rebillard *et al.* 2007). It could also result in loss of barrier integrity which is a risk factor for infection (Khan and Wingard, 2001). Severe oxidative damage to intestine could be the causative agents for the death of the animals (Arivarasu *et al.* 2007).

Among different doses of cisplatin tested, 3mg/kg bodyweight could effectively induce apoptosis without causing lethality. This is in contrast to earlier studies, wherein single dose administration of 5mg/kg cisplatin induced apoptosis, but it was on a smaller magnitude and transient, peaking at 36 h and reaching control by 48 h (Tamaki *et al.* 2003). The results of this preliminary study demonstrate that intraperitoneally administered cisplatin at a dose of 3.0 mg/kg body weight for three weeks (once in a week) is safe, well tolerated, and effective in inducing apoptosis in both villus and crypt regions.

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