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Original Article

The Study of protective effect of Nordihydroguaiaretic acid (NDGA) on cisplatin induced genotoxicity and nephrotoxicity by attenuating oxidative stress in Swiss albino mice

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ABSTRACT

Cisplatin is a magic chemotherapeutic drug, unfortunately its dose dependant dark sides may halt its magic's. It has major dark sides such as genotoxicity and nephrotoxicity. Cisplatin has a strong power to sire a great amount of free oxidative radicals, which contributes to its dark sides. The emphasis of the present study was to investigate whether the pre-treatment of NDGA had any protective consequence against cisplatin-induced genotoxicity and nephrotoxicity. In this study we evaluated the role of NDGA on cisplatin induced oxidative stress in mice through following parameters like chromosome aberrations, micronucleus assay, lipid peroxidation and glutathione depletions. The animals were divided into four treatment groups with six mice in each group (n=6). A single dose of cisplatin (7 mg/kg b.w.) has been administered on the 5th day of study to cisplatin control group. In NDGA Pre-treatment group receives cisplatin after five days NDGA pre-treatment. Then animal had been sacrificed 24 hrs after cisplatin treatment. There was a significant reduction in chromosomal aberration, glutathione level and increase in the lipid peroxidation. The anti-oxidant action of NDGA presumably modulates the oxidative stress induced by cisplatin and significantly ($p<0.05$) restore the reduced level of chromosomal aberration and anti-oxidant enzymes.

Key words: cisplatin, genotoxicity, glutathione, chromosomal aberration, anti-oxidant etc

INTRODUCTION

Cisplatin (cis-diaminedichloroplatinum, CDDP), is a more potent drug, which portrays one of the standard drug in chemotherapy. It is widely used for the management of solid tumors likes head , neck, ovarian and germ line carcinomas[1]. Nevertheless, its cumulative accumulation in the kidney is primarily responsible for its dose dependant nephrotoxicity[2]. Unfortunatally approximately 25-30 % of patients develop evidance of nephrotoxicity following a single dose of cisplatin[3]. Cisplatin is inorganic platinum derivatives, which causing the ability to cause inter and intra strand cross linking[4]. In spite of this numerous reports proved that cisplatin is a highly mutagenic and has potential to cause chromosomal abberation in peripheral blood lymphocyte of human and bone marrow of mice[5, 6]. Cisplatin is a directly acting alkylating agent which undergoes hydrolysis in aquous solution. After being hydrolysis it causes inter and intra strand crosslinking between N-7 and O-6 of the adjacent guanine molecule leads to denaturation of DNA[7]. Altogether these alterations may lead to cessation of abnormal base pairing at DNA replication.. In general, at least three types of DNA alterations have been attributed to the chemical alkylation, depurintion and single strand breakes[8]. However cisplatin was able to generate active oxygen species, like superoxide and hydroxyl radicals[9, 10] . Likewise cisplatin has a strong negative impact on the anti - oxidant profile. The anti-oxidant enzymes such as glutathione, catalase are gradually decreasing by cisplatin treatment. This substantial decrease in anti-oxidant level, which may reflect the pathogenesis of cisplatin induced renal failure[11]. Therefore, minimization of this side effect

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is requirement to improve the effectualness of cisplatin chemotherapy. Thus, much aid has been paid towards the possible role of phenolic Lignan in direction of darks face produced by cisplatin Nordihydroguaiaretic acid (NDGA) is a phenolic lignan, which obtained from the evergreen shrubs *Larreadivaricata* and *Guaiacum officinale*[12] . It is a polyphenol bearing O-hydroxyl structure, possesses four phenolic hydroxyl groups. As such, NDGA is recognized as a strong antioxidant with several beneficial health effects[13] . In ancient time various indigenous tribes of North America like seri, Pima, Yaqui, Mericopa have used extract of NDGA for treatment of copious diseases like diabetes, cancer, chicken pox, gall bladder and kidney stones [13]. Instead of that, it has brought up with a notorious source of various phytochemical like phenolic lignans, flavonoids, condensed tannins, triterpene saponines, and napthoquinone etc. The Floriano-Sanchez group demonstrated that NDGA is a potent *in vitro* scavenger of reactive oxygen species (ROS) such as peroxynitrite ($ONOO^-$), singlet oxygen ($^1O2$), hydroxyl radical (•OH), superoxide anion ($O2^{-\bullet}$), and hydrogen peroxide (H_2O_2)(13). Further more NDGA inhibitory role on generation of free redical may protect the decreasing level of anti-oxidant enzymes[14]. Therefore, the present study was undertaken to investigate the effects of the NDGA on cisplatin-induced genotoxicity and nephrotoxicity.

MATERIALS AND METHODS

Drugs and Chemicals: Cisplatin, DTNB, NDGA, bovine serum albumin (BSA), glutathione reductase, 2- thiobarbituric acid (TBA), glutathione peroxidase, reduced glutathione (GSH), catalase, superoxide dismutase(SOD) assay kit were purchased from Sigma (St. Louis,MO, USA). Giemsa solution (5 and 7%) in phosphate buffer (pH 6.8),potassium chloride, methanol, glacial acetic acid and DMSO were purchased from E. Merck, India. Blood urea nitrogen (BUN), Creatinine and Albumin kits were purchased from Accurex (Mumbai, India). All the solutions were prepared freshly before the experiment**.**

Animals: The protocol of this study was approved by the Institutional Animal Ethical Committee (IAEC) of National Institute of Pharmaceutical Education And Research (NIPER) Guwahati(351/2001/ CPCSEA), India in accordance with the CPCSEA guidelines for the safe use and care of experimental animals. Eight-week-old adult male Swiss albino mice (20–25 g) were procured from the central animal facility of the institute (NIPER, Guwahati). Animal were housed in a controlled environment (temperature, 24±3 ◦C and relative humidity, 55 ± 15%) with a 12-h light/dark cycle. During the experimental period animals were provided with normal pellet diet and water ad libitum.

Experimental design: A total of 24 adult male Swiss albino mice were used in the study .The animals were randomly divided into four groups, comprising six mice in each. The dose used for inducing nephrotoxicity and genotoxicity is based on various litreatures.Experimental design was executed as follows:

Group 1- Normal control- served as vehical control treated with 0.5 % DMSO.

Group 2- NDGA control - Mice were treated with NDGA (10 mg/kg/day i.p) which is dissolved in 0.5% for five successive days.

Group 3- Cisplatin control- Normal saline was administered intra-peritonially for 5 days and a single dose of cisplatin (7 mg/kg, i.p) dissolved in normal saline was administered on the 5th day.

Group 4- NDGA (10 mg/kg) + Cis- Mice were receive a five days pre-treatment of NDGA (10 mg/kg/day) and a single dose of cisplatin (7 mg/kg, i.p) on 5th day, 1 hour prior to NDGA dose.

Chromosomal abberation: All animals were injected intraperitoneally with 2.0 mM colchicine 90 min before being killed by decapitation, which occurred 24 h after cisplatin treatment. Bone marrow preparations for the analysis of chromosome aberrations in metaphase cells were obtained by the technique of Ford and Hamerton[15]. One hundred metaphases per animal were analysed in order to determine the frequencies of chromosome aberrations in a blind test. The mitotic index was obtained by counting the number of mitotic cells in 1000 cells analysed per animal[16].

Micronucleus assay: For this test, mice were sacrificed 24 h after treatment with a single intraperitoneal dose of cisplatin (7 mg/kg bwt). Bone marrow from femur bones was collected for the micronucleus assay. The time of peak response of induction was selected as the sacrifice time based on the preliminary assays that were managed to pick out an appropriate dose of that did not suppress cell proliferation in combination with the proper NDGA dose. The mouse bone marrow micronucleus test was carried out according to the method of Schmid[17]. Air-dried slides were stained with May–Grunwald and Giemsa as described by Schmid, made permanent, and coded. A total of 1000 polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were scored per animal by the same observer to determine the frequencies of micronucleated polychromatic erythrocytes (MnPCEs). To detect possible cytotoxic effects, the PCE:NCE ratio in 200 erythrocytes per animal was calculated according to

Gollapudi and McFadden[18]. Coded slides were scored with a light microscope at a 1000× magnification.

Assessment of renal functions: Estimation of renal functions like blood urea nitrogen (BUN) and creatinine was assessed by using multi plate moderator. The diagnostic kits for measurement of BUN and creatinine were purchased from Accurex India Limited. Tests were performed according to the manufacturer's instructions. In all the cases, a standard curve was constructed from the standards provided by the manufacturer.

Post-mitochondrial supernatant preparation: Kidneys were removed quickly, cleaned of extraneous material and immediately perfused with ice-cold saline (0.85% NaCl). The kidneys were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%) using homogenizer. The homogenate was filtered through muslin cloth and centrifuged at 800×g for 5 min at 4 °C by a REMI cooling centrifuge to separate the nuclear debris. The aliquot obtained was centrifuged at 12,000 rpm for 20 min at 4 °C to obtain PMS, which was used as a source of enzymes. All biochemical estimations were completed within 24 h of animal sacrifice[19].

Estimation of Malondialdehyde formation: The reaction mixture contained 0.60 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsomes and 0.2 ml ascorbic acid (100 mM) in a total volume of 1.0 ml. The reaction mixture was incubated at 37 °C in a shaking water bath for 1 h and stopped by adding 1.0 ml 10% trichloroacetic acid (TCA). Following the addition of 1.0 ml 0.67% thiobarbituric acid (TBA), all tubes were placed in a boiling water bath for 20 min and then shifted to a crushed ice-bath before centrifuging at 2500×g for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm against a reagent blank using a spectrophotometer (Perkin Elmer, Lamda EZ 201). The results were expressed as nmol MDA formed per hour per gram tissue at 37 °C using a molar extinction coefficient of $1.56\times105\ M^{-1}\ cm^{-120}$.

Estimation of Reduced Glutathione estimation: One-milliliter samples of PMS were precipitated with 1.0 ml sulfosalicylic acid (4%), kept at 4 °C for 1 h and then centrifuged at 1200×g for 20 min at 4 °C. The assay mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 1, 2- dithiobisnitrobenzoic acid (DTNB; 100 mM) in a total volume of 3.0 ml. The yellow color that developed was quantified at 412 nm on a spectrophotometer[21].

Estimation of Catalase activity: The reaction mixture consisted of 1.95 ml phosphate buffer (0.1 M, pH 7.4), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml 10% PMS in a final volume of 3 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed per min per mg protein[22].

Statistical analysis: The differences in the number of cells with chromosome aberrations, abnormal metaphases and mitotic index between cells treated with NDGA and/or cisplatin were analysed statistically by the Fisher exact test, with the level of significance set at (P= 0.05). Statistical analysis of the biochemical assays was performed using analysis of variance (ANOVA). Differences between treatments were determined by the Mann–Whitney test. The results were expressed as the mean±SEM of six values in each group, and a statistical probability of (P<0.05) was considered to be significant.

RESULT

Chromosomal aberrations: The result of chromosome abberation analysis in bone marrow cells of male swiss albino mice treated with cisplatin (7 mg/kg) , NDGA (10 mg/kg), and cisplarin plus NDGA are presented in Table 1. The group II treated with NDGA alone (10 mg/kg i.p body weight) did not shows any significant variation in the total number of chromosome aberrations or abnormal metaphases compared to the normal control group. But as expected, animals treated with a single dose of cisplatin in cisplatin control animal (7 mg/kg i.p. per body weight) showed an increased bone marrow cells with chromosomal aberrations ($p< 0.05$) observed 24 h after treatment with the cisplatin as compared to normal control. In general, one aberration per cell was observed in Animals treated with cisplatin, but cells with three or four aberration were also studied in table I. As result examined it was clear that the chromosome aberration detected at highest frequency were chromatic type breaks, then after isochromatid type breaks, gaps and other rearrangements such as a triradial figure. The data showed that the pretreatment of NDGA (10 mg/kg) administered presented a reduction of about 37% in cisplatin-induced chromosome abberation, when compared to the animal treated with cisplatin control group. (Table1). However, the Fisher test showed that this reduction was statistically significant. Nevertheless, there is no substantial difference in mitotic index values was observed in Group II the animal were treated with NDGA alone with compared to normal control animals. The animal treated with cisplatin presented a significantly lower mitotic index with compared to

the normal control group (p<0.05). The five days pre-treatment with NDGA significantly (p<0.05) protected against the inhibitory effect of cisplatin on mitotic index.

Micronucleus assay: The results obtained from the micronucleus study are presented in Figure I and II. Treatment of mice with NDGA did not induce any significant variation in the incidence of MNPCE as compared to the normal control animals. In additions, NDGA was not cytotoxic to the bone marrow (i.e. no statistically significant decrease in the PCE:NCE ratio) at the tested dose level. In comparing to the control group, the frequency of MNPCE was significantly(p<0.05) increased in cisplatin alone treated mice. however NDGA pre-treatment group significantly decrease the elevated level of MN as compared to cisplatin control group (p<0.05). (fig I). Nevertheless the estimated PCE: NCE ratio in bone marrow preparations shows a statistically significant reduction in the cisplatin control group with comparison to a normal control group. NDGA pre-treatment for five days significantly increased the reduced ratio of PCE: NCE as compared to the cisplatin control group (p<0.05). (fig II)

Effects of NDGA pretreatment on renal toxicity marker enzymes

Effect on BUN: The governance of a single dose of cisplatin (7 mg/kg i.p. per Body weight) leads to activation of free radical which directly or indirectly acts as a striking role in elevated stage of renal toxicity makers like BUN and creatinine. As showed in figure III , A single dose of cisplatin leads tremendous increase in BUN level in the cisplatin control group as compared to normal control animal. The five days pre-treatment of NDGA significantly diminishes the elevated level of BUN as compared to cisplatin control animal (p<0.05).

Effect on Creatinine: Likewise , there was a substantial elevation of level followed by a single dose of in a cisplatin control group with comparison to a normal control group (P<0.05). A five days pre-treatment of NDGA elicits a protective role on mice and subsequently reduce th e elevated level of creatinine with comparison to cisplatin control animals (p<0.05) (fig IV).

Protective effect of NDGA pre-treatment on anti-oxidant enzyme level

Effect on lipid peroxidation: TBARS production in the kidney tissue had been used as a measure of lipid peroxidation. The TBARS production was similar in groups treated with NDGA control and in the normal control group. The administration of NDGA alone for five consecutive days did not increase lipid peroxidation compared to the normal control group. Table II showed the results for all the groups. A single dose of cisplatin enhanced the formation of lipid peroxides in compared to the normal control group. This increase was prevented by pre-treatment with NDGA (*P*<0.05). The values of lipid peroxidation were restored to approximately normal levels in the rats pretreated with NDGA plus cisplatin**.**

Effect on renal glutathione level: The concentration of renal GSH was significantly decreased (p<0.05) in a cispltin control group with respect to normal control animal by a single dose of cisplatin. However a pre-treatment of NDGA significantly escaltes the reduced level of GSH with compared to cisplatin control group.

Effect of NDGA on catalase: A rapid depletion of catalase level was observed, which followed by a single dose of cisplatin. The single dose administration of cisplatin gradually decreases the catalog level (p<0.05) with compared to a normal control group. The depleted level of catalase was significantly (P<0.05) elevated by a five days pre-treatment with NDGA.

DISCUSSION

Chemotherapy is one of the most valuable method acting for the treatment of malignant neoplastic disease, but the cytotoxic action of chemotherapeutic drugs in normal cells can cause severe side effects such as immune-suppression, genotoxicity, nephrotoxicity, neurotoxicity, nausea, vomiting, hair loss, anemia and digestive upsets[23-26]. Hence, the damage to normal tissue by anti-tumor drugs can be a major limitation for patient's tolerance to chemotherapy. Cisplatin is one of the chemotherapeutic agent extensively used for the management of germ cell tumors, head and neck cancers, bladder caner and as a salvage treatment for other solid tumors. Although higher Doses of cisplatin are more efficient for the suppression Of cancer, but high dose therapy manifests the genotoxicity and irreversible Renal dysfunction[27, 28]. The highest concentration of cisplatin is found in cytosol, mitochondria, nuclei, and microsomes. Genomic instability is frequently thought to be the hallmark of cancer, and according to researchers it is clear that cisplatin treatment leads to genotoxicity and chromosomal abberations. These breaks are believed to result from the interaction of cisplatin with DNA through the binding with N7 of purine bases, forming cisplatin–DNA adducts[29]. The high mutagenic potency of cisplatin raises the fear that its function in cancer chemotherapy may be responsible for secondary malignancies, which have been noticed in animals and some cured patients treated with cisplatin[30, 31].

NDGA is a obtained from Creosote bush, *Larrea tridentata,* is known as chaparral or greasewood in the United States and as gobernadora or hediondilla in Mexico[14]. Many of indigenous communities used extracts and preparations of NDGA for the treatment of various disorders like chicken pox, diabetes, kidney stones, gallbladder stones and cancer[32]. The Floriano-Sanchez group demonstrated that NDGA is a potent in vitro scavenger of reactive oxygen species (ROS)[14]. Although its chemical moiety resembles the most potent anti-oxidant action[13].

Our study clearly demonstrates the antigenotoxic and anti-oxidant potential of NDGA in cisplatin-evoked genotoxicity and nephrotoxicity. The ability of cisplatin to interact with DNA is formed interstrand and intrastrand cross- connections have been suggested to play the principal character, not only in its anticancer action but also in chromosome aberrations[33]. A tremendous increase in chromatide types of break and abberant metaphases had been followed by a single dose of cisplatin in cisplartin control animals with comparision to normal control group.In general one aberration per cell was observed, but in some cases 2-3 operations were also examined. The highest frequency of chromosomal aberrations observed after the 24 hours of cisplatin administration. In our present study five days pre-treatment with NDGA significantly reduce the genotoxic damage, this is ascribable to its antigenotoxic activity. Its activity refers to its engagement as an enzyme inhibitor in the arachidonic acid cascade as well as in the *P*-450 dependent monooxigenases probably because the binding of the hydroxy groups with the catalytic sites of the enzyme and prevent further harm[(13)]. Although the mitotic index was also get disturbed due to cisplatin. However pre-treatment with NDGA significantly managed the reduced level of mitoic index.

The micronucleus assay is intellect formost for evaluating the ability of test agents to induce structural and numerical chromosomal damage[34]. Inter and intrastrand cross-linking ability of cisplatin rapidly evoked the frquency of MNPCE in cisplatin control group of animals. An increment in frequency of MNPCE in cisplatin alone treated animal it was a hallmark for induction of genotoxic damage. The pre-treatment of NDGA significantly manage the elevated level of MNPCE. Nevertheless in micronucleus assay, PCE : NCE ratio cisplatin control group and NDGA + Cis (NDGA pre-treatment) group provides cytotoxicity index. Cytotoxicity index was found to be lower in cisplatin control as comparision to normal control group. NDGA pre-treatment had significant cytotoxicity index with comparision to cisplatin control group.

An administration of cisplatin shows a substantial increment in serum creatinine and BUN concentrations with compared to normal control animals which clearly shows the intrinsic failure in acute renal failure. The renal accumulation of platinum and covalant binding of platinum with renal protein may play significant role acute renal failure[17].

Cisplatin showed the inhibitory function of anti-oxidant enzyme profiles, which may contribute to rapid generation of free radicals and reactive oxygen species[35]. This study demonstrates a reduced level of renal GSH and catalase level in a cisplatin control group with compared to a normal control group. These observations support the mechanism of nephrotoxicity induced by cisplatin in mice is partially related to the depletion of renal anti-oxidant system. Pre-treatment of NDGA for five days before cisplatin challenged significantly prevent the decrease of renal GSH and catalase level in pre-treatment group with comparison to cisplatin control group. Despite the reduced level of catalase which in turn increase the concentration of H_2O_2. Hence the concentration of MDA, as a result of lipid peroxidation, increased in the cisplatin control group as compared to a nornmal control group. A pre-treatment with NDGA prevent lipid peroxidation by enhncing the level of catalase. The outcomes of the present study concluded that a pre-treatment of NDGA reduced the total amount of chromosomes abberations, ratio in the bone marrow of mice. Also, NDGA has a potent anti-oxidant activity which partially mediated by preventing cisplatin induced decline of renal anti-oxidant enzymes and address free radical scavenging action.

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Conflict of Interest: The authors declare that they have no competing interest.

Table I: Effect of NDGA on chromosomal Aberrations induced by cisplatin in Swiss albino mice

Treatment	Mitotic Index	Types of Chromosomal Aberrations					AM	
		Gaps	Breaks		E	T	(NO.)	(%)
			C	IC				
Normal Control	2.9	4	9	0	0	0	13	2.1
NDGA Control	2.5	7	13	1	0	0	21	3.5
Cis Control	1.4[*a]	21	108	9	6	2	146[*a]	24.33
NDGA + Cis	2.3[*b]	10	78	5	2	0	93[*b]	15.5

One-hundred cells were analysed per animal for chromosome aberrations, for a total of 600 metaphases per treatment. The mitotic index was obtained by counting 6000 mitotic cells per treatment.

C=chromatid type, IC=isochromatid type, E=complex exchange, T=triradial figure, AM=abnormal metaphase.

[*a]*p* Significantly different from the Normal control group (*P*<0.05).

[*b]*p* significantly different from the Cis control group(*P*<0.05).

Table:II – Result of pre-treatment of NDGA on anti-oxidant enzymes like MDA, GSH and Catalase on cisplatin administration in kidney of Swiss albino mice.

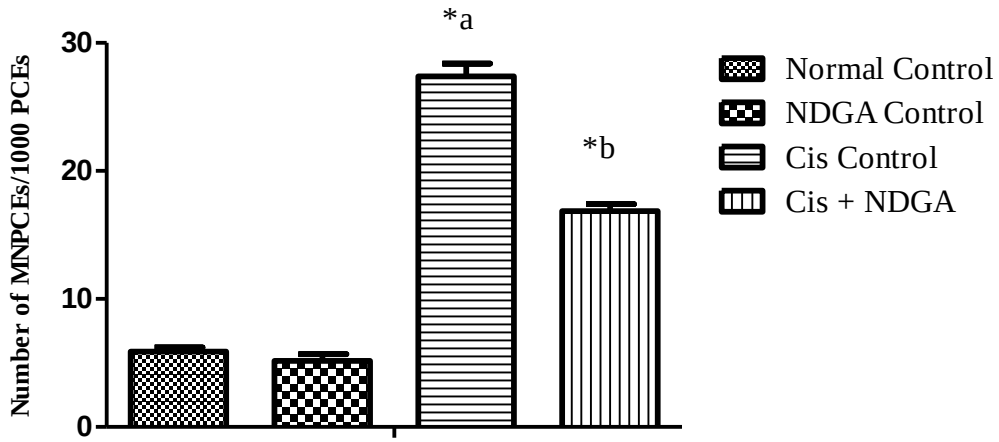
Treatment	MDA (nmol gm^{-1} tissue)	GSH (µ mol GSH g-1 of protein)	Catalase (nmol H2O2 consumed per protein)
Normal Control	118.27±1.99	33.52±0.87	122.97±1.34
NDGA Control	131.6±2.17	29.82±0.42	124.8±2.2
Cis Control	170.47±2.67[*a]	24.43±0.46[*a]	65.4±2.47[*a]
NDGA + Cis	131.6±2.17[*b]	28.43±0.48[*b]	101.23±1.92[*b]

Each value represents mean ± SEM (standard error of mean) of six animals.

[*a]*p* Significantly different from the Normal control group (*P*<0:05).

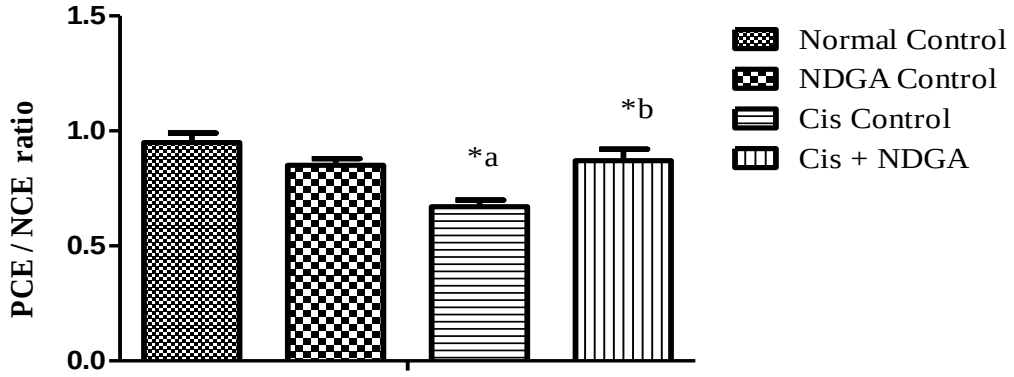
[*b]*p* Significantly different from the Cisplatin control group (*P*<0:05).

Figure I Effect of NDGA on Micronucleus induction by cisplatin in Swiss albino mice.



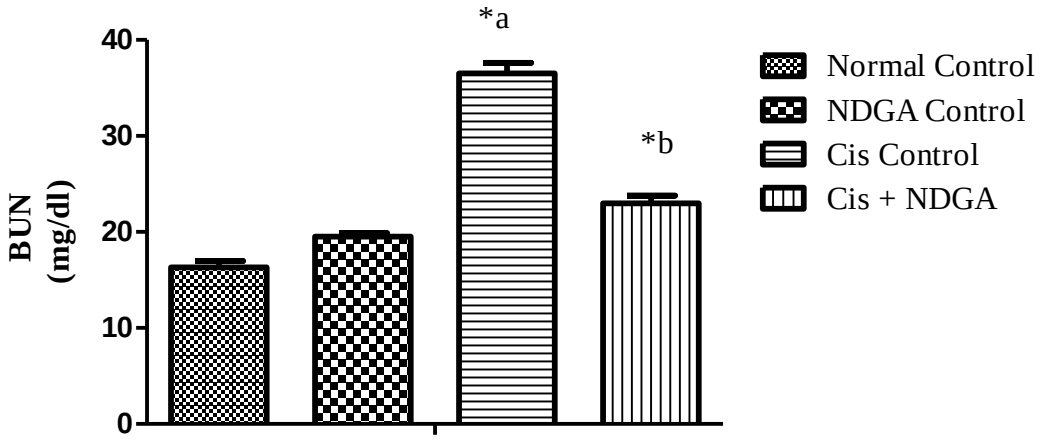
Micronucleus induction in the bone marrow of mice: there was a marked increase ($p<0.05$) in the micronucleus content of the polychromatic erythrocytes (PCE) in the bone marrow of mice due to Cisplatin treatment compared to normal controls animals. However, pre-treatment with NDGA diminish micronucleus formation significantly ($p<0.05$).

Figure II Effect of NDGA on PCE: NCE ratio in swiss albino mice.



PCE:NCE ratios in the bone marrow of mice: There was a significant decrease in the PCE:NCE ratio in the cisplatin-treated group, displaying induction of cytotoxicity in the bone marrow cells of Swiss albino mice. Pre-treatment with NDGA significantly elevates the reduced ratio of PCE:NCE.

Figure III Effect of pretreatment of NDGA on kidney toxicity marker BUN on cisplatin administration in Swiss albino mice.

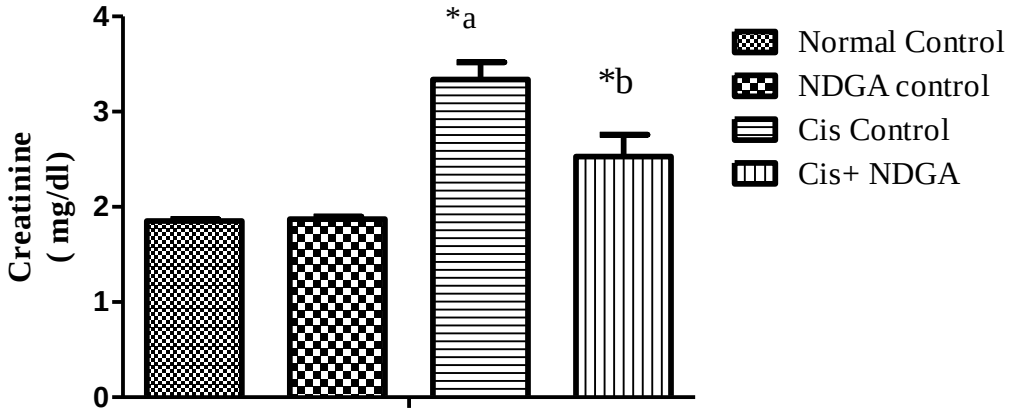


The results represent mean±SEM of six animals per group. There was a rapid, significant elevation of BUN which folowed by a single dose administration of cisplatin in the cisplatin control group as compared to normal control animals. A five days pre-treatment with NDGA signlificantly reduce the elevated level of BUN with compared to cisplatin control group.

*[a]p Significantly different from the Normal control group (P<0.05).

*[b]*p* Significantly different from the Cisplatin control group (P<0:05).

Figure IV Effect of pretreatment of NDGA on kidney toxicity marker creatinine on cisplatin administration in Swiss albino mice.



The results represent mean ± SEM of six animals per group. There was a significant elevation of creatinine level which followed by a single dose administration of cisplatin in the cisplatin control group as compared to normal control animals. A five days pre-treatment with NDGA signlificantly reduce the elevated level of creatinine with compared to cisplatin control group

*[a]p Significantly different from the Normal control group (P<0.05).

*[b]*p* significantly different from the Cisplatin control group (P<0:05).

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