Evaluation of Genotoxicity of Cisplatin in White Mice

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Abstract:

The current study was conducted to evaluate genotoxic and cytotoxic effects of cisplatin in white mice.The cytogenetics end points (micronuclei) were used as genomarker to assess the genotoxicity. Mitotic index(MI) and hematological end points (PCEs, NCEs, Hb, MCV, and Ptl) were used to assess cytotoxicity. Cisplatin is a chemotherapeutic agent for many kinds of cancer. We tested the dosages 2.0 and 10 mg/kg.b.wt. by intraperitoneal ingection. Standard methods for Micronucleus were used to assess the clastogenic and aneugenic effects of cisplatin in bone-marrow cells of white mice. The Results of this study showed elevated levels of micronucleated polychromatic erythrocytes (MnPCEs), While MI decreased significantly, (PCEs, NCEs, Hb, MCV, and Ptl increased significantly. This result lead to suggest that the tested dosages of cisplatin have genotoxic and cytotoxic effects in white mice.

Keywords: Cisplatin, genotoxicity, micronuclei, polychromatic erythrocytes, and chromosome aberration.

Note: The current study was conducted in the laboratories of college of science university of Duhok and college of science – Tikrit University – Iraq.

تقدير السمية الوراثية للعقار سيسبلاتين في الفئران البيض

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المستخلص :

أجريت هذه الدراسة في مختبرات كلية العلوم – جامعة دهوك ومختبرات كلية العلوم – جامعة تكريت – العراق لتقييم التأثيرات السمية الوراثية والسمية الخلوية للعقار المضاد للسرطان Cisplatin في الفئران البيض. تم استعمال مؤشرات الوراثة الخلوية (النوى الدقيقة لتقدير

السمية الوراثية ومعامل الانقسام الميتوزي ومؤشرات الدم (تعداد كريات الدم الحمر الناضجة وغير الناضجة وخضاب الدم ومعدل حجم الخلايا وتعداد الصفيحات الدموية) لتقدير السمية الخلوية. يعد السيسبلاتين احد العلاجات الكيميائية المستخدمة للعديد من انواع السرطان. وقد تم اختبار الجرعتين 2.0 و 10 ملغم\ كغم. وزن جسم بالحقن داخل الخلب، واستعملت الطرق القياسية لاختبار النوى الدقيقة ومعايير الدم. أظهرت النتائج ان للسيسبلاتين سمية وراثية تمثلت بارتفاع مستويات النوى الدقيقة في كريات الدم الحمر غير الناضجة وسمية خلوية تمثلت بانخفاض معنوي عند مستوى $P< 0.01$ في معامل الانقسام الميتوزي ومؤشرات الدم في المجموعتين المعاملة مقارنة بمجموعة السيطرة غير المعاملة. وهذا يقود الى الاعتقاد بان للجرعتين المستخدمة من السيسبلاتين تأثير جانبي سلبي كما اتضح من الدراسة الحالية على الفئران البيض.

Introduction

Antineoplastic drugs(ANDs) have been in clinical use for five decades. The carcinogenic properties of many of these drugs have been studied extensively in animal models and in follow-up studies of patient populations (1). Cisplatine is a chemotherapeutic drug which is used to treat cancers including: Sarcoma, small cell lung cancer, germ cell tumors, lymphoma and ovarian cancer. While it is often considered an alkylating agent, it contains no alkyl groups and does not instigate alkylating reactions, so it is properly designated as an alkylating-like drug. Cisplatin is platinum-based and was the first medicine developed in that drug class. The other names for cisplatin are diamminedichloridoplatinum (DDP), cisplatinum and cis-diamminedichloridoplatinum(II) (CDDP). By the late 1970s Cisplatin was already widely used and is still used today despite the many newer chemotherapy drugs developed over the past decades (1). The platinum-based chemotherapy drugs cisplatin, carboplatin, and oxaliplatin are among the most active and widely used agents for the treatment of malignancies, including testicular, head and neck, ovarian, lung, colorectal, and bladder cancers (2). It is generally accepted that these agents kill tumor cells primarily by creating DNA lesions, which are most cytotoxic during S-phase, probably because the lesions are potent inhibitors of DNA replication (3). In the case of bulky adducts such as intra-strand cross-links (which comprise the majority of platin-induced lesions), the stalled replication fork triggers the mono-ubiquitylation of proliferating cell nuclear antigen (4). Ubiquitylated proliferating cell nuclear antigen then recruits one or more translation synthesis (TLS) polymerases, which have active

sites that can accommodate bulky lesions, thereby allowing error-prone bypass of the lesion. In contrast, inter-strand cross-links, which account for a few percentage of cisplatin-induced DNA lesions but are far more cytotoxic, cannot be simply bypassed. Instead, their repair involves a complex interplay between a series of DNA repair pathways, including the TLS, Fanconi's anemia (FA), and homologous repair (HR) pathways (5). Although the complete mechanistic details of how these pathways accomplish this repair remain unknown, it is clear that defects in these pathways dramatically sensitize cells to agents that cause inter-strand cross-links, including the platinating agents (5). It is noteworthy that defects in these pathways are frequently found in tumor cells, raising the possibility that these repair deficiencies contribute to enhanced sensitivity of tumor cells to platinating agents (6,7).

The objective of this study was to (1) study the ability of cisplatine to induce clastogenic and aneuogenic effects in somatic cell (bone-marrow cell) and the marker used to assess this ability was the formation of micronuclei in polychromatic erythrocytes which formed by fragmented and\ or lagging chromosomes which may be seen in bone-marrow smears of white mice, (2) study cytotoxic effects of cisplatin by determination of Mitotic index in bone-marrow.

Material and methods

Animals:

Male Swiss albino mice (Mus musculus), 8–9 weeks old, weighed 25–30 g, were obtained from the animal house of University of Duhook. Animals were housed in polyplastic cages with steel wire tops in an air conditioned room (22 • ± 1ºC, 45–75% relative humidity) maintained in a controlled atmosphere of 12 h light/12 h dark cycles. Food and water were provided ad libitum.

Test compound:

Cisplatin Cis-diamminedichloroplatinum-II Cisplatin (Cis-DDP) was purchased in the form of a solution dissolved in distilled water (CAs No. 781520-03) under the trade name cisplatinum (EBEWE Pharma, Austria). The cisplatin dose was determined according to the human therapeutic dose (sub-acute dose 20 mg/m^2 and acute dose 100 mg/m^2), taking into consideration the relative body weight and surface area of the mice relative to that of an adult human being.

Treatment:

Doses and route of administration Cisplatin was administered both acutely and sub-acutely by a single intraperitoneal injection (i.p.) for five consecutive days and acutely by a single intraperitoneal injection (i.p.). In sub-acute treatment the therapeutic dose of cisplatin was 20 mg/m^2, while in acute treatment was 100 mg/m^2. The doses were adjusted for mice according to Paget and Barnes(8). The doses for mice were 2.0 mg/kg and 10 mg/kg, respectively. The control animals received an equal volume of the solvent by intra-peritoneal injection (i.p.) for five consecutive days.

Procedure:

Slides preparation for micronuclei (MN) and mitotic index (MI) The mouse micronucleus test was carried out according to Schmid (9) with some modification in fixation and staining based on the method of Heddle (10) and Hayashi (11). Animals were scarified 22 h after the last treatment either for sub-acute or acute treatment . Both the femora were removed and stripped clean of muscles. The contents of each one femur were flushed into 1.0 ml phosphate-buffered saline to prepare a marrow suspension. The bone- marrow suspension centrifuged at 1000 xg for 5 min. One drop of the pellet were placed on a microscope slide and mixed with drop of fetal calf serum. The cells were then smeared and allowed to dry. The slides were fixed in absolute methanol for 5 min and stained for 20 min in May-Grunwald and 5 min in 5% solution of Giemsa in 0.01 phosphate buffer adjusted to PH 6.8.

For mitotic index the pellet re-suspended in hypotonic KCl solution, placed in water bath at 37ºC for 20 min. Fixed with (1 volume of glacial acetic acid: 3 volumes of absolute methyl alcohol) freshly prepared, placed in refrigerator for 2hrs, two succeeded washes were done with the same fixative, centrifuged and suspension removed. 5 drops of pellet suspension were placed on clean cold slide, air dried and stained for 5 min in 5% Giemsa solution in 0.01 phosphate buffer to pH 6.8.

Scoring:

Stained preparations were coded and scored by a light microscope at 1000· magnification.

Micronucleus (MN):

One thousand polychromatic erythrocytes (PCEs) were scored per animal for determining the frequency of micro nucleated poly-chromatic erythrocytes (MnPCEs). Micronuclei were identified according to Schmid (10), Hayashi *et al*. (11) and Albanese and Middleton (12) criteria. They were morphologically identical to the normal nuclei but smaller than them (their diameter 1/5 of the main nuclei)

Mitotic index (MI):

The MI was scored in the same slides of MN by calculating the number of dividing cells (including prophase, metaphase and anaphase) in a population of 1000 cells.

Hematological end points:

Tissue sampling and sample analysis

Control and drug-treated mice were killed by IP injection of pentobarbitone sodium(Sagatal, Rhône Mérieux, Harlow, Essex, UK) and blood removed from the right ventricle following a thoracotomy incision. Blood (0.5 ml) was anti-coagulated with 1.5 mg/ml di-potassium EDTA (Teklab, Sacriston, Durham, UK). Standard laboratory methods for hematological tests were used to assess all parameters.

Statistical analysis

The student t-test analysis used for statistical analysis of the data. The results were expressed as mean± standard deviation (SD) for MN and MI data and were compared with controls by student's t-test and the minimum level of significance accepted being at $p < 0.05$.

Results

1. Body weight changes and clinical signs:

During drug administration, control, low- and high dose cisplatin mice changed in mean weight by 7.2% increase, a 3.2 decrease and 15.1 decrease respectively. In the post-dosing period (days 1-12), all animals increased in weight. mice in both cisplatin groups showed some loss of condition and reduced activity. There was no other clinical evidence of toxicity.

2. Hematological investigations:

Hematological results listed in (Table 1). In the immediate post-dosing period (days 1 and 4), in overall terms, RBC, Hb were increased. These unexpected changes were particularly evident on

day 4 at the higher cisplatin level (10 mg/kg) where the increases were 8.3 %, 11.5 % and 6.9 % above concurrent control results, respectively. At days 9 and 12, in the main, there were decreases in RBC, Hb values; these changes were significant at days 9 for the 10 mg/kg group and at day 12 for the 2 mg/kg group.

Table 1. Hematological results from male BALB/c mice treated with cisplatin at 2.0 and 10 mg/kg.b.wt over a period of 5 days and sampled at days 1, 4, 9, and 12 after final dose.

Sampling time (post-dosing)	T/ D Mg/kg.b.wt.	PCEs	RBCs	Hb	MCV	Plt
1	0 (control)	287.1	9.65	14.9	48.6	927
	2.0	34.8**	10.19*	15.6	46.9**	1343**
	105	19.5**	10.40*	16.0*	47.0*	1477**
4	0 (control)	300.6	10.30	15.6	48.1	935
	2.0	58.7**	10.17	15.4	46.6	808
	10	13.1**	11.15*	17.4**	47.6	764
9	0 (control)	307.5	10.15	15.7	48.5	980
	2.0	627.9**	9.73**	14.3**	47.3	622**
	10	185.8	10.17	15.3	46.3**	453**
12	0 (control)	295.5	10.30	16.0	49.3	1056
	2.0	518.3**	10.06	15.6	49.7	1150
	10	574.9**	9.55**	14.4**	48.4	1532**

Values are means. Control animals were not treated. There were five animals in each group at each sampling time. * P<0.05: **p<0.01: (significantly different from concurrent animals) . PCEs = polychromatic erythrocytes. RBCs = Normochromatic erythrocytes. Hb = Hemoglobin content g/ml of blood. MCV = mean packaged cell volume. Plt =platlet.

Reticulocyte (PCEs) counts were statistically significantly reduced at both cisplatin dose levels at days 1 and 4 post-dosing (Table 1). At days 9 and 12, counts suggested a return towards normal, in conjunction with a'rebound reticulocytosis', with day 12 values being statistically significantly above concurrent control data (increases were 175.4 % and 194.6 % above the control in the 2.0 and 10 mg/kg groups, respectively). At day 26, reticulocyte counts were at control baseline values(data not shown).

Cytogenetics investigations:

Micronuclei:

Both sub-acute and acute treatments of therapeutic doses, 2.0 mg/kg and 10 mg/kg, respectively, of cisplatin induced significant increase ($p < 0.001$) in the numbers of MnPCEs in comparison with the control group. Table 2 and Figure 1 shows the results of micronucleus test in control and treatments groups. we observed highly significant dose dependant at $P<0.001$ increases in number of micronucleated cells.

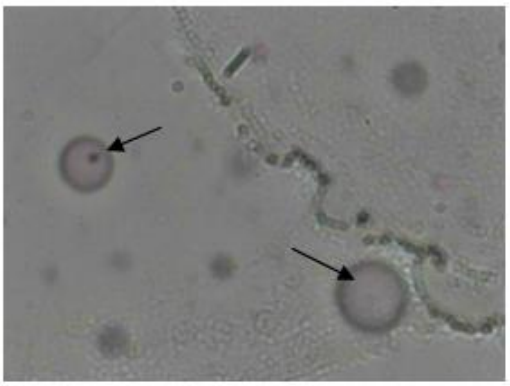

Figure 1. Polychromatic erythrocyte and micronucleated polychromatic erythrocyte. 100X geimsa and may-Grunwald stain.

Figure 2 shows normal chromosome spreads of mice in control group stained with 5% Giemsa.

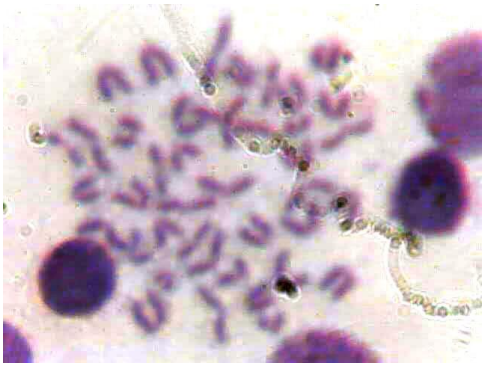

Figure 2. Mitosis chromosome spread in whit mice (n= 40) Giemsa stain, 100x.

Table 2 PCEs, MnPCEs and MN% results from male BALB/c mice treated with cisplatin at 2.0 and 10 mg/kg.b.wt over a period of 5 days and sampled at days 1, 4, 9, and 12 after final dose.

T/ D mg/kg.b. wt.	No. of mice	No. of PCEs	No. of MnPCEs	MnPCEs Mean± SD	% of MN
0.0	10	1000	235	23.50± 2.72	2.31
2.0	10	1000	861	86.1.30± 5.24**	8.61
10	10	1000	977	97.70± 5.86***	9.77

** p< 0.001 PCEs = polychromatic erythrocytes. MnPCEs = micronucleated polychromatic erythrocytes.Mn= Micronuclei.

3- Mitotic chromosomes:

Table 3 shows that percentage of dividing cells were decreased at dose- dependent manner with the increased dose of cisplatin.

Table 3. Mitotic Indices results from male BALB/c mice treated with cisplatin at 2.0 and 10 mg/kg.b.wt over a period of 5 days and sampled at days 1, 4, 9, and 12 after final dose.

T/ D mg/kg.b. wt.	No. of mice	No. of Examined cell	Total no. of Dividing cells	Mean± SD	Mitotic Index %
0.0	10	1000	549	54.90± 2.80	5.49
2.0	10	1000	213	21.30± 1.93**	2.31
10	10	1000	171	17.10± 1.35**	1.71

** p< 0.001

Discussion

All classes of ANDs have been shown to be mutagenic in *In vitro* assays[(18). Numerous ANDs have been found to be carcinogenic in animal models indications that AND-therapy was associated with second primary malignancies were initially reported in case reports and case series. In the mid-1970s, large-scale follow-up studies of cancer patients undergoing AND therapy were first reported in the literature. These reports strongly and consistently associated several alkylating agents with the development of leukemia and therapeutic application of these agents in dose dependant myelodysplastic syndromes (14, 15).

Cisplatin is a cytotoxic drug used in the treatment of metastatic ovarian and testicular carcinomas, bladder cancer, brain neoplasms and cancers of the lung, cervix, liver and pancreas. (16, 17). Cisplatin

has immunosuppressant activity and the drug is nephrotoxic, myelotoxic and ototoxic (16). Cisplatin is mutagenic and is assumed to be carcinogenic, embryotoxic and teratogenic (18) At high doses in man, Cisplatin may cause severe myelosuppression with bone marrow hypoplasia/aplasia. Dose-related changes in peripheral blood are seen with thrombocytopenia, leucopenia, granulocytopenia and anemia (19).

In the present investigations with cisplatin, during the 5-day dosing period, administration of drug at 10 mg/kg (the higher level) caused a decrease in mean body weight of 14.6% (control mice increased by 8.9%). Hematological changes (Table 1) showed that at later time points (days 26 and 61), there was no conclusive evidence of adverse late-stage/residual changes, apart from a possible effect on RBC/Hb/ at the higher cisplatine dose level.

The literature on cisplatin-induced toxicity in general and hematological toxicity in particular in
laboratory animals is not extensive. However, several authors have commented on the adverse effects of cisplatin on body weight in the mouse (20), in the rat (21, 22) . Table 1 presents results showing a generalized increase in erythrocyte values (RBC/Hb), in conjunction with reticulocytopenia at days 1 and 4, followed by an overall decrease in RBC/Hb levels at day 9 and 12. It is considered that these changes may be associated with a haemoconcentration effect and may relate to a cisplatin-induced body weight loss and dehydration in the immediate post-dosing period. Nowrousian and Schmidt (20) described an increase in RBC values in the cisplatin-treated mouse and proposed an association between this, dehydration and loss in body weight following cisplatin administration.

In the present study, i.p. doses (2.0 mg/kg and 10 mg/kg) of cisplatin were clastogenic, which was determined by the cytogenetic parameter, such as an extremely statistically significant increase in the number of micronucleated polychromatic erythrocytes (MnPCEs). These results were corroborated by previous studies on mice and rat bone marrow cells (23- 25). Low mitotic index in mice treated with high dosage of cisplatin (Table 3) consistence with the decrease of PCEs (Table 1) and this reveals cytotoxicity of cisplatin. In conclusion the two tested dosages have genotoxic and cytotoxic effects in mice.

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