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Induction of micronuclei in a transplantable murine tumor after multimodality treatment with cis-platin, radiation and hyperthermia

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Background: Tumor response after multimodality treatment using combination of radiation, chemotherapeutic drugs and hyperthermia usually assessed by parameters such as tumor growth delay, volume doubling time and regression response. The study herein was conducted to investigate the usefulness of micronucleus assay for assessing the multimodality treatment. ***Materials and Methods:*** The induction of micronuclei (MN) in a transplantable solid tumor grown in inbred Balb/c mice was analyzed after treating the tumors with cis-platin (cDDP), radiation (RT) and hyperthermia (HT). ***Results:*** The MN frequency in tumor was measured at 1, 3, 5 and 7 days of post-treatment. On day 1, all the cDDP and RT groups, except HT treatment produced significantly higher MN counts from that of the untreated tumors. Cis-platin treatment resulted in a dose-dependent linear increase in the frequency of MN induction on day one. Combination of radiation with cDDP or HT, as bimodality treatment further increased the MN counts. In the tri-modality group (cDDP+RT+HT) the MN counts were not significantly higher than the bi -modality treatments, however there was an immediate tumor shrinkage indicating the contribution of other forms of cell death. Although, MN counts were declined after day five post-treatment, remained significantly higher than the control on day seven-post treatment in hyperthermia alone or its combination with RT and RT+ cDDP groups. ***Conclusion:*** Micronucleus assay may be useful for assessing the post-treatment regression response of resistant tumors, while monitoring the response of sensitive tumors the parameters such as apoptosis and necrosis may also contribute considerably to tumor cell loss contributing immediate tumor regression. **Iran. J. Radiat. Res., 2009; 7 (3): 119-125**

Keywords: *Cis-platin, hyperthermia, multimodality treatment, micronuclei, radiation*.

INTRODUCTION

Radiotherapy is the mainstay of therapeutic modality against various cancers. However, effective control of tumors by radiotherapy alone or in combination with chemotherapy is limited due to patient intolerance and normal tissue toxicity. Therefore, in recent years, multimodality approach by the judicious combination of radiation, chemotherapeutic drugs and hyperthermia at tolerable doses are widely used for enhancing the therapeutic efficacy, reducing toxicity, and preventing or delaying development of tumor resistance. Several pre-clinical as well as clinical studies have demonstrated the efficacy of such a multimodality approach using various chemotherapeutic drugs in combination with either radiation and or hyperthermia [1-7].

The peyrone's chloride, commonly referred by the generic name, cis-platin, reported to have antitumor activity and is used in clinics extensively against bladder, head and neck, testicular, ovarian and cervical tumors. Hyperthermic sensitization of cis-platin induced enhanced cell killing, tumor regression and potential to enhance the effect of radiation have been documented earlier [1, 8, 9]. The mechanism of action of cDDP has been attributed to its ability to induce DNA cross-links [10] leading to DNA strand breaks. It is understood that non-repair or inhibition of the DNA double strand break (dsb) repair contributes to chromosomal aberrations [11, 12]. This chromosomal damage could be analyzed by doing the quantitative analysis of

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micronuclei and now considered as a very useful parameter for the prediction and prognosis of the tumor treatment response [13, 14]. Here, we report the response of a transplantable murine tumor, S-180, to cis-platin, radiation and hyperthermia as single, bi- and tri-modality treatments, using micronucleus assay as an experimental endpoint.

MATERIALS AND METHODS

The tumor Sarcoma 180 (S-180), obtained from the Advanced Center for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Center (TMC), Navi Mumbai, and maintained in our inbred BALB/c mouse colony. The animal care and handling was carried out according to the guidelines of World Health Organization, Geneva, Switzerland and Indian National Science Academy, New Delhi, India with the prior permission form the Institutional Ethical Committee. The solid tumors were produced by intra-dermal injection of viable cells (5×10^5) on the dorsum of the host animals, as described earlier. The tumors of the size 100 ± 10 mm^3, were taken for the experiments. Tumor bearing animals were anaesthetized, wherever mentioned, by intraperitoneal (ip) Ketamine, 50 mg/kg.b.wt. (Themis Chemicals Ltd. Mumbai, India) and Diazepam, 0.5 mg/animal (Ranbaxy Laboratories Ltd., India) before irradiation or hyperthermia treatment. Tumours were irradiated locally using ^{60}Co teletherapy unit (Siemens, Germany), at a dose rate of 1.0 Gy/min (RT). The dosimetric calculations were done by Prof. J.G.R. Solomon, Radiation Physicist, Department of Radiotherapy and Oncology, Kasturba Hospital, Manipal. Anaesthetized animals were treated with hyperthermia (HT) by the local exposure of the tumors, using a thermostat controlled water bath (Julabo, Germany) at 42°C or 43°C for 30min, as described earlier[1]. The different doses of cDDP (Tamilnadu Dada Pharmaceuticals Ltd. Madras, India) were dissolved in physiological saline to give a concentration of 0.5 mg/ml and injected (i.p.) 15 min before irradiation or 30 min before hyperthermia. While in the trimodality treatment, cis-platin was injected (i.p) 15 min before tumor irradiation, followed immediately by heat treatment.

Micronucleus (MN) assay

After various single, bi and tri-modality treatments and at different time points, tumors were excised into Dulbeco's Minimal Essential Medium (DMEM) and single cell suspension was prepared and processed as described earlier [1]. The MN slides were prepared by fixing the cells in Carnoy's fixative, dropping the tumor cell suspension on to grease free slides, air-dried and stained with ethidium bromide. The slides were coded to avoid the observer's bias while counting. The MN was scored in 2000 cells per animal using fluorescence microscope under UV excitation as described earlier [15]. A minimum of five animals were used per treatment group

Statistical analysis

The cDDP dose response curve for MN frequency at doses of 1, 2.5 and 5 mg/kg. b.wt. was fitted on a linear model ($Y = a+bX$) and the MN frequency was compared with the control and among the various treatment groups by One way ANOVA followed by Tukey's post hoc test and the comparison between the two treatment group was by Unpaired Student's t test using GraphPAD InStat, Software, USA.

RESULTS

The MN count at 24 hours post-treatment time point was significantly higher in all the drug and radiation groups compared to the untreated control. Among the single modality treatments the frequency of MN on day 1 was highest in the 5 mg/kg cDDP group, followed by 10 Gy gamma radiation. Cis-platin treatment showed a dose dependent increase in the

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frequency of MN induction. The data were fitted on a linear regression line with a good correlation coefficient ($r^2 = 0.9664$, figure 1).

Table 1 summarizes the frequency of micronuclei (%) 1, 3, 5 and 7 days after treatments with cDDP, radiation and hyperthermia as single or various combination treatments. There was a significant ($P<0.05$) increase in the MN induction in cDDP treatment groups when the drug dose was increased from 1 to 2.5 mg/kg and 2.5 to 5 mg/kg. In all the single modality treatments the MN increased with time after treatment and reached a maximum on day 3 or 5 days post-treatment and values were significantly ($P<0.001$) higher than the respective day control values. The MN values declined after day 5 post-treatment, though the values remained significantly higher than the control even on day 7 post – treatment only in 43°C, 30 min hyperthermia treatment group.

Bimodality treatments produced significantly higher MN counts on day one post -treatment compared to either individual agent used in the combination except for 2.5 to 5 mg/kg group compared with 2.5 to 5 mg/kg + 42°C, 30 min. A temperature dependent increase in the induction of MN was observed when 2.5 mg/kg cDDP was combined with the various doses of heat treatment (42°C or 43°C), resulting in maximum MN counts in the cDDP+43°C group. Increase in the temperature by 1°C from 42°C to 43°C in combination with cDDP resulted in a significant ($P<0.001$) increase in the percent of MN value. The induction of micronuclei was persistent even on day 7 of post treatment in the combination of radiation with 2.5 to 5 mg/kg or 43°C, 30 min.

The cDDP combination with 43°C, 30 min or RT produced higher MN counts than that of RT+43°C, 30 min treatment. Among the bimodality treatments, cDDP+10 Gy treatment resulted in the maximum number of MN counts and this was comparable to that produced by 2.5 cDDP +43°C, 30 min. Combination of cDDP with RT and HT as a trimodality treatment did not further increase the MN counts over that of cDDP+43°C, 30 min.

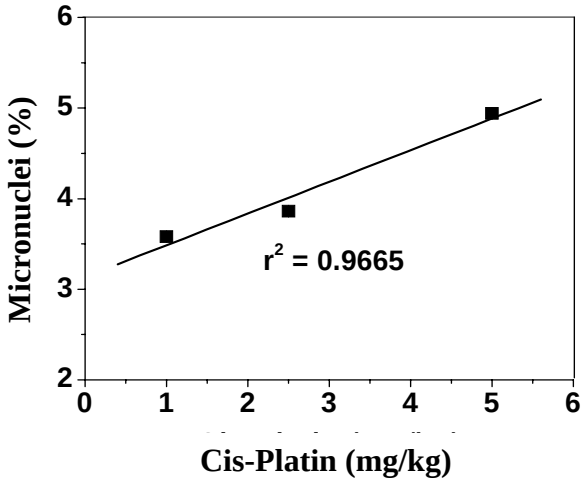


Figure 1. Dose response for micronuclei induction after cDDP treatment.

The maximum MN counts on day 5 post-treatment was observed in cDDP+43°C, 30 min combination that was significantly ($P<0.001$) higher than that produced by any of the other bimodality treatments at any post treatment times. The frequency of MN could not be followed after day one in the animals treated with cDDP+RT+43°C, 30 min due to severe necrosis and shrinkage of the tumors.

DISCUSSION

The micronuclei (MN) are formed from chromosome fragments and abnormal chromosomes which are not included in the main nucleus during mitosis [16]. It is generally accepted that mitotic divisions are necessary for the appearance of MN [17] and the cell has to undergo at least one division after irradiation or any treatment before the MN can be formed [18]. These micronucleated cells which have lost their reproductive integrity may proliferate to a limited extent [19], before they die. Micronucleus formation is considered as hallmark of genetic toxicity; as such, micronuclei are used as indicators of normal tissue genotoxicity caused by various chemical agents or environmental

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Table 1. Frequency of micronuclei (%) 1, 3, 5 and 7 days after treatments with cDDP, radiation and hyperthermia as various combination treatments (values Mean ± SE).

Treatment groups	1 day	3 days	5 days	7 days
1. Control (untreated)	3.20 ± 0.09*	3.28 ± 0.08	3.34 ± 0.10	3.50 ± 0.03
Single modality treatments				
Cis-platin alone (mg/kg.b.wt.)				
2. 1.0	3.58 ± 0.04	----------	----------	----------
3. 2.5	3.86 ± 0.05	5.60 ± 0.11	5.86 ± 0.24	4.10 ± 0.10
4. 5.0	4.94 ± 0.05	----------	----------	----------
Hyperthermia alone				
5. 42°C, 30 min	3.18 ± 0.07*	----------	----------	----------
6. 43°C, 30min	3.28 ± 0.06*	5.38 ± 0.07	5.36 ± 0.10	4.52 ± 0.22
Radiation alone				
7. 10 Gy	4.76 ± 0.23*	5.60 ± 0.12	5.92 ± 0.07	4.26± 0.19
Bimodality treatments				
8. 2.5 cis-platin +42°C, 30 min	4.10 ± 0.06	5.60 ± 0.12	5.92 ± 0.07	4.26 ± 0.19
9. 2.5 cis-platin +43°C, 30min	5.62 ± 0.07	10.16 ± 0.07	11.02 ± 0.33	4.14 ± 0.11
10. 10.0 Gy+43°C, 30min	5.38 ± 0.08*	5.72 ± 0.06	4.82 ± 0.11	4.70 ± 0.24
11. 2.5 cis - platin +10.0 Gy	5.78 ± 0.11	5.70 ± 0.07	5.66 ± 0.11	5.04 ± 0.16
Trimodality treatments				
12. 2.5 cis-platin +10 Gy+ 43°C, 30 min	5.68 ± 0.05	----------	----------	----------

All the treatment groups significant (P<0.05) when compared to the respective control groups, except on day 1, groups 5 and 6. On day 1 group 4 significant with group 2 & 3 (P<0.001); group 3 significant with group 2 (P<0.05), group 9 with 8 (P<0.001); On day 3: group 9 with groups 6, 7, 8, 10 and 11 (P<0.001), group 10 with 7 (NS); On day 5 group 9 is significant (P<0.001) compared to groups 8, 10 and 11; On day 7 all the treatment groups are not significant compared to control except group 6 (p<0.05), 10 and 11 (p<0.001).
*Data taken from Uma Devi and Rao 1993 (Reference No. 1), copyright Urban & Vogel, reproduced with permission.

toxins. Recently, there are several clinical reports on the application of MN assay as a predictive and prognostic factor after chemo - and radiotherapy [13, 14].

Earlier, Chakrabarti and Chakrabarti [20] demonstrated the presence of spontaneous micronuclei in ascites S180 untreated tumor cells *in vitro*. They showed a low frequency of MN (2%) in their studies. However, our observation in solid S180 tumors of the size 100 ± 100mm^3 growing *in vivo* showed 3.2 % MN and this spontaneous MN counts increased with the size, indicating the presence of reproductively dead cells. Similar observations were also made in xenografts of some human tumors [19, 21].

It is very well established that ionizing radiation induces different types of lesions in the DNA, including single- and double strand breaks, base damage as well as DNA cross-links. At higher radiation doses there is a high proportion of irreversible damage and reduced repair, which may contribute to the direct cell death. The relation between

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the occurrence of reproductively dead cells and the presence of chromosomal aberrations in irradiated cells has been demonstrated in earlier studies [22, 23]. A similar correlation has also been demonstrated between the frequency of MN and reproductive death [24, 25]. These micronucleated cells may be metabolically active for some time and MN may be produced in more than one post treatment mitosis [26]. Such a phenomenon can explain higher MN counts at 3 - 7 days after exposure to 10 Gy and after cDDP treatment in the present study.

The majority of the experimental data suggest that the damage to chromosomal DNA is directly responsible for the cytotoxicity of cDDP [27, 28]. It has been conclusively demonstrated that intra-and inter strand cross-links after cDDP treatment were the adducts of biological significance and responsible for cytogenetic and mutagenetic effects [29]. A fairly good correlation between cellular sensitivity and the number of inter strand cross-links exists [30-32]. In the present study cDDP show a linear increase in the frequency of MN induction after cDDP treatment. The increased frequency of MN observed in the tumors after cDDP treatment indicates the increased damage at the chromosomal level. Similarly, Adler and Tarras [33] reported a dose dependent increase in the clastogeneic effect of cDDP in mouse bone marrow and spermatogonia.

Excision repair and post-replication repair are the two types of repair processes which operate after cDDP induced DNA lesions [34, 35]. At the higher drug doses irreparable lesions or inefficient post replication repair can lead to chromosomal aberration and the resulting increase in MN. This could result in the linear drug dose dependent increase in the MN count observed in the present study. The importance of post replication repair of cDDP induced DNA strand lesions has been emphasized by Dewit [36]. Cells with residual lesions may persist for a long period before they finally die. Such cells can add to the number of cells with MN entering interphase, thus increasing the MN count at later post treatment time as observed in the present study.

Hyperthermia is known to cause conformational changes in proteins affecting the spindle apparatus. In addition, HT after drug treatment could also have an effect on the post replication repair of cDDP induced DNA lesions. Thus the individual effect of the two agents and their interaction resulted in the higher enhancement of cytogenetic damage expression as seen in the significant increase of MN frequency on day 3 and 5 after cDDP +HT. Increase in the temperature by 1°C from 42°C to 43°C in combination with cDDP resulted in a significant increase in the percentage of MN value. This increased effect may be due to the increased rate of reaction of cDDP with DNA [37].

cDDP in combination with RT increased the MN counts on day 1 and day 3. At later intervals MN frequency decreased, however, remained significantly higher than the untreated. Chadwick and co-workers [38] proposed a model for the interaction of radiation and platinum compounds on their mutual repair processes. They suggested that, a platinum compound caused a lethal event by either forming an inter-strand cross-link or two intra-strand crosslink's, one on each of the two complementary DNA strands. A supra-additive interaction of RT and platinum complex would occur when radiation induced single strand breaks (ssb) would be present opposite a platinum induced intra strand cross-link, where one lesion would prevent repair of the other. Inhibition of the repair of cross-links increases the strand lesions, leading to chromosomal aberrations, which will be expressed as MN as seen in the present observations.

Combination of the three agents, cDDP, RT and HT did not further increase the MN induction. This may be due to the severe damage to the cells resulting in an immediate cell death. It is possible that only small

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fraction of the cells entered division and contributed to MN. Even these cells may not have been able to continue proliferation's as indicated by the progressive shrinkage and complete regression of the tumor in the trimodality treatment group. The tumors which show increased sensitivity to combination treatments (like the mouse tumor (S180) used here) may result in increased cell killing as evidenced by significant increase in MN counts as well as immediate tumor shrinkage. The immediate shrinkage of the tumors in the effective treatment groups makes it difficult solely to use MN assay as a predictive parameter. Other parameters like apoptosis and necrosis may also predominantly contribute to tumor cell death and thereby to shrinkage. Therefore, while monitoring the response of sensitive tumors, especially during the multimodality approach, it may be essential to consider other parameters, such as apoptosis and necrosis, as they also contribute considerably to tumor cell loss resulting in an immediate tumor regression.

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