STUDIES ON THE MODULATORY ROLE OF ASCORBIC ACID ON ANTITUMOR ACTIVITY AND TOXICITY OF CISPLATIN IN TUMOR-BEARING MICE

Ph.D THESIS ABSTRACT
SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY

By
Amenla

DEPARTMENT OF ZOOLOGY
NORTH-EASTERN HILL UNIVERSITY
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Carcinogenesis is a multistage process by which normal cells are transformed into cancer cells. The main goals of a cancer diagnosis and treatment programme are to cure by killing or removing all cancer cells, prevent or considerably prolong the life of patients and to ensure the best possible quality of life to cancer survivors. Chemotherapy is an effective treatment for cancers and has brought considerable prognostic improvement. Chemotherapy is generally used either singly or in combination with surgery and/or radiotherapy against various types of cancers. All chemotherapeutic agents could be burdened with severe dose-dependent and dose-limiting side effects.

_Cis-_diaminedichloroplatinum (II) (cisplatin or CDDP) is a largely employed platinum based compound that exerts clinical activity against a wide spectrum of solid neoplasms, including head and neck, testicular, bladder, ovarian, colorectal, and lung cancers (Galanski, 2006). The synthesis and characterization of CDDP was first reported by Michele Peyrone in 1845, but it was not until 1965 that the medicinal application of CDDP was launched after Barnett Rosenberg and co-workers discovered during an experiment designed to elucidate the effect of electrical fields on growing bacteria _Escherichia coli_. The cells subjected to treatment formed long filaments, an observation which led to the conclusion that cell division was inhibited but cell growth was not markedly influenced. In a later experiment, the cytotoxic effect was tied to CDDP and a new class of antitumor agent for cancer chemotherapy was proclaimed (Rosenberg et al., 1969). The major cytotoxic target of CDDP in the cell is suggested to be DNA (Eastman, 1986). It is shown that the main mechanism underlying the cytotoxic effect of CDDP is the ability to react with DNA and formation of CDDP-DNA adducts with inter- and intra-strand nuclear DNA crosslinks (Florea and Büsselberg, 2011). In addition to its interaction with cellular DNA, the changes in various biochemical/enzymatic parameters, immune response, cell surface structure have also been observed which have led to propose the involvement of multistep and multilevel effects of CDDP in the tumor cells/ host (Prasad et al., 1999). However, the efficacy of CDDP is often hampered by the development of various side effects such as nephrotoxicity, myelosuppression, neurotoxicity, ototoxicity etc. in the host and acquired resistance by cancer cells (Taguchi and Razzaque, 2005; Kelland, 2007; Barabas et al., 2008). In an attempt to overcome these impediments, the uses of CDDP in combination with some modulating agents have been tried with varying success (Treskes and Van der Vijgh, 1993; Zhang et al., 2001; Sanchez-Gonzalez, 2011). In an endeavour to decrease drug-induced toxicity in the host, the use of anticancer drugs such as cyclophosphamide (Nicol and
Prasad, 2006), paclitaxel (Park et al., 2012), arsenic trioxide (Yedjou et al., 2009), mitomycin C (Mazumdar et al., 2011), adriamycin (Devi and Latha, 2011) and ifosfamide (Donya et al., 2010) in combination with ascorbic acid (AA) have also been examined.

Based on the literature survey the following objectives were undertaken for the present study:

- Evaluation of the antitumor activity and biochemical changes in the tumor-bearing hosts treated with CDDP/AA alone or in combination with AA plus CDDP.
- To determine the toxicity in the hosts treated with CDDP alone or in combination with AA.

The survival and life span of the tumor-bearing mice was increased to about 34 days and 42 days when treated with AA (Group II) or CDDP alone (Group III) respectively. Interestingly, combination treatment of mice with AA with CDDP (Group IV) further increased the host’s survivability to more than 46 days. The % ILS in the AA, CDDP and combination treated group of mice was about 79%, 122% and 142% respectively. Maximum decrease in the body weight of mice and in the tumor pH was recorded after combination treatment.

AA concentration in the serum of tumor-bearing mice was noted to decrease as compared to its normal counterpart. As compared to the corresponding control during 24-96 h of treatment, the AA alone treatment caused a time dependant significant increase in the level of AA concentration in DL cells, ascites supernatant (SN) and serum. Treatment with CDDP increased the AA concentration in DL cells (~29%) at 24 h, while in serum it was about ~11% at 96 h, however, ascites SN did not show any significant changes in the AA level. As compared to CDDP alone, combination treatment with AA plus CDDP caused a significant increase in the concentration of AA in DL cells, ascites SN and serum at corresponding time points.

Light microscopic observation of DL cells in vivo revealed that CDDP treatment (24-96 h) caused plasma membrane damage, chromatin condensation, shedding of membrane vesicles and appearance of cytoplasmic vacuoles. Combination treatment of mice with AA plus CDDP caused further membrane damage (24-48 h), with more membrane blebbing and cytoplasmic vacuolization leading to tumor cell lysis at 72-96 h of treatment. Different apoptotic features were observed in DL cells at different time intervals in all the treatment groups with maximum apoptosis being noted after combination treatment, and these include membrane blebbing, nuclear condensation and fragmentation, membrane disintegration and appearance of cytoplasmic vacuoles. Determination of apoptotic index revealed that as
compared to CDDP treatment, number of apoptotic cells significantly (P≤ 0.05) increased in AA plus CDDP treated group during 24-96 h of treatment. AA alone treatment has also caused some increase in the number of apoptotic cells in time dependent manner. Single and multiple micronuclei (MN) were observed in the DL cells in all the treatment groups with maximum frequency of MN occurring at 24 h of treatment. CDDP alone treatment revealed maximum increase in the incidence of MN, while pre-treatment of mice with AA in the combination group significantly decreased the frequency of MN at corresponding time points as compared to CDDP alone treatment. Scanning electron microscopic observation of the DL cells during 24-96 h of CDDP treatment showed a reduction in ruffles/microvilli and appearance of membrane blebs, membrane fusion and plasma membrane deformities. Combination treatment of mice with AA plus CDDP also revealed more or less similar pattern of deformities in DL cells morphology which include appearance of membrane blebs and loss in microvilli from the cell surface during 24-48 h of treatment. Severe morphological alterations with complete loss in fine membrane projections, cell shrinkage and membrane folding were observed during 72-96 h which is typical characteristic features of apoptosis. TEM study also revealed different morphological changes in the mitochondrial structure in the DL cells. CDDP treatment of mice for 24-96 h in vivo resulted in the appearance of fine microvilli-like processes on cells and disruptions in the mitochondrial membrane and changes in the arrangement of mitochondrial cristae with the development of vacuoles and elongation, thickening and reduction in the number of cristae. Combination treatment of mice with AA plus CDDP during 24-96 h revealed severe structural deformities in mitochondria with more thickened and irregular arrangement of mitochondrial cristae, mitochondrial membrane disruption, and the mitochondria acquire a roundish shape with disappearance of cellular outgrowths with reduction in the number of cristae and more pronounced vacuoles during the later periods of treatment.

Analysis of succinate dehydrogenase (SDH) activity in DL cells revealed an overall time dependant decrease in SDH activities during 24-96 h of CDDP treatment of mice. As compared to CDDP alone treatment, the combination treatment of mice with AA plus CDDP treatment caused further decrease in SDH activity in DL cells during 24-48 h of treatment.

The level of mt-LPO measured in terms of malondialdehyde concentrations in DL cells of tumor-bearing mice after CDDP treatment was noted to significantly increase in a time dependent manner during 24-96 h as compared to corresponding control. As compared to CDDP alone, combination treatment caused a significant increase in mt-LPO at 24-48 h of treatment. Comparison of LPO in the tissues of tumor-bearing mice after different treatment
conditions revealed that CDDP treatment caused a significant increase in the level of LPO in all the tissues studied (except testes) during 24-96 h, while AA alone or combination of AA plus CDDP treatment caused a significant decrease in the level of LPO in liver, kidney and testes and an overall increase in LPO in spleen and DL cells during 24-96 h. As compared to CDDP alone treatment, combination treatment revealed a decrease in the LPO level in liver and spleen during 24-96 h and in kidney, testes and DL cells during 48-96 h of treatment.

The control tumor bearing hosts showed a decrease in the protein concentration of all the tissues during tumor growth as compared to its normal counterpart. A steady increase in the protein concentration in the ascites SN was noted during tumor growth. As compared to corresponding control, a significant decrease in protein levels in liver, kidney, spleen, DL cells and in ascites SN and an increase in protein content in testes were observed during CDDP alone and combination treatment as well. As compared to CDDP alone, combination treatment of AA plus CDDP showed little improvement in the protein concentration with a significant increase in liver, kidney and spleen at 48 h and in the ascites SN during 24-96 h of treatment respectively, while DL cells showed a time dependant significant decrease in the protein concentration.

As compared to corresponding control, sialic acid in the tissues and DL cells decreased under all treatment conditions. Predominant decrease in sialic acid content in liver (~15%), kidney (~9%), spleen (~14%), testes (~26%) and DL cells (13%) was noted during 24 h, 96 h, 48 h, 48 h and 96 h of CDDP treatment respectively. As compared to CDDP alone treatment, combination treatment of AA plus CDDP caused significant increase in the concentration of sialic acid in liver and kidney at 24 h and in spleen and testes at 96 h and 24-48 h of treatment respectively. DL cells showed a time dependant significant decrease in the sialic acid concentration during 48-96 h of treatment.

As compared to the reduced glutathione (GSH) level in the tissues of normal mice, the reduced glutathione (GSH) level in the corresponding tissues of tumor-bearing control decreased. As compared to corresponding control, little/significant decrease in the GSH levels in all the tissues and DL cells were observed under all treatment conditions. However, the level of GSH in all the respective tissues was restored to approximately control levels at 96 h of combination treatment. As compared to CDDP alone, AA plus CDDP treatment caused a significant increase in GSH levels in liver and kidney at 72-96 h, and little increase in spleen and testes. DL cells showed a significant decrease in GSH levels at 24-72 h of treatment. Studies on GSH-related enzymes activities showed that CDDP alone and combination treatment both decrease the GST activity in liver and DL cells at 24-48 h and
24-96 h of treatment respectively, while it increases in kidney, spleen and testes. As compared to CDDP alone, combination treatment of mice with AA plus CDDP resulted in a significant increase in GST activity in liver and spleen while it decreases in kidney and DL cells. Under different treatment conditions, GR activity was noted to decrease significantly in liver, kidney and DL cells. As compared to CDDP alone, combination treatment revealed a significant increase in GR activity in liver (~7%) at 24 h, while a significant decrease was observed in DL cells (~12%) at 24 h of treatment. CAT activity in liver, kidney and DL cells was also found to decrease at 24-96 h, in spleen at 24 h and 72 h, and in testes during 24-72 h of CDDP treatment. As compared to CDDP alone, combination treatment significantly increase the CAT activity in liver and kidney during 24-72 h and in testes at 72 h while a significant decrease in spleen and DL cells was noted at 48 h and 24-72 h of treatment respectively.

Haematological studies showed that during tumor growth progression haemoglobin (Hb), RBC and PCV significantly decreased while WBC increased. As compared to tumor-bearing control, CDDP treatment caused a decrease in the Hb content, RBC and WBC counts and PCV. As compared to CDDP alone, combination treatment resulted in significant recovery in these haematological parameters. Also, combination treatment of AA plus CDDP significantly decreased the frequency of erythrocytes abnormalities induced by CDDP. As compared to control, CDDP treatment of tumor-bearing mice caused a significant decrease in lymphocytes, eosinophils and basophils while an increase was observed in monocytes and neutrophils. Combination treatment caused an increase in lymphocytes, eosinophils and a decrease in monocytes, neutrophils and basophils.

The frequency of chromosomal aberrations (CA) in bone marrow cells of tumor-bearing mice was noted maximum at 24 h in all the treatment groups. Various types of aberrations such as chromatid breaks, isochromatid breaks, chromosomal fragments, exchanges and sister chromatid union were observed in both CDDP alone and in combination treatment some of which were also observed in AA alone treatment. Comparative analysis at corresponding periods of treatment revealed that the CA were significantly lesser in combination of AA plus CDDP treatment group than those treated with CDDP alone. Different types of sperms abnormalities (SA) were also found to be induced after CDDP alone and combination with AA plus CDDP which include hookless head, looping mid-piece, microhead, balloon-like head, double tailed, incorrect head-neck connection, diffused head, banana head and amorphous head. Maximum abnormalities in the sperm and also maximum increase in the frequency of sperm abnormalities of tumor-bearing
mice were observed after CDDP treatment. However, pre-treatment with AA significantly decreased the frequency of sperm abnormalities induced by CDDP.

Histopathological examination of kidney, liver and testes sections from tumor-bearing mice on the 5th day after CDDP treatment showed damages in the renal tubular cells represented by glomerular atrophy, infiltration of cells and tubular congestions, destruction of the renal tubular cells in kidney; liver section showed sinusoidal distortion and remarkable locational hepatocytes damage; and testes section showed degenerations in seminiferous tubules, intercellular disassociation of germ cells, vacuolization in sertoli cells and reduction in leydig cells. AA administration prior to CDDP injection showed reduced destruction of renal tubular cells and regained glomerular attachment in kidney; amelioration in the histopathological changes showing mild to moderate damage of hepatic cell indicating recovery of the altered tissue; and in testes reduced vacuolated tubules and less deranged spermatogonial mass was observed. Renal function tests (RFT) and liver function tests (LFT) were studied to monitor/assess the changes in renal toxicity and hepatotoxicity in the hosts under different treatment conditions. As compared to that of control DL-bearing mice, CDDP induced marked elevation in serum urea and creatinine levels and in the activities of ALP, ALT and AST. The increase in serum urea and creatinine level was ~270% and ~302% respectively, while the increase in ALP, ALT and AST was ~84%, ~37% and ~601% respectively. However, as compared to CDDP alone, combination treatment of animals with AA plus CDDP significantly brought down the elevated levels of serum urea and creatinine, and in the liver enzymes as well.

From the study, it is evident that combination chemotherapy of AA plus CDDP could be very useful in enhancing CDDP-mediated therapeutic efficacy which involves induction of apoptosis in DL cells and decreasing CDDP-induced hematotoxicity, mutagenecity, hepatotoxicity and nephrotoxicity in the host signifying its protective role, thus suggesting differential effects of the combined treatment on the cancer cells and normal tissues of the host. Although the precise mechanism and kinetics through which AA mediates its protective effects remains unclear presently, the finding that AA protects cells from CDDP toxicity may indicate its ability to detoxify carcinogens by exerting its cytoprotective profile.
Conclusion

The present work describes the evaluation of anticancer efficacy and toxicity of CDDP against murine ascites Dalton’s lymphoma, and the possible role of AA involved in modulating CDDP-induced toxicity in the same tumor-bearing mice. The host survival data indicate further increase in survivability (ILS) of the hosts after treatment with AA plus CDDP suggesting better therapeutic efficacy with combination treatment against murine ascites Dalton’s lymphoma. Analyses of apoptosis indicate the higher frequency of apoptotic DL cells after treatment with AA plus CDDP. The induction of apoptosis in DL cells should be one of the factors involved in the better antitumor activity under AA plus CDDP treatment conditions. Development of abnormalities in mitochondria as well as the decrease in the mitochondrial enzyme in DL cells after CDDP or combination treatment may also play a role in CDDP-mediated cytotoxicity in DL cells. Further, the enhancement of platinum uptake by DL cells in presence of AA (i.e. AA plus CDDP combination) may also play a significant role in causing better therapeutic efficacy. As most of the sialic acid moieties are mainly confined as the mucopolysaccharides component of the plasma membrane, the observed decrease of sialic acid moieties in DL cells after the combination treatment may lead to increase the antigenecity of DL cells. The observed decrease in the cellular GSH levels and increase in LPO in DL cells after CDDP alone or combination treatment may reduce the protective ability in the tumor cells which may facilitate better cytotoxic effect under these treatment conditions. The decrease in GSH related enzymes in DL cells should be one of the possible steps involved to decrease GSH level in DL cells, thus, weakening the cellular antioxidant system. Decrease in RBCs after CDDP treatment may be correlated to cause anaemic condition in the host. However, betterment in these hematological values noted during combination treatment may suggest a protective role of AA against CDDP-induced hematotoxicity. Further, the observed increased in WBCs particularly lymphocytes after combination treatment may also play a role in the antitumor activity by enhancing antitumor immunity in the host. Moreover, study of various drug-induced mutagenic parameters such as chromosomal aberrations (CA), development of micronuclei (MN) and sperm abnormalities (SA) reveal that CDDP treatment caused an increase in all the three mutagenic parameters thereby, supporting the earlier findings of CDDP to also show mutagenic potential. However, the combination treatment of the host with AA plus CDDP showing significant decrease in these parameters suggests the protective ability of AA against CDDP-induced mutagenicity in the host. The protective role of AA is also confirmed by the histopathological observations of liver, kidney and testes.
with better tissue conditions in presence of AA plus CDDP. The changes in the pattern of histotoxicity (particularly liver and kidney) are supported by the biochemical analyses of LFT and RFT in serum, as the various marker enzymes and molecules for liver and kidney were decreased after combination treatment. Further, it may be suggested that the increase of cellular GSH level in the tissues and a decrease in LPO in the same tissues after combination treatment may also contribute to the protective ability of AA against CDDP-induced hepatotoxicity and nephrotoxicity.

From the study it is evident that combination chemotherapy of AA plus CDDP could be useful in enhancing CDDP-mediated therapeutic efficacy and at the same time decreasing its toxic effects in the host. However, the molecular mechanism behind the protective role of AA against CDDP-induced toxicity is not clearly understood at present and may be further explored.