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Riboflavin as adjuvant with cisplatin: Study in mouse skin cancer model

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1. ABSTRACT

Cisplatin used in treatment of solid tumor induces oxidative stress which leads to hepatotoxicity and nephrotoxicity. New strategies are therefore needed to combat toxicity and optimize its therapeutic potential. Riboflavin (Vitamin B_2) under photoillumination works as an anti proliferative agent and induces apoptosis. These properties of riboflavin have been exploited to mitigate cisplatin induced toxicities. 9,10-dimethylbenz(a)anthracene /12-O-tetradecanoylphorbol-13-acetate were used to induce skin tumor in Swiss albino mice. The tumor induced mice were treated with cisplatin, riboflavin as well as their combination under photo illumination. In comparison to tumor control group the cisplatin and riboflavin treated groups showed a compromised level of antioxidant enzymes, functional markers and a higher degree of lipid peroxidation. However these parameters tended towards normal in the combination treated group. The results from histopathology indicate that apoptosis was favored mode of cell death and that necrosis was reduced in combination treated groups. Our findings indicate that combination of cisplatin with riboflavin under photo illumination synergizes its anti cancer activity towards cancer cells and attenuates the cisplatin induced toxicities.

2. INTRODUCTION

Cisplatin (CP) is an effective anticancer chemotherapeutic drug widely used in the treatment of various malignant tumors including head and neck, ovarian, testicular, oesophageal and small cell lung cancer and many other solid tumors. It exerts its effect by the binding to DNA and non-DNA targets and then the induction of cell death through apoptosis, necrosis or both within the heterogeneous population of cells that forms a tumoral mass (1). It however elicits nephrotoxicity, hepatotoxicity, ototoxicity, gastrointestinal dysfunction and myelosuppression which are major setbacks in its clinical use (2). Although nephrotoxicity is considered as the dose limiting factor for CP use, hepatotoxicity can also occur at high doses (3). The

generation of reactive oxygen species (ROS) is implicated as major cytotoxic action in CP induced toxicities (4). It generates reactive oxygen species such as superoxide anion and hydroxyl radical and inhibits the activity of antioxidant enzymes in renal tissue (5-8). Studies also reveal that oxidative and nitrosative stresses are considered the most important mechanisms involved in CP induced nephrotoxicity and hepatotoxicity (9, 10). New treatment strategies where its toxicity could be lowered are therefore the requirement for its long term clinical use.

Riboflavin (RF) plays an important biological role because its isoalloxazine ring acts as a reversible redox-system in enzymes. Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are the two important derivatives of riboflavin in mammalian cells. Studies show that the singlet excited states of aqueous riboflavin can eject e-aq (solvated electrons) (11). The biological role of RF is many-sided and its nutritional deficiency has been implicated as a risk factor for various diseases including cancer. Its photosensitizing properties opened the way for the treatment of various diseases including cancer by photodynamic therapy (PDT) and ribophototherapy (RPT) (12, 13). Using the recently elucidated mechanism of apoptosis induction by RF and its photoproducts in melanoma (14) and by growing evidence of RF as potential adjuvant for anticancer drugs in cell line based studies (15, 16), we present the possible mechanisms to explain interaction between RF and CP, which leads to attenuation of CP induced toxicities.

Our lab has previously shown that RF can ameliorate cisplatin induced major toxicities in healthy mice in a dose dependent manner under photoillumination (17-19). This study tries to explore the anti cancer and ameliorative potential of the combination of RF and CP under photo illuminated conditions in two stage skin carcinogenesis induced by using DMBA/TPA.

3. MATERIALS AND METHODS

3.1. Materials

Cisplatin, riboflavin, 9,10-dimethylbenz(a) anthracene (DMBA), 12-tetradecanoylphorbol-13-acetate(TPA), normal melting agarose (NMA), ethidium bromide (EtBr), Histopaque 1077, Hank's balanced salt (HBSS), RPMI 1640, reduced and oxidized glutathione, nicotinamide adenine dinucleotide phosphate reduced (NADPH), nicotinamide adenine dinucleotide reduced (NADP) were purchased from Sigma-Aldrich Chemical Company, USA. All other chemicals used were of high analytical grade and were purchased from Sisco Research Lab, Mumbai and HiMedia Laboratories Private Limited, Mumbai.

3.2. Animal husbandry and treatment

60 adult Swiss albino male mice of 4–6 weeks old were bought from the Central Animal House of Jamia Hamdard University, New Delhi, India. These animals were housed in sufficiently large cages and treated under humane and hygienic conditions with maintained 25+/-2ºC and 12 hours day: night cycle according to 'University Guidelines for Animal Experimentation'. The animals were acclimatized on standard pellet mice diet (Ashirwad Industries, Chandigarh, India) for 10 days before the treatment and clean drinking water ad libitum. Animal experimentations were permitted by Ministry of Environment and Forests, Government of India under registration no 714/02/a/CPCSEA issued by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) dated 16th November, 2010. All experiments on animals were approved by Departmental Ethical Committee (acad/D-1188/ILK/29-09-2009). The backs of all animals were shaved 2 days prior to the start of the experiment. DMBA/TPA was used for the induction of two stage skin carcinogenesis in Swiss albino mice. Body weights of animals were recorded at the start of the experiment followed by their weekly recording till the end of the experiment. The animals were divided randomly into two groups (*Group AI, n=10 and Group B, n= 50)*. Group AI animals served as vehicle controls. These animals received acetone (200 µl /mouse) application only. Group B animals received a single topical application of DMBA in acetone (50 µg/200 µl acetone/mouse) and a week later was followed by TPA in acetone (5 µg/200 µl acetone/mouse) twice weekly till the end of the experiment. Papillomas that had diameter greater than 2 mm were considered positive. Group B that showed 100% tumor incidence by 21st week when it was further divided into 4 groups which, they were named as – group B I (Positive control), group BII (treated with riboflavin at the dose of 2 mg/kg bodyweight), group BIII (treated with cisplatin at the dose of 2 mg/kg body weight). The group given the combination of CP and RF was named as group B IV (treated with the dose of 2 mg/kg body weight of CP and with 2 mg/kg body weight of RF). Parallel to this an additional combination group BIV' was maintained without whole body photo irradiation.

Group AI and Group BI were injected with saline only in equal volume of the dose given to the treatment groups. Full body irradiation under fluorescent light (Philips, India) kept at ~10 cm distance at fluence rate of 38.6. W/m^2 for 12 hours daily during daytime was given to all animals. 1 ml syringe using saline as vehicle solution was used for dosing the animals intraperitoneally. RF was injected ½ hour prior to CP in the combination treated groups. The mice were given a daily injection for 3 days followed by a gap of a week; then again a daily dose for 3 days with a week gap and finally 3 more daily injections were given. These treatment cycles were named as T1, T2 and T3 respectively. The treatment strategy, dose and the duration of treatment were chosen carefully keeping in mind the current chemotherapy regime followed during the treatment of cancer patients. Group B I was taken as the tumor positive control for comparison with all other groups. All the animals were sacrificed by cervical dislocation method on the next day to the final dose given.

3.3. Preparation of samples

The blood was allowed to clot for sixty minutes and was centrifuged at 2500rpm (1500g) for 10 min to collect their serum which was later stored at low temperature. After the sacrifice, their kidneys, livers and non tumor /tumor skin were washed with ice-cold saline buffer. Each sample was cut with sterilized blade into two parts: quarter portions of kidney and liver and skin tumors were washed with phosphate buffered saline (pH 7.4.) and fixed in 10% formalin for histhopathological studies and the rest of the portions were kept for biochemical analysis were also homogenized separately at 5000 rpm (3000 g) in potassium-phosphate buffer (pH 7.3.6, 0.1. M) and their supernatants were taken out for biochemical estimations. Also 1 ml of homogenate of each mouse was stored for GSH and MDA estimation. After this the samples were labeled and stored at -20ºC for analysis.

3.4. Histopathological processing

The tissues in formalin were processed for paraffin embedding. These were then cut into sections of 7 µm thickness by using a rotary microtome and were stained with hematoxylin and eosin and examined under a light microscope (magnification ×400). Counting of cells was done manually under light microscopy from 5 randomly selected sections. The cells with distinct boundaries and with distinct nuclear membrane were counted as normal cells. Indistinct nucleus and sparse cytoplasm were taken as indicators of necrosis. Cells with withering or completely disappeared nucleus were considered as apoptotic cells. Grading of skin cancer was done as per guidelines laid-down by American Joint Committee on Cancer.

3.5. Biochemical estimations

Standard protocols were used to assay the activity of different antioxidant enzymes. Cu Zn superoxide dismutase (CuZnSOD) was assayed by autoxidation of pyrogallol (20) while that of catalase (CAT) was done by decomposition of hydrogen peroxide (21). Glutathione reductase (GR) activity was carried-out by the method involving oxidation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) into nicotinamide adenine dinucleotide reduced (NADP+) in presence of oxidized glutathione (22). The activity of glutathione-S-transeferase (GST) was measured by Habig *et al*. method (23). Levels of lipid peroxides were estimated by using the method of Ohkawa *et al.* (24). The level of reduced glutathione (GSH) was estimated by method of Jollow *et al* (25). The level of urea was measured according to the method of Berthelot end point assay (26) using commercially available diagnostic kits (Span Diagnostics Limited, India) and creatinine was estimated in the serum using alkaline picrate method of R.W. Bonses and H.H. Taussky (27) by the commercially available diagnostic kits (Span Diagnostics Limited, India). The activity of glutamate oxaloacetate trasaminase (GOT) and glutamate pyruvate transaminase (GPT) in the serum was assayed by commercially available estimation kits (Span Diagnostics Limited, India) based on Reitman and Frankel method (28).

3.6. Statistical analysis

Statistical analysis was performed using One ANOVA way on GraphPad Prism version 5.0.2. All data have been expressed as mean +/- SEM. Multiple comparisons between groups were determined with the Tukey test. Differences were considered significant at $p<0.0.5$. The treatment and experiments were repeated thrice to check the reproducibility of the results.

4. RESULTS

4.1. Effect of treatment on body weight and tumor volume

Animals had body weights that ranged from 25.4. +/-1.9. g to 26.7. +/-1.8. g at the beginning of experiment. By the end of the experiment the all animals had gained weight when compared to their initial weight. The Cisplatin treated group showed

Table 1. Effect of treatment on body weight and tumor volume

Group	Body weight (g)	Tumour Volume (mm^3)				
	Initial	Final	At 21st week	At end of T1 cycle	At end of T2 cycle	At end of T3 cycle
AI	26.2+/-1.1	47.5+/-2.9	-	-	-	-
BI	25.6+/-1.4	35.1+/-1.4[1]	129.1+/-2.1	129.6+/-1.4	131.1+/-2.5	130.5+/-3.2
BII	25.9+/-2.1	46.5+/-2.1[2]	130.5+/-1.4	129.2+/-1.2	127.2+/-1.6	125.2+/-3.1
BIII	26.7+/-1.8	28.1+/-1.2[2]	28.5+/-2.1	121.4+/-2.1[2]	108.6+/-2.4[2]	101.1+/-2.4[2]
BIV	25.4+/-2.7	39.8+/-2.1[3]	130.7+/-3.1	118.5+/-1.7	102.1+/-2.4[3]	96.2+/-1.7[3]
BIV'	26.4.+/-1.9	31.8.+/-1.3[3]	129.6.+/-2.8	120.4.+/-1.3	105.6.+/-1.9	100.2.+/-1.5

Data represents means+/-SEM in each group. [1]indicates significantly different from group AI at $p<0.05$. [2]indicates significantly different from group BI at $p<0.05$. [3]indicates significantly different from group BII and group BIII at $p<0.05$.

significant loss in body weight when compared to positive control group (Group BI). Group BIV that is the combination group under photoillumination shows maximum recovery of body weight when compare to all other groups. Male Swiss Albino mice showed the onset of papillomas (.2 mm) at 9 weeks in DMBA/TPA-treated mice (group B). Total tumor incidence was seen from 21st weeks onwards after which the group B was further divided according to treatment strategy. No tumor formation was seen in the vehicle-treated control group AI. Table 1 shows the tumor volume in mice after treatment with RF, CP, and combination of both. By the end of the experiment there was a considerable increase in tumor volume of positive control group BI animals when compared with RF, CP, and combination treated groups. RF and CP treated mice showed reduction in tumor volume when compared with positive control group BI. CP treated animals showed more reduction in tumor volume as compared with RF treated animals. However, RF in combination with CP under photoillumination showed maximum reduction in tumor volume as compared to CP alone treated mice. The reduction in tumor volume was less pronounced in the combination group without photoillumination. These finding suggest us that combination of RF and CP under photoillumination is more effective than individual treatment therapies.

4.2. Effect of treatment on antioxidant enzymes

The major antioxidant enzymes-CuZnSOD, CAT and GR were assayed in liver and kidney samples. It was observed that vehicle control group AI that received no treatment showed maximum activity of all the enzymes (Figure 1). The specific activity of all the enzymes decreased in the group BI, which was the tumor induced group. Also the activity of enzymes decreased most significantly in group BIII the CP treated group. The RF treated group BII showed a mild decrease in the activity of the antioxidant enzymes when compared to group BI. The combination treated groups BIV and BIV', showed increase in activities of SOD, CAT and GR in kidney, liver samples and skin tumor samples, however the recovery of antioxidant enzymes was maximum in combination group under photoillumination.

GST activity was taken as a toxicity marker in target organ because of it being a major detoxifying enzyme. This enzyme showed a visible decline in group BI .Group BIII showed an overwhelming decrease in kidney, liver and skin tumor samples when compared to group BI (Table 2). However, the groups BIV and BIV', which are the combination treated group showed an increase in its activity in all the tissues.

4.3. Effect on the levels of MDA and GSH

The level of MDA was elevated in group BI showing an increase in kidney, liver and in skin tumor samples when compared to AI (Table 2). However, the increase was most significant in group B III, where it was the highest when compared to all other groups. The MDA levels decreased in the combination treated group BIV and BIV', however the decrease was milder in group without photoillumination when compared to the group BIII. GSH levels are also affected by tumor induction showing a decline in group BI when compared to group AI. In group BIII the level of GSH showed maximum decline in all the tissues when compared to group BI. The combination treated group BIV showed a significant recovery in all the organs, while its counter group without photoillumination showed lesser recovery (Table 2).

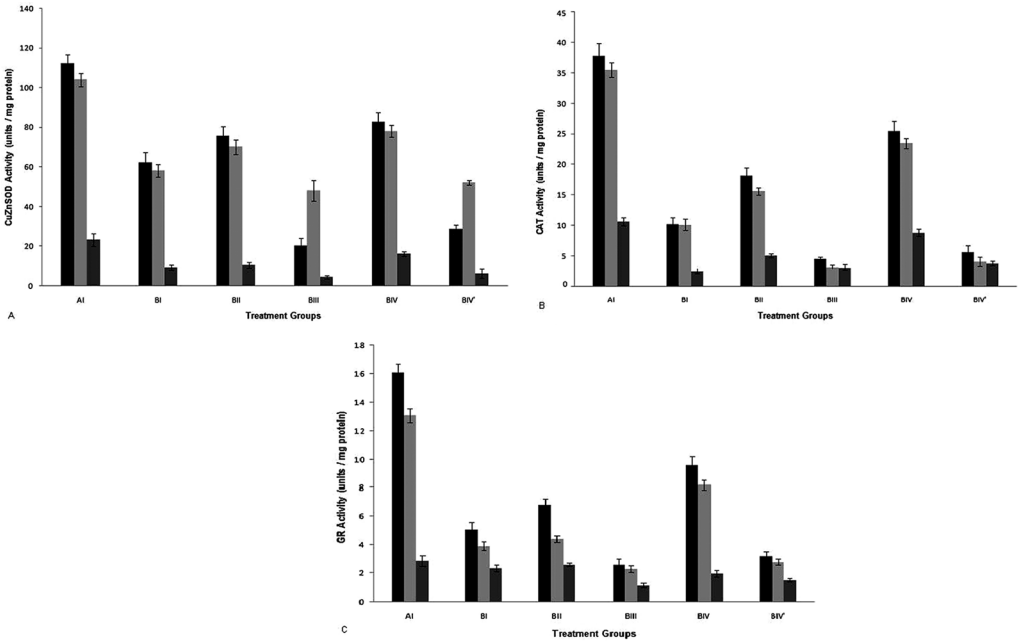

Figure 1. Effect of treatment on antioxidant enzymes A: CuZnSOD; B: CAT; C: GR. All the data have been expressed in mean +/- SEM in each group. CuZnSOD, Copper Zinc SuperOxide dismutase; CAT, catalase; GR, glutathione reductase; mg, milligram of protein in the sample. All the data have been expressed in mean +/- SEM in each group. Bar graphs with dark grey scale represent liver, light grey represent kidney and medium scale grey represent serum levels.

Table 2. Effect of treatment on (a) GST (b) MDA (c) GSH

GST (units/mg)	**AI**	**BI**	**BII**	**BIII**	**BIV**	**BIV'**
A						
Liver	91.4.+/-1.6	47.8.+/-0.9[1]	40.1.+/-1.8[2]	25.1.+/-0.8[2]	52.1+/-1.2[3]	28.3+/-1.1[3]
Kidney	85.1.+/-3.3	45.2.+/-2.1[1]	38.2.+/-1.1[2]	23.1.+/-1.7[2]	50.0+/-2.6[3]	27.2+/-1.4[3]
Non-tumor/tumor skin	10.6.+/-0.8	4.3.+/-0.1[1]	3.2.+/-0.3[2]	2.6.+/-0.1[2]	5.2+/-2.4[3]	3.1+/-0.2[3]
MDA (n mol/mg)	**AI**	**BI**	**BII**	**BIII**	**BIV**	**BIV'**
B						
Liver	2.9+/-0.13	6.3+/-0.14[1]	8.2+/-0.12[2]	11.6+/-0.13[2]	6.8.+/-0.1[3]	9.8+/-0.3[3]
Kidney	1.7+/-0.26	3.5+/-0.17[1]	5.4+/-0.14[2]	7.8+/-0.22[2]	3.9.+/-0.1[3]	8.9+/-0.3[3]
Non-tumor/tumor skin	2.1+/-0.1	3.9+/-0.24[1]	4.3+/-0.26[2]	5.1+/-0.31[2]	2.7.+/-0.1[3]	6.3+/-0.2[3]
GSH (n mol/mg)	**AI**	**BI**	**BII**	**BIII**	**BIV**	**BIV'**
C						
Liver	62.0+/-0.14	45.5+/-0.21[1]	39.4+/-0.68[2]	32.2+/-0.31[2]	51.1+/-0.11[3]	36.2+/-0.2[3]
Kidney	41.1+/-0.32	35.1+/-0.92[1]	30.2+/-2.11[2]	9.6+/-1.21[2]	24.6+/-1.43[3]	12.1+/-1.3[3]
Non-tumor/tumor skin	34.2+/-0.25	21.9+/-0.92[1]	18.3+/-0.12[2]	12.3+/-0.95[2]	27.6+/-1.23[3]	15.2+/-1.2[3]

GST, Glutathione-S-transferase; MDA, malondialdehyde; GSH, Reduced Glutathione; mg, milligram of protein in the sample; nmol/mg, nanomoles per milligram of protein in the samples. All the data have been expressed in mean+/-SEM in each group. [1]indicates significantly different from group AI at $p<0.05$. [2]indicates significantly different from group BI at $p<0.05$. [3]indicates significantly different from group BII and group BIII at $p<0.05$.

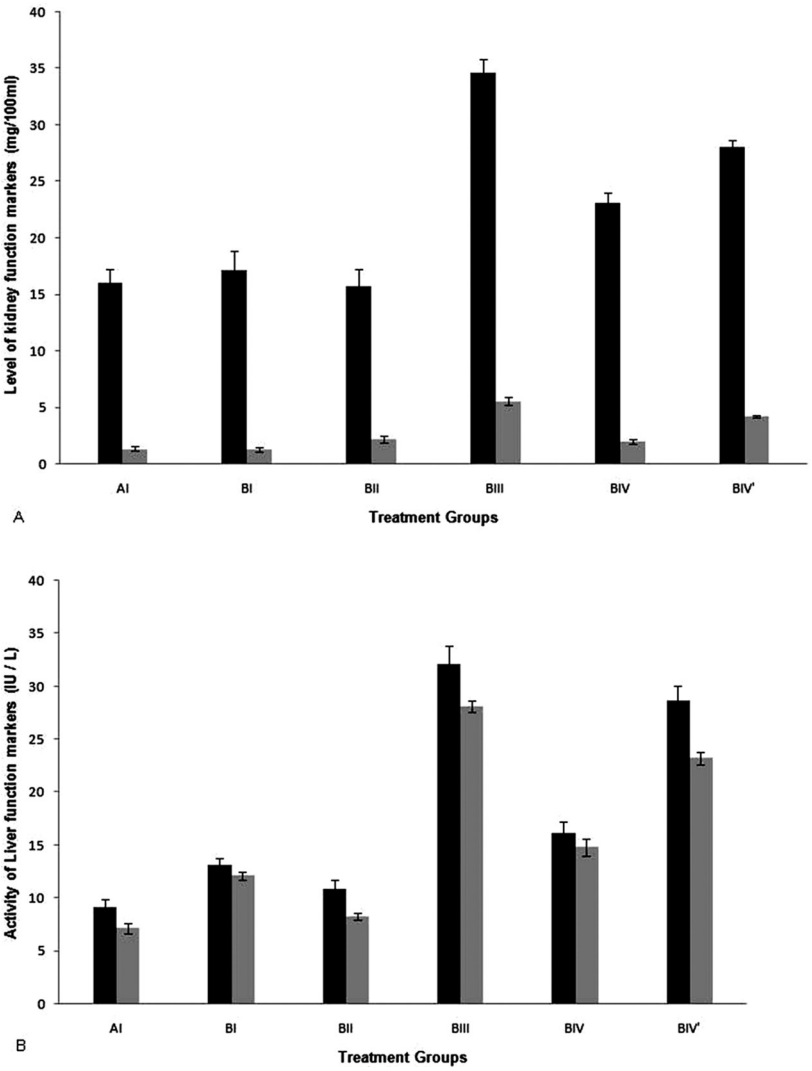

Figure 2. Effect of treatment on kidney and liver function markers in serum. A: kidney function markers, dark gray represent urea levels and light grey represent creatinine levels. I; B: liver function markers, dark gray represent GOT levels and light grey represent GPT levels. mg/100 ml, Milligram per 100 millilitres of the samples. Effect of treatment on liver function markers in serum. IU/L, international units per litre of the sample; GOT, Glutamate Oxaloacetate Transaminase; GPT, Glutamate Pyruvate Transaminase

4.4. Effect of treatment on kidney and liver function markers in serum

Urea and creatinine were taken as nephropathy markers; there levels were found to be highest in cisplatin treated group BIII when compared to all the other groups (Figure 2). The graph depicts significant nomalisation of these markers in Group BIV when compared to Group BIII. This graph depicts that the liver function markers are elevated in the serum in the tumor induced group when compared to group AI. The glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) levels were highest in the group BIII, showing hepatotoxicity caused by long term CP use (Figure 2). The RF-CP combination treatment showed their inclination towards normal when compared to CP treated group BIII. The combination-treated group BIV without light exposure also showed similar recovery patterns but the results were not as significant as its counterpart under photoillumination (Group BIV).

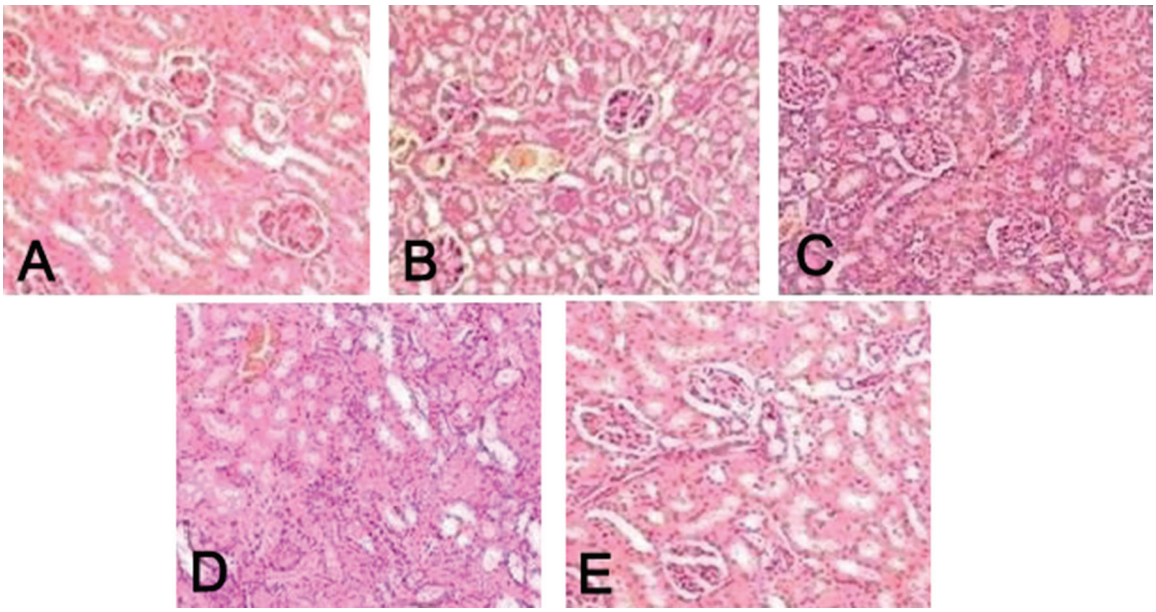

Figure 3. Hematoxylin and eosin stained kidney sections (magnification X400) of the major groups (A: Group AI, vehicle control; B: Group BI, positive control; C: Group BII, riboflavin treated group; D: Group BIII, cisplatin treated group; E: Group BIV, combination group).

4.5. Histopathology of mouse kidney, liver and skin

In group AI control mice, kidney histoarchitecture was found to be normal but in DMBA/TPA treated group BI alterations of renal histological structures were seen (Figure 3). Hematopoietic tissue and dilatation of tubules is clearly visible. The kidney section of RF treated group BII was found to be slightly better with lesser dilatation. The histomicrograph of kidney of CP treated group BIII showed structural features of acute tubular necrosis characterized by swelling. Degeneration, inflammatory cell infiltration, lesser cell density, vacuolization and loss of architecture of tubules were also seen in this group. The combination treated group BIV depicted renal tubules as normal comparable to the control, as no necrosis is seen here with minimum damage in tubules. No significant change was seen in group BIV' (Not shown).

The histopathological examination of the liver of vehicle control group AI mice showed normal appearance of hepatocytes (Figure 4). The treatment of mice with DMBA/TPA in group BI showed mostly normal histology with the aggregation of mononuclear cells in hepatic parenchyma and mild necrosis. The liver histomicrograph of RF treated group BII appeared to be slightly better when compared to positive control group BI. The liver section of CP treated group showed hemorrhages with rupture of sinusoids and broken central vein, majorly indicating necrosis. Group BIV treated group revealed structure which was closely comparable to the control group AI, with mildly swollen sinusoids. Group, BIV' without whole body photo radiation did not show any significant change when compared to CP treated group (Not shown).

The histopathological features of skin in the different treated groups are shown in Figure 5. The vehicle control group AI showed normal appearance of the epidermis and dermis of skin. DMBA/TPA treated group BII or the positive control showed squamous cell carcinoma with dysplasia and keratin pearls. The RF treated group BII showed features comparable to positive control group BI, but with lesser hyperplasia. The CP treated group BIII, showed severe degeneration and necrosis. The combination treated group BIV showed lesser degree of dysplasia and hyperplasia.

After qualitative histopathological assessment, semiquantitative analysis was performed by counting cells on five randomly selected sections of tissues of kidney, liver, and non tumor/tumor skin cells (Figure 6). The cells per renal tubular epithelium were taken as the

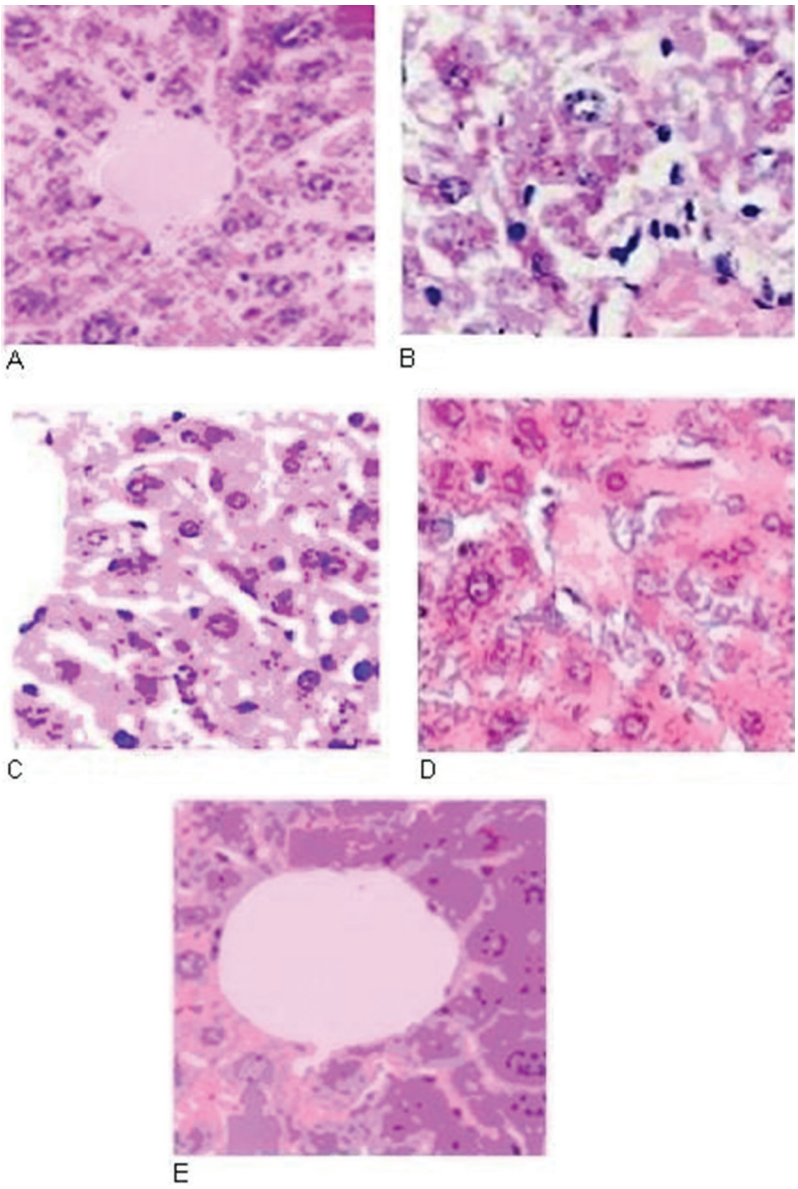

Figure 4. Hematoxylin and eosin stained liver sections (magnification X400) of the major groups (A: Group AI, vehicle control; B: Group BI, positive control; C: Group BII, riboflavin treated group; D: Group BIII, cisplatin treated group; E: Group BIV, combination group).

parameter to count the cells in kidney as its parenchyma contains diverse range of cells. Cells having necrotic features were highest in CP treated group BIII, followed by tumor induced group BI. Cells undergoing apoptosis were maximum in combination treated group BIV, this group also had minimum cells undergoing necrosis. Hepatocytes in a section were analyzed because of uniform cellular arrangement; the results followed a pattern similar to kidney tissue sections. In skin squamous cells were analysed, as DMBA/TPA causes squamous cell carcinoma. The number of normal cells declined in tumor induced group BI, which also showed necrosis. Highest necrotic cells were seen in CP treated group BIII, however the treatment with a combination of RF and CP under photoillumination caused maximum apoptosis and an increase in normal cells when compared to CP treated group.

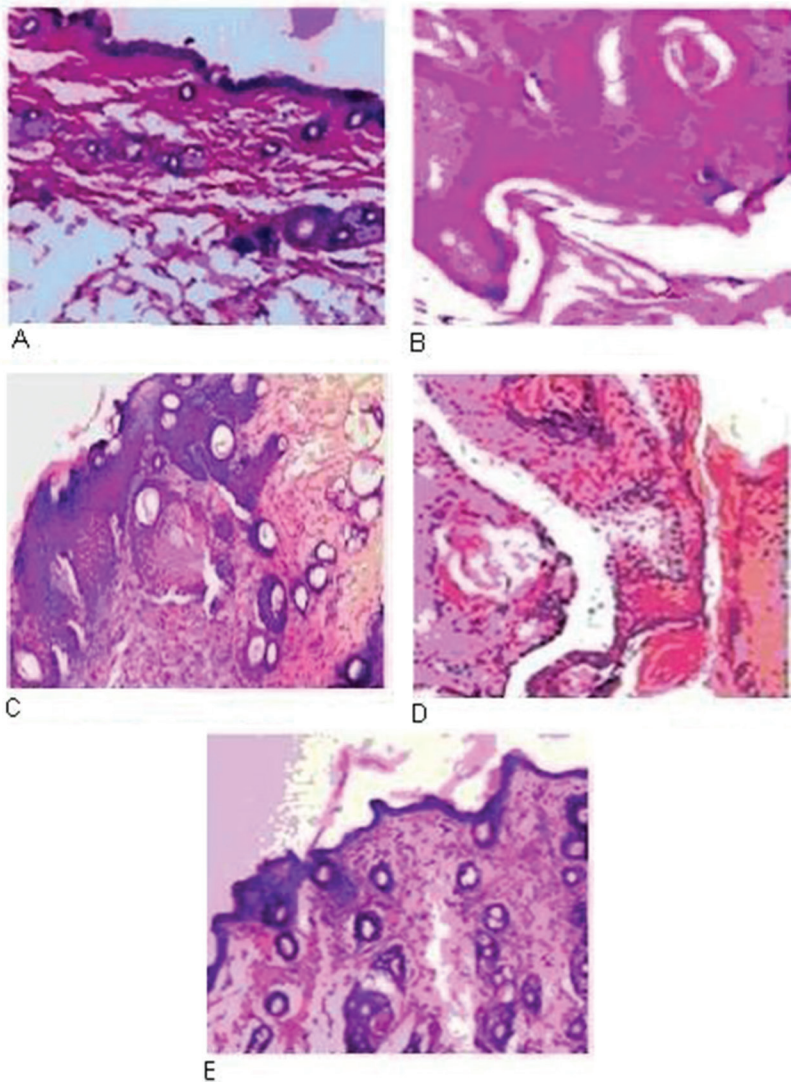

Figure 5. Hematoxylin and eosin stained non tumor/tumor skin sections (magnification X400) of the major groups (A: Group AI, vehicle control; B: Group BI, positive control; C: Group BII, riboflavin treated group; D: Group BIII, cisplatin treated group; E: Group BIV, combination group).

Grading of cancer showed that DMBA/TPA caused grade III cancer which was poorly differentiated. RF treatment showed some improvement in histology but the grade of cancer didn't change. CP treated mice had tumor of grade II, showing moderately differentiated cells. However the combination group under photoillumination, showed grade I type of cells which were well differentiated and the closest to normal tissue under microscope. These results along with results on tumor volume show that the combination suppresses progression of tumor and anticancer activity of CP is in fact potentiated in combination treated groups.

5. DISCUSSION

CP has been one of the most widely prescribed antineoplastic agents for the treatment of various cancers and solid tumors in human for nearly over three decades. Its clinical use is restricted or discontinued because of its associated side effects like nephrotoxicity, hepatotoxicity, neurotoxicity and ototoxicity during prolonged

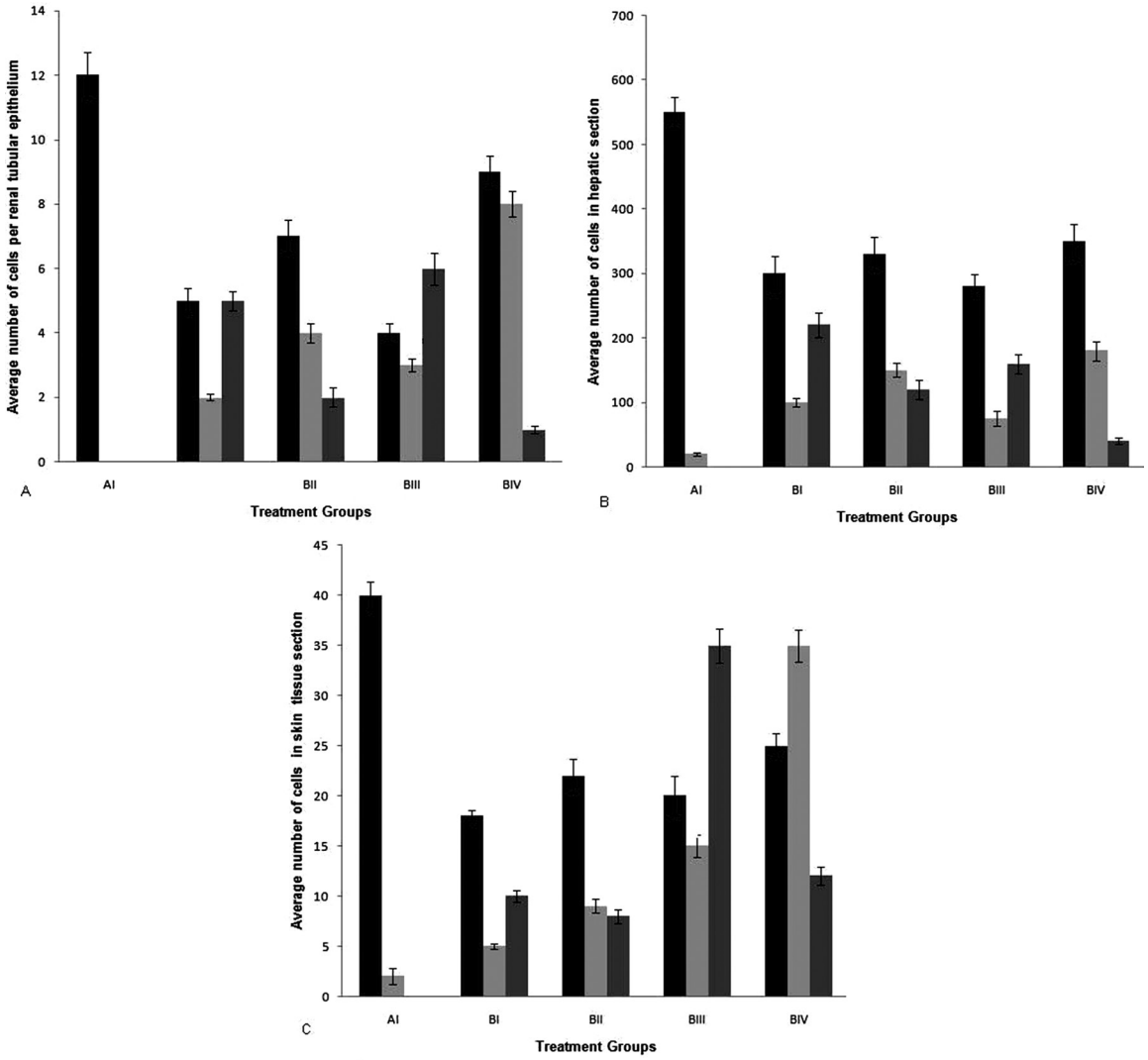

Figure 6. Semi quantitative analysis of the of the tissue sections A: Average number of cells per renal tubular epithelial cells in kidney section of various groups. B: Average number of cells in hepatic section of various groups. C: Average number of cells in hepatic section of various groups. All the data have been expressed in mean +/- SEM of 5 different preparations.

chemotherapy. Our present work is the first study that has shown the blunting effect of CP-RF combination under photo-illumination on the toxicities induced by cisplatin in two -stage mouse skin carcinogenesis induced by DMBA/TPA. The two-stage mouse skin carcinogenesis is one of the best characterized procedures and represents a well-suited model for the understanding of the multistage nature of the tumor formation. Studies show that a moderate increase of reactive oxygen species (ROS) may induce cell proliferation while excessive amounts of ROS can cause oxidative damage to lipids, proteins, and DNA, provoking oncogenic transformation, increased metabolic activity, and mitochondrial dysfunction (29-31). Many reports suggest that cancer cells are under a continuous oxidative stress (32-34). This was shown by the decreased activities of antioxidant enzymes, reduced levels of GST and GSH and elevated MDA, in the tumor induced group BI when compared to the vehicle control group AI. The higher oxidative stress observed in cancer cells may also result from the decrease in the levels or inactivation of antioxidants (35-36). The toxicity

of CP is considered to be initiated by free radical production which caused the depletion of glutathione and inactivation of antioxidant enzymes (37-38). GSH is thought to provide the first line of defense against CP induced toxicity by intercepting reactive platinum complexes before they can react with DNA and by supporting DNA repair through stabilizing the repair enzymes (39). The sharp decline in the level of GSH following treatment with CP leads to loss of integrity and selectivity of membranes of cell and their organelles. CP also exerts its cytotoxicity by forming drug-DNA adducts which can cause programmed cell death ultimately. However if the damage to cellular membrane becomes excessive it causes toxicity. It is interesting to point out that apoptosis and necrosis have been frequently observed in the same population of tumor cells treated with CP (40). Studies show that mode of cell death is decided by factors such as the availability of energy and the metabolic condition of the cell post CP treatment. Urea and creatinine levels are the index of nephrotoxicity (41). The combination of CP with RF significantly reduced cisplatin induced nephrotoxicity by lowering the levels of urea and creatinine. The treatment also affected the status of liver as evident by decrease in the level of liver function markers in serum. These results were complemented by qualitative and semiquantitanive histopathological studies. Also our initial data of MTT assay and immunoblotting (unpublished data) show that the antitumor activity is increased when CP is given in combination with RF. RF is also an apoptosis inducer and has been used as an adjuvant in treatment of wide variety of diseases including cancer (42). Our results also show that RF is also an stress inducer as shown by the levels of antioxidant enzymes, lipid peroxidation and functional markers. Various line of evidence suggest that under high level of stress, as that caused in cancer, RF can induce apoptosis via Fas–Fas ligand-mediated pathway involving caspase activation (43). Hence, treatment with RF promotes the involvement of autophagy mediated cell death along with intrinsic and extrinsic pathways of apoptosis. Hence, triggering three modes of cell death at the same time may be the reason of improved cell death by RF. Epidemiological studies show that low level of RF increases the risk of cancer (44). A recent study reveals that photoillminated RF decreases the aggressiveness of melanoma B16F10 cells *in vitro* and *in vivo* and decreases their survival by hindering mTOR as well as Src kinase. These reactions may occur without light as shown by our results but a stronger and more significant effect is seen under photoilluminated conditions because of increased proclivity of RF. In our case it appears that the free radicals generating potential of CP was quenched when given in combination. Probably CP and RF act synergistically to induce apoptosis. Our previous work also indicates that RF possibly undergoes enolization under photoillumination yielding its more reactive form (17). On the other hand, CP undergoes aquation reaction in water that ultimately forms diammoniumplatinum oxide. The Light exposure can cleave the π- bond (between Pt and oxygen of diammoniumplatinum oxide) in heterolytic fashion, making Pt an electron deficient species (Pt+). This highly unstable species can attack the lone pairs of electrons of nitrogen atoms of the isoalloxazine ring of RF. Thus, Pt+ can form four possible complexes through co-ordination bonding thereby engaging most of CP and RF in combination. Thus the excitable electrons in isoalloxazine ring of RF interact with CP at molecular level and which leads to suppression of the prooxidant potential of RF as well as decreased production of ROS by CP. Also stress level may come down to the level where apoptosis and autophagy are preferred over necrosis in the combination treated group as shown by the histopathology results indicating synergistic anti cancer role of RF and CP. Our results demonstrate the role of photoilluminated RF as a strong therapeutic adjuvant for alleviation of CP induced toxicities. Thus, modulation of cellular hepatotoxicity and nephrotoxicity induced by CP using photoilluminated RF can be employed as a complementary strategy to combat cancer in future.

6. ACKNOWLEDGEMENTS

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7. REFERENCES

1. SM Sancho-Martínez, FJ Piedrafita, JB Cannata-Andía, JM López-Novoa and FJ López-Hernández: Necrotic concentrations of cisplatin activate the apoptotic machinery but inhibit effector caspases and interfere

with the execution of apoptosis. Toxicol Sci,122(1), 73–85 (2011)
DOI: 10.1093/toxsci/kfr098

2. RY Tsang, T Al-Fayea and HJ Au: Cisplatin overdose: toxicities and management. Drug Saf, 32(12), 1109-22 (2009)
DOI:10.2165/11316640-000000000-00000

3. A Zicca, S Cafaggi, MA Mariggio, MO Vannozzi, M Ottone, V Bocchini, G Caviglioli and M Viale: Reduction of cisplatin hepatotoxicity by procainamide hydrochloride in rats. Eur J Pharmacol, 442, 265-272 (2004)
DOI: 10.1016/S0014-2999(02)01537-6

4. R Baliga, N Ueda, PD Walker and SV Shah: Oxidant mechanisms in toxic acute renal failure. Drug Metab Rev, 31, 971-997 (1999)
DOI: 10.1081/DMR-100101947

5. M Nishikawa, H Nagatomi, M Nishijima, G Ohira, BJ Chang, E Sato and M Inoue: Targeting superoxide dismutase to renal proximal tubule cells inhibits nephrotoxicity of cisplatin and increases the survival of cancer-bearing mice. Cancer Lett, 171(2), 133-8 (2001)
DOI: 10.1016/S0304-3835(01)00591-2

6. R Baliga, Z Zhang, M Baliga, N Ueda and S Shah: *In vitro* and *in vivo* evidence suggesting a role for iron in cisplatin-induced nephrotoxicity. Kidney Int, 53(2), 394-401 (1998)
DOI: 10.1046/j.1523-1755.1998.00767.x

7. H Matsushima, K Yonemura, K Ohishi and A Hishida: The role of oxygen free radicals in cisplatin induced acute renal failure in rats. J Lab Clin Med, 131(6), 518-526 (1998)
DOI: 10.1016/S0022-2143(98)90060-9

8. D Appenroth, S Frob, L Kersten, EK Splinter and K Winnefeld: Protective effects of vitamin E and C on cisplatin nephrotoxicity in developing rats. Arch Toxicol, 71(11), 677-683 (1997)
DOI: 10.1007/s002040050444

9. S Iseri, F Ercan, N Gedik, M Yuksel and I Alican: Simvastatin attenuates cisplatin-induced kidney and liver damage in rats. Toxicol, 230, 256-264 (2007)
DOI: 10.1016/j.tox.2006.11.073

10. M Nazirgolu, A Karaoglu and A. O. Aksoy: Selenium and high dose vitamin E administration protects cisplatin-induced oxidative damage to renal, liver and lens tissues in rats. Toxicol, 195, 221-230 (2004)
DOI: 10.1016/j.tox.2003.10.012

11. N Getoff, S Solar and DB McCormick: Photoejection of electrons from flavins in polar media. Science, 201, 616-618 (1978)
DOI: 10.1126/science.675244

12. Y Zhang and H Gorner: Flavin sensitized photooxidation of lysozyme and serum albumin. Photochem Photobiol, 85, 943-948 (2009)
DOI: 10.1111/j.1751-1097.2009.00547.x

13. LM Bareford, MA Phelps, AB Foraker and PW Swaan: Intracellular processing of riboflavin in human breast cancer cells. Mol Pharmacol, 5, 839-848 (2008)
DOI: 10.1021/mp800046m

14. D Machado, SM Shishido, KCS Queiroz, DN Oliveira, ALC Faria, RR Catharino, CA Spek and CV Ferreira: Irradiated Riboflavin Diminishes the Aggressiveness of Melanoma In vitro and In vivo. PLoS One, 8(1), 0054269 (2013)
DOI: 10.1371/journal.pone.0054269

15. KC DeSouzaQueiroz, WF Zambuzzi, ACSDeSouza, RAdaSilva, D Machado, GZ Justo, HF Carvalho, MP Peppelenbosch and CV Ferreira: A possible anti-proliferative and anti-metastatic effect of irradiated riboflavin in solid tumours. Cancer lett, 258, 126-134 (2007)
DOI: 10.1016/j.canlet.2007.08.024

16. MA Mu-oz, A Pacheco, MI Becker, E Silvaa, R Ebenspergera, AM Garciaa, AEDe Ioannesb and AM Edwards: Different cell death mechanisms are induced by a hydrophobic flavin in humantumor cells after visible light irradiation. J Photochem Photobiol B, 103, 57 (2011)
DOI: 10.1016/j.jphotobiol.2011.01.012

17. I Hassan, S Chibber, A. A Khan and I Naseem: Riboflavin ameliorates cisplatin induced toxicities under photoillumination. PLoS One, 7(5), 0036273 (2012)
DOI: 10.1371/journal.pone.0036273

18. I Hassan, S Chibber and I Naseem: Ameliorative effect of riboflavin on cisplatin induced nephrotoxicity and hepatotoxicity under photoillumination. Food Chem Toxicol, 48, 2052-2058 (2010)
DOI: 10.1016/j.fct.2010.05.004

19. E Husain and I Naseem: Riboflavin mediated cellular photoinhibition of cisplatin-induced oxidative DNA breakage in mice keratinocytes. Photoderm, Photoimmunol Photomed, 24, 301-30733 (2008)
DOI: 10.1111/j.1600-0781.2008.00380.x

20. S Marklund and G Marklund: Involvement of superoxide anion radical in the auto oxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem, 47, 469-474 (1974)
DOI: 10.1111/j.1432-1033.1974.tb03714.x

21. H Aebi: Catalase in vitro. Methods Enzymol, 105, 121-126 (1984)
DOI: 10.1016/S0076-6879(84)05016-3

22. I Carlberg and B Mannervik: Glutathione reductase. Methods Enzymol 113, 484-490 (1985)
DOI: 10.1016/S0076-6879(85)13062-4

23. WH Habig, MJ Pabst and WB Jokoby: Glutathione S-transferase: the first enzymatic step in mercapturic acid formation. J Biol Chem, 249(22), 7130-7139 (1975)

24. H Ohkawa, N Ohishi and K Yagi: Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem, 95, 351-358 (1979)
DOI: 10.1016/0003-2697(79)90738-3

25. DJ Jollow, JR Mitchell, N Zampaglione and JR Gillette: Bromobenzene induced liver necrosis: protective role of glutathione and evidence for 3, 4-bromobenzene oxide as hepatotoxic metabolite. Pharmacology, 11, 151-169 (1974)
DOI: 10.1159/000136485

26. M Berthelot: Repert Chem Appliq, 1, 284 (1859)
(doi not found)

27. RW Bonses and HH Taussky: On the colorimetric determination of creatinine by the Jaffe reaction. J Biol Chem, 158, 581-591 (1945)
(doi not found)

28. S Reitman and S Frankel: A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Path, 28, 56-63 (1975)

29. L Behrend, G Henderson and RM Zwacka: Reactive oxygen species in oncogenic transformation. Biochem Soc Trans, 31, 1441-1444 (2003)
DOI: 10.1042/BST0311441

30. H Pelicano, D Carney and P Huang: Ros stress in cancer cells and therapeutic implications. Drug Resist Updat, 7 (2), 97-110 (2004)
DOI: 10.1016/j.drup.2004.01.004

31. S Pervaiz and MV Clement: Tumor intracellular redox status and drug resistance serendipity or a causal relationship? Curr Pharm Des, 10, 1969-1977 (2004)
DOI: 10.2174/1381612043384411

32. PT Schumacker: Reactive oxygen species in cancer cells: live by the sword, die by the sword. Cancer cell, 10, 175-176 (2006)
DOI: 10.1016/j.ccr.2006.08.015

33. TB Kryston, AB Georgiev, P Pissis and AG Georgakilas: Role of oxidative stress and DNA damage in human carcinogenesis. Mutat Res, 3, 193-201 (2011)
DOI: 10.1016/j.mrfmmm.2010.12.016

34. JE Klauning and LM Kamendulis: The role of oxidative stress in carcinogenesis. Annu Rev Pharmacol Toxicol, 44, 239-267 (2004)
DOI: 10.1146/annurev.pharmtox.44.101802.121851

35. P Huang, L Feng, EA Oldham, MJ Keating and W Plunkett: Superoxide dismutase as

a target for the selective killing of cancer cells. Nature, 407(5), 390-395 (2003)
(doi not found)

36. KA Conklin: Free radicals: the pros and cons of antioxidants. Cancer chemotherapy and antioxidants. J Nutr, 134, 3201-3204 (2004)
(doi not found)

37. S Palipoch, C Punsawad, P Koomhin and P Suwannalert: Hepatoprotective effect of curcumin and alpha-tocopherol against cisplatin-induced oxidative stress. BMC Compl Alter Med, 14(1), 111 (2014)
DOI: 10.1186/1472-6882-14-111

38. B Fernández-Rojas, O N Medina-Campos, R Hernández-Pando, M Negrette-Guzmán, S Huerta-Yepez and J Pedraza-Chaverri: C-phycocyanin prevents cisplatin-induced nephrotoxicity through inhibition of oxidative stress. Food and Function, 5(3), 480-90 (2014)
DOI: 10.1039/c3fo60501a

39. G Chu: Cellular responses to cisplatin. J Biol Chem, 269 (2), 787-790 (1994)
(doi not found)

40. EI Montero, JM Pérez, A Schwartz, MA Fuertes, JM Malinge, C Alonso, M Leng and C Navarro-Ranninger: Apoptosis induction and DNA interstrand cross-link formation by cytotoxic trans-(PtCl2 (NH (CH3)2) (NHCH (CH3)2): cross-linking between d(G) and complementary d(C) within oligonucleotide duplexes. Chembiochem, 3, 61-67 (2002)
DOI: 10.1002/1439-7633(20020104)3:1<61::AID-CBIC61>3.0.CO;2-I

41. Y Shibayama, A Kawachi, S Onimaru, J Tokunagac, R Ikedaa, K Nishidaa, S Kuchiiwad, S Nakagawad, N Takamurac, T Motoyab, Y Takedaa and K Yamada: Effect of pre-treatment with St John's Wort on nephrotoxicity of cisplatin in rats. Life Sci, 81(2), 103-108 (2007)
DOI: 10.1016/j.lfs.2007.04.025

42. AM Edwards and E Silva: Effect of visible light on selected enzymes, vitamins and amino acids. J Photochem Photobiol B, 63, 126–131 (2001)
DOI: 10.1016/S1011-1344(01)00209-3

43. ACS DeSouza, L Kodach, FR Gadelha, CL Bos, AD Cavagis, H Aoyama, MP Peppelenbosch and CV Ferreira: A promising action of riboflavin as a mediator of leukemia cell death. Apoptosis, 11, 1761 (2006)
DOI: 10.1007/s10495-006-9549-2

44. RS Rivlin: Riboflavin and cancer: a review. Cancer Res, 33, 1977-1986 (1973)
(doi not found)

Abbreviations: CP, Cisplatin; RF, Riboflavin; CuZnSOD, Copper Zinc SuperOxide dismutase; CAT, Catalase; GR, Glutathione reductase; ROS, Reactive Oxygen Species; PDT, Photodynamic Therapy; RPT, Ribophototherapy; GST, Glutathione-S-transferase; MDA, Malondialdehyde; GSH, Reduced Glutathione; DMBA, 9,10-dimethylbenz(a)anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate

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