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A STUDY ON CISPLATIN-INDUCED HEPATOTOXICITY AND PROTECTIVE ROLE OF TURMERIC IN RATS

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ABSTRACT

Toxic effects of cisplatin and protective role of turmeric was assessed on hepatic biomarkers. A total of 48 rats were divided into 4 groups of 12 rats each. Group 1 was maintain ed as sham control, 2 was treated with cisplatin (@ 2 mg/kg b.wt, intraperitoneally on day 1, 7, 14 and 28), 3 was treated with turmeric (@ 0.05 mg/kg b.wt. p.o. once daily for 28 days) and 4 was treated with cisplatin + turmeric (as per above schedule). Blood was collected at fortnight intervals and serum was separated for estimation of hepatic bio markers. Six rats in each group were then euthanized on day 14 and 28 for histopathology, while tissue parameters were assayed on day 28. Serum AST and GGT were significantly ($P < 0.05$) increased, while serum protein was significantly ($P < 0.05$) reduced in group 2. The tissue enzyme assays revealed a significant ($P < 0.05$) increase in CYP_{450}, TBARS and protein carbonyls and significant ($P < 0.05$) decrease of GSH, SOD, catalase, GST, GPX, G6PD, Na+ /K+ ATPase and Mg2 + ATPase in group 2 as compared to other groups. Sections of liver showed marked central vein and sinusoidal congestion, bile duct hyperplasia, few hepatocytes with hydropic degeneration and other degenerative changes. It concluded that the ameliorative group 4 showed mild to moderate improvement in all parameters in comparison to group 2. It is concluded that supplementation of turmeric was found beneficial in countering the toxic effects of cisplatin on liver.

KEY WORDS: ATPases, cisplatin, hepatotoxicity, oxidative stress, turmeric

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INTRODUCTION

Cancer is one of the most dreadful diseases and currently taking the heaviest toll of human lives, with distant hope of finding an effective and complete cure unless detected and treated in early stages[1]. Cancer prevalence in India is estimated to be around 2.5 million with over 8, 00,000newcasesand 50,000 deaths occurring each year [2]. Cisplatin is known as penicillin of cancer drugs as it was prescribed widely for the first and effective treatment of cancer. It is a plat inum containing drug used in the treatment of var ious types of cancers[3]. Similar to the adverse effects of other anti-cancer agents, cisplatin has its own dose-dependent cytotoxicity on liver and other organs [4, 5].

Derivatives of turmeric namely, curcumin, demethoxycurcumin and bis-demethoxycurcumin have antioxidant effects. Curcumin exerts powerful inhibitory effect against H_2O_2-induced damage in human keratinocytes and fibroblasts and in NG 108-15 cells. Curcumin reduces oxidized proteins in amyloid pathology in Alzheimer transgenic mice. It also decreases lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates. This is brought about by maintaining the activities of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase. Since ROS have been implicated in the development of various pathological conditions, turmeric has the potential to control the diseases through its potent antioxidant activity. Therefore, this study was undertaken to evaluate the protective role of turmeric in ameliorating the hepatotoxic effects induced by cisplatin in rats [6].

MATERIALS AND METHODS

i. Chemical Reagents

Cisplatin was obtained from VHB Life Sciences Ltd.,Mumbai. Fresh turmeric was procured from farm and was shade dried and made into powder. Other chemicals and reagents were obtained from Qualigens Pvt. Ltd., Mumbai, India.

ii. Animals

Female rats of *Sprague dawley* strain 3 months old (weighing 150-200g) were procured from National Institute of Nutrition, Hyderabad Andhra Pradesh. The animals used in this study were approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

iii. Experimental procedure

A total of 48 *Sprague dawley* female rats were randomly divided into 4 groups consisting of 12 in each group. Six rats in each group were euthanized on day 14. All the groups were maintained as per the following treatment schedule.

Group 1: Control diet.

Group-2: Control diet+ Cisplatin @ 2 mg / kg body wt. by intraperitoneal injection weekly once for 4 weeks.

Group-3: Control diet+ Turmeric control @ 0.05 ppm by oral gavage needle daily.

Group-4: Control diet+ Cisplatin @ 2 mg/kg body wt. by intraperitoneal injection weekly once for 4 weeks + turmeric @ 0.05 ppm body wt. by oral gavage needle daily.

Turmeric was administered by dissolving in DMSO.

iv. Collection and preparation of samples

Liver tissues were collected at the end of 28th day. Rats were fasted overnight and sacrificed by cervical decapitation, removed the brain, weighed, dissected and washed in ice-cold saline. Some pieces were immediately homogenized (1:10, w/v) in a cold (4^{0}c) buffer containing Tris base (20 mmol/L), EDTA (1 mmol/L), and sucrose (0.5 mmol/L), KCl (150 mmol/L) with the pH adjusted to 7.4. Homogenates were centrifuged at 4000 g for 20 min at 4^{0}c, and collected clear supernatant for estimation of lipid peroxidation levels and proteins using serum bovine albumin as the standard as per the procedure described by[7].

v. Measurement of Glutathione (GSH)

Brain GSH levels were measured as per the procedure described by[8]. 100 µl of 25% trichloroacetic acid was added to 400 µl of homogenate, centrifuged and collected supernatant and used as sample. To 2.0 ml of 0.6 mM 5-5' dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate (pH 8), 0.1 ml of sample and 0.9 ml of 0.2 M phosphate buffer was added and the absorbance was read at 412 nm against a reagent blank. The standards (0.05-5 mg/ml) were also treated in the same way.

vi. Measurement of Glutathione S-transferase (GST)

Brain GST levels were measured as per the procedure described by[9]. Homogenate was centrifuged at 1, 05,000 g for 60 min and supernatant was used as enzyme source. 1 ml of phosphate buffer, 0.1 ml of 1-Chloro-2, 4-dinitrobenzene (CDNB) and 0.1 ml of supernatant was taken into a cuvette and adjusted the volume to 2.9 ml with distilled water. The mixture was incubated for 5 min at 37^0 C. The reaction was started by addition of 0.1 ml of glutathione and absorbance was read for 5 min at 340 nm. Reaction mixture without homogenate was used as blank.

vii. Measurement of Thiobarbituric acid reacting substances (TBARS)

Brain TBARS levels were measured as per the procedure described by[10]. 500 µl of supernatant from the homogenate, 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid were taken in a tightly Stoppard tube. The tube was heated to boiling temperature for 45 min. cool the tube and the contents were centrifuged. The supernatant was read at 532 nm against blank. The concentration of test samples was obtained using molar extinction coefficient of MDA.

viii. Measurement of protein carbonyls

Brain protein carbonyl levels were measured as per the procedure described by[11]. 1 ml of homogenate supernatant containing 1 mg protein was added to 4 ml of 10 mM dinitrophenyl hydrazine (DNPH) in 2.5 M HCl. Samples were vortexed and incubated at room temperature for 1 hour in dark. Then protein was precipitated by adding 5 ml of 20% trichloroacetic acid and centrifuged at 3000 rpm for 10 min, supernatant was discarded to collect protein precipitate, which was washed thrice with 4 ml of ethanol: ethyl acetate (1:1) solution. Final protein precipitate was re-dissolved in 2 ml of 6 M guanidine HCl in 20 mM of potassium phosphate and kept at 37^0C for 10 min, and centrifuged to remove the insoluble substances and the absorbance was read at 372 nm against 2.5 M HCl blank. Known concentration of bovine serum albumin dissolved in 6 M guanidine HCl in 20 mM of potassium phosphate was used as standard.

ix. Measurement of Na^+/K^+ and Mg^{2+} ATPase

Na^+/K^+ and Mg^{2+} ATPase measured as per procedure described by [12]. Enzyme preparation (microsomal preparation) and ATP solution were thawed and kept on ice. Reaction mixture (0.5 ml) was taken into 2 test tubes. 0.2 ml of 10 mM ouabain was added to one tube, while equal amount of distilled water was added to the other. 100 µl of microsomal preparation (containing 3 mg protein/ml) was added to both the tubes and pre-incubated for 5 min. To make total volume of incubation mixture to 1 ml, 100 µl of water was added to both the tubes. Reaction was initiated by adding 100 µl of 30 mM ATP solution at room temperature. The reaction was terminated by adding 1 ml of 10% TCA after 30 min. Tubes were immediately transferred to ice. After 10 min, the tubes were centrifuged for 5 min to remove the precipitate. The supernatant was used for estimation of phosphate by the method[33]. The difference in the activity between the absence and presence of ouabain was taken as Na^+-K^+ ATPase activity. Activity in the presence of ouabain was taken as Mg^{2+} ATPase activity.The

liberated Pi is estimated by the method of [13].0.5 ml of microsomal preparation supernatant was made to a total volume of 4 ml with distilled water. 4 ml of reagent A was pipetted into each test tube, capped with parafilm, mixed and kept for 1.5 - 2 h at 37^{o}C. Then it was removed, allowed a few min to cool to room temperature and the absorbance at 820 nm was read against distilled water blank.

x. Glucose 6 phosphate dehydrogenase (G6PD)

Glucose 6 phosphate dehydrogenase measured as per procedure described by [14]. A reaction mixture was prepared by pipetting 21 ml deionized water,5ml Reagent A (buffer), 1ml of Reagent B (G 6-P), 1 ml of Reagent C (β- NADP) and 1ml of Reagent D ($MgCl_2$ into a beaker mixed and equilibrated to 25^{0}C. The pH was adjusted to 7.4 with 1 M NaOH. 2.9 ml of reaction mixture was pipetted into test and blank cuvettes each and equilibrated at 25^{0}C, absorbance was monitored at 340 nm until it was constant. Then 0.1 ml of reagent A (buffer) added to blank and 0.1 ml of homogenate added to test cuvette. Then immediately mixed by inversion and recorded the increase in absorbance for approximately 5 min, and obtained the change in absorbance/min.

xi. Cytochrome P_{450} activity (Cyt P_{450})

Cytochrome P450 activity measured as per procedure described by[15]. The microsomes were diluted with a phosphate glycerol buffer (0.01 M potassium phosphate, pH 7.4, 20% glycerol) to 1.5 mg protein/ml. One ml aliquots were taken into 2 separate cuvettes. One was designated as reduced P450 (P) and other as reduced P450-CO complex (PCO). The PC cuvette was bubbled with carbon monoxide (CO) gas for 2 min. Both were reduced by adding 50 µl of 0.5 M sodium dithionite solution (made fresh). At this stage, the yellow colour of the PCO sample was visible, whereas the P sample remained colourless or turned pale pink. The difference in absorbance of the samples at 450 and 490 nm was measured spectro-photometrically.

xii. Estimation of Superoxide dismutase (SOD)

Superoxide dismutase measured as per procedure described by [16].Three test tubes labeled as Blank (B), Standard (S) and Test (T). 0.65 ml of PBS, 30µl of MTT was added in all three test tubes. Then 10µl of sample added to Test and 75µl of pyrogallol was added to Test and Standard Then mixed well and incubated at room temperature. Exactly after 5 min, added 0.75 ml of DMSO to the test, standard and blank. Then measured the absorbance of the purple colour formed at 570nm.

xiii. Estimation of Catalase

Catalase measured as per procedure described by[17]. To assay mixture containing 0.4 ml of 0.2 M H_2O_2 and 0.5 ml of 0.01 M phosphate buffer (pH 7), 0.1 ml of haemolysate was added and mixed well. Into this, 2ml of dichromate acetic acid solution was blown exactly after 60 sec. Then kept in boiling water bath for 10 min. Read the absorbance of green coloured chromic acetate at 570 nm against reagent blank containing 0.4 ml of 0.2 M H_2O_2 and 0.5 ml of 0.01 M phosphate buffer (pH 7)

xiv. Estimation of Glutathione Peroxidase (GPX)

Glutathione Peroxidase measured as per procedure described by[18.] Four test tubes each with 2 ml of phosphate buffer (0.1M), 0.1 ml of haemolysate, 0.1 ml of reduced glutathione and 0.1 ml of H_2O_2 respectively were incubated at 25^{0}C for 5 min, following which 0.1 ml NADPH was added and the enzyme activity was monitored at 60 sec interwals for 5 min at 320 nm wave length.

xv. Estimation of proteins

Proteins measured as per procedure described by[7] . 100 µl of sample was made

up to 1.0 ml with distilled water. To this, 5 ml of freshly prepared alkaline copper sulphate solution (a mixture of 50 ml of 2% sodium carbonate in 0.1 N sodium hydroxide and 1.0 ml of 0.5% copper sulphate in 1% potassium sodium tartrate) was added and kept for 10 min at room temperature. 0.5 ml of Folin-ciocalteu reagent was added and allowed to stand at dark for 30 min. The resultant blue colour was read at 660 nm against distilled water blank. Known concentrations of bovine serum albumin ranging from 5 -50 mg/ml added in the place of sample was used as standard.

xvi. Estimation of serum biomarkers

Serum was separated from whole blood by centrifugation.AST [19]and GGT were estimated by enzymatic method using standard kits supplied by Qualigens Pvt.Ltd., Mumbai.

xvii. Histopathology

Six rats in each group were then euthanized on day 14 and 28 for histopathology. Approximately 0.2g of liver tissue was preserved in 10% Neutral buffer formalin for histopathological study, the fixed tissues were processed and stained with Hematoxylin and Eosin (H & E) stain as described by [20]

xviii. Analysis of variance

Results were expressed as mean ± S.E. One-way analysis of variance (ANOVA) by SPSS (Statistical Package for Social Sciences) (Ver. 10.00) followed by Duncan test was used to analyze the results with $p < 0.05$ considered significance

RESULTS

i. Oxidative stress parameters

The indicators of oxidation of proteins, protein carbonyls, were increased (6.98±0.27 n moles/mg protein) in group 2 on day 28 (Table 1). There is a significant ($p<0.05$) increase in the activity of CYP_{450} in group 2 (4.88±0.11 n moles/mg microsomal protein; Table 3). The concentration of TBARS (lipid peroxisation index) was also significantly ($p<0.05$) increased in group 2 (0.89±0.06 n moles MDA/mg protein) as compared to the remaining groups (Table 1), while group 4 revealed a significant ($p<0.05$) reduction in the above parameters that was treated with turmeric, owing to its antioxidant properties.

Table 1
Parameters of oxidative stress and antioxidant defenses in liver homogenate of different groups of rats

Group	TBARS (n moles MDA/ mg protein)	Protein carbonyls (n moles/mg protein)	Reduced glutathione (µ moles/mg protein)	GST activity (µ moles/ min/ mg protein)	GPX activity (µ moles/min/ mg protein)
1. Control	0.32± 0.02[a]	3.11± 0.10[a]	254.61±8.11[a]	1.43±0.04[a]	0.0081±0.001[a]
2.Cisplatin	0.89±0.06[b]	6.98 ±0.27[b]	96.70± 5.36[b]	0.89± 0.01[b]	0.0029± 0.000[b]
3.Turmeric	0.24 ±0.01[a]	2.81 ±0.16[a]	261.2±4 9.7[a]	1.51± 0.06[a]	0.0093± 0.001[a]
4.Cisplatin+ Turmeric	0.51 ±0.01[c]	4.07± 0.36[c]	201.82± 7.51[c]	1.06 ±0.07[c]	0.0057±0.001[c]

Values are Mean ± SE (n = 6); One way ANOVA (SPSS)
Means with different alphabets as superscripts differ significantly (P<0.05).

Total protein, GSH (non-enzymatic antioxidant), SOD, catalase, GST and GPX (enzymatic antioxidants) were significantly ($P < 0.05$) decreased (Table 1 & 2) in group 2 suggesting ongoing oxidative stress..G6PD was found to be decreased (1.16±0.05 U/mg protein) (Table 2), while there is increase in above parameters in group 4 owing to antioxidant properties of turmeric.

Cisplatin interferes with cellular Na^+/K^+ ATPase and Mg^{2+}ATPase activity and hence the activities of these membrane enzymes were reduced in the present study (7.01± 0.91 and 6.91± 0.77 µ moles of Pi liberated/mg microsomal protein/30 min, respectively) (Table 3) in group 2 as compared to the remaining groups (Table 3).

Table 2
Antioxidant and metabolizing enzymes in liver homogenate of different groups of rats

Group	Superoxide dismutase activity (µ moles/mg protein)	Catalase activity (µ moles/mg protein/ min)	G6PD activity (U/mg protein)
1. Control	15.43±0.24[a]	14.26±0.31[a]	3.72±0.07[a]
2.Cisplatin	6.89± 0.40[b]	9.68± 0.43[b]	1.16± 0.05[b]
3.Turmeric	15.51± 0.60[a]	15.71± 0.57[a]	4.05± 0.10[a]
4.Cisplatin+ Turmeric	10.06 ±0.37[c]	12.01 ±0.62[ab]	2.46 ±0.12[c]

Values are Mean ± SE (n = 6); One way ANOVA (SPSS)
Means with different alphabets as superscripts differ significantly (P<0.05).

Table 3
ATPases and CYP_{450} in liver homogenate of different groups of rats

Group	Na^+-K^+ ATPase activity (µ moles of Pi liberated/mg microsomal protein/30 min)	Mg^{+2} ATPase activity(µ moles of Pi liberated/mg microsomal protein/30 min)	CYP_{450} activity (n moles/mg microsomal protein)
1. Control	13.16± 1.07[a]	11.02±0.51[a]	3.06± 0.07[a]
2.Cisplatin	7.01±0.91[b]	6.91±0.77[b]	4.88±0.11[b]
3.Turmeric	13.51 ±1.29[a]	12.51±0.89[a]	3.31 ±0.09[a]
4.Cisplatin+ Turmeric	11.77 ±1.10[a]	10.07±0.73[a]	4.01 ±0.10[ab]

Values are Mean ± SE (n = 6); One way ANOVA (SPSS)
Means with different alphabets as superscripts differ significantly (P<0.05).

ii. Serum Biomarkers

There was significant ($P < 0.05$) increase in the activity of liver biomarker enzymes such as serum AST (33.49±3.13 IU/L) and GGT (31.65±4.30 IU/L) on day 14 with a similar trend on day 28 in group 2 as compared to the remaining groups (Table 4).

Table 4
Serum biomarkers in different groups of rats

Group	AST (IU/L)		GGT (IU/L)		Total protein concentration(g/dl)	
	Day 14	Day 28	Day 14	Day 28	Day 14	Day 28
1. Control	17.56 ±0.75[a]	16.26±0.63[a]	11.19 ±0.77[a]	11.00±0.98[a]	8.15±0.50[a]	7.95±0.23[a]
2.Cisplatin	33.49±3.13[b]	50.05±5.39[b]	31.65±4.30[b]	40.72±1.25[b]	5.43±0.45[b]	4.86±0.31[b]
3.Turmeric	17.45±1.16[a]	10.81±1.26[d]	10.81±1.18[a]	10.81±0.93[a]	7.90±0.39[a]	8.00±0.13[a]
4.Cisplatin + Turmeric	22.40±1.00[c]	28.37±2.55[c]	22.39±3.37[c]	28.37±1.36[c]	6.41±0.42[c]	5.99±0.15[c]

Values are Mean ± SE (n = 6); One way ANOVA (SPSS)
Means with different alphabets as superscripts differ significantly (P<0.05).

iii. Gross and Histological changes

Gross pathological changes in group 2 rats revealed atrophied/ shrunken liver with abscess (Fig. 1). Histological sections of liver revealed marked central vein congestion (Fig.2), moderate bile duct hyperplasia and sinusoidal congestion in group 2 on day 14 (Fig.3). Few sections also revealed marked central vein congestion, sinusoidal dilatation and bile duct hyperplasia (Fig.4). The sections of liver revealed marked degenerative changes in hepatocytes in addition to central vein congestion and bile duct hyperplasia on day 28 (Fig. 5).

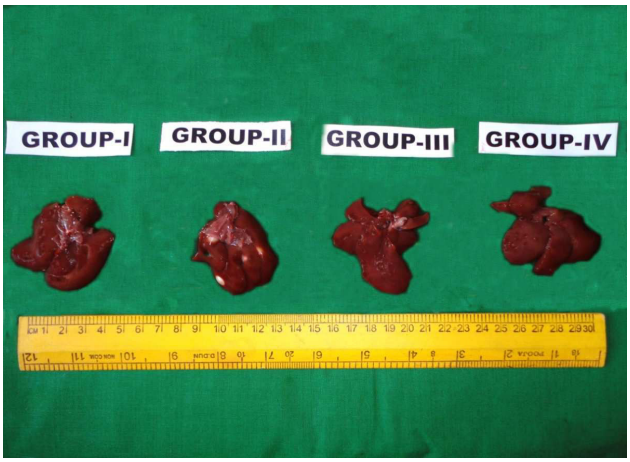

Figure 1
Photograph of rat liver showing shrunken liver with abscess (group II, day 28)

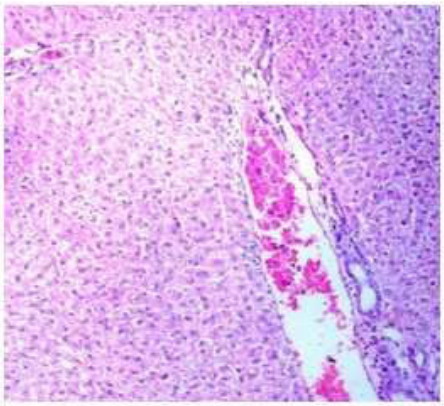

Figure 2
Photomicrograph of liver showing marked central vein congestion (Group 2, day14). H&E X100

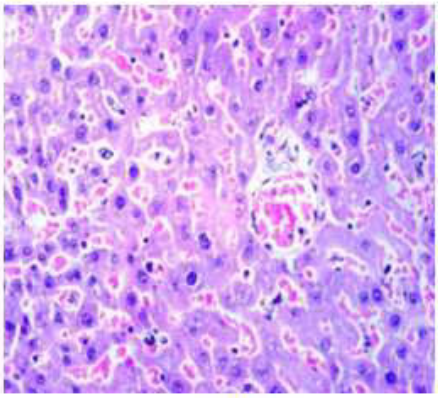

Figure 3
Photomicrograph of liver showing moderate bile duct hyperplasia and sinusoidal congestion (Group 2, day 14). H&E X 200

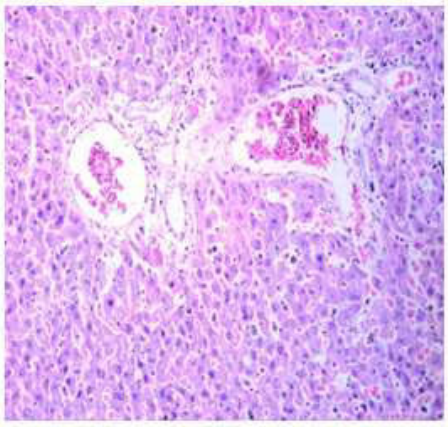

Figure 4
Photomicrograph of liver showing marked central vein congestion, sinusoidal dilation and bile duct hyperplasia (Group 2, day 14). H&E X 100

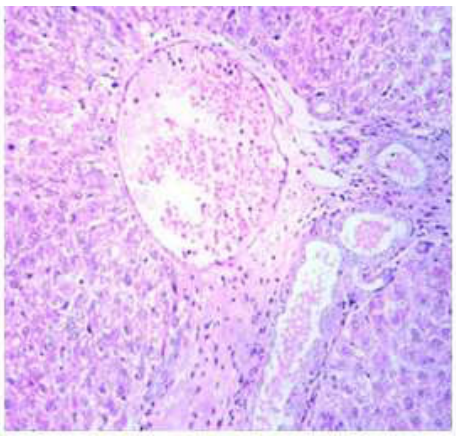

Figure 5
Photomicrograph of liver showing marked degenerative changes, central vein congestion and bile duct hyperplasia (Group 2, day 28). H&E X100

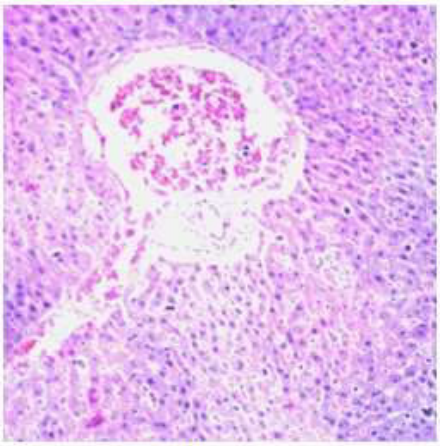

Figure 6
Photomicrograph of liver showing moderate central vein congestion in (Group 4 day 14). H&E X100

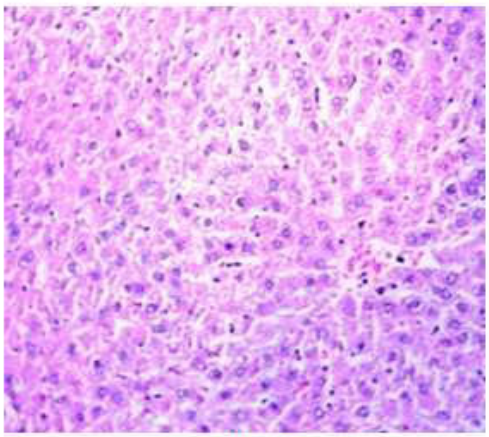

Figure 7
Photomicrograph of liver showing mild dilation of sinusoids (Group 4, day 14). H&E X100

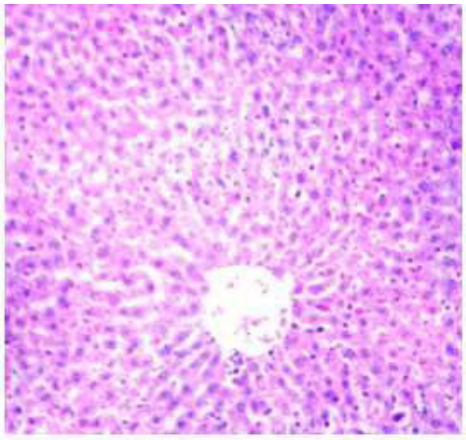

Figure 8
Photomicrograph of liver showing very mild central vein congestion (Group 4, day28).H&E X100

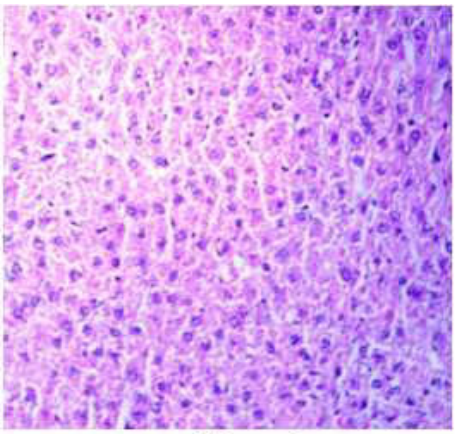

Figure 9
Photomicrograph of liver showing normal architecture (Group 3, day 28) H&E X100

DISCUSSIONS

In rats of group 2, GSH, SOD, catalase, GST, GPX, G6PD, Na^+/K^+ ATPase and Mg^{2+}ATPase were significantly decreased while CYP_{450}, TBARS, protein carbonyls were significantly increased. This can be attributed to imbalance between oxidant and antioxidant system mediated by cisplatin. Further there was increase in serum AST and GGT which were markers of hepatotoxicity. These findings were in accordance with[21]. Active metabolites of cisplatin react with thiols in glutathione and small proteins metallothionein and caused cell death. These findings were on par with [22].Cisplatin also affects enzymes of carbohydrate metabolism thus G6PD was found to be decreased and protein carbonyls were increased due to enhancement of lipid peroxidation[5]. CYP_{450} in cisplatin treated rats poorly coupled with NADPH- CYP_{450} and enhances hepatotoxicity either by free radical production or through Fas agonist which caused elevation of CYP_{450}. This study was in correlation with [23]. Cisplatin interferes with mitochondrial function thus Na^+/K^+ ATPase and Mg^{2+}ATPase activities were reduced. These findings were on par with [4].

Histopathological sections revealed sinusoidal dilation, congestion and other degenerative changes which might be due to r elease of toxic metabolites of cisplatin.
These findings were on par with [24]. Significant improvement in group 4 in all parameters was observed compared to group 2 as turmeric had antioxidant and anticarcinogenic properties [6].

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