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Effect of supplementation of black tea extract on indomethacin induced alteration of pathophysiology of rat liver

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ABSTRACT

Cyclooxygenase-2 (COX-2) expression and prostaglandin production are suggested to play important, complex roles in the pathogenesis of various liver diseases. Indomethacin generates free radicals and induce oxidative and nitrosative stress with depletion of antioxidants. In this study, we have evaluated the beneficial effects of black tea extract against indomethacin induced liver toxicity. We studied the effect of supplementation of Black tea extract (BTE, 2.5 g tea leaf/dL of water, i.e., 2.5% of aqueous BTE, orally) on indomethacin (5mg/kg body weight, i.p.) induced liver oxidative and nitrosative stress in Wister strain male albino rats. Hematological parameters, serum nitric oxide, L-ascorbic acid and serum malondialdehyde concentrations were evaluated. We also evaluated total protein, SGOT, SGPT and histopathology of liver. Indomethacin treated rats showed significantly decreased body weight, hepatosomatic index, hematological parameters, L-ascorbic acid concentrations, nitric oxide, total protein levels and increase in malondialdehyde SGOT and SGPT parameters as compared to their controls. However, simultaneous treatment with indomethacin and black tea extract produced a remarkable improvement of all the above parameters when compared with treatment with indomethacin drug alone. However, simultaneous treatment with indomethacin and black tea extract significantly improve the serum nitric oxide, L-ascorbic acid and malondialdehyde concentrations, as compared to indomethacin treatment alone. Histopathology of the liver revealed swollen hepatocyte, foci of fatty changes and ballooning degeneration in indomethacin treated rats which almost reverse back in BTE supplemented indomethacin group. Results indicate that BTE is beneficial in preventing NSAID induced hepatocellular damages.

Keywords: Indomethacin, free radicals, black tea extract, antioxidants, liver histopathology.

INTRODUCTION

Injury to hepatocytes by drug or toxicant stimulates the release of a cascade of factors by the cells in the liver. Immediately following injury, formation of arachidonic acid (AA) metabolites in the liver contributes to the initiation of acute inflammation [1]. Prostaglandins are generated from arachidonate by the action of cyclooxygenase isoenzymes, and their biosynthesis is blocked by nonsteroidal anti-inflammatory drugs, including those selective for inhibition of cyclooxygenase-2 (COX 2). Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) that was developed specifically to abate the inflammatory responses to the indolic hormones, tryptophan and serotonin [2]. Inhibition of COX with indomethacin blocks the downstream production of prostanoid vasodilatory metabolites. In the liver, endothelial and Kupffer cells are considered the major cellular sources of prostaglandins, while

hepatocytes rapidly metabolize COX products [3]. Indomethacin leads to mitochondrial oxidative stress associated with the generation of intra-mitochondrial reactive oxygen species (ROS), which induces imbalance of oxidants and antioxidants status in living system [4]. Reactive oxygen species (ROS) and lipid peroxidation (LP) products impaired the respiratory chain in hepatocytes either directly or indirectly through oxidative damage to the mitochondrial genome [5]. The ROS generated could nonselectively damage DNA, possibly resulting in genetic changes in active genes [6]. These features, in turn, lead to the generation of more ROS, and a vicious cycle ensues. Reduction in the production of nitric oxide is the first step for the occurrence of many diseases [7]. Indomethacin stimulates significantly less amount of nitrite and nitrate levels in cirrhotic liver and characterized by endothelial dysfunction, that results in impaired release of endothelial derived relaxing factors (EDRF) including NO [8]. Black tea extract is one of the most effective drink in terms of antioxidant properties and can serve as natural sources to the free radical scavengers and antioxidant agents [9]. Black tea leaves can be considered as promising sources of natural antioxidants and as possible preventative agents of some common human health disorders.

In view of free radical scavenging properties of black tea extracts the present study was aimed to assess the possible amelioratic effects of black tea extracts on indomethacin induced hepatotoxicities in male albino rats.

EXPERIMENTAL SECTION

Experimental design

Laboratory-bred adult male albino Wister rats fed with laboratory stock diet (Hindustan lever, Mumbai, India) and water *ad libitum*, and weighing 150 – 170 gm were used. They acclimatized a week to the laboratory conditions at 22 – 24 ° C and a 12 h light: dark (circadian) cycle. The acclimatized animals divided into four groups of six animals each and three animals were kept in each metabolic wire cage (60 cm × 30 cm × 20 cm). Group I rats were healthy controls. Group II rats were treated i.p, with Indomethacin NSAID drug (Yarrow chemical limited, Mumbai, India) at a dose of 5 mg/kg of body weight for 21 days [10]. Group III (BTE dose of 2.5 g tea leaf/dl of water that is 2.5% of aqueous BTE also for 21 days orally) and Group IV rats were given both indomethacin and black tea extract for the same period. All the animals were sacrificed at the end of the last dose after an overnight fast. All the experimental procedures followed were performed in accordance with the approval of the Institutional Animal Ethics Committee (1169/ ac/08/CPCSEA) under strict compliance of Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines for the experimental studies.

Preparation of 2.5% aqueous BTE

The black tea (*Camellia sinensis*) extract was prepared from cut, tear and crush broken orange pickoe grade black clonal tea. It was processed and supplied by Tocklai Experimental Station, Jorthat, Assam. A fresh 2.5% aqueous BTE was prepared every day following the method of Wei et al [11]. Twenty-five gram of black tea was added to 500 mL of boiling water and was steeped for 15 min. The infusion will be cooled to room temperature and then filtered. The tea leaves were extracted a second time with 500 mL of boiling water and filtered, and the two filtrates were combined to obtain a 2.5% aqueous BTE (2.5 g of tea leaf/100 mL water).

Gravimetry

The body weights of all animals were recorded on the 1st day of the indomethacin treatment and the day of sacrifice. The liver weight was determined after dissecting out, and washing in ice cold saline in a single-pan balance (ATCO. M. No. D2RS02-W). Hepatosomatic index was the ratio of liver weight to body weight of rats before sacrifice.

Animal sacrifice and blood collection

At the end of the last dose, the animals were sacrificed after overnight fasting by cervical dislocation between 09.00 AM and 11.30 AM to avert the circadian influences on the animals [12]. Blood was collected in centrifuge tubes,(plane and EDTA) kept at room temperature for about 2 h and centrifuged at 3000 rpm for 15 min to collect serum. EDTA tubes were used for hematology investigations.

Determination of hematological parameters:

About 2 ml of blood were collected in commercial tubes containing about 40 µl of potassium salt of EDTA as anticoagulant and analyzed within 24 h by fully automated hematological cell counter (Sysmax K-4500 of Transasia Ltd.)[13]. The parameters measured were hemoglobin (Hb) concentration, white blood cell (WBC) count, red blood cell (RBC) count, packed cell volume (PCV%), mean cell volume (MCV), platelet count. The values of the mean corpuscular hemoglobin (MCH) and MCHC were calculated.

Biochemical determination of markers:
Serum should be free of hemolysis and separated from blood cells as soon as possible after collection. 2-3 ml of blood samples were centrifuged at 3000 rpm for 15 min and later all the parameters were analyzed. Estimation of serum nitric oxide has been measured by Moshage et al [14]. L-ascorbic acid level measured by the method of Roe and Koether [15]. Lipid peroxidation was estimated by measuring thiobarbituric acid (TBA) and was expressed in terms of malondialdehyde (MDA) content according to the method of Kie Satoh [16]. Estimation of serum total protein by Biuret method with end point colorimetry according to manufacturer's protocol using semi automated analyzer (Chem Chek, AGAPPE) [17]. The serum SGOT and SGPT activity has been measured by UV (IFCC), kinetic assay using commercial kit (Cliniquant-FSR IFCC method Meril diagnostics) according to the manufacturer's protocol [18].

Histopathological studies
After sacrifice, liver of the experimental rats were dissected out and adhering blood and tissue fluid were removed by blotting and then stored in 10% formalin fixative solution and processed. Fixed tissues were cut and stained with hematoxylin and eosin accordingly [19]. The sections were examined under a light microscope, and photomicrographs were taken by using USB MIPS (NO. 70.0343) with a connected personal computer.

Statistical analysis
Data were expressed as mean ± standard deviation of the mean. Statistical comparisons were performed by one-way ANOVA, followed by *post-hoc t*-test, and the values were considered as statistically significant when $P < 0.05$**.**

RESULTS

Gravimetry
No death was observed in any of the experimental groups. All the rats in groups I, III, and IV remained active and healthy with normal feeding behavior. Their mean body weight at the end of experiment is shown in table 1. However, rats treated with indomethacin (group II) were found to be lethargic and their % body weight gain was found to be the lowest among all groups. Table 1 shows that group II rats were having only 17.5% of percentage body weight gain from its initial body weight as compared to group I (43.72%). However, in case of group IV (indomethacin + black tea extracts) an increase of 33.43% has been observed. Table 1 also shows that indomethacin treated (group II) rats had a significant decrease in hepatosomatic index as compared to healthy controls but simultaneous treatment with black tea extracts (group IV) showed a remarkable improvement.

Table 1: Gravimetric Parameters in Male Albino Rats after Black Tea Extract (BTE) Indomethacin (NSAID) Treatment

Parameters (Units)	Group I	Group II	Group III	Group IV
Initial body weight(gm)	164.00±2.52^a	165.00±5.51^a	162.00±1.41^a	164.00±1.67^a
Final body weight(gm)	235.6±3.88^a	193.8±4.40^b	239.3±4.32^a	218.8±2.40^c
% weight gain(gms)	43.72±3.19^a	17.51±1.89^b	47.73±2.27^a	33.43±1.65^c
Hepato-somatic index (gm)	0.0255±0.0016^a	0.0239±0.0023^b	0.0256±0.0016^a	0.0242±0.0013^c

Group I: Control, group II: Indomethacin treated, group III: BTE treated, group IV: BTE +Indomethacin treated. Each value is mean±SEM of six observations in each group. In each row, values with different superscripts (a, b, c, d) were significantly different from each other (P<0.05). Post-hoc t-test analysis was used to test for differences among the means when ANOVA indicated a significant P<0.05.

Changes in hematological and biochemical parameters:
Table 2 shows that indomethacin administration resulted in significant decrease of RBC count, hematocrit value (PCV %) and Hb concentration in indomethacin treated rats (group II) when compared to untreated control (group I). Although indomethacin plus Black tea extract treated group IV rats also showed a significant decrease of RBC count, PCV % and Hb concentration in comparison with their control but when compared with indomethacin alone treated rats (group II), a significant increase of all the above mentioned parameters were noticed. No significant alterations of any of those parameters were found in case of only black tea extract treated rats when compared with untreated control rats. From table 3, it may be observed that indomethacin significantly decreases serum total protein, vitamin C and nitric oxide levels whereas an increase in the MDA, SGOT and SGPT levels were noticed in group II rats. However; supplementation of BTE on indomethacin treated rats (group IV) showed an improvement of the nitric oxide, vitamin C, serum protein, SGOT and SGPT levels as compared to group II rats.

Table 2: Changes in Hematological Parameters of Male Rats after Indomethacin Treatment Alone or In Supplementation with Black Tea Extract

Parameters (Units)	Group I	Group II	Group III	Group IV	F Ratio	P value
Hb(gm %)	13.70±0.3[a]	9.21±0.7[b]	14.53±0.5[a]	12.96±0.6[a]	93.982	0.0000
TLC (10^3cell/µL)	10.8±3.3[a]	5.6±6.2[b]	11.38±4.8[c]	9.3±5.5[d]	167.438	0.0000
DC (Nutrophil) (%)	16.0±0.1[a]	22.50±3.2[b]	16±1.7[a]	16.33±2.5[a]	10.330	0.0000
Lymphocyte (%)	78.6±2.5[a]	70.0±2.7[b]	78.5±1.6[a]	76.50±2.7[a]	16.213	0.0000
Esinophil (%)	2.66±0.8[a]	5.0±0.8[b]	3.3±0.5[a]	4.1±1.1[a]	7.939	0.0001
Monocyte (%)	2.6±1.0[a]	2.5±1.3[a]	2.1±1.3[a]	3.0 ±1.4[a]	0.429	0.734
RBC (10^6cell/µL)	7.33±0.3[a]	4.35± 0.6[b]	7.62±0.5[a]	4.96±0.5[c]	55.833	0.0000
MCV (fl)	58.66± 4.7[a]	79.33±4.6[b]	62.83±1.8[a]	65.50±2.3[c]	36.574	0.0000
MCH (pg)	17.66±1.5[a]	29.5±4.9[b]	19.33±2.4[a]	25±3.2[c]	16.148	0.0000
MCHC (g/dL)	36.16±2.5[a]	32.66±2.2[a]	36.83±2.5[a]	33.16±2.4[a]	4.318	0.017
Platelet count (10^3cell/µL)	6.315 ±0.9[a]	9.456±0.7[b]	6.261±0.4[a]	7.811±0.5[c]	28.789	0.0000
PCV (%)	49.46± 3.0[a]	41.56±4.7[b]	49.83±5.5[a]	46.50±1.8[c]	5.249	0.0000

Group I: Control, group II: Indomethacin treated, group III: BTE treated, group IV: BTE +Indomethacin treated. Each value is mean ±SEM of six observations in each group. In each row, values with different superscripts (a, b, c, d) were significantly different from each other (P<0.05). Post-hoc t-test analysis was used to test for differences among the means when ANOVA indicated a significant P<0.05.

Table 3: Effect of BTE on Biochemical Parameters in Male Albino Rats after Indomethacin (NSAID) Treatment

Biochemical Parameters	Group I	Group II	Group III	Group IV	F ratio	P Value
Total proteins(gm/dl)	7.2±0.3[a]	4.0±0.2[b]	8.0±0.1[a]	5.0±0.1[c]	328.026	0.0000
SGOT (U/L)	53.83±4.9[a]	288.5±43.33[b]	51.66±2.9[a]	190.3±6.3[c]	162.579	0.0000
SGPT (U/L)	40.83±2.2[a]	90±2.828[b]	40.66±2.0[a]	70.66±2.1[c]	640.638	0.0000
MDA (µmol/L)	1.806±0.4[a]	7.946±0.8[b]	2.053±0.2[a]	3.955±0.4[c]	174.265	0.0000
Vitamin C (µg/ml)	76.70±1.6[a]	49.63±2.6[b]	77.34±1.3[a]	70.18±1.4[c]	245.605	0.0000
Nitric Oxide(µmol/L)	44.68 ±1.6[a]	23.26±1.0[b]	45.16±0.9[a]	35.26±1.3[c]	385.209	0.0000

Group I: Control, group II: Indomethacin treated, group III: BTE treated, group IV: BTE + Indomethacin treated. Each value is mean±SEM of six observations in each group. In each row, values with different superscripts (a, b, c, d) were significantly different from each other (P<0.05). Post-hoc t-test analysis was used to test for differences among the means when ANOVA indicated a significant P<0.05.

Histopathological studies

The histological structure of normal untreated rat liver (group I) showed normal architecture (fig 1a and 1b). In group II (Indomethacin) showed majority of the distorted 'lobular' architecture of liver parenchyma, little swollen hepatocytes, vacuolated microvesicular and eosinophilic cytoplasm beside increase in number of mitotic figures. Foci of fatty change and ballooning degeneration with necrosis of hepatocytes were found in zone 3 (centrilobular) areas (fig.2 a & b). Moderate proliferation of portal area along with fibrous tissue and infiltration of mixed acute and chronic inflammatory cells were also noticed (fig.2 a & b). Group II also shows variable widening of sinusoidal spaces and Kupffer cell hyperplasia along with abnormal dilatation and congestion of central vein (fig.2a & b). In group III (BTE) showed normal hepatic parenchymal tissue which is composed of numerous hexagonal to pyramidal 'lobules' (fig.3a & b) In group IV (Indomethacin + BTE) showed near normal architecture of liver parenchymal tissues. Hepatocytes also showed mild variation in cellular size and shape along with mild dilatation and congestion of central vein (fig 4a and b).

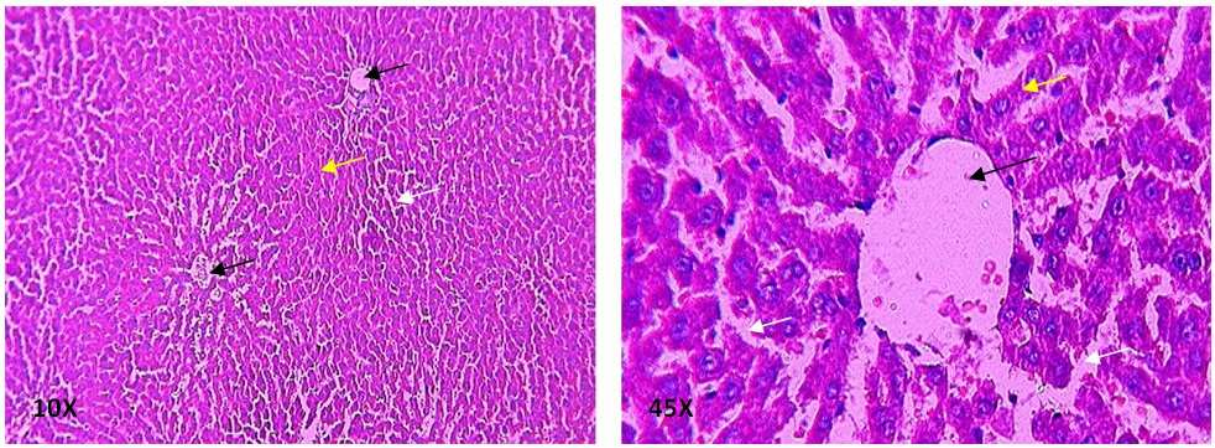

Fig.1: Liver section of Control rats (Group I)

Normal hepatic parenchymal tissue showing numerous hexagonal to pyramidal 'lobules' (yellow arrow) and central vein (black arrow) appeared normal and sinusoids present between lobules (white arrows).

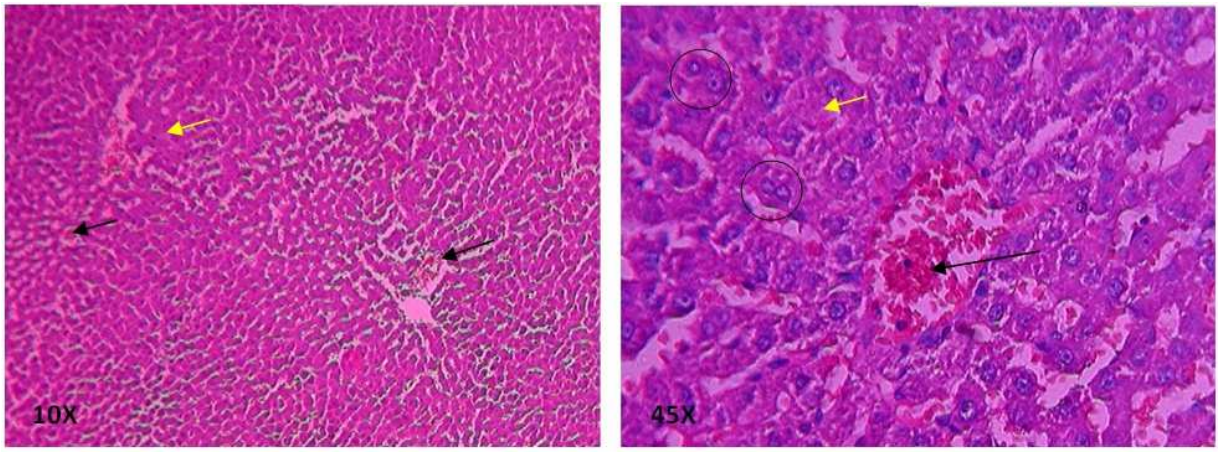

Fig. 2: Liver section of Indomethacin (group II)

Majority of the distorted 'lobular' architecture of liver parenchyma (yellow arrows), Increase in number of mitotic figures (circles). Moderate proliferation of portal area with fibrous tissue with infiltration of mixed acute and chronic inflammatory cells (black arrow).

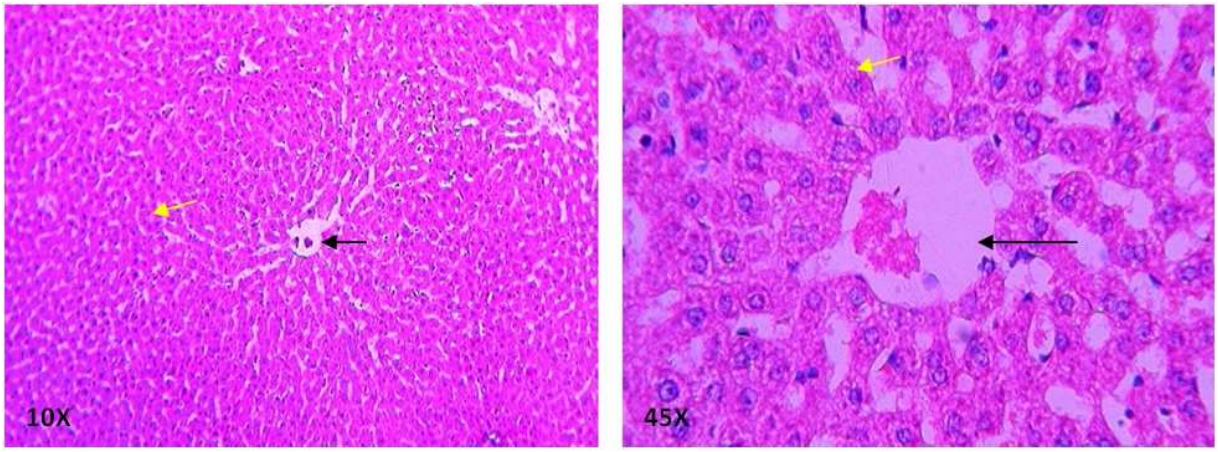

Fig.3: Liver section of black tea extracts (group III)

Normal hepatic parenchymal tissue which is composed of numerous hexagonal to pyramidal 'lobules' (yellow arrow) and central vein (black arrow) appeared normal.

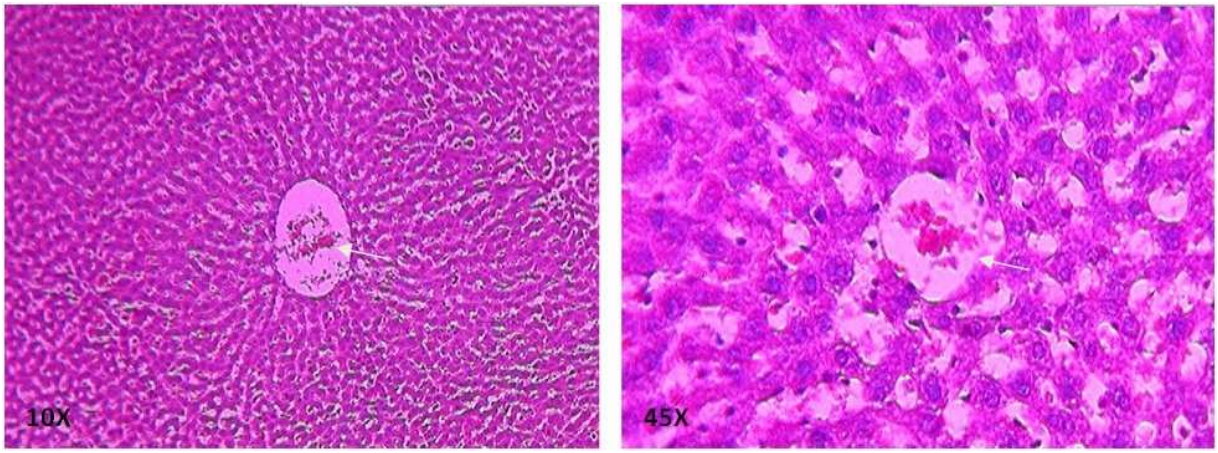

Fig. 4: Liver section of Indomethacin and black tea extract (group IV)

Normal architecture of liver parenchymal hepatocytes showed mild variation in cellular size and shape and mild dilatation and congestion of central vein (white arrow).

DISCUSSION

Gravimetry

Rats exposed to indomethacin had a significant decrease of hepatosomatic index as compared to controls. This clearly reflects the adverse effect of indomethacin on body weight gain and hepatocyte development. Simultaneous supplementation of black tea extract improved the growth and developmental status of the liver in indomethacin treated rats. The reduction of liver weight after indomethacin treatment may be due degenerative effect on hepatic tissue, as it is a major site of metabolism.

Hematological parameters

Changes in hematological picture conclude to anemia and it may be due to loss of blood during gastrointestinal bleeding and release of immature RBCs in circulation. However in our study the decrease in hemoglobin concentration, RBCs count, WBCs count and PCV% may be due to non-regenerative anemia arising from indomethacin induced direct injury of hematopoietic stem cells resulting in decreased erythrocyte, leukocyte and

platelet count [20]. Simultaneous treatment of black tea extract decreased the toxic effects of indomethacin on hematological values and also showed a protective role in anemia and leucopenia.

Biochemical parameters

Increased activity of both SGOT and SGPT after indomethacin treatment may be due to leakage of enzymes from liver cytosol into the blood stream giving an indication on the hepatotoxic effect of indomethacin [21]. Following cell damage, the membranes become permeable and enzyme activity is found in the extra cellular fluid and serum, so the highest activity of SGOT was recorded in the serum. The improvement of SGOT activity towards control value in the rats simultaneously treated with black tea extract proved the hepatoprotective effect of black tea extract due to its antioxidant properties. Decreased protein concentration induces reduction of body weight, organ weight, reflecting biochemical defects, structural disorders, and altered physiologic functions [22].

Reactive nitrogen species and reactive oxygen species (ROS) play roles of both deleterious and beneficial species. Excessive production of ROS results in oxidative stress, a deleterious process which can be an important mediator of damage to macromolecules and cell structures, including membrane lipids and proteins, mitochondria, and DNA [23]. Increased in the generation of ROS and enhanced lipid peroxidation are considered responsible for the toxicity of a wide range of compounds [24]. MDA is the major product of peroxidized poly unsaturated fatty acids and increased erythrocyte MDA concentrations of rats definitely accompanied by increased ROS formation and enhanced lipid peroxidation DNA damage altered calcium and sulfhydryl homeostasis as well as marked disturbances of anti oxidant defense system [16]. In our study indomethacin induced significant increase in melondialdehyde concentrations observed in the serum (Table 3). The generation of reactive oxygen species (ROS) and the release of proteins from the mitochondria lead to the activation of different pathways of cell death [25]. Increased ROS generated by mitochondria can cause oxidative damage of cellular macromolecules, including nucleic acids, lipids, and proteins along with depletion of cellular antioxidants, leading to cellular injury [26]. Ascorbate is a well-known antioxidant required by all mammalian cells for proper functioning to control various biochemical reactions. The decreased intracellular ascorbate has profound effect on cellular and tissue metabolic pathways [27]. In our study l-ascorbic acid level was significantly decreased in serum was may be due to imbalance in the anti oxidant and prooxidant balance in blood (Table3). Nitric oxide is the fundamental signaling device. When it becomes toxic, it reacts with superoxide and is converted to peroxynitrite, nitrosative stress may lead to nitrosylation reactions, which can alter the structure of proteins and so inhibit their normal function. Peroxynitrite initiates lipid peroxidation *via* a reaction of lipids with its decomposition products, the hydroxyl radical and nitrogen dioxide [28]. Indomethacin stimulates significantly less amount of nitrite and nitrate levels in cirrhotic liver and characterized by endothelial dysfunction that results in impaired release of endothelial relaxing factors including NO [8], this may be due to altered gene expression [22]. In our study indomethacin induced significant decrease in nitric oxide concentrations observed in the serum (Table 3). The aqueous extract of black tea has shown to quench reactive oxygen species (ROS) such as singlet oxygen, superoxide and hydroxyl radicals, prevent the oxidative cross-linking of test proteins and inhibit single-strand breakage of DNA in whole cells.

Histopathological studies

We observed in histological studies that indomethacin can cross the liver sinusoidal barrier, and produce degeneration of the liver revealed swollen hepatocyte, foci of fatty changes, ballooning degeneration and necrosis of hepatocytes. Moderately enlarged portal area and proliferation fibrous tissue with infiltration of mixed acute and chronic inflammatory cells. The sinusoidal spaces were variably widened with increase in number of Kupffer cells, abnormal dilation and congestion of portal vein.

It may be concluded from present findings that indomethacin induces oxidative damage in erythrocytes and liver. This results in disruption of overall hematology and liver metabolic function and also disrupts serum antioxidant defense system. But simultaneous treatment with black tea extract may protect against toxic influence on above stated hematological, biochemical and anti oxidant parameters.

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