ORIGINAL ARTICLE

HEPATOPROTECTIVE EFFECT OF SEA BUCKTHORN BERRY SEED OIL IN CYCLOPHOSPHAMIDE-INDUCED HEPATIC TOXICITY IN BALB/c MICE

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Background: Chemotherapy-induced hepatotoxicity can be mitigated by use of antioxidants, which may help the liver to recover its endogenous antioxidant mechanism. The objective of this study was to determine the effectiveness of sea buckthorn berry seed oil (SBO) in attenuating cyclophosphamide-induced changes in liver enzymes and liver histology in BALB/c mice. **Methods:** This experimental study was conducted in Physiology Department of Foundation University School of Health Sciences (FUSH), in collaboration with Pathology Department, FUSH and National Institute of Health, Islamabad from Jan 2018 to Jun 2019. Thirty healthy male BALB/c mice were divided into 3 groups of 10 each. Group-1 served as control. Group-2 received cyclophosphamide (25 mg/Kg body weight) intraperitoneally for 10 days. Group-3 was co-administered cyclophosphamide (same dose) with of sea buckthorn berry seed oil (40 mg/Kg body weight) orally for ten days. All animals were sacrificed on 11th day. Serum levels of liver enzymes as liver injury biomarkers were assayed. Liver histopathology was carried out for evidence of hepatic injury and recovery. **Results:** Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) increased significantly in Group-2 (p<0.05). In Group-3, the rise in hepatic injury serum markers was significantly less than Group-2 (p<0.05). Upon histological examination, Group-2 was grade 5 according to Kleiner's scoring system for steatohepatitis, and had grade 2 sinusoidal dilatation on Rubbia-Brandt grading system. These changes were significantly less in Group-3 (p<0.05). **Conclusion:** Co-administration of SBO mitigated the CP-induced rise in hepatic injury biomarkers and sinusoidal injury.

Keywords: Cyclophosphamide, sea buckthorn berry seed oil, chemotherapy-induced hepatotoxicity, cellular antioxidants, oxidative stress

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INTRODUCTION

Traditional cytotoxic agents remain the mainstay of cancer treatment, despite recent progress in cancer therapeutics with the advent of targeted therapies and immunotherapies.[1] Cyclophosphamide (CP) is a synthetic alkylating agent commonly used as a chemotherapeutic and immunosuppressive drug.[2] It is an inactive cytostatic, which undergoes metabolic activation catalysed by the hepatic cytochrome P450 (CYP450) monoxygenase systems into active metabolites, phospharamide mustard and acrolein. Phospharamide mustard is responsible for anti-tumour effects while acrolein contributes to cytotoxicity and carcinogenicity of CP.[3]

During metabolic activation, reactive oxygen species (ROS) are also formed. Side effects of CP, mediated by generation of ROS, direct depletion of cellular antioxidants and the resultant oxidative stress, form the major limitation of using CP.[4] These side effects include pulmonary fibrosis, haemorrhagic cystitis, gastrointestinal bleeding, and irreversible azospermia in men, etc. Microvascular fatty changes in liver also form a part of toxicity spectrum.[5] CP is used in most myeloablative regimen, and when combined with total body irradiation, has been reported to result in veno-occlusive disease (VOD) in 38% of the patients. The hepatotoxicity appeared to be greatly potentiated by radiation.[6]

Chemotherapy-induced hepatotoxicity can be mitigated by use of antioxidants, which may help the liver to recover its endogenous antioxidant mechanism (reduced glutathione- GSH).[7] Consequently, combining a treatment regimen with effective and safe antioxidants could be the appropriate approach to allay cancer therapy-induced toxicity.[8]

Sea buckthorn is a natural source of antioxidants with documented free radical scavenging abilities.[9] Sea buckthorn seed and pulp oil are rich source of mono- and polyunsaturated fatty acids, carotenoids, phytosterols, vitamins E, K and 28 trace elements like zinc, iron, sulphur, calcium, magnesium, selenium, copper etc.[10] Selenium present in sea buckthorn extract may help in biosynthesis of glutathione peroxidase, which is crucial for the degradation of lipid hydroperoxides.[8–11]

Previous research has documented significant antioxidant effect of sea buckthorn extract against free radical production, oxidative damage, and lipid peroxidation resulting from acetaminophen, chromium, sodium nitroprusside, nicotine, CCl_4, hypoxia and radiation.[12–14] Few studies have been published till now to assess the antioxidant effect of sea buckthorn berry

seed oil (SBO) on CP-induced hepatic damage in mice. This study was designed to evaluate the influence of sea buckthorn berry oil on cyclophosphamide-induced rise in liver injury serum markers and cytoarchitecture damage in BALB/c mice.

MATERIAL AND METHODS

Healthy male BALB/c mice from inbred colony maintained at National Institute of Health (NIH) Islamabad were used for the study. Sample size was calculated using Resource Equation method[15]. Thirty adult mice with average weight of 30±5 g were maintained in well ventilated polypropylene cages (with 5 animals each) containing wood shavings. Animals were kept at 25±2 °C with relative humidity of 50–60% and on 12-hour light/12-hour dark cycle. Rodent feed was given to animals. The pellet diet consisted of 65% carbohydrate, 25% protein and 10% fat. Feed and tap water were given *ad libitum*. The study and all procedures were approved by the Institutional Ethical Review Committee (Ref: 217/FF/FUMC/ERC). The animals were cared for in accordance with Guide for the Care and Use of Laboratory Animals (8th ed., 2010).[16]

Injection cyclophosphamide 1 gm (Cyclomide 1,000 mg) was procured from Pharmedic Laboratories, Pakistan. Commercially available preparation of sea buckthorn seed oil was procured from SIBU Sea Berry Therapy, Utah, USA. Phosphate-buffered saline (PBS) (0.1M, *p*H 7.4) that contained KCl (1.17% w/v) was freshly made in Physiology Lab at FUSH. All other chemicals used for experiment were of analytical grade.

Animals were divided into 3 groups of 10 mice each. Group-1 was negative control; group-2 was positive control and group-3 was experimental group. Group-1 mice were given normal saline (0.65%) 1 ml/Kg body weight (i.p.) daily for 10 days. Group 2 mice were given CP 25 mg/Kg body weight (i.p.) for 10 consecutive days to induce the liver damage.[8] Group 3 animals were given CP 25 mg/Kg body weight (i.p.) along with SBO 40 mg/Kg body weight (orally) for 10 consecutive days.[17]

On 11th day, after ensuring proper anaesthesia, intra-cardiac blood sampling was done for assessments of liver injury serum markers. Samples were centrifuged at room temperature at 4,000 rpm for 15 minutes. Serum was used to assess alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin by commercial kits on auto-analyser (Selectra E Fully Automatic Chemistry Analyser).[7] A one-centimetre sample of each liver was preserved in a 10% formalin solution for 24 hours before slides were prepared for histological examination. Five µm thickness sections were cut and fixed on slides, stained with haematoxylin and eosin and observed under compound light microscope for histopathological analysis and comparison among the three groups.[18] The grading for hepatic injury, as depicted by hepatocellular and sinusoidal damage, was done by using NAFLD Activity Score (NAS) proposed by Kleiner *et al*[19], for hepatocellular damage, and grading system of sinusoidal dilation given by Rubbia-Brandt *et al*[20], for sinusoidal damage, as adapted from Vauthey *et al*[21].

The statistical analysis was done using SPSS-24. Values were expressed as Mean±SD. The statistical significance of the differences of various quantitative changes between the experimental and control groups were evaluated using one-way ANOVA followed by Tukey's HSD (Honestly Significant Difference) post hoc test for multiple comparisons. The difference was regarded statistically significant at $p \leq 0.05$.

RESULTS

The liver enzymes, AST, ALT and ALP, levels increased significantly in Group-2. Serum alkaline phosphatase (ALP) was more than 3 times the control average value and ALT/ALP ratio was less than 2. In Group-3, the rise in hepatic injury serum enzyme markers was significantly less than Group-2. A significant increase in the serum bilirubin was seen in Group-2 compared to Group-1 after CP administration (89.75%, $p<0.001$). In Group-3, the increase in the serum bilirubin was significantly less as compared to Group-2 (18.6%, $p<0.001$). The bilirubin levels of Group-1 and Group-3 were not significantly different ($p=0.242$). (Table-1).

Microscopic examination of the sections of Group-1 showed normal histological structure like hepatic lobes containing cords of hepatocytes with sinusoids between these cords. The central vein was normal, and the portal triads also appeared normal. In Group-2, there was diffuse fatty infiltration, central vein congestion, loss of hepatocyte architecture, damage to endothelium of sinusoids with sinusoidal congestion and dilatation. Upon grading of steatohepatitis by the NAFLD Activity Score (NAS)[19], Group-2 had a score of 5 and was graded as steatohepatitis. Sinusoidal injury was graded as moderate based on grading system of sinusoidal dilation given by Rubbia-Brandt *et al*[20]. Based on these observations, Group-2 was classified as having hepatic injury, defined as steatohepatitis Kleiner score ≥4, and/or grade 2 to 3 sinusoidal dilation, as adapted from Vauthey *et al*[21].

All these changes were less in Group-3 compared to Group-2, with NAFLD Activity Score (NAS) of 3 and was graded as borderline steatohepatitis. Sinusoidal injury was scored as grade 1 (mild)[20]. With steatohepatitis NAS equal to 3 and grade 1 sinusoidal dilation, Group-3 did not qualify as having hepatic injury. The photomicrographs of histological changes in Group-2 and Group-3 are given in Figure-1 and Figure-2 respectively. The parameters used for grading are given in Table-2 and Table-3.

Table-1: The effects of CP, and CP+SBO on serum levels of total bilirubin, ALT, AST, and ALP

Groups	Total bilirubin (mg/dL)	ALT (SGPT) (u/L)	AST (SGOT) (u/L)	Alkaline phosphatase (u/L)
Group-1 (Control)	0.39±0.09	56.5±11.37	140.2±5.02	257.5±2.36
Group-2 (CP)	1.02± 0.14[a]	115.8±7.84[a]	221.9±19.45[a]	788.1±5.66[a]
Group-3 (CP+SBO)	0.47±0.06[b]	74.9±7.73[b]	177.4±5.79[b]	380.7±4.24[b]

a=p<0.05 Group-1 *vs* Group-2, b=p<0.05 Group-2 *vs* Group-3

Table-2: Grading of steatohepatitis by NAFLD Activity Score (NAS)

Parameters	Group-1	Group-2	Group-3
Steatosis (Kleiner score)	0 (≤5%)	1 (5%–35%)	0 (≤5%)
Lobular inflammation (foci/×200 field)	0 (no foci)	2 (2–4 foci)	1 (≤2 foci)
Ballooning	0 (none)	2 (many cells)	1 (few cells)
NAFLD Activity Score	0	5	3
Grading	none	Steatohepatitis	Borderline steatohepatitis

NAFLD: nonalcoholic fatty liver disease

Table-3: Grading of sinusoidal injury

Parameter	Group-1	Group-2	Group-3
Sinusoidal dilatation	0 (absent)	2 (moderate-centrilobular involvement extending in two-thirds of the lobular surface)	1 (mild-centrilobular involvement limited to one-third of the lobular surface)

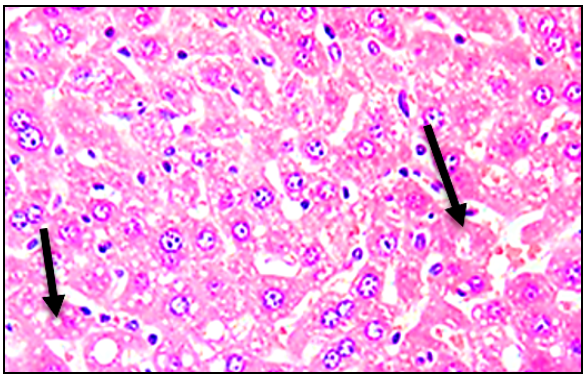

Figure-1: Photomicrograph of histological changes in hepatic tissue in Group-2

Sinusoidal dilation with congestion and fatty changes with disruption of hepatic architecture in liver tissue (×400)

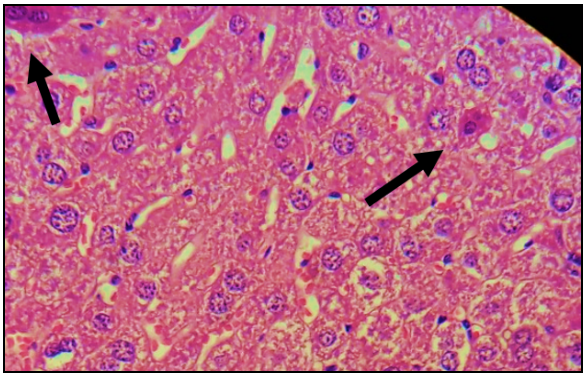

Figure-2: Photomicrograph of histological changes in hepatic tissue in Group-3

Multinucleated giant cells as a sign of regenerative changes in liver tissue (×400)

DISCUSSION

Recently there has been a revival of interest in chemoprotective and antioxidant activities of plant extracts used in traditional medicine.[22] A study was conducted by Nafees *et al*[23] to demonstrate the protective effects of rutin, a naturally occurring bioflavonoid derived from buck wheat, against the hepatotoxicity induced by CP. They concluded that the hepatoprotective effects of rutin were associated with up-regulation of antioxidant enzyme activities that counter the increased amounts of oxidants (ROS) generated by CP and resulted in down regulation of serum toxicity markers.

In the present study, sea buckthorn seed oil (SBO) was evaluated for the antioxidant activity against the CP-induced oxidative stress, specifically hepatotoxicity, in male BALB/c mice. CP administration causes oxidative injury to the liver tissue and leads to the leakage of the marker enzymes such as AST, ALT, ALP and bilirubin in serum because of its metabolite acrolein, which damages cell membrane and inhibits transport proteins. The rise in ALP was around 3-times more than the upper limit of normal/control value and ALT/ALP ratio was <2, which is suggestive of cholestatic damage.[24] Co-administration of SBO with CP significantly reduced/haulted the rise in serum AST, ALT, ALP, and bilirubin levels found in CP group, indicating a protective effect.

Our findings are supported by previously published work. Gupta *et al*[25] reported that sea buckthorn (SBT) extract (500 mg/Kg for 10 days) was hepatoprotective in arsenic toxicity (induced with 25 ppm in drinking water for 3 months) with significant reversal of AST, ALT and ALP, although not to the control levels. In their study, as well as ours, the reversal of hepatic injury markers was not complete. As the duration of SBT administration is same, it suggests that full benefits of SBT are not realized in short term use.

A 2011 study[12] investigating the effect of SBT administration on CCl_4 induced oxidative stress, reported CCl_4 induced elevation of serum AST (171%), ALT (215.3%) and bilirubin (232.6%). Pre-treatment by oral administration of SBT extract (25–75 mg/Kg body weight) for 7 days significantly protected from CCl_4 induced elevation in serum ALT, AST and bilirubin.[12] When compared to our study, there was a greater elevation (more than twice) in the serum levels of liver enzymes upon induction of hepatic injury. The reason for this may be the toxin itself. CCl_4 toxicity is a research model used to produce acute hepatocellular injury, while CP in therapeutic doses is reported to cause more sinusoidal and ductal damage and relatively less hepatocellular damage.[12]

Hepatoprotective effect of SBT has also been studied on chromium-induced oxidative stress in Sprague-Dawley rats by Geetha *et al*[26]. They reported

that potassium dichromate treatment for 30 days significantly increased serum AST and ALT levels. Co-administration of SBT leaf extract in 100 and 250 mg/Kg of body weight doses for the same duration preserved the serum ALT and AST levels at control values. They concluded that the leaf extract of SBT protected the rats from the chromium induced oxidative injury.[26] The fact that in their study the liver enzymes remained unchanged in the face of oxidative stress when given SBT supplementation, suggests that a higher dose than that used in the current study, given for a longer duration, may curtail oxidative stress damage.

The mechanism of injury is probably due to damage to sinusoidal endothelium of liver because of toxic metabolites of CP, with generation of ROS and depletion of glutathione from sinusoidal endothelial cells, causing their necrosis, obstruction and obliteration of hepatic veins.[5] Initial injury to the sinusoidal endothelial cells leads to extravasation of RBCs into the sub-endothelial space of Disse, emboli formation and blocking of venous out-flow, resulting in hepatic congestion and sinusoidal dilatation. At later stages, fibrosis occurs which causes destruction of central venules, leading to hepatic sinusoidal obstructive syndrome (SOS) or veno-occlusive disease (VOD).[27]

Intensive chemotherapy, often with cyclophosphamide, is closely associated with endothelial cell injury leading to rapidly progressive, occlusive disease of small hepatic venules.[28] When high doses of CP are used, as chemotherapy in cancer or as myeloablation therapy in combination with total body irradiation, in preparation for bone marrow haemopoietic cell transplantation, acute liver failure and death can occur.[5] The observation of sinusoidal endothelial damage with sinusoidal congestion in Group-2 (grade-2) and concurrent steatohepatitis in our study correlates with the observations made in the clinical setting and earlier animal studies.

Khan *et al*[5] looked at the effect of CP on the micro-anatomy of liver at various doses. They reported that CP-induced histological changes like central vein congestion and fatty infiltration in the liver were dose related and manifested earlier when larger doses were used and later when low doses were given for longer durations. Other studies looking at the effect of antioxidant supplement on toxin-induced steatohepatitis and sinusoidal injury support the lessening of damage seen in our study.

A study by Sheweita *et al*[7] aimed to investigate the role of essential oils extracts (fennel, cumin and clove) as a source of natural antioxidants in the mitigation of CP-induced hepatotoxicity. They treated male mice with CP (2.5 mg/Kg body weight/day) for 28 days, and on histological examination of livers, inflammation in portal tract and hepatocytes, swelling and dilation in sinusoidal space, as well as hyperplasia in Kupffer cells were observed.[7] They found that co-administration of the essential oils with CP lessened the changes caused by CP to a significant extent. They observed that quercetin present in essential oils was found to act as scavenger of ROS/RNS radicals, inhibiting the oxidative DNA damage induced by H_2O_2 and preventing free radical-mediated cytotoxicity. This observation supports the protective effect of SBO seen in our study, as SBO is documented[29] to be rich in flavonols like quercetin and isorahmnetin.

Deleve *et al*[30] investigated the cellular mechanism of action of CP in rodent liver along with the consequences of supplementation with methionine and serine on CP toxicity. They observed that CP caused hepatic VOD, and the damage was inflicted indirectly because CP requires activation by the hepatocytes with generation of acrolein. In co-culture of hepatocytes and endothelial cells, sinusoidal endothelial cells (SEC) were significantly more susceptible to CP toxicity with acrolein-induced depletion of cellular glutathione (GSH) preceding the cell death. They also probed the consequences of supplementation with serine and methionine on CP toxicity in co-culture. Serine is precursor of methionine, which is precursor for cysteine, a thiol-containing, semi-essential proteinogenic amino acid in hepatocyte GSH. They reported that hepatocytes in supplemented medium when exposed to CP maintained GSH levels at ~80% of the control level and both hepatocytes and SEC were protected from CP toxicity. They concluded that the protection afforded by the serine/methionine supplement was due to increase in hepatocyte GSH level.[30] This observation supports our results of lesser sinusoidal damage (grade-1) seen in SBO supplemented group compared to grade-2 in CP group, with high levels of antioxidants (vitamins A, C, E, and K, carotenoids and trace elements copper, zinc and selenium) in SBO probably replenishing the endogenous cellular GSH.

CONCLUSION

Our study demonstrates the protective effect of SBO against CP-induced liver damage, which reflects its antioxidant properties. This evidence can be used to counter the hepatotoxic potential of CP, especially in myeloablative regimes of cancer therapy.

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GNS: Concept and design of work, acquisition, analysis and interpretation of data, drafting and agreement to be accountable for all aspects of work.
SA: Contribution to design of work, revising it critically for intellectual content and final approval of draft.
MS: Contribution acquisition, analysis of data, revising it critically and accountable for all aspects of work.

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