Agricultural Chromium Contaminated Water may Induced Hepatic Toxicity and their Amelioration by Morus nigra Fruit Extract

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Abstract

The uneducated farmer does not know the chemical quality of soil and water which is used for irrigation. Sometimes the soil/water may contain some toxic chemicals, which may cause anomalies in their consumers. The present study reveals the histopathological and micrometric changes of hexavalent chromium Cr(VI) exposure and their ameliorations upon post-treatment of Morus nigra fruit pulp extract (M). Thirty male mice were equally distributed as control (C) without any treatment, chromium (Cr) and chromium morus (CrM) treated group, were given 50ppm Cr for 10 days in drinking water but the CrM group was additionally given 0.2ml M / 12 hourly for next 5days. Results indicate that Cr exposure leads to pathological signs in the hepatic architecture of hepatic cords and necrosis of the hepatocytes leading to fibrosis. The numbers, mean relative area and CSA of hepatocytes decreased while liver fractional weight, CSA of hepatocytes nuclei, central veins, sinusoidal spaces, and numbers of kupffer cells significantly increased in Cr treated group but there were signs of recovery like the hepatoblastic proliferation and rehabilitation of hepatic cords in CrM treated group. The histological, haematological, and statistically analyzed micrometric data show that post-treatment of M convincingly recovers the hepatic pathologies.

Keywords: histopathological, amelioration, hepatocyte, kupffer cells, necrosis

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Introduction:

Endocrine disruption by environmental pollutants has raised serious concern and the peak activities were observed in locations adjacent to industrial and shipping activities. According to the recommendations of the National Research Council, the essential dietary intake of chromium is 50-200µg/day (Bogden and Klevay, 2000), and its deficiency causes diabetes and cardiovascular diseases (Kobla and Volpe, 2000). Cr (VI) is a widely used industrial chemical (1-3) a highly toxic metal and an environmental pollutant (4-6). Readily taken up by the cells (7-11) and induces the generation of reactive species of oxygen and nitrogen (12) causing tissue and DNA damage (13). Acute chromium toxicity affects the vital organs and normal behaviour which may be deleterious for life especially in cancer production (14-16) and adversely affects the male and female reproductive systems and rate of fertility (17).

Such factors lead to impairment of the antioxidant defence systems and increased toxic effects, causing alterations in the cytoplasmic signalling process and producing damage at macromolecular levels (Al-Gubory *et al.,* 2010), like alteration in body weight (Lukacinova *et al.,* 2011), variation in blood profiles and denaturation of liver enzymes (Serezli *et al*., 2011).

The liver is the most complex organ (18, 19) populated by two major types of cells, parenchymal and non-parenchymal (20). Non-parenchyma cells include Sinusoidal endothelial cells and Kupffer cells (20). Exposure to Cr(VI) compounds may result in hepatotoxicity (21). It causes hepatocellular apoptosis and hepatocytes atrophy (22). It damages the hepatocytes by causing an increase in cell cytoplasm and lysis of nuclei (23). Cr(VI) promotes oxidative stress in the liver, and hepatocytic necrosis and disturbs the level of enzyme activities (16, 21). Chromium widens the sinusoid space of hepatocytes, hepatocytes inflammation, hepatocytes atrophy, and cytoplasmic vacuolization in the liver (Lushchak *et al.,* 2011). Lipid peroxidation is the indicator of free radicals formation alterations of mitochondrial electron transport chain in aerobic respiration and oxido-reductase enzymes (Laura *et al.,* 2012).

Many plants and herbs from Pakistan have been investigated for their fruit extracts have free radicals scavenger ability and metal chelating activity against toxicants (Hussain *et al.,* 2012). Heavy metals induce histological changes in the testis and liver (Sharma *et al.,* 2013), while the plant extracts have ameliorative competency against free radicals and heavy metals (Batool *et al.,* 2010; Swami *et al.,* 2012; Okon *et al.,* 2013).

In nature, protective antioxidant mechanisms exist as many medicinal plants are known to possess antioxidant activity (24-27). Mulberry has medicinal value and possesses antioxidant potential due to anthocyanins (28-32). The anthocyanins and antioxidants combat the free radicals and prevent the body from lipid peroxidation (Ozsahin *et al.,* 2012).

It can inhibit the Low-Density Lipoprotein oxidation (33). Berry anthocyanins belong to the family of compounds known as flavonoids and have active free radical scavenging ability (34-37). Mulberry fruit is abundantly used in natural medicine against illnesses like sore throat, fever, hypertension, and anaemia (38, 39). The utilization of mulberry fruit keeps away from liver and kidney damage, strengthens the joints, improves eyesight, and has anti-ageing effects (40). Leaves, branches, bark, and roots of *Morus nigra* also have ameliorative potentials against lipid-related anomalies (41-44).

By considering the beneficial medical aspects of *Morus nigra* the present study is undertaken to investigate its possible ameliorative effects on oxidative damage to the liver, resulting from exposure of normal male mice to Cr(VI) toxicity.

Materials and methods:

Ripe fruits of *Morus nigra* were purchased from the local market and berries were carefully selected, washed thoroughly in cooled boiled drinking water for 5 minutes, air dried, and finally the pulp was softened. 100g of the pulp was blended with an electric juicer in 100 mL of cooled boiled drinking water for 5 minutes. The resulting juicy material was

centrifuged at 500rpm for 10 minutes to separate the deep purplish supernatants from the bottom-settled fibrous pulpy mass. The supernatants were immediately placed at –30°C in sterilized 5mL ice cube dishes. The frozen cubes (one each) were then placed in sterilized (air-tight) plastic bags and stored at –30°C. For each treatment, extract from a freshly thawed (at room temperature) cube was used.

A 1000ppm Cr(VI) solution was prepared by dissolving 2.282g of $K_2Cr_2O_7$ in 1000ml water as a stock solution, which diluted to get the 50ppm required solution.

Thirty Swiss Webster male mice ranging in weight from 25-30g, aging between 3-4 months were randomly divided (n=10) into 3 groups, provided with Cr-free water as the control group, 50ppm Cr(VI) solution in drinking water (*ad-labitum*) for 10 days to Cr and CrM groups while CrM was additionally given fruit pulp extract through oral gavage 0.2ml/12h daily for next 5 days. The ambient temperature and humidity of the animal house were maintained at 23±3C˚ and 45% respectively. The dark and light cycle was maintained for 12-12 hours. The animals were weighed daily before feeding throughout the study to record the body weight variations during this exposure.

Before recovery each animal was weighted and then anaesthetized with chloroform, the bellies were carefully opened with forceps and scissors. The intact liver was removed and weighed to calculate the hepato-somatic index. The liver was finally fixed in Buoine's and after fixation for 48 hours the organs were processed for wax embedding. Serial sections, 4-6 microns thick, were obtained on a rotary microtome (ERMA TOKYO 422). The sections were further treated with Hematoxylin and Eosin using standard protocol for permanent record and histopathological and micrometric studies at 40×, 100× and 400× magnifications by trinocular research microscope (Labomed CXR_2) attached to a 7.2-megapixel digital camera (Sony DSC-W35).

To highlight the pathological outcomes, digital photographs of selected sections were improved in corelDRAW11 for color, contrast, cropping, and addition of highlighting signs and presented in the result section. The calibrations for each working magnification were made separately with the help of digital photo-shots of the stage micrometer on the same magnifications. The data obtained was pooled to obtain mean values in such a way that each animal was represented as a unit. Thus measurement of the mean Cross Sectional Area (CSA) of hepatocytes, hepatocytic nucleus, central hepatic vein, width of sinusoidal spaces, and the mean number of kupffer cells and hepatocytes per unit area moreover relative area occupied by hepatocytes was obtained. For each of these structures, diameters were obtained with the help of right-angle perpendicular lines drawn across them passing through the center on the projected image in corelDRAW11 to calculate CSA.

Data obtained based on histology and micrometry etc. was analyzed through ANOVA and Duncan's Multiple Range Test.

Results

Histological observations:

All typical signs of normal histological dispositions such as the hepatic lobules with centrally placed lobular vein and hepatocytes arranged in hepatic cords radiating from the central vein properly lined with kupffer cells and showing hepatic sinusoids in between were visible in the C group (Fig: 1 C).

Signs of extreme hepatic lobular necrosis were evident in Cr group. These include disaligned hepatic cords, hepatocytic necrosis, the presence of debris of hepatocytes, and widened intracellular spaces. Signs of connective tissue development at the expense of hepatocytic involutions were also visible. The signs of necrosis of hepatocytes include disfigured nuclei, irregular cellular margins, and cytoplasmic fusions between adjusted hepatocytes. Endothelial cell infestation from the lobular vein into the lobules was also been seen (Fig: 1 Cr).

Obvious signs of liver regeneration that include hepatoblastic mitosis and the rehabilitation of hepatic cords were clearly visible in the CrM group (Fig: 1 CrM).

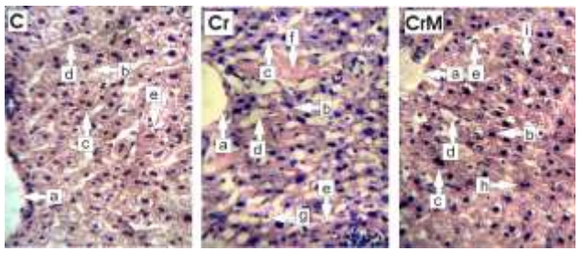

Fig 1: Histological sections of Mice liver Control (C), Chromium (Cr), and Chromium + Morus (CrM) groups at 400×.

a: Central vein, b: Uninucleatedhepatocyte, c: inucleated, patocyte, d: Sinosoidal space, e: Kupffer cell,

f: Hepatocytic debris, g: Hepatocyte ecrosis, h: Hepatoblast-proliferation, i: Emerging hepatic cord

Anatomical and Micrometrical ameliorative observations: There was significant variation among the organ index indicate the mean fractional weight of the liver had non-significant variation among control (06g±0.33) and CrM group (05g±0.139) while Cr group (11.54g±1.25) show significant variation from C and CrM groups. The mean CSA of hepatocytes at 400× indicates the significant difference between C (236.38μ²±9.82), Cr (161.18μ²±13.01) and CrM group (133.81μ²±04.04) likewise their nuclei CSA also significantly fluctuate among C (32.07μ²±01.19), Cr (41.94μ²±01.53) and CrM group (28.03μ²±01.09). The mean CSA of central veins at 40× specifies the significant disparity among C (2900.86μ²±296.49), Cr (3779.05μ²±605.19), and CrM group (1956.34μ²±256.32) correspondingly the width of Sinusoidal spaces at 400× also significantly differ among C (6.16μ±0.28), Cr (7.15μ±0.35) and CrM group (5.17μ±0.31). The mean numbers of Kupffer cells at 400× in 14400μ²area indicate the non-significant variation between C (10.83±0.34) and CrM group (11.42±0.48) while Cr group (15.5±0.85) significantly differ from C and CrM groups. The mean numbers of hepatocytes at 100× in 46225μ²area show a significant disparity among C (112.13±6.08), Cr (80.63±3.46), and CrM group (56.75±5.47). The mean relative area of hepatocytes at 100× in 46225μ²area indicates the significant variations among C (26504.11μ²±1437.45), Cr (12995.14μ²±558.29), and CrM group (7593.72μ²±732.36) seen in Tab 1.

Tab: 1 Anatomical and Micrometrical amelioration of fruit against chromium exposure in mice

Parameters	C	Cr	CrM
Mean fractional weight of Liver (%) ***	06±0.33[a]	11.54±1.25[b]	05±0.139 [a]
Mean CSA of hepatocytes (μ²)***(400×)	236.38±9.82[c]	161.18±13.01[b]	133.81±04.04[a]
Mean CSA of the hepatocytic nucleus (μ²)***(400×)	32.07±01.19[b]	41.94±01.53[c]	28.03±01.09[a]
Mean CSA of central vein(μ²)** (40×)	2900.86±296.49[b]	3779.05±605.19[c]	1956.34±256.32[a]
Mean width of Sinusoidal spaces (μ)***(400×)	6.16±0.28[b]	7.15±0.35[c]	5.17±0.31[a]
Mean number of Kupffer cells in 14400μ² area*** (400×)	10.83±0.34[a]	15.5±0.85[b]	11.42±0.48[a]
Mean number of hepatocytes in 46225μ² area***(100×)	112.13±6.08	80.63±3.46	56.75±5.47
MRA of hepatocytes in 46225μ² area***(100×)	26504.11±1437.45[c]	12995.14±558.29[b]	7593.72±732.36[a]

C; control group, Cr; chromium group, CrM; chromium + morus group,
CSA; cross sectional area, MRA; mean relative area, μ; micrometer

Hematological Observations

Preliminary phytochemical protective activities were studied against Cr-induced anomalies in mice concerning blood profile changes concerning bilirubin, total protein, and globulin changes as shown in (Tab 2).

Tab 2: Ameliorative effects of Mulberry on Cr induced anomalies on Blood Profile.

PARAMETERS	C	Cr	Cr- M
RBC ($\times10^6$/ul)*	7.97±0.16^{a}	8.02±0.01^{b}	7.84±0.08^{a}
TLC ($\times10^3$/ul)*	8.06±0.42^{a}	6.35±0.74^{b}	7.47±0.07^{a}
%Neutrophil***	11.05±0.86^{a}	43.02±2.09^{c}	11.45±0.61^{a}
%Lymphocytes ***	67.06±7.39^{a}	42.7±3.9^{b}	83.04±1.04^{c}
%Monocytes *	2.07±0.15^{ac}	2.03±0.15^{a}	2.06±0.22^{ab}
%Eosinophil *	1.55±0.14^{ad}	1.02±0.01^{b}	0.95±0.14^{c}
Hb (g/dl) **	13.06±0.16^{a}	10.39±0.95^{b}	12.86±0.29^{a}
PCV% **	49.23±1.35^{a}	38.28±2.65^{b}	46.16±2.37^{c}
MCV fl ***	58.93±0.09^{a}	51.26±1.17^{b}	52.05±0.87^{b}
MCH (pg) ***	16.58±0.03^{a}	15.2±0.18^{b}	16.46±0.28^{a}
MCHC(g/dl)***	27.65±1.01^{a}	30.39±0.37^{b}	31.32±0.24^{c}
Platelet($\times10^3$/ul)***	807.01±16.09^{a}	989.2±39.09^{c}	907.00±13.07^{b}

C: control. Cr: chromium treated, Cr-M: chromium+morus,

Values are mean ± SEM, N= 10, RBC: Red Blood Cell, Hb: Hemoglobin, PCV: Pack Cell Volume, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Concentration of Hb in RBC. Statistical analysis (ANOVA: two factors without replication)*:p≤0.05-0.01; **:p ≤ 0.001; ***:p ≤ .0001, group means ±SEM, $^{a\,b\,c\,d}$: Anyone two groups not sharing a lower case letters differ significantly from each other (Duncan's Multiple Range comparison-post hoc analysis).

The highest mean RBC was detected in Cr (8.2±0.1 $\times10^6$/ul) followed by control and Cr-M groups 7.97±0.16, 7.84±0.08 $\times10^6$/ul respectively. Similarly highest mean TLC was recorded in the control group (8.6±0.42$\times10^3$/ul) followed by Cr-M and Cr (7.47±0.7 and 6.35±0.74 $\times10^3$/ul). The highest mean value of Neutrophil was observed in the Cr^{+6} treated group (43.2±2.9%) followed by C and Cr-M groups (13.4±1 and 11.45±0.61%). The mean value of Lymphocytes was noted in Cr-M, C, and Cr groups (83.4±1.04, 67.6±7.39, and 42.7±3.9% respectively). The mean value of Monocyte was recorded in C, Cr-M, and Cr groups (2.7±0.15, 2.6±0.22 and 2.3±0.15% respectively). The highest mean value of Eosinophil was studied in the control group (1.55±0.14%) followed by Cr and Cr-M groups (1.2±0.1 and 0.95±0.14% respectively). The mean value of Hb was noted in C, Cr-M and Cr groups (13.06±0.16, 12.86±0.29 and 10.39±0.95g/dl respectively). Highest mean value of PCV was observed in the control group (49.23±1.35%) followed by Cr-M, and Cr groups (46.16±2.37 and 38.28±2.65% respectively). The highest mean value of MCV was quantified in the control group (58.93±0.9 fl) followed by Cr-M and Cr groups (52.05 ± 0.87 and 51.26±1.17fl respectively). The highest and lowest mean MCH levels were noted in Cr^{+6} treated groups (15.2±0.18pg). The other groups like C and Cr-M have 16.58±0.3 and 16.46±0.28 respectively.

The mean value of MCHC was measured and observed in Cr-M, Cr, and C groups (31.32±0.24, 30.39±0.37, and 27.65±1.1g/dl respectively). The highest and lowest mean Platelets levels were noted in Cr^{+6} treated groups (989.2±39.96$\times10^3$/ul). The Cr-M and C are 907±13.7 and 807.1±16.9 $\times10^3$/ul respectively. Statistical analysis (ANOVA) has shown highly significant variation among the groups (P ≤ 0.0001). A comparison between the groups (Duncan's multiple range test) has shown a significant (p ≤ 0.05) difference was observed among group C (Tab 2.

Discussion

Chromium although a micronutrient, is toxic at higher doses particularly in its hexavalent form (45-47). On the other hand, Morus fruit extract has long been used to cure various pathological and allergic conditions (48-50).

In the present study, we investigated the curative potential of *Morus nigra* upon the hepto-histopathological manifestations of Cr exposure in mice. Results indicate that Cr exposure caused rapid weight loss in the exposed animals while post-treatment of Morus extract was resulted in a secondary weight gain. Simultaneously the mean percentile fractional weight of the liver was increased in just 10 days of Cr exposure than that of

the control, indicating hepatic inflammation. This condition was reversed just within 5 days of Morus post-treatment indicating its detoxifying and health-promoting capabilities.

Histologically it was seen with great concern that chromium exposure brings about various pathological changes in mice liver that include hepatocytic necrosis with an increased deposition of debris. These findings are well in line with the available literature (23, 51, 52). While the post-treatment of Morus fruit extract led to hepatoblastic proliferation and rehabilitation of the hepatic cords.

Micrometric data shows a significant decline in the area occupied by the hepatocytes, the number of hepatocytes per unit area, and the mean CSA of the hepatocytes on Cr treatment with a concurrent increase in sinusoidal spaces and mean caliber of the centrilobular veins. These findings indicate that Cr exposure causes hepatocytic necrosis leading to an increase in the sinusoidal spaces.

Cr(VI) treatment significantly increases RBC, neutrophil, MCHC, and platelets and a significant decrease in TLC, lymphocytes, monocytes, eosinophil, Hb, PCV, MCV, and MCH as other metals exactly like the toxicity of copper (Ajani and Akpoilih, 2010). Cr deposition in organs causes the loss of blood plasma and water depletion, resulting in the appearance of higher levels of RBC (Bassett *et al.,* 1986) also associated with fibrosis (Buchman *et al.,* 2001). The neutrophils increased by toxification of Cr(VI) treated as compared to control untreated normal as Pb, Cu, Hg, and Cd in humans also indicate the causative agents of fibrosis (Mushtakova *et al.,* 2005). Cr(VI) induces pericellular fibrosis, vacuolation of hepatocytes, and portal tract indicated by dense mononuclear inflammatory cells (Accion *et al.,* 2006). The fibrosis distorts the hepatic vessels and leads to increased intrahepatic resistance and portal hypertension. Damage to liver hepatocytes in the Cr^{+6} treated group causes impaired liver function and the liver becomes unable to detoxify the toxicants in blood.

The area occupied by the hepatocytes, the number of hepatocytes per unit area, and the CSA of the hepatocytes show a further decline in post-treatment of Morus extract. These findings can be misleading if not seen in conjuncture with the histological findings. The number of functional hepatocytes was lesser than Cr in the CrM group, while clear signs of removal of debris with simultaneous hepatoblastic proliferation were seen histologically; indicating liver regeneration upon Morus extract as post-treatment. Secondary decline in sinusoidal breadth and the mean caliber of the centri-lobular veins also indicate rehabilitation of normal physiological status of the liver post-treatment of Morus fruit extract.

Amelioration of Hepatocellular Fibrosis

The plants (Sodipo *et al.,* 2013) are responsible for increasing MCV; the same tendency was evident in MFE against Cr-induce fibrosis. The decline of MCV was not only inhibited but also activated MCV by MFE, indicating the ability to block the toxicity of Cr(VI).

The novel extract of Morus showed excellent results in blocking the heavy metals toxicity by improving MCV. The MFE increased the MCH and brought it approximately equal to the normal control values, ameliorated the Cr-induced anomalies, and cured the fibrosis by up-regulating MCH. The plant extracts in MFE maintain RBC which is associated with fibrosis; indicating the clue about detoxification against heavy metals due to the presence of medicinal phytochemicals (Okon *et al.,* 2013). MFE significantly increases PCV and reverses the Cr+6-induced oxidative thresh and incongruities (Suiying *et al.,* 2000) (Tab 2).

Mulberry antioxidants play a significant role in the treatment of Cr-induced oxidative stress as metal-chelating activities like other medicinal plants (Atef and Al-Attar, 2011). Morus fruit extract also has radical scavenging, regulating cell cycle, and apoptosis-preventing abilities (Lim *et al.,* 2013).

Blueberry L-carnitine contains anthocyanins which is the main ingredient of vitamin A, and can increase energy and boost metabolism. Natural fruit extract without any side effects indicates that there is a positive link between fruit extract and carnitine without alteration in normal cecal microbial composition. The phytochemicals do not destroy the intestinal microbiota, which is essential for the carnitine palmitoyl transferase-1

pathway during rehabilitation and proper lipid metabolism (Koeth *et al*., 2013).

Our results indicate that hexavalent chromium is injurious to general health and particularly hepatotoxic causing various histopathological and micrometric changes in the liver, while Morus fruit extract surely bears curative potentials against such pathological manifestations. These findings indicate that Morus fruit extract bears nutraceutical capabilities against noxious environmental toxicants particularly heavy metals such as Hexavalent chromium.

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