ORIGINAL ARTICLE

Antioxidative Effects of Vitamin C on Methotrexate Induced Hepatic Damage in Rat

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ABSTRACT

Objective: This study aimed to explore the protective effects of vitamin C against Methotrexate induced biochemical and histological changes in rats.

Study Design: Experimental study.

Place and Duration of Study: The study was conducted at the Department of Anatomy, Baqai Medical University Karachi, Pakistan from June 2017 to July 2018.

Methods: This study included 45 adult male albino Wistar rats aged between 10-12 weeks and weighing around 180-200g. The rats were randomly divided into 3 groups: Group A, received no intervention and was kept on standard diet for 10 days; Group B, received 20mg/kg intraperitoneal single dose of Methotrexate; Group C received 200 mg/kg oral tablets of vitamin C for seven days and 20mg/kg intraperitoneal single dose of Methotrexate. At the end of the study period, animals were anesthetized with ether, cardiac blood samples were collected for enzyme estimation and organs were removed for histopathological examination.

Results: Liver sections from Group A demonstrated normal parenchyma with cords of hepatocytes radiating from the central vein with portal triads at the ends of hepatic lobules while Methotrexate treated Group B showed severely degenerated architecture along with hemorrhagic parenchyma with dilatation of central vein, and sinusoids. Comparatively, liver sections from Group C displayed less deranged histologic and morphologic changes. A significant increase in serum levels of AST, ALT and ALP and albumin and reduction in hepatocyte count, increase in hepatocyte diameter and a decrease in hepatocyte nuclear diameter occurred in group B, while above parameters improved in vitamin C protected group C.

Conclusion: This study exhibited that the potential biochemical and histological alterations caused by Methotrexate can be attenuated by antioxidant effects of Vitamin C.

Keywords: *Hepatotoxicity, Liver Enzymes, Methotrexate, Oxidative Stress, Vitamin C.*

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Introduction

Acute liver failure is common not only in the developed world but also in other parts of the world. Drug induced hepatitis remains at top of the list of causes of acute liver failures.[1] Liver, a vital internal organ, is responsible for metabolic functions of the body.[2] It is involved in the chemically induced biotransformation of drugs and serves as the target site for drug metabolism, rendering it most susceptible for drug injury.[3] More than 1000 drugs are known to cause liver injury.[4] Drug induced liver injury (DILI) accounts for 5% of all hospital admissions and 50% of all acute hepatic failures.[5] Drugs or their active metabolites can directly damage the hepatocytes and/or the bile ducts and mediate an inflammatory response.[6] The progression of liver damage is also accelerated by the hepatic cells, including hepatocytes, stellate cells and Kupffer cells, which produce exosomes.[7]

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Methotrexate, a widely used anti-folate agent, was initially developed as an anticancer drug. It is given in high doses for several cancers of breast, prostate, urinary bladder, head and neck and acute leukemia. It is now used in low doses for long durations as an immunosuppressive and anti-inflammatory drug for rheumatoid arthritis, psoriasis, SLE and sarcoidosis.[8] 20 to 30% of patients discontinue Methotrexate, in the initial phase of treatment due to its unbearable side effects.[9] It characteristically disturbs cellular metabolism by inhibiting dihydrofolate reductase enzyme reversibly. This hinders nucleotide biosynthesis, thus inhibiting DNA synthesis leading to reduction in cellular growth and proliferation necessary for tumor suppression. It also affects rapidly dividing cells of the body like RBCs, intestinal lining cells and hepatocytes, causing myelosuppression and hepatotoxicity.[8]

Once Methotrexate enters the hepatocytes, it is converted into Methotrexate polyglutamates. This metabolite's intracellular accumulation triggers lipid peroxidation within hepatocytes, generating ROS leading to oxidative burden on the cells. Proinflammatory signaling pathways are induced and cytokines (e.g. TNFα and IL6) are released causing inflammation and steatosis. This results in hepatocyte hypertrophy, hence larger hepatocyte diameters. The hypertrophy can also be a result of microsomal enzyme induction. The overall weight of liver is also increased. With further accumulation of harmful metabolites, fibrosis, and ultimately apoptosis of hepatocytes occurs. Apoptotic cells have condensed cytoplasm with pyknotic and fragmented nuclei.[10,11]

Research has pointed towards hepatoprotective potency of many chemicals and herbs due to their antioxidant effects.[12] Vitamin C or ascorbic acid is an essential factor of human diet. It is water soluble and has well-documented antioxidant effects.[13] Studies suggest that it has been given as a supplement to protect against heart diseases, chronic diseases like cancer, and neurodegenerative diseases, specially memory loss and has also served as an immunity booster.[13,14]

This study was designed to observe methotrexate induced cell damage and to analyze the possible protective role of Vitamin C against oxidative stress in hepatocytes by using histological and biochemical parameters.

Methods

The study was conducted at the Department of Anatomy, Baqai Medical University Karachi, Pakistan from June 2017 to July 2018 after taking approval and acceptance from the Ethical Committee and Board of Advance Research and Studies of University on dated: 08th May 2017 vide letter no: BMU-EC/2017-04. This study was performed at the Animal House and Department of Anatomy according to the below mentioned protocols.

Animals Inclusion-Exclusion

45 Wistar albino rats (adult/male) were bought from the Animal House of Baqai Medical University Karachi, Pakistan. The Department of Hematology's animal house facility was used to breed and raise animals. The animals included in this study were between 180 and 200 g in weight and 10 to 12 weeks of age. Animals which were underweight, diseased or those previously subjected to experimentations were excluded. Also, female rats were not included to avoid effects of reproductive cycles.

Animal Housing

The animals were randomly split up into 3 groups, each with 15 rats. They were kept in a facility with a controlled temperature between 25 to 30 °C and humidity between 40 to 70%. Following the weight assessment, the rats were housed in clear plastic cages (5 animals per cage) with soft wood-chip bedding. A 12-hour natural cycle of day and night was made available to them. They were fed a conventional laboratory pellet diet and given unlimited access to water. For acclimatization phase, the rats were monitored for 10 days to rule out any health-related difficulties. All animals were taken care of according to the ethical standards of Pakistan.

Chemicals

Chemicals used in this study were Methotrexate 50 mg injection (Unitraxate®) manufactured by Al Habib pharmaceuticals and Vitamin C 500 mg tablets (cecon®) manufactured by Abbott Laboratories. They were purchased from a local pharmacy.

Grouping

The grouping was done in the following manner:

Group A; received no intervention and was kept on a standard diet for 10 days and were served as control

group.

Group B; received 20mg/kg dose of Methotrexate intraperitoneally on 4th day of study.[15]

Group C; received 200 mg/kg tablets of Vitamin C orally for 7 days along with 20mg/kg dose of Methotrexate intraperitoneally on 4th day of study.

Dosing

For dosing, animals were starved overnight and were given an oral dose of Vitamin C the next morning between 10-11 am. It was crushed and dissolved in distilled water then given through gastric gavage. For concomitant Vitamin C and Methotrexate dosing, first Vitamin C was given orally then an hour later, Methotrexate was administered through intra-peritoneal injection with insulin needle.

Data collection

Animal weighing was practiced daily, and rats were kept separate and labelled according to groupings. On the eighth day of the study, animals were administered ether anesthesia in a glass container and weighed for the final time. A midline incision was made anteriorly to expose internal organs.

Blood collection

Blood samples were drawn from cardiac punctures using a 5cc syringe and then stored in Serum separator tube (SST) for biochemical estimations of liver enzymes; serum glutamate pyruvate transaminase or alanine transaminase (SGPT or ALT), serum glutamic-oxaloacetic transaminase, or (SGOT or AST), alkaline phosphatase (ALP); and serum albumin.

Weight calculation and Tissue harvesting

Liver was dissected out and grossly examined. The absolute liver weight, that is the dry weight of liver, was taken. The relative liver weight was calculated with the formula; absolute weight/body weight *100.

The liver was then washed with normal saline and stored in 10% formalin. A sample was cut and placed on plastic cassettes. It was then processed through tissue processor and paraffin blocks were made. Block was sectioned through rotatory microtome. 5 micrometer thin sections were picked up on glass slides through water bath and H & E staining of slides were performed. The tissue was then observed under the microscope at 40X and 100X magnifications.

Micrometry

Micrometry was carried out by observing the H & E-stained liver slides under 400X magnification. The ocular reticule and scale were calibrated using a stage micrometer. Hepatic cells count per reticule was carried out using ocular reticule. Mean of two perpendicular lines, passing through the center of the hepatocyte and nucleus from one edge of the cell and nuclear membrane to the other, was calculated to measure the hepatocyte and nuclear diameters respectively. The measurements of 10 cells and nuclei each were recorded per field. 5 fields were taken of each slide, to ensure accuracy and account for variability, and their mean diameter and hepatocyte counts were then calculated.

Data analysis

For the statistical analysis of data, SPSS (Statistical Packager for Social Sciences) version 25.0 was used. The means of quantitative variables were calculated and were expressed as mean and standard deviation (Mean ± SD). One-way analysis of variance (ANOVA) was applied with post hoc Tukey's HSD (honest significant difference) test, for the statistical analysis to compare the means of the three study groups. To find mean difference between groups, 95 % confidence interval was employed and the *P* Value less than 0.05 was considered significant.

Results

Animal behavior and body weights

All animals in control group A were active and receptive to external stimuli. However, the animals in Methotrexate treated group B were weak and sluggish, mildly disoriented, and less responsive to external stimuli. The animals in Vitamin C protected group C were also weak and less active as they did not show considerable reactions to external stimuli as those of control group A. Mean values of initial and final body weight with standard deviations are shown in table-1. The final body weight of Methotrexate treated group B decreased significantly while that of Vitamin C group increased significantly in comparison to group B. Table-2.

Gross examination of Liver and weights

Control A group animals had normal sized liver with reddish brown appearance, soft textured with smooth edges whereas group B livers were hard in consistency, edematous and slightly distorted edges.

Table-1: Descriptive Statistics of weights, hepatic morphometry, and hematological parameters of Different groups

Parameters	A Control	B Methotrexate -treated Mean ± Standard Deviation	C Methotrexate + Vitamin C
Initial Weight (gm)	187.01 ± 2.59	183.84 ± 2.31	181.95 ± 2.58
Final Weight (gm)	201 ± 2.58	162 ± 2.58	175.94 ± 2.5
Absolute Liver Weight (gm)	6.1 ± 1.51	9.55 ± 1.42	6.84 ± 1.48
Relative Liver Weight (gm)	3.04 ± 0.76	5.9 ± 0.91	3.89 ± 0.85
Hepatocyte Count Per reticule	16.12 ± 1.51	7.18 ± 1.36	11.25 ± 1.51
Hepatocyte Diameter (µM)	13.34 ± 0.66	17.6 ± 1.51	14.72 ± 0.58
Nuclear Diameters (µM)	7.28 ± 1.51	5.29 ± 1.41	6.27 ± 1.41
Serum Levels of ALT (µ/L)	33.7 ± 1.51	51.27 ± 15.35	43.8 ± 1.51
Serum Levels of AST (µ/L)	59.96 ± 2.47	103.41 ± 2.68	60.18 ± 2.81
Serum Level of ALP (µ/L)	190.71 ± 2.33	353.64 ± 2.54	209.02 ± 2.58
Serum Levels of Albumin (g/dL)	3.22 ± 0.49	4.19 ± 5.36	3.84 ± 0.47

**Statistically Significant*

Table-2: Statistical analysis (Analytical statistics) of weights, hepatic morphometry, and hematological parameters among different groups

Comparative groups	A vs B Control vs Methotrexate Treated	A vs C Control vs Methotrexate +Vitamin C	B vs C Methotrexate vs Methotrexate +Vitamin C
Parameters	**Difference of Means between different parameters (*P*-value)**		
Final body weight (gm)	39 (0.01)*	25.06 (0.01)*	-13.94 (0.01)*
Absolute Liver Weight (gm)	-3.45 (0.01)*	-0.74 (0.57)	2.71 (0.01)*
Relative Liver Weight (gm)	-2,86 (0.01)*	-0.85 (0.03)*	2.01 (0.01)*
Hepatocyte Count Per reticule	8.94 (0.01)*	4.87 (0.01)*	-4.13 (0.01)*
Hepatocyte Diameter (µM)	-4.26 (0.01)*	-1.38 (0.01)*	3.4 (0.01)*
Nuclear Diameters (µM)	1,99 (0.01)*	1.01 (0.23)	-0.38 (0.28)
Serum Levels of ALT (µ/L)	-17.57 (0.01)*	-10.1 (0.01)*	7.47 (0.01)*
Serum Levels of AST (µ/L)	-43.45 (0.01)*	-0.22 (0.99)	43.24 (0.01)*
Serum Level of ALP (µ/L)	-162.93 (0.01)*	-18.31 (0.01)*	144.62 (0.01)*
Serum Levels of Albumin (g/dL)	-0.97 (0.82)	-0.62 (0.96)	0.35 (1.00)

The livers of group C were less edematous, slightly large with regular borders. Mean values with SD of absolute and relative liver weight are shown in table-1. Absolute and relative spleen weights increased significantly in group B and decreased significantly in group C as compared to group B. Table-2.

Microscopic examination

The H & E-stained liver section of animals of group A at 40X magnification showed normal parenchyma of hepatic lobules, with radial arrangement of hepatocytes as hepatic cords, connecting peripheral portal triad to the central vein. Narrow blood sinusoids are present between the hepatic chords, lined by endothelium, containing flattened nuclei, and Kupffer cells, irregular in shape with ovoid nuclei. Hepatocytes were polyhedral in shape, with acidophilic cytoplasm evenly distributed around darkly pigmented nuclei. The portal triad's structure included the hepatic artery, portal vein, and one or two bile ducts. Figure.1 and 2.

The liver section of animals in Methotrexate treated group B at 100X magnification showed distorted hepatic parenchyma with areas of hemorrhage within hepatic cords, sinusoids, and central vein. The central vein was dilated and congested. The peri-central vein area was infiltrated with inflammatory

cells as exhibited in figure.3. Methotrexate and Vitamin C protected livers at 100X magnification showed mild architectural disruptions. Mild dilation of central vein with few hemorrhagic areas and almost normal sinusoids was observed in figure.4.

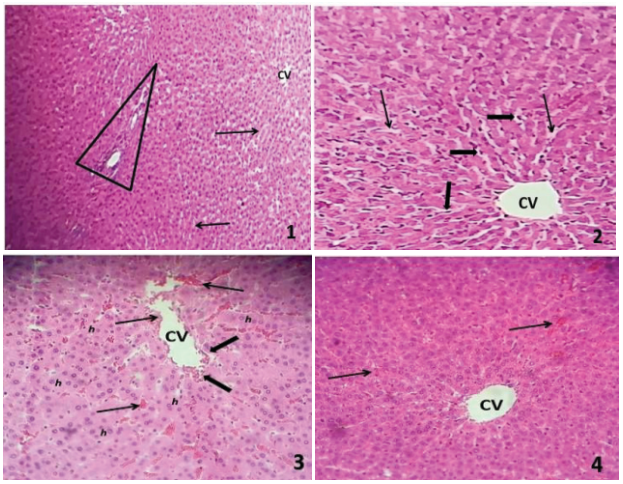

Fig.1: Group A control liver showing normal architecture. Single cell thick cords of hepatocytes (thin arrows) connecting central vein (CV) with the Portal Triad (triangle) at the periphery of lobule. H & E 40X

Fig.2: Group A control liver showing cords of hepatocytes arranged around the central vein (CV) with intervening narrow sinusoids (thin arrows) lined by endothelium and containing Kupffer cells (thick arrows) H & E 400X.

Fig.3: Group B Methotrexate treated liver showing disturbed architecture. Hemorrhagic areas within hepatic cords and sinusoids (thin arrows) and in dilated central vein (CV). Hepatocytes (h) are enlarged with vesicular nuclei. Peri-central areas of inflammatory cells infiltration are also seen (Thick arrow). H & E 100X

Fig.4: Group C Methotrexate with Vitamin C liver showing mild disruption in architecture. Mildly dilated central vein (CV) with few Hemorrhagic areas within hepatic cords and sinusoids (thin arrows). H & E 100X

Micrometric analysis

Mean values with SD of hepatocyte count per reticule, hepatocyte diameter, and nuclear diameter, are shown in table-1. The hepatocyte count per reticule in groups B and C was significantly decreased than that in group A, while it significantly increased in group C when compared with group B. The hepatocyte diameter between groups B and C was significantly increased than that in group A, but decreased significantly in group C when compared with group B. The nuclear diameter in groups B, but not group C, was significantly decreased in comparison to group A.

Biochemical Analysis of liver enzymes and serum Albumin

The Mean values with SD of serum levels of ALT, AST, ALP, and albumin are shown in table-1. Mean serum levels of ALT, AST and ALP are comparatively increased in group B and C than that of group A while group C decreased in comparison to group B. Mean serum level of albumin increased in groups B and C than that of group A and decreased in group C as compared to group B but the results remain insignificant.

Discussion

The aim of this study was to examine the methotrexate-induced hepatocyte injury and to analyze the protective role of Vitamin C against oxidative stress by using histological and biochemical parameters in rat model.

The use of rats in biomedical research and solving basic sciences problems dates back to the 1940s. They are still considered to be the best biological models because of their relevance to humans. Research in the fields of physiology, pathology, immunology, pharmacology, nutrition, toxicology and behavioral sciences depends mainly on rats.[16] The liver is the main metabolic center of the human and rat body alike. It is primarily responsible for the metabolic elimination of endogenous compounds like steroids, fatty acids and bile acids, and is accountable for the metabolic biotransformation of almost all the drugs and xenobiotics that are consumed.[3] Biotransformation of these xenobiotics renders them hydrophilic thus excretable, but many a times lethal for the hepatic cells. Likewise, biotransformation of Methotrexate produces its less soluble and toxic metabolites, which are responsible for its hepatotoxic effects. The intracellular accumulation of these metabolites triggers lipid peroxidation within hepatocytes, generating ROS.[11] Present study showed Methotrexate treated rats to be sluggish in reaction to external stimuli. which was also observed in other studies which showed cognitive impairment with methotrexate injections.[17] The reason for this could be that Methotrexate administration causes decrease food intake, gastrointestinal toxicity and diarrhea.[18] The weight of the rats in this study were reduced by

Methotrexate, which was same as seen by Ilamkar et al. in their mice.[19] While, Vitamin C was able to minimize the loss in body weight, similar to a study with paracetamol-induced body weight loss.[20]

This study demonstrated that methotrexate increased absolute and relative liver weights which corresponded with similar research conducted by Abdul-Hamid et al., and Sundus S.et al. Hepatotoxic agents cause inflammation, leading to swelling and fluid accumulation, which increases the liver weights.[21,22] Khaldoun Oularbi, et al also documented in their study the same reversal of liver weight gain with Vitamin C, seen in our study.[23]

The most sensitive marker of organ toxicity way before any observable morphological change is its weight. Absolute organ weight alone is a comparatively insensitive parameter as many factors like animal age, sex, stress, housing conditions and experimental settings can affect it. Thus, absolute organ weight should be standardized with the body weight. This standardized parameter is the relative organ weight or the organ to body weight index.[24]

Similar to the observations recorded in current study, many other studies have shown toxic effects of methotrexate on liver architecture: hepatocyte degeneration, hemorrhages, congestion of central vein and sinusoidal spaces and mononuclear cell infiltration.[25,26] These changes were ameliorated with the addition of Vitamin C; Al Sammak, et al also demonstrated similar results.[27] Morphometric analysis showed hepatotoxicity with decreased hepatocyte counts, increased hepatocyte and decreased nuclear diameter in methotrexate group. These results were same as the toxic effects of carbamazepine in another study, which also showed reversal of these histomorphometric changes by inclusion of another antioxidant, Vitamin E.[28]

Methotrexate caused elevation in serum levels of AST, ALT, ALP, and albumin in this study. The same increase had been observed with methotrexate in different studies as well.[17,18] This could be due to hepatocytic inflammation secondary to oxidative stress. Oxidative stress within the body is a result of imbalance between the generation and degradation of reactive oxygen species (ROS). ROS are highly reactive metabolic end products of oxygen, present in the form of free radicals in the tissue. They induce enzymatic reactions within the mitochondria, leading to cellular damage.[29] The tissues with high metabolic turnover like liver are prone to this kind of damage.[8]

In current study, Vitamin C prevented liver injury, demonstrated by reducing elevated serum levels of ALT, AST, ALP, and albumin. Similarly in other studies, Vitamin C was able to reverse the effects of Paracetamol and ibuprofen induced increase in ALT, ASP and ALP enzymes and albumin.[20,30] Vitamin C, which is a chain breaking antioxidant, reduces ROS free radicals by donating electrons and itself gets oxidized, to become dehydroascorbate.[13,14] The main antioxidative mechanism of Vitamin C in hepatocytes against Methotrexate is the inhibition of lipid peroxidation and proinflammatory cytokines.[9]

Conclusion

This study presented the encouraging antioxidant effects of Vitamin C in amelioration of hepatic histological, histomorphometric and biochemical changes caused by Methotrexate in rat model. Adjuvant treatment of Methotrexate with Vitamin C in patients can prove beneficial in long term treatment regimes to avoid oxidative stress induced side effects especially in the liver of patients. Future studies should be conducted to elaborate the transcriptomes and metabolomes involved in antioxidative pathways of Vitamin C.

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Authors Contribution

NY: Idea conception, study designing, data analysis, results and interpretation, manuscript writing and proofreading
TK: Idea conception, study designing, data collection, data analysis, results and interpretation, manuscript writing and proofreading
RA: Data collection, data analysis, results and interpretation, manuscript writing and proofreading
ABA: Study designing, data analysis, results and interpretation, manuscript writing and proofreading
AR: Data collection, data analysis, results and interpretation, manuscript writing and proofreading
SM: Study designing, data analysis, results and interpretation, manuscript writing and proofreading
RN: Idea conception, data analysis, results and interpretation, manuscript writing and proofreading