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Whey Protein Concentrate Ameliorates the Methotrexate-Induced Liver and Kidney Damage

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Abstract

Methotrexate (MTX) is a cytotoxic immunosuppressant that is widely used in the treatment of tumours, rheumatoid arthritis, and psoriasis. This study aims to evaluate the effects of whey proteins on MTX-induced liver and kidney damage by focusing on oxidant-antioxidant systems and eating habits. The study was conducted in four groups of 30 Sprague-Dawley rats (control, control+whey protein concentrate (WPC), methotrexate, methotrexate+WPC. A single dose of 20 mg/kg MTX was administered intraperitoneally to the MTX groups. Control and MTX groups were given 2 g/kg WPC by oral gavage every day for ten days. At the end of day 10, blood samples were drawn and liver and kidney tissues were removed. MTX administration increased the lipid peroxidation level and decreased glutathione level, superoxide dismutase and glutathione-S-transferase activities in the liver and kidney. Administration of WPC significantly reduced the damage caused by MTX in the liver and kidney. While a decrease in serum urea level and an increase in serum creatinine level were detected in the MTX group, WPC administration reversed these results up to control group levels. Administration of WPC to the MTX group significantly reversed the histopathological damage scores of the liver and kidney. Whey protein concentrate administration ameliorated the MTX-induced oxidative damage in the liver and kidney tissues due to its antioxidant properties. Liver and kidney damage can be prevented by using whey proteins as a nutraceutical in MTX therapy.

In conclusion, whey proteins demonstrated a protective effect against MTX-induced liver and kidney damage.

Keywords: Methotrexate, whey proteins, oxidative stress, liver, kidney

INTRODUCTION

Methotrexate (MTX, 4-amino-N10-methyl folic acid), an antagonist of folic acid, is commonly used as a chemotherapeutic drug to treat a variety of cancers and inflammatory diseases [(1)]. It is converted to 7-hydroxy MTX in the liver. Both MTX and 7-hydroxy MTX are excreted by the kidneys and a small part is also excreted in the bile [(2)]. MTX is used in dose-dependent manner, which demonstrates that the treatment dose varies depending on the disease to be treated. [(3)]. Due to dose-dependent hepatotoxicity and nephrotoxicity, MTX's clinical usage is restricted. Even at modest dosages, MTX can cause hepatic fibrosis and cirrhosis as a significant adverse effect. The use of low-dose MTX in the treatment of psoriasis was found to increase the risk of cirrhosis by 7%. In 8% of the patients, transaminase levels were found to be three times higher than usual [(4; 5)]. The pathogenesis of MTX-induced renal impairment is thought to be mediated by either MTX and its metabolites precipitating in the renal tubules or MTX's direct toxic impact on the renal tubules [(6)]. Although the actual mechanisms of MTX-induced liver and kidney damage are unknown, one of the hypotheses is the breakdown in cellular antioxidant defences, which increases the production of reactive oxygen (ROS) and nitrogen species, inhibits cytosolic NADP-dependent dehydrogenase and NADP malic enzyme, lowers glutathione, superoxide dismutase, and catalase levels, and reduces the effectiveness of the antioxidant defence system protecting the cell against ROS [(7)]. Endogenous antioxidant defence systems against oxidative stress appear to benefit from dietary antioxidant consumption [(8)]. Milk proteins can produce peptides with antioxidant properties. Whey (also known as lactoserum) is a turbid pale yellow-green liquid formed after the casein in milk has been coagulated by the action of a protease enzyme or by acid treatment [(9)]. Whey fraction of milk contains whey proteins (β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin, glycomacropeptide, lactoferrin and lactoperoxidase) [(10)] and bioactive peptides [(11)]. Whey proteins can be utilized as functional foods to improve human health and prevent diseases like cancer, cardiovascular disease, diabetes mellitus, gut function disturbances, obesity management, and muscle synthesis augmentation [(12; 13)]. Mansour et al reported that whey protein isolate ameliorates cyclophosphamide-induced liver and kidney damage in rats via its antioxidant and anti-inflammatory activities [(14)]. Athira et al. demonstrated the protective effect of whey protein hydrolyzate against oxidative damage caused by paracetamol [(15)]. In these studies, the mechanism for the antioxidant effects of whey proteins has been related to the inhibition of lipid peroxidation, scavenging of reactive oxygen species, and chelation of transition metals.

Accordingly, this study aimed to investigate the possible antioxidant mechanism of whey protein concentrate against MTX-induced hepatic and renal damage in rats.

MATERIALS AND METHODS

Materials

Methotrexate was obtained from David Bull Laboratories, Mulgrave-Victoria, Australia. Whey protein beverage (Tazelen) was obtained from Kaanlar Food Industry and Trade, Turkey, and standard rat chow was purchased from MBD Feed Manufacturer, Turkey. All chemicals used were in analytical grade and were obtained from Sigma-Aldrich.

Animals and Experimental Design

The study was approved by the Marmara University School of Medicine Animal Care and Use Committee (Protocol Number: 55.2021.mar). Male Sprague-Dawley rats weighing 250 to 300 g were housed in wire-bottom cages in a constant temperature room of 22 ± 2°C with 12-hour light and dark cycles and fed standard rat chow.

The thirty rats were divided into the following four groups control (C), whey protein concentrate-treated control (C+WPC), methotrexate administration (MTX), whey protein concentrate treated methotrexate administered (MTX+WPC) groups. In experimental models for the induction of tissue damage, 20 mg/kg methotrexate was a commonly used dose for rats [16; 17; 18; 19; 20].

Following MTX injection (in physiological saline, 20 mg/kg, single dose), either saline (MTX group, $n = 8$) or whey protein concentrate (2 g/kg, oral gavage, MTX + WPC group; $n = 8$) was administered for the consecutive 10 days. In other rats, saline (C group, $n = 6$) or whey protein concentrate (2 g/kg, oral gavage C+WPC group, $n = 8$) was administered for 10 days, following a single dose of saline injection. Trunk blood samples were drawn and liver and kidney tissue samples were taken on day 10.

Preparation of Whey Protein Concentrate

The whey protein beverage was lyophilized using a VIRTIS (SP Industries Inc., USA) freeze drier at -50°C under a vacuum. 50 ml whey protein beverage was approximately 20 g after lyophilization. WPC was orally administered in a dose of 2 g/ kg. [21; 22]. The administered dose of WPC provides less than 1% contribution to the daily energy intake of rats.

Biochemical Analysis

Serum urea and creatinine levels were measured according to the methods of Rosenthal and Slot [23; 24]. Liver and kidney tissues were homogenized in physiological saline. A motor-driven tissue homogenizer (IKA Ultra-Turrax T25 Basic; Labortechnic, Staufen, Germany) was used to homogenize the tissue samples. The homogenates were centrifuged at 3000 x g for 10 minutes at 4 ^{o}C. The supernatant was used for the analysis of lipid peroxidation (LPO) [25], nitric oxide (NO) [26], glutathione (GSH) [27], glutathione-S-transferase (GST) [28], superoxide dismutase (SOD) [29], tissue factor (TF) [30] activities. SDS-polyacrylamide gel electrophoresis was also carried out in the liver and kidney tissues according to the method of Laemmli [31].

Histological Analysis

For light microscopic investigation, liver and kidney tissue samples were fixed in 10% neutral buffered formaldehyde. After routine tissue processing, samples were embedded in paraffin. Approximately 5-µm-thick sections were stained with hematoxylin and eosin (HE) and at least five microscopic areas in each sample were examined for morphologic analysis. Histopathologic scoring for the liver was performed by the following criteria: degeneration of hepatocytes, vascular congestion and sinusoidal dilatation, inflammatory cell infiltration and the number of activated Kupffer cells. For the kidney, degeneration of glomerular structure and dilatation of Bowman's space, degeneration of proximal and distal tubule, vascular congestion and inflammatory cell infiltration were taken into consideration.

The microscopic scores of liver and kidney tissues were calculated as the total of the scores given to each criterion. Each of the criteria was scored semiquantitatively as 0: none; 1: mild; 2: moderate, 3: severe). The maximum total score for both tissues was calculated as "12". Staining sections were examined and photographed with a digital camera (Olympus DP72, Tokyo, Japan) attached to a photomicroscope (Olympus BX51, Tokyo, Japan).

Statistical analysis

Statistical analyzes were performed using GraphPad Prism 6.0 package program (GraphPad Software, San Diego, CA, USA). Results were presented as mean and standard deviation (*SD*). The normality of the distribution of all data was determined. The data showed a normal distribution, thus parametric tests were used. "One-Way Analysis of Variance" (One-Way ANOVA) was used to compare the means of more than two groups and identify differences. The post hoc Tukey test was also utilized to analyze the differences in variable subgroups.

The cutoff point for significance was applied to interpretations, where $p < 0.05$ was regarded as significant.

RESULTS

Nutritional Analysis of Whey Protein Concentrate

The nutritional components of whey protein concentrate were presented in Table 1.

Rat Chow Consumption and Body Weights

The chow consumption of rats significantly decreased in the MTX group compared to the control group. Whey protein concentrate administration to the MTX group caused a significant increase in chow consumption compared to the MTX group. Administration of whey protein concentrate to the control group also caused a significant increase in chow consumption (Table 2).

There was no significant difference between the weights of the animals at the beginning of the experiment. When the weights were evaluated on day 10, MTX administration caused a significant weight reduction compared to the control group. WPC administration to the MTX group caused significant weight gain (Table 2). Although WPC administration caused weight gain in the MTX group, this increase was significantly lower than in the WPC-given control group (Table 2).

Biochemical Analysis

Serum urea and creatinine levels

While the serum urea level of the MTX group significantly decreased, the serum creatinine level significantly increased compared to the control group (Table 2). Administration of WPC to the MTX group brought the urea and creatinine levels to the levels of the C+WPC group. The application of whey protein concentrate to the control group significantly decreased the urea and creatinine levels compared to the control group but the urea and creatinine levels were still within the normal range (Table 3).

Liver Results

When compared to the control group, the liver LPO level significantly increased in the MTX group. GSH level, SOD and GST activities were significantly decreased, and NO level and TF activity did not significantly change in the MTX group compared to the control group. WPC administration to both MTX and C groups significantly decreased the LPO level. The LPO level of the MTX+WPC group was significantly higher than the C+WPC group. Whey

protein concentrate administration to the MTX group also significantly increased GSH level, SOD, GST and TF activities and significantly decreased the NO level compared to the MTX group. Whey protein administration to the C group also increased the GSH level, SOD, GST and TF activities compared to the control group (Fig 1).

Kidney Results

LPO level was significantly increased and GSH level, SOD and GST activities significantly decreased in the MTX group compared to the C group. WPC administration to control group decreased NO level, increased GSH level and GST activity. WPC administration to the MTX group significantly decreased LPO level and TF activity, and significantly increased GSH level, SOD and GST activities (Fig 2).

SDS Polyacrylamide Gel Electrophoresis

Fig. 3 shows the electrophoretic patterns of liver and kidney tissue in all groups. While no significant changes were detected in the intensity of the liver protein bands, differences in the density of the kidney protein bands were detected in the electrophoretic examination of the kidney and liver tissue. Changes in the protein profile of the kidney tissue occurred in the form of decreased protein band density with MTX administration and increased protein band density with the WPC administration in the control and MTC groups.

Histological Analysis

Light microscopic evaluation of the control and C+WPC groups revealed normal liver morphology including the regular arrangement of hepatocytes and sinusoids (Fig. 4A and 4B). In the MTX group, severe vacuolar degeneration and pyknotic nucleus in hepatocytes, increased number of activated Kupffer cells, inflammatory cell infiltration and marked sinusoidal dilatation and congestion were prominent features of the morphologic damage (Fig. 4C). On the other hand, reduction in the number of activated Kupffer cells and inflammatory cell infiltration, diminished sinusoidal dilatation and congestion in addition to mild vacuolar degeneration in hepatocytes were observed in the MTX+WPC group (Fig. 4D). MTX treatment significantly increased the histopathologic score of liver tissue compared with control and C+WPC groups ($p<0.05$; Fig. 4I). A higher histopathologic score of liver tissue in the MTX group was significantly reduced by treatment with WPC ($p <0.05$; Fig. 4I). In control and C+WPC groups, regular kidney parenchyma including glomeruli and tubules were observed. (Fig. 4E and 4F). Methotrexate treatment caused a prominent dilatation of tubules and degeneration in tubular cells, marked vascular and glomerular congestion,

dilatation in Bowman's space, and inflammatory cell infiltration in the interstitium (Fig 4G). On the other hand, mild glomerular and vascular congestion, regression in the dilatation of Bowman's space and inflammatory cell inflammation in the interstitium and improvement in dilated tubules and degenerated tubular cells were observed in the MTX+WPC group (Fig 4H). The histopathologic score of kidney tissue in the MTX group was significantly increased compared with control and C+WPC groups ($p < 0.05$; Fig. 4J). Administration of WPC to MTX group significantly reversed this histopathologic score ($p < 0.05$; Fig. 4J).

DISCUSSION

The findings of this study show that MTX treatment causes oxidative tissue damage in the liver and kidney, as measured by increased lipid peroxidation and decreased GSH levels, SOD and GST activities whereas whey protein administration protects against this oxidative damage. Histological findings also supported these findings, demonstrating the severity of the damage induced by MTX and the ameliorative effect of whey proteins on this damage. Histological findings supported these findings, demonstrating the severity of the MTX-induced damage and the ameliorative effect of whey proteins on this damage.

The anti-metabolite drug MTX is frequently used to treat cancer and inhibit the immune system, however, organ damage during MTX treatment reduces its therapeutic effectiveness[32]. There are many potential processes underlying MTX toxicity[33] but no effective treatments to ameliorate the condition exist. In addition to being administered as a drug to cause oxidative stress, MTX has also been used to induce cachexia, anorexia, intestinal mucositis, and digestive absorption problem in experimental animal models [34; 35; 36]. There are some findings regarding the effects of MTX on the gastrointestinal system. Fox et al. reported that MTX caused weight loss in rats without causing enteritis, while Jahovic et al. reported that MTX caused weight loss due to malabsorption and enterocolitis in rats [37; 38]. In this study, while enteritis was not observed in MTX-treated rats, severe appetite loss was. WPC administration increased the feed consumption in MTX-treated rats. As a result of the prevention of appetite loss, weight gain has been observed in WPC-administered MTX-treated rats. Giving WPC to healthy rats also increased chow consumption.

While there are studies on the role of MTX-induced oxidative stress in liver and kidney damage [38; 39; 40; 41] there is no study related to the effect of whey proteins on MTX-induced liver and kidney damage.

In this study, MTX administration increased lipid peroxidation and decreased GSH levels, SOD and GST activities and did not change NO levels in liver and kidney tissues. It is thought that the reason for detecting the NO level as unchanged may be related to the administration of a single dose of MTX or the 10-day trial period. Regarding liver and kidney damage in MTX treatment, Bedoui stated that MTX hepatotoxicity may occur due to the depletion of folate reserves and methotrexate-polyglutamate formation in the liver [(42)]. Li et al also stated that MTX-induced oxidative stress may cause glomerular and tubular damage by causing MTX accumulation in the kidney [(43)].

Since MTX induces oxidative stress by increasing reactive oxygen species in tissues [(38; 40; 44)] the effects of various antioxidant substances against MTX-induced oxidative stress have been investigated [(7; 38; 44; 45; 46)].

Jahovic et al. determined that melatonin decreased lipid peroxidation levels and increased GSH levels in hepatorenal oxidative damage induced by MTX [(38)]. In the study of Çetinkaya et al., they stated that N-acetylcysteine decreased lipid peroxidation in the liver tissue and increased GSH level and SOD activity in the MTX-induced oxidative stress [(47)] Abdel-Daim et al. showed that MTX-induced liver, kidney and heart damage was ameliorated with a flavonoid derivative diosmin [(48)]. Similar to these studies, there are studies in which vitamin C [(7)], resveratrol [(45)], quercetin [(46)], berberine [(49)] and gallic acid [(44)] were used to treat MTX-induced tissue damage. In this study whey proteins, which are known to have antioxidant properties, ameliorated MTX-induced oxidative damage by decreasing MDA level, increasing GSH level, SOD and GST activities. This progress was also supported by the histological findings. In addition, it was determined that the whey proteins given to the control group rats did not adversely affect the liver and kidney tissues.

TF activity was another parameter investigated in this study. Tissue factor (FIII) is a coagulation protein that is involved in the coagulation mechanism's extrinsic pathway. A variety of clinical conditions can cause TF expression in monocytes and endothelium, which can lead to thrombotic consequences. Pathological conditions caused by infection or disease can raise TF levels in the blood, which can then activate the coagulation mechanism [(50)]. In this study, TF activity did not change in MTX-induced liver and kidney damage, whey protein application to this group increased liver TF activity and decreased kidney TF activity. Since there is no study related to the effect of whey proteins on TF activity in MTX-induced liver and kidney damage, the obtained TF activity findings should be taken into consideration during whey protein use.

In many experimental animal models, MTX has been found to increase serum urea and creatinine levels [16; 51; 52], but there are also studies showing that MTX reduces serum urea concentration [53; 54; 55]. According to Severin et al., serum urea concentration increased for the first four days after MTX administration, then decreased and returned to normal levels by the eighth day. The low urea level, obtained at the end of 10 days of this study, is consistent with the findings of Severin et al. In this study, MTX administration decreased serum urea levels while increasing serum creatinine levels compared to the control group. Although the serum urea concentration appeared to be lower than in the control, it was still within the normal range. Low blood urea levels are often associated with malnutrition but liver damage can also decrease urea synthesis, as urea synthesis occurs in the liver. The administration of whey proteins restored the urea level, which had been decreased as a result of MTX administration, and also reduced the increased creatinine level.

Boukhettala et al. revealed that MTX treatment altered protein metabolism [34]. In their study, MTX administration reduced protein synthesis in the jejunal mucosa of rats; it also increased proteolysis, particularly in the lysosomal pathway. In this study, when the protein profile of the liver and kidney were investigated with SDS-PAGE, MTX did not change the liver protein profile, but it caused a decrease in some protein bands in the kidney tissue. WPC administration to the MTX group improved the deteriorated protein profile of the kidney tissue and did not change the liver protein profile. This finding indicates that whey proteins help the compensation of the decreased kidney proteins.

It is thought that bioactive peptides derived from whey proteins as a result of gastrointestinal digestion or bioactive peptides in the whey protein mixture with antioxidant properties may also play a role in improving liver and kidney damage caused by MTX.

In conclusion, whey proteins demonstrated a protective effect against MTX-induced liver and kidney damage. Whey proteins and their bioactive protein contents can provide nutritional support to MTX treatment.

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Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

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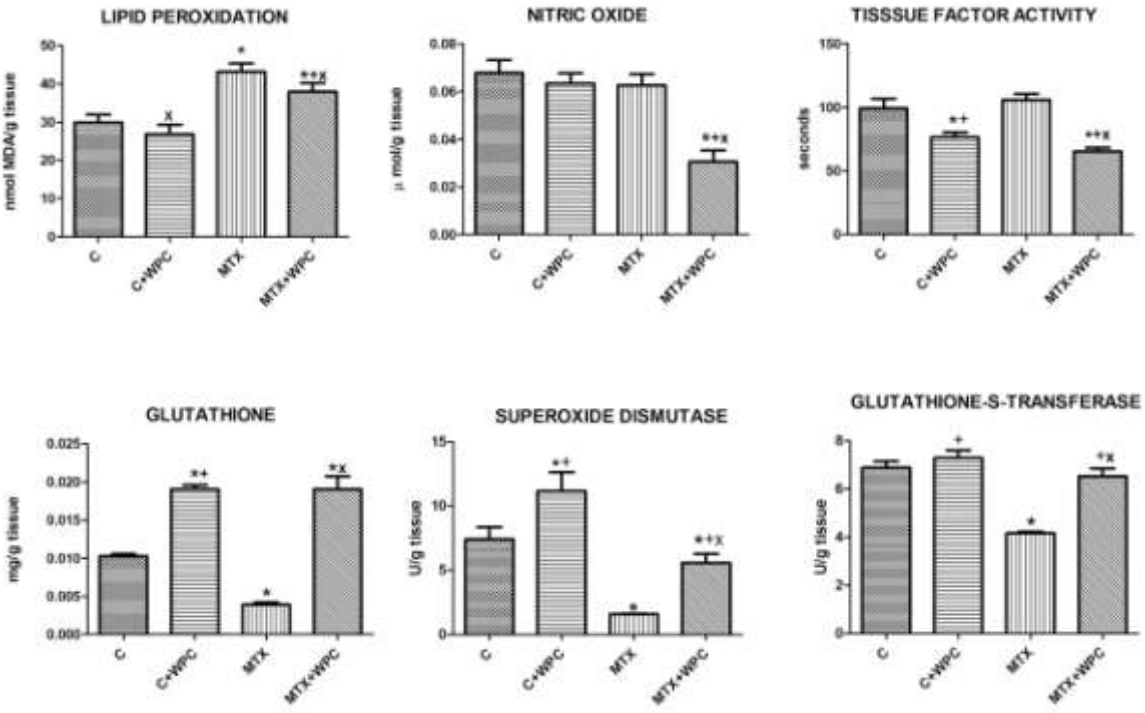

Figure 1: MDA, GSH, NO levels, SOD, GST and TF activities of liver tissue

C: Control group**, C+WPC:** Whey protein concentrate administered control group **MTX:** Methotrexate administered group**, MTX+WPC:** Methotrexate and whey protein concentrate administered group **MDA:** Malondialdehyde, **GSH:** Glutathione, **SOD:** Superoxide dismutase,

GST: Glutathione-S-transferase, **NO:** Nitric oxide, **TF:** Tissue factor

(*): $p<0.05$ compared to control group, **(x):** $p<0.05$compared to MTX group, **(+):** $p<0.05$ compared to C+WPC group, n=8.

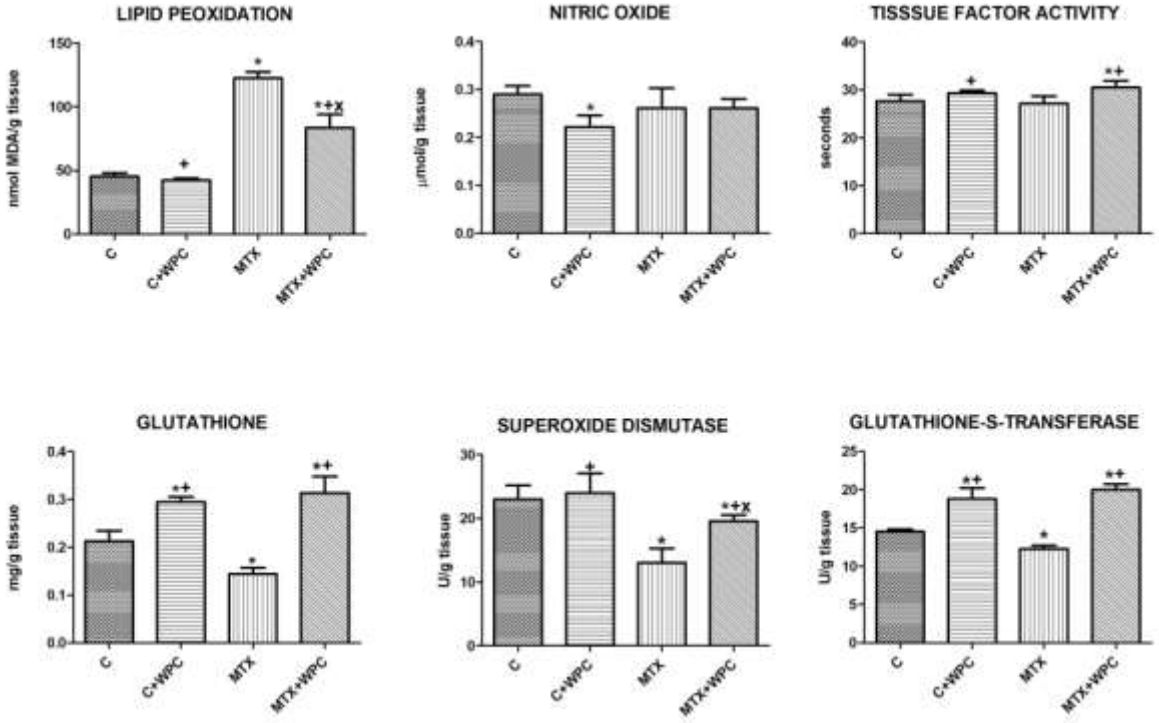

Figure 2: MDA, GSH, NO levels, SOD, GST and TF activities of kidney tissue

C: Control group**, C+WPC:** Whey protein concentrate administered control group **MTX:** Methotrexate administered group**, MTX+WPC:** Methotrexate and whey protein concentrate administered group, **MDA:** Malondialdehyde, **GSH:** Glutathione, **SOD:** Superoxide dismutase, **GST:** Glutathione-S-transferase, **NO:** Nitric oxide, **TF:** Tissue factor

(*): $p<0.05$ compared to control group, **(x):** $p<0.05$compared to MTX group, **(+):** $p<0.05$ compared to C+WPC group, n=8.

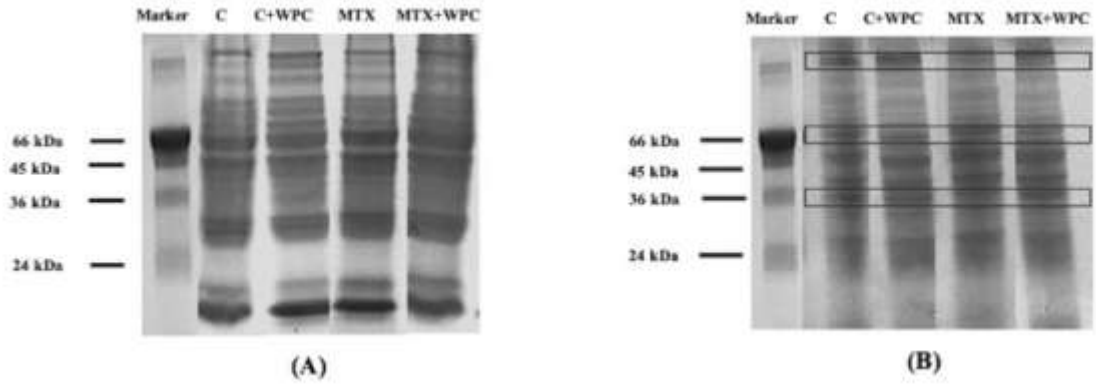

Figure 3: Electrophoretic Pattern of Methotrexate and Whey Protein Concentrate administered liver and kidney proteins

C: Control group**, C+WPC:** Whey protein concentrate administered control group **MTX:** Methotrexate administered group**, MTX+WPC:** Methotrexate and whey protein concentrate administered group

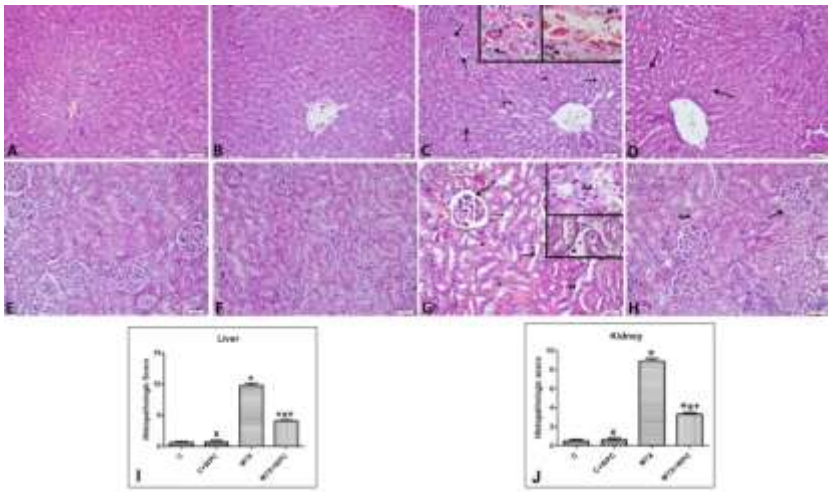

Figure 4: Representative light micrographs of liver and kidney tissues in experimental groups. Regular liver parenchyma in control and C+WPC groups (**A and B**). In MTX group (**C**)**,** degenerated hepatocytes (arrows), marked sinusoidal dilatation and congestion (**), increased number of activated Kupffer cells (broken arrows), inflammatory cell infiltration (arrowheads). In the MTX+WPC group (**D**), improvement in hepatocyte structure (arrows) besides mild vacuolar degeneration in hepatocytes in some regions of the liver parenchyma. Regular kidney morphology in control and C+WPC groups (**E, F**). In the MTX group (**G**), marked glomerular congestion and dilatation in Bowman's space (arrow), inflammatory cell inflammation (arrowhead), severe vascular congestion (*) and tubular degeneration (broken arrows). In the MTX+WPC group (**H**) normal glomerular (arrow) and tubular (broken arrow) structures in most regions of the kidney. A-H; HE staining, bar: 50 μm inset: 20 μm. The graph of the histopathologic score of the liver (**I**) and kidney (**J**) tissue in experimental groups.

(*): p<0.05 compared to control group, **(x):** p<0.05compared to MTX group, **(+):** p<0.05 compared to C+WPC group**,** n=8

Table 1: Nutritional Components of Whey Protein Concentrate

Nutritional components	**Concentrations**
Energy	70 kcal/100 g
Protein	10,40 g/100g
Carbohydrate	5.79 g/100g
Fat	0.44 g/100g
Diet Fiber	0.78 %
Ash	0.95 %
L-Alanine (Ala)	455 (mg/100g)
L-Aspartic Acid (Asp)	915 (mg/100g)
L-Methionine (Met)	166 mg/100g
L-Glutamic Acid (Glu)	1197 mg/100g
L-Phenyalanine (Phe)	218 mg/100g
L-Lysine (Lys)	1179 mg/100g
L-Histidine (His)	224 mg/100g
L-Tyrosine (Tyr)	201 mg/100g
Glycine (Gly)	166 mg/100g
L-Valine (Val)	344 mg/100g
L-Leucine (Leu)	725 mg/100g
L-Isoleucine (Ile)	435 mg/100g
L-Threonine (Thr)	758 mg/100g
L-Serine (Ser)	486 mg/100g
L-Proline (Pro)	387 mg/100g
L-Arginine (Arg)	153 mg/100g
Vitamin A	21.9 µg/100g
Vitamin E	0.77 mg/100g
Vitamin B6	0.022 mg/100g
Calcium (Ca)	517.6 mg/kg

Table 2: Rat Chow Consumption and Body Weights

	C (n=6)		**C+WPC (n=8)**		**MTX (n=8)**		**MTX+WPC (n=8)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Rat Chow Consumption (g/animal)	17.56	0.44	24,45	0,85*^{x}	15,41	0,27*	23,36	0,89 *^{x}
Body Weights (g)	264	5.5	262	13.4^{x}	229.5	17.7*	250.7	4.7^{x}

SD: Standard deviation **C:** Control group**, C+WPC:** Whey protein concentrate administered control group **MTX:** Methotrexate administered group**, MTX+WPC:** Methotrexate and whey protein concentrate administered group

(*): $p<0.05$ compared to control group, **(x):** $p<0.05$compared to MTX group.

Table 3: Serum Urea and Creatinine levels

	C (n=6)		C+WPC (n=8)		MTX (n=8)		MTX+WPC (n=8)	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Urea (mg/dL)	28.1	0.83	23.0	1.41 *+	20.2 *	1.17	24.0 +*	0.32* x
Creatinine (mg/dL)	0.77	0.04	0.47	0.03 *+	1.03 *	0.06	0.51	0.07* x +

SD: Standard deviation **C:** Control group**, C+WPC:** Whey protein concentrate administered control group **MTX:** Methotrexate administered group**, MTX+WPC:** Methotrexate and whey protein concentrate administered group

(*): $p<0.05$ compared to control group, **(x):** $p<0.05$compared to MTX group.