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Histological and Histochemical Examination of the Liver in Male Rats Following Whey Protein Feeding

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***Abstract*—** Whey protein is widely used as a nutritional supplement because of its high biological value, rapid digestibility, and rich content of essential and branched-chain amino acids. However, its hepatic effects may vary according to dose and duration of administration. This study aimed to evaluate the histological and histochemical alterations in the liver of adult male rats following six weeks of whey protein feeding. Fifteen adult male albino rats were randomly divided into three equal groups (n = 5 per group): a control group receiving distilled water, a low-dose whey protein group receiving 5 g/kg body weight, and a high-dose whey protein group receiving 10 g/kg body weight. Liver specimens were processed using the routine paraffin technique. Hematoxylin and Eosin staining was used to assess general hepatic architecture, hepatocyte arrangement, steatosis, inflammatory infiltration, and cellular alterations, while Masson's Trichrome staining was applied to evaluate collagen deposition and fibrotic remodeling. The control group showed well-preserved hepatic lobular architecture, regularly arranged hepatocyte cords radiating from the central vein, and no evidence of steatosis, inflammation, necrosis, or fibrotic change. In the 5 g/kg whey protein group, liver sections showed mild to moderate macrovesicular steatosis with focal inflammatory cell infiltration, while the general hepatic architecture remained relatively preserved. Masson's Trichrome staining revealed moderate collagen deposition, mainly in the perisinusoidal and portal/periportal regions. In contrast, the 10 g/kg whey protein group exhibited marked macrovesicular steatosis, large cytoplasmic vacuolation, peripheral displacement or fading of hepatocyte nuclei, extensive inflammatory infiltration, disruption of hepatocyte cords, and more advanced collagen deposition with bridging fibrotic changes. These findings indicate that six weeks of whey protein feeding induced dose-dependent histological and histochemical liver alterations in male rats. The lower dose produced moderate steatotic, inflammatory, and fibrotic changes, whereas the higher dose caused more severe hepatic tissue injury, architectural disruption, and advanced fibrotic remodeling. The results suggest that excessive whey protein intake may impose a progressive hepatic tissue burden under the conditions of this experimental model.

Keywords **—** Whey protein, liver, male rats, histology, histochemistry, Hematoxylin and Eosin, Masson's Trichrome, steatosis, collagen deposition, fibrosis.

INTRODUCTION

The liver is one of the most metabolically active organs in mammals and is a main player in carbohydrate, lipid and protein metabolism, detoxification, bile production, plasma protein synthesis as well as regulation of systemic energy balance. Due to these functions, the hepatic tissue is extremely sensitive to the changes that occur in the nutritional status and may present early microscopic changes in response to nutritional supplementation. The rats are a popular laboratory model for experimental study as they are age-controlled, weight-controlled, and the amount of food they eat can be controlled and physiological responses can be measured easily to investigate organ-specific responses to nutritional interventions (1, 2). The liver is a highly organized organ in terms of its microarchitecture, which is related to the metabolic and detoxifying functions. The liver lobule is built around a central vein and the hepatocytes are in the form of plates or cords, between which are hepatic sinusoids. In this manner, efficient exchange of nutrients, oxygen, metabolites and waste products can occur between blood and the hepatocytes. Thus, alterations in hepatocyte organization, sinusoidal spaces, infiltration of inflammatory cells, cytoplasmic vacuolation, and connective tissue deposition can be a sign of hepatic damage or adaptation before there are any detectable biochemical changes (2).

Protein is an indispensable macronutrient which is necessary for tissue growth and repair, enzyme production, immune function and nitrogen balance. The quality of dietary protein is based on its amino acid composition, digestibility and bioavailability. Whey protein is a nutritional supplement with a high biological value, is very easy to digest and provides a large amount of essential amino acids, particularly branched-chain amino acids. But, dosage, period of treatment, and form of supplement, and metabolic state of the organism may influence the physiological response to whey protein

(3,4). While whey protein has shown some beneficial metabolic, antioxidant and lipid-regulatory effects in some models, excessive or prolonged consumption could be a metabolic burden to the liver. Amino acid catabolism, nitrogen disposal, lipid handling and oxidative balance are the major functions of the liver. Thus, it is possible that under specific experimental conditions high dose protein supplementation can increase the workload of the liver, and it can lead to cellular stress, oxidative damage, fat deposition in the liver, inflammatory response, and fibrotic remodeling (4, 5). The effect of whey protein on the liver has been previously demonstrated in various experiments to be context-dependent. In another rat study, whey protein supplementation was shown to have a protective effect on liver damage scores in rats fed a high-fat/high-fructose diet under certain metabolic conditions (6). However, other experimental studies have shown that the use of whey protein can also be linked to inflammatory and apoptotic liver reactions in particular when whey protein is not modified by physical exercise and when high doses of whey protein are taken (5). The results suggest that whey protein should not be viewed as generally beneficial or detrimental as its hepatic impact is dependent on dosage, duration of consumption, metabolic status and experimental design. Histological and histochemical techniques are very important for the assessment of direct tissue effect of nutritional supplementation on the liver. Hematoxylin and Eosin staining can be used to determine the hepatic architecture, hepatocyte cords, central vein areas, steatosis, inflammatory infiltrate and cell damage. Masson's Trichrome, on the other hand, is a histochemical stain that can be used to assess collagen deposition and fibrotic remodeling (2). Collagen staining is useful to detect early and advanced fibrotic changes in the hepatic tissue, as hepatic fibrosis is largely associated with activation of hepatic stellate cells and excessive extracellular matrix deposition (7). In the present study, the effect of whey protein supplementation on the liver of male rats was studied histologically and histochemically. Liver sections were stained with Hematoxylin and Eosin stain (HE) to assess general tissue architecture and hepatocellular changes, and Masson's Trichrome stain (MT) for assessment of deposition of collagen fibers and remodeling of fibrosis. The two doses of whey protein were 5 g/kg and 10 g/kg body weight for 6 weeks. This design was employed to establish if there are dose-dependent changes in the liver at the microscopic and histochemical levels due to whey protein.

In the present study, the objective was to assess the histological and histochemical effects of whey protein supplementation in adult male rats, after 6 weeks of oral administration of two different doses.

MATERIALS AND MTHODS

Study Design and Experimental Animals

This experimental study was designed to evaluate the histological and histochemical effects of whey protein feeding on liver tissue in adult male albino rats. A total of fifteen adult male albino rats were used in the final analysis and randomly allocated into three equal experimental groups, with five rats in each group (n = 5/group). The first group served as the control group and received distilled water as vehicle only. The second group received whey protein at a dose of 5 g/kg body weight, while the third group received whey protein at a dose of 10 g/kg body weight. Whey protein was freshly dissolved in distilled water before administration and given orally by gastric gavage once daily for six consecutive weeks. The administered dose was calculated according to the body weight of each animal.

Table 1. Experimental design and animal grouping

Group	Treatment	Dose	Route of administration	Duration	Number of rats
Control group	Distilled water	Vehicle only	Oral gavage	6 weeks	5
Whey protein 5 g/kg group	Whey protein dissolved in distilled water	5 g/kg body weight	Oral gavage	6 weeks	5
Whey protein 10 g/kg group	Whey protein dissolved in distilled water	10 g/kg body weight	Oral gavage	6 weeks	5

Ethical Considerations

All the animal procedures were performed following institutional guidelines for care and use of laboratory animals after ethical approval obtained from Al-Qasim Green University. Animal welfare was kept to a minimum and the number of animals used to an absolute minimum. The animal care and reporting were conducted in accordance with accepted guidance on the care of laboratory animals and ARRIVE 2.0.

Liver Tissue Collection

After the 6-week experimental period, animals were fasted for 8-12 hours and on the day of the experiment, animals were anesthetized with ketamine 80 mg/kg and xylazine 10 mg/kg intraperitoneally. Liver samples were immediately taken after sacrifice and used for histological and histochemical analysis. Small samples of representative liver tissue were removed, washed in normal saline to remove blood residues and impurities and then fixed.

Histological Tissue Processing

In order to preserve the architecture of the liver specimens and avoid postmortem autolysis, liver specimens were fixed in 10% neutral buffered formalin for 24-48 hours. The samples were fixed, then dehydrated in increasing alcohol (70, 80, 90 and two changes of absolute alcohol). The dehydrated specimens were then put into xylene for about 30 minutes to 1 hour until the tissue became transparent for paraffin infiltration. After clearing, the specimens were infiltrated with molten paraffin wax in an oven at 58°C for two hours with two changes of paraffin wax. The tissues were then put into fresh molten paraffin wax, oriented, and left to cool at room temperature and then stored until sectioning.

The sectioning and preparation of slides

Using a rotary microtome, the paraffin blocks were sectioned. Serial sections of liver were cut at 5-7 micron and

floated on warm water bath to remove wrinkles and mounted. The sections were then placed on clean glass slides which had been treated with Mayer's albumin adhesive. To make sure tissue sections were well dried and mounted, the slides were placed in an oven at 40°C for 24 hours before staining.

Hematoxylin and Eosin Staining

Liver samples were stained with Harris hematoxylin and eosin for routine histological examination. Sections were initially deparaffinized in xylene and rehydrated by a series of decreasing alcohol concentrations to water. The nuclei were stained with hematoxylin, washed and differentiated as necessary. Eosin was then employed as counterstain for showing cytoplasmic and extracellular components. The sections were stained and then dehydrated using ascending grades of alcohol, cleared in xylene and permanently mounted in DPX. The general hepatic architecture, hepatic parenchyma, hepatocytes, central vein region, hepatocyte cord arrangement, cytoplasmic vacuolation, steatosis, infiltration of inflammatory cells and other microscopic tissue changes were evaluated by H&E staining.

Histochemical Study

Histochemical study of paraffin sections of liver was done to demonstrate connective tissue elements and to study the distribution of collagen fibers among the experimental groups. Masson's Trichrome stain was used as the special histochemical stain as it distinguishes the hepatocyte (parenchymal cells) from fibrous connective tissue. The distribution of collagen and the fibrotic remodeling in the liver tissue was evaluated by this stain, which highlights collagen in perivascular, periportal, peri-sinusoidal and bridging fibrotic regions.

Masson's Trichrome Staining

The paraffin liver sections were stained with Masson's Trichrome and were 5–7 µm thick. The sections were deparaffinized in two changes of xylene (5min each) and rehydrated in descending grades of ethyl alcohol (100%, 90%, 80% and 70%) for 2-3min in each grade and washed in distilled water. To increase the staining reaction and better differentiate the connective tissue elements, the sections were mordanted in Bouin's solution at 56°C for 45–60 mins. The slides were then cooled and washed with running tap water until the yellow colouration was removed.

The nuclei were stained in Weigert's iron hematoxylin for 10 minutes then washed in running tap water for 5 minutes. Staining was then done with Biebrich scarlet-acid fuchsin solution for 10-15 minutes to show the cytoplasmic and muscular elements. Differentiation was carried out in phosphomolybdic-phosphotungstic acid solution for about 10 minutes and staining was done with aniline blue solution for 5–10 minutes for the demonstration of collagen fibers. These sections were then treated with 1% acetic acid for 2-5 minutes to enhance color differentiation, quickly dehydrated by an ascending series of alcohol, cleared in xylene and permanently mounted in DPX. Collagen fibres were blue/blue green, cytoplasm/ muscle fibres were red/pink and nuclei were dark brown/ black in this stain.

Microscopic Examination and Image Documentation

The H&E- and Masson's Trichrome-stained liver sections were examined using a light microscope. Representative microscopic fields were selected from each experimental group and photographed using a Zeiss Primo Star 3 microscope equipped with a Zeiss Axiocam 208 color digital camera. Appropriate magnifications were used to document the histological and histochemical features of the liver sections.

Validity and Reliability of Histological and Histochemical Assessment

The validity of the histological assessment was supported by the use of standard and widely accepted staining techniques. Hematoxylin and Eosin staining was used for routine evaluation of hepatic architecture, hepatocyte arrangement, cytoplasmic vacuolation, steatosis, inflammatory infiltration, and cellular alterations. Masson's Trichrome staining was used as a specific histochemical method for demonstrating collagen fiber deposition and fibrotic remodeling. To improve reliability, all tissue samples were processed under the same fixation, dehydration, embedding, sectioning, staining, and mounting conditions. Liver sections were prepared at comparable thickness, examined under the same microscopic settings, and representative fields were selected consistently from each group. Fibrotic changes were evaluated on Masson's Trichrome-stained sections according to the NASH-CRN/Kleiner fibrosis staging system, based on the distribution and severity of collagen deposition in perisinusoidal, periportal, bridging, and nodular regions.

Fibrosis Assessment

Fibrosis was primarily evaluated on Masson's Trichrome stained sections based on NASH-CRN/Kleiner fibrosis staging system. The presence, distribution and severity of the collagen fiber deposition in the hepatic parenchyma, perisinusoidal areas, periportal areas, bridging regions and cirrhotic nodular formations were the criteria used in the evaluation. The fibrosis staging was defined as follows: Stage 1a/1c: Mild or delicate perisinusoidal fibrosis; stage 1b: Dense perisinusoidal fibrosis; stage 2: Perisinusoidal and periportal fibrosis; stage 3: Bridging fibrosis linking central veins or portal tracts; and stage 4: Cirrhosis with nodule formation.

RESULT

In Figure (1) liver sections of control group revealed normal hepatic organization with preserved lobular architecture. Hepatic sinusoids were well-distributed between the hepatocyte plates and the hepatocyte cords were arranged in regular radiating pattern around the central vein. Hepatocytes had normal polygonal shape with eosinophilic cytoplasm and central nuclei with no sign of fatty change, inflammatory infiltration, necrosis or architectural distortion. The results showed that the liver tissue of control group had normal microscopic characteristics and was used as a control group for comparison with the treated group. The liver sections of whey protein group at 5 g/kg showed mild to moderate fatty changes in the hepatic parenchyma. The vacuolar changes were predominantly focally distributed as areas of clear cytoplasm in individual hepatocytes with the general lobular architecture being largely retained. The

hepatocyte cords and central vein were still discernible and the fatty change did not cause significant disturbance of the hepatic architecture. This pattern indicates an early/early-mid adaptive response of the liver to the dose of whey protein used, with limited steatotic involvement when compared to the higher dose group as shown in Figure (2). The 5 g/kg whey protein group in Figure (3) showed inflammatory cell aggregation in the liver also in foci. These inflammatory foci were small and limited and did not show much invasion of surrounding hepatocyte cords. Although lobular inflammation was present, the overall lobular architecture was preserved in most instances and the parenchymal reaction was not as prominent as in the 10 g/kg group. This result suggests that the lower dose caused a mild inflammatory reaction related to focal involvement of the parenchymal tissue and not to diffuse damage of the liver. In Figure (4), fatty degeneration of liver sections of 10 g/kg group of whey protein was more evident than 5 g/kg group. The steatotic changes were widespread and affected a higher percentage of hepatocytes, with prominent clear cytoplasmic vacuoles, filling a large portion of hepatocyte cytoplasm. In some places, the vacuoles led to the dislodgement of nuclei towards the cell periphery, which is typical of macrovesicular steatosis. The increased density and distribution of vacuolated hepatocytes suggest a dose-dependent increase in the accumulation of lipids in the liver and alteration of the parenchymal structure. The inflammatory response in the 10 g/kg whey protein group was more severe and widespread than in the lower dose group as shown in Figure (5). In the affected lobules, large inflammatory cell aggregates were observed that disrupted the normal hepatocyte cord pattern, producing multifocal aggregates. Marked parenchymal involvement was associated with the inflammatory infiltrate, which was greater in response to the higher dose. The above modifications indicate that the higher dose of whey protein was positively related to a higher inflammatory response in the liver and a higher alteration of the normal microarchitecture.powder samples.

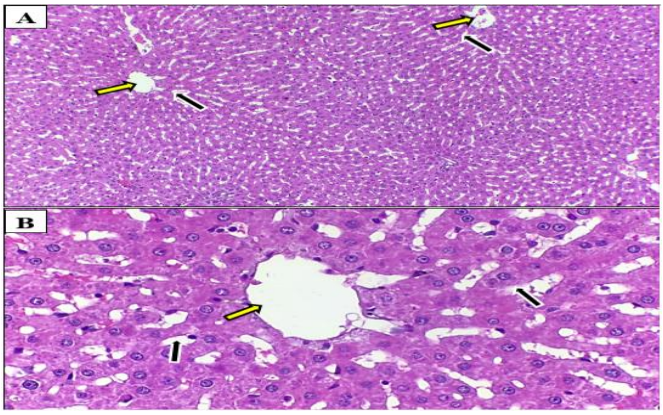

Figure 1. Cross section of liver tissue from the control group rat. A and B: Normal hepatic histological architecture was preserved. The hepatic lobular organization appeared clear, with hepatocyte cords (black arrow) radiating from the central vein (yellow arrow) toward the portal areas. H&E stain. A: 100×; B: 400×.

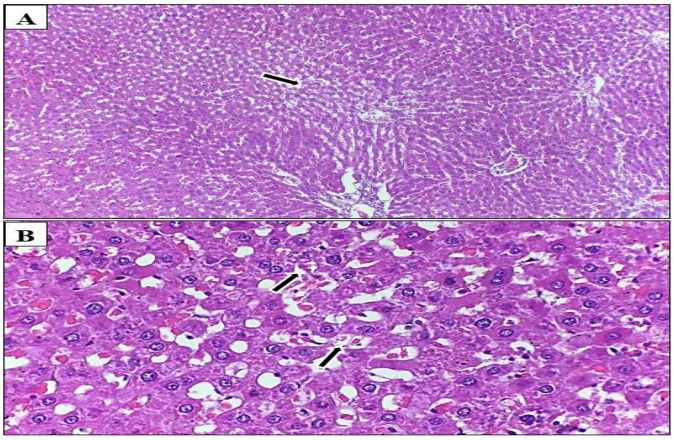

Figure 2 . Cross section of liver tissue from the whey protein 5 g/kg group rat. A: Mild to moderate fatty changes (black arrow) are evident as small clear spaces and cytoplasmic vacuoles scattered within the affected lobules. The lesion shows a focal distribution, with most of the hepatic architecture remaining preserved. B: Macrovesicular steatosis (black arrow) is characterized by distinct cytoplasmic vacuoles within hepatocytes. The degree of vacuolation is less severe than that observed in the 10 g/kg group. H&E stain. A: 100×; B: 400×.

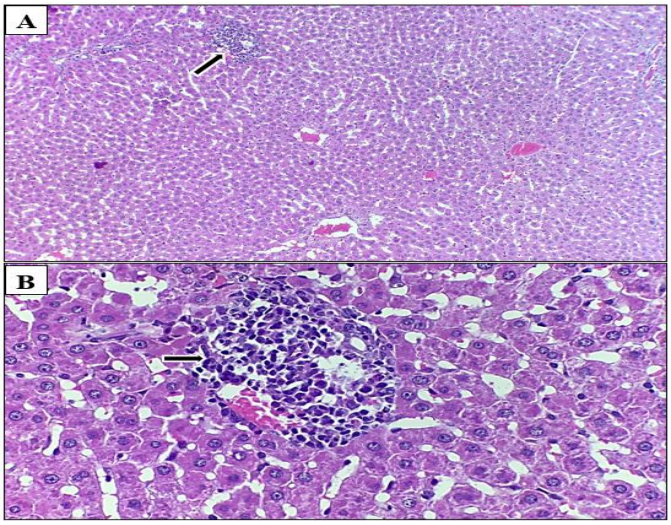

Figure 3. Cross section of liver tissue from the whey protein 5 g/kg group rat. A and B: Focal inflammatory cell aggregations (black arrow) are observed within the hepatic parenchyma. The inflammatory reaction is localized and limited, while the hepatic architecture remains mostly preserved compared with the 10 g/kg group. H&E stain. A: 100×; B: 400×.

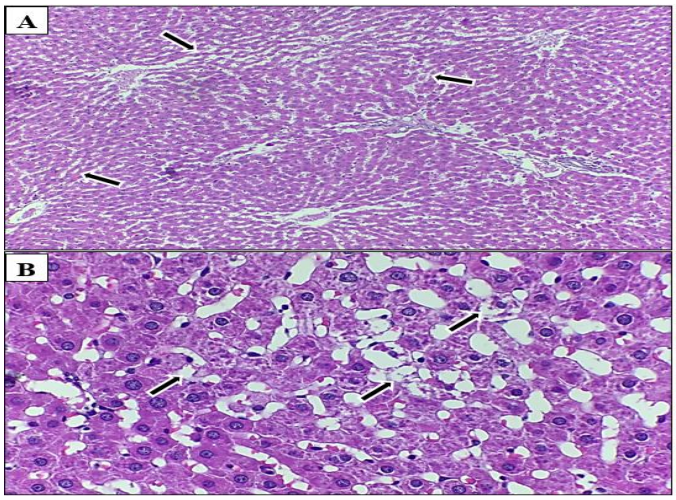

Figure 4. Cross section of liver tissue from the whey protein 10 g/kg group rat. A: Marked fatty liver changes (black arrow) are evident and involve a larger proportion of the hepatic parenchyma compared with the 5 g/kg group. B: Prominent macrovesicular steatosis (black arrow) is characterized by large clear vacuoles occupying most of the hepatocyte cytoplasm, frequently causing peripheral displacement of nuclei. H&E stain. A: 100×; B: 400×.

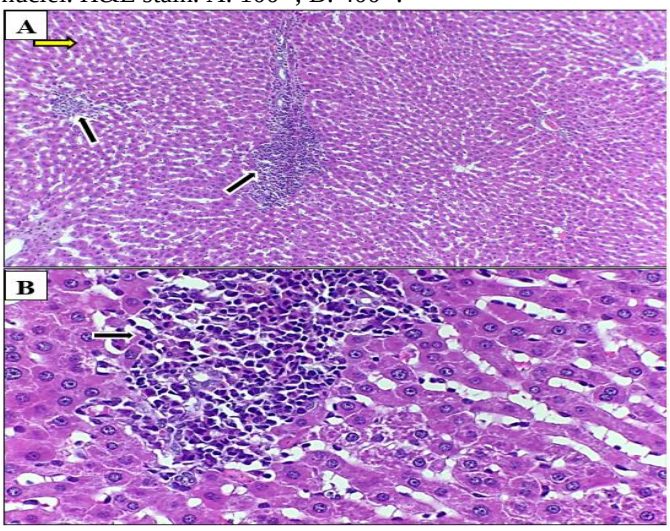

Figure 5. Cross section of liver tissue from the whey protein 10 g/kg group rat. A and B: Marked inflammatory cell infiltration (black arrow) is observed as large multifocal aggregates within the hepatic parenchyma. The inflammatory foci are more extensive than those observed in the 5 g/kg group and are associated with disruption of the normal hepatocyte cord arrangement. H&E stain. A: 100×; B: 400×.

Histochemical Findings of Liver Sections Stained with Masson's Trichrome

Masson's trichrome stained sections of control group (Figure 6) showed normal distribution of connective tissue in the liver. Abnormal extension of fine collagen fibers was confined to the subendothelial region around the central vein and to normal supporting stromal areas and not into the hepatic parenchyma. The hepatocyte cords were found to be well organized and no perisinusoidal, periportal or bridging fibrosis was seen. This finding assures that there was no pathological fibrous deposition in the control liver and serves as normal histochemical reference to compare to the treated liver groups. Moderate deposition of collagen fibers was observed in the perisinusoidal and portal or periportal regions in Masson's Trichrome stained sections of the whey protein group 5 g/kg in Figure (7). The fibrous tissue was seen as clearly demarcated blue-stained areas emanating from portal tracts and sometimes involving adjacent parenchymal areas but not with frequent and complete bridging. This pattern is seen in moderate hepatic fibrosis (NASH-CRN/Kleiner Stage F2) with fibrosis in both perisinusoidal and periportal compartments. The result shows an association with measurable fibrous expansion with the 5 g/kg dose, with a partial preservation of basic hepatic architecture. The fibrosis stage relied on the study relies on the extent and degree of collagen deposition seen in Masson's Trichrome sections. In the group of 5 g/kg whey protein, a moderate macrovesicular steatosis was observed in association with some inflammatory cell infiltration in foci (Figure (8). Lipid vacuoles were seen in a significant proportion of hepatocytes but lipid vacuoles in almost all parenchymal cells were not seen. In many hepatocytes, the nuclei were still recognizable and the outlines of the cells were also partially preserved. Also, there were some inflammatory foci scattered within the lobules, mainly consisting of mononuclear cells. From these results, it is concluded that the low dose produced combined steatotic and inflammatory changes, which were also somewhat less destructive than in the high dose group, but were also moderately distributed. As shown in Figure (9), Masson's trichrome stained sections in the whey protein group (10 g/kg) showed that there was extensive deposition of collagen and severe hepatic fibrosis. There was marked remodeling of the lobular architecture with the formation of fibrous tissue with blue staining bridging the portal areas and the central veins and adjacent portal tracts, forming bridge-like septa. This pattern is that of NASH-CRN (stage F3) with areas of bridging fibrosis without definite cirrhotic nodule formation. Broad fibrous bands and extension of these bands into the parenchyma are an advanced fibrotic response to the higher dose of whey protein. The fibrosis was more extensive and disturbed the normal hepatic organization more compared with the 5 g/kg group. In Figure (10) macrovesicular steatosis was observed in the liver sections of the 10 g/kg whey protein group with severe inflammatory infiltration. The majority of the damaged hepatocytes had large clear cytoplasmic vacuoles and some hepatocytes had displaced nuclei, fading nuclei or apparently lost nuclei suggestive of later stages of cellular damage. The inflammatory infiltrate was abundant and large, and it seemed to be originating from large areas of liver vein and spreading to the surrounding liver tissue with disruption of the hepatocyte cords. These results show that the high dose resulted in the most pronounced changes on the histological level, including steatosis, inflammatory infiltration, hepatocellular damages and architectural changes.

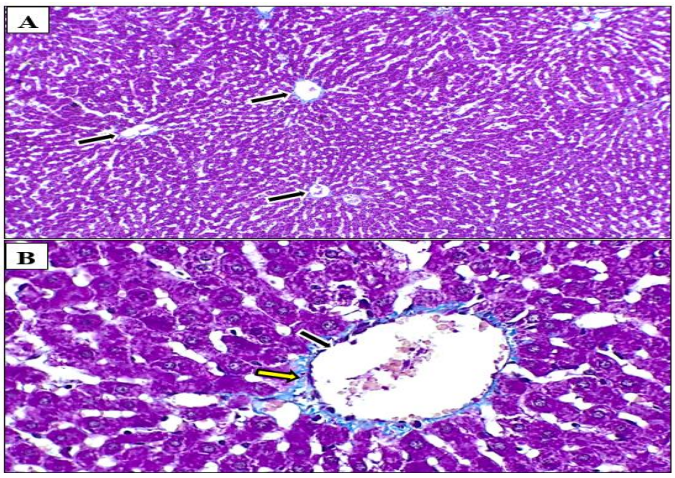

Figure 6 . Cross section of liver tissue from the control group rat. A and B: Normal hepatic histological architecture was preserved. The hepatocyte cords radiated normally from the central vein toward the portal areas. The central vein (black arrow) was lined by a continuous endothelial layer, and a thin subendothelial connective tissue layer composed of fine collagen fibers (yellow arrow) was observed. Masson's Trichrome stain. A: 100×; B: 400×.

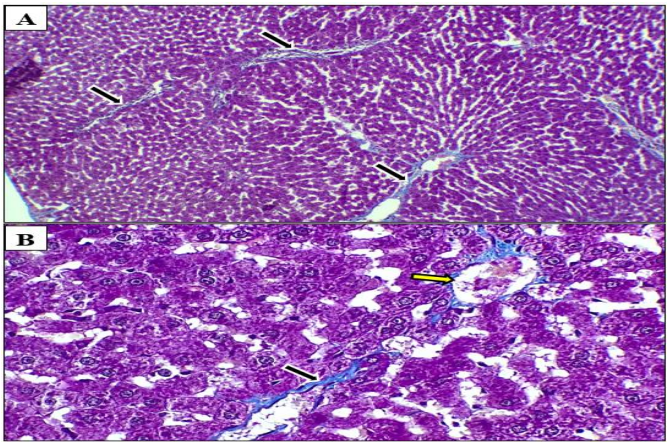

Figure 7 . Cross section of liver tissue from the whey protein 5 g/kg group rat. A and B: Moderate hepatic fibrosis, corresponding to NASH-CRN/Kleiner stage F2, is observed. The lesion is characterized by distinct perisinusoidal and portal/periportal collagen deposition (black arrow), with fibrous expansion of portal areas and occasional short septa extending into the hepatic parenchyma, without frequent complete bridging. Masson'sTrichrome stain. A: 100×; B: 400×.

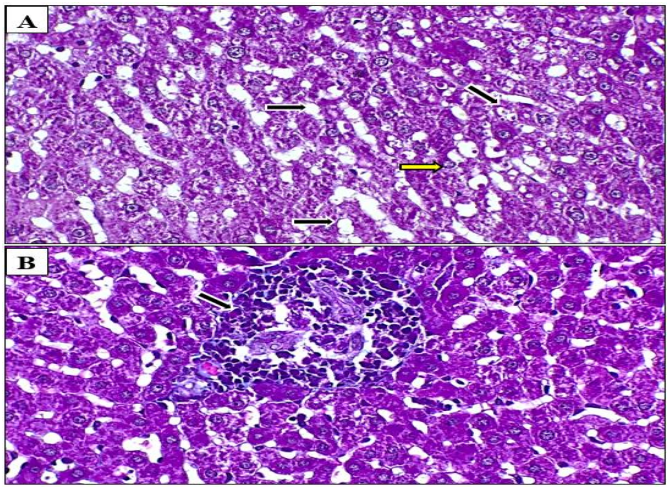

Figure 8 . Cross section of liver tissue from the whey protein 5 g/kg group rat. A: Moderate macrovesicular steatosis (black arrow) is observed, with large lipid vacuoles involving a considerable proportion of hepatocytes. Some vacuoles cause peripheral displacement of hepatocyte nuclei (yellow arrow), while the overall hepatic architecture remains partially preserved. B: Focal inflammatory cell aggregation (black arrow) is observed within the hepatic parenchyma, mainly as discrete mononuclear inflammatory foci. Masson's Trichrome stain. A: 100×; B: 400×.

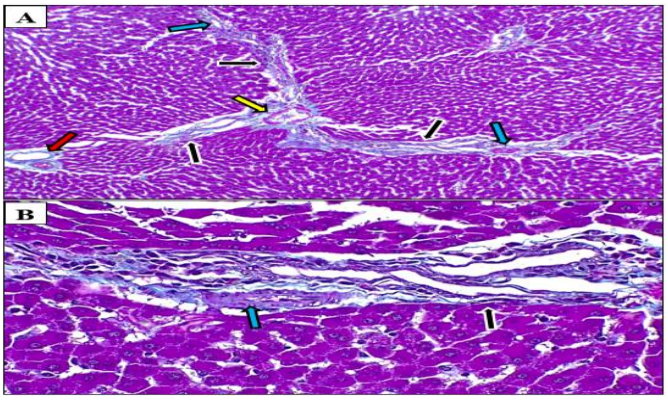

Figure 9 . Cross section of liver tissue from the whey protein 10 g/kg group rat. A and B: Severe hepatic fibrosis, corresponding to NASH-CRN/Kleiner stage F3, is observed in lobules showing marked steatotic degeneration. Extensive collagen deposition (black arrow) forms confluent bridging septa connecting portal tracts (yellow arrow), central veins (blue arrow), and adjacent portal areas (red arrow). These fibrous bands markedly disrupt the normal lobular architecture. Masson's Trichrome stain. A: 100×; B: 400×.

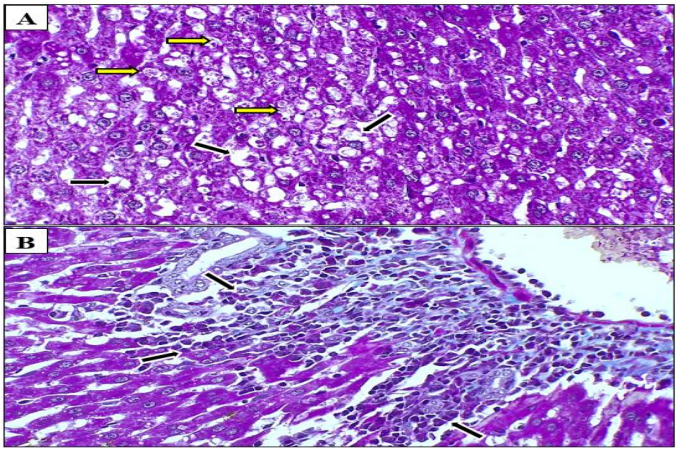

Figure 9 . Cross section of liver tissue from the whey protein 10 g/kg group rat. A and B: Severe hepatic fibrosis, corresponding to NASH-CRN/Kleiner stage F3, is observed in lobules showing marked steatotic degeneration. Extensive collagen deposition (black arrow) forms confluent bridging septa connecting portal tracts (yellow arrow), central veins (blue arrow), and adjacent portal areas (red arrow). These fibrous bands markedly disrupt the normal lobular architecture. Masson's Trichrome stain. A: 100×; B: 400×.

Table 2. Summary of histological and histochemical liver findings in the experimental groups

Group	H&E findings	Masson's Trichrome findings	Fibrosis stage	explanation
Control group	Normal hepatic lobular architecture; regularly arranged hepatocyte cords; normal central vein region; no steatosis, inflammation, necrosis, or architectural distortion	Normal collagen distribution limited to expected vascular and supporting stromal areas; no abnormal perisinusoidal, periportal, or bridging fibrosis	F0	Normal hepatic structure
Whey protein 5 g/kg group	Mild to moderate macrovesicular steatosis; focal inflammatory cell infiltration; hepatic architecture mostly preserved	Moderate collagen deposition in perisinusoidal and portal/periportal regions; occasional short septa without frequent complete bridging	F2	Moderate steatotic, inflammatory, and fibrotic alterations
Whey protein 10 g/kg group	Marked macrovesicular steatosis; large cytoplasmic vacuoles; peripheral nuclear displacement or nuclear fading; extensive inflammatory infiltration; disruption of hepatocyte cords	Marked collagen deposition with bridging fibrous septa connecting portal tracts and central veins; evident architectural remodeling	F3	Severe hepatic tissue injury with advanced fibrotic remodeling

Table note: H&E, Hematoxylin and Eosin; F0, no fibrosis; F2, perisinusoidal and portal/periportal fibrosis; F3, bridging fibrosis without definite cirrhotic nodule formation. Fibrosis staging was based on Masson's Trichrome-stained sections using the NASH-CRN/Kleiner fibrosis staging approach.

DISCUSSION

In the present study, whey protein feeding for six weeks induced dose-dependent histological and histochemical alterations in the liver of adult male rats. The control group showed preserved hepatic lobular architecture, regularly arranged hepatocyte cords, normal central vein regions, and no evidence of steatosis, inflammatory infiltration, necrosis, or abnormal collagen deposition. In contrast, the 5 g/kg whey protein group showed mild to moderate macrovesicular steatosis with focal inflammatory cell infiltration, while the 10 g/kg group exhibited more severe hepatic injury, characterized by marked macrovesicular steatosis, large cytoplasmic vacuolation, peripheral displacement or fading of hepatocyte nuclei, extensive inflammatory infiltration, and disruption of hepatocyte cord arrangement. These findings indicate that the hepatic response to whey protein was dose-dependent, with the higher dose producing more pronounced microscopic tissue injury. The steatotic changes observed in the treated groups may be related to the central role of the liver in amino acid catabolism, lipid metabolism, nitrogen disposal, and energy homeostasis. Excessive protein intake may increase hepatic metabolic workload, particularly when high doses are administered for a prolonged period. The presence of macrovesicular steatosis in both whey protein-treated groups suggests disturbance in hepatic lipid handling, in cluding imbalance between lipid uptake, de novo lipogenesis, mitochondrial oxidation, and lipid export from hepatocytes. The more extensive steatosis observed in the 10 g/kg group supports the interpretation that the higher dose imposed a greater metabolic burden on hepatocytes. The present findings agree with Gürgen et al., (5), who reported that whey protein supplementation may induce inflammatory and apoptotic responses in liver tissue, particularly in non-exercising rats. This agreement supports the possibility that excessive whey protein intake, especially without accompanying metabolic adaptation such as exercise, may be associated with hepatic stress and tissue injury. The inflammatory cell infiltration observed in the present study was focal and limited in the 5 g/kg group but became more extensive and associated with hepatocyte cord disruption in the 10 g/kg group. This dose-related inflammatory pattern strengthens the interpretation that high-dose whey protein feeding may shift the hepatic response from mild adaptive alteration to more evident tissue damage. However, the present findings disagree with studies that reported hepatoprotective effects of whey protein under specific experimental conditions. Yiğit Ziolkowski et al., (6) found that whey protein supplementation reduced liver

damage scores in rats exposed to a high-fat/high-fructose diet. Similarly, Aydın et al., (10) reported that whey protein showed protective effects against acrolein-induced oxidative damage in liver mitochondria. This disagreement may be explained by differences in experimental design, animal metabolic status, background diet, dose, duration, and the presence or absence of pre-existing hepatic injury. Whey protein may exert antioxidant or protective effects in models of metabolic or toxic injury, whereas high-dose administration in otherwise healthy animals may increase hepatic metabolic load and contribute to tissue injury. Therefore, whey protein should not be interpreted as uniformly beneficial or harmful; its hepatic effect appears to depend strongly on dose, duration, physiological condition, and experimental context.

The histochemical findings using Masson's Trichrome staining provided additional evidence of progressive hepatic remodeling. The control group showed normal collagen distribution limited to expected vascular and stromal regions. In the 5 g/kg group, moderate collagen deposition was observed mainly in perisinusoidal and portal/periportal regions, corresponding to moderate fibrotic remodeling. In the 10 g/kg group, collagen deposition was more extensive and formed bridging fibrous septa connecting portal tracts and central veins, indicating more advanced pre-cirrhotic fibrotic remodeling. These findings demonstrate that high-dose whey protein feeding did not only produce steatosis and inflammation but also promoted extracellular matrix deposition and architectural remodeling. The progressive collagen deposition observed in the treated groups may be explained by activation of hepatic stellate cells following repeated hepatocellular injury. Oxidative stress, inflammatory mediators, and hepatocyte damage can stimulate hepatic stellate cells to transform into myofibroblast-like cells, which produce collagen and other extracellular matrix components. This interpretation agrees with Kim and Kim, (7), who described hepatic stellate cell activation as a central mechanism in liver fibrosis, involving several signaling pathways, including TGF-β/Smad, MAPK, PI3K/AKT, Wnt/β-catenin, NF-κB, and AMPK pathways. The bridging fibrosis observed in the 10 g/kg group may therefore represent the structural consequence of persistent hepatocellular stress, inflammatory activation, sinusoidal disturbance, and extracellular matrix accumulation.The present histochemical findings also agree with Abdulmajeed and Sergi (12), who emphasized the importance of sinusoidal endothelial dysfunction and collagenization in fatty liver injury. The coexistence of steatosis, inflammatory infiltration, and collagen deposition in the whey protein-treated groups supports the concept that lipid accumulation and inflammatory injury can be linked to fibrotic remodeling. In the 5 g/kg group, the changes were moderate and partially preserved the hepatic architecture. In contrast, the 10 g/kg group showed bridging fibrosis and more severe architectural disruption, suggesting progression from early tissue reaction toward advanced remodeling. The combined use of H&E and Masson's Trichrome staining strengthened the interpretation of dose-dependent hepatic injury. H&E staining demonstrated the cellular components of damage, including steatosis, cytoplasmic vacuolation, inflammatory infiltration, nuclear changes, and disruption of hepatocyte cords. Masson's Trichrome staining demonstrated the connective tissue response, including collagen deposition and fibrotic remodeling. This combined pattern is consistent with the pathological sequence in which metabolic stress and lipid accumulation initiate hepatocellular damage and inflammation, followed by extracellular matrix deposition and fibrosis. It also agrees with current histopathological principles in liver disease, where fibrosis stage is considered an important indicator of disease severity and progression (14).The present study supports the hypothesis that whey protein feeding can produce dose-dependent hepatic alterations under the conditions of this experimental model. The lower dose caused moderate steatotic, inflammatory, and fibrotic changes, while the higher dose produced more severe hepatocellular damage and advanced fibrotic remodeling. The findings agree with studies reporting inflammatory liver responses after whey protein use, but disagree with studies showing protective effects in metabolically challenged or toxic injury models. These differences confirm that the biological effect of whey protein on the liver is context-dependent and influenced by dose, duration, dietary background, physical activity, and baseline hepatic condition.

CONCLUSION

The present study demonstrated that six weeks of whey protein feeding induced dose-dependent histological and histochemical alterations in the liver of adult male rats. The 5 g/kg dose produced moderate hepatic changes, including macrovesicular steatosis, focal inflammatory cell infiltration, and moderate collagen deposition, while the 10 g/kg dose caused more severe hepatic injury characterized by marked steatosis, extensive inflammatory infiltration, hepatocyte cord disruption, nuclear alterations, and bridging fibrotic remodeling. These findings indicate that high-dose whey protein intake may impose a progressive hepatic tissue burden and promote collagen-associated fibrotic remodeling under the conditions of this experimental model. Further studies using larger sample sizes, longer follow-up periods, biochemical markers, and immunohistochemical assessment are recommended to determine the reversibility, mechanism, and clinical relevance of these hepatic alterations.

RECOMMENDATIONS

Further studies are recommended using larger sample sizes and longer experimental periods to determine whether the hepatic changes induced by whey protein feeding are reversible, progressive, or permanent. Future investigations should include additional experimental time points to assess the chronological development of steatosis, inflammatory infiltration, collagen deposition, and fibrotic remodeling.

It is also recommended to compare different forms of whey protein, including whey protein concentrate, whey protein isolate, and whey protein hydrolysate, because differences in purity, peptide composition, processing method, and additive content may influence the severity of hepatic microscopic

alterations. Including dose-response designs with intermediate doses may also help identify a safer exposure range.

Additional histochemical and immunohistochemical assessments should be included in future studies to clarify the underlying mechanisms of liver injury and fibrosis. Sirius Red staining may be used for more specific collagen assessment, while immunohistochemical markers such as α-SMA, TGF-β, collagen I, collagen III, TNF-α, and IL-6 may provide further information about hepatic stellate cell activation, extracellular matrix deposition, and inflammatory signaling.

Future studies should also compare sedentary and exercise-trained animals, since physical activity may modify the hepatic response to high-dose protein supplementation. Combining histological findings with biochemical liver function tests, oxidative stress markers, lipid profile parameters, and molecular inflammatory markers would provide a more comprehensive evaluation of the hepatic effects of whey protein feeding.

Study Limitations

The present study has some limitations that should be considered when interpreting the findings. First, the assessment was based mainly on routine histological examination using Hematoxylin and Eosin staining and histochemical evaluation using Masson's Trichrome staining. Although these methods clearly demonstrated steatosis, inflammatory infiltration, nuclear alterations, hepatocyte cord disruption, collagen deposition, and fibrotic remodeling, immunohistochemical confirmation of hepatic stellate cell activation and inflammatory signaling was not performed.

Second, the study evaluated only one experimental duration of six weeks. Therefore, it was not possible to determine whether the observed hepatic alterations were reversible, progressive, or permanent over longer follow-up periods. Third, the study did not include biochemical liver function markers, oxidative stress parameters, lipid profile measurements, or molecular inflammatory markers, which could provide additional functional correlation with the microscopic findings.

Fourth, only two whey protein doses were evaluated, and the study did not compare different commercial forms of whey protein, such as whey protein concentrate, whey protein isolate, and whey protein hydrolysate. These products may differ in purity, peptide composition, additives, processing methods, and biological effects. Finally, the relatively small number of animals in each group may limit the generalization of the findings. Therefore, future studies with larger sample sizes, additional doses, longer experimental periods, biochemical markers, and immunohistochemical or molecular assessments are required to confirm and expand the present findings.

The study has several strengths

The present study has several strengths. First, it used a controlled experimental design with two different doses of whey protein, allowing evaluation of dose-dependent hepatic alterations. Second, the study combined routine histological examination using Hematoxylin and Eosin staining with histochemical evaluation using Masson's Trichrome staining, which enabled assessment of both cellular injury and collagen-associated fibrotic remodeling. Third, the comparison between the control, 5 g/kg, and 10 g/kg groups provided clear microscopic evidence of progressive hepatic changes in relation to dose.

Another strength of this study is the direct microscopic documentation of hepatic architecture, steatosis, inflammatory cell infiltration, hepatocyte cord disruption, nuclear alterations, collagen deposition, and bridging fibrotic changes. The use of a recognized fibrosis staging approach also improved the interpretive value of the histochemical findings. Therefore, the study provides useful preliminary evidence about the possible hepatic tissue effects of high-dose whey protein feeding in adult male rats.

Histological and histochemical explanation.

Histological results seem to suggest that the alterations in the liver started as cellular damage, characterized by macrovesicular steatosis, cytoplasmic vacuolation and the presence of inflammation in the 5 g/kg group. These changes were more pronounced in the higher dose (10 g/kg) and consisted of increased inflammatory infiltration, pushing of the nuclei or fading of the hepatocyte and disruption of hepatocyte cords. The histochemical results supported the presence of progressive deposition of collagen with this tissue injury. Moderate collagen deposition in the 5 g/kg group indicates early to moderate fibrotic remodeling and bridging fibrous septa in the 10 g/kg group indicate advanced pre-cirrhotic fibrosis. This implies that the histochemical reaction was not independent of the histological damage but rather was the connective tissue reaction of repeated damage to the hepatocytes.

Practical Implication

In conclusion, the results indicate that the histological and histochemical analysis should be included as a part of the assessment of liver safety of high dose protein supplementation. Routine H&E staining can be used to see cellular injury, steatosis, inflammation and architectural disturbance and Masson's Trichrome staining can be used to see collagen deposition and fibrotic remodeling. Thus, the use of gross appearance or a simple biochemical screening alone may underestimate early or progressive changes at the tissue level in the liver.

Ethical Approval

All experimental procedures involving animals were conducted in accordance with institutional guidelines for the care and use of laboratory animals. Ethical approval was obtained from Al-Qasim Green University before the beginning of the study. Animal handling, anesthesia, tissue collection, and sacrifice were performed with consideration of animal welfare, and all efforts were made to minimize animal suffering and to use the minimum number of animals required to achieve the study objectives.

Conflict of Interest

The authors declare that there is no conflict of interest related to this study.

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Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

All authors contributed to the study design, animal handling, tissue processing, histological staining, histochemical staining, microscopic examination, interpretation of liver sections, manuscript preparation, revision, and approval of the final version of the manuscript.

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