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DIETARY ADMINISTRATION OF UNDENATURED WHEY PROTEIN DECREASED THE HISTOPATHOLOGICAL CHANGES INDUCED BY CIPROFLOXACIN IN HEPATIC, RENAL AND ILAEL TISSUES OF MICE

(With 2 Tables and 9 Figures)

By

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إعطاء بروتين الشرش غير المدنتر في الوجبات قلل التغيرات النسيجية المرضية الناتجة عن إعطاء سبروفولكساسين في أنسجة الكبد والكلى واللفائفى للجرذان البيضاء

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يحتوى بروتين الشرش غير المدنتر Undenatured whey protein على كل الأحماض الامينية الأساسية وغير الأساسية اللازمة لنمو الخلايا بشكل طبيعي. استهدف هذا البحث دراسة ما إذا كان بروتين الشرش غير المدنتر له قدرة علي التقليل من آثار المضاد الحيوي سبروفولكساسين Ciprofloxacin. استخدمت بعض التقنيات المناعية والفسيولوجية والنسيجية المرضية لهذا الغرض. تم تقسيم الجرذان المستخدمة في هذا البحث إلي مجموعة ضابطة (المجموعة الأولي) (I) وثلاث مجاميع معالجة. أعطيت المجموعة الثانية (II) سبروفولكساسين يوميا لمدة ٧ أيام. أعطيت المجموعة الثالثة (III) المضاد الحيوي يوميا لمدة ٧ أيام بالتوازي مع بروتين الشرش غير المدنتر لنفس المدة. وأعطيت المجموعة الرابعة (IV) المضاد الحيوي وبروتين الشرش غير المدنتر بنفس طريقة المجموعة الثالثة غير أن مدة البروتين زيدت ٨ أيام أخري لتصل إلي إجمالي ١٥ يوما. أوضحت القطاعات النسيجية في الكبد (II) عدم وضوح حدود الخلايا الكبدية مع ضيق في الجيوب الدموية وكذلك زيادة حجم وعدد خلايا كبفر Kupffer cells. من ناحية أخري بينت الصبغات الهستوكيميائية انخفاضا واضحا في كمية الجليكوجين في الخلايا الكبدية في هذه المجموعة (II) مع تحسنا طفيفا في محتوي كل من الجليكوجين والبروتين في المجموعة الرابعة (IV). علي مستوي النسيج الكلوي بينت النتائج وجود التهابات خلوية وضمورا واضحا في الكبة الدموية في المجموعة الثانية (II) وصحب ذلك انخفاض في المستوي الكربوهيدراتي والمستوي البروتيني. تغيرت هذه المؤشرات السلبية مع إعطاء الجرذان بروتين الشرش غير المدنتر حيث أبدت الكبة الدموية والتركيب النسيجي العام تحسنا واضحا خصوصا في المجموعة

الرابعة (IV). في المجموعة الثانية (II) تميز نسيج اللفائفي بزيادة كبيرة وواضحة في سمك الطبقة العضلية Muscularis وكهوف ليبركين rypts of Lieberkuhn والألياف Fibers لكن تحسنا نسيجيا ملحوظا وجد في نسيج اللفائفي للمجموعة الثالثة (III) وزاد هذا التحسن مع زيادة مدة إعطاء بروتين الشرش غير المدنتر في المجموعة الرابعة (IV). تم هذا التحسن النسيجي بالتوازي مع زيادة واضحة في نسبة الكربوهيدرات فى الخلايا الكأسية وحواف الخملات. جاءت المؤشرات المناعية (Phagocytic activity and chemkinetic index) والفسيولوجية (البروتين الكلى والامينوجلوبيولين الكلى) لتبدى نفس التراجع في المجموعة الثانية (II) وتحسنا في المجموعة الثالثة (III) وتحسنا واضحا في المجموعة الرابعة (IV) عاكسة بذلك النتائج الهستولوجية والهستوكيمائية. الخلاصة أن بروتين الشرش غير المدنتر قلل من آثار سبروفولكساسين ومع استمرار إعطائه لمدة أطول, حسن التركيب النسيجى لكل من اللفائفى والكلى, لكنهما مع ذلك لم يصلا إلى التركيب النسيجى الأمثل. أيضا اثر هذا البروتين ايجابيا وبوضوح في المؤشرات المناعية والفسيولوجية وربما كان ذلك هو العامل الفعال فيما أضفاه هذا البروتين من تحسن نسيجى واضح في هذه الدراسة.

SUMMARY

Whey protein contains all amino acids that are necessary for normal cell growth. This work aimed to study whether whey protein can decrease the effects induced by Ciprofloxacin. Histological, histochemical and immunological methods were used. Mice were classified into a control (group I) and three treated groups. Group II was daily administrated Ciprofloxacin for 7 days. Group III was administrated Ciprofloxacin and synergistically mice administrated whey protein for the 7 days. Mice of group IV were treated in the same way like as those of group III with the whey protein continue for 8 days more (whey protein was administrated 15 days in group IV instead of 7 days in group III). Liver sections of group II revealed that cell boundaries of hepatocytes became unclear, blood sinusoids became narrow and Kupffer cells increased in both size and number. PAS stain showed a reduced carbohydrate content in group II, while it showed a little improved in group IV. A moderate improvement in the protein content was observed in this group. Kidney sections of group II appeared with some cellular inflammations, edematous renal cells and glomeruli were shrunken. Also a decrease in carbohydrate and protein contents was observed. An obvious effect of the whey protein had obtained of the renal tissues in group IV, since glumeruli, renal tubules and the general histological architecture became similar to the normal. Ileum sections revealed a thickness in the muscularis and increasing in crypts of Lieberkuhn and in fibers. An improvement was noted in group III and increased as the time of whey protein prolonged in group IV. There was an observed decrease of the

carbohydrate contents in both group II and group III. This carbohydrate contents and intensity was obviously increased in goblet cells and brush border of group IV. The intensity of proteins in the tissues of treated groups was nearly as that in control. The physiological and immunological indices reflected the same severe effect of the antibiotic. An observed improvement was achieved in the phagocytic activity and the chemokinetic index after administration of the undenatured whey protein. In conclusion, these findings showed severe effects of the Ciprofloxacin on the histological architecture of studied organs. After 15 days (group IV) an improvement was observed in both renal and ileal tissues but could not completely restore their normal histological architecture. This protein obviously affected the physiological and the immunological responses, and this might be the potential factor in initiating the normalizing processes of the histological architecture structures.

Key words: *Undenatured whey protein, Ciprofloxacin, histopathological changes, Immunological response, mice.*

INTRODUCTION

Undenatured whey protein has been found to have a number of bioactive properties. Normal processes to extract whey component from the other constituents lead to significant denaturing of the bioactive whey proteins. Undenatured whey protein isolates utilize proprietary processes to attain a protein containing over 90% undenatured whey protein. Bioactive components include lactoferrin, lysozyme, lactoperoxidase, glycomacropeptide, alpha-lactalbumin, bovine serum albumin, various growth factors and immunoglobulins. Lactoferrin exhibits anticancer, antiviral, antibacterial, and antifungal activity. It plays active roles in iron transport and in the cytotoxic defenses of neutrophils, and it scavenges free iron which acts as a free radical (Barta *et al.*, 1991; Kawasaki 1993; Marchetti 1994; Wong and Watson 1995).

Whey protein is believed to be the highest quality protein available when compared to different proteins. It contains all essential and non-essential amino acids and is a good source of glutamine and the branched-chain amino acids that are necessary for cell growth (David 1999). These amino acids include arginine, glycine, leucine, isoleucine and valine which are essential to different tissues (Ganong 1997). The present work aimed to investigate the effect of the undenatured whey protein on decreasing the harmful effects of the antibiotic, Ciprofloxacin

on the histological structure of some tissues and some immune parameters.

MATERIALS and METHODS

Preparation of the undenatured whey protein

Undenatured whey protein was prepared from sweat cheese whey by adding ammonium sulfate to 3.3 M/l. The precipitated proteins were collected and dialyzed against water to remove the residual amount of the salt. Protein content was determined according to the method of Ling (1963).

Experimental diet

To prepare 500g of the diet, 5g vitamins, 25g mineral salts, 40g fats, 50g sucrose, 100g protein and 280g starch were mixed (20% protein). The diet was kept at 4ºC until use. Animals were freely fed in a rate of 3g / 25g body weight of the mouse per day (Bounous 1999 and 2000). Whey protein is much superior to casein in terms of protein quality. Therefore, the effect of the undenatured whey protein could be easily monitored by the comparison with the casein. Whey protein supplementation for 2 weeks can increase lymphocyte GSH (oxidized and reduced) by 24% (Zavorsky *et al.*, 2007).

Experimental animals

Forty adult male Swiss mice, weight 25-30g were purchased from the Biological Supply Center, Theodore Bilharz Research Institute; TBRI, Cairo, Egypt and used in this study. Animals were in the laboratory under constant conditions for at least one week before use.

Animal groups

Animals were classified into a control group (group I) and other three treated groups. Casein was the protein fraction in the experimental diets of both control and group II. The undenatured whey protein which was prepared as mentioned above was the protein part of the diets administrated by groups III and IV. All treated mice (groups II, III and IV) were challenged with an oral administration of the antibiotic, Ciprofloxacin suspended in PBS for 7 days at a therapeutic dose of 0.7 mg/1ml PBS /25 g every day for 7 days. Groups were as the following:

1- Control mice (groups I) were administrated the same amount of PBS for 7 days.

2- Group II was orally administrated Ciprofloxacin for 7 days.

3- Group III administrated the antibiotic in the same way as the second one and synergistically administrated a complete diet containing the

undenatured whey protein for 7 days.

4- Group IV was administrated the antibiotic and the whey protein in the same way like the third group and then they continue the administration of the undenatured whey protein for 8 days more before sacrificing.

Mice were sacrificed under mild diethyl ether anesthesia and blood samples without anticoagulants were collected from each animal. The serum from the non-heparinized blood sample was used in order to measure enzymes, total protein and total Ig. Serum was collected and preserved at -20 °C until use.

Histological examination

Organs were collected at the end of the experiment and tissues were fixed in Bouin's fixative, processed into paraffin, and 4 micrometre thick sections were prepared. Sections were stained with Haematoxylin and Eosin (H&E) for general histological architecture, Periodic-Acid Schiff's (PAS) reagent was used to stain the general carbohydrates and the Mercuric Bromophenol Blue (HgBPB) stain was applies to detect proteins. Slides from each animal were examined for the histopathological changes.

Chemokinetic assay

A modified Boyden chamber assay was applied in which cells migrate from one compartment, through a microscope filter, towards a compartment containing chemoattractant. In this method of Gearing and Rimmer, (1985), the two compartments were formed by the wells of two microtiteration plates on top of each other with cellulose acetates filters (5μm in diameter from Millipore 67/20 Molsheim, France) partitioning the wells. The lower test wells were filled with chemoattractants (*Aspergillus niger* in 0.9% saline) and in case of control, the lower wells were filled with 0.9% saline solution. Millipore filters were placed overall the lower wells. The upper plate was inverted and placed over the lower plate. 0.3 ml of tested spleen was introduced into each chamber through a hole made in the base of each well of the upper plate. The migrating cells were fixed with methanol, followed by 10% formalin for 20 min. each. Filters were stained with H&E for 5 min. and then washed with distilled water. The chemokinetic effect obtained was expressed by chemokinetic index calculated: Chemokinetic index = N ÷ N`. N and N` are the mean number of cells per high power field with chemokinetic stimulus and the mean number of cells per high power field with control stimulus, respectively.

Phagocytosis

The phagocytic activity of macrophages as well as other phagocytic cells in spleen and superior mesenteric lymph node was monitored using the phase contrast microscopy. A piece of spleen was removed and was kept in cold 0.9 % saline. The piece of spleen was teased carefully with two fine needles in 0.1 ml cold saline. Then, one drop of each teased sample was mixed with a drop of paraffin oil and examined with the aid of phase contrast microscopy. Percentages of phagocytosis were calculated and any remarkable phagocytic state was photographed.

Statistical analysis

Statistical analysis was undertaken using MINITAB software (MINITAB, State College, PA, Version 13.1, 2002). Data from the total protein, the total Ig, enzymes and phagocytosis experiments were first tested for normality using Anderson Darling test, and for variances homogeneity prior to any further statistical analysis. Data were normally distributed, and variances were homogeneous, thus, One-way ANOVA was used to determine overall effects of treatments followed by individual comparison using Tukey's Pairwise comparison.

RESULTS

Sections from the liver of control mice showed the normal hepatic tissues (Fig. 1A). Investigation of the sections obtained from mice administrated antibiotic (group II) revealed that the cell boundaries of the hepatocytes became unclear. The nuclei were faintly stained containing less chromatin granules. The blood sinusoids became very narrow. Kupffer cells are increased in both size and number (Fig. 1B). Administration of undenatured whey protein for 7 days (group III) did not show any observed improvement. In this group, the cytoplasm of hepatocytes appeared highly vacuolated and tends to adhere to the cell membrane. Some of the nuclei disappeared, while others were presented with faintly stained (Fig. 1C). Although, in case of group IV, most hepatocytes still had irregular shapes with irregular nuclei, administration of undenatured for showed an observed effect. The cytoplasm of some hepatocytes appeared vacuolated, while some other hepatocytes, especially those near the central vein, appeared with normally distributed cytoplasm (Fig. 1D).

Sections from the liver of control mice stained with PAS reagent show great amount of carbohydrate content as shown by their strong

positive reaction. It appears as fine granules stained dark pink, uniformly distributed in the cytoplasm, nuclei and cell membranes of the hepatocytes (Fig. 1E). Sections from group II showed a highly reduction of carbohydrate content and granulation of the cytoplasm disappeared as seen in Figure 1F by taking weak PAS stain. Also a decrease in glycogen content with little amount of granules in their cytoplasm in sections from groups III and IV (Fig. 1G and 1H). Sections from the liver of control mice stained with HgBPB showed a high quantity of protein content. It appears as dark blue granules homogeneously distributed in the cytoplasm of the hepatocytes (Fig. 2A). A clear depletion of protein content in some hepatocytes in groups II and III (Fig. 2B and 2C), while, the cytoplasm of some hepatic cells in group IV showed a moderate amount of proteins (Fig. 2D) what it means that undenatured whey protein caused an improvement.

Sections from the kidney of control mice are shown in Figure 3A. In groups II, the glomerulus became shrinkage with appearing wide urinary space. Many of swollen cells can be seen in the renal cortical tubules in group III. The lumen of the renal tubules was obviously narrow. The cell boundaries of the renal tubules became unclear with faintly stained nuclei that contain less chromatin material (Fig. 3B). A significant swelling of the renal tubular epithelial cells under the capsule was observed resulting in a narrow tubular lumen. There was a clear vaculation in the cells lining the tubules under the capsule (Fig. 3C). An observed improvement was occurred of the architecture of the glomerulus and the cortical tissue in group IV (Fig. 3D).

Sections from kidney of control mice stained with PAS showed a considerable amount of carbohydrate (Fig. 3E). Cortex of groups II, III and IV showed a significant decrease in carbohydrate content especially in glomerulii, brush borders and cell membrane of renal tubules (Fig. 3F-3H). The protein content of the control kidney which stained with HgBPB appeared as relatively dark blue granules show great amount of proteins in glomeruli, nuclei, cytoplasm and cell membranes of renal tubules (Fig. 4A). Cortex of groups II, III and IV showed a slightly decrease in protein content in glomeruli, nuclei, cytoplasm and basement membranes of renal tubules (Fig. 4B-4D).

Section from the ileum of control mice is shown in Figure 5A. In group II, an increase in thickness of both longitudinal and circular muscle layers was observed. Crypts of lieberkuhn increased in number. Many muscle fibers, lymphocytes and fibroblasts appeared in the lamina propria. The submucosa became slightly wide with relatively wide blood

vessels in groups (Fig. 5B). Section of group III indicated that a slight increasing in thickness of muscularis, but became thinner than in group II, and layers became more regular. The submucosa is wide containing very large blood vessels. Crypts of lieberkuhn decreased in number (Fig. 5C). The villi became slightly tall containing lymphocytes, fibroblasts and large lymphatic lacteal. In group IV, the submucosa is still slightly wide with the blood vessels became narrow than in groups II and III. The lamina propria still rich in lymphocytes and fibroblasts (Fig. 5D). A great number of goblet cells were detected.

Sections from ileum of control mice revealed that the carbohydrate materials are mainly concentrated in the goblet cells of both crypts of lieberkuhn and villi as well as the brush border of the villi (Fig. 6A). In group II and group III there was a decrease of the carbohydrate intensity inside the goblet cells and along the brush border of the villi (Fig. 6B and 6C). The goblet cells, the brush border as well as the basement membrane of the epithelial lining attained highly positive carbohydrate materials in group IV (Fig. 6D). Therefore, there was a clear increase of the carbohydrate materials in sections of group IV in comparison to groups II and III. Sections of mice stained with HgBPB revealed that the protein contents are mainly concentrated in the serosa, muscularis layer, the cellular component of lamina propria and the epithelial cells of the villi (Fig. 7A). Sections of groups II, III and IV showed that the intensity of proteins in the tissues was nearly as that in control group (Fig. 7B-7D).

Furthermore, results showed that groups III and IV had significantly higher protein levels comparing with that of the control one (8A). The same group recorded also a significantly higher value of the total Ig (8B). This can be due to the administration of whey protein which incorporated in the body protein synthesis in order to repair the tissue damage. Alkaline phosphatase was significantly elevated in both group II and group III. This enzyme was significantly decreased in mice of group IV (Fig. 9A). No significant changes were occurred for both the hepatic enzymes, GPT and GOT (Fig. 9B). As shown in Table 1 and Table 2, group II recorded lower phagocytic activities comparing with the control group, while the third and the forth groups revealed significant higher phagocytic activities. Similar results were obtained in the case of the chemokinetic index except that group II achieved a higher value beside groups III and IV.

Table 1: The phagocytic activity of spleen and superior mesenteric lymph node cells of the control and the different treated mice groups.

	Groups			
	Control (Group I)	Group II	Group III	Group IV
Phagocytic activity of spleen cells	45±7	35±5	60±4*	77±9*
Phagocytic activity of a lymph node cells (%)	42±5	41±3	59±6*	85±11*

Values are mean ± SE. of ten determinants. Statistical significance (*P < 0.05) is shown on comparing treated samples versus the control ones.

Table 2: Chemokinetic index of the control and the different treated mice groups. Migrating spleen cells were stained with H & E as mentioned in the materials and methods.

	Groups			
	Control (G I)	Group II	Group III	Group IV
Chemokinetic index	2.2	2.5	3	3

LEGENDS OF FIGURES

Fig. 1: A: Liver of a control animal (group I) showing normal hepatic histological structures, blood sinusoids (bs) and the normal hepatocytes (h) (H and E x630). **B:** Liver of 7 days antibiotic (group II) showing the vacuolated hepatocytes (vh) and the blood haemorhage (hb) in the dilated central vein. The blood siusoids are narrow and the Kubffer's cells are increased (H and E x630). **C:** Liver of 7 days antibiotic and 7 days undenatured whey protein (group III) showing the highly vacuolated hepatocytes (hv) in all cells of the hepatic unit (H and E x630). **D:** Liver of 7 days antibiotic and 15 days undenatured whey protein (IV) showing a decrease in the vacuolated hepatocytes (h), especially near the central vein while many are still vacuolated (vh) (H & E x630). **E, F, G & H:** Liver of control (group I), 7 days antibiotic (group II), 7 days antibiotic and 7 day undenatured whey protein (group III), of 7 days antibiotic and 15 day whey protein (IV), respectively, showing the general carbohydrates content and the carbohydrate granules (cg) (PAS x200).

Fig. 2: A, B, C & D: Liver of control (group I), 7 days antibiotic (group II), 7 days antibiotic and 7 day undenatured whey protein (group III), 7 days antibiotic and 15 day undenatured whey protein (IV), respectively, showing the protein content (HgBPB x200).

Fig. 3: A: Kidney of control (group I) showing the cortex with the renal corpuscles (c) and the renal tubules (t) (H and E x 630). **B:** Kidney of 7 days antibiotic (group II) showing the cortex layer with the shrunken renal corpuscle (c) and the wide blood vessel (bv) (H and E

x 630). **C:** Kidney of 7 days antibiotic and 7 days undenatured whey protein (group III) showing the cortex layer with a significant swelling and a clear vacuolation in cytoplasm (arrows) of the cells of the renal tubular epithelium under the capsule (H and E x 630). **D:** Kidney of 7 days antibiotic and 15 days undenatured whey protein (IV) showing an improvement in the general architecture of the renal tissue (H & E x630). **E, F, G & H: :** Kidney of control (group I), 7 days antibiotic (group II), 7 days antibiotic and 7 days undenatured whey protein (group III), Kidney of 7 days antibiotic and 15 day whey protein (IV), respectively, showing the general carbohydrates content (PAS x200).

Fig. 4: A, B, C & D: Kidney of control (group I), 7 days antibiotic (group II), 7 days antibiotic and 7 day undenatured whey protein (group III), Kidney of 7 days antibiotic and 15 day undenatured whey protein (IV), respectively, showing the protein content (HgBPB x200).

Fig. 5: A: Ileum of control (group I) showing the layers of serosa, muscularis and submucosa (H & E x630). **B:** Ileum of 7 days antibiotic (group II) showing the very thik muscularis (m) and the dialated blood vessels (bv) in the submucosa (H & E x 630). **C:** Ileum of 7 days antibiotic and 7 days undenatured whey protein (group III) showing the very wide blood vessels (bv) in the submucosa (H & E x 630). **D:** Ileum of 7 days antibiotic and 15 day undenatured whey protein (group VI) showing the dilated blood vessels (bv) in the submucosa (H and E x630).

Fig. 6: Carbohydrate staining of ileum of control (A), group II (B), group III (C) and group IV (D) (PAS x200).

Fig. 7: Ileum of control **(A)**, group II **(B)**, group III **(C)** and group VI **(D)** showing the protein staining (HgBPB x200).

Fig. 8: Total protein and albumin (A) and total Ig (B) levels in the sera of the control and the different treated groups. Values are mean ± SE. of ten determinants. Statistical significance (*P < 0.05) is shown on comparing treated samples versus the control ones.

Fig. 9: Alkaline phosphatase levels (A) and GPT and GOT (B) in the sera of control and different treated groups. Values are mean ± SE. of ten determinants. Statistical significance (*P < 0.05) is shown on comparing treated samples versus the control ones.

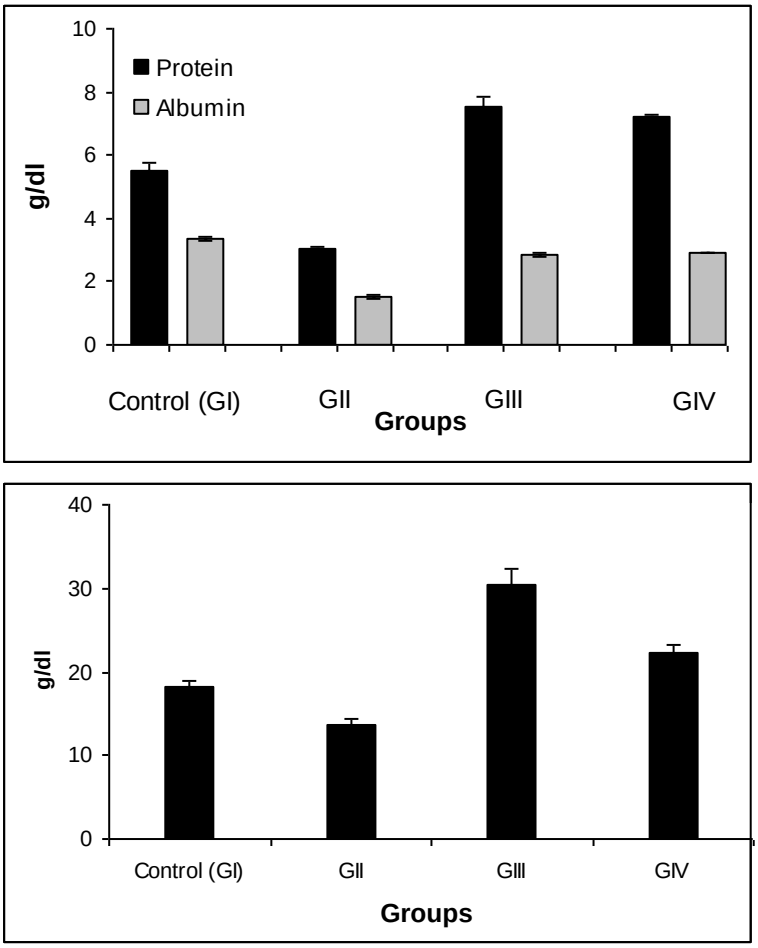

Fig. 8: Total protein and albumin (A) and total Ig (B) levels in the sera of the control and the different treated groups. Values are mean ± SE. of ten determinants. Statistical significance (*P < 0.05) is shown on comparing treated samples versus the control ones.

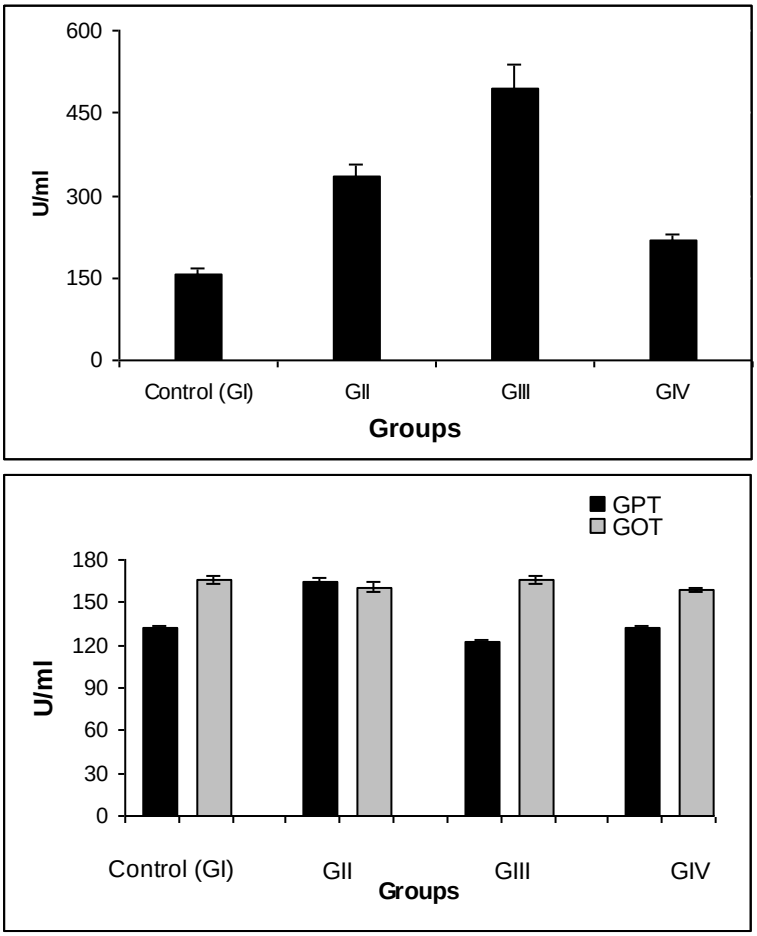

Fig. 9: Alkaline phosphatase levels (A) and GPT and GOT (B) in the sera of control and different treated groups. Values are mean ± SE. of ten determinants. Statistical significance (*P < 0.05) is shown on comparing treated samples versus the control ones.

DISCUSSION

Results of the liver showed a severe effect of the Ciprofloxacin on its histological architecture. Kupffer's cells were increased in number in the treated mice. The increased number of Kupffer's cells might be attributed to the increase of foreign substances which should be cleared up by phagocytosis (Henell *et al.,* 1983). Results showed also a cellular infiltration in the hepatic tissue. Abundance of leucocytes and lymphocytes in particular are a prominent response of body tissues facing any injurious impacts (El-Banhawy *et al.,* 1993). The drug

induced water retention followed by vaculation in the hepatocytes and appearance of inflammatory cellular infiltration. Vaculation and damage of the mammalian liver cells were noticed by other investigators following treatment with differenet agents such as rodenticides (El-Banhawy *et al.,* 1993) and antibiotics (Zhang and Wang 1984). Retention of water inside the hepatocytes resulted in their oedema. Zandg and Wang (1984) suggested that vaculation is mainly a consequence of considerable disturbances in lipid inclusions and fat metabolism occurring under pathological cases. The vaculation of liver cells appeared at first in the peripheral hepatocytes, extending gradually towards its center. This may be due to the direction of the lobular blood supply. Therefore, peripheral heptocytes are firstly, subjected to high concentration of the antibiotic, then, antibiotic concentration decreases gradually toward the center. So, the central hepatocytes are lesser affected (Elewa 1996, Amer 1998; Ebaid *et al.,* 2007). Therefore, after administration of undenatured whey protein for 15 days, it was noticed that cytoplasm of few hepatocytes, particularly the central ones, became approximately normally distributed.

Previous studies reported hepatic tissue adaptation to the injury produced by non-steroidal anti-inflammatory drugs (Skeljo *et al.,* 1996; Ibrahim 1999). In contrary, although the highly regulative capacity of the hepatic cells, a low restoration has been achieved even after supplementation with the whey protein in the present work. This may indicate a low significant role of the whey protein in recompense of the hepatic tissues. However, appearance of few hepatocytes with normal cytoplasm after supplementation with whey protein for 15 days indicated the beginning of a curative stage in the hepatic tissue. Because the liver is the central organ in the metabolism, it is engaged to form antibiotic intermediates. Thus, liver was expressed to more severe tissue damage than renal and ileal tissues and the recovery in hepatic tissue delayed. In the present work, whey protein supplementation was for about 2 weeks as suggested by Zavorsky *et al.,* (2007). This period was enough for renal and ileal tissues to achieve an advanced tissue repair as mentioned below. It seemed that this period was enough only for the beginning of hepatic tissue repair. This confirmed that 15 days supplementation with the whey protein is not really enough for all different tissues subjected to the antibiotic stress. Therefore, further time might be needed in this case for hepatic tissue in order to achieve an advanced restore stage as renal and ileal tissues did, especially that Kume *et al.* (2006) confirmed the role of whey protein in preventing hepatitis and portal fibrosis.

The elevation of the drug level in the kidney circulation must be faced by a capillary constriction to decrease the filterate containing the drug to minimize its effect and protect the tubular cells. At the same time the mesangial cell processes may be retracted due to the contraction of their filaments which may be stimulated by angiotensin II present in these cells (Stevens and Lowe 1997). This may explain the atrophy of the glomerular tissue. Reducing the harm effects of the antibiotic was obvious in group III, where it obtained whey protein simultaneously with the antibiotic for 7 days. An observed improvement was occurred of the glomerulus and the general histological architecture of the renal tissues in group IV (Fig. 3D).

Robbe *et al.* (2001) revealed the structural diversity and specific distribution of O-glycans (O-specific carbohydrate structures) in normal human mucin along the intestinal tract. Thus, PAS stain reflects the intestinal mucus content. There was a decrease of the carbohydrate intensity inside the goblet cells and along the brush border of the villi in group II and group III (Fig. 5E). However, the goblet cells, the brush border as well as the basement membrane of the epithelial lining attained highly positive carbohydrate materials in group IV (Fig. 5F). Therefore, mucus production was increased after administration of whey protein. Also mucin increased in intestinal mice after treatment with inositol hexaphosphates in a mechanism to prevent the infection with *Aeromonas hydrophila* (Ahmed and Ebaid 2008). Mucin allows the maintenance of the normal intestinal flora by attachement sites (Robbe *et al.* 2001). Mucin also protects the mucosa from the pathogenic bacterial-overgrowth and penetration. Thus the increased amount of mucin in the present study can be an attempt to protect and/or maintain the enterocytes after the antibiotic stress. This is also supported by the H and E sections which revealed an observed improvement in the ileal histological structure.

After 15 days a great improvement was obtained in both renal and ileal tissues but could not completely restore their normal histological architecture. This improvement may by achieved by a mechanism that undenatured whey protein induced a glutathione, thereby reducing cellular damage during antibiotic stress (group III) and improving the intracellular function after stopping this stress (group IV). Glutathione is a potent intracellular tripeptide which vigorously binds damaging free radical molecules that would otherwise harm the cell. Therefore, glutathione is needed in greater quantities during stress, and the whey protein is a potent inducer of glutathione [Bonnous 2000;

Middleton 2004]. Recently, Zavorsky *et al.* (2007) found a significant relationship between the dosage of supplementation and the change in lymphocyte GSH levels. Furthermore, the increase in GSH was linear with the amount of whey protein ingested.

The recovery obtained in the histological structure my influenced also by the immunological response which was remarkably enhanced after supplementation with the whey protein. An observed improvement was achieved in the phagocytic activity and the chemokinetic index. Similar results were obtained by Ebaid *et al.* (2005) when they applied the undenatured whey protein in a wound healing process in mice. This can be due to the fact that the peptides and the amino acids incorporated in the whey protein can influence the immune response in different ways, and minor changes in the dietary amino acid profile may modulate the immune response (Belokrylov 1992). Therefore, it is possible that, unique amino acid groups or peptides derived from the whey proteins after ingestion may have significant immunomodulatory activities. Whey protein enhanced host humoral immune response via an important role of glutathione of the deitary whey protein (Bonnous and Batist 1989). A diet containing whey protein could enhance the IgM plaque-forming cell response in mouse spleen and this can increase its opsonic activity toward different antigens (Bonnous *et al.,* 1988; Roitt *et al.,* 1993). Therefore, it was likely that the phagocytic activity and chemokinetic indices increased in mice administrated whey protein.

Glutathione is necessary for lymphocyte proliferation (Wong and Watson 1995; Bonnous and Batist 1989]. Whey protein increased lymphocyte number (Ebaid *et al.,* 2005), and this in turn may be accompanied by increasing in Ig concentration. This indicated why the total Ig in group III and group IV was increased.

The pathological changes of the liver might led to impair its functions and this in turn, interferes with the secretion of plasma proteins. Thus, a significant decrease in these proteins and also in immunoglobulins was recorded in group II. This can decrease the blood osmotic pressure, consequently drainage of tissue fluids decreased, explaining the oedema and congestion observed in the hepatic and renal tissues (Fig. 1C and 3C). Group III and group IV recorded significantly higher protein levels than that of the control group (Fig. 7A). This increase in the plasma protein was faced with an increase in the protein content of the hepatic tissue, especially in group IV. This can be due to the role of whey protein in the protein synthesis in order to restore the tissue damage. The undenatured whey protein contains all essential and

non-essential amino acids and is a good source of the branched-chain amino acids that are necessary for cell growth (David 1999). Alkaline phosphatase was significantly elevated in group II which showed the most severe tissue damage. This enzyme was also significantly elevated in group III in which tissue was still partially damaged. A significant decline in this enzyme value as the time of supplementation with whey protein prolonged (group IV). This is seemed more likely, since most tissues became at least partially normalized in this group.

In conclusion, results indicated an obvious role of the undenatured whey protein in improving the immune response and its subsequent tissue restoration after the antibiotic stress. The improvement process was advanced as the time prolonged. Also, our previous results showed a significant role of the whey protein in accelerating different stages of wound healing processes (Ebaid *et al.*, 2005). The role of the undenatured whey protein in tissue restore can be based on providing body with both essential and non-essential amino acids, and also with the strong anti-oxidant, glutathione. However, more studies are needed to insight the detailed mechanism which enables the undenatured whey protein of the incorporation in the cell growth process and in the different cellular functions.

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