

## Histological Study on the Effect of protein malnutrition on liver and Jejunal mucosa of young rat and role of Soymilk Administration

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**Abstract: Background:** Protein malnutrition is one of the most common causes of death and illness among children under five years in developing countries. The gastrointestinal tract is the first part of the body to be affected in such condition. Substitution of animal protein intake by vegetarian protein as soymilk could be a life saving solution. **Aim of the work:** This study aimed to monitor the effect of protein malnutrition on the jejunal mucosa and liver of young rats, and to evaluate the possible beneficial effect of soymilk rehabilitation. **Materials and Methods:** Forty young albino rats were used in this study. They were divided into three groups. Group I served as a control group. Group II rats were subjected to protein malnutrition for two weeks. In group III, rats were subjected to protein malnutrition for two weeks followed by rehabilitation by soymilk intake for four weeks. The weight of animals was monitored in each group. Jejunal and liver specimens were taken, processed either for light microscopic, scanning or transmission electron microscopic examination. Morphometric and statistical studies were also done. **Results and conclusion:** Protein malnutrition resulted in major degenerative changes in the jejunal mucosa and liver structure. Rehabilitation by soymilk successfully attenuated this degenerative condition.

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**Keywords:** Malnutrition, soymilk, young rat, jejunum, liver.

### 1. Introduction:

Malnutrition is a broad term commonly used as an alternative to under nutrition. People are malnourished if their diet does not provide adequate calories and protein for growth and maintenance. Malnutrition could be attributed to the inability to fully utilize eaten food due to illness [1]. The World Health Organization (WHO) defines malnutrition as the cellular imbalance between the supply of nutrients and energy and the body's demand for them to ensure growth, maintenance, and specific functions [2].

Malnutrition in developing countries was found to affect 165 million children under five years of age [3]. These children exhibit stunted growth, with especially increasing number of stunted children in some parts of Africa due to population increase. It was estimated that malnutrition results in approximately 1 million deaths every year [4]. Millions of Egyptian children are born into poverty; where under nutrition at a young age translates into lifelong health problems. The results of Egypt Demographic and Health Surveys (EDHS), 2008, indicated that 6% of children under five years of age are underweight with 1% being severely underweight. The incidence of underweight children was the same in urban and rural areas. Not only children are affected but also adults [5].

Proteins play various roles in the human body providing structure and aid in transportation of nutrients. They also help the immune system fighting infection. Additionally, they help maintaining fluid

balance and the proper acid- base balance. Proteins are also used as a source of energy when carbohydrates are unavailable. Inappropriate low content of amino acids in diet with increased presence of carbohydrates and fiber contents have negative effect on the digestive system. Histological analysis of the digestive system is considered a good indicator of the nutritional status in animals and human. Particularly, intestine and liver are the most important organs in digestion and absorption of nutrients from food, and therefore monitoring of these organs is considered necessary in protein malnutrition [6].

The gastrointestinal tract and its associated glands are responsible for around 20 to 35% of the protein turnover as well as the energy expenditure of the entire body. Histological analysis of the digestive system is a good indicator of the nutritional status in any species. The intestine and the liver are the most important organs in digestion and absorption of nutrients from food, and therefore monitoring these organs is considered necessary in protein malnutrition [7]. Among different parts of small intestine, previous studies proved that the jejunum is the first and most affected small intestinal segment [8]. This justifies our choice of histological examination of jejunal mucosa and liver structure in protein malnutrition.

The relatively expensive proteins of animal sources in under-developed countries had focused attention on the possibilities of utilizing the vegetable resources, which are relatively inexpensive, in diet of

infants [9]. Soy protein was found to be the most inexpensive source of high-nutritional quality protein. Therefore, it is the world's predominant commercially available vegetable protein. Additionally, several health beneficial substances (such as isoflavone, saponin, oligosaccharide, phospholipid, polypeptide and dietary fibers) have been identified in soybeans. This led to an increased interest and demand for soybean and soy-based products. Soymilk is an aqueous extract of whole soybean, and its potential role in prevention and treatment of chronic diseases has long been known [10]. It is a popular beverage with abundant vegetable protein especially in Asian countries [11].

Since research should be linked to actual clinical health problems, this work aimed to assess the histological effects of protein malnutrition on the jejunal mucosa and hepatic structure. In addition, this study aimed to evaluate the efficiency of soymilk as a main protein source in diet in rehabilitation of such conditions.

## **2. Materials and methods:**

The experiment was done in the Medical Research Center, Faculty of medicine, Ain Shams University using forty male albino rats, three weeks old, of weight ranging between 60- 80 gm. They were divided into three main groups:

**Group I (control group):** Served as a control group. Rats of this group (n=20) were fed a standard chow diet containing cow milk, carbohydrates and fresh vegetables. They were provided food and water *ad-libitum*. They were subdivided into two equal subgroups (n=10):

**Subgroup IA:** Comprised control rats that were sacrificed after two weeks.

**Subgroup IB:** Comprised control rats that were sacrificed after six weeks.

**Group II (protein malnutrition group):** Rats of this group (n=10) were subjected to chronic protein malnutrition for two consecutive weeks. They were fed a protein-deficient diet served as corn flour and water (every 20 mg was dissolved in 200 ml water) [12], and fresh vegetables. They were provided this food and water *ad-libitum*. They were sacrificed after two weeks.

**Group III (soymilk rehabilitation group):** These rats (n=10) were subjected first to 2 weeks of protein malnutrition as in group II. Rehabilitation was then done using soymilk (20 mg of corn flour was dissolved in 200 ml of soymilk) [12], lactasoy®, (lactasoy Co. Ltd, Thailand). They were provided this food and water *ad-libitum*. The nutritional rehabilitation period lasted 4 consecutive weeks after which they were sacrificed.

Weight of all animals was measured before starting the experiment and just before sacrifice. All animals were anesthetized with intraperitoneal sodium thiopental (45 mg/kg) [13]. Jejunal specimens -one centimeter after the end of the c-shaped duodenum and the right lobes of the liver were taken and cut into small pieces. The jejunal specimens were cut longitudinally and their mucosa was washed thoroughly with saline to remove the debris. Both jejunum and liver specimens were then put in the proper fixative:

### **1- Light microscopic study (LM):**

Specimens of Jejunum and liver were fixed in 10% neutral buffered formalin and processed to obtain paraffin blocks. Serial sections of both organs, 5µm-thickness were prepared and subjected to Hematoxylin and Eosin stain (H&E) and periodic acid- Schiff stain (PAS) [14].

Jejunal specimens were also subjected to immunohistochemical staining for immunoglobulin A (IgA) using Avidin - Biotin peroxidase technique. The antibody was purchased as Monoclonal Anti-Rat IgA, Product Number R0636 (dilution of 1:500), Sigma-Aldrich, Saint Louis, Missouri, USA. Sections were counterstained with hematoxylin, dehydrated, cleared and mounted. The reaction appeared as brownish cytoplasmic granules. Positive controls were done on tonsil specimens. Negative control was done after omitting the primary antibody [14].

### **2- Scanning electron microscopic study (SEM):**

Small pieces of the jejunum were fixed in 2.5% glutaraldehyde in Phosphate buffered saline (pH=7.4) for two hours at room temperature. Tissues were then processed and gold-coated using sputter coated SCD/005. Tissues were mounted on copper stub and viewed using scanning electron microscope (XL30) in the Anatomy Department, Faculty of Medicine, Ain Shams University [15].

### **3-Transmission electron microscopic study (TEM):**

Small liver specimens (1 mm<sup>3</sup>) were fixed in phosphate-buffered glutaraldehyde and processed to form capsules. Ultrathin sections (50–60 nm thickness) were cut using an ultramicrotome. Sections were mounted on copper grids and stained with a saturated solution of uranyl acetate followed by lead citrate. Ultra-thin sections were examined and photographed by JEM-1200EXII transmission electron microscope in Faculty of Agriculture, Cairo University [14].

### **4-Morphometric and Statistical studies:**

**a)** Height of the enterocytes of the jejunal villi in H&E-stained sections.

**b)** Number of goblet cells in jejunal villi in PAS-stained sections.

c) Number of intraepithelial lymphocytes in mucosa of villi in H&E-stained sections.

d) Number of IgA positive cells in lamina propria of jejunal villi in immunohistochemically-stained sections.

The measurements were performed using Leica Qwin software program installed on a Dell PC (Texas, USA). The PC was connected to a microscope (Leica microsystem, Heerbrugg, Switzerland) in the Histology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt. Measurements were taken from five different sections (five high power fields/section) from eight animals in each group. The mean values were estimated and the standard deviation (SD) was calculated using SPSS statistical program version 17; IBM Corporation, NY 10589. Data were evaluated using the one-way analysis of variance test (ANOVA). The least significant level of probability used was at *P* value less than 0.05.

### **3. Results:**

#### **Weight and behavior of rats:**

Rats of the control group (group I) gained weight progressively throughout the study. They reached an average weight of  $110.25 \pm 3.3$  (Mean $\pm$ SD) after two weeks from the beginning of the experiment. The rats continued to increase in weight that reached an average of  $150.33 \pm 5.9$  gm at the end of the experiment after six weeks.

In group II, a significant reduction of rats' weight was detected after two weeks as compared to the control rats, with an average of  $70.67 \pm 7.77$  gm, *P* < 0.05. Moreover, protein malnourished rats presented severe wasting, fatigue, lack of motor activity, changing in behavior up to eating each others. Two of them died during the experiment.

Soy milk rehabilitation group (group III) presented a catch up growth after the administration of soy milk. There was a significant increase of animal weight as compared to malnutrition group ( $117.67 \pm 14.22$  gm, *P* < 0.05). However, this was non-significantly decreased as compared to control group. In addition the rats' behavior much improved with disappearance of aggressiveness and lethargy seen in the protein malnourished rats.

#### **Jejunal structure:**

Both subgroups of the control group showed the same histological appearance in all studies performed.

#### **A- Light microscopic examination results:**

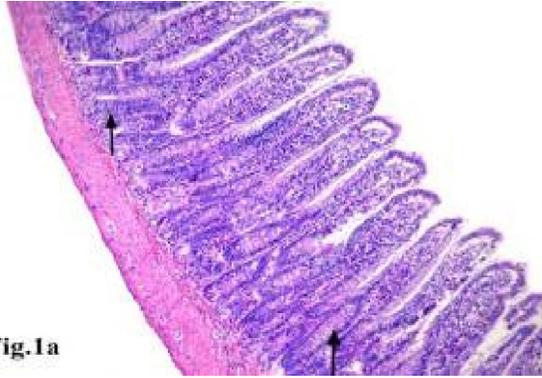
##### **I- The H&E-stained sections:**

Sections of the jejunum of control group (group I) showed long fingerlike villi mucosal extensions enclosing a core of lamina propria. They were continuous with intestinal glands (crypts of Lieberkühn) that were seen spanning the lamina

propria and limited inferiorly with the muscularis mucosa (Fig.1a). The surface of the villi and crypts was covered by continuous layer of enterocytes and intervening goblet cells. The enterocytes appeared acidophilic, columnar in shape with basal oval nuclei (Fig.1b) with mean height of  $26.30 \pm 2.33$  (Mean $\pm$ SD) (histogram 1). Intact acidophilic brush border was seen on the enterocytes' apical surfaces (Fig.1b). The goblet cells appeared distended by pale basophilic foamy secretion (Fig.1b). Scattered intraepithelial lymphocytes were detected (Fig.1b); with a mean number of  $6.60 \pm 1.82$  (histogram 1). The core of the villi illustrated loose connective tissue (CT) containing central lacteal, mononuclear cells and blood capillaries (Fig.1b).

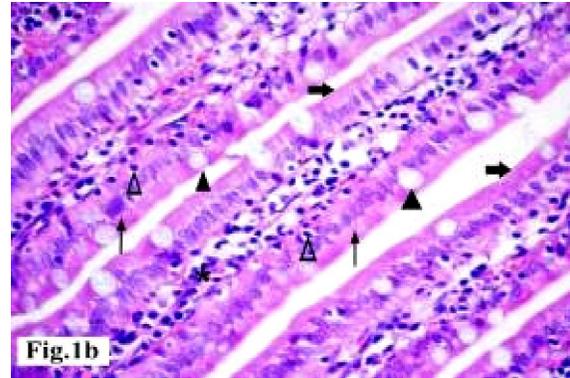
Examination of H&E-stained sections of the jejunum of protein malnourished rats (group II) revealed that whole jejunal wall thickness was apparently decreased as compared to that of the control group. Severe histological alteration of the mucosal architecture was also evident. The villi appeared distorted and shorter when compared to control group. Multiple focal areas showed sloughed villi tips (Fig. 2a). Many enterocytes appeared cubical with significant decrease in height as compared to the control group ( $19.55 \pm 0.94$   $\mu$ m, *P* < 0.05, histogram 1). They appeared darkly acidophilic with dense irregular nuclei. Other enterocytes appeared ballooned with ill-defined borders, having vacuolated cytoplasm and pale rounded nuclei. Interrupted brush border was seen on the enterocytes' apical surfaces. Few goblet cells were seen. Many intraepithelial lymphocytes were seen in-between the enterocytes. They were significantly increased as compared to the control group, with a mean number of  $16.00 \pm 2.45$ , *p* < 0.05 (histogram 1). The core of the villi showed congested blood vessels and mononuclear cells (Fig.2b).

The H&E-stained sections of the jejunum of the soy milk rehabilitated group showed marked improvement of jejunal structure as compared to the malnourished group. The mucosa restored normal appearance with regular, long fingerlike villi. However, some villi showed sloughing of their apices (Fig.3a). The enterocytes regained a well defined columnar shape, with acidophilic cytoplasm and oval vesicular nuclei. Intact acidophilic brush border was seen on the enterocytes' apical surfaces. Significant increase in height as compared to the malnutrition group was well noticed ( $29.0 \pm 3.62$   $\mu$ m, *p* < 0.05, histogram 1). Intraepithelial lymphocytes were observed, with a mean number of  $(8.2 \pm 1.10)$  which was significantly decreased as compared to group II, *p* < 0.05. However, it was non significantly increased as compared to the control group (*P* > 0.05, histogram 1).



**Fig.1a**  
Jejunal mucosa thrown into regular tall finger-like villi with a core of lamina propria. Crypts of Lieberkühn are seen spanning the lamina propria (↑).

*Control group, H&E x 250*



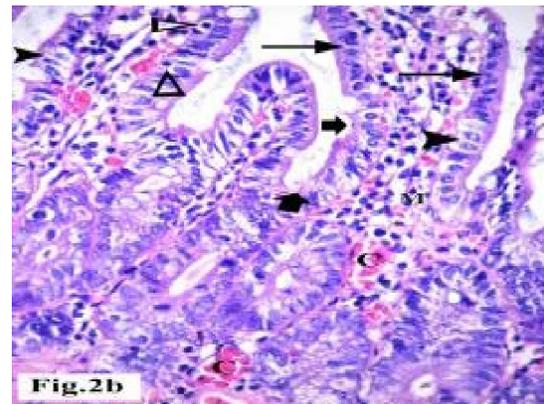
**Fig.1b**  
Columnar acidophilic enterocytes with basal oval nuclei covering the villi and lining the crypts (↑) can be seen. Notice the intact brush border of the enterocytes (thick arrow). Intervening goblet cells are seen expanded with pale basophilic foamy secretion (▶). Scattered intraepithelial lymphocytes can be observed (Δ). Note the mononuclear cells in the core of villi (\*).

*Control group, H&E x 400*



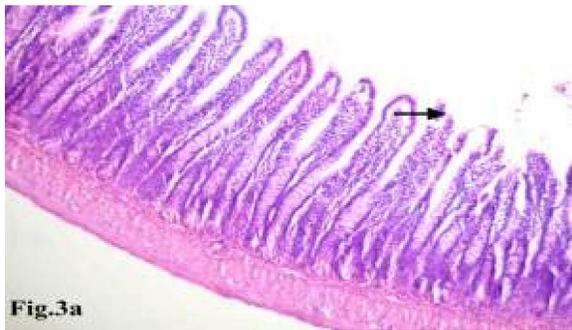
**Fig.2a**  
Overall decrease in the jejunal wall thickness can be seen. Notice the distortion of the villi which appear short and atrophic. Most of the villi are seen with sloughed tips (↓).

*Group II, H&E x 250*



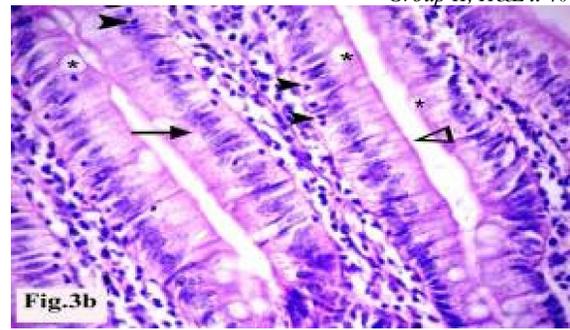
**Fig.2b**  
Ballooned vacuolated enterocytes with ill-defined borders and karyolytic nuclei (▶). Other enterocytes appear cubical with darkly acidophilic cytoplasm and dense irregular nuclei (→). Notice the intraepithelial lymphocytes (Δ), mononuclear cells (M) and congested capillaries (C) in the lamina propria.

*Group II, H&E x 400*



**Fig.3a**  
Jejunal villi appear tall and regular. Few villi with sloughed tips can still be seen (→).

*Group III, H&E x 250*



**Fig.3b**  
The enterocytes appear columnar with acidophilic cytoplasm and regular nuclei (→). Intact brush border can be seen (Δ). Note the presence of many goblet cells (\*), and few intraepithelial lymphocytes (▶).

*Group III, H&E x 640*

**II- PAS-stained sections:**

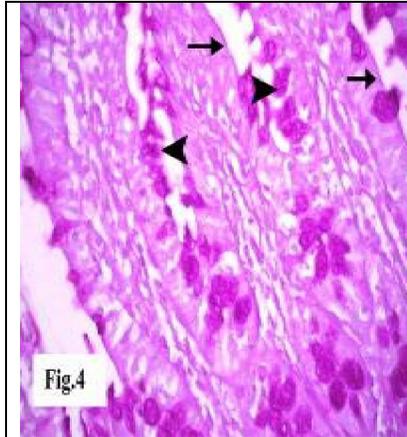
Sections of the control group demonstrated intact brush border of enterocytes. It also showed numerous

goblet cells distended with magenta red secretion. A thin film of mucous was seen covering the luminal border of enterocytes and goblet cells (Fig.4). Mean number of goblet cells in the villi was  $49.13 \pm 18.01$  (histogram 1).

The protein malnourished group (group II) showed interrupted brush border of most of the enterocytes. Many goblet cells appeared empty, while others appeared with less amount of mucin content reflected by pale magenta red vacuolated secretion (Fig.5). Significant decreased in number of goblet

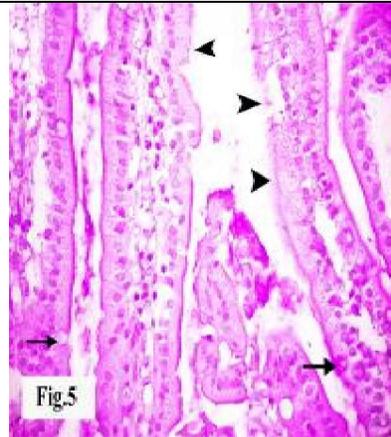
cells in villi ( $22.93 \pm 4.91$ ,  $p < 0.05$ ) was evident as compared to that of the control (histogram 1).

The soymilk rehabilitated group showed that the brush border of many enterocytes regained its integrity. The goblet cells were seen distended by mucin (Fig.6). A non significant increase in mean number of goblet cells in the villi was detected as compared to group II ( $28.73 \pm 6.06$ ,  $p > 0.05$ ). However, they were significantly decreased,  $p < 0.05$ , as compared to the control (histogram 1).



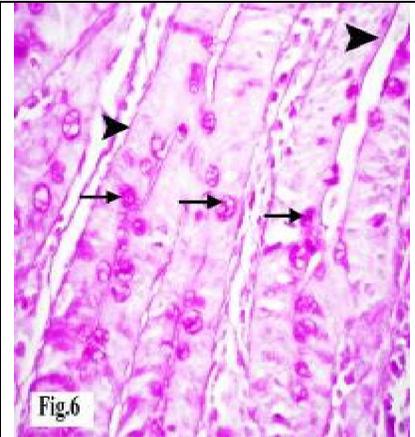
**Fig.4**  
Regular intact brush border can be seen (▶). Goblet cells appear with its magenta mucin content (→).

*Control group, PAS x 400*



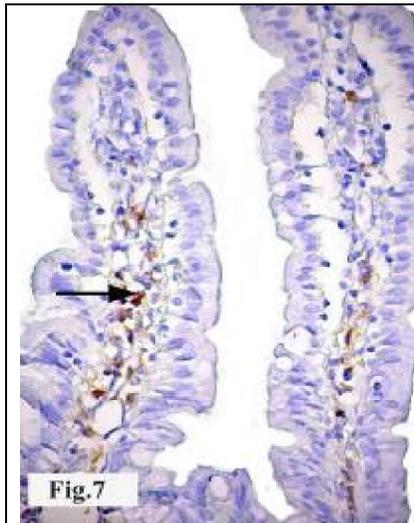
**Fig.5**  
Interrupted brush border can be seen in most of the enterocytes (▲). Few goblet cells with decreased mucin content can be noticed (→).

*Group II, PAS x 400*



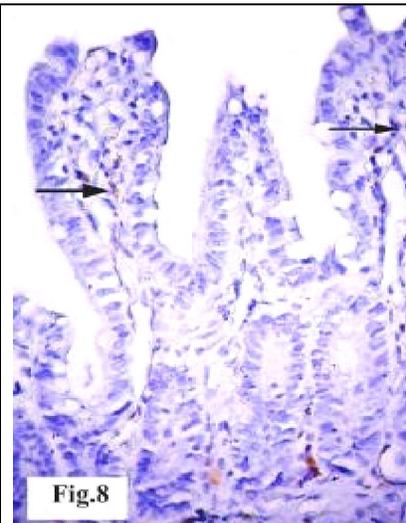
**Fig.6**  
Intact brush border can be seen (▲). Note the increase in goblet cell number and mucin content (→)

*Group III, PAS x 400*



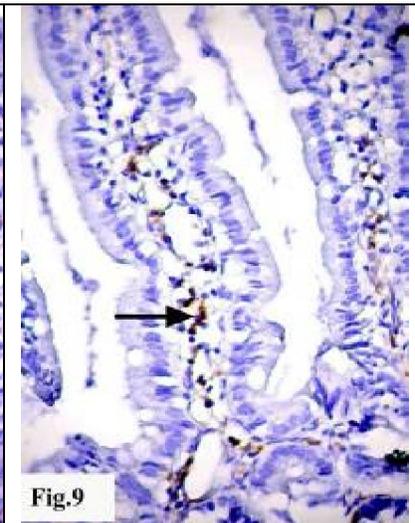
**Fig.7**  
Few IgA immunohistochemically positive cells appear clearly in the core of jejunal villi (→).

*Control group, Avidine-Biotin peroxidase technique x 400*



**Fig.8**  
Minimal IgA immunohistochemically positive cells in the core of villi (→).

*Group II, Avidine-Biotin peroxidase technique x 400*



**Fig.9**  
Few IgA immunohistochemically-stained cells in the core of villi appear (→).

*Group III, Avidine-Biotin peroxidase technique x400*

### III- Immunohistochemically- stained sections for IgA:

Minimal positive reaction of IgA-secreting plasma cells in the CT core of intestinal villi was detected in both subgroups of the control group (Fig.7), with mean number of  $(10.7 \pm 1.89, \text{histogram } 1)$ .

Sections of the protein malnourished group (group II) revealed a significant decrease in mean number of IgA positive cells as compared to the control group  $(6.3 \pm 5.62, p < 0.05, \text{histogram } 1, \text{Fig. } 8)$ .

Significant increase in mean number of IgA positive cells was detected in soymilk rehabilitated group (group III) as compared to the malnutrition group  $(11.8 \pm 1.81, p < 0.05, \text{histogram } 1)$ . They were non-significantly different from the control group (Fig.9).

### B- The scanning electron microscopic results:

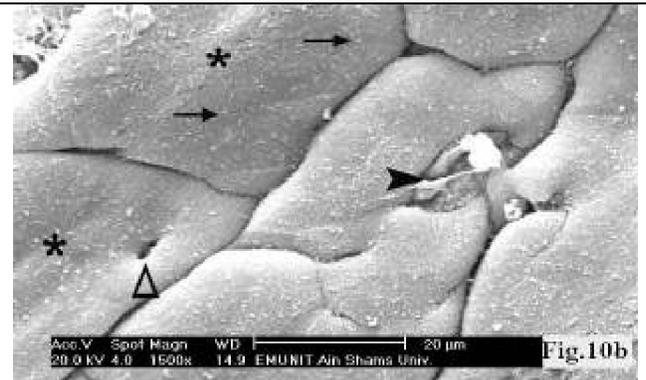
The control group exhibited regular villi of the jejunal mucosa with narrow intervillous spaces (Fig.10a). The villi were seen covered by intact enterocytes with their hexagonal pattern and scattered mucous secretions on their surfaces. Microvilli were seen on the enterocytes' surface. Goblet cells' orifices appeared either devoid of secretion or covered by protruding mucin secretion (Figs.10a, 10b).

Examination of the protein malnourished group revealed disturbed architecture and ulcerations of most of the villi. Other villi appeared devoid of microvilli with smooth surface and loss of the normal hexagonal pattern of the enterocytes. Apparent reduction in number of goblet cells' orifices was evident as compared to the control group and they were mostly seen depleted from mucous (Fig.11a). The ulcerated surface of some villi showed extrusion of necrotic material at their tip with appearance of red blood cells leaking from the ulcer (Fig.11b).



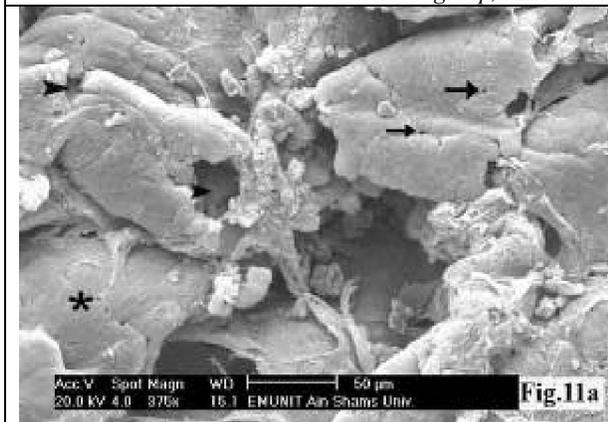
Regular villi (→), with narrow intervillous spaces (\*). Notice the mucous secretion on the surface and sides of the villi (▲).

Control group, SEM x 375



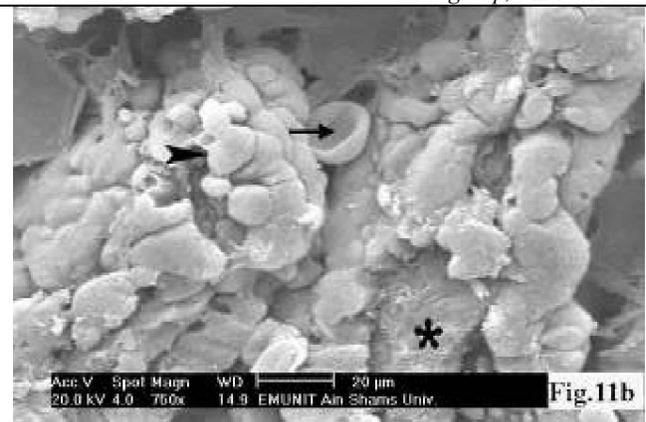
Intact hexagonal-shaped enterocytes (\*) covered by microvilli (→). Goblet cells' orifices are seen either protruding secretion (▶) or devoid of secretion (Δ).

Control group, SEM x 1500



Ulcerated areas of many villi (▶). Other villi appeared with smooth surface with loss of the hexagonal pattern of the enterocytes (\*). Notice the apparently decreased number of goblet cells with depletion of their mucin content (→).

Group II, SEM x 375



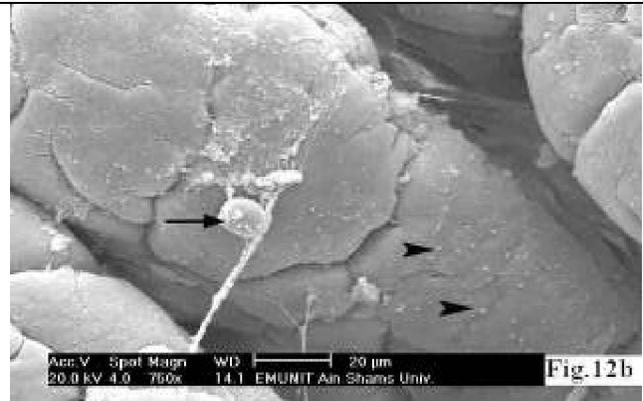
Extruded necrotic material (▶) at the tip of some ulcerated villi (\*). Red blood cells are seen leaking from the ulcer (→).

Group II, SEM x 750



Restoration of the regular arrangement of villi covered by intact enterocytes with regular hexagonal appearance.

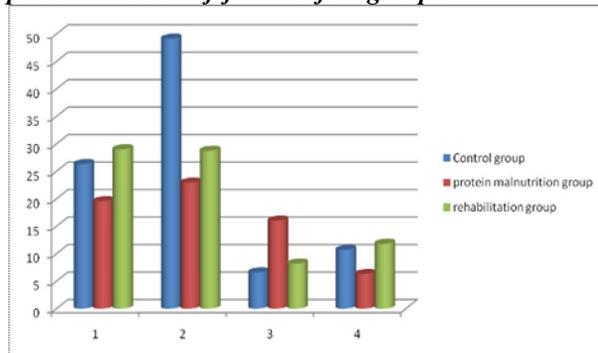
Group III, SEM x 188



Secretion extruded from a goblet cell orifice is seen (→). Notice the reappearance of microvilli (▶).

Group III, SEM x 750

**Histogram 1: Means of measurements of different parameters in the jejunum of all groups:**



- 1= Height of enterocytes of jejunal villi (in  $\mu\text{m}$ ).  
 2 = Number of goblet cells in jejunal villi.  
 3= Number of intraepithelial lymphocytes.  
 4= Number of IgA positive cells in jejunal villi.

In the soymilk rehabilitated group, examination by SEM showed restoration of the regular arrangement of the villi with intact epithelial covering cells (Fig.12a). Enterocytes appeared intact with regular hexagonal appearance. Reappearance of microvilli was evident in focal areas. Goblet cells' orifices appeared distended with secretion (Fig.12b).

#### Liver structure:

Both subgroups of the control group showed the same histological appearance in all studies performed.

#### A- Light microscopic results:

##### I- The H&E-stained sections:

Examination of H&E-stained hepatic sections of the control group showed branching and anastomosing plates of hepatocytes radiating from the central vein with intervening blood sinusoids (Fig.13a). Portal areas were seen at the periphery of the hepatic lobules (Fig.13b). The hepatocytes appeared polyhedral with acidophilic cytoplasm and central rounded vesicular nuclei. Binucleated cells were frequently encountered (Figs.13a, Fig.13b).

In the protein malnourished group, focal histopathological changes were seen in all of the sections examined. Many hepatocytes appeared with vacuolated with halos around the nuclei (Fig.14a). Others were seen degenerated with vacuolated cytoplasm containing acidophilic Mallory bodies and karyolytic nuclei. These cells were ballooned and located mainly in the periportal areas (Fig.14b), although were found shrunken and scattered in other areas (Figs.14a). On the other hand, many cells appeared degenerated mainly in the periportal areas with deeply acidophilic cytoplasm and pyknotic nuclei (Fig.14b).

Many sections exhibited margination of chromatin and concomitant central pallor in the nuclei of most of the hepatocytes (Figs.14a, 14b). Acidophilic hyaline exudates were mostly seen in central veins (Fig.14a) and in the portal venules (Fig.14b). Congested central veins were also observed (Fig.14c).

H&E stained-sections revealed apparent degree of restoration of hepatic architecture in the soymilk rehabilitated group. Regular arrangement of hepatocytes into plates of cells radiating from the central vein or in the periportal areas was evident. The cytoplasm of most of liver cells appeared acidophilic with central rounded nucleus. Most of the nuclei showed regular chromatin pattern (Figs.15a, 15b).

##### II- The PAS-stained sections:

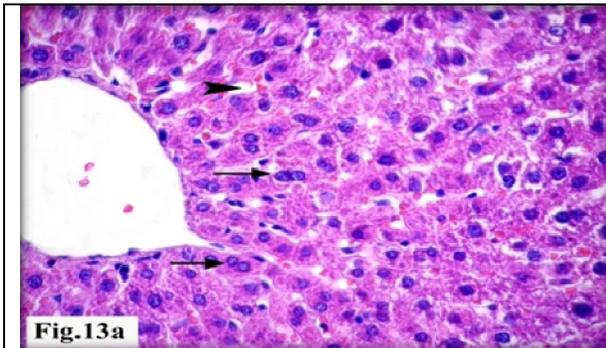
Hepatic sections of the control group stained with PAS showed the irregularly distributed glycogen granules' content of the hepatocytes (Fig.16).

The PAS stained-sections of the protein malnourished group showed multiple focal areas with apparently increased glycogen granule content, mostly in zone I and to a lesser extent in zone III (Fig.17).

In the soymilk rehabilitated group, the PAS stained-liver sections revealed apparent decrease in glycogen content of hepatocytes. This was more

evident in zone I when compared to the protein

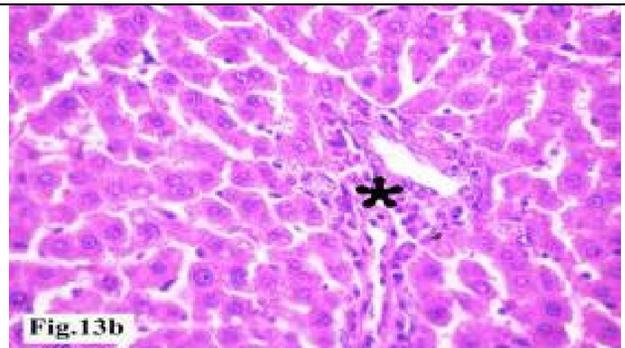
malnourished group (Fig.18).



**Fig.13a**

Hepatocytes with acidophilic cytoplasm and central rounded vesicular nucleus with intervening hepatic sinusoids (▶). Binucleated cells can be seen (→).

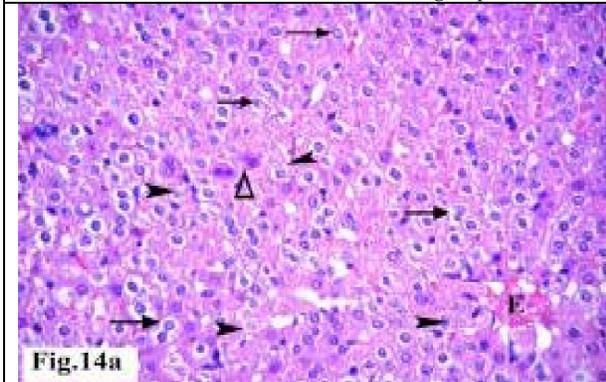
*Control group, H&E x 400*



**Fig.13b**

The portal area at the periphery of the lobule (\*). Notice the hepatocytes with acidophilic cytoplasm and regular vesicular nuclei (→).

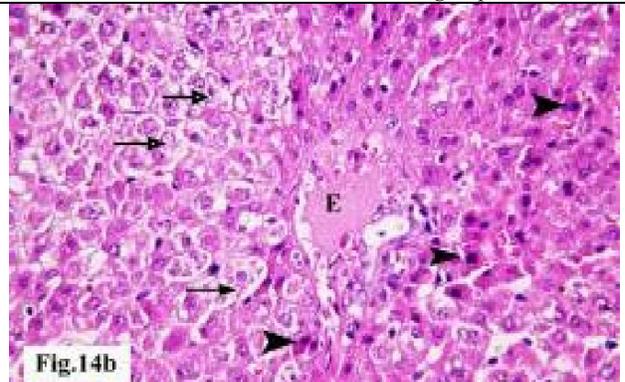
*Control group, H&E x 400*



**Fig.14a**

Vacuolated hepatocytes with halos around the centrally-pale nuclei can be seen (→). Some vacuolated hepatocytes with karyolytic nuclei can be noticed (▶). Other hepatocytes are seen with deeply acidophilic cytoplasm and deeply stained nuclei (Δ). Note the acidophilic exudate in the central vein (E).

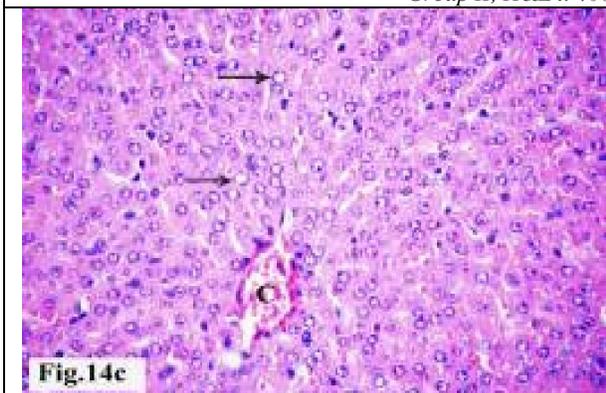
*Group II, H&E x 400*



**Fig.14b**

Many of the hepatocytes in the periportal area are ballooned, vacuolated, full of Mallory bodies and karyolytic nuclei (→). Other hepatocytes have deeply acidophilic cytoplasm and pyknotic nuclei (▲). Note the acidophilic exudate in the portal venule (E).

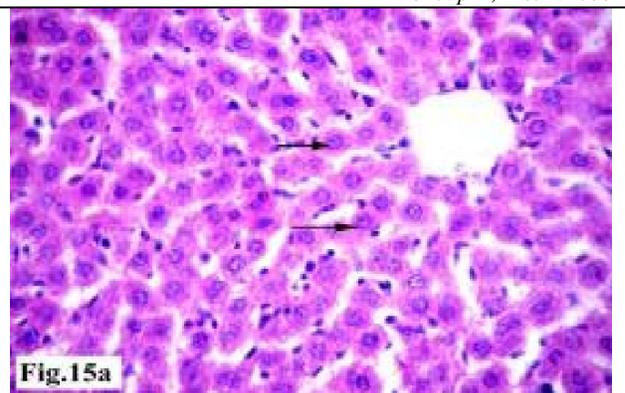
*Group II, H&E x 400*



**Fig.14c**

Margination of chromatin with concomitant central pallor is seen in nuclei of most of the hepatocytes (→). Note the congested central vein (C)

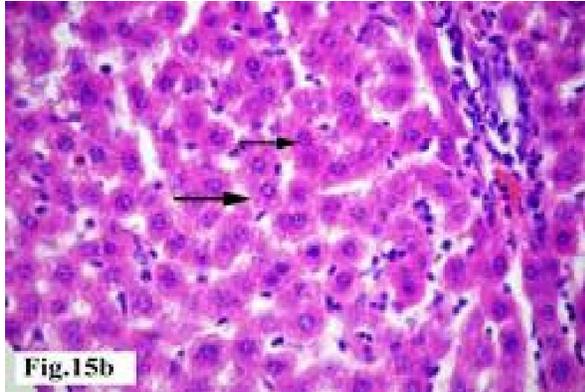
*Group II, H&E x 400*



**Fig.15a**

Regularly arranged hepatocytes can be seen. The cytoplasm appears mildly acidophilic with central regular vesicular nuclei. Regular chromatin pattern is noticed in most of the hepatocytes' nuclei (→).

*Group III, H&E x 400*



Regularly arranged hepatocytes in the periportal area. They are acidophilic with central regular vesicular nuclei (→).

Group III, H&E x 400

### B- Transmission electron microscopic results:

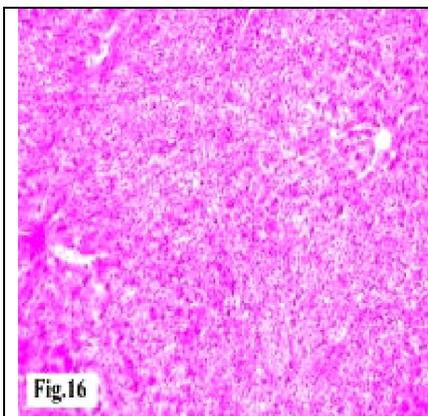
By TEM, hepatocytes of the control group appeared studded with mitochondria with apparent cristae, rough endoplasmic reticulum (rER) and euchromatic nuclei. Scattered  $\alpha$  glycogen rosettes were seen (Fig.19).

On examination of the protein malnourished group by TEM, Bizarre-shaped mitochondria with lost cristae were frequently seen. The mitochondria were irregular either short (Figs.20a, 20b, 20c) or highly elongated (Figs.20a, 20b). Curved and circled around highly elongated mitochondria could be easily seen (Fig.20b). Small electron lucent droplets were seen inside most of the mitochondria (Figs.20a, 20b, 20c). The rER often appeared scanty and fragmented. They were frequently seen tethered to the elongated mitochondria (Figs.20a, 20b, 20c). Many peroxisomes could be also observed (Fig.20a). Multiple tiny

moderately dense fat droplets were seen intermingled with enormous amount of glycogen granules that were not only seen as typically aggregated  $\alpha$  rosettes, but also as individual  $\beta$  particles (Figs.20a, 20b, 20c, 20d). Some nuclear envelopes were seen regular and intact (Fig.20a).

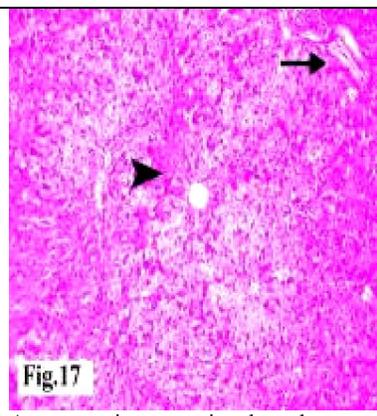
Others were totally ill-defined in shrunken nuclei, showing clearly visible regular uniformly-sized nuclear pores (Fig.20c, 20d). The nuclear pores on the periphery of these nuclei exhibited moderately electron dense granular content, unlike the ones toward the centre of the nuclei which demonstrated electron lucent content (Fig. 20d). Abnormal chromatin organization was clearly observed in the nuclei. Little amount of chromatin was seen in the center of the nuclei while heterochromatin was seen condensed at the inner nuclear membrane (Figs. 20a, 20c, 20d). Nucleoli were often unrecognizable in most of the nuclei (Figs.20a, 20c, 20d). Glycogen aggregations could be observed inside the nuclei (Fig.20c, 20d).

By TEM examination, rehabilitation by soymilk showed great improvement of the hepatocytes structure. The cytoplasm of hepatocytes contained plenty of regular oval or round mitochondria with plenty intact rER. Fat droplets were apparently largely decreased in number as compared to the malnutrition group and were gathered into medium-sized droplets. Glycogen appeared less in amount as compared to the malnourished group. It was focally present as  $\alpha$  rosettes in-between the organelles. The nuclei appeared with intact envelope, regular chromatin organization and visible nucleoli (Figs.21).



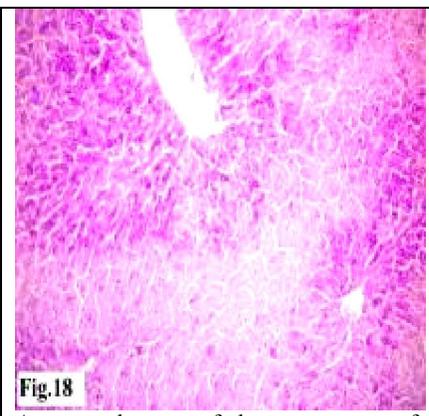
Irregular distribution of glycogen granules in the hepatocytes.

Control group, PAS x 200



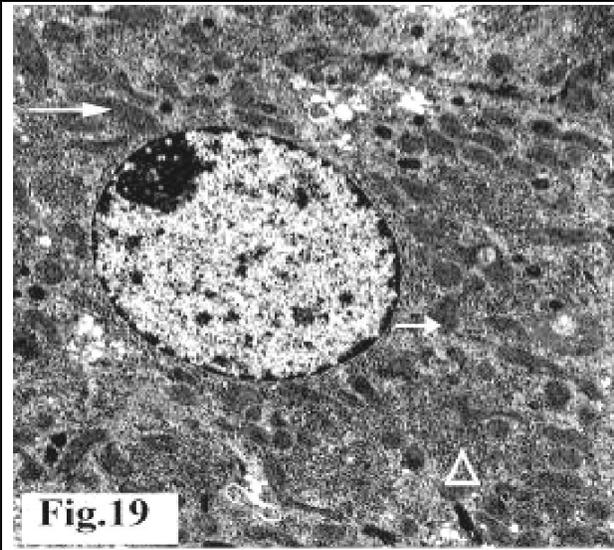
Apparent increase in the glycogen content of the hepatocytes in mostly in zones I (→) and to a lesser extent in zone III (▶).

Group II, PAS x 200



Apparent decrease of glycogen content of most of the hepatocytes.

Group III, PAS x 200



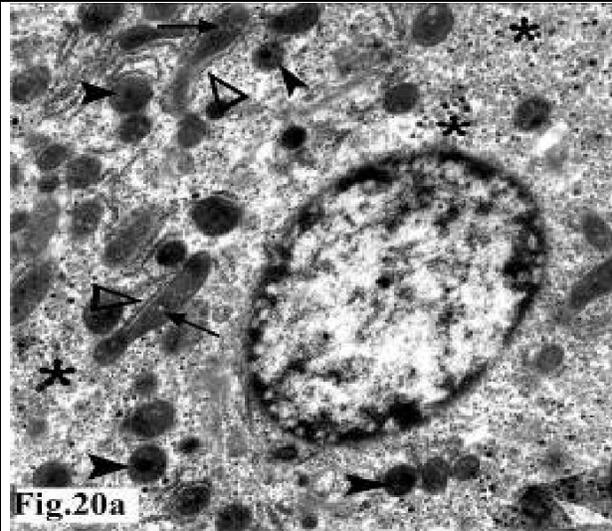
**Fig.19**

L:1.tif  
Print Mag: 10500x @ 211 nm  
TEM Mode: Imaging

2 microm  
HV=80.0kV  
Direct Mag: 5500x

A hepatocyte studded with mitochondria (→) and rough endoplasmic reticulum (Δ). The nucleus appears regular and euchromatic. Note the scattered α glycogen rosettes (▲).

*Control group, TEM x 5000*



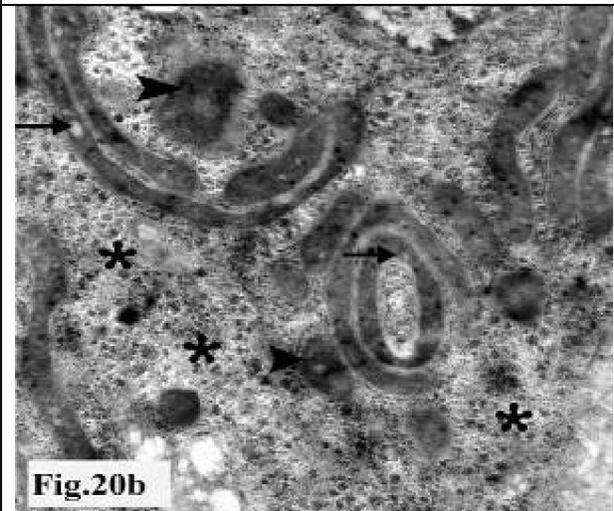
**Fig.20a**

L:1.tif  
Print Mag: 21000x @ 211 nm  
TEM Mode: Imaging

500 nm  
HV=80.0kV  
Direct Mag: 10000x

Elongated mitochondria with lost cristae, containing small electron lucent droplets (→) can be seen. Mitochondria are seen tethered to scanty rER (Δ). Many peroxisomes can be easily noticed (▲). Dispersed numerous glycogen granules intermingled with tiny fat droplets are seen throughout the cytoplasm (\*). The nucleus appear with little amount of chromatin in its center. Most of the heterochromatin is adherent to the inner nuclear membrane. Note the disappearance of the nucleolus.

*Group II, TEM x 10000*



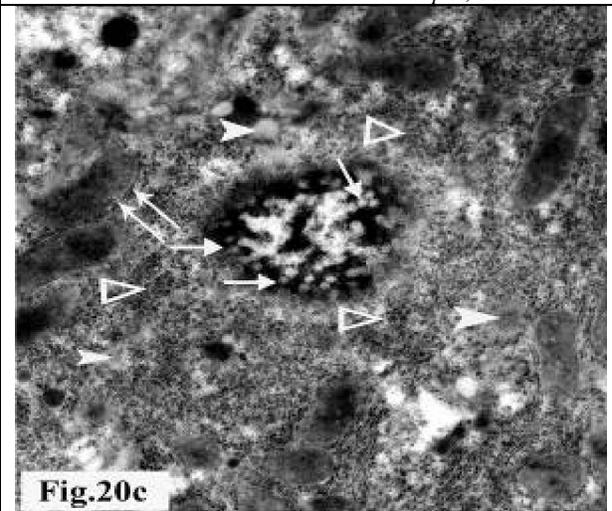
**Fig.20b**

L:1.tif  
Print Mag: 36000x @ 211 nm  
TEM Mode: Imaging

500 nm  
HV=80.0kV  
Direct Mag: 15000x

Irregular elongated and circled mitochondria containing electron lucent droplets (→). Short mitochondria can be also seen (▶). Note the accumulated fat droplets and glycogen granules in the cytoplasm (\*).

*Group II, TEM x 15000*



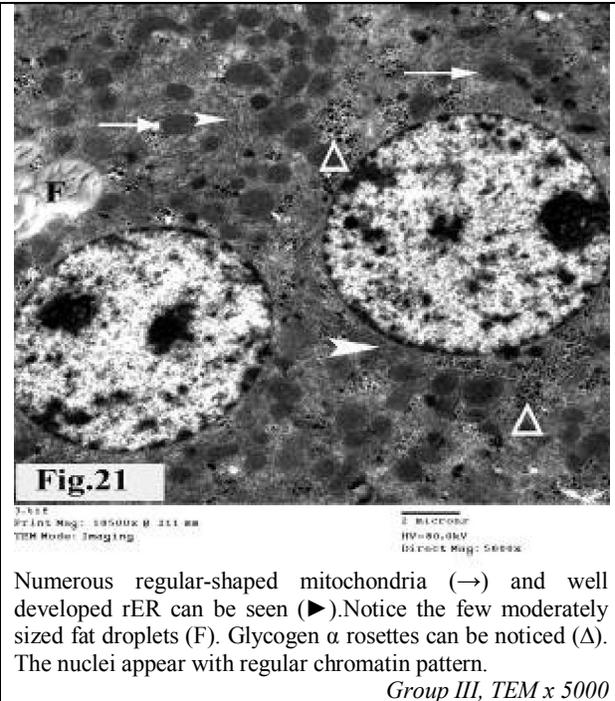
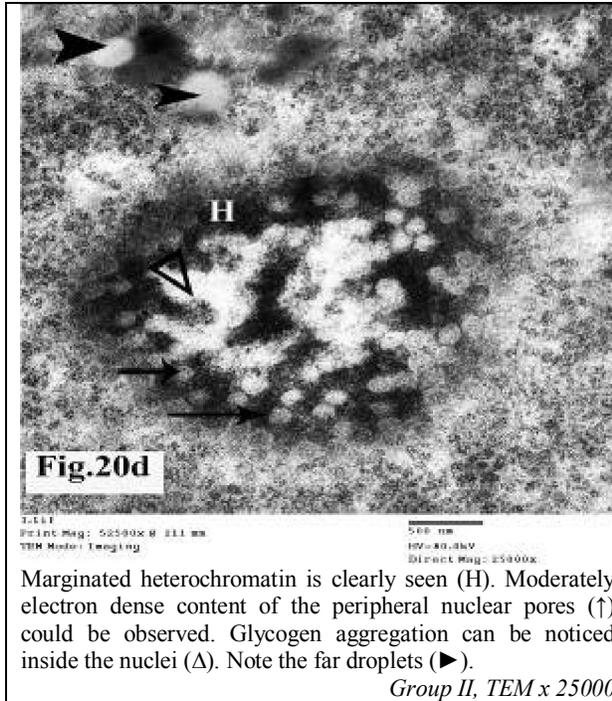
**Fig.20c**

L:1.tif  
Print Mag: 20000x @ 211 nm  
TEM Mode: Imaging

500 nm  
HV=80.0kV  
Direct Mag: 12000x

Shrunken nucleus with ill-defined nuclear envelope and condensed marginated heterochromatin (Thick arrow). Nuclear pores can be observed (→). Note the fat droplets (▶) and the glycogen granules (Δ). Notice the rER tethered to the elongated mitochondria (↑↑).

*Group II, TEM x 12000*



#### 4. Discussion:

In the present study, aggressive behavior of the protein malnourished rats was noticed. A link between aggressiveness and poor nutrition, either prenatally or postnatally was previously reported. It was stated that malnutrition effects brain development. Moreover, Malnutrition was considered, per se, a stressful situation for juvenile rats resulting in alteration of their social behavior [16].

In the present work, protein malnourished rats showed significant weight loss when compared to control group. This result was similar to that reported by several researchers [7, 12, 16]. It was stated that loss of weight is the first clinically apparent feature in all protein malnourished species [7].

Protein malnutrition had a negative impact on the height of jejunal villi which were ulcerated with sloughed tips. Atrophic, stunted villi were known as well documented features of protein malnutrition in rats [7, 8, 18]. In addition, enterocytes significantly decreased in height after protein malnutrition in the present work, together with thin or interrupted brush border of most of them. Such decrease in height could correspond to a compensatory mechanism aiming at approximating the nutrients of the intestinal lumen of the blood vessels within the lamina propria. This approximation was found to contribute for the increase of nutrients' diffusion and, consequently, absorption [8]. Coinciding, it was stated that a new equilibrium might be achieved between cell size and diminished nutrition. They added that atrophy in malnutrition represents a retreat by the cells to a

smaller size at which survival could be still possible [19]. Protein malnutrition might also decrease the epithelial cell plasma membrane biogenesis. Multiple mechanisms were employed to explain this phenomenon. Decreased cellular mitotic activity was proposed as a cause. It was attributed mainly to the deficiency of essential amino acids and micronutrients necessary for the protein synthesis of mitotic spindles [12, 20, 21]. As a consequence, decrease rates of production, maturation, and migration of intestinal cells would result. In addition, it was also suggested that decreased villous growth factor in bile and pancreatic enzymes in protein malnutrition could affect the villous height [18]. Moreover, the expected decrease in cell membrane biogenesis could alter the expression of glycoprotein receptors in the enterocyte membrane. This in turn might modify the amount of mucin that is retained on epithelial surfaces [22]. This can explain the interruption and thinness of brush border of the enterocytes seen in the current study.

In the present study, goblet cells were significantly decreased in number. This coincides with the previously reported decrease in cellular proliferation in cases of protein malnutrition [12]. Moreover, apparently little mucin content of goblet cells was obvious in protein malnourished rats. Cystein deficiency might be contributing significantly to reduced secretory mucin in malnutrition owing to the fact that mucin proteins are rich in cysteines, which would be deficient in protein malnutrition [23]. Reduced mucin production might result in decrease of one of the most important first line intestinal

protective mucosal barrier. This disrupted barrier would increase the liability to intestinal invasion by micro-organisms [24]. That might be augmented by the compromise of innate immune responses in malnutrition [25].

Intraepithelial lymphocytes (iELs) were significantly increased in the protein malnourished group of the present study. It was reported that iELs are the closest populations of immune cells to the host-lumen interface [26]. These cells were known to be of the T lymphocyte family, possessing  $\gamma\delta$  T cell receptor (TCR $\gamma\delta$ ). The intimate association of TCR $\gamma\delta$  iELs with the epithelium of the small intestine is indicative of their involvement in maintaining intestinal barrier function [27]. Both, enterocytes and iELs found to participate in immediate host defense; limiting pathogen translocation across the intestinal epithelium [28]. Cross-talk was confirmed to occur between epithelium and intraepithelial lymphocytes. This might occur through specific cytokine receptor signaling. Interleukin 23 (IL23) was found to be expressed by enterocytes upon pathogenic or commensal challenges, leading to IL22 expression by iELs. This resulted in upregulation of the bactericidal Angiogenin 4 production by Paneth cells, maintaining intestinal microbial homeostasis and mucosal defense of the gastrointestinal tract [27].

The IgA-secreting plasma cells play an important role in the local immunological defense against pathogens and intestinal damage [29]. Protein energy malnutrition was documented to impair IgA production by plasma cells and its secretion into the lumen of the intestine [12, 30]. Results reported in the present study confirmed these previous researches, since a significant decrease of IgA producing cells in the jejunal lamina propria was detected during the experimental protein malnutrition in group II. It was suggested that this could be due to impairment of the ability of the cells IgA producing-plasma cells to home to mucosal tissues [31].

Light microscopic examination of liver sections of the protein malnourished group of this study showed many ballooned degenerated hepatocytes full of Mallory bodies (Mallory hyaline). These Mallory bodies were previously described as aggregations of damaged cytokeratin intermediate filaments mainly of types 8 and 18. Other hepatocytes showed apoptotic changes in the form of deeply acidophilic cytoplasm with deeply-stained nuclei. Both findings were previously reported as characteristic features of non alcoholic fatty liver disease (NAFLD) [19].

Abnormally elongated mitochondria were clearly seen by TEM in the protein malnourished group in the present study. Mitochondrial elongation in response to starvation was observed in cell cultures and in vivo in muscle and liver from mice fasted for 12 h. Elongation

of mitochondria was hypothesized to increase the activity of ATP synthase, optimizing ATP production in times of nutrient restriction. Otherwise, when the damage is beyond the repairing capacity of the hepatocytes, cellular apoptosis or even necrosis would occur. Starvation was reported to cause unopposed fusion and elongation of mitochondria by increasing cyclic AMP (cAMP) levels and subsequent activation of protein kinase A [32].

In close relation, starvation was also found to induce macroautophagy; a catabolic process that allows the recycling of intracellular components under conditions of nutrient depletion. During early macroautophagy unopposed mitochondrial fusion occur leading to their elongation both in vitro and in vivo. Longer mitochondria were supposed to be protected from being degraded that is critical to sustain cell viability. Macroautophagy was reported to be initiated by multiple protein complexes that sense nutrition deprivation. The starved cells eat their own components and recycle the contents to provide themselves with nutrients and energy in an attempt to survive nutritional deficiency [19]. Moreover, mitochondria were suggested to participate in the formation of the autophagosomal membrane, in a process that depends on the tethering of mitochondria to the endoplasmic reticulum [32]. It was suggested rER provides the mitochondria with essential elements necessary for the extension, curvature and closure of the isolation membrane to form autophagosomes [33]. This was in line with the results of the present study seen by TEM where mitochondria were frequently seen associated to or even surrounded by rER.

Multiple electron lucent bodies were detected inside the mitochondria in the current study. In normal condition, it was stated that fatty acids reach the mitochondrial matrix where the enzymes responsible for beta oxidation and ATP production (oxidative phosphorylation) are present. In NAFLD, mitochondria usually adapt by increasing rates of beta-oxidation. However, this could lead to higher production of reactive oxygen species (ROS). This in turn would likely play an important role in the development of insulin resistance and in the same time, leads to mitochondrial DNA damage and functional decline. This will result in accumulation of partially oxidized intermediates which further exacerbate insulin resistance and leads to the progression of NAFLD [34]. Moreover, mitochondrial-derived ROS was reported to contribute to the amplification of autophagy [32] Thus, in our opinion, the electron lucent bodies found in the current study might be excess translocated fat that crossed the mitochondrial membranes in their way to be beta-oxidized. These bodies might also be

accumulated partially oxidized intermediates resulting from increased rate of beta-oxidation process.

Moreover, the TEM results of the present study where tiny fat droplets were seen accumulating in the hepatocytes' cytoplasm. It could be one of the causes of vacuolated hepatocytes' cytoplasm seen by H&E. Fat accumulation was also reported as a well known reversible condition NAFLD [35]. It was suggested that decreased release of fats from the liver in the form of lipoproteins might contribute to fatty liver formation. It is known that apolipoprotein B-100 (apo B-100), in plasma, carries fat out of the liver on very-low-density lipoprotein (VLDL). Apo B-100 is a very large molecule, rich in leucine. Because plasma leucine is much reduced in protein malnutrition, one can visualize how synthesis of lipoprotein is impaired [36]. It is noteworthy that more fat content would have been seen inside the hepatocytes if the duration of the protein malnourishment was more than the two weeks employed in the present study. This could also be justified by the known slowly progressive course of the NAFLD [19].

It was also reported that hepatic steatosis represented a "first hit" rendering the liver more susceptible to liver injury for a "second hit" such as oxidative stress. Additionally, free fatty acids confirmed to induce endoplasmic reticulum stress (ER stress). This ER stress could mediate insulin resistance and predispose to type II diabetes and obesity in adult life [35]. The ER stress might be the cause of their scanty and fragmented appearance as seen by TEM in protein malnourished group of the present study.

Accumulation of huge amount of glycogen in the cytoplasm as well as in the nuclei of hepatocytes was evident in the protein malnourished group of the present study. They were not seen only as typically aggregated  $\alpha$  rosettes, but also as individual  $\beta$  particles. This glycogen accumulation in the malnourished animals despite their weight loss could be considered as an indicator of insulin resistance where the liver cannot utilize its stored glycogen in response to insulin secretion during high blood sugar situations [37]. In addition, glycogen granules and marginated chromatin hepatocytes' nuclei was previously reported in many liver diseases and was termed "glycogenated or glycogen nuclei" [38, 39, 40]. It was reported that glycogen intranuclear inclusions were most prevalent in liver sections containing the highest levels of cytoplasmic glycogen [39]. Some theories have been suggested for explanation of these glycogen nuclei. It was hypothesized that glycogen could be synthesized in situ within the interchromatin regions of the nucleus by nuclear glycogen synthase enzyme [40]. Others previously suggested that glycogen might be translocated from the cytoplasm across the nuclear

membrane and accumulated in the nucleus [38]. It was noted that moderately electron dense nuclear pores were visible at the periphery of the shrunken nuclei in the protein malnourished group of the present study. It might be possible that glycogen granules got access to the nuclei in the present study through these pores.

Protein-energy malnutrition is still highly prevalent in the developing countries resulting in stunted growth, behavioral changes, high prevalence of infections, fatty liver and even death. Hence, an ideal food for the prevention and management of malnutrition should be of high nutritive value, acceptable to the children and their mothers. Additionally, it must be readily available at a cheap rate, easy to prepare and well tolerated both in health and disease. Soymilk was found to fulfill these conditions, as its protein value is essentially equivalent to that of food proteins of animal origin. It is furthermore easily processed and can be combined with a variety of food to increase nutritive value and make them ideal both as a staple and as a weaning food. Correction of protein starvation by soymilk was recommended to be the solution of choice as a life saving solution in developing countries where prolonged protein starvation can lead to death [9].

Rehabilitation of malnourished animals by complementing the restricted diet with soymilk in the present study allowed a catch-up phenomenon of weight gain. This was in accordance with the results of a previous study [10]. Significant but incomplete weight recovery was detected. Although it was non significant from the control values, however, it might take more than the four weeks rehabilitation period to the rats to fully reach the desired weights.

Significant increase in enterocytes' height was detected in the present study in the soymilk rehabilitated group. This finding goes well with the results of previous studies [12, 20, 21]. Soymilk, by its content of all essential amino acids could have induced this tissue structural recovery to almost normal values.

Results of the current work showed that the rehabilitation with soymilk-complemented corn flour increased notably the mucosal immune function by significantly increasing the number of IgA-secreting plasma cells. These data would suggest that high quality soymilk micronutrients might enhance or preserve immune function. It was hypothesized that soymilk could induce T cells to produce Th2-IgA stimulating cytokines, within the lamina propria, thus could modulate IgA production by B cells [12]. Moreover, soy oligosaccharides (raffinose and stachyose) could have a prebiotic effect. Prebiotics are indigestible food ingredients that can beneficially affect the host by selectively stimulating the growth and/or activity of certain endogenous microbial

population groups such as bifidobacteria and lactobacilli [41]. It has been reported that bifidobacteria enhances immune responses by increasing antibody production and proliferation of B cells in Peyer's patches [42].

Marked improvement at the cytoplasmic as well as nuclear level in both light microscopic and electron microscopic examination of jejunal mucosa and liver was detected after rehabilitation by soymilk in the present study. It was reported that dietary soy protein reduces the hepatic triglyceride level more than dietary proteins of animal origin such as casein. This was attributed to the suppression of fatty acids biosynthesis [43]. Several researchers have shown that dietary soy protein reduces the hepatic sterol regulatory element binding protein (SREBP-1) responsible for activation of the genes involved in fatty acids biosynthesis [44, 45].

Soy isoflavones, present in soybean products, were reported to exert a beneficial effect on lipid and glucose metabolism [46] maintaining normal insulinemia [44]. This might occur through activation of the peroxisome-proliferator activated receptors (PPAR) that participate in cellular lipid homeostasis and insulin action [46]. Hence, these effects might explain the obvious decrease in cytoplasmic glycogen granules and improvement of the nuclear structure after using soymilk in the present study.

Polyphenols content of soymilk has been reported to function as potent free radical scavengers within the body, where they can neutralize free radicals before they can cause cellular damage [47]. Various soy products were reported to decrease serum liver enzyme levels as indication of their ability to protect the hepatocytes from oxidative damage [48]. In addition, soy food and vegetables had been reported to be rich in many phenols such as flavonoid. Flavonoids have antioxidants capacity that is much stronger than those of vitamins C and E, reportedly used to prevent free radical production [49]. Soymilk was also found to increase protein synthesis and accelerate regeneration of cells [50].

Taken together, the present study confirmed that protein malnutrition had a negative impact on the histological structure of jejunum and liver of young rats. Nutritional rehabilitation by soymilk met with success and managed to reverse these structural alterations. It is recommended to use the highly nutritive -yet cheap- soymilk to overcome the serious problem of protein malnutrition in young population.

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