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RESEARCH ARTICLE

# Effect of a normal protein diet on oxidative stress and organ damage in malnourished rats

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**BACKGROUND:** We investigated the effects of three weeks of renutrition with a normal protein diet on oxidant/antioxidant status in malnourished rats using biochemistry and histology.
**METHODS:** Eighteen young Wistar rats were divided into three groups: control group was fed on a normal protein diet; malnourished group was fed on low protein diet and renourished group was fed on low protein diet followed by a normal protein diet. Serum albumin was evaluated. Malondialdehyde, protein carbonyl, superoxide dismutase and catalase levels were determined in the intestine, muscle and liver. Intestinal and hepatic damage were assessed by histological examination.
**RESULTS:** Protein malnutrition resulted in a significant decrease of body weight, albumin level, villus length, intraepithelial lymphocytes counts (IELC) and superoxide dismutase level (liver and muscle). However, catalase activity increased significantly in muscle and gut but there was no difference in liver. In all organs, malondialdehyde and protein carbonyl content of malnourished group showed a significant increase. Interestingly, a normal protein diet for three weeks resulted in a return to normal levels of superoxide dismutase, albumin, malondialdehyde and protein carbonyl in all organs. Catalase activity decreased in the muscle and gut and exhibited no significant difference in the liver. The renutrition diet enhanced also the recovery of intestinal epithelium by increasing villus length. Hepatic damage of rats fed normal protein diet was markedly reduced (macrovesicular steatosis decreased by 45%).
**CONCLUSION:** The normal protein diet could improve the oxidant/antioxidant imbalance and organ damage induced by protein malnutrition.

**Keywords** normal protein diet, organ damage, oxidative stress, protein malnutrition, rat

## Introduction

Protein-energy malnutrition has been recognized as the most widespread nutritional problem around the world (FAO, 2004; UNICEF, 2009). The term Kwashiorkor refers to the disease that occurs when there is inadequate protein intake with reasonable calorie (energy) intake (Ibukun-Olu, 2001). Protein malnutrition (PM) contributes to growth failure, hypoproteinemia, odema, fatty liver (Keusch, 2003) and can impair intestinal architecture and the host's immune system, resulting in increased vulnerability to infection (Guerrant et al., 1990; Coutinho et al., 2008; Guerrant et al., 2008). These alterations vary in accordance with the severity and duration of the nutritional deficiency (Muller and Krawinkel, 2005).

There has been growing evidence showing that protein malnutrition gives rise to oxidant stress and cell injury (Ashour et al., 1999; Tatli et al., 2000; Catal et al., 2007). Previous studies found that protein malnutrition can lead to change the antioxidant capacity of tissues, such as reducing the concentrations of glutathione (GSH) and activity of superoxide dismutase (SOD) (Ogasawara et al.,1989), and further arouse an oxidative stress (Marks et al., 1996; Li et al., 2002), which retarded the development of tissues or organs though changing digestion and absorption (Li et al., 2010), nutrient metabolism (Robertson et al., 2003) and immune function (Tohyama et al., 2004). Oxidative stress is a general term used to describe the steady state level of oxidative

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damage in cells, tissues or organs caused by an imbalance between the production of reactive oxygen species and biological systems ability to readily detoxify the reactive intermediates or easily repair the resulting damage (Akinola et al., 2010).

An ideal food for the prevention and management of malnutrition should be of high nutritive value. Periodic high-dose vitamin A supplementation (Stevens et al., 2015), oral zinc supplementation (Lamberti et al., 2013), and iodized salt (Zimmermann, 2013) have improved the health and survival of millions of malnourished children. However, micronutrients have little to no effect on linear growth in children (Ramakrishnan et al., 2009; Mayo-Wilson et al., 2014; Stammers et al., 2015). Recent trials of lipid-based nutrient supplements in complementary feeding have also shown little to no effect upon linear growth (van der Merwe et al., 2013; Ashorn et al., 2015). It is recently apparent that quality protein and essential amino acids are missing in the diet and may have adverse consequences for child growth and the reduction of child stunting (Semba, 2016). Therefore, the aim of the present study is to explore the effect of three weeks of renutrition with a normal protein diet (23.5% + 0.3% of methionine) on oxidant/antioxidant status and organ damage of liver, muscle and gut in protein malnourished rats.

## Materials and methods

### Animals and diets

The experiments described in this study comply with the current Algerian legislation covering the protection of animals.

Five- to six-week-old Wistar rats weighing 103±8.98 g purchased from Pasteur Institute of Algiers were kept in a laboratory environment of light-and-dark cycles. They were housed individually in cages and divided into three groups ($n$ = 6 rats for each group): control group (CG) was fed on a normal protein diet (23.5% casein + 0.3% methionine) for 42 days (1st group); malnourished group (MG) was fed on low protein diet (3% casein) for 21 days (2nd group) and renourished group (RG) was fed on low protein diet (3% casein) for 21 days followed by a normal protein diet (23.5% casein + 0.3% methionine) until the 42nd day (3rd group). The composition of diets is shown in Table 1 (Kheroua and Belleville, 1981). The body weight of all groups was registered every 3 days during the experimental period. At the end of the experiment, all animals were anesthetized with sodium pentothal (5 mg/kg). Blood, intestine, muscle and liver were collected. All tissues were washed with phosphate buffered saline (PBS) solution. One part of the intestine and liver were fixed in 10% buffered formalin solution for histopathological investigations. The other part was stored at-80°C until analysis.

### Albumin assay

Serum albumin was determined by a colorimetric method Biosystems Kit (COD 11547, Spain). Albumin in the sample reacts with bromocresol green in acid medium forming a colored complex that can be measured by spectrophotometry (Doumas et al. 1971) at 630 nm.

Sample preparation for biochemical assay. 25 mg of each tissue was lysed in 250 µL of RIPA buffer (Item No. 10010263) with protease inhibitors (Trypsin 10µg/ml) on ice and sonicated for 15 seconds at 40V. The homogenate was centrifuged at 1600 × g for 10 min at 4°C. The supernatants were collected for MDA-TBA analysis.

For protein carbonyl and catalase activity analysis, 100 mg of each tissue sample was homogenized with 1 ml of cold buffer (50 mM potassium phosphate, pH 6.7 containing 1 mM EDTA) and centrifuged at 10.000 × g for 15 min at 4°C. The supernatants were used.

For analysis of superoxide dismutase, 100 mg of each tissue sample was homogenized in 1 ml of cold 20 mM HEPES buffer, pH 7.2 containing 1 mM EGTA, 210 M manitol and 70 mM sucrose. The homogenates were centrifuged at 1500 × $g$ for 5 min at 4°C. The supernatants were used.

### Oxidant and antioxidant capacity

*Lipid peroxidation and protein oxidation*

The malondialdehyde (MDA) (Cayman Chimical Company, 10009055, USA) and the protein carbonyl (PC) (Cayman Chimical Company, 10005020, USA) in the liver, muscle and gut were determined through colorimetric methods according to the commercial kits, respectively. Briefly, the concentration of MDAwas analyzed using the thiobarbituric acid (TBA)

**Table 1** Diet compositions in ponderal and energetic percentages according to Kheroua and Belleville (1981).

| Diet | Protein (Casein) | | Lipid | | carbohydrates | | Minirals + vitamins | Agar | Cal/100g of diet |
|---|---|---|---|---|---|---|---|---|---|
| | p.100 ponderal | p.100 energetic | p.100 ponderal | p.100 energetic | p.100 ponderal | p.100 energetic | p.100 ponderal | p.100 ponderal | |
| Control diet (+ 0.3% Met) | 23.5 | 25 | 5 | 12 | 59.5 | 63 | 8 | 5 | 373 |
| Low protein diet | 3 | 3.2 | 5 | 12 | 80 | 84.8 | 8 | 4 | 377 |

method to generate a colored product absorbing at 540 nm. The protein carbonyl colorimetric assay utilizes the 2,4–dinitrophenylhydrazine (DNPH) reaction to measure the PC content in organs at an absorbance of 360 nm.

*Catalase activity (CAT)*

Catalase activity was determined by Cayman Kit (707002, USA). The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of $H_2O_2$. The absorbance was recorded at 540 nm.

*Superoxide dismutase (SOD)*

The superoxide dismutase levels were determined using Cayman Kit (706002, USA) which utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The absorbance was read at 440 nm.

*Total protein*

The proteins content was determined using Lowry's method measuring absorbance at 750 nm (Lowry et al., 1951). Bovine serum albumin was used as a standard.

### Histology

Segments of intestine and liver were fixed in 10% buffered formalin solution, embedded in paraffin and stained with hematoxylin and eosin for light microscopic examination. The measurements of villi length and intraepithelial lymphocytes counts (IELC) were made using an optical microscope equipped with a micrometer.

### Statistical analysis

All data are expressed as mean±standard error (SE). Statistical analysis was performed using Student t-test or ANOVA with $p < 0.05$ as the minimal level of significance.

## Results

### Body weight

The mean weight of animals during malnutrition phase was reduced significantly during 21 days ($p < 0.001$) when compared to the control group. After 21 days of renutrition, the body weight was significantly increased ($p < 0.001$) (Fig. 1).

### Albumin level

Compared to the control group, the level of albumin was significantly reduced in the malnourished group ($p < 0.001$). However, a significant increase in the level of albumin was found after 3 weeks of renutrition ($p < 0.05$) (Fig. 2).

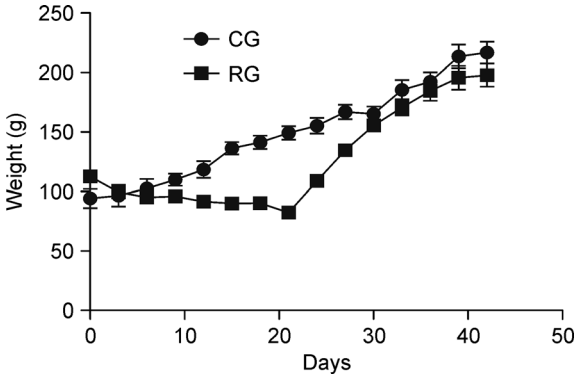


**Figure 1** Body weight of rats during malnutrition and renutrition phase. Values are given as mean±SE (standard error) from six independent measurements. CG: Control group, RG: Renourished group. MG vs CG $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. RG vs CG $^{***}p < 0.001$, RG (42 day) vs RG (21day) $^{\#\#\#}p < 0.001$.

### Oxidant and antioxidant capacity

The mean oxidant damage products (MDA and PC) levels were significantly increased in liver ($p < 0.001$), muscle ($p < 0.001$) and intestine (MDA: $p < 0.01$; PC: $p < 0.001$) in malnourished group compared to the control group. After 21 days of renutrition, the level of MDA and PC decreased significantly in liver ($p < 0.001$), muscle ($p < 0.001$) and gut (MDA: $p < 0.01$; PC: $p < 0.001$) compared with malnourished group.

Protein restriction increased the activity of CAT in muscle ($p < 0.001$) and intestine ($p < 0.001$) but there was no difference in liver. After renutrition period, the activity of the CAT decreased significantly in muscle and gut compared with malnourished group ($p < 0.001$). However, in liver there was no difference. Moreover, protein malnutrition decreased significantly the level of SOD in liver ($p < 0.01$) and muscle ($p < 0.01$). In contrast, a significant increase in the level of

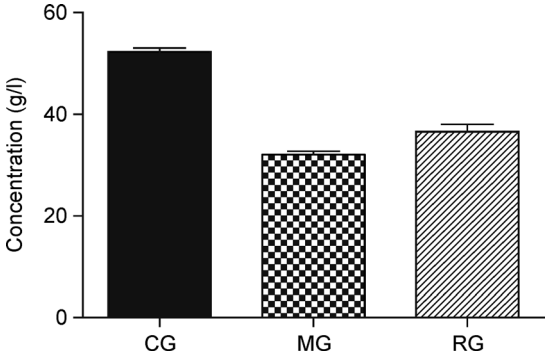


**Figure 2** Albumin concentration of all groups. Values are given as mean±SE (standard error) from six independent measurements. CG: Control group, MG: Protein malnourished group, RG: Renourished group. MG vs CG $^{***}p < 0.001$, RG vs MG $^{\#}p < 0.05$.

SOD was found in the intestine ($p < 0.001$). After three weeks of renutrition, the concentration of SOD was significantly increased in all organs: liver ($p < 0.001$), muscle ($p < 0.05$) and gut ($p < 0.001$) (Table 2).

### Histology

The microscopic images revealed regular villi with unistratified epithelium and polymorphous lamina propria in the control group (Fig. 3A). However, in the malnourished group, a clear atrophy with the distortion of epithelium architecture and enlarged villi were observed (Fig. 3B). The normal protein diet used in our study clearly reduced the microscopic lesions caused by protein malnutrition (Fig. 3C).

*Villus length and intraepithelial lymphocyte counts (IELC)*
After protein malnutrition, the villus length and IELC were significantly reduced compared to the control group ($p < 0.001$). When rats were renourished for 3 weeks, a marked recovery in the villus length (Fig. 4A) and IELC (Fig. 4B) were seen ($p < 0.001$).

## Discussion

In this study, protein malnutrition (3% of casein) for three weeks reduced substantially the body weight of rats, which was in line with other reports (Carreira et al., 1996; Gourine et al., 2015). After 3 weeks of renutrition (23% of casein + 0.3% methionine), all animals developed a significant weight gain.

In our study, the level of albumin was significantly reduced after protein malnutrition. However, a significant increase in the level of albumin was found after 3 weeks of renutrition diet. Prada et al. (2007) reported that after one month of recovery with a normal protein diet this metabolic alteration was reversed. It was shown that protein deficiencies led to hypoalbuminemia (Perampalli et al., 2010). It is considered as one of the hallmarks of protein malnutrition. It is assumed that albumin has a potential to protect tissues against oxidant injury and is a substantial contributor to the total serum antioxidant status.

In malnourished group, the MDA-TBA level of all organs was significantly increased. MDA is a useful indicator of membrane damage (Esrefoglu et al., 2016). Jimoh et al. (2005) demonstrated that the increase in the concentration of MDA in tissues of animals fed lower protein diet is an indication of increased lipid peroxidation. In the present study, an increased PC levels was found in all organs during protein malnutrition. The same result was found in intestine rat (Bodiga et al., 2005), in liver rat (Cho et al., 2000) and in serum malnourished children (Khare et al., 2014) suggesting that protein malnutrition is characterized by a significant increase of PC.

In the present study, the normal protein diet (23.5% + 0.3% of methionine) was used for the synthesis of methionyl-transfer RNA, which is the cofactor for the initiation of synthesis of all body proteins (Roediger et al., 1995). Green et al. (2014) hypothesized that methionine supplementation during the early phase of treatment would increase the cysteine availability through enhanced protein turnover and *de novo* synthesis, which in turn, would enhance glutathione synthesis, thereby quickly restoring the antioxidant capacity.

In our experimental design, protein malnutrition produced

**Table 2** Oxidant and antioxidant capacity of liver, muscle and gut of different groups.

| | | | |
|---|---|---|---|
| **Liver** | | | |
| | CG ($n = 6$) | MG ($n = 6$) | RG ($n = 6$) |
| MDA (μM/mg protein) | 0.392±0.028 | 0.869±0.066*** | 0.433±0.016### |
| PC (nmol/mg protein) | 1.462±0.106 | 3.211±0.028*** | 1.224±0.059 ### |
| SOD (U/mg protein) | 72.187±1.969 | 55.056±4.362** | 82.349±3.729### |
| CAT (nmol/min/mg protein) | 0.32±0.003 | 0.314±0.013 | 0.317±0.001 |
| **Muscle** | | | |
| | CG ($n = 6$) | MG ($n = 6$) | RG ($n = 6$) |
| MDA (μM/mg protein) | 0.681±0.031 | 1.887±0.068*** | 0.796±0.057 ### |
| PC (nmol/mg protein) | 0.746±0.003 | 3.986±0.008*** | 0.861±0.023### |
| SOD (U/mg protein) | 68.657±1.664 | 56.586±1.031** | 64.678±2.836# |
| CAT (nmol/min/mg protein) | 0.258±0.002 | 0.35±0.005*** | 0.209±0.002### |
| **Gut** | | | |
| | CG ($n = 6$) | MG ($n = 6$) | RG ($n = 6$) |
| MDA (μM/mg protein) | 0.458±0.007 | 2.36±0.359** | 0.753±0.017## |
| PC (nmol/mg protein) | 5.39±0.45 | 13.585±0.399*** | 4.662±0.074### |
| SOD (U/mg protein) | 28.534±1.99 | 46.141±2.989*** | 73.564±4.236### |
| CAT (nmol/min/mg protein) | 0.345±0.002 | 0.513±0.005*** | 0.358±0.003### |

Values are given as mean±SE (standard error) from six independent measurements. CG: Control group. MG: Protein malnourished group. RG: Renourished group. MG vs CG **$p < 0.01$, ***$p < 0.001$. RG vs MG #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$.

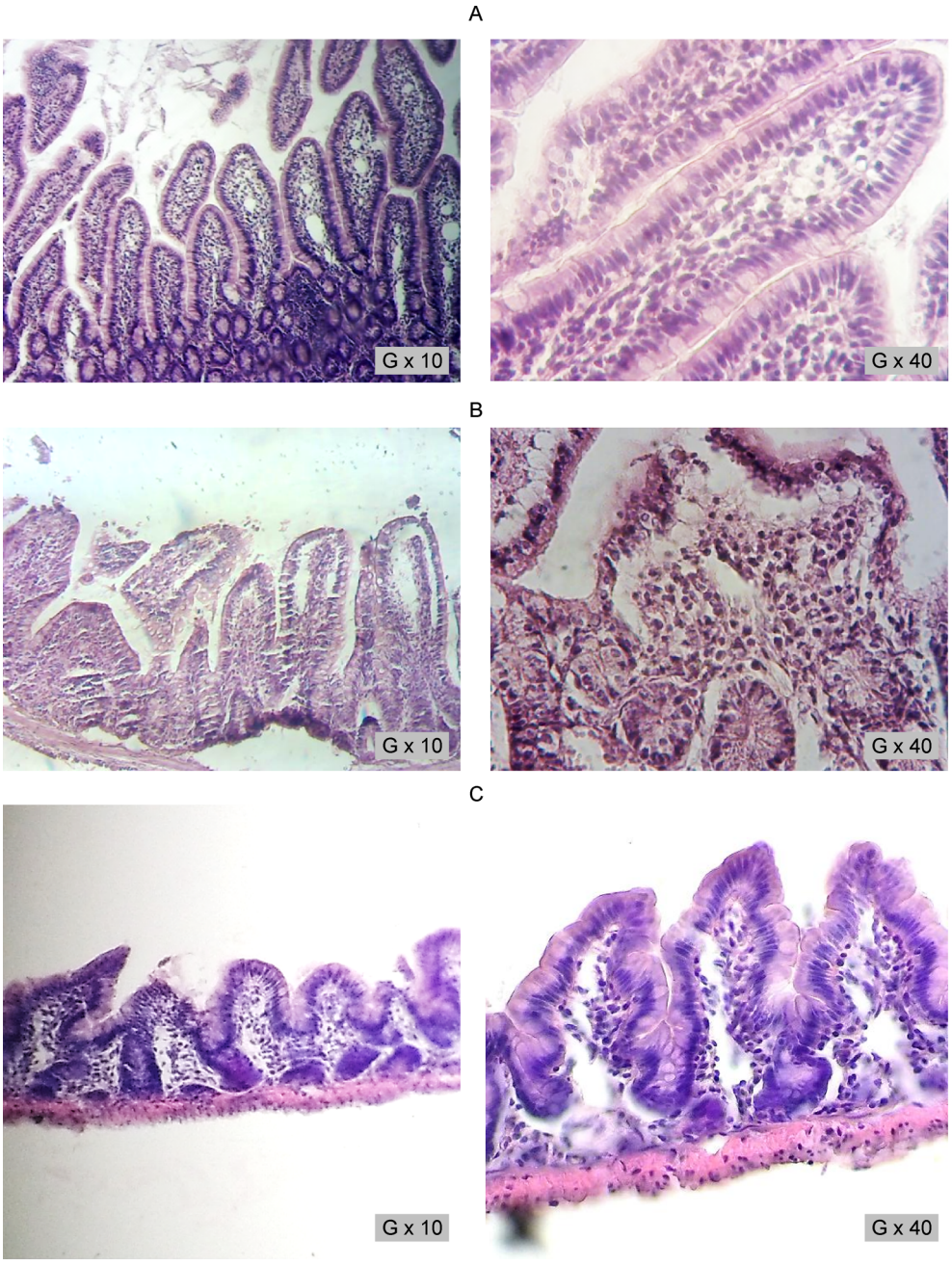


**Figure 3** Light microscopy (G × 10 and G × 40) showing intestinal villi stained with hematoxylin-eosin. Intestinal tissues were obtained from rats of CG (A), MG (B) and RG (C). CG: Control group, MG: Protein malnourished group, RG: Renourished group.

a significant decrease of SOD activity in liver and muscle. This could be due to a reduction of its synthesis as reported by (Kumary et al., 1993; Sies1999) and/or due to a decrease of its activity as noted by (Tatli et al., 2000; Yang et al., 2012). However, in gut, the level of this enzyme was increased by protein malnutrition. This result was not consistent with those of Nieto et al. (2000) who started malnutrition after rats weaning, at the age of 3 weeks. Indeed, an increasing number of studies have shown that weaning decreased the antioxidant capacity and increased free radicals (Buch et al., 2017; Burke et al., 2009; Luo et al., 2016).

The significant increase in catalase activity in gut and muscle found in our study might be explained by a compensatory response to reduce the oxidative stress caused by malnutrition (Jimoh et al., 2005; Tisan et al., 1995). Indeed, a high catalase activity in malnourished rats may reflect the chronic exposure of cells to oxidative stress (Akinola et al., 2010).

In the present study, there was no difference in liver catalase concentration. Liver is more vulnerable to damage under particular kinds of stress (Ying et al., 2015). This thermogenic organ contains a large number of mitochondria

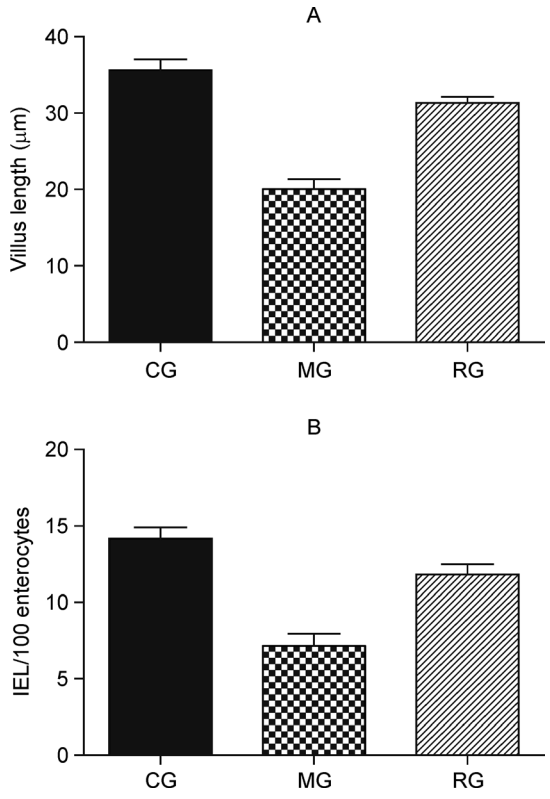


**Figure 4** Villus length (μm) (A) and IEL counts (B) in all groups during protein malnutrition and renutrition period. Values group, RG: Renourished group. MG vs CG ***$p < 0.001$, RG vs MG $^{###}p < 0.001$.As shown in Fig. 5A, livers from the control group showed normal hepatic cells with well-preserved cytoplasm and well-defined nucleus and nucleoli. However, the histological architecture of liver sections of malnourished rats showed the coexistence of a macrovesicular (50%) and a microvesicular steatosis (50%) characterized by multiple lipid droplets in the hepatocyte (Fig. 5B). In this group, the liver cells have a clarified appearance. After 3 weeks of renutrition, the normal protein diet used in this study led to 45% decrease of the macrovesicular steatosis. However, we observed the presence of some pseudocysts and portal inflammatory cells (Fig. 5C).

in mammals, which is the place with high oxygen consumption and reactive oxygen species (ROS) formation (Turrens, 2003; Assaad et al., 2014).

The negative correlation between SOD and CAT levels observed in malnourished rat muscle supports that the superoxide anion ($O_2^{\bullet-}$) could be converted to hydrogen peroxide ($H_2O_2$) not only by SOD but also by spontaneous dismutation (Brookes, 2005).

After 3 weeks of renutrition, we have observed an increase in the SOD level in all organs. We suggested that proteins and methionine contained in the renutrition diet probably boost the synthesis of the SOD. It is well known that protein had major importance as a source of amino acids and essential nutrients, being the precursor of neurotransmitters, structural proteins, enzymes and other vital proteins (Siegel, 1999). Methionine deficiency affects the biosynthesis of proteins not merely due to the lack of this amino acid in protein chains, but it is also fundamental for the initiation of protein synthesis in ribosomes (Yang et al., 2012). However, the activity of CAT decreased that is probably due to the reduction in the concentration of free radicals.

In our study, malnourished rats presented a significant decrease of villus length and IELC. It was shown that childhood protein deficiency impairs intestinal epithelial stem cells functions, compromising the host's ability to repair the intestinal epithelium upon intestinal injury or infection (Liu et al., 2016). Previous investigations showed that diet with protein restriction can not only decreased IELC (Gendrel et al., 1992; Leitch et al., 1993) but induced also a reduction in intestinal epithelial cell proliferation and mucosal atrophy (Bodiga et al., 2005; Ueno et al., 2011; Vlasova et al., 2017). According to literature, these alterations have a profound effect on brush-border enzymatic activity (Chappell et al., 2003) and mucosal mass and mucosal integrity (Nunez et al., 1996). In our study, experimental malnutrition was associated with concomitant sign of intestinal free radical damage. Oxidative stress is partly responsible for the intestinal dysfunction observed in malnutrition (Akinola et al., 2010). Lipid peroxidation affects membrane integrity and stability; therefore, cell membranes from malnourished animals, already altered under basal conditions, may be more sensitive when exposed to oxidative alterations (Gao et al., 2010).

After 3 weeks of renutrition, villus length of malnourished group was significantly increased. The proliferative/regenerative capacity of the intestinal epithelium, as both is highly dependent on protein availability and efficient synthesis (Mudd et al., 2007; Badaloo et al., 2012). Indeed, numerous studies suggested that the amount of food consumed and quality of dietary nitrogen play a key role in the recovery of malnourished patients (Poullain et al., 1991; Lochs et al., 2006).

In the malnourished group, the histological architecture of liver sections showed the coexistence of a macrovesicular and a microvesicular steatosis. The first scientific description of the direct consequences of malnutrition on liver function dates back to Hughes (1945), when the accumulation of hepatic fat has been described in children affected by severe protein–calorie malnutrition. Badaloo et al. (2005) suggested that protein malnutrition can lead to decreased synthesis of VLDLs and inhibited their transport, which resulted in hepatic fat accumulation. Additionally, there is abundant evidence suggested that increased liver triglycerides led to increased oxidative stress in the hepatocytes of animals and humans (Araya et al., 2004; Hensley et al., 2000; Lieber, 2004; Seki et al., 2002; Yang et al., 2000). On the other hand, it was shown that peroxidation of PUFAs attenuated the secretion of VLDL in rodents (Pan et al., 2004), which could contribute to the accumulation of triglycerides in the liver.

In our study, the macrovesicular steatosis was reduced to below 45% after renutrition with normal protein diet. Diets containing 40–50 en% protein clearly decrease hepatic lipid

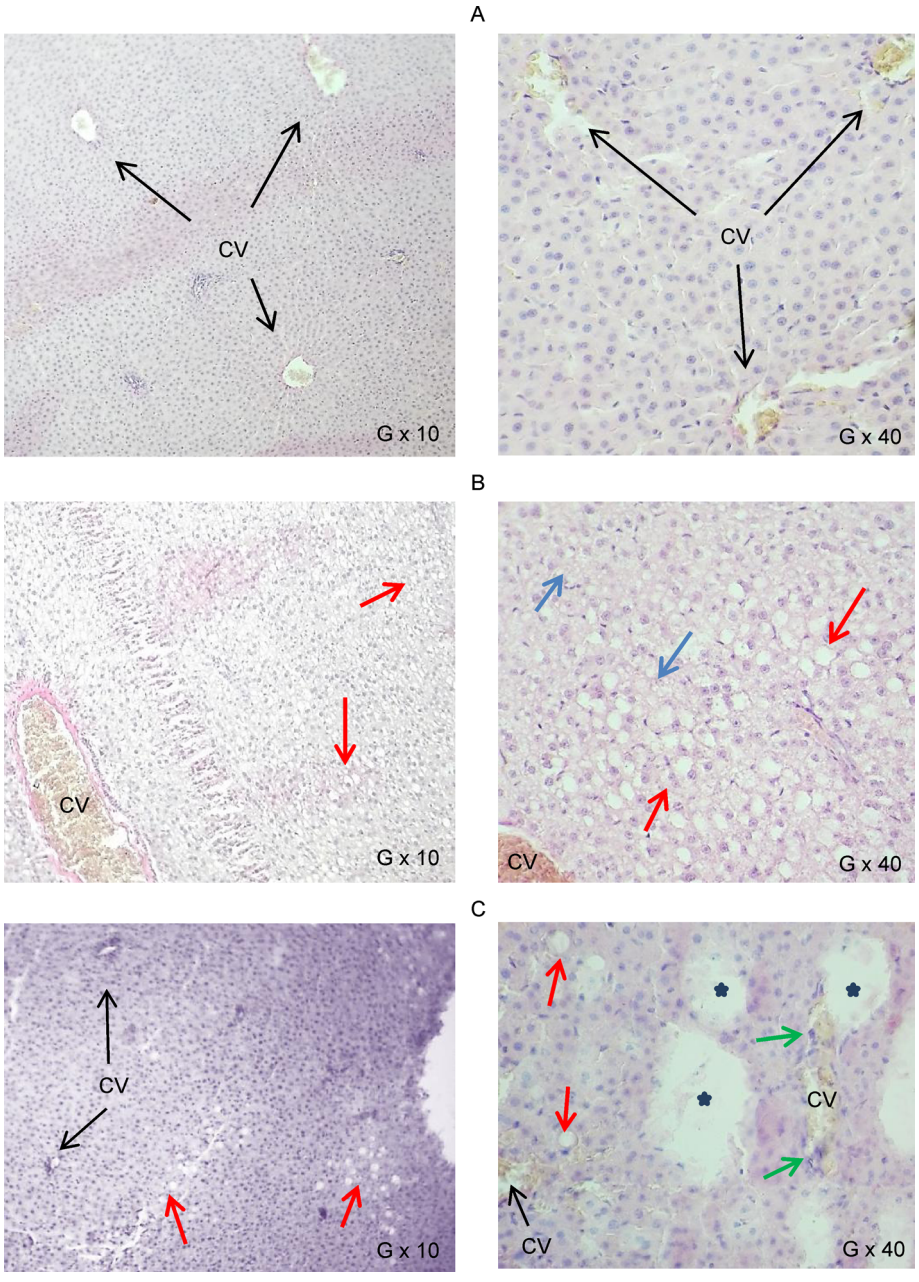


**Figure 5** Light microscopy (G × 10 and G × 40) showing histological structure of liver stained with hematoxylin-eosin. Livers were obtained from rats of CG (A), MG (B) and RG (C). CG: Control group, MG: Protein malnourished group, RG: Renourished group. →: macrovesicular steatosis, →: microvesicular steatosis, ★: pseudocysts, →: portal inflammatory cells.

content to a minimum, irrespective of the carbohydrate (CHO) or fat content of that diet (Freudenberg et al., 2012; Schwarz et al., 2012; Garcia Caraballo et al., 2017), while diets containing 20–35 en% protein are optimal for weight maintenance (Peters and Harper, 1985; Moundras et al., 1993; Noguchi et al., 2008).

## Conclusion

We demonstrated that protein malnutrition reduced the body weight, albumin level, villus length, IELC and disrupted the oxidants/antioxidants balance. We also demonstrated that renutrition with a normal protein diet for three weeks induced not only, the restoration of the body weight, the albumin level and the organ damage but also improved the oxidant/antioxidant imbalance induced by protein malnutrition.

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## Declaration of interest

The author confirms that this article content has no conflict of interest.

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