# Histological and Immunohistochemical Study of the Effect of Prenatal Zinc Deficiency on Postnatal Development of Rat Liver

**Original Article**

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## ABSTRACT

**Introduction:** It is generally recognised that zinc is an important trace element with crucial functions in cellular metabolism. The liver is responsible for zinc metabolism. Zinc deficiency can cause growth compromised effects in many organs.
**Objectives:** Study of the effects of zinc deficiency on the postnatal development of rat liver using histological and immunohistochemical evaluation.
**Materials and Methods:** Female adult rats, after matting, were allocated randomly into two groups: the control group (24 rats received a single i.p. injection of distilled water on day 9 of gestation) and the experimental group (24 rats i.p. injected 1.10 phenanthroline (zinc chelating agent) in a single dose of 30 mg/kg on day 9 of gestation). Zinc deficiency was confirmed by measuring the serum zinc level in both groups of pregnant females on the 10th day of gestation. Male offspring of treated and control rats were sacrificed at the following postnatal ages: newborn, 15 days, and three months. Samples from the liver were then processed for histological study using light and electron microscopic examination and immunohistochemical evaluation for Bcl2 expression.
**Results:** Light microscopic examination of the treated groups showed a disorganized hepatic architecture with apoptosis of hepatocytes. There was noticeable dilatation and congestion of blood sinusoids and central and portal vessels. Masson's trichrome stain revealed remarkable fibrosis in the treated groups' portal triad. The immunohistochemical study showed weak immunoreactivity for Bcl-2 in zinc deficient groups. The ultrastructural study showed degenerated hepatocytes with cytoplasmic vacuolations. The mitochondria appeared swollen with cristolysis and an electron-dense matrix.
**Conclusion:** Zinc deficiency resulted in deleterious postnatal structural effects on hepatic tissue that affected all age groups and even extended to the adult age.

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**Key Words:** Histopathological; immunohistochemistry; liver development; postnatal; zinc.

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## INTRODUCTION

Zinc is the second most prevalent essential trace element in the human body with various biological effects[1]. Zinc plays a crucial part in various cell functions including cell proliferation and apoptosis, defence against reactive oxygen species, and DNA damage repair since more than 300 proteins contain domains with zinc, which are critical for controlling cellular processes integrity. CuZn superoxide dismutase (SOD) is a key first-line defence enzyme against oxygen radical species[2,3].

Zinc deficiency is increasingly recognised as a very widespread public health issue, particularly in poor populations. A zinc shortage is thought to impact 2 billion individuals globally. Zinc insufficiency is the fifth most common reason for the loss of healthy life years in developing countries[4]. According to several reports, zinc deficiency can cause a variety of issues, such as immune system weakness, development disorders, and cognitive impairment[5,6]. Low circulating zinc concentrations have been linked to an elevated risk of cancer, according to epidemiological research, lack of zinc weakens the host's defences against DNA damage, that makes people more vulnerable to DNA-damaging substances, and eventually raises their risk of developing cancer[7].

Low levels of cellular zinc may worsen oxidative stress and interfere with p53's ability to bind DNA and repair DNA[8]. Therefore, processes resulting in decreased DNA integrity with zinc shortage in *vivo* may involve numbers of distinct mechanisms. A zinc deficiency could directly damages DNA, as well as DNA damage repair mechanisms[8]. Additionally, accumulating evidence points to zinc's antioxidant capabilities and its ability to shield tissue from oxidative damage[9].

The liver is the primary organ involved in the metabolism of zinc and is essential in preserving the homeostasis of systemic zinc, which is susceptible to disorders of the liver. However, a zinc deficiency may change the way liver functioning and how the immune system reacts[1,10].

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It was found that zinc deficiency impaired immune system function and produced numbers of metabolic problems, such as insulin resistance, hepatic steatosis, hepatic encephalopathy, and others[11,12].

To the best of our knowledge, according to the available literature, no previous study has been carried out to evaluate the effects of induced zinc deficiency on the postnatal development of the liver. So, this study was planned as a trial to assess the effects of zinc deficiency as a well-known an essential trace element required for normal cell function and integrity on the liver structure at different stages of postnatal development using histological (using light and transmission electron microscope) and immunohistochemical methods.

## MATERIAL AND METHODS

### *Animals*

#### Ethics declarations

The experimental protocols were in acquiescence with the National Institutes of Health Guidelines for Human Treatment of Animals, with agreement from the Institutional Animal Care and Use Committee of Faculty of Medicine, Assiut University. The study was approved by the ethics committee of the Faculty of Medicine, Assiut University, Egypt. This study was performed following the principles of the Declaration of Helsinki.

The experiment was conducted at the Anatomy and embryology Department, Faculty of Medicine, Assuit University, Egypt. A total of 48 adult female and 12 adult male (200-250) gm albino rats were obtained from Animal House, Faculty of Medicine, Assiut University. The animals were housed in a room with a 12:12 hr light: dark cycle and were kept under controlled temperature. Food and water were available ad libitum. A glass rod was gently inserted into the vagina to take a vaginal smear, which was then spread out on a slide and stained with shori stain. Cornified non-nucleated epithelial cells without leucocytes are hallmark of the estrus phase. Male and female rats were permitted to mate overnight (not more than three adult females with one adult male). On the second day of mating, the presence of the mucous plug and sperms in the vaginal smear indicate day zero of pregnancy[13].

### *Experimental Design*

Female rats, after matting, were singly housed and randomly allocated into two groups:

**Group A:** the control group (n= 24) adult female rats received a single intraperitoneal injection of distilled water on day 9 of gestation.

**Group B:** the experimental group (n= 24) adult female rats received intraperitoneal injection of 1.10 phenanthroline in a single dose of 30 mg/kg on day 9 of gestation. Phenanthroline is a zinc chelator reported to decrease the zinc level in the body) and was purchased as a powder from Sigma Company, utilized as aqueous preparation in a ratio of 3 mg/ml of distilled water. It was used because of its availability and low cost. The used drug and the dose in this work were according to[14]. The occurrence of zinc deficiency was confirmed by measuring the serum zinc level in both groups of pregnant female rats on the 10$^{th}$ day of gestation. It was measured by standard atomic absorption technique in Clinical Toxicology and Forensic Chemistry Laboratory, Faculty of Medicine, Assiut University. Sixty-four male offspring of the treated and control rats were sacrificed at the following postnatal ages: newborn, 15 days, and three months. Ages were chosen as that is the period of lactation as weaning occurs on the twenty-first day[15]. These postnatal ages were chosen to detect the effect of the prenatal zinc deficiency exposure of pregnant females and their milk on the structural development of the liver of the offspring and to investigate if this effect will extend to the adult age or not. Numerous researchers carried out experimental studies on albino rats and determined that, generally, a human month is comparable to a laboratory rat's daily existence when taking into account their complete lifespan[16]. The animals at the following ages newborn, 15 days, and 90 days postnatally, were anesthetized by inhalation of ether. Liver samples were processed for both light and transmission electron microscopic study.

### *Tissue Sampling*

At the end of experiment, rats of each group were anesthetized with diethyl ether. The rats were sacrificed; their hearts were perfused with isotonic saline through the left ventricle until the flowing blood (cut at the right atrium) was cleared. To get the liver samples, a vertical ventral midline incision was performed in the abdominal wall. The samples were collected and prepared for histological and immunohistochemical investigation.

### *Histological study*

For the light microscopic study, the samples were preserved in 10% formalin for 24 hours, processed for paraffin sections, cut serially (5µm- thickness), then Hematoxylin and eosin (H&E) and Masson's trichrome staining were done[17].

For ultrastructural analysis the specimens were divided into 1m3 long fragments. After they were fixed in cold 3.5% glutaraldehyde in 0.1 M phosphate buffer (PH: 7.2), after which they fixed with 1% osmium tetraoxide (OsO4). Finally, were processed, embedded in epon. Toluidine blue stain was applied to semithin sections thick before being inspected under a light microscope. The copper grids were mounted, and ultrathin slices (50–70nm thick) were cut and dyed with lead citrate and uranyl acetate[18]. Sections were examined and then photographed by transmission electron microscope (Jeol-JEM-100 CXII Jeol, Tokyo, Japan) in the Electron Microscopic Unit, Assiut University.

### *Immunohistochemical study*

Using the avidin-biotin peroxidase technique was used for detecting the antiapoptotic protein Bcl2 expression

(B-cell lymphoma-2), the primary antibody used was a rabbit polyclonal antibody. The cellular reaction was cytoplasmic. Deparaffinized paraffin sections were rehydrated in decreasing alcohol concentrations before being treated with the primary antibody (diluted 1:100) overnight at 4°C ( New Markers, Lab Vision, Fremont California, USA). The sections were rinsed three times with phosphate-buffered saline (PBS), then treated for an hour with a secondary antibody conjugated to peroxidase. Utilizing 3, 3′-diaminobenzidine hydrogen peroxide as a chromogen, the immunoreactivity was made visible. Finally, slides were counterstained with Mayer's hematoxylin[19,20].

## RESULTS

### *Light Microscopic Examination*

**Using H&E and toluidine blue staining**

In the new born group, examination of liver sections stained with H&E and toluidine blue of the control group revealed classical lobules with radiating and anastomosing cords of hepatocytes arising from the central vein. The hepatocytes appeared polyhedral, with rounded central vesicular nuclei and acidophilic finely vacuolated cytoplasm. Some hepatocytes appeared binucleated. The cords of hepatocytes were separated by hepatic sinusoids which are lined by endothelial and Kupffer cells. The portal area revealed stroma with branches of the portal vein, hepatic artery, and bile duct (Figures 1A,1B). Light microscopic examination of sections in the liver of zinc deficient newborn rats stained with H&E revealed extensive disrupted architecture of the hepatic lobules and markedly congested dilated portal vein and dilated bile duct. Most of the hepatocytes degenerated with an apparent reduction in their number. The blood sinusoids revealed marked dilatation and congestion (Figure 1C). Examination of semithin liver sections of zinc deficient newborns' declared severe liver architecture disruption. The hepatocytes showed pronounced cytoplasmic vacuolation, and rarefied cytoplasm. Some cells appeared as Ghost with absent nuclei. Some hepatocytes revealed darkly stained binucleated nuclei, others appeared amalgamated and darkly stained. Very few hepatocytes still showed vesicular nuclei surrounded by vacuolated cytoplasm. The sections also revealed remarkable dilated congested central veins (Figure 1D).

In 15 days old age, the liver lobules of control animals examined by light microscopy using H&E and toluidine blue stains demonstrated portal triad, branch of the portal vein, hepatic artery, and bile canaliculi. The lobules were formed of plates of hepatocytes that are separated by blood sinusoids and radiating from the central vein. The walls of the sinusoids are lined with endothelial and Kupffer cells. The hepatocytes cytoplasm appeared finely granulated, and the nuclei seemed vesicular, lightly stained with prominent nucleoli. The Kupffer cells were small with pale cytoplasm and small nuclei (Figures 2A,2B). Examination of the Zinc deficient group using the light microscope stained with H&E showed portal vein appeared markedly congested. The bile duct showed noticeable hyperplasia, and thickened hepatic artery in the portal triad among disrupted hepatocytes. There was also an area of haemorrhage and necrosis (Figure 2C). Semithin section examination showed severely disrupted liver architecture with deleterious degeneration of the hepatocytes with marked vacuolation. Some hepatocytes revealed rarefied cytoplasm. The blood sinusoids appeared markedly dilated and congested with a noticeable congested portal vein and dilated bile duct (Figure 2D).

In Adult Age, Hematoxylin and eosin and toluidine blue staining of the control group revealed a normal hepatic architecture, with the liver divided into ill-defined traditional hepatic lobules. Hepatocyte cords that extended from the central vein to the lobule's edge made up each one. Narrow blood sinusoids with Kupffer and endothelial cell linings separated the cords. The hepatocytes had a polyhedral form, spherical vesicular nuclei, and finely vacuolated acidophilic cytoplasm (Figures 3A,3B). Examination of H&E-stained sections in the liver of zinc-deficient group adult rats showed severe disruption in its architecture and extensive vacuolization in the liver parenchyma. The portal triad showed a severely dilated congested portal vein, noticeable dilated bile duct, and great thickening of the hepatic artery. There was also extensive periportal infiltration with inflammatory cells and marked fibrosis. Most hepatocytes were degenerated with absent nuclei (Figure 3C). Examination of the semithin sections of the liver of zinc deficient adult rats showed the same picture as with H&E examination of marked disruption of liver structure. Most hepatocytes revealed degenerated appearance with extensive intercellular vacuolization in liver parenchyma with multiple spaces between these distorted cells. The hepatocyte showed condensed cytoplasm with nuclei that appeared greatly distorted, shrunken, very pale, and sometimes with the absence of surrounding cytoplasm. Others hepatocytes were markedly disfigured , degenerated with absent nuclei. Some cells appeared as ghost (Figures 3D,3E,3F).

**Masson’s trichrome staining**

In new born age group, using Masson’s trichrome stain, light microscopic examination of the section from a control rat liver exhibited few collagen fibers in the central vein area (Figure 4A). In contrast, zinc deficient rats showed increased collagen fibers deposition around the central vein area (Figure 4B).

In 15 days old age, control liver sections stained with Masson’s trichrome revealed a minimal amount of collagen fibers that are accumulated in the portal tract area and in the perisinusoidal space (Figure 4C), while there was a greater increase in the deposition of collagen fibers in the portal tract areas and the perisinusoidal spaces of the zinc-deficient group compared with the control group (Figure 4D).

In Adult age, Masson’s trichrome-stained sections for the control group showed little amount of collagen fibers

around the central vein (Figure 4E), while the zinc deficient group declares pronounced deposition of collagen fibers in the portal tract areas compared with the control group. Also, perisinusoidal collagen deposition was noticed (Figure 4F).

### *Transmission electron microscopic examination of the liver specimens*

**Transmission electron microscopic examination of the hepatocytes and Kupffer cells of new born age group**

**Hepatocytes**

Ultrastructural examination in the liver of the control newborn indicated polyhedral liver cells. The cytoplasm showed numerous mitochondria, membranes of rough endoplasmic reticulum and glycogen granules. The nucleus appeared spherical, with dispersed chromatin inside prominent nucleolus and many lysosomes (Figure 5A). Electron microscopic examination of a hepatocyte of zinc deficient newborn rat showed electron-dense heterochromatic nucleus with abnormal shaped prominent nucleolus, rarefied cytoplasm with multiple variable-sized vacuoles. The mitochondria appeared severely distorted, some appeared swollen with cristolysis, and others appeared with a disrupted membrane (Figure 5B). Other hepatocytes revealed irregular- nuclei with clumps of chromatin and indented interrupted outline. The mitochondria appeared swollen with severe crystolysis. The rough endoplasmic reticulum appeared dilated with the appearance of phagolysosomes (Figure 5C).

**Kupffer cells**

Examination of liver ultrathin sections of the control newborn group displayed Kupffer cell as the irregular-shaped cell with an indented nucleus, peripheral chromatin condensation, and multiple mitochondria with the appearance of red blood cells in the liver sinusoids (Figure 5D). Examination of a Kupffer cell of zinc deficient newborn showed heterochromatic oval nucleus with clusters of chromatin and peripheral chromatin condensation. Dilated rough endoplasmic reticulum and multiple phagolysosomes were noticed (Figure 5E).

**Transmission electron microscopic examination of the hepatocytes and Kupffer cells of 15 days old age group**

**Hepatocytes**

Transmission electron micrograph of the hepatic sections of the control rat (15 days) showed that the hepatocyte exhibited euchromatic rounded nuclei with regular nuclear envelopes, prominent two nucleoli, rough endoplasmic reticulum and mitochondria (Figure 6A). Electron microscopic examination of the liver of zinc-deficient rats 15 days old declared some hepatocytes with shrunken apoptotic nucleus, swollen distorted mitochondria, multiple lipid globules, multiple lysosomes, dilated smooth endoplasmic reticulum. Other hepatocytes declared rounded nuclei and prominent nucleoli. The nucleus contains dense clumps of heterochromatin. The cytoplasm showed multiple varied-sized vacuoles within rarefied cytoplasm (Figures 6B,6C).

**Kupffer cell**

The Kupffer cell of the control group appeared lining the hepatic sinusoid and exhibit thin filipodia, cytoplasmic phagolysosomes, rough endoplasmic reticulum, and heterochromatic nucleus with peripheral chromatin condensation. (Figure 6D). The Kupffer cell of the zinc-deficient group revealed an irregular-shaped nucleus with an indented outline, dilated rough endoplasmic reticulum and phagolysosomes were observed (Figure 6 E).

**Transmission electron microscopic examination of the liver specimens of the hepatocytes and Kupffer cells of adult group**

**Hepatocytes**

Ultrastructural study of control adult rat liver showed hepatocyte with an euchromatic rounded nucleus and prominent nucleolus. The cytoplasm displayed polymorphic mitochondria, rough endoplasmic reticulum (Figure 7A). Electron microscopic examination in zinc-deficient group of a hepatocyte revealed some with severe disruption of the normal appearance. The cell showed a shrunken nucleus with an indented nuclear membrane, multiple variable-sized vacuoles, markedly destructed mitochondria, and noticeable dilated smooth endoplasmic reticulum. The rough endoplasmic reticulum cisternae appeared dilated (Figure 7B). Other hepatocytes appeared apoptotic, exhibiting small, deformed nuclei with defected irregular nuclear membrane, and severely disrupted mitochondria with the complete destruction of the cristae (Figure 7C).

**Kupffer cells**

The Kupffer cell of the control group showed lysosomes and a large heterochromatic nucleus in the wall of the blood sinusoid (Figure 7D). In the zinc deficient group, the Kupffer cell revealed disfigured, abnormal, darkly stained nucleus that showed a damaged nuclear membrane (Figure 7 E).

### *Bcl-2 immunohistochemical staining*

In new born group, Examination of sections from a control rat liver of newborn age, showed an intense positive cytoplasmic immune reaction for Bcl-2 (Figure 8A). While Bcl-2 immunostained sections in the newborn zinc deficient rat liver showed a weak cytoplasmic Bcl-2 immunoexpression (Figure 8B).

In 15 days old age group, Bcl2-immunostained liver section of the control group declared intense immunostained reaction in the 15 days old age (Figure 8C), while zinc deficient group showed a very weak Bcl-2 immune reaction (Figure 8D).

In Adult age, Bcl2-immunostained liver section in the control adult group revealed a prominent Bcl2 immunoexpression reaction (Figure 8E), while the treated rat showed a noticeable weak Bcl-2 immunoexpression in the cytoplasm (Figure 8F).

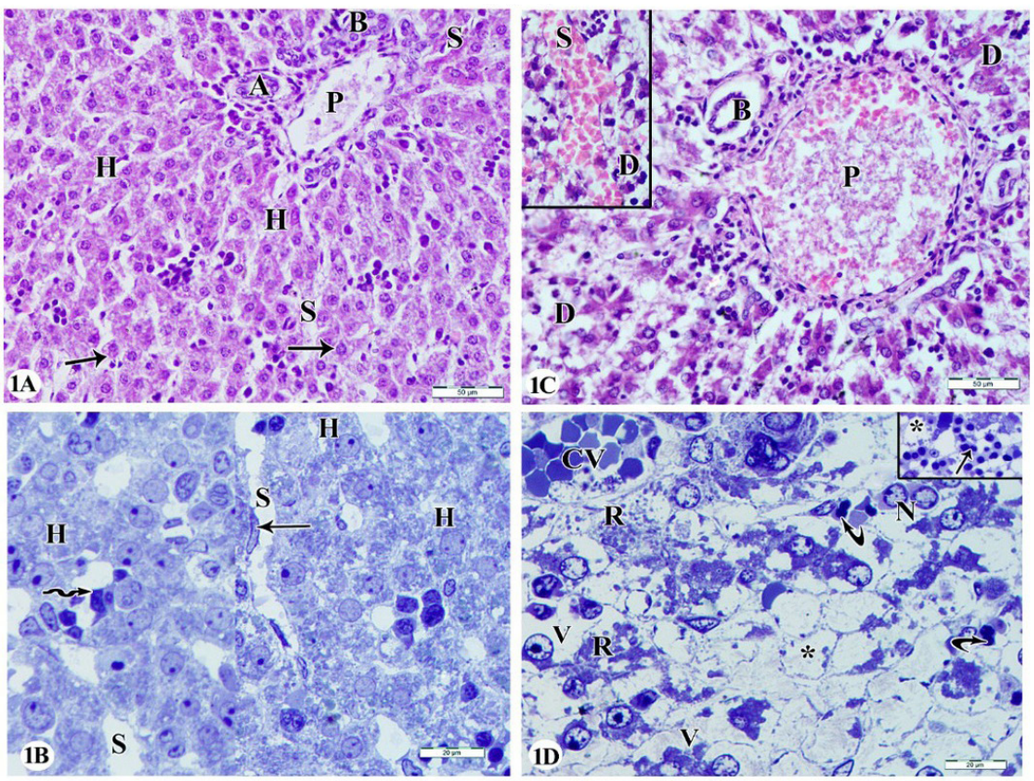


**Fig. 1A:** A photomicrograph of section in liver of control rat of new born age. It shows classical lobule with radiating and anastomosing cords of hepatocytes (H) arising from the central vein. The hepatocytes are polyhedral, with central rounded vesicular nuclei and an acidophilic finely vacuolated cytoplasm; some hepatocytes appear binucleated (arrows). The cords of hepatocytes are separated by hepatic sinusoids (S), Notice the portal triad with a clear branch of portal vein (P), hepatic artery (A) and bile duct (B). (H&E X 400). **Fig. 1B:** A photomicrograph of semithin section of the liver of control new born group showing cords of hepatocytes (H) which is separated by sinusoids (S). The sinusoids lined by Kupffer cells (wavy arrow) and endothelial cells (arrow). (Toluidine blue x1000). **Fig.1C:** A photomicrograph of a section in the liver of zinc deficient new born rats showing extensive disrupted architecture of the hepatic lobules, and markedly dilated congested portal vein (P) and dilated bile duct (B) Most hepatocytes are degenerated with apparent reduction of their number (D). Inset reveals the dilated congested blood sinusoids (S). (H &E x 400). **Fig.1D:** A Photomicrograph of semithin liver sections of zinc deficient newborn showing sever disruption of the liver architecture. The hepatocytes revealed pronounced cytoplasmic vacuolation (V), rarefied cytoplasm (R). Some cells appeared as Ghost with absent nuclei (*). Some hepatocytes revealed darkly stained binucleated nuclei (curved arrow). Very few hepatocytes still show vesicular nuclei (N) surrounded by vacuolated cytoplasm. The section also revealed remarkable dilated congested central vein (CV). Inset, showing amalgamated darkly stained cells (arrow). (Toluidine blue ×1000)

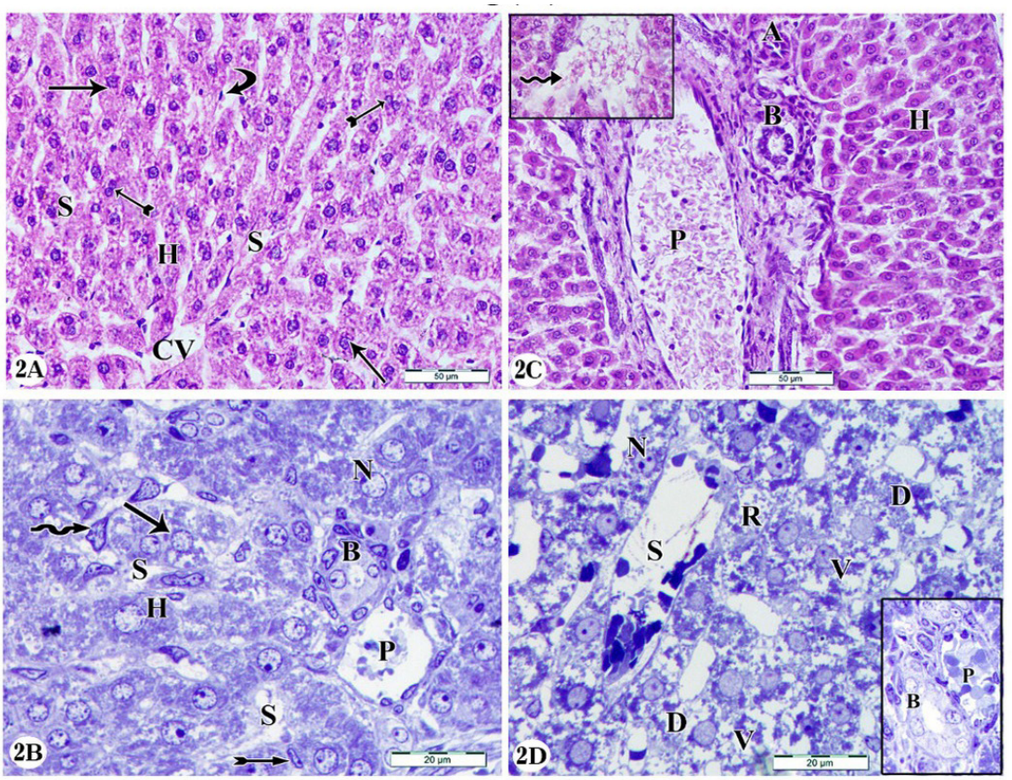


**Fig. 2A:** Sections a control rat liver of 15 days age showing cords of hepatocytes (H) with central vesicular nuclei (arrow) arising from normal central vein (CV). Among these hepatocytes appear the hepatic sinusoids (S) with thin lining of endothelium (curved arrow). Some hepatocytes are binucleated (tailed arrows). (H&E X400). **Fig .2B:** A photomicrographs of semithin sections of the liver of a control 15 days rat showing hepatocytes (H)) with the vesicular nuclei (N) and prominent nucleoli. Cords of hepatocytes are separated by blood sinusoids (S) which are lined by endothelial cells (tailed arrow)) and Kupffer cells (wavy arrow). Note the portal vein (P) and bile duct (B) and hepatocytes that are binucleated (arrow). (Toluidine blue X1000). **Fig.2C:** A photomicrograph of section in liver of 15 days zinc deficient rats. It shows the portal vein (P) markedly dilated and congested. The bile duct reveals hyperplasia (B), thickened hepatic artery is noticed (A) in the portal triad among the disrupted hepatocytes (H). Inset: showing area of haemorrhage and necrosis (wavy arrow). (H& E x 400). **Fig. 2D:** A Photomicrograph of semithin sections in the liver of 15 days old zinc deficient rats showing sever disrupted liver architecture with deleterious degeneration (D) of the hepatocytes with marked vacuolation (V). Some hepatocytes revealed rarefied cytoplasm (R). Few hepatocytes revealed vesicular nuclei and prominent nucleoli (N). The blood sinusoids (S) appeared markedly dilated and congested. Inset: showing congested portal vein (P) and obviously dilated bile duct (B). (Toluidine X 1000).

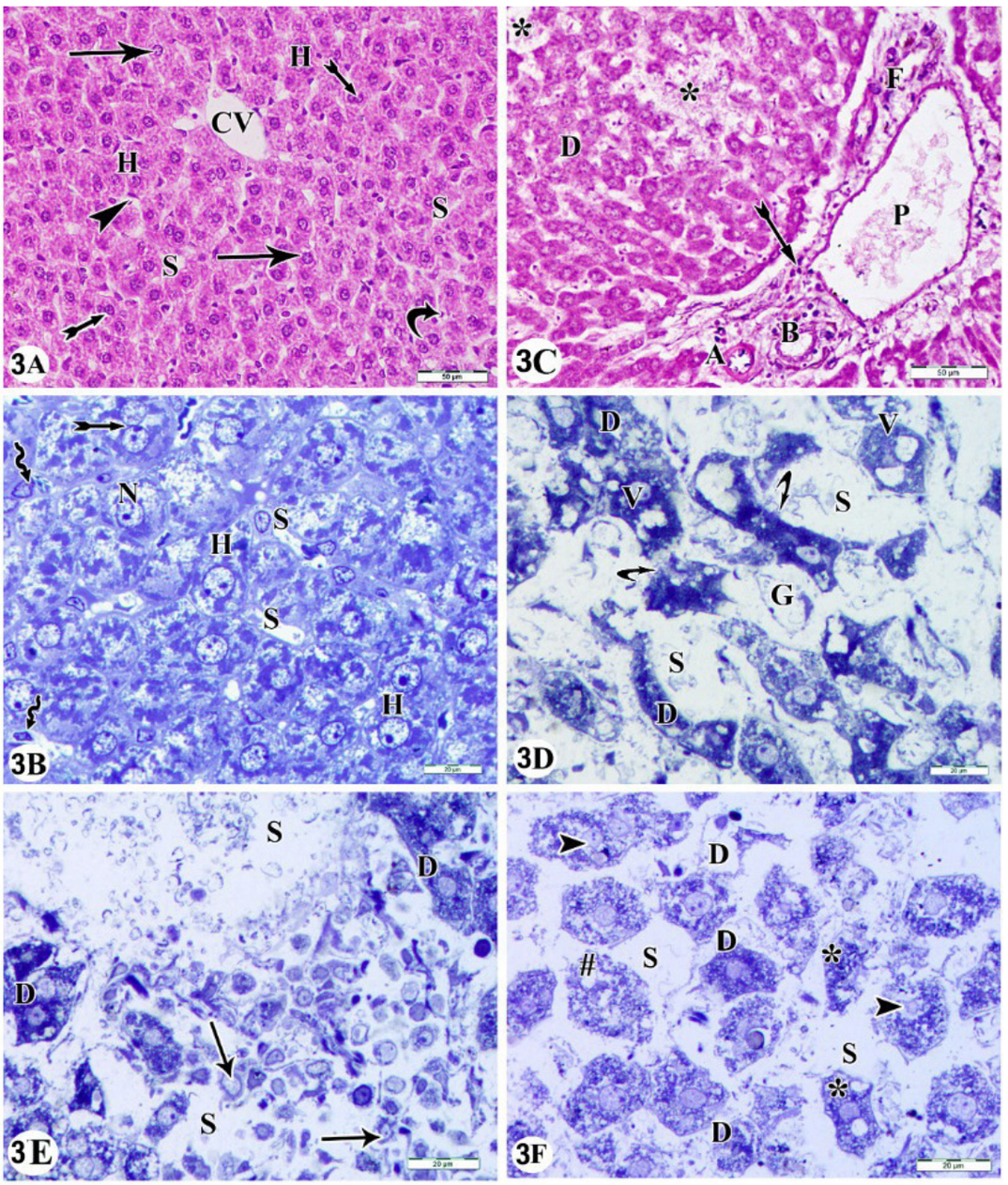


**Fig. 3A:** Light micrograph of liver section from control adult rat showing the normal histological structure of hepatocytes (H) with central spherical nucleus (arrow) radiating from central vein (CV). Some hepatocytes appeared binucleated (tailed arrow). The cords of hepatocytes are separated by hepatic sinusoids (S), which are lined by Kupffer cells (Curved arrow) and endothelial cells (head arrow). (H&E X400). **Fig. 3B:** A photomicrograph of semithin sections of a control adult rat liver showing cords of hepatocytes (H) separated by blood sinusoids (S). Hepatocytes appear polyhedral with rounded vesicular nuclei (N), prominent nucleoli and granular cytoplasm. Some hepatocytes appear binucleated (tailed arrow). Note Kupffer cells (wavy arrow) hanging in the blood sinusoids. (Toluidine blue, × 1000). **Fig. 3C:** A photomicrograph of a section in the liver of zinc deficient adult rats showing sever disruption in the hepatic architecture with extensive vacuolization and necrosis in the liver parenchyma (*). The portal triad shows severely dilated congested portal vein (P), noticeable dilated bile duct (B) and great thickening of the hepatic artery (A). There is also extensive periportal infiltration with inflammatory cells (tailed arrow) and marked fibrosis (F). Most of the hepatocytes are degenerated with absent nuclei (D). (H&E X 400). **Fig. 3D:** A photomicrograph of semithin sections in the liver of zinc deficient adult rats reveals marked disruption of liver architecture. Most hepatocytes are degenerated (D) with extensive intercellular vacuolization (V) in the liver parenchyma with appearance of multiple spaces (S) in between these distorted cells. It declares also marked disrupted appearance of degenerated hepatocyte with condensed cytoplasm (curved arrow). Some hepatocytes appear as ghost (G). (Toluidine blue x1000). **Fig. 3E**: A photomicrograph of semithin sections in the liver of treated adult rats shows sever disorganization of liver architecture. Most hepatocytes are degenerated (D) with the nuclei appear greatly distorted, shrunken very pale (Arrow) and replacement of hepatocytes by multiple spaces (S). (Toluidine blue x1000). **Fig. 3F:** A photomicrograph of semithin sections in the liver of zinc deficient adult rats declares remarkable destruction of o liver architecture. The hepatocytes appear markedly degenerated (D) with extensive cytoplasmic vacuolization (*) in the liver cells with appearance of multiple spaces (S) in between these disrupted cells. The nuclei appear greatly distorted, shrunken and very pale (head arrow). Some hepatocytes show big vacuoles (#) within the cytoplasm. (Toluidine blue x1000).

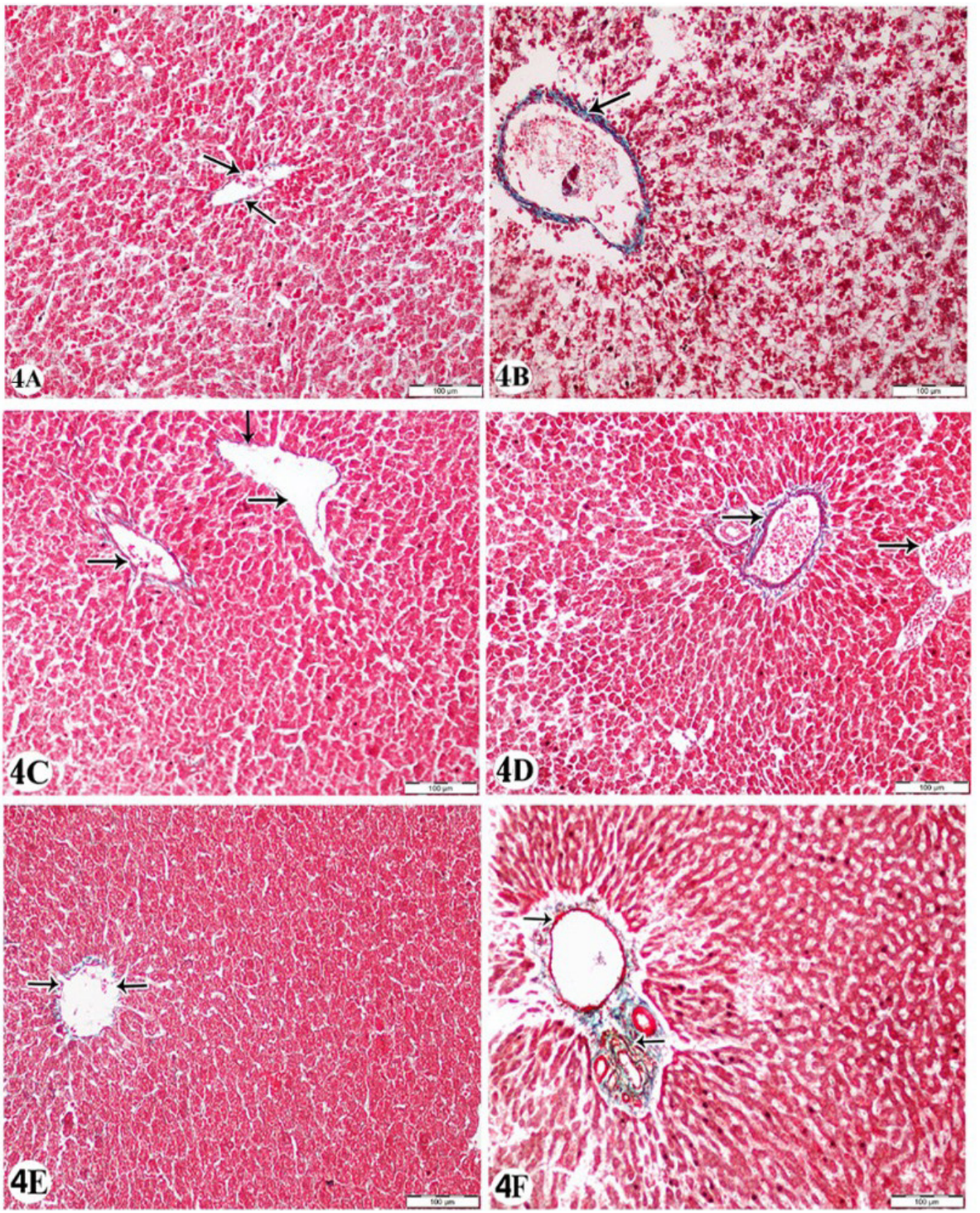


**Fig. 4A:** A photomicrograph of a section from a control rat liver of newborn age, showing few collagen fibers in the portal tract area (arrow). (Masson's trichrome stain, × 200). **Fig. 4B:** A photomicrograph of a section of the liver from zinc deficient new born rat showing increased deposition of collagen fibers in the portal tract area (arrow). (Masson's trichrome stain, × 200). **Fig.4C:** A photomicrograph of a section of the liver from 15 days old age control rat showing minimal amount of collagen fibers deposited in the portal tract area (arrow). (Masson's trichrome stain, × 200). **Fig.4D:** A photomicrograph of a section of the liver from 15 days old age zinc deficient rats showing an increase in the deposition of collagen fibers in the portal tract area (arrow). (Masson's trichrome stain, × 200). **Fig. 4E:** A photomicrograph of a section of the liver from adult age control rat showing little amount of collagen fibers in the portal tract area (arrow). (Masson's trichrome stain, × 200). **Fig. 4F:** A photomicrograph of a section of the liver from adult age zinc deficient rat showing pronounced increase in the deposition of collagen fibers in the portal tract area (arrow). (Masson's trichrome stain, × 200).

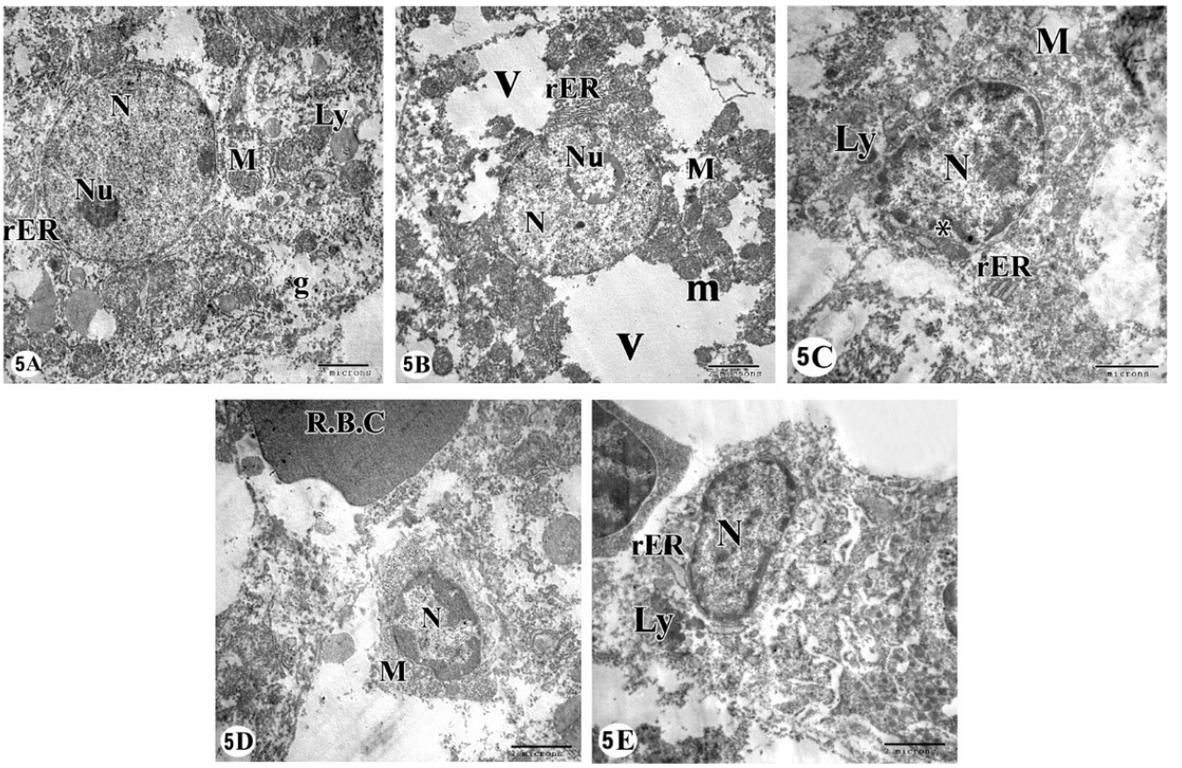


**Fig. 5A:** An electron photomicrograph of ultrathin section in the liver of control newborn showing the liver cell as polyhedral. The cytoplasm shows membranes of rough endoplasmic reticulum (rER), numerous mitochondria (M) and glycogen granules (g). The nucleus (N) appears spherical, with dispersed chromatin inside prominent nucleolus (Nu), and many lysosomes (Ly). (x 5800)

**Fig.5B:** An electron micrograph of a hepatocyte of zinc deficient newborn rat showing electron dense heterochromatic nucleus (N) with abnormal shaped prominent nucleolus (Nu), rarefied cytoplasm with multiple variable-sized vacuoles (V). The mitochondria appear severely distorted, some appears swollen with cristolysis (M) and others appear with disrupted membrane (m). Note the rough endoplasmic reticulum (rER). (X 5800). **Fig.5C:** An electron micrograph of the zinc deficient liver sections of new born age. The hepatocyte reveals markedly irregular shaped nucleus with clumps of chromatin (N), and indented interrupted outline (*). The mitochondria appear swollen with sever cristolysis (M). Note dilated rough endoplasmic reticulum (rER) and phagolysosomes (Ly). (X 7200) **Fig. 5D:** An electron micrographs of liver ultrathin sections of control new born group showing Kupffer cell appears as irregular shaped cell with indented nucleus (N), peripheral chromatin condensation and multiple mitochondria(M), Notice red blood cells (R.B.C) in the liver sinusoids. (X 7200). Fig. 5E: An electron micrograph of a Kupffer cell of zinc deficient new born showing heterochromatic oval nucleus (N) with clusters of chromatin and peripheral chromatin condensation dilated rough endoplasmic reticulum (rER) and multiple phagolysosomes (Ly). (x7200)

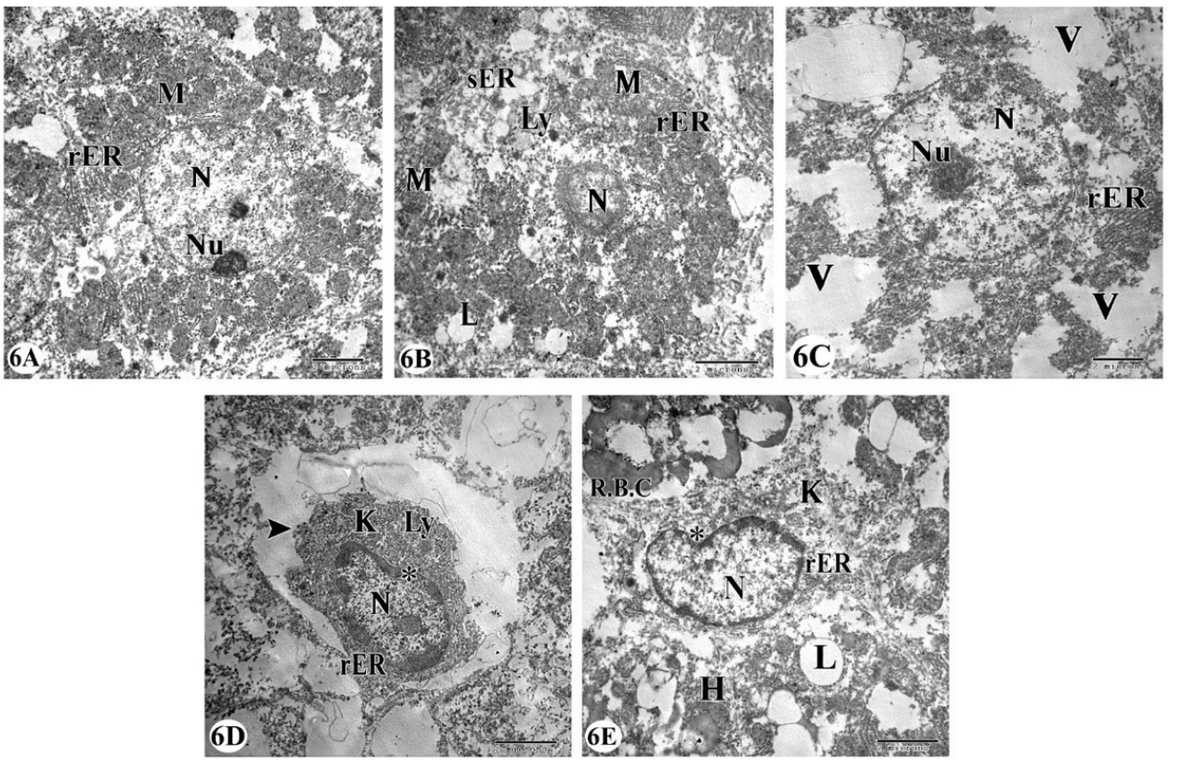


**Fig. 6A:** An electron micrograph of liver section of control rat (15 days) showing the hepatocyte exhibiting rounded euchromatic nuclei (N) with regular nuclear envelopes, prominent two nucleoli (Nu), mitochondria (M), and rough endoplasmic reticulum (rER) . (x5800). **Fig. 6B:** An electron micrograph of the liver of zinc deficient 15 days rats showing hepatocyte revealed shrunken apoptotic nucleus (N), swollen distorted mitochondria (M), multiple lipid globules (L), multiple lysosomes (Ly), dilated smooth endoplasmic reticulum (sER). Note rough endoplasmic reticulum (rER). (X 7200). **Fig. 6 C:** An electron micrograph of the zinc deficient liver sections (15 days) showing a hepatocyte with rounded nucleus (N) and prominent nucleolus (Nu). The nucleus (N) contains dense clumps of heterochromatin. The cytoplasm shows multiple varied sized vacuoles (V), and rough endoplasmic reticulum (rER) within rarefied cytoplasm. (X 5800). **Fig.6 D:** An electron micrograph of liver section of control rat 15 days showing the Kupffer cell (K) lining hepatic sinusoid and exhibiting thin filipodia (arrow head), cytoplasmic phagolysosomes (Ly), rough endoplasmic reticulum (rER) and heterochromatic nucleus (N) with peripheral chromatin condensation (*). (X7200). **Fig.6 E:** An electron micrograph of the zinc deficient rat's liver of 15 days group showing Kupffer cell (K) lining blood sinusoids. It shows irregular shaped nucleus (N) with peripheral chromatin condensation and indented outline (*), dilated rough endoplasmic reticulum (rER). Note the red blood cells (R.B. C) in the blood sinusoids that are surrounded by degenerated hepatocytes (H) that reveals multiple lipid globules (L). (X7200)

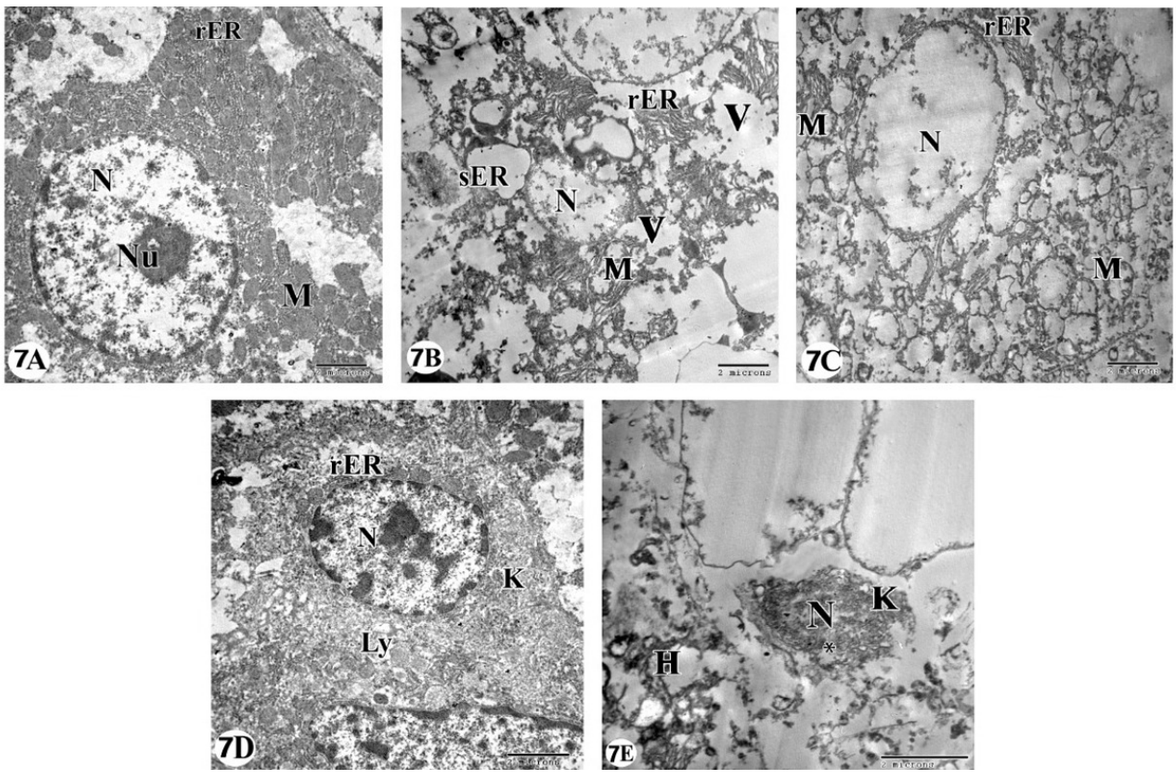


**Fig. 7A:** An electron micrograph of control adult rat liver showing hepatocyte with rounded euchromatic nucleus (N) and prominent nucleolus (Nu). The cytoplasm shows polymorphic mitochondria (M), rough endoplasmic reticulum (rER). (× 5800) **Fig. 7B:** A, an electron photomicrograph of a hepatocyte of adult zinc deficient group showing severely disruption of the cell. It reveals a shrunken nucleus (N) with indented nuclear membrane, multiple variable-sized vacuoles (V), markedly destructed mitochondria (M), and noticeable dilated smooth endoplasmic reticulum (sER.) Note the dilated rough endoplasmic reticulum cisternae (rER). (X 5800). **Fig. 7C:** An electron micrograph treated adult rat liver showing an apoptotic hepatocyte exhibiting small, deformed nucleus (N) with defected irregular nuclear membrane, severely disrupted mitochondria (M) with complete destruction of the cristae. Note rough endoplasmic reticulum (rER). (X 5800). **Fig.7 D:** An electron micrograph of control adult rat liver showing a Kupffer cell (K) observed in the wall of the blood sinusoid containing lysosomes (arrows), rough endoplasmic reticulum (rER) and large heterochromatic nucleus (N). (x 7200). **Fig. 7 E:** An electron micrograph of a section of zinc deficient adult rat liver showing disfigured Kupffer cell (K) containing abnormal darkly stained nucleus (N) that reveals damaged nuclear membrane (*). The cell is surrounded by severely damaged degenerated hepatocyte (H). (x7200)

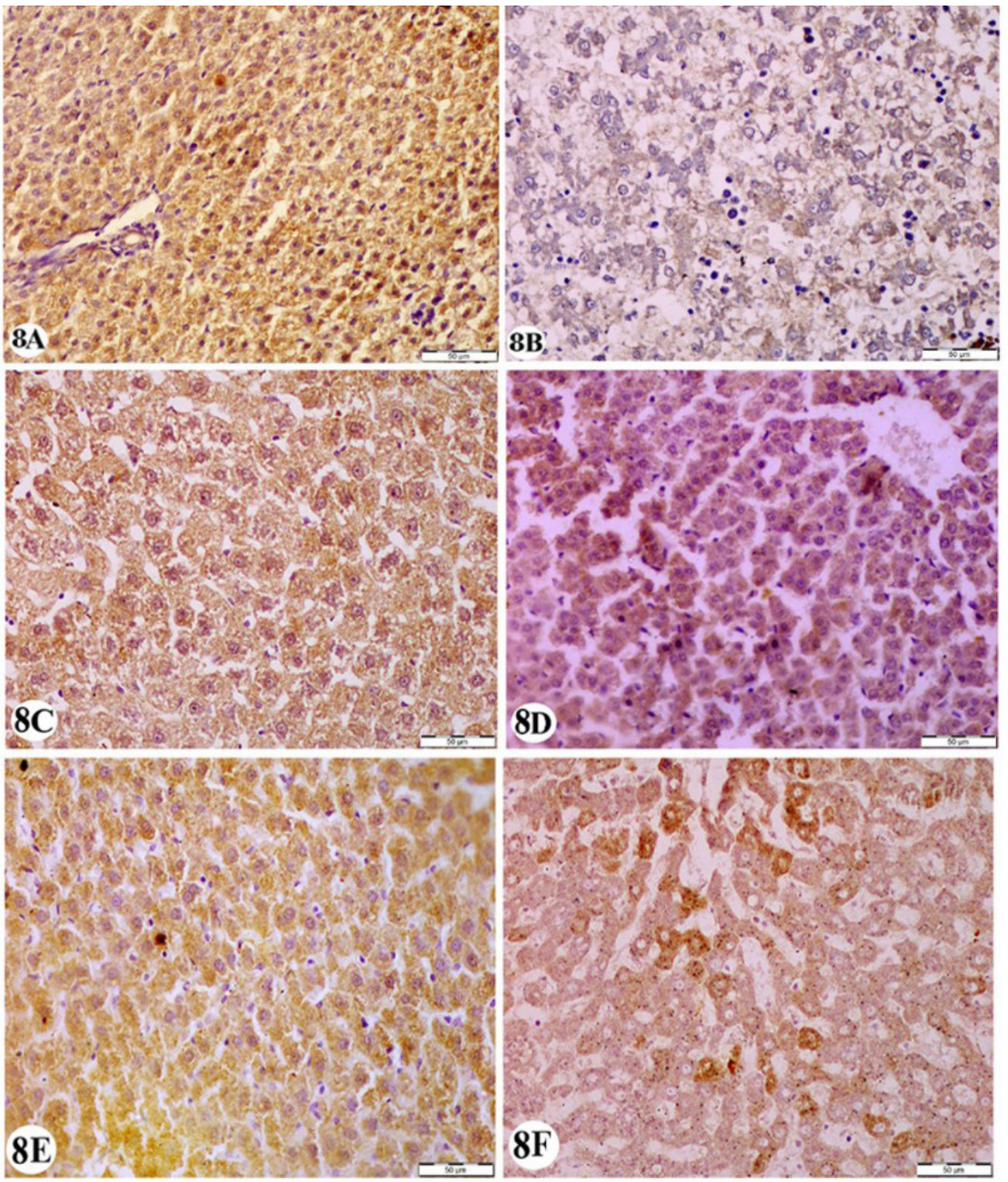


**Fig. 8A:** A photomicrograph of a section from a control rat liver of newborn age, showing an intense positive nuclear immune reaction for Bcl-2. (Immunostaining for Bcl-2, × 400). **Fig. 8B:** A photomicrograph of a Bcl-2 immunostained section in the zinc deficient new born rat liver showing a weak Bcl-2 immunoexpression cytoplasm. (Bcl2 immunostaining, × 400). **Fig. 8C:** photomicrograph of a Bcl2immunostained section in the 15 days old age control rat liver showing intense Bcl-2 immunoexpression cytoplasm (Bcl-2 immunostaining, × 400). **Fig. 8D:** photomicrograph of a Bcl2immunostained section in the 15 days old age zinc deficient rats liver showing a very weak Bcl-2 immunoexpression cytoplasm. (Bcl-2 immunostaining, × 400) **Fig. 8 E:** A photomicrograph of a Bcl2immunostained section in the adult control rat liver showing a prominent Bcl-2 immunoexpression cytoplasm. (Bcl-2 immunostaining, × 400) **Fig. 8 F:** A photomicrograph of a Bcl2immunostained section in the adult zinc deficient rat liver showing a noticeable weak Bcl-2 immunoexpression cytoplasm. (Bcl-2 immunostaining, × 400).

## DISCUSSION

Various studies confirmed that zinc deficiency is a worldwide public health problem[21]. Around 80% of pregnant women worldwide are thought to eat less zinc than is advised by nutritionists. In one of the investigations done in Egypt, 53.5% of the sample had low levels of zinc[22]. The vital role zinc plays during the first stage of life, including embryogenesis and fetal life, is now well established. While appropriate zinc supplementation during pregnancy may reduce the incidence of preterm birth, maternal zinc limitation during pregnancy affects fetal growth[23]. Despite this consistent number of articles on the role of zinc in early life for the fetus, no available studies dealt with the effect of prenatal zinc deficiency on the histological structure of the liver in early as well as late postnatal life. From these considerations, this study was carried out to elucidate more light on morphological changes both at the light and ultra-structural levels with respect to the age of the developing rat.

As zinc supplementation was proved to have a hepatoprotective effect against many toxic insults[24-26]; it could be postulated that zinc deficiency induced in the present study exerted a damaging effect on the postnatal developmental processes of rat hepatocytes resulting in the alteration of the structural integrity of hepatocytes at both light and microscopic levels. The present results revealed severe deleterious effects of zinc deficiency on the liver architecture for all age groups. Light microscopic examination showed severely disrupted architecture of the hepatic lobules, markedly dilated congested portal, and central veins. Most hepatocytes degenerated with areas of hemorrhage, necrosis, and vacuolization. Hepatocytes with darkly stained degenerated nuclei were frequently seen in most groups. Electron microscopic examination of hepatocytes of the treated groups provided more details about light microscopic observation regarding the shrunken apoptotic hepatocytes where nuclei showed heterochromatin condensation. Many hepatocytes revealed multiple variable-sized vacuoles within rarefied cytoplasm. Markedly swollen distorted mitochondria with cristolysis were observed. Multiple lipid globules, multiple lysosomes, and noticeable dilated smooth endoplasmic reticulum were declared.

All previous alterations denoted cellular injury; linked to induced prenatal zinc deficiency in pregnant rats. It is evidenced that low zinc levels could be connected to decreased activity of key antioxidant enzymes such as Cu/Zn-specific SOD which could be the possible sources of reactive oxygen species (ROS)[27]. Free radicles possess damaging effects on cytoplasmic biomolecules as lipids, proteins, and DNA leading to cell death and apoptosis[28]. Also, ROS are produced in the cytoplasm as a result of changes in the endoplasmic reticulum (ER) and mitochondria which are severely affected in the current study based on electron microscopic observation of zinc deficiency groups[29,30]. In addition to experimental chelation of zinc done in the present study, more decrease in Zinc concentrations could be occurred because of its being excessively utilized in providing an antioxidant defense mechanism[31].

The results of numerous studies strongly showed that zinc may be crucial for preserving the body's equilibrium of ROS. Zinc reduced oxidative injury, proinflammatory cytokine release, and inducible NO synthase (iNOS) in human subjects, according to cell culture models and clinical trials involving normal healthy people, elderly people, and people with sickle cell anemia[32]. Prior studies have shown that even dietary deficiencies in zinc, an important antioxidant, cause rats' lipid peroxidation to rise. Zinc functions as a free radical scavenger and has been demonstrated to cause oxidative stress in the testes of rats with zinc deficiency[33,34].

In the current work, Masson's trichrome-stained sections revealed marked fibrosis in the portal areas of the liver and around the blood vessels in all ages of albino rats born to zinc deficient female rats. In the light of what was proved earlier from releasing of free radicles with zinc deficiency, the current finding could come in agreement with previous studies[35,36], which explained the increased collagen formation to be linked to hydroxyl reactive oxidizing molecules production in the biological system led to lipid peroxidation. The latter caused damage to proteins and nucleic acids. Another researcher showed that ROS increase collagen synthesis, which increases hepatic fibrosis because they encourage the proliferation of hepatic stellate cells. Collagen type I was deposited as a result of activated stellate cells[37].

Similarly, numerous investigations using animal models and some clinical trials have shown that zinc deficiency may be linked to fibrosis in chronic inflammatory illnesses, such as liver, myocardial, and cystic fibrosis[38-40]. On the other hand, other studies have shown that supplementing with zinc can enhance liver function in both alcoholic liver disease and hepatitis C patients, with improved fibrosis markers in those who have the chronic form of the disease[41].

Regarding congested portal and central veins observed herein metabolic disturbance resulting from zinc deficiency was described to be associated with distinct blood vessel dilatation[12]. The activation of Kupffer cells with subsequent increase and production of nitric oxide, which is thought to be a contributing factor for arterial dilatation, could be another explanation for the vascular congestion and dilatation observed in treated rats[42]. A finding connected to portal hypertension and possibly a result of liver damage brought on by zinc deficiency was described by Hu, *et al*.[43] as dilated and congested central vein and blood sinusoids with separation of the central vein's endothelial lining. On the other hand, Puche, *et al*.[44] attributed sinusoidal dilatation to the activation of perisinusoidal cells that had contractile properties.

The current study declared remarkable vacuolation of hepatocytes at both light and electron microscopic levels;

this was explained by Barabara and Mindigulo[45], who reported that zinc deficiency results in lipid deposition in hepatocytes; a clinical condition known as nonalcoholic fatty liver diseases. They also added that dietary zinc deficiency results in endoplasmic reticulum stress with dysfunction of enzymes needed for hepatic lipid metabolism with subsequent fatty liver development, which may end to liver fibrosis[46]. In view of such findings, most light microscopic observations, including vacuolation and ultrastructural demonstration of lipid deposition within hepatocytes in all age groups suffering from zinc deficiency, could be explained.

In the present work, some hepatocytes in the treated group have darkly stained shrunken nuclei with either clump of chromatin or peripheral heterochromatin condensation. This finding is in accordance with Taylor *et al* and Pedrycz *et al*,[47,48], who stated that hyperchromatic nuclei were a degenerative change denoting apoptosis. Zinc is proven to be an anti-apoptotic agent, so with zinc deficiency, it is well justifiable to see such apoptotic changes in the cells. Furthermore, these findings were clearly confirmed with the decrease in Bcl2 immunostaining of hepatocytes in zinc deficient groups observed in the current study. Omu *et al*. reported that zinc deficiency is associated with reduced Bcl2 expression, which is similar to the finding reported in the current study. Also, they documented a decrease in testosterone production, increased oxidative stress, and apoptosis. These findings suggest that zinc has a role in male reproduction[49].

In the current study, EM investigation of the treated groups revealed degenerative alterations in the liver tissue, including many vacuoles and lipid droplets in the cytoplasm of the hepatocytes. Some possessed nuclear chromatin that was peripherally condensed, while others had shrunken, electron-dense nuclei. Severe destructive changes in the mitochondria were observed. These deleterious changes were suggested to be related to mitochondrial dysfunction with ATP depletion[50]. Furthermore, hepatocytes or their cytoplasmic organelles, including mitochondria, could be injured as a result of abnormalities in cell respiration and changes in intracellular and extracellular transport. Hepatocyte necrosis, parenchymal degeneration, or abnormalities in the action of metabolic enzymes are signs of this injury[51,52]. Besides mitochondrial alteration, the current study declared dilated endoplasmic reticulum. This comes in harmony with Ellis *et al*.[53], who proved that the buildup of misfolded proteins that create a vicious cycle of ER stress and oxidative damage observed with zinc deficiency that causes ER stress response.

The present study revealed obvious destruction of the cell membrane. This comes in line with Bettger and Dell,[54], who stated that zinc plays a critical function in maintaining membrane stability. Given that zinc deficiency has been shown to increase lipid peroxidation and damage to lipid membranes. Additionally, zinc is a well-known necessary component of coenzymes and metalloenzymes such alkaline phosphatase and Cu/Zn-SOD, which works in numerous aspects of cellular metabolism and is crucial for maintaining the integrity of cell membranes[55]. The present study declared the appearance of lipid globules which comes in harmony with[56], who stated that zinc deficiency inhibited lipolysis and postulated that zinc restriction could lead to a rise in triglyceride due to alterations in lipid metabolism in the adipose tissue and liver.

The current study declared hyperplasia of the bile duct and around the hepatic artery. This comes in line with several studies that found that zinc deficiency stimulates cell proliferation by upregulating gene expression of enzymes involved in DNA synthesis, such as deoxythymidine kinase[57].

Kupffer cells are resident macrophages located in the sinusoidal space[58]. Activation of these cells is an early key issue in liver tissue damage and repair. In this study, an apparent increase was noticed in the number of Kupffer cells in the zinc-deficient groups. In the light of what is known about the role of these cells in the protection of the hepatic tissue against any injury, these cells increased to put more support and protection of liver tissue[59]. Ultra-structurally, the current study declared alteration of Kupffer cells in all zinc deficient groups. The cells were declared heterochromatic oval nuclei. Others revealed irregular shapes with an indented outline, dilated rough endoplasmic reticulum, and multiple phagolysosomes. Kupffer cells may play a crucial role as an initial cytotoxic cell type and are likely to be a source of ROS and proinflammatory mediators, according to several data. These activated cells also increased the cellular damage of hypoxic hepatocytes[60].

It is clearly pronounced in this study findings that prenatal zinc deficiency induced a long-term effect on the offspring which extend to be highly obvious in adult age, this comes in accordance with[61,62] who proposed hypothesis links poor foetal and infant growth with subsequent development of diabetes, cardiovascular disease and long-term irreversible impact on the offspring's brain leading to neurological and behavioural disorders later in life.

## CONCLUSION

In summary, the study results clearly declared the deleterious effects of zinc deficiency on the liver cell architecture at different stages of postnatal liver development. Further studies are needed using zinc supplements to assure the expected improvement of the liver architecture. Moreover, well-designed clinical trials are needed to fully appreciate the benefits of zinc as an antioxidative and anti-inflammatory agent on these structural abnormalities.

## CONFLICT OF INTERESTS

There are no conflicts of interest.

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**الملخص العربى**

# دراسة نسيجية وهستوكيميائية مناعية على تأثير نقص الزنك قبل الولادة على نمو كبد الجرذان بعد الولادة

**امل راتب[١]، هبة كمال[١،٢]**

**[١]قسم التشريح الآدمى وعلم الأجنة – كلية الطب - جامعة اسيوط**

**[٢]قسم التشريح الآدمى وعلم الأجنة جامعة سفنكس**

**المقدمة:** من المعروف ان الزنك عنصر هام وله وظائف أساسية في التمثيل الغذائي الخلوي.ويمكن أن يسبب نقصه آثارًا تضر بالنمو في عديد من الأعضاء ويعد الكبد مسؤولا عن استقلاب الزنك.

**الهدف من الدراسة:** دراسة آثار نقص الزنك في الجرذان الحامل على نموالكبد بعد الولادة باستخدام التقييم النسيجي والهستوكيميائي المناعي

**المواد والطرق:** تم تقسيم إناث الجرذان البالغة بشكل عشوائي بعد التأكد من حملهم إلى مجموعتين: المجموعة الضابطة (٢٤ جرذًا تلقت حقنة من الماء المقطر واحدة داخل التجويف البريتونى فى اليوم التاسع من الحمل) والمجموعة التجريبية (٢٤ جرذًا تلقت حقنة داخل التجويف البريتونى من ١,١٠ الفينانثرولين (عامل مزيل للزنك) بجرعة واحدة ٣٠ مجم / كجم في اليوم التاسع من الحمل). تم تأكيد نقص الزنك عن طريق قياس مستوى الزنك في الدم في كلتا المجموعتين من الإناث الحوامل في اليوم العاشر من الحمل. تم التضحية بنسل ذكور الجرذان المعالجة والضابطة في الأعمار التالية للولادة: حديثي الولادة ، ١٥ يومًا ،ثلاثة شهورثم تم أخذ عينات الكبد وتجهيزها للمجهر الضوئى وإلالكترونى بالإضافة إلى تقنية صبغات هستوكيميائية مناعية بروتين ورم الغدد لليمفاوية (Bcl-2).

**النتائج:** أظهر الفحص المجهري الضوئى للمجموعات التى عانت من نقص عنصر الزنك تهدم واضح فى الخلايا الكبدية مع الموت المبرمج لتلك الخلايا. فضلا عن حدوث إحتقان وإتساع ملحوظ في الجيوب الدموية والأوعية المركزية والوريد البابى وكذلك كشفت صبغة ماسون ثلاثية الألوان عن تليف ملحوظ حول الأوردة. ايضا أظهرت الدراسة الهيستوكيميائية المناعية ضعف النشاط المناعي (Bcl-2) في المجموعات التى تعرضت لنقص الزنك. وعلى مستوى التركيب الدقيق فقد ظهرت خلايا الكبد متدهورة مع وجود فجوات حشوية أيضا لوحظ تورم وتلف وتحلل للميتوكوندريا.

**الخلاصة:** أدى نقص الزنك إلى آثارتركيبية ملحوظة فى أنسجة الكبد والتي أثرت على جميع الفئات العمرية بعد الولادة حتى انها امتدت إلى سن البلوغ.