

# Research Journal of Pharmaceutical, Biological and Chemical Sciences

## Hypervitaminosis A induced Histological, Histochemical and Immunohistochemical alterations in the liver of albino mice.

Seham A Ibrahim<sup>1</sup>, and Yosry A Okdah<sup>2</sup>.

<sup>1</sup>Zoology Department, Faculty of Science, Benha University

<sup>2</sup>Zoology Department, Faculty of Science, Menoufia University, Egypt

### ABSTRACT

Vitamin "A" intake in excess doses of the animal requirement results in a toxic syndrome known as hypervitaminosis . The present study was conducted to evaluate the effect of hypervitaminosis A on liver of mice. Fifty Swiss albino mice were randomly divided into four groups Swiss albino mice of 10 animals each group and dosed as follows:Group I, which was a control ,Group II, III were i.p. administered vitamin A (500 I U/kg body weight/day/mice for 2 and 3 ,repectively.Histological examination of liver of hypervitaminosis showed congestion and dilation of blood vessels,leucocytic infiltrations, cytoplasmic vacuolation of the hepatocytes and fatty degeneration. Histochemical observations showed depletion of polysaccharides.Expression of Bcl-2 and PCNA was recorded in hypervitaminosis A- treated hepatocytes.Aspartate transaminase (AST), alanine transaminase(ALT) and alkaline phosphatase (ALP) activity were significantly higher in sera of hypervitaminosis A animals as compared to control Group. It concluded that intake of vitamin A for three weeks is associated with increased risk hepatotoxicity in albino mice.

**Keywords:** Hypervitaminosis A, Mice, Liver, Histology , Histochemistry,Immunochemistry

*\*Corresponding author*

## INTRODUCTION

Vitamin A (retinoid) are fat-soluble vitamins, essential nutrient for human and are involved in a wide variety of biochemical functions including embryogenesis, vision reproduction, skeletal development, neurodevelopment, growth maintenance of epithelial tissues, cellular proliferation and differentiation processes. The vitamin is stored primarily in the liver and transported in plasma bound to a specific retinol-binding protein (RBP) [1]. Vitamin A and its derivative retinoic acid regulate various aspects of cell behavior as growth, differentiation, and proliferation. Retinoic acid derivatives have been suggested to play a role in processes such as hepatic regeneration and fibrosis[2,3]. Hypervitaminosis A is caused by excessive consumption of vitamin A, typically as vitamin concentrate, supplement or vitamin-rich liver. Both acute and chronic forms of hyper vitaminosis A occur naturally in man and animal. The incidence of chronic hypervitaminosis A is becoming more frequent problem worldwide due to the increasing use of vitamin A supplements in children of developing countries where vitamin A deficiency is commonplace. It has been shown that vitamin A administration in large doses can be hepatotoxic[4,5]. The reported changes in the liver ranged from the apparent normal histology up to established cirrhosis[6,7].

Galal et al.[8] found that ,the maximum effects were shown in the periphery of the hepatic lobules with focal areas of necrosis and pyknotic nuclei . The hepatocytes showed marked vacuolation with depletion of glycogen and fat droplets .Large Ito cells were numerous and demonstrated near the blood sinusoids. Mononuclear and lymphocytic infiltrations were observed in the portal tract, around the central veins and in the interlobular septa. The reticular fibers were increased in the portal tract and became longer and more branching . No changes were seen in the collagenous fibers.Choudhary and Swami[9] concluded that ,the chronic intake of vitamin A for twelve weeks or more is associated with increased risk for hepatotoxicity in Swiss albino mice then acute toxicity.Lind et al.[10]reported that the excessive intake of vitamin A has been associated with an increased risk of fractures in humans. In animals, a high vitamin A intake leads to reduction of long bone diameter and spontaneous fractures. The present work studied the histological, histochemical and immunohistochemical alterations in liver of mice exposed to hypervitaminosis A.

## MATERIAL AND METHODS

### Animals

Healthy male Wistar albino mice weighing between 25-30g.were obtained from the Animal House of the Faculty of Veterinary Medicine in Moshtohor city, Benha University, Benha, Egypt. Animals were kept in 12 h light dark cycle and provided *libitum* with water and dietad. A balanced standard diet composed of dried skim milk (5%), fish meal (10%), soybean meal (12%), alfalfa meal (4%), corn gluten meal (3%), ground corn (24.5%), ground hard winter wheat (23%), wheat middlings (10%), Brewer's yeast (2%), molasses (1.5%), soybean oil (2.5%), plus minerals and vitamins prepared at Ministry of Agriculture, Egypt. Forty rats were used in this study. They were divided into three groups .

**Group I** : Control group given sesame oil ( 10 animals ).

**Group II**: Animals of this group (15 rats) were injected with vitamin A at a dose level of 500I.U. interaperitoneally daily for two weeks.

**Group III**: Animals of this group (15 rats)were injected with vitamin A at a dose level of 500I.U. interaperitoneally daily for three weeks.

### Histological and Histochemical Investigation

Animals of different groups were dissected out after two and three weeks and their liver were carefully separated and washed in normal saline. Specimens were fixed in 10% phosphate buffered formalin (pH 7.4). Fixed materials were embedded in paraffin wax and sections of 5 micrometer thickness were cut. Slides were stained with haematoxylin and eosin for histological examination. For histochemical demonstration of total carbohydrates periodic acid Schiff's technique (PAS) [11] was used. Total proteins were detected using the mercury bromophenol blue method[12].

### Immunohistochemical studies

The immunostaining was performed using the avidinbiotin complex (ABC) method and an automatic autostainer (CODE-ON Immuno/DNA slide stainer: Biotek solution, Santa Barbara, CA). Formalin-fixed Slides were deparaffinized and blocked for endogenous peroxidase with 1.75% hydrogen peroxide in methanol for 20 min, antigen retrieval for 15 min using Biogenex Antigen Retrieval Citra solution in 90°C water bath for 30 min. The slides were allowed to cool for 20 min before continuing. Slides were then blocked by normal horse serum for 5 min at 37°C. The monoclonal antibody was applied overnight in humid medium at room temperature followed by the biotinylated secondary antibody for 15 min at 37°C and the ABC complex for 15 min at 37°C (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) was applied for 20 min at room temperature as chromogenic slides were counterstained with hematoxylin, dehydrated, and covered by coverslips. In negative control slides, the same system was applied with replacement of the monoclonal antibody by diluted normal bovine serum. PCNA- immunostaining was performed using polyclonal rabbit-anti-human (A3533 1 g fraction; DAKO, Glostrup, Denmark). Bcl<sub>2</sub> was detected using a Bcl<sub>2</sub> monoclonal antibody (Dako A/S, Glostrup, Denmark).

### Biochemical studies

For the biochemical study, sera were obtained by centrifuging the blood samples and storing them at 20°C until the assays could be completed. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined colourimetrically according to Reitman and Frankel[13]. Alkaline phosphatase (ALP) activity was determined according to the method of Belfield and Goldberg[14] using reagent kits obtained from Bio-Merieux Chemical Company ( France).

### Statistical Analysis

Data were expressed as mean values  $\pm$  SD. All statistical analyses were performed using SPSS statistical version 16 software package (SPSS® 4 Inc., USA).

## RESULTS

### Histological results

Histological examination of the liver sections in the control mice showed a normal histological picture. The central vein lies at the center of the lobule surrounded by the hepatocytes with strongly eosinophilic granulated cytoplasm and distinct nuclei. In addition between the strands of hepatocytes the hepatic sinusoids are exhibited as shown in Fig.1 a. Treating animals with vitamin A for 2 weeks revealed congestion of blood vessels and leucocytic infiltrations (Fig.1b&c). The hepatocytes appeared with cytoplasmic vacuolation and pyknotic nuclei (Fig.2a). After 3 weeks, these histopathological changes became severe. Large masses of leucocytic infiltrations was observed and extended to the sinusoidal spaces (Fig.2b). Fatty infiltrations composed of fat droplets with different sized were abundant (Fig.2c).

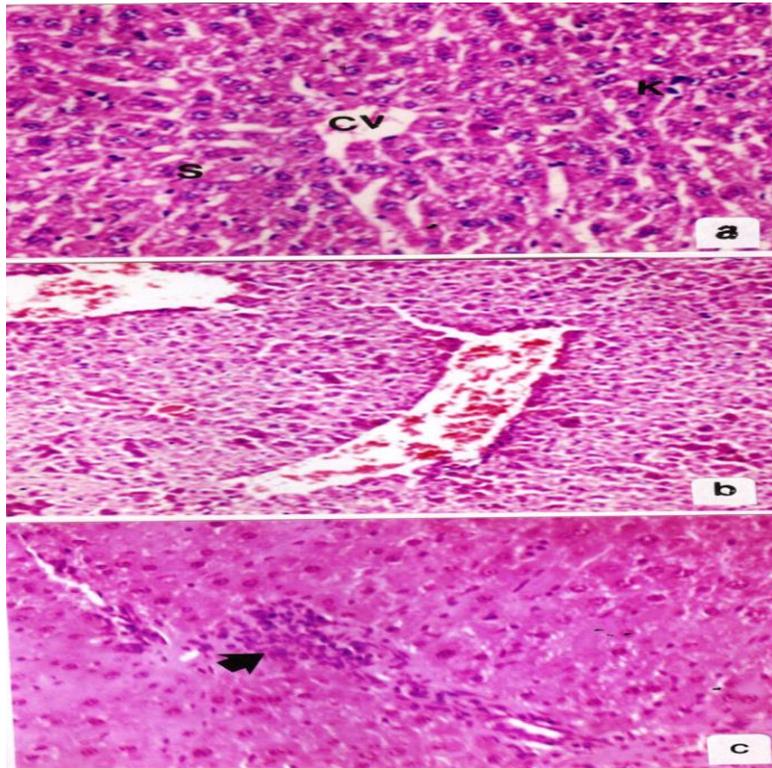


Fig. 1(a):Section in liver of a control mouse showing central vein (CV),sinusoid (S) and kupffer cell (K) ,  
 (b)hypervitaminosis A treated mouse after 2 weeks showing congested and dilated blood vessel,H&E X400.  
 (c)hypervitaminosis A treated mouse after 2 weeks showing leucocytes infiltration (arrow),H&E X400.

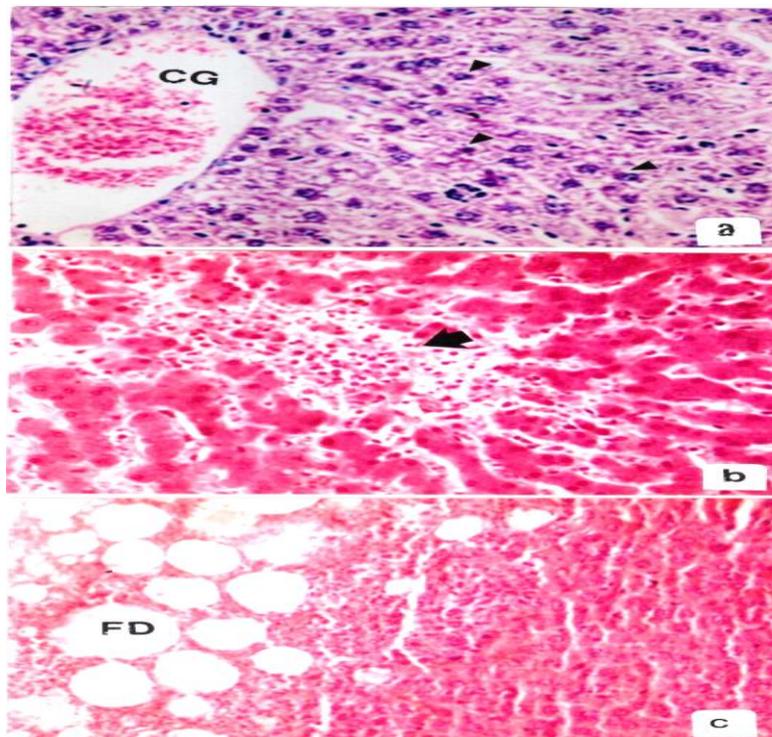
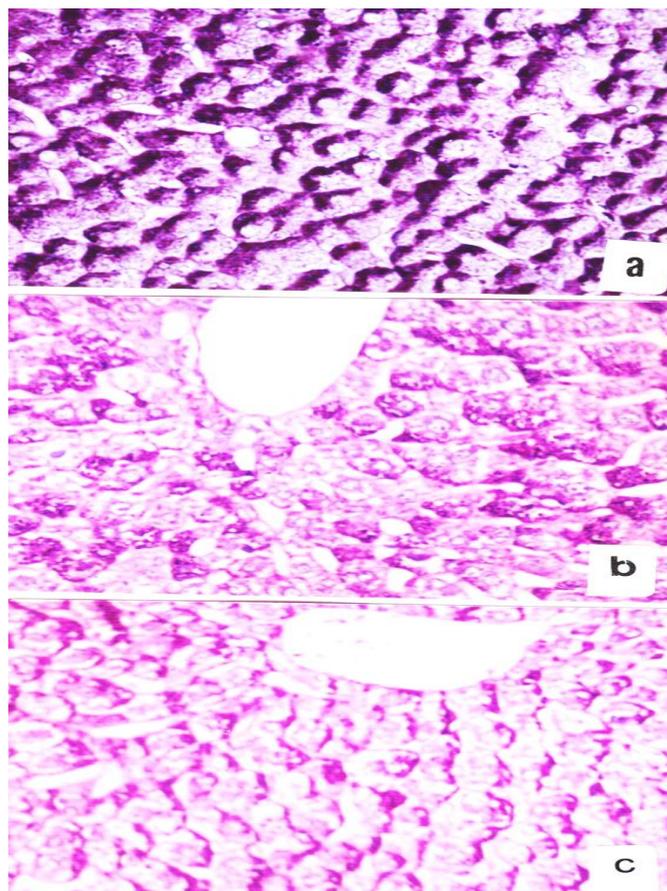


Fig 2.(a) hypervitaminosis A treated mouse after 2weeks showing enlarged and congested central vein and cytoplasmic vacuolation of the hepatocytes (arrow heads),H&E. X400.  
 (b)hypervitaminosis treated mouse after3 weeks showing luocytic infiltrations,( arrow) and widened sinusoids,H&E.X400.  
 (c) hypervitaminosis A treated mouse after 3 weeks showing fatty degeneration,FD: fat droplets,H&E. X400.

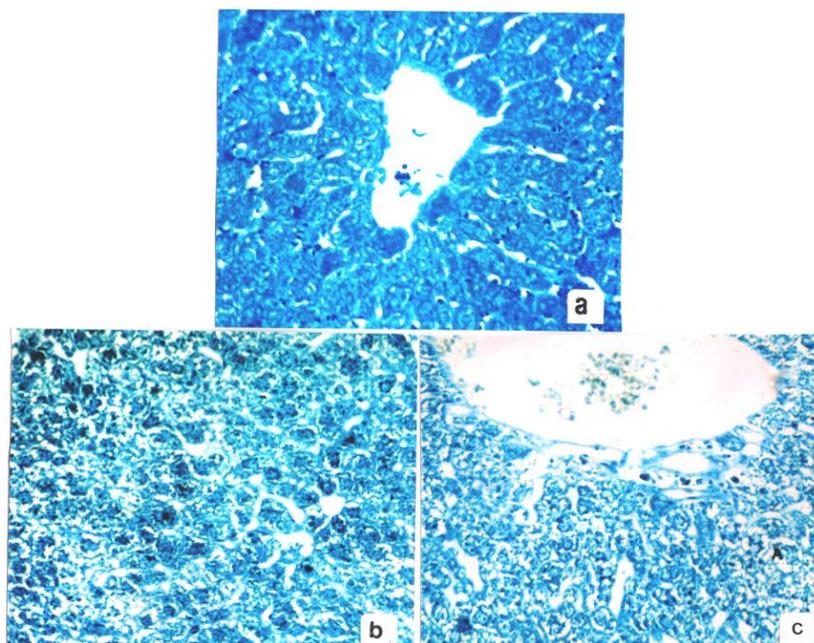


**Fig. 3(a).** Section in liver of a control mouse,PAS X400 (b) hypervitaminosis A treated mouse after 2 weeks showing decrease of polysaccharides,PAS X400 (c)hypervitaminosis A treated mouse after 3 weeks showing marked decrease of total polysaccharides, PASX400.

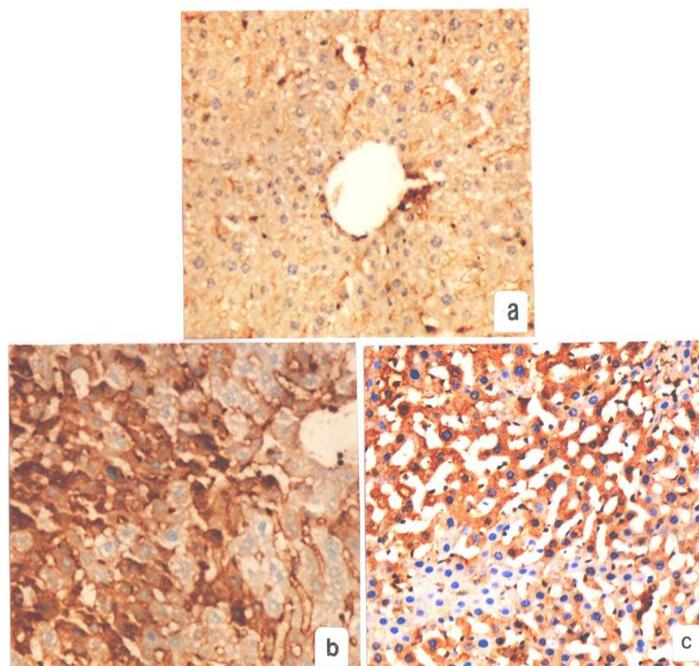
**Histochemical observations**

PAS-positive polysaccharide granules were found in the cytoplasm of most hepatocytes as intense coarse granules displaced to one pole of the cell during fixation period (glycogen flight phenomenon). The nuclei are negatively stained (Fig.1).A marked depletion of the polysaccharides was noticed in most of the hepatic cells treated with vitamin A (Fig.3b).Marked depletion of polysaccharides was observed in hepatocytes after 3 weeks of treatment with vitamin A (Fig.3c).

Total protein contents of the liver cells of control mice are positively reflected by the appearance of blue color after staining with bromophenol blue. Generally, hepatic tissue cytoplasm contains excessive amount of total proteins in the form of the fine granules. Positive reactivity observed in cell membrane and nuclear membrane acquiring an intense satiability denoting their protein richness .In addition, both chromatin bodies and nucleoli exhibiting deep coloration. Also the walls of blood vessels exhibited strong attainability(Fig.4a). Application of vitamin A for 2 weeks induced decrease in the total protein contents in the liver cells (Fig.4b). Sections obtained from animals following this course of treatment for three weeks indicated marked decrease in the protein content of hepatocytes in conjunction with the cytoplasmic vacuolization (Fig.4c).



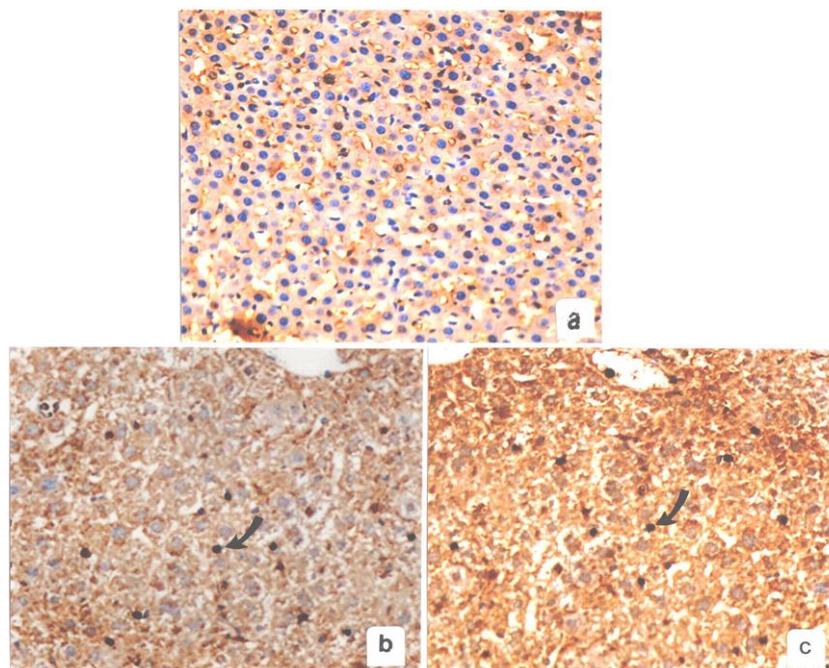
**Fig.4(a).** Section in liver of a control mouse, bromophenol blue.X400 (b) hypervitaminosis A treated mouse after 2 weeks showing decrease in total proteins,bromophenol blue. X400 (c)hypervitaminosis A treated mouse after 3 weeks showing marked decrease of total proteins,bromophenol blue.X400.



**Fig. 5(a)** Section of liver a control mouse X400,(b) hypervitaminosis A treated mouse after 2 weeks showing increase of expression of Bcl<sub>2</sub> , X400 (c)hypervitaminosis A treated mouse after 3 weeks showing highly expression of Bcl<sub>2</sub> X400.

**Immunohistochemical results**

Figure 5a showed negative expression of Bcl<sub>2</sub>in liver cells of control mice. The number of the Bcl<sub>2</sub>positive staining cells increased in liver cells of rats treated with hypervitaminosis A for 2 weeks (Fig.5b).Such increase became marked after 3 weeks of exposing animals to hypervitaminosis A (Fig.5c).Immunohistochemical examination of liver for expression of PCNA showed negative expression in hepatocytes of control mice (Fig.6a). The hepatocytes of hypervitaminosis A -treated rats revealed a positive expression of PCNA after 2 and 3 weeks (Fig. 6 b&c).



**Fig 6 (a) Section of liver a control mouse X400 b) hypervitaminosis A treated mouse after 2 weeks showing PCNA positive cells ( arrow) , X400 (c)hypervitaminosis A treated mouse after 3 weeks showing increase of PCNA-positive staining cells(arrow) X400**

**Biochemical results**

Table (1) show comparative data for different levels of ALT, AST and ALP in control mice and mice treated with hypervitaminosis A for 2 and 3 weeks period. The data showed that the level of ALT, AST and ALP were increased compared to their corresponding control at the end of the experimental period. Highly significant increase ( $P < 0.01$ ) were indicated after three weeks from injected with hypervitaminosis A.

**Table 1: Biochemical parameters in control and hypervitaminosis A groups**

Parameter	Control			2 Weeks			3 Weeks			F-value	Sig.
	mean	±	SE	mean	±	SE	mean	±	SE		
ALT (µ/ml)	12.480	±	0.811	16.378	±	0.488*	18.093	±	0.574**	20.265	0.000
AST (µ/ml)	8.185	±	0.553	10.875	±	0.123*	12.343	±	0.423**	26.645	0.000
ALP (µ/ml)	24.700	±	0.878	33.935	±	0.917*	41.438	±	2.189**	32.932	0.000

F-value = ANOVA F-test. Sig. = significance level.

\* (significant difference,  $P < 0.05$ ). \*\* (highly significant difference,  $P < 0.01$ ).

**DISCUSSION**

In the present results, hypervitaminosis A caused histopathological alterations in liver of mice. These results are in agreement with Galal et al.[8], who reported that hypervitaminosis A induced many histological changes in the liver of rats including leucocytic infiltrations, necrosis and fatty degeneration. The picture of chronic hypervitaminosis A is highly variable, hepatosplenomegaly are the most manifestations [15]. The hepatotoxicity of hypervitaminosis was studied by other investigators [16,17]. Cytoplasmic vacuolation was recorded in hepatocytes of hypervitaminosis-treated mice. According to Lane [18], the vacuolation of the liver cells can be attributed to swelling of the mitochondria and proliferation of the endoplasmic reticulum. Leucocytic infiltrations was recorded in the present work. Leo et al.[19] showed that vitamin A caused necrosis in liver of rats and they added that the necrotic material may have a chemotactic effect leading to lymphocytic and macrophage infiltration of the portal tract.

Histochemical results revealed depletion in polysaccharides in liver of hypervitaminosis A mice. In agreement with this finding, Leo et al. [19] reported that the glycogen depletion noticed in liver cells particularly in the peripheral zone might be due to enhancing glycogenolysis after hypervitaminosis. Total proteins decreased in liver cells after treatment with hypervitaminosis A. De Duve et al [20] demonstrated that the addition of retinol to mitochondrial fraction of rat liver homogenate increases the rate of release of lysosomal Phosphatase into the incubation medium. Thus it is likely that the decrease in total proteins may be due to the release of lysosomal enzymes under the effect of hyper vitaminosis.

The regulation of programmed cell death or apoptosis, is an essential determinant of the cell life span. The mammalian Bcl-2 family of apoptosis-associated proteins consists of members that inhibit apoptosis (Bcl-2, Bclxl, Mcl-1, A1, etc) and others that induce apoptosis (Bax, Bak, Bad, Bcl-xs, Bik, etc), and the balance between pro-apoptotic and anti apoptotic members determines the fate of the cells in many systems [21]. The present study showed over expression of Bcl-2 in hepatic cells of hypervitaminosis A mice. Osman et al. [22] reported that Bcl-2 is elevated in chronic hepatitis. Rodriguez et al. [23] added that over-expression of either Bcl-2 or Bcl-xL in mouse liver protects hepatocytes from Fas-induced apoptosis and liver destruction in a dose-dependent manner.

Proliferating cell nuclear antigen (PCNA), an essential regulator of the cell cycle, is a 36kDa molecule which is highly conserved among species. It has been shown that PCNA serves as a co-factor for DNA polymerase delta in S-phase and is involved in DNA repair during DNA synthesis [24]. The temporal pattern of PCNA expression makes it a useful tool to study cell proliferation. It starts to accumulate in the G1 phase of the cell cycle, reaches the highest level during the S phase and decreases during the G2/M phase [25]. Expression of PCNA increased in hepatic cells of hypervitaminosis mice. Similarly, Lettinga et al. [26] detected an increase in the number of PCNA-positive sinusoidal cells in liver of retinol-treated rats in comparison with control livers.

The present results showed that ALT, AST and ALP elevated in sera of hypervitaminosis animals. Similarly, Choudhary and Swami [9] reported that chronic intake of vitamin A for twelve weeks or more is associated with increased risk for hepatotoxicity in Swiss albino mice and leads to elevation of ALT, AST, and ALP. Increased ALP has been reported in high dose of vitamin A-treated rats [26]. The liver functional transaminases (AST and ALT) and alkaline phosphatase (ALP) enzymes activity in serum are most frequently measured for diagnosis of liver diseases particularly infective hepatitis, alcoholic cirrhosis, biliary obstruction, toxic hepatitis and liver cancer [27,28]. These liver functional enzymes are not secreted into the blood, any elevation of their activities in blood is resulted from leakage of liver damage cells and from the disturbance and dysfunctions in liver functional enzymes [29,30]. It concluded that intake of vitamin A for three weeks is associated with increased risk hepatotoxicity in albino mice.

#### REFERENCES

- [1] Bendich A., Langseth L. (1989): Safety of vitamin A. *Am J Clin Nutr.* ; 49:358-371.
- [2] Batia W., Iris B., Nicholas O., Iris G., Zippi B., Ehad M., Anita J. and Ram R. (2010): vitamin A deficiency associated with enhanced Proliferation of Bile duct epithelial cells in the rat. *IMAJ.* 12 :82-86.
- [3] Rosewicz S, Stier U, Brembeck F, et al. Retinoids (1995): effect on growth, differentiation, and nuclear receptor expression in human pancreatic carcinoma cell lines. *Gastroenterology* 109: 1646-1660.
- [4] Russel, R.M.; Boyer, J.L.; Ba-gheri, S.A. and Hruban, Z. (1974): Hepatic injury from chronic hypervitaminosis A resulting in portal hypertension and ascites. *N. Engl. J.M.*, 291: 435-440.
- [5] Farrell, G.C.; Bathal, P.S. and Powell, L.W. (1977): Abnormal liver function in chronic hypervitaminosis A. *Dig. Dis.*, 22:724-728.
- [6] Rubin, E.; Florman, A.L.; Degnan, T. and Diaz, J. (1970) : Hepatic injury in chronic hypervitaminosis A. *Am J .Dis. Child.* , 119: 132-138.
- [7] Muentner, M.D.; Perry, H.O. and Ludwig, J. (1971) : Chronic vitamin A intoxication in adults . Hepatic neurologic and dermatologic complications. *Am. J. Med.*, 50:129-136.
- [8] Galal G.G., Ibrahim S.N., EL-Sady E.A. and Hamdi K.N. (1991): Hstological and Histochemical studies on the toxic effects of hypervitaminosis A on the liver and spleen of the albino rat. *Sci. Med. J. Cai. Med. Synd.* , 3(2):171-187.
- [9] Choudhary, P. R. , Kamlesh Kumar Swami, K.K. (2012): Hypervitaminosis -A is associated with increased risk of hepatotoxicity in swiss albino mice . *Int. J. Biol Med Res.*; 3(1): 1311-1314.
- [10] Lind T., Anders Sundqvist, Lijuan Hu., Gunnar Pejler, Goran Andersson, Annica Jacobson and Hakan

- Melhus (2013) : Vitamin A is a negative regulator of osteoblast mineralization. Vitamin A Inhibits Osteoblasts. *J. Cell. Physiol.* 114(2):doi:10.1371 .
- [11] Kiernan ,J. A.(1981).*Histological and Histochemical Methods, Theory and Practice*. Pergamon, Press. New York USA
- [12] Pearse, A. G. E. (1972). *Histochemistry, Theoretical and Applied*, 3rd.,vol. 2. Churchill Livingstone. London .
- [13] Reitman,S. and Frankel,S.( 1957).Alanin aminotransferase and Aspartate aminotransferase-Colorimetric methods of determination. *Amer. J. Clin. Path.*, 28:56.
- [14] Belfield, A. and Goldberg, D.M. (1971).Revised assay for serum phenyl phosphatase activity using 4-amino- antipyrine.*Enzyme*,12(5) :561-573.
- [15] Helsing E.Vitamins in: *Dukes MNG.(1988) Meyler's side effects of drugs*. 11th edition, Elsevier, Amsterdam.
- [16] David M., Hodak E. & Lowe NJ. (1988).Adverse effects of retinoids. *Med.Toxico.* 3: 273-288 .
- [17] McEvoy GK. Vitamin A. Drug information (19 88).American Hospital Formulary Service. The American Society of Hospital Pharmacists ,Montgomery Avenue, Bethesda, Maryland, USA. 2090-2093.
- [18] Lane BP. (1968): Hepatic microanatomy in hypervitaminosis A in man and rat. *Am J Pathol* 53:591–598.
- [19] Leo, M. A.; Arai, M.; Sato, M. and Lieber. C.S. (1982): Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 82: 194-205.
- [20] DeDuve, C., Wittiaux, R. and Wibo,M. *Biochem.Pharm.*, 9,97(1962).
- [21] Kroemer G. (1997). The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nat Med.* , 3: 614-620.
- [22] Osman HG., Gabr OM., Lotfy S., Gabr S.(2007) .Serum levels of Bcl-2 and cellular oxidative stress in patients with viral hepatitis. *IndianJ. Med. Microbiol.* , 25 (4): 323-329.
- [23] Rodriguez I., Matsuura K., Khatib K., Reed JC., Nagata S., Vassalli P.(1996). A bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destruction but not from rapid death induced by anti-Fas antibody injection. *J Exp Med.* , 183: 1031-1036.
- [24] Bravo R., Macdonald-Bravo H. (1987). Existence of two populations of cyclin proliferating cell nuclear antigen during the cell-cycle-association with DNA-replication sites. *J Cell Biol.* 105: 1549-54.
- [25] Tan CK, Sullivan K, Li XY, Tan EM., Downey KM., So AG. (1987). Autoantibody to the proliferating cell nuclear antigen neutralizes the activity of the auxiliary protein for DNA-polymerase delta. *Nucleic Acids Res.*, 15: 9299-308.
- [26] Lettinga, K.D. Gutte r, W., van Noorden, C.J.F., Schellens, J. P. M. and Frederiks ,W.N. 1996. Early effects of high dose of retinol (vitamin A) on the *in situ* cellular metabolism in rat liver. *Liver* 16:1-11.
- [27] Zaahkoug, S. A.M., Helal E. G. E. , Abd-Rabo T. E. I. and Rashed, 2000. Carbamate toxicity and protective effect of vit. A and vit. E on some biochemical aspects of male albino rats. *Egypt. J. Hosp. Med.*, 1: 60-77.
- [28] Abdel-Wahhab, M.A., Abdel-Galil M.M. , Hassan A.M. , Hassan N.H., Nada S.A., Saeed A. and El-Sayed M.M. ( 2007) *Zizyphus spina-christi* extract protects against aflatoxin B1-initiated hepatic carcinogenicity. *Afr. J Trad., CAM* 4(3): 248-256.
- [29] Kaneko, J., Haevey W. and Bruss, L. 1997. *Clinical Biochemistry of Domestic Animals*. 5th ed., Academic press, Inc. San Diego, London, New York.
- [30] Attia, A.M. and H.M. Nassr, 2009. Dimethoate-induced changes in biochemical parameters of experimental rat serum and its neutralization by black seed ( *Nigella Sativa* L. ) oil. *Slovak J. Anim. Sci.*, 42 (2) : 87-94.