

Histopathological Studies on the Modulating Effect of Carnosine in Phenytoin Induced Teratogenesis in Rat

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Abstract: In the present study administration of Phenytoin (PHT), in pregnant rats induced degenerative changes in the liver. These degenerative changes could be modulated by co administration of carnosine as antioxidant in two doses (10 and 5 mg). The result of both carnosine doses proved its protective role of carnosine against teratogenic effect of PHT on liver of rats in a dose dependent manner.

Key words: Carnosine, Phenytoin Teratogenesis, Liver.

INTRODUCTION

Many substances were used for the control of epilepsy, but the best and earliest hope was offered by the recognition of bromide's antiepileptic properties and by the synthesis of phenobarbital. The search for better antiepileptic drugs continued until the discovery of the anticonvulsant effect of phenytoin (PHT)^[13]. PHT became one of the most widely used anticonvulsants, since it could produce effective anticonvulsant action without significant sedation. PHT was also used as one of the most effective and widely used compounds for treating generalized tonic-clonic seizures^[17]. Based largely on their chemical structure, anticonvulsant drugs were classified into six groups namely, Barbiturates, hydantion, succinimide, oxazolinediones, benzodiazepines and miscellaneous group^[39].

The use of PHT as a treatment of epilepsy had to face considerable pharmacokinetic limitations that impaired the quality of patient's lives; it had teratogenic potential and a negative effect on cognitive functions on the offspring. Teratogenic effect of PHT associated with modulation of reactive-intermediates-scavenging enzyme activities, and provided further support for role of generation of reactive intermediates in PHT induced teratogenesis^[2,8].

Ruprah *et al.*^[38] found that PHT and other anticonvulsants were highly protein bound. The majority of PHT; 90% was bound to plasma proteins mostly albumin. On the contrary, in pregnant animals, the proportion of plasma unbound drug was increased. This was attributed to the decreasing albumin levels with the progress of pregnancy. Hence the free drug crossed plasma membranes, blood brain barrier and the

placenta barrier. In document, Yerby *et al.*^[60], reported that the total concentration of PHT decreased significantly during pregnancy, but the free drug concentration did not. This was primarily due to dynamic changes in plasma protein binding.

Speidel and Meadow^[52] and Olsen *et al.*^[35] recorded Microcephaly, cleft lip without cleft palate, congenital heart disease and risk for cancers of the liver and biliary apparatus were the commonest anomalies found.

PHT treatment was reported to cause renal, neural impairment and multiple biochemical hepatotoxic actions that were proportional to dose, age and treatment duration^[8,11,12].

Carnosine, β -alanyl histidine dipeptide, is a unique mammalian compound. It is present in tissues of many animals, Especially long-lived species. It is widely distributed in tissues; CNS, muscle, liver, lung and blood. Its highest concentration is found in skeletal muscular tissues^[35,49,66].

Carnosine was reported to have many biochemical functions. It acted as a cytosolic proton buffering agent^[10]. It had antioxidant property capable of inhibiting the harmful oxidizing actions of reactive oxygen species (ROS) on different macromolecules. It protected tissue lipids against malonaldehyde-induced peroxidations^[61]. Further, it also uniquely repaired accumulation of oxidized products derived from lipid components of biological membranes^[16]. The dipeptide protected proteins against glycosylation^[25]. Recently; it was found to improve oxidative phosphorylation and normalize energy charge in insulted tissue^[13,45]. It had anti-shock effect by decreasing histamine release from mast cells^[42]. Based on the broad bioactivities

carnosine, the present study was conducted to elucidate the effect of this compound on teratogenesis following administration of PHT.

Investigating many biochemical actions of carnosine, Soliman *et al.*^[44,46,47,48,49,51] recorded the dipeptide corrective actions in different hepatic insults; chronic infection, chronic ethanol intoxication and hypercholesterolemia. The degree of correction of liver pathology varied from moderate to complete cure.

MATERIAL AND METHODS

Material: In the present study a total of 60 adult albino rats were used, 45 were females and 15 were males. The adult rats weighted about 200 grams. Female rats were separated from males for two weeks before the beginning of the experiment. Every three female rats were kept overnight in a cage with a single male in a ratio of 3:1. Next morning, 42 of females that showed a positive vaginal smear were considered to be in the first day of pregnancy and were kept in separate cages. The animals were maintained all over the period of experiment on balanced diet containing bread, green vegetables and water. The pregnant female rats were divided into six groups each composed of 7 pregnant female rats: -

1. The first group after 3 week of gestation of female rats (21 day), livers of their offspring at birth were used as normal control specimens.
2. The second group pregnant female rats were treated orally with single daily dose of 0.9 mg/ rat of phenytoin throughout their three weeks of gestation (21 day).
3. The third group pregnant female rats were injected intraperitoneal with daily dose of 10mg/kg body weight of carnosine throughout their three weeks of gestation.
4. The fourth group pregnant female rats were injected intraperitoneal with daily dose of 5mg/kg body weight of carnosine throughout their three weeks of gestation.
5. The fifth group pregnant female rats were injected intraperitoneal with single daily dose of 10 mg/kg body weight of carnosine and phenytoin 0.9 mg /rat throughout their three weeks of gestation.
6. The sixth group pregnant female rats were injected intraperitoneal with single daily dose of 5 mg of carnosine/kg body weight and phenytoin 0.9 mg/ rat throughout their three weeks of gestation. The experiment conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC).

Methods:

Calculation of Phenytoin (Phentoin) Dose: Phenytoin presents in form of capsule (was obtained from Sigma Chemical Company) contained 50 mg of phenytoin. The human average therapeutic dose of phenytoin for an adult weighting 70 kg is 50 mg/day^[2,41]. The equivalent therapeutic dose for adult rat was calculated by using the formula of Paget and Barnes^[36] as follow:

$$50 \times 18 \\ \text{————} = 0.9 \text{ mg Phenytoin per rat} \\ 1000$$

Calculation of Carnosine Dose: Carnosine in form of white powder was purchased from Sigma chemical Co. The dipeptide carnosine dissolved in 1 cc of distilled water and given intraperitoneal in a dose of 10mg / kg body weight /day (in a ratio of 1: 10 w/v) for third group and 5mg /kg body weight / day (in a ratio of 1: 5 w/v) for fourth group^[42,43].

Drug Administration: Phenytoin is available form of capsule each capsule contained 50 mg which dissolved in 1cc of distilled water; each pregnant female rat treated orally with 0.9mg/rat / day. Carnosine had given intraperitoneal in a dose of 10 and 5mg / kg body weight /day.

Collection of the Specimens: The offspring of experimental animals were anaesthetized lightly by diethyl ether inhalation and the livers were collected at birth.

Preparation of Paraffin Sections: All the specimens were fixed by immersion in formal saline solution 10% for 3 days. The specimens were dehydrated in ascending grades of ethyl alcohol (70% - 90% - 100%) and cleared in benzene. The specimens were impregnated for three changes in paraffin and were finally embedded in paraffin wax. The paraffin blocks were cut by rotary microtome into serial transverse sections at 8 μ -thick.

Staining Techniques for Paraffin Sections: The following stains were used for this work:

1. Haematoxylin and eosin stain^[20] for the study of general structures.
2. Masson's trichrome stain^[20] for collagen fibers.
3. Periodic acid Schiff reaction (PAS)^[5] for the study of mucopolysaccharides and polysaccharides.

Morphometric Analysis:

1. Calculation the number and percentage of dead offspring in all groups.

2. The body weight and length of offspring were measured in all groups.
3. Morphometric measurements were performed by using (leica Qwin 500 c) image analyzer computer system (England). It was used to measure perimeter of the nucleus in the hepatocytes of liver. Also to detect the mean gray of PAS positive material in the hepatocytes .The image analyzer was first calibrated automatically to convert the measurement units (pixels) which produced by the image analyzer program into actual micrometer units.

I) Measuring Perimeter of the Nucleus: The perimeter nucleus of hepatocytes in the liver was measured in transverse sections of liver.

Four slides were taken from equidistant points along each serially sectioned specimen. Then the nucleus was measured in five randomly chosen high power fields x400 in a standard frame of cm² in each of these slides, Then the data obtained were subjected to statistical analysis to obtain the cumulative mean number of nucleus perimeter in the liver of three animals in each group (Fig. 1).

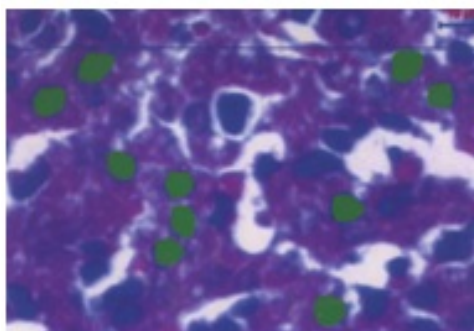


Fig. 1: A copy of display seen in the monitor's screen of the image analyzer for measuring of the nucleus of hepatocytes in the liver of control group in a high power field x400.

II) Morphometric Analysis of the Mean Gray of PAS Positive Material in the Hepatocytes of Liver:

It was done on sections stained with periodic acid Schiff reaction. The pink color of PAS stain was masked by gray binary color to determine the mean gray of PAS,positive material in the hepatocytes of liver (Figs 2, 3 and 4). Ten high power microscopic fields at x 200 magnification were randomly examined within a small measuring frame of known area in the hepatocytes of liver in a slide for each animal, so 30 readings were obtained for three animals in each group. Gray measurements gave information about the gray levels (i.e. the brightness) of all the pixels in the image.

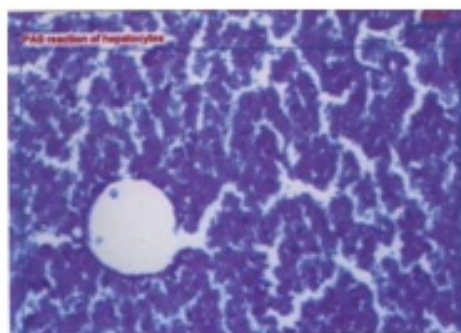


Fig. 2: The PAS reaction in the hepatocytes of the control group in a power field at magnification x 400.

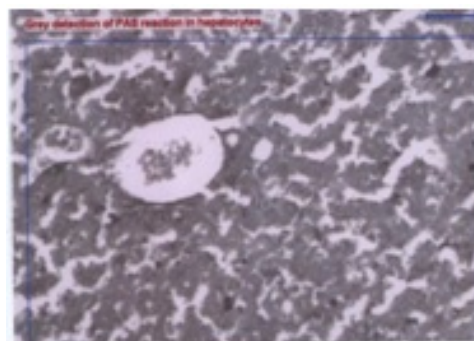


Fig. 3: The previous field after being transformed into a gray delineated image.

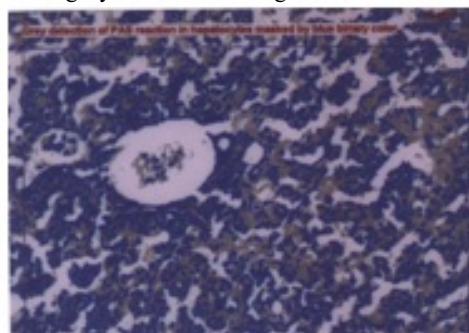


Fig. 4: A small known measuring frame at the hepatocytes of the pervious figure masked by a binary colour.

$$\text{Mean gray of PAS} = \frac{\text{Sum of gray positive material}}{\text{Number of pixels measured}}$$

Statistical Analysis: The results were collected, tabulated, statistically analyzed. ANOVA test (paired simple T test) was the statistical test of choice. It is a sort global test which was performed to test the significance of difference between means of more than

two groups^[3,2]. If the degree of probability (P) is more than 0.05 the results will be insignificant statistically. The (P) value is the degree of significant and expressed as 0.01, 0.001, 0.0001 ...etc

RESULTS AND DISCUSSION

Microanatomy of the Normal Neonatal Rat's Liver and Those Injected Intraperitoneal with 5 & 10 Mg Carnosine: Examination of transverse sections of the normal neonatal rat's livers and those injected different doses of carnosine showed non significant changes and demonstrated that a thin connective tissue capsule covered the liver which had flat cells with flat nuclei. The liver consisted of ill-defined hepatic lobules each lobules has a central vein and hepatic cords. The hepatic cords were irregularly radiating from the central vein towards the periphery and were separated by the blood sinusoids and the haemopoietic cells. The portal tracts and the central veins were clearly apparent (Figs. 5, 6&7). The hepatic cords appeared as irregular groups of hepatocytes. They were arranged as intercommunicating of cells plates irregularly radiating from the central vein. The hepatic cords were separated by primitive vascular blood sinusoids.

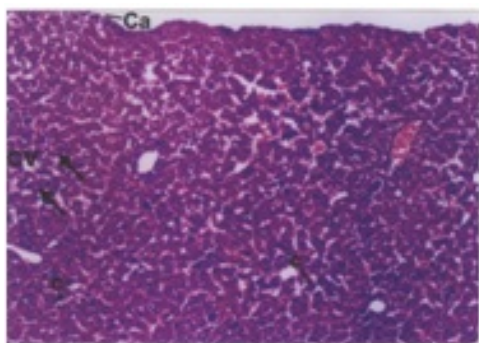


Fig. 5: A photomicrograph of a transverse section of the liver of control albino rat at birth showing that the liver is covered by a thin capsule (Ca) and is formed of ill-defined hepatic lobules. Notice the presence of small haemopoietic cells (c), central veins (CV) and hepatic cords (arrow), which were irregular radiating from central vein. (HX&E X100).

The Blood sinusoids were lined with flat endothelial cells and Von kupffer cells. The flat endothelial cells had dark flat nuclei while the Kupffer cells were large fusiform in shape with dark oval nuclei. The haemopoietic cells were found widely dispersed throughout the liver and some of them were arranged as small islets. They consisted of cells at various stages of erythropoiesis. The central vein was lined with a single layer of flat endothelial cells

containing flat nuclei and acidophilic cytoplasm (Fig. 7).

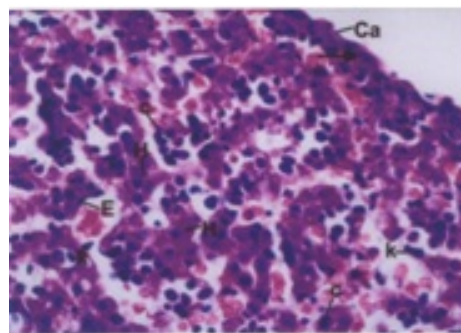


Fig. 6: A photomicrograph of a transverse section of the liver of control albino rat at birth showing that the liver is covered with fibrous capsule (Ca) which have a flat cell with eosinophilic cytoplasm (arrow). The hepatocytes (H) have a large vesicular nucleus and dark eosinophilic cytoplasm. Notice the blood sinusoids are lined with flat endothelial cells (E) and Von Kupffer cells (k). Notice also the presence of numerous haemopoietic cells (c) (HX&E X400).

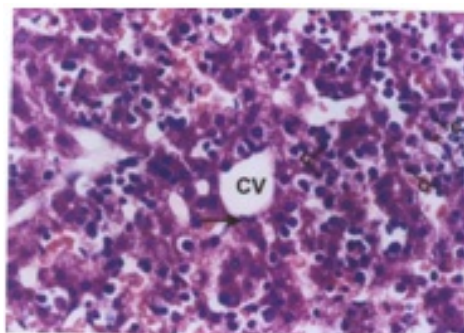


Fig. 7: A photomicrograph of a transverse section of the liver of control albino rat at birth showing that the hepatic cords are irregularly radiating from the central vein (CV). The central vein lined with flat endothelial cells. Notice the presence of numerous haemopoietic cells (c). (HX&E X400).

The hepatocytes were polyhedral in shape. They had eosinophilic granular cytoplasm and vesicular basophilic nuclei (Figs. 6, 7). In the different groups, the portal tract was formed of a branch of portal vein, a branch of bile duct and a branch of hepatic artery. The portal vein was lined with a simple squamous epithelium and the bile duct was lined with one layer of cubical epithelium while the hepatic artery was lined with a single layer of flattened endothelial cells resting on a basal lamina (Fig. 8). In addition, Masson's trichrome stain showed the normal distribution of the

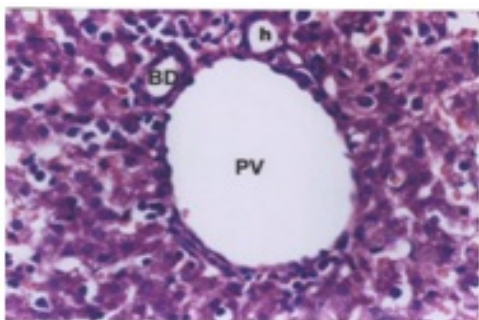


Fig. 8: A photomicrograph of a transverse section of the liver of control albino rat at birth showing that the portal tract is containing a branch of portal vein (PV) and a branch of bile duct (BD) and a branch of hepatic artery (h). (HX&E X400).

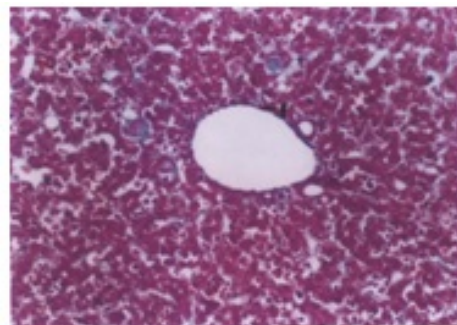


Fig. 10: A photomicrograph of a transverse section of the liver of control albino rat at the birth shows the normal distribution of the collagen fibers (f) around the portal tract. (Masson's trichrome X 200).

collagen fibers in the capsule and portal area (Figs. 9 and 10). Also in the above various demonstrated groups, periodic acid Schiff reaction showed the normal PAS positive material in the hepatocytes (Fig. 11).

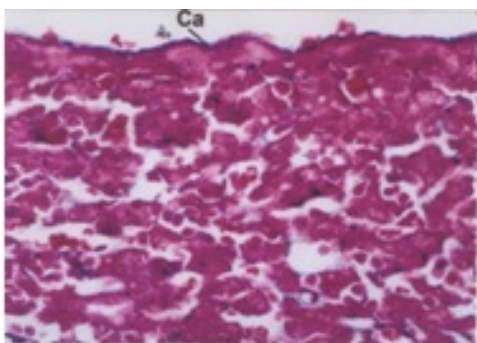


Fig. 9: A photomicrograph of a transverse section of the liver of control albino rat at the birth shows the normal distribution of the collagen fibers in the capsule (Ca) (Masson's trichrome X 400).

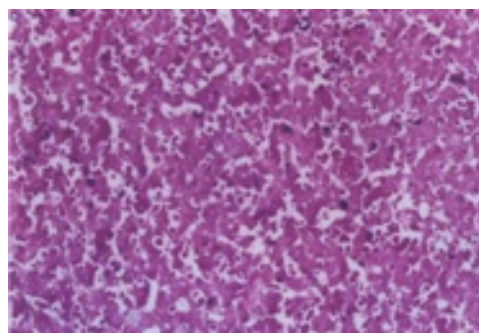


Fig. 11: A photomicrograph of a transverse section of the liver of control albino rat at the birth shows the normal distribution of the PAS positive material in the hepatocytes. (P A S X 200).

I- Microanatomy of the Neonatal Rat's Liver of Treated Mothers with Phenytoin:

The effect of maternal treatment with phenytoin on the neonatal rats varied from small sized completely distorted non viable one to other with manifest gross congenital malformation which appeared in clubbing of foot (Fig 12). The number and percentage of dead offspring about 10% in this group (Table 1). The body weight and length of neonatal rats in this group decreased than control one (Tables 2 & 3).

Examination of serial transverse sections of the neonatal rat's liver at birth from treated mothers showed marked degenerative changes. The hepatic capsule became separated from liver tissue. (Figs. 13, 14 & 16).



Fig. 12: A photograph showing a small sized completely distorted none viable albino rat at birth of treated mother with phenytoin (arrow). Other with normal size and has clubbing foot (head of arrow).

The hepatic cords became disorganized. Most of the hepatocytes showed variable signs of marked degeneration. Most of hepatocytes showed marked cytoplasmic vacuolations. Their nuclei appeared faint

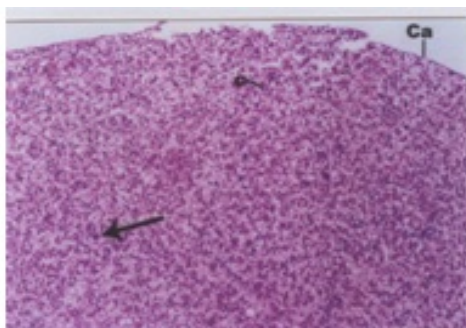


Fig. 13: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin showing that the liver covered by destructive capsule (Ca) and it is formed of ill –defined hepatic lobules. Notice presence of the haemopoietic cells (c) and giant cells (arrow). (HX&E X100).

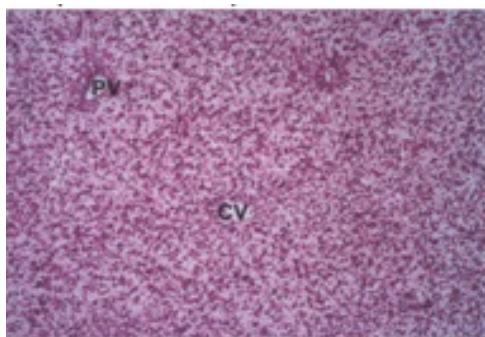


Fig. 14: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin showing that the central vein (CV) and portal vein (PV). (HX&E X100).

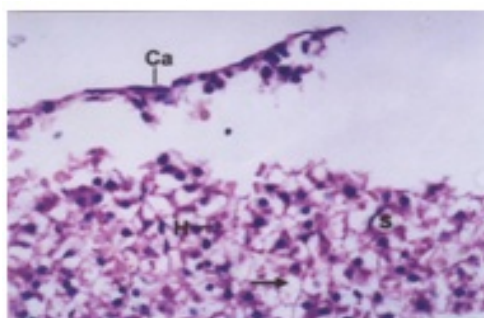


Fig. 15: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin showing that the capsule (Ca) is separated from the liver's tissue. Most of the hepatocytes (H) have vacuolated cytoplasm; most of their nucleus is lost or faint (arrow). Notice also the blood sinusoids (s) are hardly be detected. (HX&E X400).

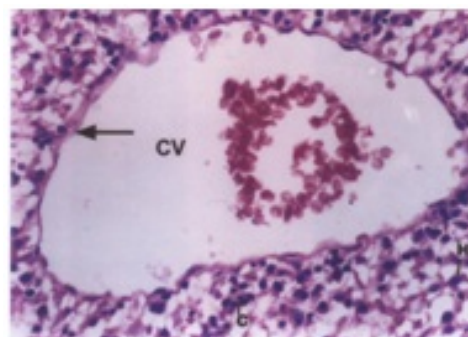


Fig. 16: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin showing that markedly vacuolated hepatocytes (H). Notice also the central vein is congested and dilated (CV) with disturbed endothelial lining (arrow). Notice presence of numerous haemopoietic cells ©. (HX&E X 400).

Table 1: Number and percentage of dead rats at birth in different studied groups.

Groups	Total number of offspring	Dead number of offspring	%
Control	70	--	--
PHT	50	5	10%
Cam 10 mg	70	-	-
Cam5 mg	70	-	-
PHT+cam10 mg	60	--	--
PHT+ cam 5mg	60	--	---

Table 2: Comparative studies of mean body weight of offspring at birth in different studied groups.

Groups	Mean + SD	Significant
Control	6.98+0.17	--
PHT	5.658+0.35	**0.001
Cam 10 mg	7.00+0.20	0.19
Cam 5mg	7.00+0.1 7	0.19
PHT+cam10mg	7.288+0.25	*0.04
PHT+ cam 5mg	7.38+0.27	0.059

Table 3: Comparative studies of mean body length of offspring at birth in different studied groups.

groups	Mean + SD	Significant
Control	5.20+0.380	--
PHT	4.20+0.353	**0.003
Cam10 mg	5.23+0.21	0.12
Cam 5 mg	5.21+0.11	0.11
PHT+cam10mg	4.88+0.389	*0.04
PHT+ cam 5mg	5.60+0.234	0.19

or small but some cells loss of their nuclei (Figs. 15&16). Giant cells formation could also be detected (Fig. 13). The blood sinusoids hardly detected. The haemopoietic cells appeared as the control one (Figs. 13, 15, 16, 5&7). In addition, the central vein appeared congested which had disturbed endothelial lining as compared with the control one (Figs. 16&7).The portal area showed congested and dilated portal vein which surrounded with infiltrated cells as compared with the

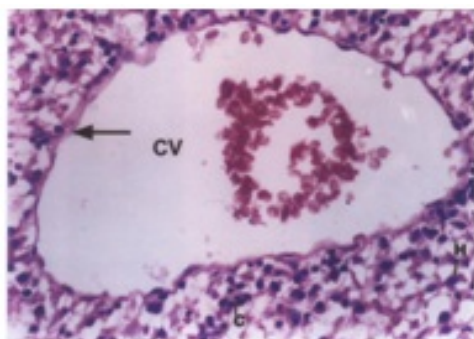


Fig. 17: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin shows the portal area. Notice dilation and congestion of portal vein (PV). (HX&E X 400).

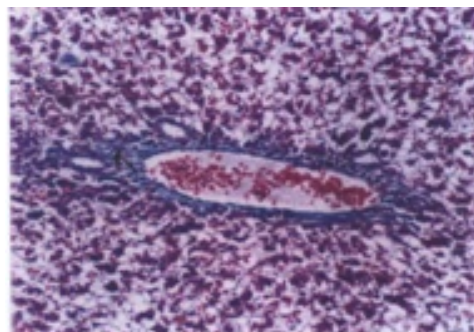


Fig. 19: A Photomicrograph of a transverse section of the liver of albino rat at birth of treated mothers with phenytoin shows a markedly increase in the collagen fibers (f) deposition around portal tract. (Masson's trichrome X 200).

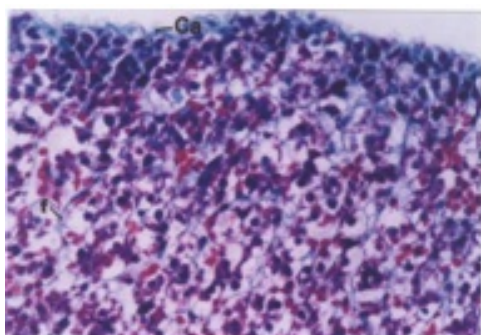


Fig. 18: A Photomicrograph of a transverse section of the liver of albino rat at birth of treated mothers with phenytoin shows markedly increase in the collagen fibers (f) deposition in the capsule (Ca) and in between the hepatocytes. (Masson's trichrome X 400)

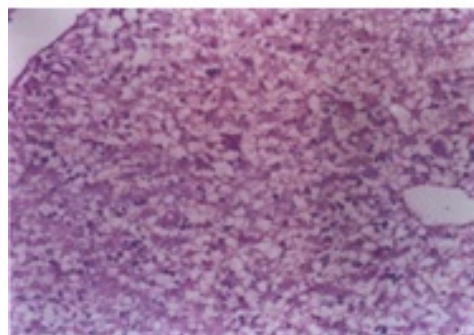


Fig. 20: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mothers with phenytoin shows a marked reduction in the P A S positive reaction of the hepatocytes. (P A S X200).

control one (Figs.17& 8). Masson's trichrome stain showed marked increased of the collagen fibers deposition in the capsule, in between the hepatocytes and around the portal tract as compared with the control one (Figs. 18,19,9&10). Periodic acid Schiff reaction showed that the PAS positive material was markedly reduced than that in the control one (Figs. 20&11).

III- Microanatomy of the Neonatal Rat's Liver of Treated Mothers with Phenytoin and Carnosine 10mg: Examination of transverse sections of the rat's liver at birth of treated mothers with phenytoin and carnosine 10 mg showed mild changes in the liver parenchyma which appeared more or less as the control. The liver covered with capsule which had flat cells with flat nuclei. The liver appeared consisted of ill-defined hepatic lobules. The hepatic cords were irregularly radiating from the central vein

towards the periphery and were separated by the blood sinusoids and the haemopoietic islets appeared clearly. The central veins and the portal tracts were clearly seen (Fig. 21).

The hepatic cords appeared as intercommunicating plates of cells which were irregularly radiating from the central vein which appeared more or less as the control (Figs. 22, 5, 6&7).

The hepatocytes were polyhedral in shape. They had light eosinophilic granular cytoplasm and large vesicular basophilic nuclei with one or more nucleoli. Some hepatocytes had small vacuolated areas in their cytoplasm (Figs 22 & 23). The blood sinusoids were lined with flat endothelial cells and large Von kupffer cells. The sinusoids appeared small in size compared to the control. The central vein was lined with a single layer of flat endothelial cells with flat nuclei and acidophilic cytoplasm, which appeared more or less as the control (Figs. 23, 5, 6 & 7). Although the

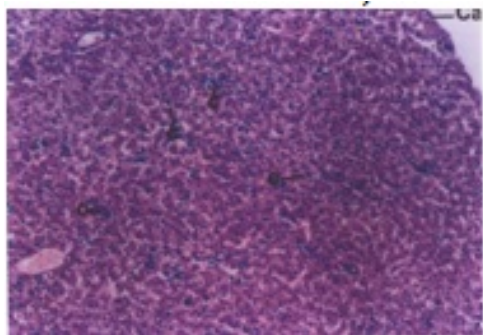


Fig. 21: A photomicrograph of a transverse section of the liver albino rat at birth of treated mother with phenytoin and carnosine 10 mg shows that the liver is formed of ill-defined hepatic lobules. Notice the presence of haemopoietic islets (c). Notice also liver is covered by a thin capsule (Ca). (HX&E X100).

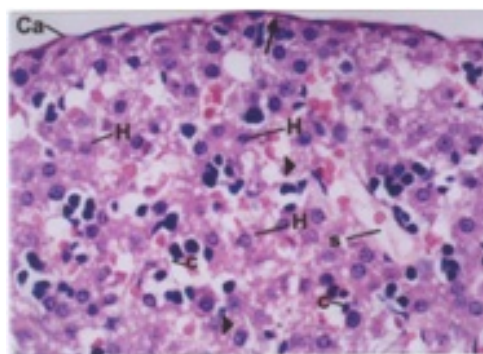


Fig. 22: A photomicrograph of a transverse section of the liver albino rat at birth of treated mother with phenytoin and carnosine 10 mg shows the liver covered with fibrous capsule (Ca) which have a flat cell with eosinophilic cytoplasm (arrow). The hepatocytes (H) have a large and vesicular nuclei and light eosinophilic cytoplasm, some of hepatocytes have small vacuolated areas in their cytoplasm (head of arrow). Notice the blood sinusoids are lined with flat endothelial cells (E) and Von Kupffer cells (k). Notice also the presence of large numerous haemopoietic cells (c). (HX&E X400)

haemopoietic cells were aggregated in large islets, which were evenly distributed throughout the liver which appeared more or less as the control (Figs 22, 23&8).

The portal tract consisted of a branch of portal vein which showed slightly congestion and a branch of bile duct, which appeared more or less as the control while the cells of bile duct have a large vesicular nucleus (Figs. 24&9).

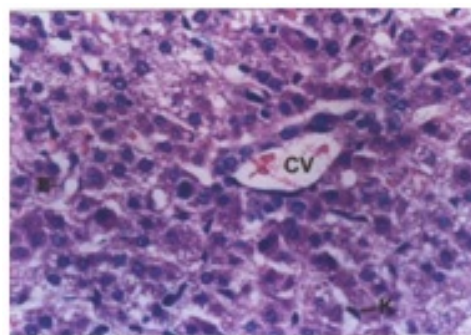


Fig. 23: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin and carnosine 10 mg shows that the central vein (CV) is surrounded by irregular hepatic cords. Notice that the blood sinusoids are easily detected and small in size and are lined with large Kupffer cells (K) and flat endothelial cells (E). (HX&E x400).

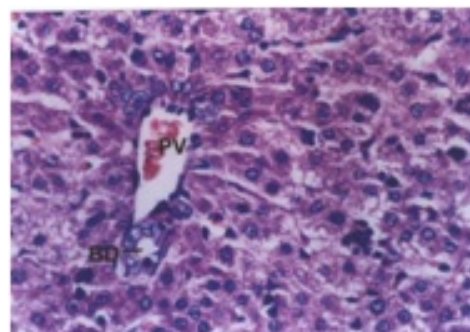


Fig. 24: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin and carnosine 10 mg showing that the portal tract contains a branch of portal vein which appear slightly congested (PV) and branches of bile duct (BD). Notice the cells of bile duct have a large vesicular nucleus. (HX&E x400).

Masson's trichrome stain showed the distribution of the collagen fibers in the capsule and around the portal tract, in between the hepatocytes and in the wall of sinusoids appeared mild increased than the control (Figs.25, 26, 9&10).

Periodic acid Schiff reaction showed marked increased distribution of PAS positive material in the hepatocytes as compared to the control (Figs. 27& 11).

IV- Microanatomy of the Neonatal Rat's Liver of Treated Mothers with Phenytoin and Carnosine 5 mg: Examination of transverse sections of the rat's liver at birth of treated mothers with phenytoin and

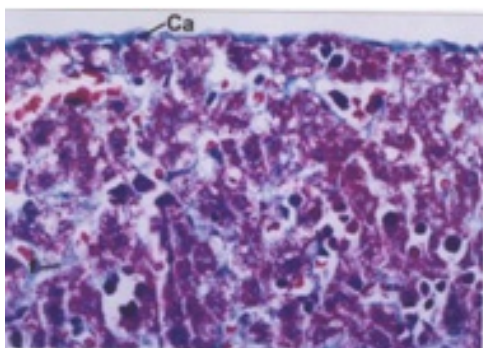


Fig. 25: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin and carnosine 10 mg shows the distribution of the collagen fibers (f) mild increase in the capsule (Ca), in between the hepatocytes and in the wall of (Masson's trichrome X 400).

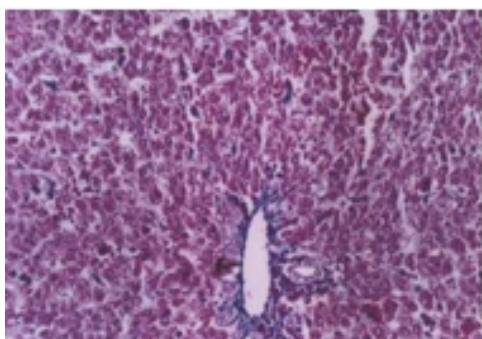


Fig. 26: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin and carnosine 10 mg shows mild increase of the collagen fibers (f) distribution around the portal tract. (Masson's trichrome x200).

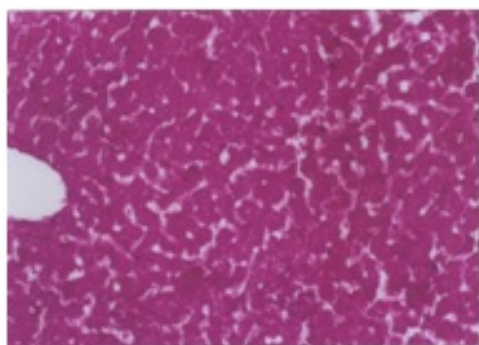


Fig. 27: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin and carnosine 10 mg shows increase PAS positive material in the hepatocytes. (PAS x200).

carnosine 5 mg showed mild changes in the liver parenchyma which appeared more or less as the control. The liver appeared consisted of ill-defined hepatic lobules covered by a thin capsule which had flat cells (Fig. 28). The hepatic cords were irregularly radiating from the central vein towards the periphery and were separated by the blood sinusoids and the haemopoietic islets appeared clearly. The central veins and the portal tracts were clearly seen (Fig. 29). The hepatic cords appeared as intercommunicating plates of cells which were still irregularly radiating from the central vein which more or less as the control in size (Figs. 30,5, 6&7). The hepatocytes were polyhedral in shape. They had distended with eosinophilic granular cytoplasm and large vesicular basophilic nuclei with one or more nucleoli (Figs. 29 & 30).

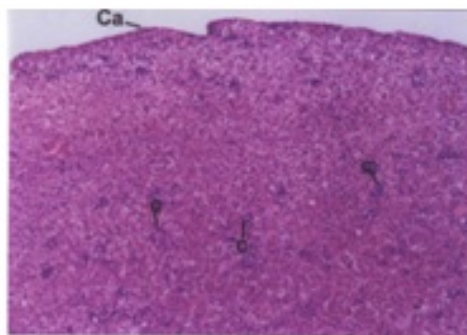


Fig. 28: A photomicrograph of a transverse section of the liver albino rat at birth of treated mother with phenytoin and carnosine 5 mg shows that the liver is formed of ill-defined hepatic lobules. Notice the presence of haemopoietic islets (c). Notice also liver is covered by a thin capsule (Ca). (HX&E X100)

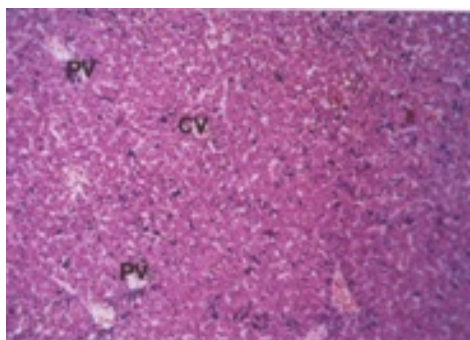


Fig. 29: A photomicrograph of a transverse section of the liver albino rat at birth of treated mother with phenytoin and carnosine 5 mg shows the central vein (CV) and portal vein are congested (PV). (HX&E X100)

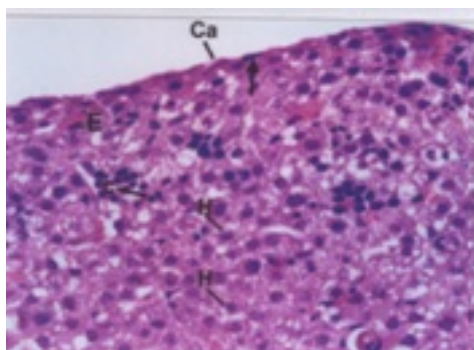


Fig. 30: A photomicrograph of a transverse section of the liver albino rat at birth of treated mother with phenytoin and carnosine 5 mg shows the liver covered with fibrous (Ca) which have a flat cell with eosinophilic cytoplasm (arrow). The hepatocytes (H) have a large and vesicular nucleus and eosinophilic cytoplasm. Notice the blood sinusoids are decrease in size and congested with blood, it lined with flat endothelial cells (E) and Von Kupffer cells (k). Notice also the presence of numerous haemopoietic cells (c). (HX&E X400).

The blood sinusoids were lined with flat endothelial cells and Von kupffer cells, which appeared congested and small in size. Although the haemopoietic cells were aggregated in large islets, which were evenly distributed throughout the liver (Figs. 28, 29& 30). The central vein was lined with a single layer of flat endothelial cells with flat nuclei and acidophilic cytoplasm, which appeared congested and slightly dilated compared to the control (Figs. 30, 5, 6&7).

The portal tract consisted of a branch of portal vein, which appeared congested, and branch of bile duct, while the cells of bile duct have a large vesicular nucleus compared to the control (Figs.31&8). Masson's trichrome stain showed the mild increased distribution of the collagen fibers in the capsule, in between hepatocytes and markedly increased around the portal tract compared to the control (Figs.32, 33, 9&10). Periodic acid Schiff reaction showed marked increased distribution of PAS positive material in the hepatocytes compared to the control (Figs.34&11).

Measuring Perimeter of the Nucleus of Hepatocyte:

Application of paired simple T test (Table 4) showed that there was a highly significant decrease ($p < 0.010$) of the perimeter of the nucleus in the hepatocytes of the liver in the group treated with PHT ($14.49 + 2.1$). On the other hand, There was no significant difference ($P > 0.05$) in the perimeter of the nucleus of hepatocytes in carnosine 10 mg with phenytoin treated group ($22.31 + 1.54$). Also There was no significant difference

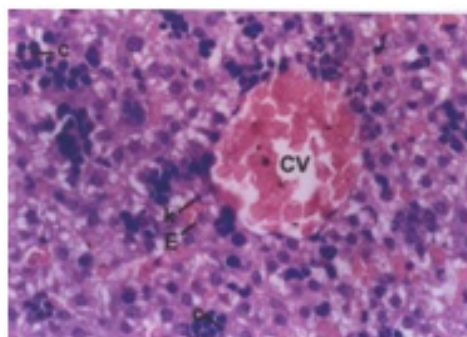


Fig. 31: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin and carnosine 5 mg shows the central vein (CV) is congested and slightly dilated which surrounded with irregular hepatic cords. Notice that the blood sinusoids are congested and are lined with Kupffer cells (K) and flat endothelial cells (E). Notice also the presence of large haemopoietic islets (c). (HX&E x400).

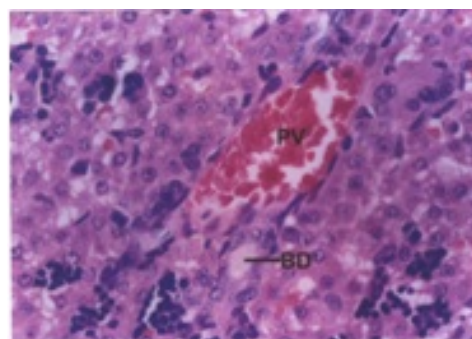


Fig. 32: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin and carnosine 5 showing that the portal tract contains a branch of portal vein (PV) which is congested and branches of bile duct (BD). Notice the cells of bile duct have a large vesicular nucleus. (HX&E x400).

($P > 0.05$) in the perimeter of hepatocytes in carnosine 5mg with phenytoin treated group ($22.23 + 1.24$) either compared with normal control ($21.66 + 1.34$) or those receiving 5 and 10 mg carnosine ($22.00 + 1.23$ and $22.19 + 1.34$ respectively) groups.

Measuring of Optical Density of Hepatocyte:

Application of paired simple T test (Table5) showed that there was a highly significant increase ($p < 0.010$) of the PAS optical density of hepatocyte treated with PHT ($6.690 + 1.023$). On the other hands, there was

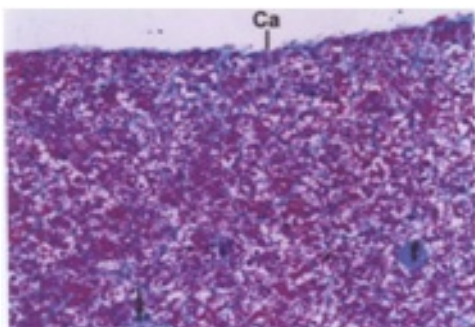


Fig. 33: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin and carnosine 5mg shows mild increase distribution of the collagen fibers(f) in the capsule (Ca) in between hepatocytes and in the wall of blood (Masson's trichrome X 200).

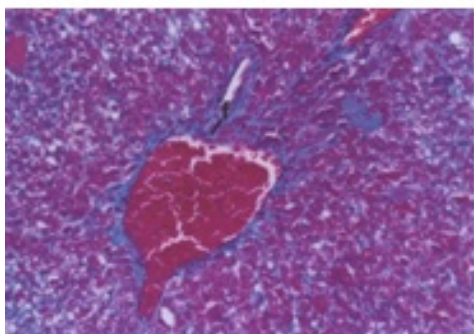


Fig. 34: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin and carnosine 5mg shows marked increase the distribution of the collagen fibers (f) around the portal tract. (Masson's trichrome x200).

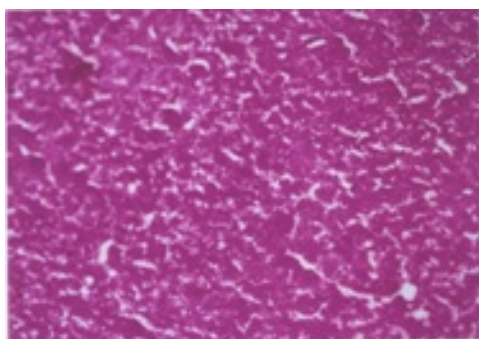


Fig. 35: A photomicrograph of a transverse section of the liver of albino rat at birth of treated mother with phenytoin and carnosine 5 mg shows increase PAS positive material in the hepatocytes. (PAS x200).

Table 4: Comparative studies of nuclear perimeter in hepatocyte in all studied groups.

Groups	Mean + SD	Significant
Control	21.66+1.134	--
PHT	14.49+2.117	0.000*
Cam10 mg	22.19+1.34	0.17
Cam 5 mg	22.00+1.23	0.16
PHT+cam10mg	23.31+1.514	0.29
PHT+ cam 5mg	22.23+1.249	0.17

Table 5: Comparative studies of PAS optical density of hepatocyte in different studied groups.

Groups	Mean + SD	Significant
Control	0.732.49+1.032	--
PHT	6.690+1.033	0.000**
Cam10 mg	0.733+0.89	0.11
Cam 5 mg	0.700+0.87	0.12
PHT+cam10mg	0.790+1.494	0.000**
PHT+ cam 5mg	0.800+1.470	0.000**

T. test = comparison between each treated group & control group; P > 0.05 = insignificant, P < 0.05 = significant (*), P < 0.01 = highly significant (**)

significant decrease ($p < 0.010$) in the optical density of hepatocytes in carnosine 5 and 10 mg with phenytoin treated groups (0.800+1.470 and 0.790 +1.49 respectively) either compared with normal control group (0.732 +1.03) or 5 and 10 mg carnosine receiving groups (0.700+0.87 and 0.733+0.89 respectively).

Discussion: The present study of PHT treated group revealed teratogenic effect in the neonates' rats of treated mothers. The teratogenicity varied from complete resorption (undifferentiated tissue mass), incomplete resorption (unviable head and body with no limbs), to clubbing of one foot. A significant reduction in neonatal weight and length was presented in treated group with PHT. This teratogenicity could fairly be attributed to PHT affecting the neonatal metabolism causing increased ROS as proved by Liu -ling and Wells-Peter^[31], Wells *et al*^[57] and Winn & Wells^[58] they found PHT might initiate oxidative damage to lipids and proteins in embryonic tissues of mice. Protein oxidation was significantly increased at 3 h after PHT treatment in maternal hepatic and embryonic microsomes. PHT caused cellular damage and increased protein degradation in the muscle.

Examination of liver serial transverse sections of neonatal rats' from these PHT- treated mothers at the birth, showed marked degenerative changes. The hepatic cords as well as the vascular architecture became disorganized.

The hepatocyte nuclei were small, morphometric and statistical studies confirmed highly significant decrease of the nuclei perimeter that could be attributed to PHT initiating DNA oxidation. Kim *et al*^[27] and Wells *et al*^[57] reported that PHT enhanced micro nuclear formation in skin fibroblasts and ROS covalently bind to or oxidize DNA of embryos of mice

and rats and these micronuclei were a genotoxic sign. Most of hepatocytes showed marked cytoplasmic vacuolations. These vacuolations caused by PHT treatment could be attributed to permeability disorders of the cell membrane^[9]. The author stated that vacuolations were one of the structural indications of permeability disorders of the membranes causing enhanced transport of water and electrolytes into the cell. Halliwell and Chirico^[23] reported that the permeability disorder was attributed to many cellular membrane insults caused by excess ROS-mediated formation of lipid peroxides, which ultimately generated self-sustaining lipid peroxidation. Cellular macromolecules; lipids, polysaccharides, proteins and DNA, are the potential targets in oxidative stress. Abdel Hamid^[1] stated that by light microscopic examination, the fetal liver sections of the mice of PHT-treated mothers showed generalized vacuolated distorted hepatocytes. Larrey and Pageaux^[30] recorded that the hepatotoxicity of PHT causing cytoplasmic vacuolations could be referred to the drug itself or its chemically reactive metabolites that could bind covalently to hepatic macromolecules leading to idiosyncratic, toxic or immune allergic hepatitis.

Testing cytoplasmic glycogen by PAS stain revealed its remarkable depletion. Further confirmation by morphometric and statistical studies showed highly significant decrease of the mean specific color of PAS positive material. In document, Abdel Hamid^[1] showed marked decrease of PAS positive material in liver hepatocytes in fetal mice of mothers treated with PHT. The glycogen depletion could fairly be explained by PHT therapy causing low blood glucose which instantaneously leads to decreased hepatic glycogen. In assurance, PHT was reported to induce hypoglycemia in patients treated for epilepsy^[18,34,55].

The distortion of vascular architecture in PHT liver showed marked dilatation and congestion of both central and portal veins. In addition, the central vein showed disturbed endothelial lining while the portal area was surrounded with infiltrating cells and moderate fibrosis. These observations of the dilatation of both veins could be supported and explained by Ober *et al.*^[33] and Walter and Isreal^[56] stating that dilatation of the two vein systems was due to PHT increasing prostaglandin synthesis that induced smooth muscle relaxation and consequent vasodilatation. This relaxation of the smooth muscle of hepatic veins was also recorded by Bacon and Britton^[3] and Poli and Parolia^[37] reported that formation of hydroxyl radicals and other highly reactive oxidizing molecules in biological system led to lipid peroxidation, that caused damage of proteins and nucleic acids. The end results of these reactions increased the collagen and ground substance formation. The congestion of dilated blood

vessels were explained by increased vascular permeability which leads to loss of fluid from the blood, so the vessels got engorged with RBCs. However, Kumar *et al.*^[29] contradicted this opinion of RBCs involvement. They stated a leukocyte dependent mechanism for this vascular congestion and endothelial injury was found as a consequence of leukocyte accumulation during a PHT inflammatory response. Leukocytes could release excess ROS and proteolytic enzymes which then caused endothelial injury or detachment. In contrast, Woolf^[59] referred the congestion of both vessels to the increased blood amount than normal either to an inflammatory increased blood flow causing vascular dilation or to decreased out flow due to obstruction.

Microscopic examination of transverse hepatic sections of the neonatal rats at birth showed marked improvement following treatment of PHT group with 10mg carnosine compared with PHT alone treated group. The architecture of both hepatic cords and the vascular elements appeared normal. The hepatocytes were polyhedral in shape and engorged with eosinophilic granular cytoplasm. The nuclei were considerably large presenting normal vesicular basophilic appearance and having one or more nucleoli. These histological findings were confirmed with both morphometric and statistical studies which showed significant increase of the perimeter of hepatocytes nuclei. These nuclear results could be explained by increased mitotic activity in hepatocytes caused by the antioxidant effect of carnosine. Silaeva *et al.*^[41] recorded increased both nuclear diameter and mitotic activities of cultured hepatocytes by carnosine. Badway^[4] histologically and morphometrically studied the effect of the PHT on mice liver in doses 200 and 400 mg of PHT/kg. Both doses produced an apparent increase in the number of binucleated hepatocytes. Additional enlargement of the hepatocytes was produced by doubling the dose (400mg/kg).

Instead of the marked cytoplasmic vacuolations in PHT animals, the prophylactic carnosine conjoined PHT showed scanty vacuoles of extremely minute size. Further there was cytoplasmic engorgement. The cytoplasmic engorgement by carnosine was proved by PAS stain to be due to excess glycogen instead of the depletion state in PHT animals. Further morphometric and statistical studies showed highly significant increase of the mean PAS positive color in the hepatocytes denoting increased glycogen storage. Soliman *et al.*^[43] and Fouad *et al.*^[61] further proved that carnosine, normalized the low blood glucose level in chronic infection.

The corrective action of conjoined carnosine-PHT treatment on vascular architecture involved the 3 vascular elements distorted in PHT group. The

sinusoidal blood spaces became narrower than control that could be due to cytoplasmic engorgement with glycogen. The sinusoids were lined with flat endothelial cells and large Von Kupffer cells. This result could be explained by increased phagocytic and secretory activities of Kupffer cells. Silaeva *et al.*^[41] and Shen *et al.*^[63] supported this present finding by stating that carnosine activated the phagocytic and secretory activities of Kupffer cells and increased mitotic activity in hepatocytes. They explained this pronounced effect of carnosine by its ability to bind to macrophages and to stimulate their synthetic and secretory abilities leading to liberation of more cellular growth and proliferation factors. The interaction of these factors with lymphocytes strongly activates the natural systems of body immune resistance.

The size of both central and portal veins in 10 mg carnosine conjoined with PHT group showed much decrease than in PHT group. The central vein was also normally lined; with a single layer of flat endothelial cells having flat nuclei and acidophilic cytoplasm. The portal tract was found normal, consisting of a branch of portal vein and a branch of bile duct. The cells of bile duct have vesicular nuclei larger than normal. The haemopoietic cells showed normal aggregations in large islets, evenly distributed throughout the liver.

This improvement with carnosine co-administered with PHT might be attributed to the antioxidant effect of carnosine and its ability to prevent membrane lipid and other cellular macromolecules insults by ROS (peroxidation and degradation) thus, stabilizing cell membranes and membrane bound-enzymes^[14,64]. In support many researches proved the beneficial action of carnosine not only in alleviating oxidative toxicity but also improving levels of other antioxidant factors^[21,22,47,48,65].

The distribution of the collagen fibers around the portal tract and in the capsule appeared slightly increased as shown by Masson's trichrome stain. These findings were similar to the results of Soliman *et al.*^[43] and Talbot *et al.*^[54] showing improvement of portal tract fibrosis in rats treated with carnosine after infestation with *Shistosoma mansoni* and on fetal fibroblast (BFF) cell lines.

Regarding to 5 mg carnosine conjoined with PHT, microscopic examination of transverse hepatic sections of neonatal rats showed marked improvement with 5 mg carnosine treatment similar to those of carnosine big dose (10 mg). However, few histological deviations from those of the big carnosine dose were found. There was more glycogen engorgement as well as more vascular congestion. The amount of blood cells in hepatic vessels was comparable to that present in PHT sections. Considering the size variation of vessels in either group, congestion was more evident and this

small dose did not oppose PHT. Thus, it was only the big carnosine dose that was capable of nullifying PHT toxicity on the vessels. These data indicating dose dependent action of carnosine on vessels. In supporting, Cho- Ch *et al.*^[15], was used 3, 10 & 30 mg/ kg carnosine in treating stress-induced gastric hemorrhagic lesions in rats. The author recorded the dose dependent effect of carnosine.

Carnosine was proved to be a strong antioxidant presenting a unique double mechanism. It was not only able to scavenge excess ROS liberated, but also to uniquely reversing peroxidized lipids to its native form^[16]. In addition, the antioxidants action of carnosine was reported to prevent glycation that disturbed protein structure^[31] and to stabilize membrane fluidity^[19]. Kucuk *et al.*^[28] recorded that antioxidant administration of carnosine decreased oxidative damage caused by ROS liberation. All these actions of carnosine could maintain normal biological activity of cells and consequently, participate in carnosine prophylactic and curative actions.

From the results of the present study it can be concluded that administration of PHT in pregnant rats induced degenerative changes in the liver. These degenerative changes could be modulated by co-administration of carnosine as antioxidant. These results of both carnosine doses proved the protective role of carnosine against teratogenic effect of PHT on liver of rat in a dose dependent manner. Thus, collectively the conjoined treatment of the big dose carnosine is better used for conjunction with PHT than the small dose. Further this treatment should be investigated in more variable doses of carnosine as a safe therapy to epileptic patients avoiding much of PHT disadvantageous toxicity. So, in spite of the species differences, PHT must be taken with an antioxidant as carnosine for mothers suffering from epilepsy.

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