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EFFECT OF SODIUM NITRATES AND NITRITES ON THE LIVER MORPHOLOGY IN BROILER CHICKS

(With 13 figures)

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تأثيرنترات ونيتريت الصوديوم على مورفولوجية كبد كتاكيت إنتاج اللحم

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الهدف من إجراء هذا البحث هو دراسة تأثير زيادة نسبة نترات ونيتريت الصوديوم فى ماء الشرب(٢٥% فوق المعدل المسموح به) على مورفولوجية كبد كتاكيت إنتاج اللحم. استخدم فى هذا البحث مائة وثمانون كتكوتا من عمر يوم واحد حتى وصلت إلى عمر ستة أسابيع. تم تقسيم الكتاكيت إلى ثلاث مجموعات، مجموعة ضابطة ومجموعة معاملة بنترات الصوديوم والثالثة معاملة بنيتريت الصوديوم. فى نهاية الأسبوع الثاني والرابع والسادس أخذت عينات من كبد أربعة كتاكيت من داخل كل مجموعة وبطريقة عشوائية، حيث تم تمرير ها للفص المجهري الضوئي والإلكتروني النافذ. يتكون كبد الكتاكيت من صفائح من الخلايا الكبدية، كل منها مكون من صفين من الخلايا. ولقد بينت الدراسة وجود حبيبات دهنية وحويصلات متفاوتة الحجم فى كبد المجموعات المعاملة بالنترات والنيتريت والتى لم يتم مشاهدتها فى المجموعة الضابطة. هذا وقد وصلت هذه الحبيبات والحويصلات إلى أعلى نسبة لها (٦,٤% من جملة النسيج الكبدى) بعد مرور أربعة أسابيع فى المجموعة المعاملة بالنيتريت ثم إنخفضت بعد ذلك. أما فى المجموعة المعاملة بالنترات فوصلت نسبة هذه الحبيبات والحويصلات إلى ٢,٢٨% فقط بعد مرور ستة أسابيع على بداية التجربة. لوحظ أيضا وجود عدد من الخلايا الكبدية فى درجات متفاوتة من التحلل حيث شوهد تغير فى شكل النواة وتراكم بعض العضيات الخلوية المتحللة. يتضح من هذا أن كبد كتاكيت إنتاج اللحم قد تأثر بزيادة نترات ونيتريت الصوديوم بنسبة ٢٥% فوق النسبه المسموح بها فى ماء الشرب وكان تأثير النيتريت سريعا وأكثر وضوحا من النترات.

SUMMARY

The effects of a moderate increase in sodium nitrate and nitrite in drinking water (25% over the permissible level) on the liver morphology in newly hatched chicks were studied. A total of 180 one-day old newly hatched commercial meat-type unsexed chicks were used in a 6 weeks trail. Chicks were allocated to 3 treatments (control, sodium nitrate- and nitrite-treated groups) with three replicates (20 chicks each). At the end of the experiment, liver samples were collected from 4 chicks from each group and processed for both light and electron microscopy. The parenchyma of the liver of control chicks was generally composed of hexagonal shaped or polygonal hepatocytes arranged in hepatic laminae, each formed of two rows. Light and electron microscopy ascertained the accumulation of lipid droplets and/or globules as well as variably sized vacuoles within the cytoplasm of hepatocytes in treated groups, that were not detected in control group. Degenerative changes in many liver cells were recorded. Additionally and on the ultrastructural level, deformity of the nuclear membrane and presence of degenerated cell organelles were observed. The morphometrical data showed that the area percent of lipid inclusions and vacuoles reached their highest value (6.4% of the liver parenchyma) after 4 weeks and declined afterwards in nitrite-treated group. On the other hand, the area percent of these inclusions and vacuoles were ill-measurable until the end of the fourth week in nitrate-treated group and became 3.28% of the liver parenchyma at the end of the experiment. In conclusion, small doses of nitrite and nitrate in drinking water (25% over the permissible level in water) leaded to moderate alteration in hepatocytes morphology that appeared quickly in nitrite treated group and decreased by prolonged treatment. Nitrate treatment resulted in mild effect on the liver parenchyma that started later than that observed in nitrite treatment.

Key Words: *Nitrate – Nitrite – Morphology – Liver – Chicks.*

INTRODUCTION

It should be noted that farm animals poisoning by nitrates and nitrites is fairly frequent today, due to the increased use of nitrogenous fertilizers. Large numbers of ruminants, pigs and poultry may be affected (Bartik and Piskac, 1981). Martins & Probst (1991) stated that

the water of river Nile in Egypt has the highest dissolved salt content compared with that of the major African rivers. This was partially explained as a result of irrigation system and industrial activities.

Several investigations, concerning the toxic effects of nitrites and nitrates on animal and human life, have been reported by Sell and Roberts (1963), Sunde (1964), Marrett & Sunde (1968), Osweiller et al. (1985) and Walker (1990). Additionally, excessive dietary nitrate and nitrite lead to depressing the rate of growth in swine (Tollet et al., 1960), rat (Smith et al., 1961) and cattle (Weichenthal et al., 1963). Atef et al. (1991) added that the nitrates and nitrites were environmental pollutants for feed and water and might contribute to the etiology of liver and kidney diseases and problems related to failure of the immunity in domestic fowl.

Although the nitrates and nitrites toxicity in different mammalian species is well documented, little is known about their effects in domestic fowls. Previous investigations in domestic fowls and other avian species were mainly concerned with the effects of nitrates and nitrites on growth and survival (Adams et al., 1966 & 1969; Arends et al., 1967; Marrett & Sunde, 1968 and Adams, 1974). In addition, one part per million (ppm) is well bellow the level which would have any effect on human health, the permissible level of nitrate was reported to be 40 ppm. Therefore, this investigation was performed to describe the effects of sodium nitrates and nitrites administration continuously in drinking water at a moderate dose on the hepatocytes in newly hatched meat-type chicks.

MATERIAL and METHODS

A total of 180 one-day old newly hatched commercial meat-type unsexed chicks were used in a 6 weeks trail. At one-day old, chicks were wing-banded and randomly allocated to 3 treatments (control, nitrate- and nitrite-treated groups) with three replicate (20 chicks each). Birds were housed in pen floor brooders with heat controlled environment and a constant illumination (23L:1D). A basal corn-soybean diet that meets all nutrients requirement, as recommended by the NRC (1994), were fed to chicks for ad libitum consumption in mash form, and birds had free access to water. The experiment was carried out in the Farm of Poultry Research Unit of Assiut University.

The source of nitrates and nitrites, used in this experiment, was reagent grade sodium nitrate and nitrite (Fisher Scientific) dissolved in water to reach final concentration of 50 ppm of either nitrates or nitrites for treated groups (25% over the permissible level). The control group received tap water with no supplementation of nitrate or nitrite salts. The tap water was analyzed for total concentration of NO_3 and NO_2 at the beginning and at the end of the experiment. Values obtained for NO_3 and NO_2 were 6.2 ± 1.73 and 0.0 ppm respectively.

For light and electron microscopical examinations, 4 chicks from each group (control, nitrate and nitrite groups) at 2 weeks, 4 weeks and 6 weeks of age were killed under anaethesa. Samples of the liver were fixed in solution formed of paraformaldehyde – gluteraldehyde at pH 7.3 as described by Karnoveskey (1965). After fixation, the samples were washed by 0.1M caccodylate buffer (pH 7.3) followd by immersion in 1% osmiun tetraoxide for 2 hours. After osmication, the samples were washed carefully in 0.1 M caccodylate buffer (pH 7.3) and dehydrated in ascending grades of ethanol then washed by propylene oxide. The samples were embedded in epon-araldite mixture. Semithin sections were cut and stained with toluidine blue for light microscopical examination. Ultrathin sections were cut and mounted on copper nets. After staining the ultrathin section with uranyl acetate and lead citrate (Reynolds, 1967), examination have been performed by transmission electron microscope in the Unit of Electron Microscope in Assiut University. The morphometrical measurments were carried out on semithin section using Leica image analysis system (Leica, Germany).

RESULTS

Light microscopical observations:

In control group (Fig. 1), the semithin sections of parenchyma of the liver showed nearly the same morphological features at two, four and sixth weeks of age. The parenchyma was generally composed of hexagonal shaped or polygonal hepatocytes arranged in hepatic laminae, each one formed of two rows. These laminae appeared irregular or zigzag-like in shape and separated from each other by irregular blood sinusoids of variable sizes, which were continuous with a central vein. The hepatocytes were usually appeared darkly basophilic by toluidine blue. Their nuclei were large in size containing one or two prominent nucleoli.

In treated groups, after two weeks from the beginning of the experiment, the liver parenchyma of sodium nitrate–treated group showed no remarkable morphological differences from that of control group. The sodium nitrite-treated chicks revealed the presence of very small lipid droplets that appeared as small vacuoles within the cytoplasm of hepatocytes (Fig. 2).

At the end of the 4th week, in sodium nitrite-treated group, the cytoplasm of most hepatic cells appeared vacuolated (Fig. 3). Within the hepatocyte-cytoplasm numerous rounded clear vacuoles of variable sizes were observed. In some hepatocytes, vacuoles appeared in close association and start to coalesce with each other (Fig. 4). In sodium nitrate- treatred group, these cytoplasmic inclusions have been also observed, but they were relatively few in number and smaller in size when compared with that treated with sodium nitrite.

At the end of the 6th week of the experiment, the morphology of the hepatocytes in sodium nitrite-treated group resembled nearly the picture described at the end of the 4th week with moderate reduction in the cytoplasmic vacuoles. In the sodium nitrate-treated group, the hepatocytes demonstrated remarkable increase in the number and size of the cytoplasmic vacuoles (Fig. 5). Intracellular spaces with variable sizes and irregular outlines were seen in some areas of the liver parenchyma in the last treated group (Fig. 6).

In addition, the liver parenchyma of chicks in both treated groups displayed the presence of lightly stained hepatocytes at the end of the fourth and sixth weeks (Fig. 5). These light cells appeared at different stages of degeneration. Their nuclei demonstrated peripheral grouping of chromatin. They showed frequently shrinked cytoplasm leaving clear surrounding spaces.

Morphometrically, although small vacuoles were seen in the liver parenchyma in the sodium nitrite-treated group after two weeks, their area percent was difficult to be measured. At the end of the fourth week, the area percent of cytoplasmic vacuoles was about 6.40% of the total surface area of the liver parenchyma in sodium nitrite - treated group. At the same time, the area percent of these cytoplasmic inclusions was still ill-measurable in sodium nitrate treated group. In 6 weeks old chicks, the area percent of the intracellular inclusions were remarkably increased in the nitrate treated group. It reached about 3.28% of the total surface area of the liver parenchyma, while the area percent of these vacuoles decreased in nitrite treated group and became about 2.41% of the surface area of the liver parenchyma.

Electron microscopical observations:

In control group (Fig. 7), the ultrastructural characteristic features of the hepatocytes along the period of experiment was mainly represented by the presence of a large number of mitochondria in close association with short lamellae of rough ER occupying most of the cytoplasm. The mitochondria appeared frequently large in size and oval or round in shape with lightly stained matrix and less distinct mitochondrial cristae. The hepatocytes showed also abundant free ribosomes, while the Golgi-apparatus was ill-distinct. Few fine vesicles with clear content can be seen between the cytoplasmic cell organelles. In the center of the cell, a large rounded nucleus was located. The nucleus contained few amount of heterochrmatin in the form of small clumps or fine granular substance. They attached to the nuclear membrane or scattered in the karyolymph.

The electron microscopical examination of the sodium nitrate- and sodium nitrite- treated groups displayed clearly the steps of development of the intracellular vacuoles in hepatocytes.

At the end of the 2nd week, the hepatocytes in chicks treated with sodium nitrites displayed numerous fine micro-vesicles within the short lamellae of rough ER, that found in close association with the mitochondria or scattered freely within the cytoplasm. These vesicles were also found coalescent with each other to form larger vesicles or vacuoles (Fig. 8). They contained electron lucent substance and sometimes filled with less electron dense or milky colored substance. Small fat droplets appeared also in close association with the rough ER. Accumulated fatty substance within the hepatocytes can be seen as a large fat globule with irregular borders (Fig. 8). At the end of the 4th week, signs of cell organelles degeneration within the hepatocytes were also observed. Autophagosomes appeared as vacuoles of different sizes containing cell organelles in variable degrees of autolysis (Fig. 9). In addition, other hepatocytes, containing variable number of round vacuoles with less electron dense substance, were demonstrated (Fig. 10).

At the end of the 6th week, these large vacuoles were frequently observed (Fig. 11). They appeared coalescing or partially fusing to form large irregular spaces that occupied supranuclear or apicolateral postion leaving small area for other cell organelles (Fig.12). In addition, hepatocytes showing signs of degeneration were demonstrated (Fig. 13). Their heterochromatin was grouped under the nuclear envelope. The perinuclear space was swollen forming a large clear vesicle. The outer

nuclear membrane appeared irregular in shape. Few small vesicles appeared in close contact with the outer nuclear membrane. Irregular clear spaces and collapsed or condensed cytoplasmic material could be identified (Fig. 13).

DISCUSSION

The present study indicated clearly that the liver in chicks is affected by pollution of drinking water with 25% over the permissible level of sodium nitrates and nitrites. These findings confirm the previous investigations dealing with the effect of nitrates and nitrites on the liver in some mammals (Shirley, 1975; Zimmerman, 1990 and Atef et al., 1991).

The current investigation revealed the presence of variable-sized vacuoles with regular outlines in the sodium nitrate- and nitrite-treated groups. The regular contour of these vacuoles suggests strongly that they are fat inclusions. The unstained contents of these vacuoles on the ultrastructural level may be a result of dissolution of the lipid content of the vacuoles during the preparation. However, stained lipid droplets and globules were demonstrated in some hepatocytes in both treated groups. The accumulation of triglycerides in the liver results from an imbalance between uptake of fatty acids and their secretion as very low-density lipoprotein (Popp and Cattley, 1991). Additionally, ultrastructural investigation supports the previous statement of Popp and Cattley (1991) who reported that the lipid inclusions of hepatocytes develop near, and possibly from, endoplasmic reticulum and may coalesce until they are visible as clear vacuoles by light microscope.

The morphometrical data emphasized that the area percent of lipid inclusions and vacuoles reached their highest value (6.4% of the liver parenchyma) after 4 weeks and declined afterwards in nitrite-treated group. On the other hand, the area percent of these inclusions and vacuoles were ill-measurable until the end of the fourth week in nitrate-treated group and became 3.28% of the liver parenchyma at the end of the experiment. These findings may be explained by the notion of Bartik and Piskac (1981) who mentioned that nitrites are much more toxic than nitrates, where nitrates are reduced to nitrite as a result of the enzymatic activity of the gut microflora. The decrease in lipid inclusions and vacuoles observed at the end of the experiment in nitrite-treated group may be attributed to the animal adaptation to changed conditions and by

its ability to detoxicate a greater amount of toxic substance (Bartik and Piskac, 1981).

The current work showed the presence of cells in different stages of degeneration in treated groups. At first these cells displayed lightly stained cytoplasm and peripheral grouping of chromatin in their nuclei. Progress of the process leads to shrinkage of the cytoplasmic mass and condensation of the nucleus. In the same concern, Popp and cattley (1991) mentioned that apoptotic bodies are observed as individual hepatocytes that have undergone programmed shrinkage and are histologically characterized as small dense structures that may have dense, sometimes fragmented, chromatin. Additionally and on the ultrastructural level, some hepatocytes displayed the occurrence of autophagosomes. Such picture suggests that the increase of sodium nitrates and nitrites 25% over the permissible level in drinking water may enhance the apoptosis in the liver parenchyma.

In conclusion, small doses of sodium nitrates and nitrites in drinking water (50 ppm) led to a moderate alteration in hepatocytes morphology in chicks, that appeared quickly in sodium nitrite treated group and decreased by prolonged treatment. Sodium nitrate pollution, on the other hand, resulted in mild effect on the liver parenchyma that started later than that observed in sodium nitrite pollution.

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LEGENDS

Fig. 1: Liver in control group (2 weeks of age) showing hepatocytes forming irregular laminae (arrowhead), separated by blood sinusoids (empty arrow). Arrow points to the central vein. Semithin section, toluidine blue stain. X 400.

Fig. 2: Hepatocytes showing few intracellular small vacuoles (arrows) in sodium nitrite–treated group (2 weeks of age). Semithin section, toluidine blue stain. X1000.

Fig. 3: Hepatocytes in sodium nitrite-treated group at 4 weeks of age showing numerous vacuoles (arrows) of variable sizes. Arrowhead points to the central vein. Semithin section, toluidine blue stain. X400.

Fig. 4: Higher magnification of liver parenchyma in sodium nitrite-treated group at 4 weeks of age showing some intracellular vacuoles starting to coalesce with each other. Empty arrow points to the central vein. Semithin section, toluidine blue stain. X1000.

Fig. 5: Parenchyma of liver in sodium nitrate-treated group at 6 weeks of age showing distributed intracellular vacuoles (double arrows). Notice the presence of lightly stained hepatocytes (arrows). Semithin section, toluidine blue stain. X1000.

Fig. 6: Arrowheads point to the intracellular irregular spaces within hepatocytes of the sodium nitrate -treated group at 6 weeks of age. Semithin section, toluidine blue stain. X1000.

Fig. 7: Electron micrograph of hepatocytes in control group (2 weeks of age) showing a large number of mitochondria (m) in close association with the lamellae of rough ER (arrows). Nucleus (N). X 5000.

Fig. 8: Electron micrograph of hepatocytes in sodium nitrite - treated group (2 weeks of age) showing fine micro-vesicles (empty arrowheads) within the lamellae of rough ER (double arrow). Many micro-vesicles (arrows) coalesce together forming large vesicle (empty arrow). Large lipid globule (arrowhead), lipid droplets (L) and mitochondria (m). X10000.

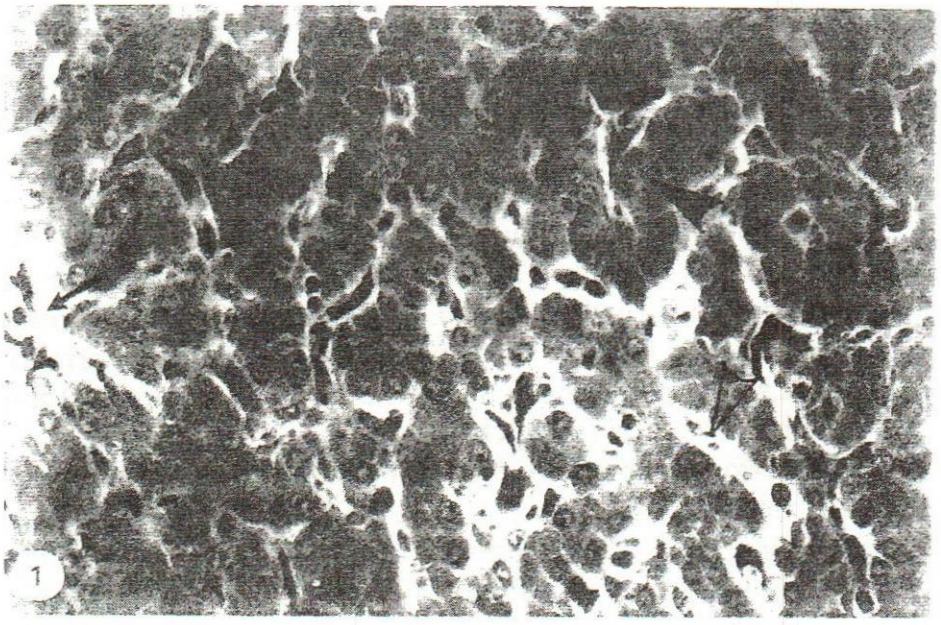
Fig. 9: Electron micrograph of hepatocytes in sodium nitrite - treated group (4 weeks of age) showing autophagosomes (arrow). Bile ductule (arrowheads), Golgi-area (G), mitochondria (m) and nucleus (N). X5000.

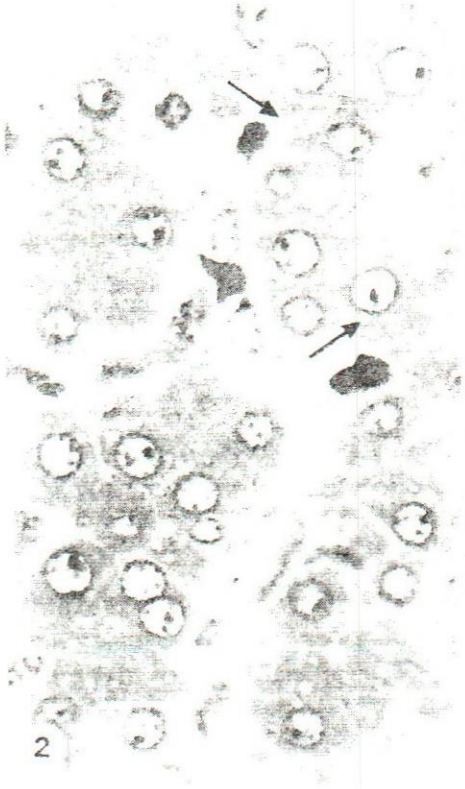
Fig. 10: Electron micrograph of hepatocytes in sodium nitrite - treated group (4 weeks of age) displaying several intracellular vacuoles (V) of different sizes. Nucleus (N). X5000.

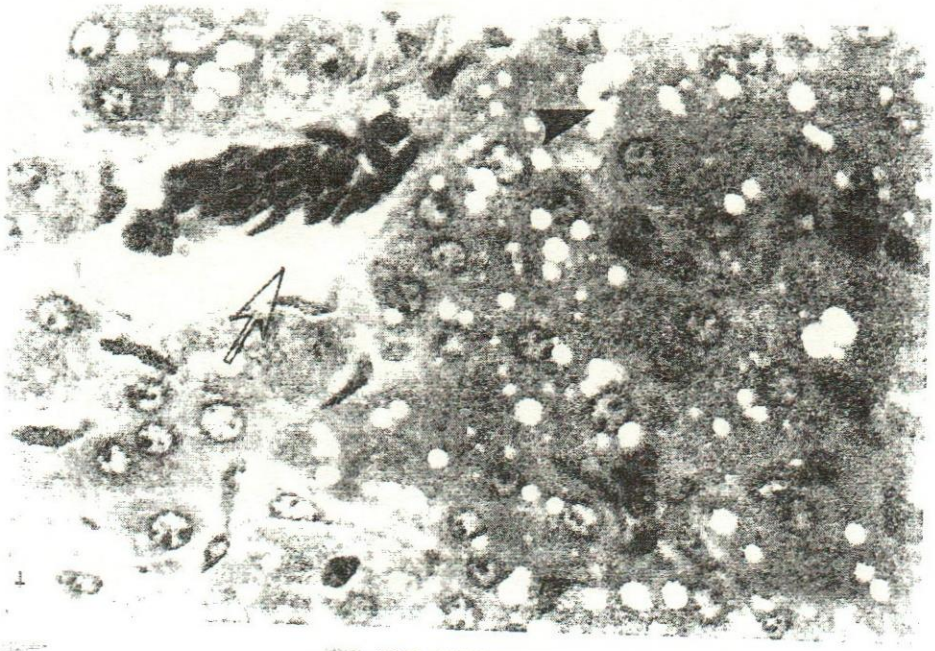
Fig. 11: Electron micrographs of hepatocytes in sodium nitrate - treated group (6 weeks of age) showing intracellular rounded vacuoles (V) containing remnant of fat substance. X5.000.

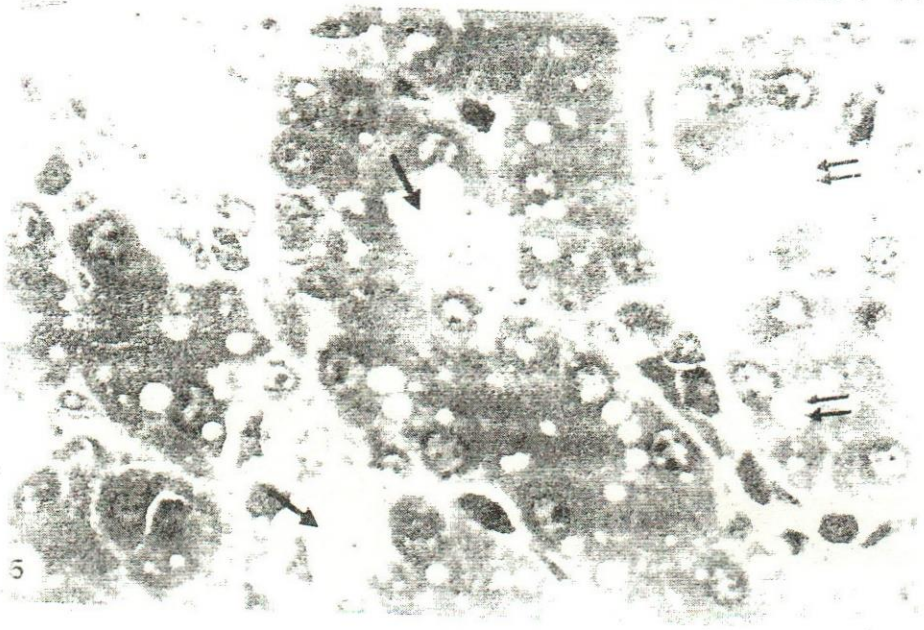
Fig. 12: Electron micrograph of hepatocyte in sodium nitrate - treated group (6 weeks of age) showing fusion of several vacuoles (V) containing fine less-dense material. X6700.

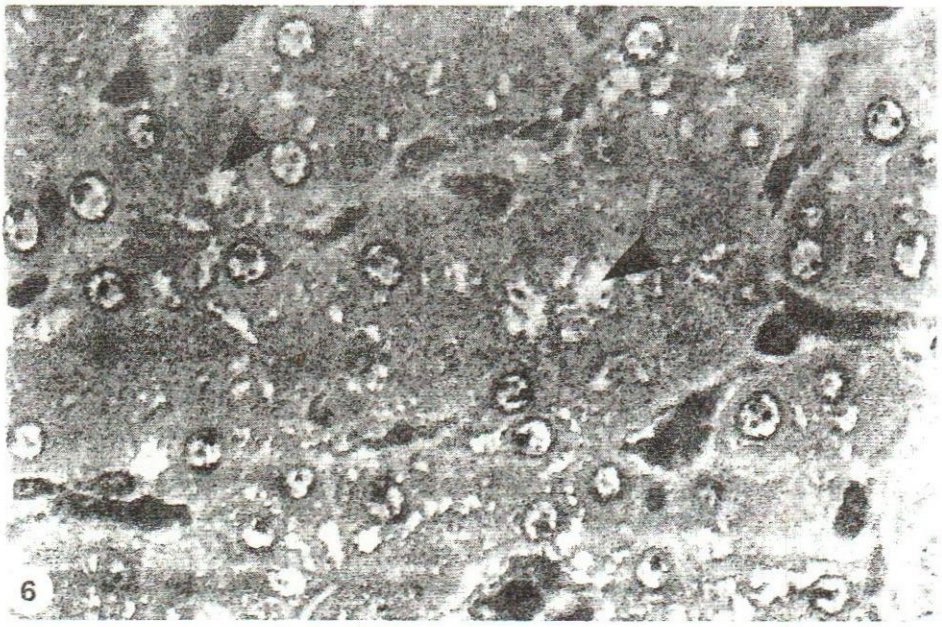
Fig. 13: Electron micrograph of degenerated hepatocyte in sodium nitrite –treated group (6 weeks of age) showing the degenerated nucleus (arrow), condensed cytoplasmic material (arrowhead) and irregular clear spaces (arrows). X6700.











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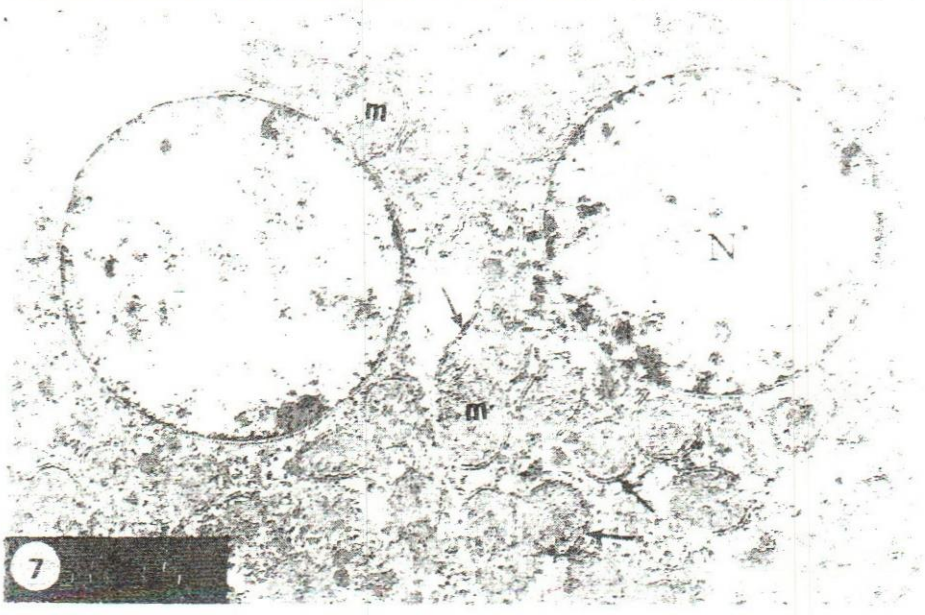

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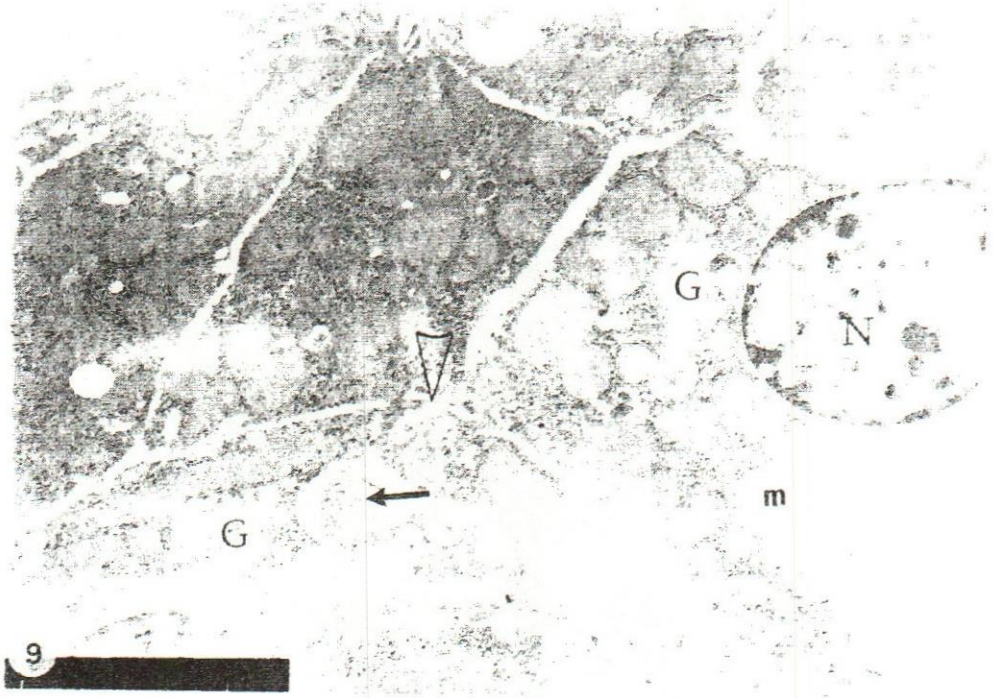

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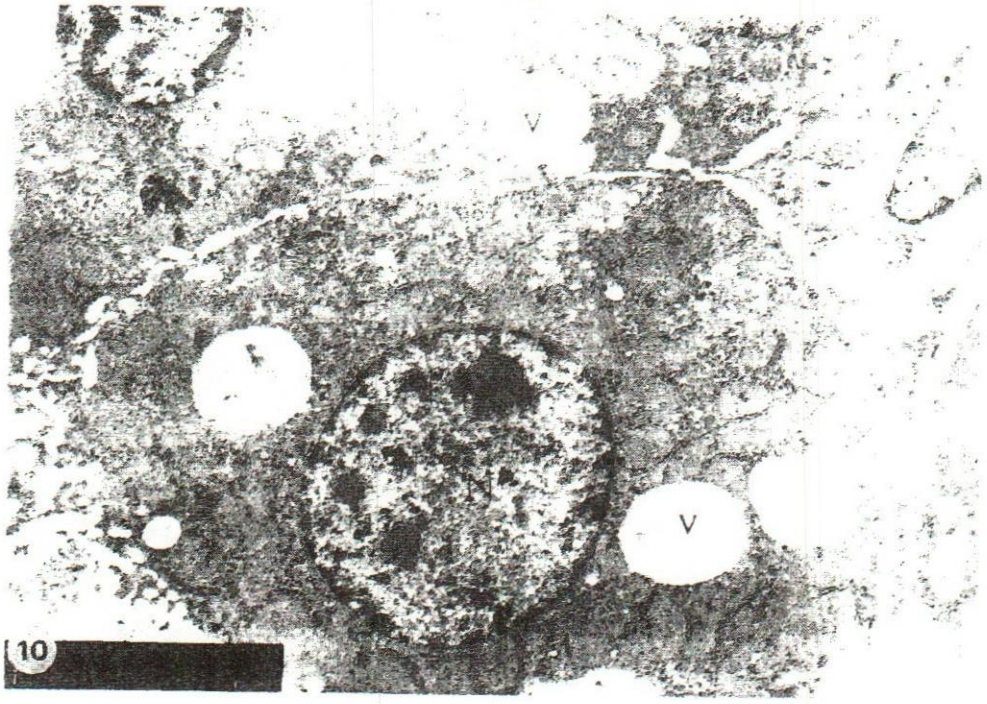

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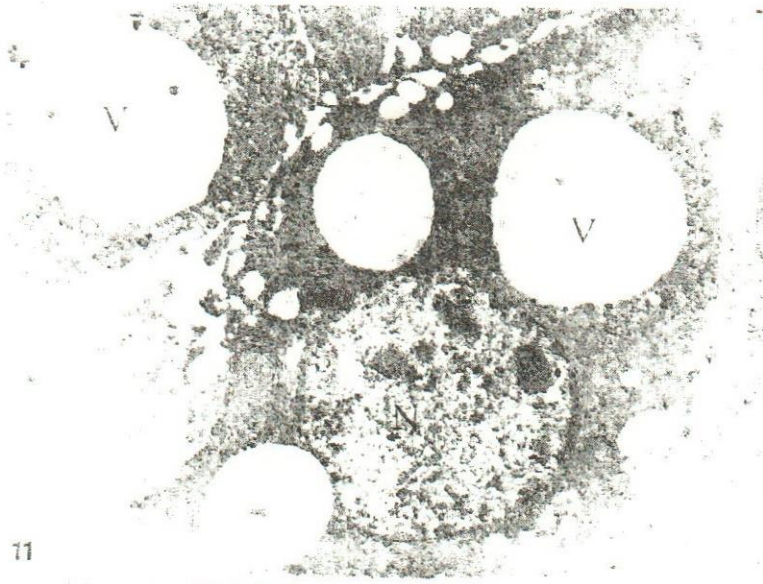

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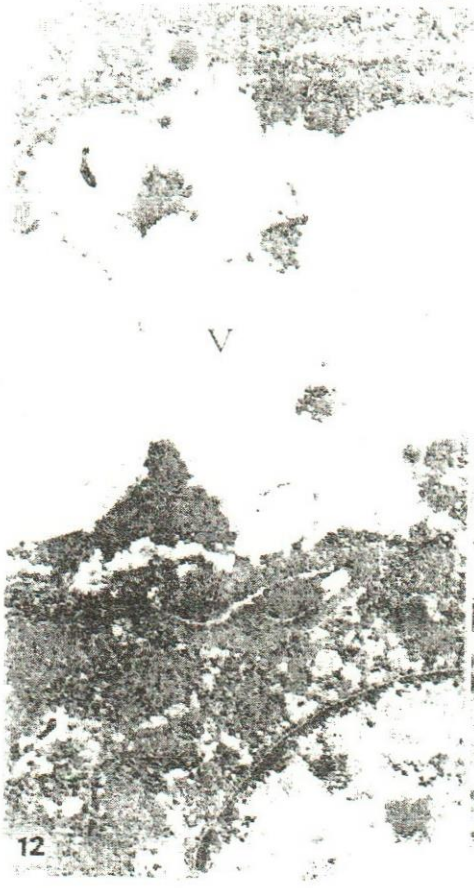

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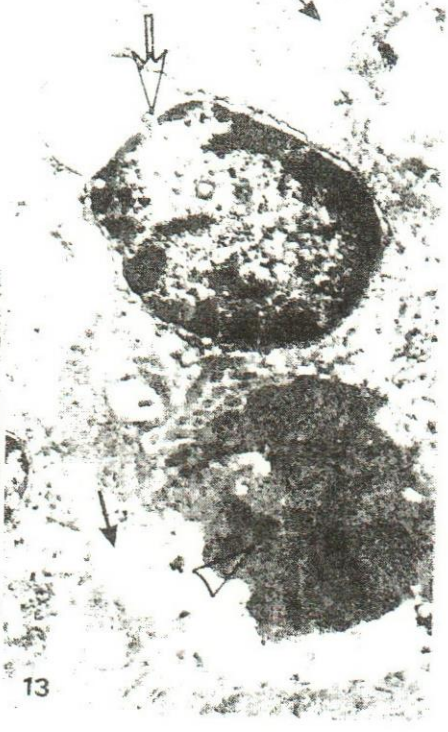

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