

**ASETAMİNOFENİN YENİ-DOĞAN VE ERİŞKİN SIÇANLARDA
KARŞILAŞTIRMALI HEPATOTOKSİK ETKİSİ: IŞIK VE ELEKTRON
MİKROSKOBİK BİR ÇALIŞMA**

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**ACETAMINOPHEN-INDUCED
HEPATOTOXICITY IN NEW-BORN RATS
COMPARED TO ADULTS: A LIGHT AND
ELECTRON MICROSCOPIC STUDY**

ÖZET

Bu çalışmada oniki erişkin ve oniki yenidoğan Wistar albino türü sıçan kullanıldı. Her gruptan beş havana 300 mg/kg ve beş hayvana 500 mg/kg dozunda asetaminofen intraperitoneal olarak uygulandı. Sekizinci saatte hayvanlar dekapite edildiler. 300 mg/kg asetaminofen uygulanmış yeni-doğan sıcağların karaciğerlerinde az sayıda nekrotik hücre izlenirken, 500 mg/kg uygulanmış sıcağların karaciğerlerinde az sayıda nekrotik hücre izlenirken, 500 mg/kg uygulanmış olanlarda hafif hepatik nekroz gözlemlendi. 300 mg/kg asetaminofen uygulanan erişkin sıcağlarda orta derecede ve 500 mg/kg asetaminofen uygulanmış olanlarda ise ciddi hepatik nekroz izlendi. Böylece asetaminofen hepatotoksitesinin yaşa ve doza bağlı olduğu gözlemlendi. Ultrastrüktürel olarak toksik dozlarda asetaminofen uygulanmış yeni-doğan sıcağların karaciğerlerinde belirgin intersellüler alanlar izlendi. Her iki yaş grubunda hepatositlerde şişme, sitoplazmik membranlarda parçalanma ve hücre içi yapılarda dağılım gözlemlendi. Endoplazma retikulumu parçalanmış ve membranları dağılmıştı. Membran artıkları intersellüler alanlarda izleniyordu. 500 mg/kg asetaminofen uygulanan grupta sitoplazmik membran hasarı ve hücre şişmesi oldukça belirgindi ve sitozol kaybı izlendi. Literatür araştırmalarımıza göre çalışmamız yeni-doğan döneminde toksik dozda asetaminofenin karaciğerde oluşturduğu değişiklikleri incelemek için yapılmış ilk elektron mikroskopik çalışmadır. Asetaminofenin daha önce rapor edilenden daha erken bir postnatal dönemde, daha düşük dozlarda ve daha kısa sürede hepatik nekroza ve irreversible değişikliklere neden olduğu kanısındayız.

Anahtar Kelimeler: Asetaminofen, Hepatotoksite, Yeni-doğan, Erişkin.

SUMMARY

Twelve adult and twelve new-born male Wistar albino rats were used in this study. In each group, five rats received 300 mg/kg and five 500 mg/kg doses of acetaminophen intraperitoneally. After 8 hours animals were killed with decapitation. There was small number of necrotic cells in the livers of 300 mg/kg acetaminophen administered new-born rats whereas 500 mg/kg acetaminophen administered rats developed mild hepatic necrosis. In adult rats received 300 mg/kg of acetaminophen there was moderate and received 500 mg/kg of acetaminophen there was severe hepatic necrosis. So, the hepatotoxicity of acetaminophen was age and dose-dependent. Ultrastructurally, the liver of the new-born rats exposed to toxic doses of acetaminophen contained areas of prominent intercellular spaces. In both groups the hepatocytes were swollen and had ruptured cytoplasmic membranes and disrupted internal structures. Membranes of endoplasmic reticulum were disrupted and endoplasmic reticulum was fragmented. Membranous debris was present in the intercellular spaces. In the 500 mg/kg acetaminophen group cytoplasmic membrane damage and the cell swelling were greatly increased and loss of cytosol was observed. To the best of our knowledge, this is the first ultrastructural study performed to investigate the alterations caused by acetaminophen in new-born liver. We suggest that acetaminophen causes hepatic necrosis and irreversible changes at an earlier postnatal age within a shorter time and by administering lower toxic doses than that of previously reported.

Key words: Acetaminophen, Hepatototoxicity, New-born, Adult, Rat.

Not: Bu çalışma 5.Karadeniz Tıp Günlerinde Türkçe Olarak Sunulmuştur.

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INTRODUCTION

The liver frequently is a site of acute cellular damage caused by chemicals; because it usually is the organ that is exposed to the highest concentrations of xenobiotics; and it contains large quantities of enzymes that metabolize these.^{1,2} The analgesic and antipyretic acetaminophen (APAP) is generally considered to be safe when taken appropriately.³⁻⁵ But following overdose, it may be toxic. The most commonly reported toxicity is fulminating centrilobular hepatic necrosis, which also occurs in laboratory animals.^{6,7} Damage to the liver caused by APAP is thought to be a result of the conversion of a fraction of the dose to chemically reactive metabolites which can covalently bind to cellular macromolecules.^{2,7,8} Current evidence suggests that the hepatotoxicity is a result of conversion of the drug to the reactive arylating metabolite, N-acetyl-p-benzoquinone imine (NAPQI), by the cytochrome P450 mixed function oxidase system.^{6,9} NAPQI is an intermediate both indirectly and directly and is shown to be a potent hepatocellular toxicant that acts as both a powerful electrophile and an oxidant.² At therapeutic doses, this metabolite is efficiently detoxified by conjugation with glutathione, however hepatic glutathione is depleted by larger doses and the metabolite binds to protein sulfhydryl groups. Binding correlates with the development of the toxicity.^{6,8-10} More recent studies suggest that the acetaminophen-induced hepatotoxicity is likely due to cellular oxidative stress, resulting in lipid peroxidation, protein thiol oxidation and changes in the intercellular calcium hemostasis, all processes being potentially fatal to cells.^{2,5}

It is generally accepted that children are less susceptible to APAP than adults.^{4,11,12} Adults and adolescents account for most serious and fatal cases of APAP poisoning. Adolescents and adults are twice as likely to develop plasma levels in the toxic range as children younger than 6 years of age.¹¹ Clinical experience suggests that if an adult consumes more than 7.5-15 g (150 to 200 mg/kg) of APAP as a single dose or a child ingests 150 mg/kg of body weight hepatotoxicity may occur, doses of 20 to 25 g. or more are potentially fatal.^{11,13,14}

In the present study, we have investigated the hepatotoxicity of APAP in 2-week and 6-month-old rats comparatively. To the best of our knowledge, there is no reported ultrastructural study about hepatotoxicity of APAP in new-borns, we used electron

microscope to define the APAP-induced alterations in new-born liver.

METHODS

Twelve 2-week-old (weighing 17-20 gr) and twelve 6 month-old (weighing 220-260 gr) male Wistar albino rats were used in this study. Purified acetaminophen was obtained from Saba pharmaceuticals. Five to six rats were housed together at 23-25 °C and 60 % humidity with a 12/12 hour light and dark cycle. One day before the experiment rats were weighed and housed two per cage. Rats were given rat chow and tap water ad libitum. In both adult and new-born rat groups, five rats received 300 mg/kg of APAP and five received 500 mg/kg of APAP intraperitoneally in saline in a volume of 10 ml/kg. These doses were chosen on the basis of previous experience to be a threshold toxic dose and an LD50 dose, respectively, in young adults.¹⁵ In each group, two control animals were injected with an equal volume of saline. Two-week-old rats were on breast feeding and they stayed with their mothers until the end of the study. Clinical observation was performed during the experience and eight hours following the injections the animals were killed by decapitation. Livers were removed and placed in 10 % buffered formal saline, then embedded in paraffin wax. Sections (5 µm) cut from paraffin blocks and mounted on glass slides were stained with hepatoxilin and eosin for histopathological examination. Hepatic sections were examined for morphologic changes and the pictures were taken with Olympus BH-2 photo microscope. Evidence of cellular necrosis was determined solely on the basis of nuclear characteristics (i.e. pyknosis of nuclei). By this grading system the percentage necrosis was quantified: 0: absent, minimal: necrosis of less than 5% of hepatocytes, mild: necrosis of 5-20 % of hepatocytes, moderate: necrosis of 20-40 % of hepatocytes, and severe necrosis of greater than 40 % of hepatocytes. On the other hand, the livers of two rats received 300 mg/kg and two rats received 500 mg/kg of APAP and of two control rats were prepared for the electron microscopic examination. These samples were cut into smaller pieces and fixed in 3% glutaraldehyde buffered with 0.2 M NaH₂PO₄ + NaHPO₄ (pH= 7.2-7.3), postfixed in 2 % OsO₄ buffered with 0.2 M NaH₂PO₄ + NaHPO₄ (pH= 7.2-7.3). Specimens were dehydrated in acetone and embedded in Araldite CY212. Semithin sections were studied

with toluidin blue for orientation purpose. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Jeol 100S X electron microscope.

RESULTS

The criteria used to assess the APAP toxicity in the new-born and adult rats were based on two lines of evidence: lethality data and histopathological evidence of liver injury.

At the 300 mg/kg and 500 mg/kg doses, all adult and new-born rats survived for 8 hours; there was no spontaneous death in our study. But at 500 mg/kg dose, adult rats were near death (moribund and shallow respiration) were recorded). But at the 300 mg/kg and 500 mg/kg doses, new-borns were active and no physical difference was observed. The histopathological changes were as follows.

New-born rat received 300 mg/kg acetaminophen. The light microscopic changes common to this group were mild cellular swelling, sinusoidal congestion and in a few area, hepatocytic vacuolation. There was small number of necrotic hepatocytes. The damage could not be assigned to one lobular region (i.e. focal necrotic changes).

New-born rat received 500 mg/kg acetaminophen. In this group, all animals developed mild centrilobular hepatic necrosis (Figure 1). The other light microscopic changes were hepatocytic vacuolation, sinusoidal congestion and mild hepatocytic fatty change.

In the livers of acetaminophen administered new-born rats, in some areas there was no pathological alterations within the hepatocytes. Some of the nuclei were pyknotic. In some nuclei lipid droplets were observed. In the livers of 300 and 500 mg/kg APAP administered rats the hepatocytes were swollen and had ruptured cytoplasmic membranes and disrupted internal structures. Membranes of endoplasmic reticulum were disrupted and endoplasmic reticulum was fragmented. Membranous debris was present in the intercellular spaces. Cellular membranous organelles were scattered throughout the intercellular spaces (Figure 2-4). In the 500 mg/kg APAP group cytoplasmic membrane damage and the cell swelling were greatly increased and loss of cytosol was observed. The cristae mitochondriales were generally not observed and the internal structure of mitochondria was electron dense (Figure 4,5). Occasionally necrosis resulted in focal haemorrhages in affected areas (Figure 3,5).

Adult rat received 300 mg/kg acetaminophen. In the liver of drug administered rats some of the lobules were morphologically deranged. Moderate centrilobular hepatic necrosis was a feature of this group. The other light microscopic alterations included sinusoidal dilatation, congestion (Figure 6).

Adult rat received 500 mg/kg acetaminophen. Most of the lobules were morphologically deranged. Severe centrilobular hepatic necrosis, sinusoidal dilatation and congestion was observed (Figure 7).

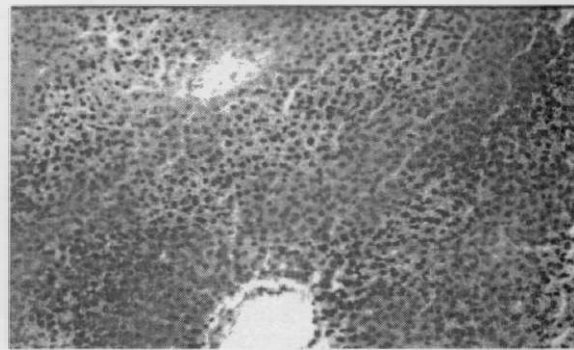


Figure 1. New-born liver received 500 mg/kg of APAP. Mild hepatic necrosis is seen. Hematoxylin and eosin X250

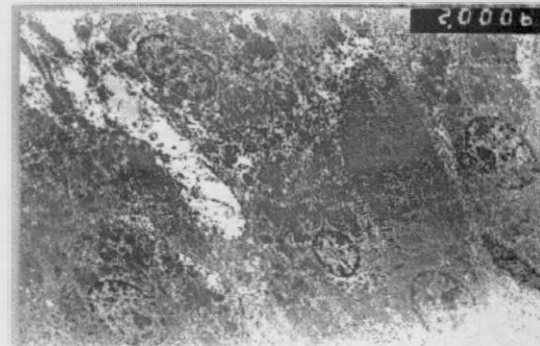


Figure 2. New-born liver received 300 mg/kg of APAP. Some of the hepatocytes are separated from each others by large spaces. Cytoplasmic membranes are ruptured. Some of the nuclei are pyknotic. Uranyl acetate and lead citrate X2000.

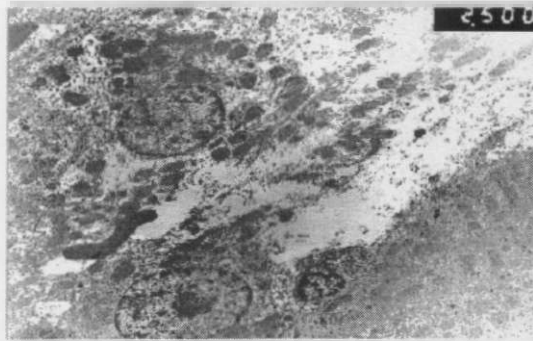


Figure 3. New-born liver received 500 mg/kg of APAP. Cytoplasmic membranes of many hepatocytes are ruptured. Mitochondria, membranes of endoplasmic reticulum and ribosomes are scattered throughout the prominent intercellular spaces. Erythrocytes demonstrating focal hemorrhages are scattered. Uranyl acetate and lead citrate X2500.



Figure 4. New-born liver received 500 mg/kg of APAP. Cytoplasmic membranes of the hepatocytes are ruptured. Mitochondria and cellular debris are seen in the intercellular spaces. Uranyl acetate and lead citrate X2000.

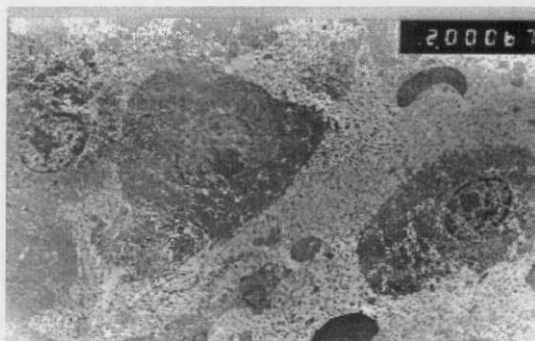


Figure 5. The liver of new-born liver rat received 500 mg/kg of APAP. Cytoplasmic membranes of many of the hepatocytes are ruptured. Erythrocytes demonstrating focal hemorrhages are scattered. Uranyl acetate and lead citrate X2000.

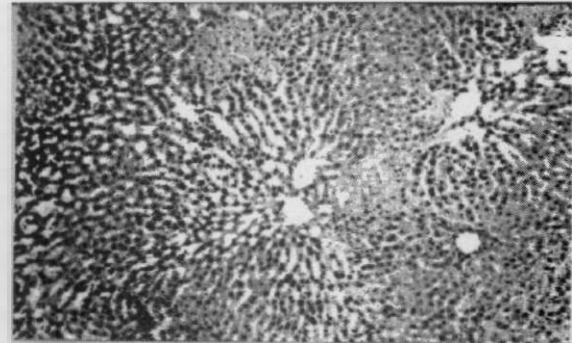


Figure 6. The liver of adult rat received 300 mg/kg of APAP. Note the moderate hepatic necrosis. Sinusoidal dilatation and congestion are observed. Hematoxylin-eosin X100.

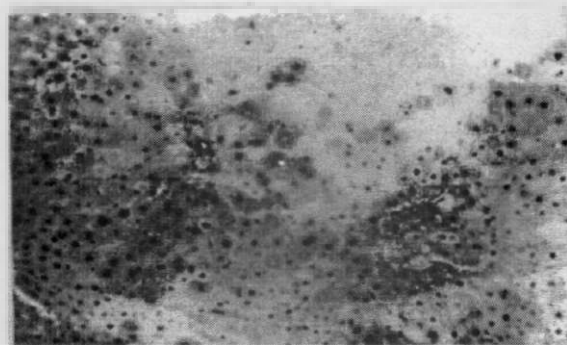


Figure 7. The liver of adult rat received 500 mg/kg of APAP. Severe hepatic necrosis is observed. Hematoxylin-eosin X100.

DISCUSSION

It is generally accepted that children are less susceptible to acetaminophen toxicity than are adults.^{4,11,12} In our study at the 300 mg/kg and the 500 mg/kg doses, all adult and new-born rats survived until decapitation. In our 500 mg/kg adults rats, though there was no death, moribund and shallow respiration were observed. But new-born rats were highly active and there was no sign of death clinically. Our observation is consistent with the fact that the LD50 dose for APAP in neonatal rats is slightly higher than that observed in adult animals.¹⁶

The histopathological criteria of the hepatic injury of APAP is centrilobular hepatic necrosis, centrilobular hepatocytic atrophy, hepatocytic vaculation, hepatocytic fatty change, loss of sinusoidal cells, focal peliosis, sinusoidal congestion and inflammatory infiltration.^{14,19} In the present study, centrilobular hepatic necrosis was a common feature found in adult rats received 300 mg/kg and 500 mg/kg APAP. In 500 mg/kg APAP group, the severity of necrosis was higher than that of the 300 mg/kg group. The other alterations including derangement of the lobules, sinusoidal congestion and loss of sinusoidal cells were more pronounced in rats receiving higher dose of APAP. Adamson et.al. (1991) found that, morphologic alterations produced by APAP were more significant in animals receiving higher doses.¹⁵ At normal doses acetaminophen is readily detoxified largely by glucuronidation and sulphation in the liver. A small fraction of the dose is oxidized in the liver by cytochrome P-450 dependent monooxygenase system to a highly reactive metabolite; NABQI, this is very effectively detoxified by conjugation with glutathione. However as the dose of acetaminophen increases, the amount and the proportion of the administered dose that undergoes oxidation to NABQI increase due to the depletion of the sulphate pool. The levels of NABQI increase to an extent such that synthesis of glutathione is exceeded and the intermediate escapes detoxification. NABQI is also potential agent so that when it interacts with thiol groups, two reactions can occur: the thiol groups can be oxidized to disulphide, one of the mechanisms of detoxification by glutathione; at least until the levels of hepatic glutathione are low; or they can undergo nucleophilic addition resulting in covalent binding to reactive metabolite of cellular proteins.⁶⁻¹⁰ Thus, the extent of the hepatic damage produced by APAP is dose-dependent.

In the new-born rats at 300 mg/kg dose of APAP, the morphologic changes in the liver was minimal. There was small number of necrotic cells at this dose group. In the same group at 500 mg/kg dose, mild centrilobular necrosis was observed. So, the range of necrosis and the other morphologic alterations were lower in new-born rats compared to adults. Thus, our study indicates that 2-week-old rats are remarkably resistant to the hepatotoxic effects of high doses of APAP when compared with older animals. There are some theories about the mechanism why postnatal animals have less susceptibility to APAP toxicity. Young animals have higher levels of APAP covalently bound to hepatic

macromolecules and excrete higher amounts of mercapturic acid conjugate compared to adult animals. So the lower susceptibility of neonates appears to be due to a relative inability of the young animals to metabolically activate the drug.^{4,20} For many drug substrates both human and rodent neonates have poorly developed drug metabolizing systems, the activity of which increases with maturation. Hence, the young may be less affected by agents requiring metabolic activation, such as APAP.³ It is likely that these toxic processes are quenched by superior defence mechanism in the postnatal liver cells. It has been shown that the activities of hepatoprotective enzymes, glutathione peroxidase and glutathione reductase were markedly higher in liver of 2-week-old mice than in adults. APAP is primarily metabolized to the sulphate of glucuronide (94 %) and the shift from sulphate to glucuronide predominance between ages 9-12 year parallels the change in degree of toxicity at these ages. A small amount of APAP is excreted unchanged and the remaining approximately 4 % is metabolized via cytochrom P450 and glutathione to the mercapturic acid conjugate.¹⁵ Accidental ingestion of an overdose of APAP in children has not frequently been reported to produce hepatotoxic reactions, in fact, there is only a few case reports in the literature.^{12,21} This rarity is in contrast to the more frequent ingestion of overdoses of APAP in the adolescent age group where several reports of hepatotoxic reaction have appeared.¹⁷ Beierschmitt et.al. (1989) reported that 75 % of 3-month-old mice, but only 14-29 % of 1-1.5 and 2-month old mice had centrilobular necrosis after a toxic dose of APAP.³ In all of these studies, age-related differences in APAP toxicity have been observed. Age-related toxicity have been shown in human, too. ^{4,11,12} No significant APAP induced hepatic necrosis was reported in the laboratory animals younger than 19 day old. Using light microscope Adamson et.al.(1991) observed no hepatic necrosis in 2-week-old mice received 300 and 500 mg/kg doses of APAP within 8 hours.¹⁵ Green et al (1984) reported that morphologic parameters in 11 day old rats given toxic doses of APAP (750-1250 mg/kg) were not different from controls' whereas older animals developed hepatic centrilobular damage.¹⁶ In the previous studies hepatic necrosis was observed within 20-24 hours after drug administration using higher doses than those we administered. Since we observed centrilobular necrosis even in 300 mg/kg dose group, we think that smaller doses of APAP than that of previously reported causes

hepatic necrosis in new-borns. So we decided to investigate the ultrastructural alterations caused by APAP in new-born rat liver. No previous electron microscopic study performed to investigate the APAP-induced hepatotoxicity in new-borns was encountered in the literature.

Early, reversible light microscopic changes are difficult to detect. Ultrastructural hepatocellular changes induced by APAP were more prominent than light microscopic alterations in our study. By electron microscope in some areas pathological change was not observed. But generally many of the hepatocytes were swollen and had ruptured cytoplasmic membranes and disrupted internal structures. Organelles were spilled out and were found lying in the intercellular spaces. In the previous studies, it is reported that APAP induces severe hepatic necrosis in adults. The prominent microscopic changes induced by APAP are revealed glycogen depletion, loss of ribosomes, cytoplasmic matrix swelling, increased vacuolation of the endoplasmic reticulum, centrilobular mitochondrial injury and bleb formation.^{12,19,22} Ferguson et al. (1990) observed pyknotic nuclei, disrupted cell membranes and swollen and fragmented endoplasmic reticulum and later, the membranous organelles and granules in the intercellular spaces in adult mice pancreas after 500 mg/kg APAP administration²¹

The first changes in the cells undergoing necrosis are mild cytoplasmic swelling, dilatation of the smooth endoplasmic reticulum and the loss of ribosomes from rough endoplasmic reticulum. A further characteristic change is blebbing from plasma membrane of cytoplasmic fragment that include cytosol but not the larger organelles such as mitochondria and endoplasmic reticulum.²³ The intracellular calcium homeostasis is susceptible to depletion of glutathione level in cells, as glutathione plays an essential role in regulation of the cellular protein thiol disulphide status. The cascade of events proposed to lead to the cytotoxic effect due to a disturbance of the calcium homeostasis, therefore, follows primarily upon depletion of cellular glutathione. Under these circumstances a loss of critical protein thiol groups may occur causing a disruption of the intracellular calcium homeostasis which results in an increase of the cytosolic calcium level. An elevated cytosolic calcium level has been associated with the activation of certain degradative enzymes, such as proteases, endonucleases and lipases and alterations of hepatocyte morphology, visible as surface membrane blebs. These blebs have been shown to

precede cell death due to exposure of hepatocytes to cytotoxic chemicals as APAP.⁵ Cell death itself is only structurally recognizable after the event, by degree of disruption of cellular morphology so gross as to be clearly irreversible. This will usually include disintegration of the cell membrane, disorganization of the cytoplasmic organelles and shrinkage or fragmentation of the nucleus. The entire cell may become shrunken and condensed its organelles barely recognizable. Dying cells often become disconnected from their neighbours and detached or extruded from their position particularly in epithelial sheets which may in turn lead to disordered function and to the triggering of the defensive mechanisms of inflammation and reparative fibrosis. Cell injury may therefore be defined as a failure of the cell, on challenge, to maintain itself within homeostatic tolerance limits. This may be an acute rapidly developing abnormality, such as the distension of intracytoplasmic membrane-limited spaces, sometimes accompanied by condensation of the intervening cytoplasmic matrix.²⁴ An important question is the nature of the "point of no return" at which there is irreversible commitment to necrosis. This commitment coincides closely with two mitochondrial changes: a violent dilatation called "high amplitude swelling" and appearance of matrix densities. The latter are usually flocculent and probably represent denatured proteins. The necrotic cells swell rapidly and both plasma and internal membranes begin to rupture. As a result loss of cytosol occurs. Organelles spill out and are found lying in the intercellular spaces. Nuclei may swell prior to rupture in some forms of lethal injury but more often the nuclei of degenerating cells become condensed and shrunken. Membrane rupture and dispersal of organelles as we observed are irreversible changes.²³

We concluded that new-born rats had low susceptibility to APAP hepatotoxicity compared to adults. In addition we observed that APAP causes hepatic necrosis and irreversible changes at an earlier postnatal age within a shorter time and with lower toxic doses than that of previously reported. To the best of our knowledge, this is the first ultrastructural study performed to investigate the alterations caused by toxic dose of APAP in new-born liver.

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