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Ultrastructural studies on the testes and liver of mice after treatment with different doses of Diclofenac Sodium.

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Abstract : Diclofenac sodium is one of the most common non-steroidal compounds of phenyl acetic acid class, binds extensively to plasma albumin and having inhibitory effects on prostaglandin biosynthesis. The present investigation was conducted to reveal the effect of diclofenac sodium (4mg/kg body weight and 14 mg/kg body weight) on testes and liver of mice at ultrastructural level. Electron microscopic study revealed changes in testes seminiferous tubules such as degenerative changes in mitochondria, endoplasmic reticulum, Golgi bodies etc. Spermatogonia showed apoptotic changes in the form of nuclear chromatin condensation and deeply stained cytoplasm. Similar changes were also observed in the hepatocytes of liver.

Key words: Diclofenac, Prostaglandins, Ultrastructure, inflammation and hepatocytes.

INTRODUCTION

The non- steroidal anti-inflammatory drugs are the heterogeneous group of compounds, chemically unrelated, but mostly organic acids.[1]These are most frequently prescribed therapeutic agents used for the treatment of rheumatic diseases, due to their analgesic, antipyretic and anti-inflammatory actions. NSAIDs are most commonly used pharmaceutical products contributing to morbidity worldwide.Diclofenac sodium is one of the most common non-steroidal compounds of phenyl acetic acid class, binds extensively to plasma albumin, having inhibitory effects on prostaglandin biosynthesis.Inhibition of prostaglandin synthesis is considered to be fundamental to the mechanism of action of diclofenac sodium.[2]There is growing interest in the toxicity of diclofenac because of its clinical use and for the study of mechanism of hepatotoxicity, renal dysfunction, testicular toxicity and hypersensitivity reactions. This non- steroidal drug has been found to be a prime suspect in causing cell injury due to its ability to bind covalently to macromolecules in situations where intracellular levels of NADH, NADPH, GSH and other reducing agents are very low.[3]The mechanism by which diclofenac causes liver alterations in certain individuals is not fully understood and both formation of toxic metabolite and covalent binding of the drug to hepatic proteins have been invoked to explain its toxicity.The frequent target of the drug is mitochondrion and the alterations of its function have immediate effects on the energetic balance of cells.[4] Mitochondrial injury, apoptosis, necrosis and liver damage associated with diclofenac sodium was suggested to be based on peroxide catalyzed production of NSAID radicals which resulted in oxidized GSH and NADPH.[5]

Spermatogenesis is a complex and dynamic process that results in the continual production of spermatozoa in mammals. Apoptosis of the germ cells that occurs in the testicular epithelium serves as a mechanism to reduce the germ cell population to the levels which can be supported by the Sertoli cells. Diclofenac administration in mice

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results in severe changes in the morphology of different types of cells in the testes including the Sertoli cells. Necrotic cells characterized by plasma membrane rupture, marginated chromatin along the nuclear membrane, swollen mitochondria and vacuolization within the cytoplasm are the consequence of deleterious effects of the drug.

MATERIAL AND METHODS

The protocol of the present investigation was approved by Institutional Animals Ethics Committee (IAEC approval no. IAEC/Bio/5/2011-H.P.U.) Himachal Pradesh University, Shimla. Healthy, pathogen free Swiss albino mice of Balb C strain weighing 22-25 g were procured from Central Research Institute (CRI) Kasauli, Himachal Pradesh. These were maintained in the animal house of the department of Biosciences, H.P. University, Shimla under suitable hygienic conditions with 16 hr. day light and temperature of 24± 20C. Animals were caged in polypropylene cages (six mice/cage) on soft chip bedding. Animals were provided with commercial feed (Hindustan Lever Ltd. New Delhi, India) and were given water ad libitium. Each animal was assigned a unique identification number by individual marking on fur.Oral administration was selected as it is one of the proposed routes for toxicity and canular feeding was preferred for accuracy.

The animals were divided into three groups, as

1. The mice of the first group served as normal (control) animals.
2. The mice of the second group were administered a daily dose of diclofenac sodium (4mg/kg body weight) for 28 days.
3. The mice of the third group were given a daily dose of diclofenac sodium (14 mg/kg body weight) for 28 days.

Mice were sacrificed after 7^{th}, 14^{th}, 21^{st} and 28^{th} day of drug therapy by cervical dislocation; testes and liver were taken out and proceeded for ultrastructural studies.

Transmission Electron Microscopy

Mice tissues (Liver and testes) were carefully excised and fixed in fixative for 12 hr. at 4^0C. The fixed tissues were then washed four times in 0.2 M phosphate buffer and post fixed in 1% osmium tetroxide for 2 hours. They were dehydrated in graded acetone steps and embedded in araldite CY212.Transmission Electron Microscopy was performed at AIIMS, New Delhi.

Staining of ultrathin sections

A double staining method using uranyl acetate and lead citrate were used to obtain a good contrast of sections. 50 µl of uranyl acetate was taken on a piece of parafilm, kept inside a petridish. The grid was floated upon the stain droplet with its section side facing down followed by first placing the petridish cover and then one dark cover upon it as the staining is found to be more effective when carried out in dark. Staining was done for 10-15 minutes. Grid was then washed twice in double distilled water with continuous agitation and dried carefully on a filter paper. A small droplet of lead citrate was pipetted on a piece of parafilm kept in a petridish and again the grid was placed with its section side facing down on to the stain. Staining was done for 5-10 minutes. Each grid was washed briefly in 0.2M NaOH and then twice in double distilled water and dried. The grids were stored in grid box or in petri dish. The sections were observed under Transmission Electron Microscope Morgagni 268 (Fie Company), Netherland.

RESULTS AND DISCUSSION

Testes

Ultrastructurally, the seminiferous tubules being composed of large number of spermatogonial cells normally of two types A and B. The type A spermatogonium, which are the predominant ones have a large oval nuclei with fine euchromatin and prominent nucleoli (Fig.1). The Sertoli cells rest on the basement membrane of the tubule and extends to the lumen. These extend from the basement membrane to the luminal surface of the seminiferous epithelium. They are elongated or pyramidal in shape and exhibit ill-defined outlines. They have lightly stained cytoplasm and large nucleus located at the base of the cell and showed indentation in the nuclear membrane with homogeneous nucleoplasm (Fig.2).Lateral processes of the Sertoli cells are interconnected by tight junctions, which are likely to be the structural basis for blood testes barrier.Treatment with diclofenac for 14 days in the low dose group (4mg/kg body weight), led to some degenerative changes with nucleus becoming pycnotic. The cells appeared vacuolated and number of apoptotic bodies seen in the cytoplasm (Fig.3). Vesiculation in mitochondria was a commonly observed feature. Thickening and irregularities of the basement membrane

were observed in some tubules. Spermatogonia showed apoptotic changes in the form of nuclear chromatin condensation (Fig.3) and deeply stained cytoplasm. Some of the other changes observed were the presence of empty vesicular spaces, loss of cristae in the mitochondria and stacking of endoplasmic reticulum.Some of the apoptotic changes associated with the testicular parenchyma resulted in the appearance of large number of dense bodies in the cytoplasm and degenerated areas in the form of cytoplasmic vesicles. Mitochondria showed enlargement with lysed cristae and cytoplasm showed large number of apoptotic bodies and Vesiculation after 21 days of treatment in high dose group (Fig. 4).

Diclofenac treatment for 28 days caused extensive testicular tissue damage. Dilated cisternae of endoplasmic reticulum, vesiculated mitochondria with degeneration and loss of cristae and blebbing of nuclear membrane with cytoplasmic vacuolations were observed in the spermatocytes. The spermatocytes also showed chromatin condensation and margination. The spermatocytes displayed morphological characteristics of apoptosis including cell shrinkage and presence of residual bodies in the cytoplasm (Fig.5). The Golgi apparatus appears swollen and irregular dense fibres in sperm tail showed vesiculated mitochondria around. There was complete degeneration of spermatid nucleus and other cell organelles resulting in the accumulation of large number of apoptotic bodies. Some of the other changes noticed were heterochromatized nucleus, tubular mitochondria and disorganised cell organelle (Fig.6).

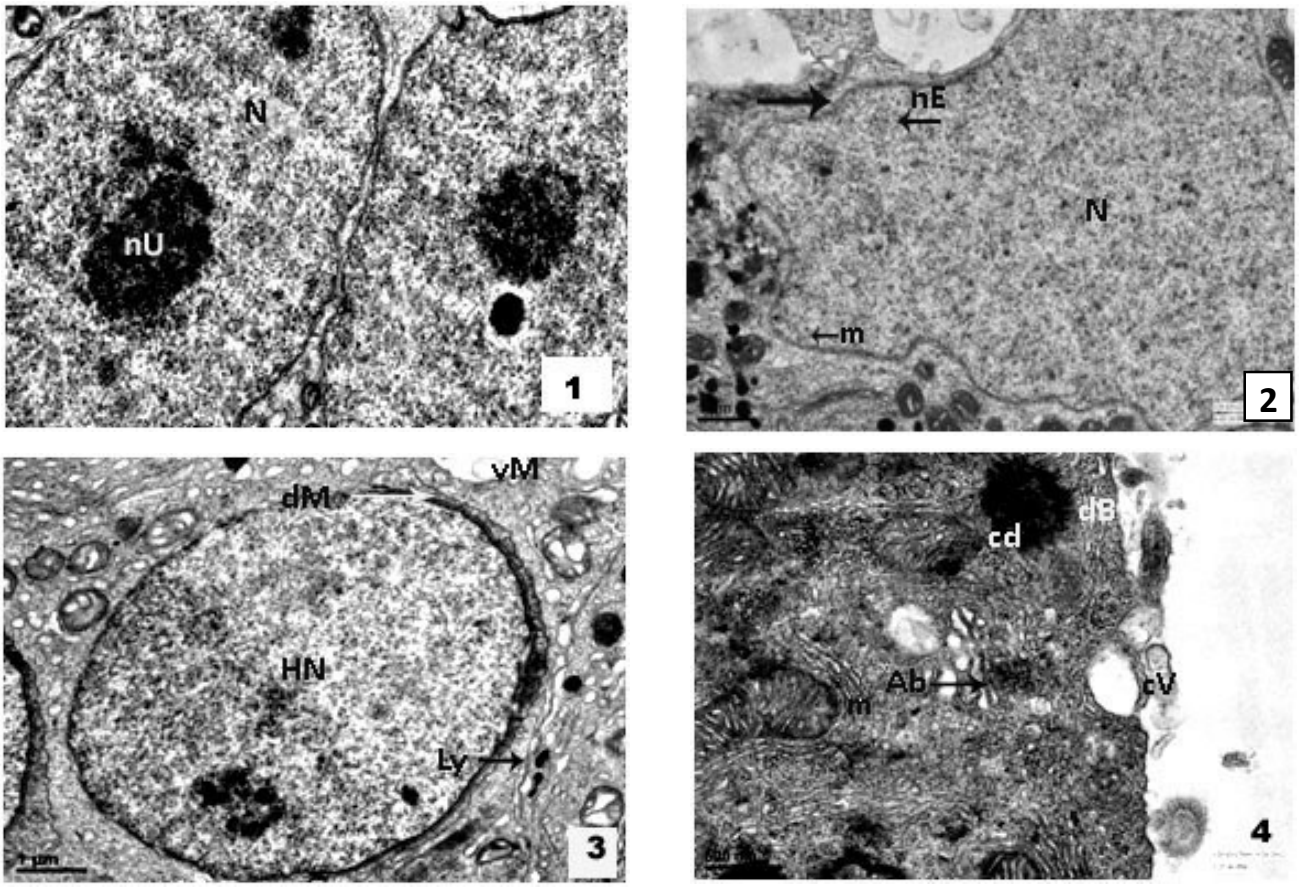
Diclofenac toxicity caused numerous ultrastructural changes in testes leading to alteration in the normal architecture. Most of these changes appeared degenerative showing distension of mitochondria, presence of varied number of abnormal vesicles in Sertoli cells, disrupted nuclear membrane in the spermatogonia and marginated chromatin along the nuclear membrane. Administration of drug seemed most likely to induce disorganization of the mitochondria resulting in their dispersion throughout the cytoplasm followed by vesiculation of endoplasmic reticulum and its dilation leading to vacuolization of cytoplasm. Similar ultrastructural changes were observed in rat testes after treatment with melatonin.[6]Sertoli cells revealed some morphological alterations and were characterized by a ruptured nucleus, shrinkage of cytoplasm and nucleus. Necrosis of these cells was evident from plasma membrane rupture, swollen mitochondria and vacuolization within the cytoplasm. Similar observations were also made in Sertoli and spermatogenic cells of Shiba goats after treatment with Bisphenol- A.[7]

Liver

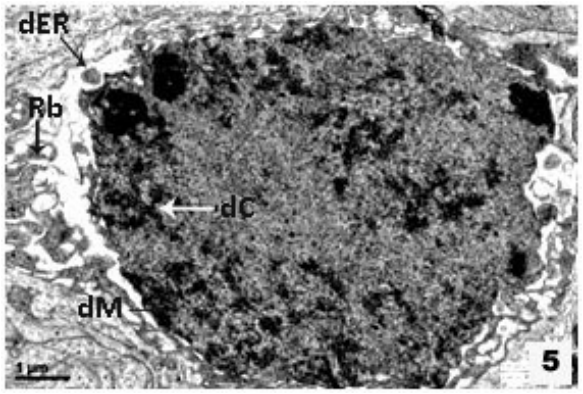
Electron microscopic examination of the control mice liver sections showed the normal ultrastructure. The hepatocytes contained normal euchromatic nuclei surrounded by cytoplasm and contained different cell organelles including mitochondria, cisternae of rough and smooth endoplasmic reticulum (Fig.7). Normal liver cells were provided with microvilli on the surfaces which formed the walls of bile canaliculi.

After 14 days of drug therapy the ultrastructural alterations were quite pronounced in the mitochondria and endoplasmic reticulum. The mitochondria were long and swollen lacking cristae. The mitochondrial membrane was disintegrated and the endoplasmic reticulum showed Vesiculation(Fig.8). Cytoplasmic accumulations of granular, electron dense material and concentrically laminated arrays of material are indicative of bile substances.Meanwhile, the mice administered with diclofenac for 28 days in the low dose group showed severe changes in the mitochondria and other cell organelles. There was accumulation of large number of lipid droplets and apoptotic bodies in the cytoplasm (Fig.9).After 14 days of diclofenac treatment in the high dose group, electron microscope examination of mice hepatocytes showed corrugated nuclei. Many areas of cytoplasm turned into electron lucent spaces separated with areas of aggregated strands endoplasmic reticulum and mitochondria (Fig.10). Some of the mitochondria appeared irregular with ruptured membranes. Kupffer cell hyperplasia was noticed and cytoplasm was filled with many cytoplasmic organelles, vacuoles and extravasated RBC's.There appeared large number of cytoplasmic vacuoles showing extreme degeneration of most of the cytoplasmic organelles after 28 days of treatment (Fig.11,12). The degenerated mitochondria were tube like showing swelling and were without membranes. In some hepatocytes the microvilli from adjacent hepatocytes were seen projecting into the intercellular canals.

Diclofenac and ibuprofen are known to cause severe ultrastructural changes in liver of rats.[8]Although, several ultrastructural changes with diclofenac treatment were noticed throughout the experiment but after prolonged administration the extent and severity of the changes were noteworthy.Most of the NSAIDs have been reported to target renal tissues.[9] The ultrastructural changes involved enlarged mitochondria, irregular and ruptured mitochondrial membrane, altered rough endoplasmic reticulum, destruction of mitochondrial cristae, and hypertrophied Golgi apparatus and nuclear changes in the form of distortion and destruction of nuclei. Mitochondria and endoplasmic reticulum were sensitive indicators to the slightest pathological changes induced by drug administration, which might lead to dysfunction. The mechanism of diclofenac induced mitochondrial injury involves generation of reactive oxygen species resulting in oxidative stress to hepatocytes[10]. Several reports have confirmed the vulnerability of mitochondria to diclofenac as evidenced by mitochondrial swelling and cristolysis.[11,12]Large prominent lysosomes including autophagic vacuoles and residual bodies observed have also been seen in a number of pathologic states of liver.[13,14,15] An increase in the number of autophagic vacuoles is seen as an indicator of focal cytoplasmic injury. The presence of these structures in large numbers indicated a more severe liver injury. Intact lysosomes seen occasionally showed advanced autolytic changes. These observations might confirm earlier reports on cells undergoing necrosis in vitro, where release of hydrolytic enzymes from lysosomes did not appear to play a significant role in initiating degenerative cytoplasmic alterations.[16]



Figures 1-4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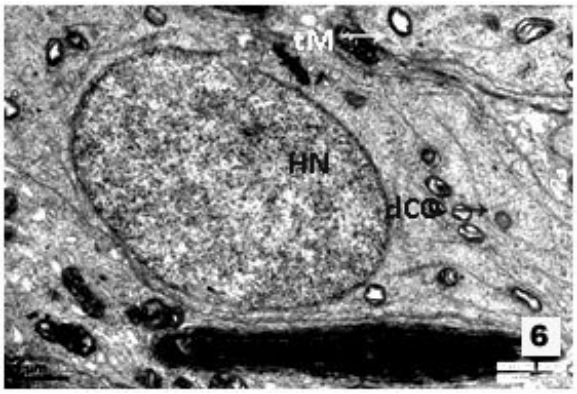
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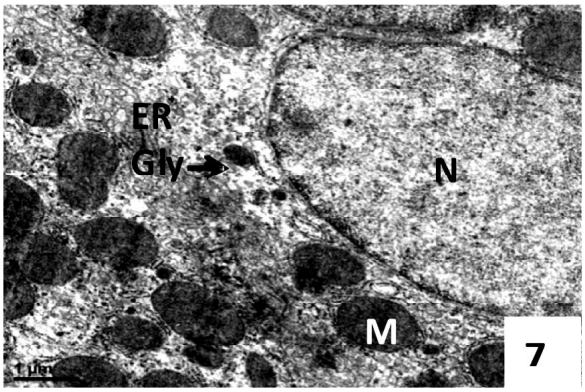
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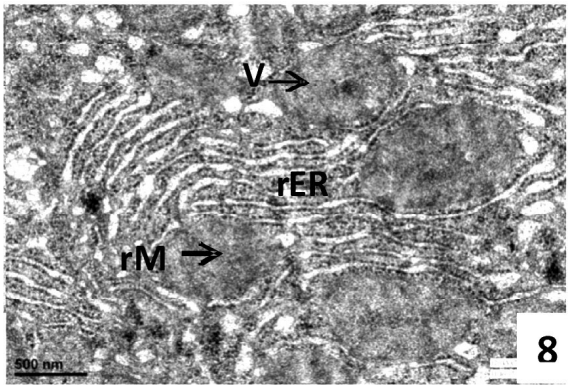
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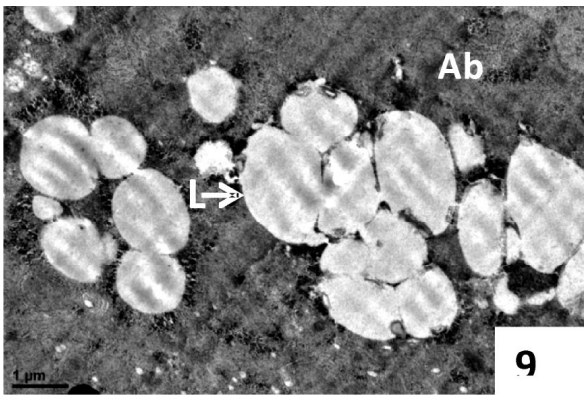
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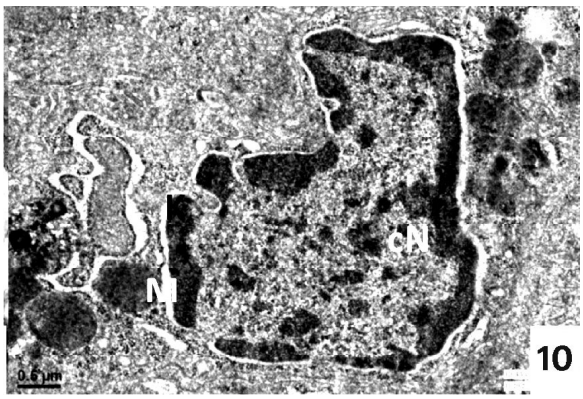
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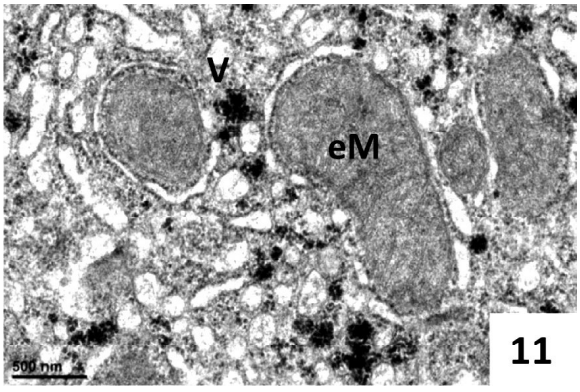
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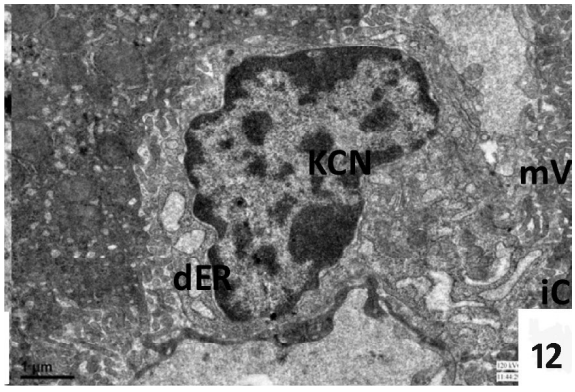
KCN
mV
dER
iC
12

Figures 5-6

Fig.1: Transmission electron micrographs of control mice testes showing normal type A spermatogonium with large euchromatic nucleus (N) having centrally placed nucleoli (nU).

Fig.2: Transmission electron micrograph of control mice testes with normal Sertoli cell nucleus having nuclear envelope (nE), normal mitochondria (m) and tight junctions (N).

Fig.3: Transmission electron micrograph of diclofenac administered mice testes (4mg/kg body weight) after 21 days depicting heterochromatic nucleus (HN), slightly degenerated nuclear membrane (dM), vesiculated mitochondria (vM) and multiple lysosomes (Ly).

Fig.4: Transmission electron micrograph of diclofenac administered mice testes (14mg/kg body weight) after 21 days exhibiting mitochondria with degenerated cristae (cd), cytoplasmic vesicles (cV), presence of dense bodies (dB) and apoptotic bodies (Ab),

Fig.5: Transmission electron micrograph of a diclofenac administered mice testes (14mg/kg body weight) after 28 days showing degenerated nuclear membrane (dM), dilated endoplasmic reticulum (dER) and multiple residual bodies (Rb) in the cytoplasm.

Fig.6: Transmission electron micrograph of mice testes (14mg/kg body weight) after 28 days showing tubular mitochondria(tM), disorganised cellular organelles (dCO) and chromatin material in the heterochromatized nuclei (HN).

Fig.7: Transmission electron micrograph of liver hepatocyte of control mice exhibiting hepatocyte with euchromatic nuclei (N). The cytoplasm contains electron dense mitochondria (M) with intact cristae, cisternae of endoplasmic reticulum (ER) and sparse glycogen (Gly).

Fig.8: Transmission electron micrograph of mice liver of treated group (4mg/kg b.w.) after 14 days showing ruptured mitochondrial membrane (rM) and stacked rough endoplasmic reticulum (rER). Cytoplasmic accumulations and vacuoles (V) a sign of degenerated hepatocytes can be seen.

Fig.9: Transmission electron micrograph of liver of treated group (4 mg/kg b.w.) after 28 days exhibiting the presence of large number of lipid droplets (L) and apoptotic bodies (Ab).

Fig.10: Transmission electron micrograph of mice liver of treated group (14mg/b.w.) after 14 days showing the presence of corrugated nucleus (cN), degenerated mitochondria (M). The presence of Kupffer cell (K) can also be noticed.

Fig.11: Transmission electron micrograph of mice liver of treated group (14mg/kg b.w.) after 28 days revealing degenerated and enlarged mitochondria (eM), large number of vacuoles in the cytoplasm (V).

Fig.12: Transmission electron micrograph of mice liver of treated group (14mg/kg b.w.) after 28 days exhibiting heterochromatized Kupffer cell nucleus (KCN), microvilli (mV) projecting into canals and intercellular canals (iC). Notice the presence of dilated cisternae of endoplasmic reticulum (dER).

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REFERENCES

1. **Brooks, P. M. and Day, R. O. 1991**.Nonsteroidal anti-inflammatory Drugs, differences and similarities. *N. Engl. J. Med*. **324:** 1716-1725.

2. **Kirchner, T., Aparicio, H., Argentieri, D. C., Lau, C. Y. and Ritchie, D. M. 1997**. Effect of tepoxalin, a dual inhibitor of cyclooxygenase/ 5 –lipoxygenase on events associated with NSAID induced gastrointestinal inflammation. *ProstaglandinsLeukot.Essent.Fatty Acids*.**56(6):** 417-423.

3. **Subramanian, S. 2009**. Diclofenac induced toxic manifestations in adjuvant induced arthritic rats peripheral and reproductive organs of male wistar rats *Rattusnorvagicus*. *J. Toxicol. Environ. Health Sci.* **1(1):** 12-21.

4. **Rosser, B. G. and Gores, G. J. 1995**. Liver cell necrosis: cellular mechanisms and clinical implications. *Gastroenterol.***108:** 252-275.

5. **Galati, G., Tafazoli, S., Sabzevari, O. and Chan, T. 2002**. Idiosyncratic NSAID drug induced oxidative stress. *Chem. Biol. Interact.* **142(1-2):** 25-41.

6. **Rashed- Morad, A., Sherif, H. and Ihab, K. 2011**. Electron

microscopic study on the effects of melatonin on early spermatids in rat testes.*J. Ani. Vet. Adv*. **10(16):** 2140-2148.

7. **Andriana, B. B., Tay, T. W., Hiramatsu, R., Awal, M. A., Kanai, Y., Kurohmaru, M. and Hayashi, Y. 2010**. Bisphenol-A induced morphological alterations in Sertoli and Spermatogenic cells of immature Shiba goats in vitro: An ultrastructural study. *Reprod. Med. & Biol*. **3:** 205-210.

8. **Moorthy, M., Fakurazi, S. and Ithnin, H. 2009**. The histomorphological analysis of liver following administration of low doses of diclofenac and ibuprofen.*J. Biol. Sci*. **9(7):** 676-681.

9. **Palmer, B. F. 1995**. Renal complications associated with use of non-steroidal anti-inflammatory agents. *J. Invest. Me*d. **43:** 516-553.

10. **Sokol, R. J., Straka, M. S., Daha, R., Devereaux, M. W., Yerushalmi, B. and Gumpricht, E. 2001**. Role of oxidant stress in the permeability transition induced in rat hepatic mitochondria by hydrophobic bile acid. *Pediatric Res*. **49:** 519–31.

11. **Somasundram, S., Rafi, S., Hayllar, J. and Sigthorsson, G. 1997**. Mitochondrial damage: a possible mechanism of the tropical phase of NSAID induced injury to the rat intestine. *Gut***41:** 344-353.

12. **Taib, N. T., Jarrar, B. M. and Mubarak, M. M. 2004**. Ultrastructural alterations in renal tissues of rabbit inducesd by Diclofenac sodium (Voltaren).*Saudi Med. J*. **25(10):** 1360-1365.

13. **Ashford, T. P. and Porter, K. R. 1962**. Cytoplasmic components in hepatic cell lysosomes.*J. Cell Biol.* **12:** 198-202.

14. **Stenger, R. J. 1963**. Hepatic parenchymal cell alterations after long term carbon tetrachloride administration.*Am. J. Pathol*. **43:** 867-895.

15. **Confer, D. B. and Stenger, R. J. 1964.** The evolution of lysosomes in hypoxic liver parenchyma as seen with electron microscope.*Am. J. Path*. **45:** 533-646.

16. **Trump, B. F., Goldblatt, P. J. and Stowell, R. E. 1962.** An electron microscopic study of early cytoplasmic alterations in hepatic parenchymal cells of mouse liver during necrosis in vitro (autolysis).*Lab. Invest*., **11:** 986-1015.

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