



Hepatotoxic Effect of Bisphenol A in Rats, an Immunohistochemical and Ultrastructural Study

P. Amaravathi^{*1}, Ch. Srilatha², V. Ramadevi³, D. Sreenivasulu⁴, P. Eswara Prasad⁵ and K. Sujatha²

¹State Level Diagnostic Laboratory, SVVU, Tirupati, INDIA

²Department of Veterinary Pathology, College of Veterinary Science, Tirupati, INDIA

³Department of Veterinary Pathology, College of Veterinary Science, Gannavaram, INDIA

⁴Dean of Extension, Sri Venkateswara Veterinary University, Tirupati, INDIA

⁵Associate Dean, College of Veterinary Science, Tirupati, INDIA

^{*}Corresponding author: P Amaravathi; Email: amarapvet@gmail.com

Received: 06 June, 2017

Revised: 10 Sept., 2017

Accepted: 20 Sept., 2017

ABSTRACT

Bisphenol A (BPA) is one of the common environmental endocrine disruptors with estrogenic properties and is the building block of carbonate plastic and a component of resin coatings. The present experiment was designed to make a systematic study of experimentally induced BPA toxicity in both male and female Wistar albino rats at 500 and 250 mg / Kg b.wt. to groups II, V and III, VI respectively by mixing in sunflower oil for 12 weeks. In the present study significant increase in thiobarbituric acid reactive substances and decrease in antioxidant enzyme levels like catalase, superoxide dismutase, reduced glutathione and glutathione peroxidase were observed in liver of all BPA treated rats when compared to corresponding controls. Histopathologically, the liver revealed binucleated cells, hyper chromatic nuclei, karyomegaly, extensive bile duct proliferation with dysplasia and proliferation of endothelial cells in BPA treated groups in dose dependent manner. Histochemically more intense alkaline phosphatase reaction was noticed in hepatocytes around central vein, Immunohistochemically increased expression of VEGF was observed in hepatocytes around central vein, Ultra structurally, hepatocytes of BPA treated groups revealed decreased mitochondria with degeneration, fragmented endoplasmic reticulum and clumping of nuclear chromatin.

Keywords: Bisphenol A, hepatotoxic, antioxidant enzymes, histopathology, VEGF

Bisphenol A (BPA) is one of the common environmental endocrine disruptors with estrogenic properties and is the building block of carbonate plastic and a component of resin coatings. It is being used in a wide variety of consumer products, including food and beverage packaging, compact disks, eye glass lenses, dental sealants, artificial teeth, cans, drums, reinforced pipes, adhesives, nail polish and carbonless papers used in receipts making BPA a ubiquitous part of our daily life (Richard *et al.*, 1987; Vandenberg *et al.*, 2007). Wide spread use of BPA in consumer products has led to great public concern since adverse effects of BPA on human and animal reproduction are suspected due to its estrogenic activity. BPA has high affinity to estrogen related receptor (ERR - γ) which may be related to its ability to function as endocrine disruptor

(WHO, 2009). Considering the limited availability of information the present study was undertaken to know the toxicological effects of BPA on liver in rats.

MATERIALS AND METHODS

Wistar albino rats with body weight around 150g (procured from Laboratory animal facility, Chennai) were used for the present experiment. Rats were acclimatized to the experimental conditions for one week, after acclimatization the animals were grouped and housed in standard poly propylene rat cages (three rats in 1 cage) during the experiment. They were maintained at 25 $\pm$ 1°C and a 12:12 hour interval light/dark cycle throughout the experimental period for 12 weeks by taking necessary



precautions and providing standard laboratory hygienic conditions and laboratory animal feed and water *adlibitum*. The approval of the institutional animal ethical committee was obtained prior to commencement of the experiment.

The Bisphenol A (4, 4' – dihydroxy – 2, 2 – diphenyl propane) with a laboratory reagent grade was procured from the Sd Fine chemicals, Bombay with 98.70% purity. In the present experiment, BPA was fed to Wistar albino rats at 500 mg / Kg b.wt. and 250 mg / Kg b.wt. to male (Groups II & III) and female rats (Groups V & VI) respectively by mixing in refined sun flower oil for 12 weeks. To the Groups I (male) and IV (female) rats, sunflower oil was given and were kept as controls. Six rats from each group were randomly sacrificed at every fortnight intervals after starting the experiment *i.e.* 2nd, 4th, 6th, 8th, 10th and 12th weeks.

Oxidative stress

At each sacrifice, tissue pieces of liver were collected and stored at –20°C until use to analyse oxidative stress.

Tissue preparation

Tissue pieces of liver were minced separately and homogenized in 0.05M ice cold phosphate buffer (pH 7.4) by using a virtis homogenizer to make 10% homogenate. The homogenate was mixed with 10% trichloroacetic acid in the ratio of 1:1, centrifuged at 15,000 rpm for 60 min at 4°C and the supernatant obtained was used for estimation of thiobarbituric acid reactive substances (Yagi, 1976), super oxide dismutase (Marklund and Marklund, 1974), catalase (Caliborne, 1985), reduced glutathione (Moron *et al.*, 1979) and glutathione peroxidase (Rotruck *et al.*, 1973) in liver of all rats in all groups.

Gross and histopathology

A detailed postmortem examination was conducted on all the sacrificed rats in all the experimental groups. The gross lesions were recorded and representative tissue pieces from liver were collected and preserved in 10% neutral buffered formalin for histopathological studies. Fixed tissues were processed by routine paraffin embedding technique. Sections of 5 to 6 microns thickness were cut and were stained with routine Haematoxylin and Eosin method (H&E) (Culling, 1974).

Histochemistry

Pieces of liver from both experimental and control groups were collected in chilled neutral buffered formalin. Frozen sections were taken and the alkaline phosphatase activity in liver was demonstrated by the Gomori's method (Bancroft and Cook, 1994).

Immunohistochemical studies

VEGF (Vascular Endothelial Growth Factor) marker was used to know the endothelial proliferation in liver of both experimental and control groups. For the immunohistochemical studies, the primary and secondary antibodies were procured from BioGenex company. (Sujatha *et al.*, 2013).

Electron microscopic studies

The specimens (subjected for TEM examination) were rinsed in 0.1M phosphate buffer pH 7.2 (PB) to remove blood from the surface. Liver tissues greater than 2 cm long were minced into smaller pieces of approximately 3 × 3 mm and were fixed in 3% glutaraldehyde, buffered with phosphate buffer for 3 hours. It was rinsed twice with phosphate buffer for 10 minutes per rinse. The tissues were then fixed in 2% aqueous osmium tetroxide for 2 hrs and rinsed in 3 changes of distilled water for 10 minutes. Each dehydration was accomplished by immersion in a graded series of ethanol solutions of 25, 50, 75, 95 and 100%. Infiltration with propylene oxide and embedding with increasing concentrations of propylene oxide followed by dehydration were carried. Thin sections (600 nm) were obtained by use of Ultra microtome and were placed on a copper 200 – mesh grid. They were stained with uranyl acetate and lead citrate. Two blocks were prepared for each specimen and two grids/ blocks were observed. Thereafter, four grids were observed for each specimen.

Statistical analysis

The results were analyzed statistically by performing one way ANOVA (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Oxidative damage

There was a significant ($P < 0.05$) increase in Thiobarbituric

Acid reactive substances (TBARS) and decrease in catalase, super oxide Dismutase (SOD), reduced glutathione and glutathione Peroxidase (GPx) values in BPA treated groups in dose dependent manner in both male and female rats when compared to controls (Group I and IV) and the results were shown in Table 1. There was no significant difference between corresponding male and female BPA treated rats.

Similar results were reported by Bindhumol *et al.* (2003), Chitra *et al.* (2003) and Kabuto *et al.* (2003). The decrease in antioxidant enzymes activity might be due to BPA induced generation of reactive oxygen species (ROS).

Gross and histopathology

In the present study liver revealed moderate enlargement during 2nd and 4th weeks. Moderate to severe enlargement of the liver with paleness (Fig. 1) and focal areas of hemorrhages were observed from 6th week onwards in all BPA treated groups in a dose dependent manner. European Union (2003) studies also revealed pale livers in BPA treated rats.

Microscopically, liver revealed diffuse areas of congestion, thrombus formation, sinusoidal dilatation and sinusoidal congestion in majority of rats throughout the experimental period. By the end of 2nd week, livers of Group II and V rats revealed mild sinusoidal dilatation with congestion, degenerated hepatocytes, focal loss of hepatocytes with MNC (Mononuclear cell) aggregates, mild to moderate

perivascular and periductular infiltration of MNCs and mild periductular fibrosis.

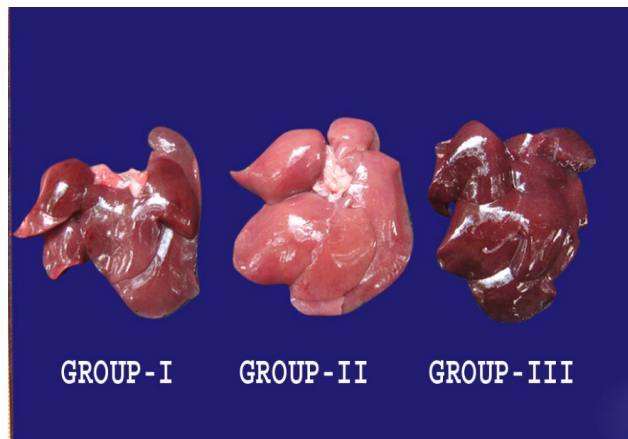


Fig. 1: Liver: Group II to III: Mild to moderate enlargement and paleness

In addition, binucleated hepatocytes, anisokaryosis, karyomegaly, mild vesicular fatty change in paracentral hepatocytes, mild proliferation of bile ducts, moderate proliferation of Kupffer cells and perivascular infiltration of MNCs and periductular and perivascular fibrosis were more prominent in all rats and were more evident in some of the rats by the end of 4th and 6th week. At 8th week, diffuse areas of congestion and binucleated hepatocytes with hyper chromatic nuclei were noticed in majority of the rats. Moderate diffuse vesicular fatty changes, focal areas of necrosis, increased number of apoptotic bodies,

Table 1: Effect of BPA on oxidative stress in liver

Groups	TBARS (nM of MDA / g of tissue)	Catalase (nM of H ₂ O ₂ decomposed / min/mg of protein)	SOD (U/min/mg of protein)	Reduced glutathione (µg GSH / g tissue)	GPx (U/min/mg of protein)
I	494.22 ± 22.93 ^a	0.27 ± 0.02 ^a	14.34 ± 0.52 ^a	518.65 ± 13.94 ^a	29.14 ± 1.52 ^a
II	1100.23 ± 165.18 ^e	0.11 ± 0.03 ^{de}	7.55 ± 1.17 ^{de}	294.43 ± 38.31 ^e	15.84 ± 2.15 ^e
III	879.45 ± 113.05 ^{bcd}	0.14 ± 0.03 ^{cde}	8.87 ± 1.35 ^{bcd}	355.18 ± 39.57 ^{cde}	16.95 ± 2.00 ^{cde}
IV	504.4 ± 24.36 ^a	0.26 ± 0.01 ^a	13.18 ± 0.26 ^a	520.20 ± 14.34 ^a	27.76 ± 1.64 ^a
V	1081.38 ± 155.72 ^{de}	0.11 ± 0.03 ^e	7.09 ± 1.6 ^e	306.86 ± 34.35 ^{de}	16.15 ± 1.78 ^{de}
VI	923.87 ± 141.15 ^{bcd}	0.15 ± 0.03 ^{bcd}	8.58 ± 1.45 ^{bcd}	357.84 ± 33.08 ^{bcd}	17.71 ± 1.64 ^{bcd}

Mean values with different subscripts differ significantly (P<0.05)

SE - standard Error



severe sinusoid dilatation with hemorrhages, extensive bile ductular proliferation with dysplastic changes around bile ducts as well as in parenchyma (Fig. 2), periportal fibroblast proliferation and MNC infiltration were observed during 10th week of BPA feeding. In addition, severe vesicular fatty change as well as at places focal areas of necrosis, perivascular MNC infiltration, prominent apoptosis, proliferating blood capillaries and proliferation of endothelial cells were evident prominently. When frozen sections were stained with Oil Red 'O' stain, the areas of fat filled hepatocytes and Kupffer cells were stained red indicating fatty change and it was more conspicuous in all the rats by the end of 12th week.

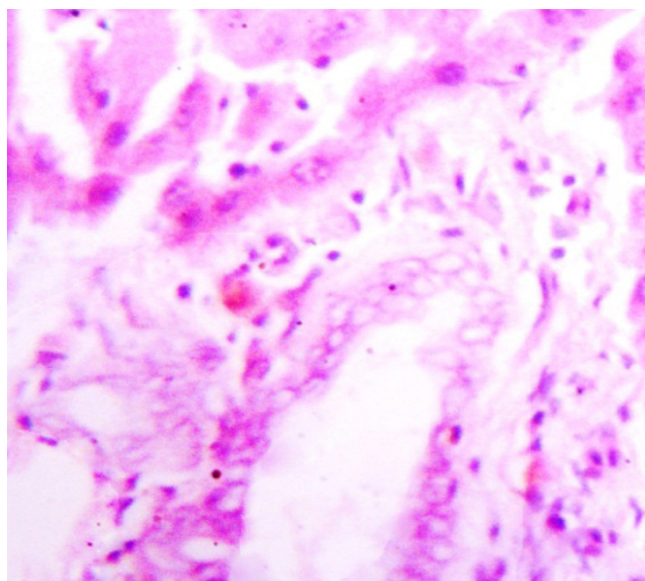


Fig. 2: Liver: Group II: Section showing extensive bile ductular proliferation with dysplasia. H & E: $\times 280$

Histopathologically, by the end of 2nd and 4th weeks the livers of majority group III and VI rats, revealed focal areas of congestion, mild sinusoidal congestion and dilatation, binucleated hepatocytes, hyper chromatic nuclei, anisokaryosis, karyomegaly, periportal infiltration and mild bile ductular proliferation and the lesions were more conspicuous in 6th and 8th weeks. Dilated sinusoids with moderate congestion, bile ductular proliferation with dysplasia and periportal fibrous tissue proliferation were more evident in the rats exposed for 10 weeks. Mild to moderate vesicular fatty change (Fig. 3), Kupffer cell proliferation, apoptosis and endothelial cell proliferation were more striking by the end of 12 weeks. There was

no difference in histopathological changes between corresponding male and female BPA treated rats.

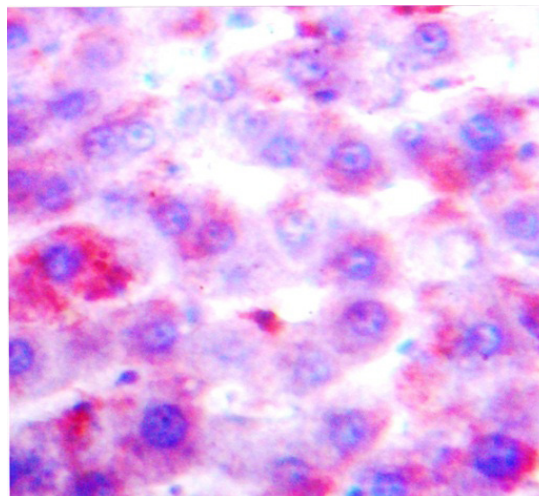


Fig. 3: Liver: Group II: Section showing vesicular fatty change in hepatocytes and Kupffer cells. Oil Red 'O' stain: $\times 280$

NTP (1982) observed congestion, inflammation, fibrosis, fatty metamorphosis, focal cellular change, eosinophilic cytochange, multinucleated giant hepatocytes, hyperplasia and inflammation of bile duct in rats treated with BPA. Tyl *et al.* (2002) and Yamasaki *et al.* (2002) observed chronic hepatic inflammation in rats. Increased proliferation/ apoptosis ratio in both epithelial and stromal compartments in rats, an increased number of hyperplastic ducts, augmented stromal nuclear activity in liver and stroma associated with hyperplastic ducts with signs of desmoplasia and presence of increased number of mast cells suggesting a heightened risk of neoplastic transformation were noticed by Milena *et al.* (2007). These changes might be due to increased lipid peroxidation in liver by the BPA metabolites and decreased antioxidant enzymes.

Histochemistry

Alkaline phosphatase activity was detected in liver using Gomori method. In sections, areas with brownish to black color development indicate enzyme activity. In present study, more intense reaction was observed in hepatocytes around central vein (Fig. 4). Increased activity of alkaline phosphatase in hepatocytes around central vein might be due to increased oxidative damage caused by BPA.

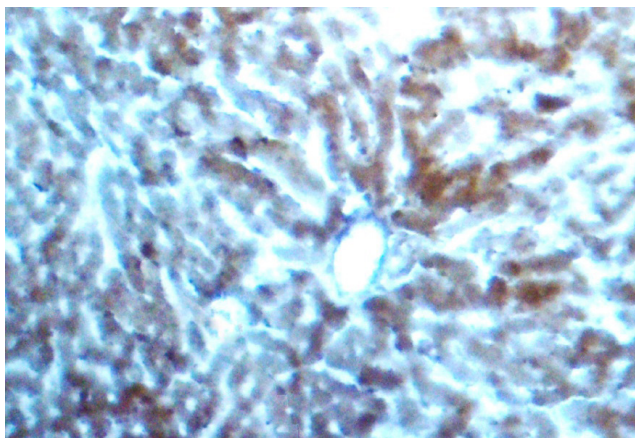


Fig. 4: Liver: Alkaline phosphatase:Group II: Note intense reaction in hepatocytes around central vein. Gomori stain: $\times 70$

Immunohistochemistry

Immunohistochemistry was carried out for detection of VEGF using monoclonal antibodies against VEGF and a place where the brown color develops indicate the presence of antigen. The intensity was higher in cytoplasm of hepatocytes around central vein and peripheral hepatocytes (Fig. 5).

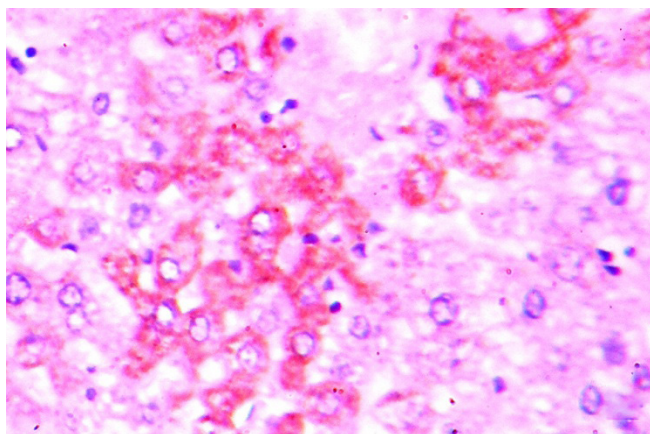


Fig. 5: Liver: Immunohistochemistry: Group II: Note reaction in hepatocytes in hepatic lobule. VEGF: $\times 280$

This increase could be related to hypoxia caused by oxidative damage which results in releasing of hypoxia inhibition factor (HIF) that in turn stimulate the release of VEGF that causes neovascularization. This VEGF plays an important role in aiding growth of some tumors by promoting both tumor angiogenesis and stromal generation (Nagy *et al.*, 1998; Dvork *et al.*, 1991) and also

involved in developing of striking alterations in vascular permeability and changes in neovascularisation in the processes such as embryonic development, neoplasia and wound healing (Brygida *et al.*, 1992; Xinghua *et al.*, 2001). Increased capillary proliferation was noticed prominently and consistently in almost all organs of BPA treated rats.

Ultra structural studies

Examination of transmission electron microscopy of Group II and V hepatocytes revealed detached cytoplasmic organelle from cell membrane (Fig. 6), decrease in the number and size of endoplasmic reticulum (Fig. 7), reduced size of mitochondria and degenerated mitochondria with chemical deposition in cytoplasm (Fig. 8), cytoplasmic vacuolation in both hepatocytes and Kupffer cells and disrupted nuclear membrane, clumping and margination of nuclear chromatin in majority of hepatocytes and Kupffer cells (Fig. 9). Whereas in Groups III and VI rats, few hepatic cells revealed reduced size of mitochondria and decreased endoplasmic reticulum in majority of cells.

Examination of transmission electron microscopy of hepatocytes in higher dose of BPA fed rats (Groups II and V) revealed detached cytoplasmic organelles from cell membrane, decreased quantity and size of endoplasmic reticulum, reduced size of mitochondria and degenerated mitochondria, cytoplasmic vacuolation in both hepatocytes and Kupffer cells. Majority of hepatocytes and endothelial cells revealed disrupted nuclear membrane, clumping and margination of nuclear chromatin, whereas in lower dose of BPA fed rats (Groups III and VI), few hepatic cells revealed reduced size of mitochondria and decreased ER in majority of cells. All the results might be due to ER stress. The ER stress might be due ROS production and is independent of estrogen receptors (Junpei *et al.*, 2010).

In the present study toxic changes were noticed in liver. Severe congestion, proliferation of endothelial cells of blood vessels and increased expression of VEGF indicates that BPA is having effect on expression of endothelial cells. Ultra structural changes in liver indicated the toxic effects of BPA at sub cellular level. It was concluded that BPA at 500mg/Kg b.wt was highly toxic to rats. More extensive studies are required to know the molecular mechanisms involving in the production of these changes in liver caused by BPA.

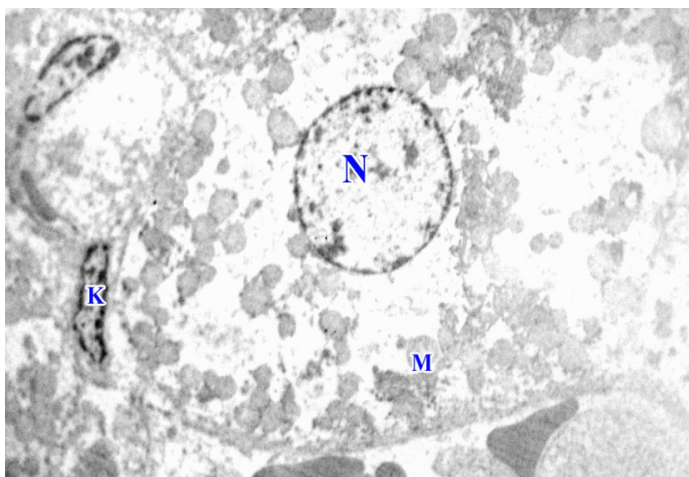


Fig. 6: Liver: Transmission Electron Microscopy: Group II: Note decreased number of mitochondria (M), margination of nucleus (N) and Kupffer cell (K). Uranyl acetate: $\times 2685$

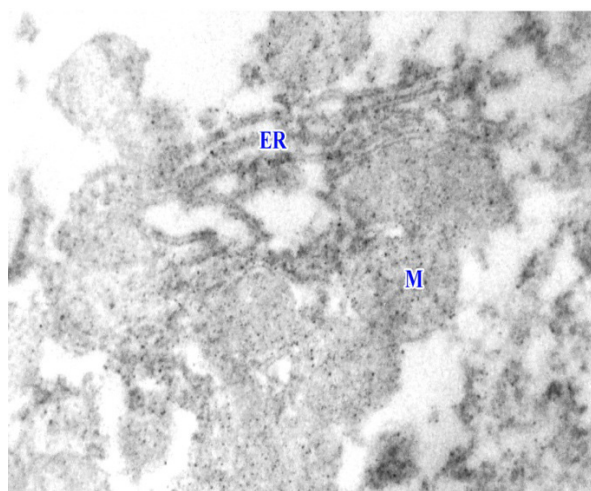


Fig. 7: Liver: Transmission Electron Microscopy: Group II: Section showing decreased quantity of Endoplasmic reticulum (ER). Uranyl acetate: $\times 17900$

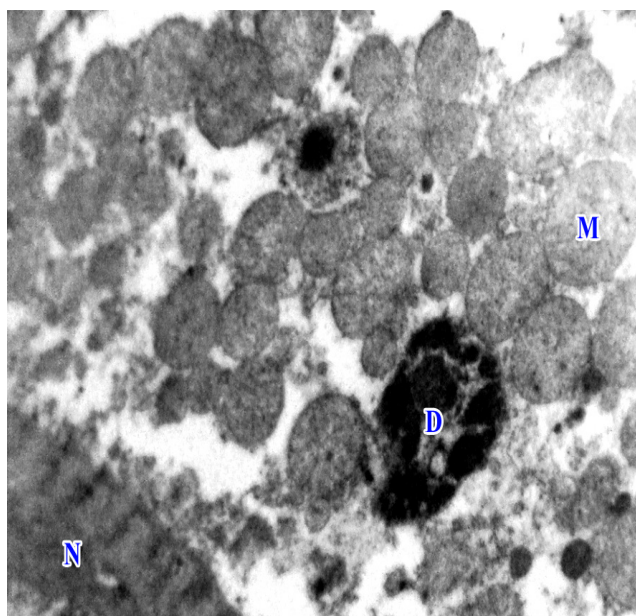


Fig. 8: Liver: Transmission Electron Microscopy: Group II: Note reduced size of mitochondria (M) and degenerated mitochondria with chemical deposition (D) in cytoplasm. Uranyl acetate: $\times 5370$

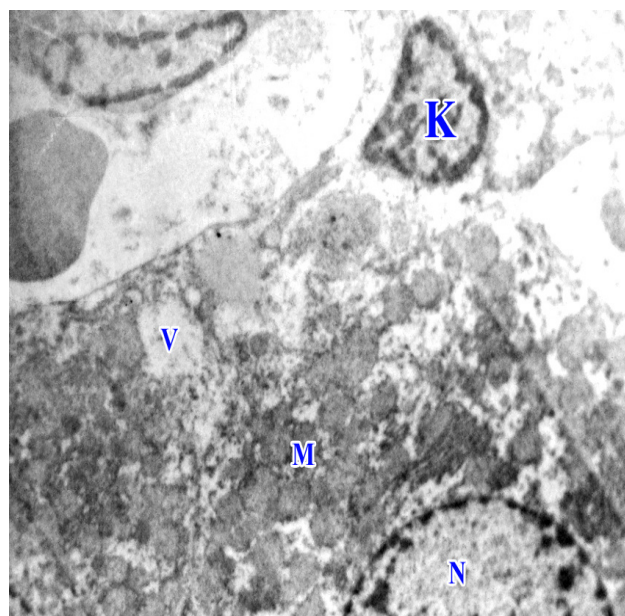


Fig. 9: Liver: Transmission Electron Microscopy: Group II: Section showing cytoplasmic vacuolation (V) in both hepatocytes and Kupffer cells and disrupted nuclear membrane, clumping and margination of nuclear chromatin in of hepatocytes and Kupffer cells (K). Uranyl acetate: $\times 4060$

ACKNOWLEDGEMENTS

The authors are thankful to Sri Venkateswara Veterinary University for providing facilities to carry out this work.

REFERENCES

- Bancroft, D.J. and Cook, C.H. 1994. Manual of Histological Techniques and their Diagnostic Application. Churchill Livingstone, pp. 297-300. Medical Division of Longman Group UK Limited.
- Bindhumol, V., Chitra, K.C. and Mathur, P.P. 2003. Bisphenol A induces reactive oxygen species generation in the liver of male rats. *Toxicology*, **188** (2-3): 117-124.
- Brygida Berse, Lawrence, F.B., Livingston Van De Water, Harold, F.D. and Donald, R.S. 1992. Vascular permeability factor (Vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages and tumors. *Mol. Biol. Cell.*, **3**: 211 – 220.
- Caliborne, A.L. 1985. Assay of catalase. In Handbook of Methods for Oxygen Radical Research. Ed. Greenward, R.A. pp. 283-284. CRC Press, Baco- Raton.
- Chitra, K.C., Ramachandra Rao. and Muthus, P.P. 2003. Effect of bisphenol A and co-administration of bisphenol A and vitamin C on epididymis of adult rats: A histological and biochemical study. *Asian. J. Androl.*, **5**: 203-208.
- Culling, C.F.A. 1974. Hand Book of Histopathological and Histochemical Techniques (Including Museum Techniques) 3rd Edition pp. 361. The University of Michigan, Butterworth.
- Dvorak, H.F., Sioussat, T., Brown, L.F., Berse, B., Nagy, J.A., Sotrel, A., Manseau, E.J., Van De Water, L. and Senger, D.R. 1991. Distribution of Vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. *J. Exp. Med.*, **174**: 1275 – 1278.
- European Union. 2003. Risk assessment of report – 4, 4' – isopropylidenediphenol (Bisphenol A).
- Junpei Asahi., Hanako Kamo., Ryoko Baba., Yoshiaki Doi., Akihisa Yamashita., Daisuke Murakami., Ayuko Hanada. and Takeshi Hirano. 2010. Bisphenol A induces endoplasmic reticulum stress associated apoptosis in mouse non – parenchymal hepatocytes. *Life Sci.*, **87**(13 – 14): 431 – 438.
- Kabuto, H., Hasuike, S., Minagawa, N. and Shishibori, T. 2003. Effects of bisphenol A on the metabolism of active oxygen species in mouse tissues. *Environ. Res.*, **93**(1): 31 – 35.
- Marklund, S.L. and Marklund, G. 1974. Involvement of superoxide anion radical in the auto oxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.*, **47**: 496 – 474.
- Milena Durando., Laura Kass., Julio Piva., Carlos Sonnenschein., Ana M.S., Enrique H.L. and Minica Munoz de Toro. 2007. Prenatal Bisphenol A exposure induces preneoplastic lesions in the mammary gland in Wistar rats. *Environ. Health. Persp.*, **115**(1): 80 – 86.
- Moron, M.S., Depierre, J.W. and Mannervik, B. 1979. Levels of glutathione, glutathione reductase and glutathione- S-transferase activities in rat lung and liver. *Acta. Biochem. Biophys.*, **582**: 67- 78
- Nagy, J.A., Brown, L.F., Senger, D.R., Lanir, N., Van De Water, L., Dvorak, A.M. and Dvorak, H.F. 1998. Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. *Biochem. Biophys. Acta.*, **948**: 305 – 326.
- NTP (National Toxicology Program). 1982. Carcinogenesis bioassay of Bisphenol A in F344 rats and B6C3F1 mice (Feed study) technical report studies No. 215.
- Richard, E.M., Julia, D.G., Catherine, J.P., Rochelle, W.T., Melissa, C.M. and Carole, A.K. 1987. The developmental toxicity of Bisphenol A in rats and mice. *Fund. Appl. Toxicol.*, **8**: 571 – 582.
- Rotruck, J.D., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G. and Hekstra. 1973. Selenium, biochemical role as a component of glutathione peroxidase and assay. *Science.*, **179**: 588 -590.
- Snedecor, W.G. and Cochran, G.W. 1967. Statistical Methods, 6th Edition, pp. 258 – 268. Oxford and IBH Publishing Company, New Delhi.
- Sujatha, K., Srilatha, Ch., Anjaneyulu, Y., Chandra Sekhar Rao, T.S., Sreenivasulu, D. and Amaravathi, P. 2013. Expression of BaX and Bcl2 in lead induced apoptosis in rat liver and kidney and its amelioration with *Ocimum Sanctum*: A leaf extract. *J. Toxicol. Health. Photon.*, **103**: 145-149.
- Tyl, R.W., Myers, C.B., Marr, M.C., Thomas, B.F., Keimowitz, A.R., Brine, D.R., Veselica, M.M., Fail, P.A., Chang, T.Y., Seely, J. C., Joiner, R. L., Butala, J.H., Dimond, S.S., Cagen, S.Z., Shiotsuka, R.N., Stropp, G.D. and Waechter, J.M. 2002. Three generation reproductive toxicity study of bisphenol A in CD Sprague - Dawley rats. *Toxicol. Sci.*, **68**: 121 – 146.
- Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N. and Welshons, W.V. 2007. Human exposure to Bisphenol A (BPA). *Reprod. Toxicol.*, **24**: 139 – 177.
- WHO. 2009. Bisphenol A (BPA) – Current state of knowledge and future actions by WHO and FAO.
- Xinghua Long., Kathleen, A.B., Robert, M.B. and Kenneth, P.N. 2001. Effects of the xenoestrogen Bisphenol A on expression of vascular endothelial growth factor (VEGF) in the rat. *Exp. Biol. Med.*, **226** (5): 477 – 483.



- Yagi, K. 1976. Simple fluorimetric assay for lipid peroxides in blood plasma. *Biochem. Med.*, **15**: 212 – 216.
- Yamasaki, K., Sawaki, M., Noda, S., Imatanaka, N. and Takatsuki, M. 2002. Subacute oral toxicity study of ethynylestradiol and Bisphenol A, based on the draft protocol for the “Enhanced OECD test guideline no. 407. *Arch. Toxicol.*, **76**: 65 – 74.