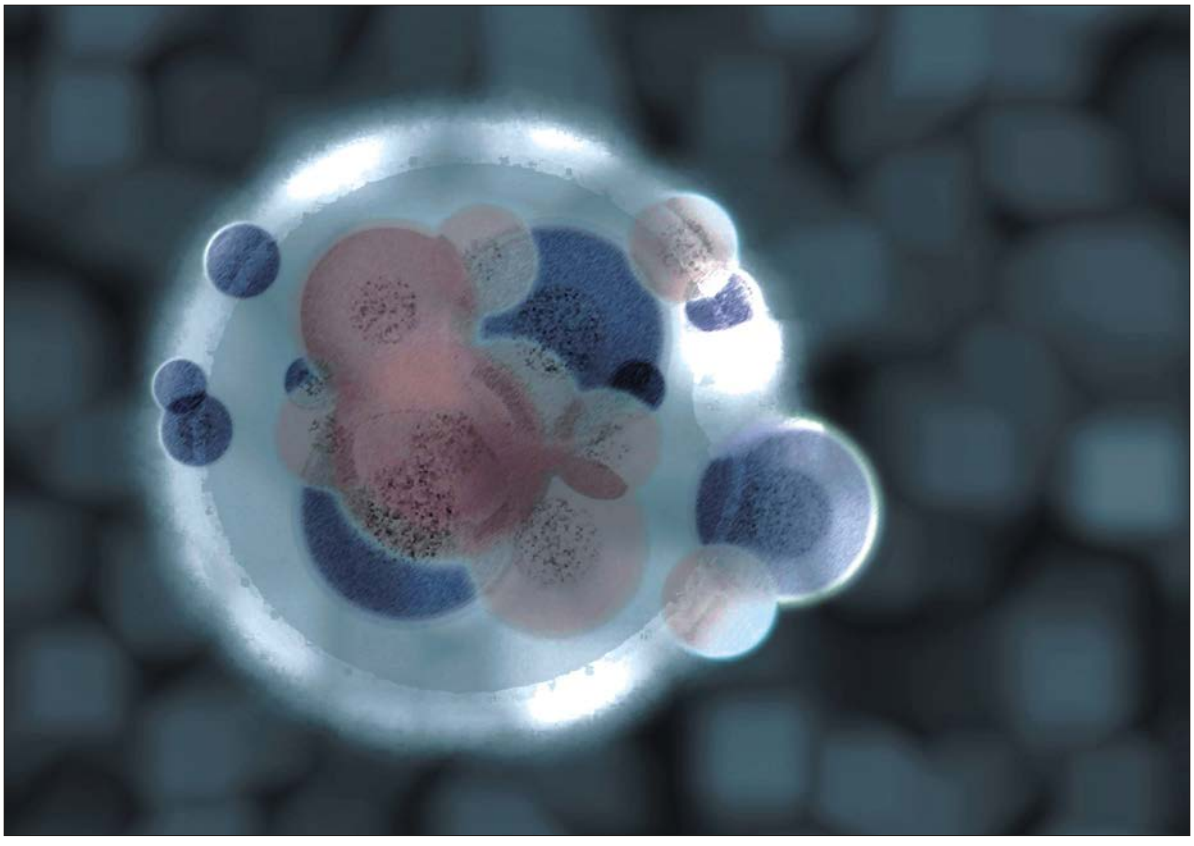

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 9 (7): 676-681, 2009
ISSN 1727-3048

The Histomorphological Analysis of Liver Following Administration of Low Doses of Diclofenac and Ibuprofen

[1]M. Moorthy, [1]S. Fakurazi and [2]H. Ithnin
[1]Department of Human Anatomy,
[2]Department of Patology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia

Abstract: This study was conducted to investigate and to compare liver perturbation following administration of low doses of diclofenac and ibuprofen to rats. Hundred and forty-four male Sprague Dawley rats were dosed with 3, 5 and 10 mg kg^{-1} diclofenac and ibuprofen in saline via intraperitoneal injection for 15 days. The control group was administered with saline in a similar manner. Four rats were euthanised every three days until day 15. Livers were removed, cleaned and a section across the right lobe was taken and fixed in 10% formalin for light microscopy and TUNEL assay. One-way ANOVA was used to analyse the data. $p<0.05$ was accepted as significant in this study. Three, 5 and 10 mg kg^{-1} diclofenac-treated groups and 5, 10 mg kg^{-1} ibuprofen administered groups showed significant changes compared to saline-treated group at day 15. The changes include presence of focal infiltration by neutrophils and lymphocytes and mild focal necrosis. In 5 and 10 mg kg^{-1} diclofenac administered groups and 10 mg kg^{-1} ibuprofen-treated group, apoptotic cells were seen around the perivenular regions (PV) only at day 15. However, not all the PVs were present with apoptotic cells. This study has shown that, diclofenac is probably more potent in inducing histomorphological changes at low doses. Both the drugs seem to exert time and dose dependent liver morphological alterations to the treated animals.

Key words: Non-steroidal anti-inflammatory drugs, hepatocytes, apoptosis

INTRODUCTION

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are group of widely used drugs for the treatment of rheumatoid diseases and to relief pain and inflammation due to their analgesic, anti-pyretic and anti-inflammatory properties (Galati *et al*., 2002; O'Connor *et al*., 2003; Teoh and Farrell, 2003). They were identified as the major group of drugs in causing hepatic injury in United States of America (Laine, 2001; Galati *et al*., 2002).

Diclofenac, is a commonly used NSAID in the treatment of rheumatic diseases (Boelsterli, 2003; Bort *et al*., 1999). Significant hepatotoxicity were seen in 1-5 per 100,000 patients consuming this drug (Tolman, 1998). The hepatotoxicity is believed to be due to either metabolic or immunologic idiosyncrasy (Boelsterli, 2003). Metabolites of diclofenac were identified as toxicants that can undergo covalent binding with various nonprotein or protein groups (Boelsterli, 2003). Many findings also indicate that the metabolites are capable of causing apoptosis of hepatocytes (Gomez-Lechon *et al*., 2003a, b; Masubuchi *et al*., 2002). This was identified to be related to the ability of the drug to cause oxidative stress that is followed by mitochondrial permeability transition (MPT) (Masubuchi *et al*., 2002). MPT was found to cause leakage of cytochrome c and other apoptotic components from mitochondria into the cytosol, leading to activation of caspase cascade. This even ends with apoptosis of hepatocytes.

Ibuprofen-induced hepatotoxicity was related to the widespread use of the drug (Al-Nasser, 2000). An analysis on risk of individual NSAIDs for liver disease resulting in hospital admission indicated 44.6 per 100,000 patient-years for ibuprofen (Rubeinstein and Laine, 2004). Ibuprofen-induced hepatocellular injury could be related to the formation of acyl glucuronide (Castillo *et al*., 1995), since covalent binding of ibuprofen acyl glucuronide to plasma protein was found in all patients taking the drug (Castillo *et al*., 1995). However, the mechanism by which liver injury is inflicted is still unclear. As with diclofenac, recent study with ibuprofen was also found to induce apoptosis in cell line (Campos *et al*., 2004). It could be

Corresponding Author: Mohanambal Moorthy, Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia Tel: 012-3236537

related to the ability of this drug to induce MPT, since it has been shown to target mitochondria (Al-Nasser, 2000). The exact underlying mechanism is still not clear and under extensive study.

This study was conducted to investigate and to compare liver perturbation following administration of low doses of diclofenac and ibuprofen to rats.

MATERIALS AND METHODS

Animal study: One hundred and forty four male Sprague Dawley rats were purchased from the Faculty of Veterinary Medicine, Universiti Putra Malaysia. The rats were then acclimatized under controlled condition of humidity with regular light/dark cycle and free access to feed and water for one week. The rats were randomly distributed into 3, 5 and 10 mg kg^{-1} diclofenac and ibuprofen groups and control group. The treatment groups were administered with diclofenac and ibuprofen in saline intraperitoneally at 0.5 mL/rat/day. The control group was given saline in a similar manner. Four rats from each group were euthanised every 3 days until day 15. Upon euthanisation, the livers were removed and cleaned in normal saline. A section across the right lobe was taken and fixed in 10% (v/v) formalin for further analysis.

Harris Hematoxylene and Eosin (H and E) staining: Ten percent formalin fixed liver samples were processed in graded ethanol and xylene and embedded with paraffin. Sections of 3-4 μm were made and fixed on microscope slides. These slides were then stained with Harris Hematoxylene and Eosin (H and E) stain and mounted with DPX. The prepared slides were viewed under light microscope and scored according to Table 1.

TUNEL assay: This assay was carried out with DeadEnd Colorimetric Tunel System (Promega). Paraffin embedded sections on poly-L-lysine coated slides were treated according to the manufacturer's instruction.

Statistical analysis: One-way ANOVA was used to analyse the data. $p<0.05$ was accepted as significant in this study.

Table 1: Lesion scoring table for morphological analysis of H and E stained liver sections

Description	Score
Preservation of normal liver architecture	0
Hydrophic degeneration	1
Fatty change	2
Infiltration by lypmphocytes	3
Infiltration by neutrophils	4
Necrosis	5

Adapted and modified from Aydin *et al*. (2003)

RESULTS

Rats administered with 3 mg kg^{-1} diclofenac for 15 days showed significant presence of lymphocytic infiltration as shown in Fig. 1 with the scoring of above 3.0. Lesion scoring analysis for 5 mg kg^{-1} diclofenac-treated rats revealed scoring of 4.3. Liver sections revealed similar observation to 3 mg kg^{-1} diclofenac with the addition of neutrophilic infiltrations mainly at PV regions at day 15 as indicated in Fig. 2. Liver sections obtained from rats administered with 10 mg kg^{-1} diclofenac showed significant difference in hepatocellular morphology compared to saline on day 15 with the scoring of 4.7. The observation was similar to the lower

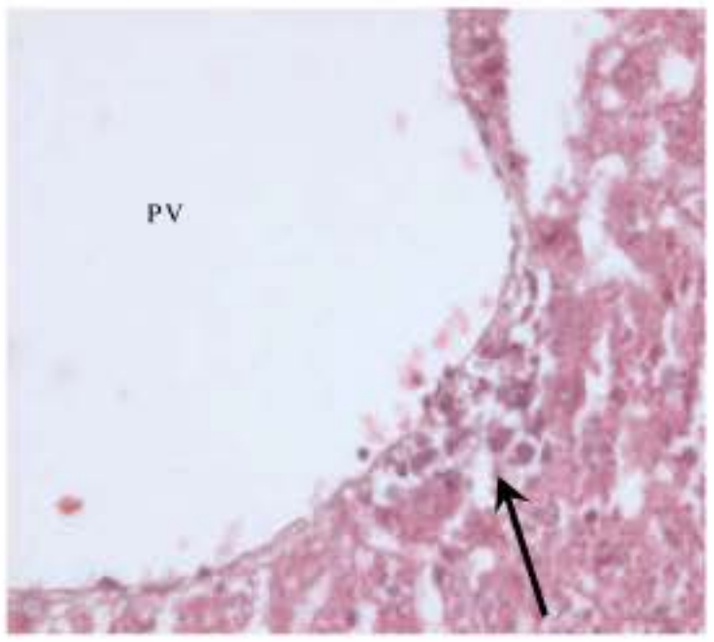

Fig. 1: Photomicrograph shows liver section of 3 mg kg^{-1} diclofenac-treated rat. Arrow indicates mild focal infiltration by lymphocytes around PV at day 15. Magnification: 400x. H and E staining

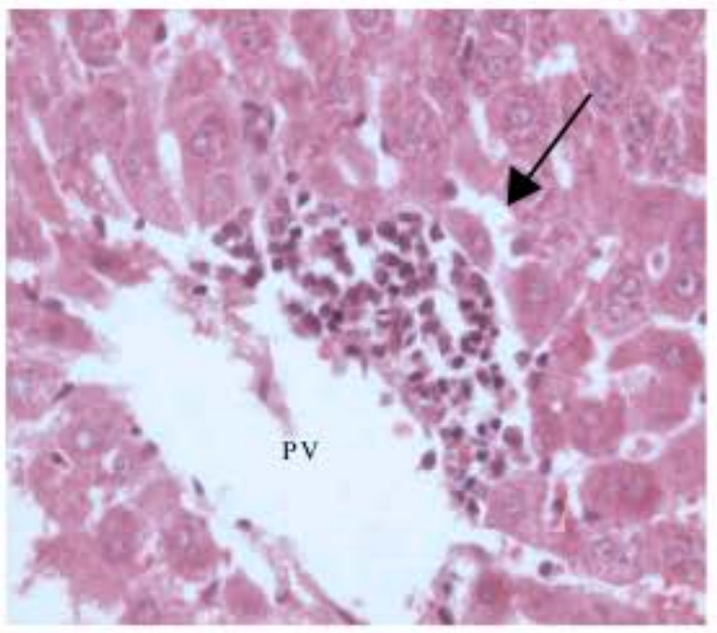

Fig. 2: Photomicrograph shows liver section of rat administered with 5 mg kg^{-1} diclofenac at day 15. Arrow indicates mild lymphocytic and neutrophilic infiltration around PV. Magnification 400x. H and E staining

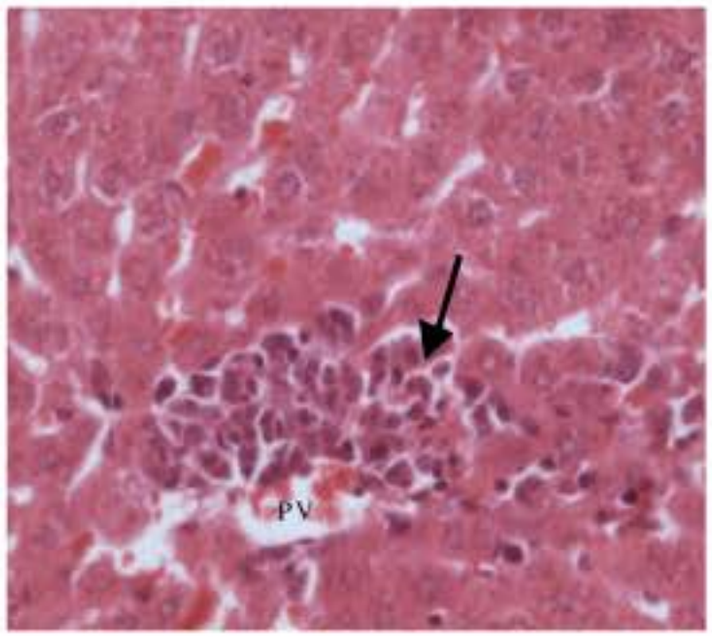

Fig. 3: Photomicrograph shows liver section of rat administered with 10 mg kg^{-1} diclofenac at day 15. Arrow indicates mild focal necrosis around PV. Magnification 400x. H and E staining

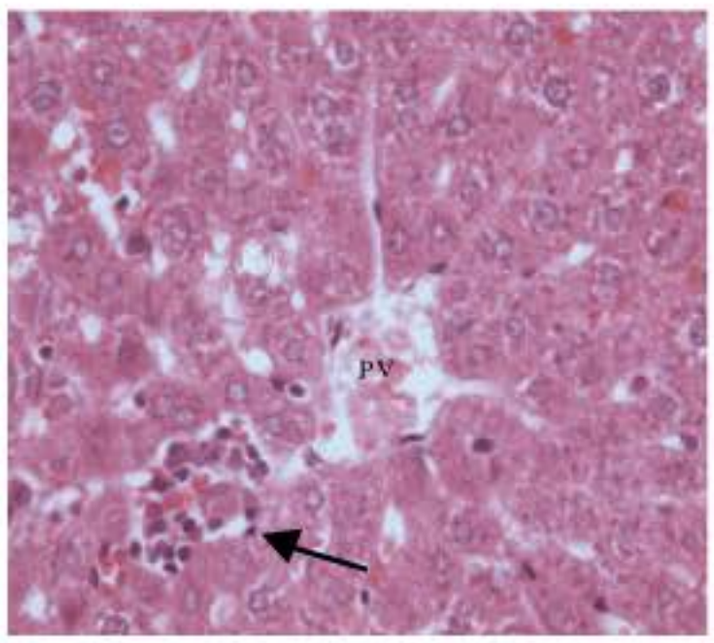

Fig. 4: Photomicrograph shows liver section of rat administered with 5 mg kg^{-1} ibuprofen at day 15. Arrow indicates mild focal lymphocytic and neutrophilic infiltration around PV. Magnification 400x. H and E staining

doses with mild focal hepatocyte necrosis as seen in Fig. 3. The changes in these groups were mainly seen around PV regions and to a lesser extent around periportal (PT) regions. Treatments of less than 15 days possess insignificant changes.

Meanwhile, both doses of 5 and 10 mg kg^{-1} ibuprofen, induced significant hepatocellular changes compared to livers from rats treated with saline on day 15. The main changes were the presence of mild focal lymphocytic and neutrophilic infiltrations which were mainly around PV regions as revealed by Fig. 4 and 5. While, lower dose and time points showed insignificant changes compared to saline. Lesion scoring mean value

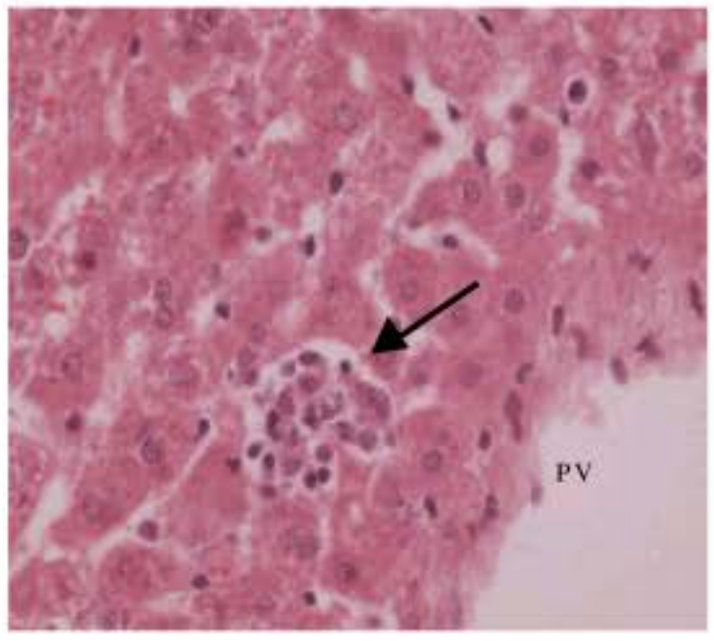

Fig. 5: Photomicrograph shows liver section of rat administered with 10 mg kg^{-1} ibuprofen at day 15. Arrow indicates lymphocytic and neutrophilic infiltration around PV. Magnification 400x. H and E staining

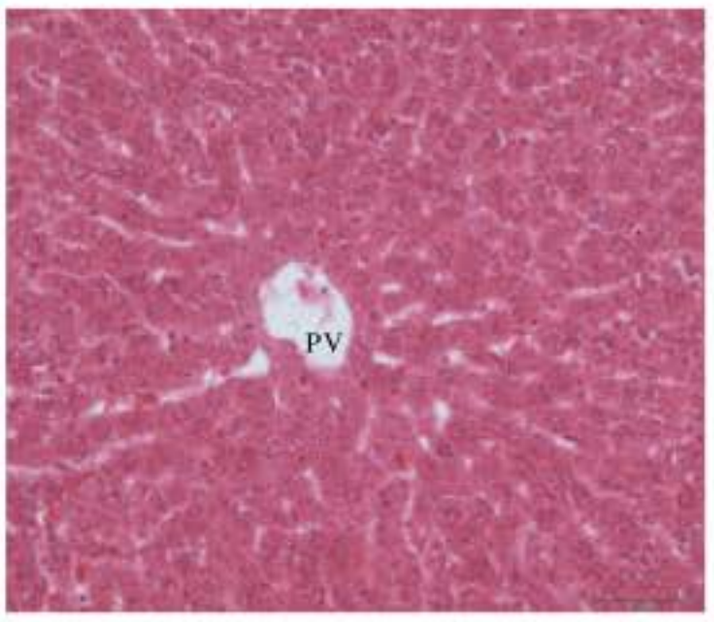

Fig. 6: Photomicrograph of liver section from saline-treated rat at day 15. The section reveals normal liver architecture. PV: Perivenular region Magnification: 200x

Table 2: Lesion scoring mean value of diclofenac, ibuprofen and saline-administered groups on day 15

Treatment groups	Score (mean±SEM)
Saline	1.1±0.4
3 mg kg^{-1} diclofenac	3.3±0.6*
5 mg kg^{-1} diclofenac	4.3±0.3*
10 mg kg^{-1} diclofenac	4.7±0.1*
3 mg kg^{-1} ibuprofen	2.0±0.4
5 mg kg^{-1} ibuprofen	2.8±0.3*
10 mg kg^{-1} ibuprofen	2.9±0.4*

*Indicate significance compared to saline at $p<0.05$

of diclofenac, ibuprofen and saline-administered groups on day 15 shown in Table 2.

Detection of apoptotic cells -TUNEL assay: Colorimetric TUNEL assay revealed the presence of apoptotic cells around some PV regions in 5 (Fig. 7) and 10 mg kg^{-1}

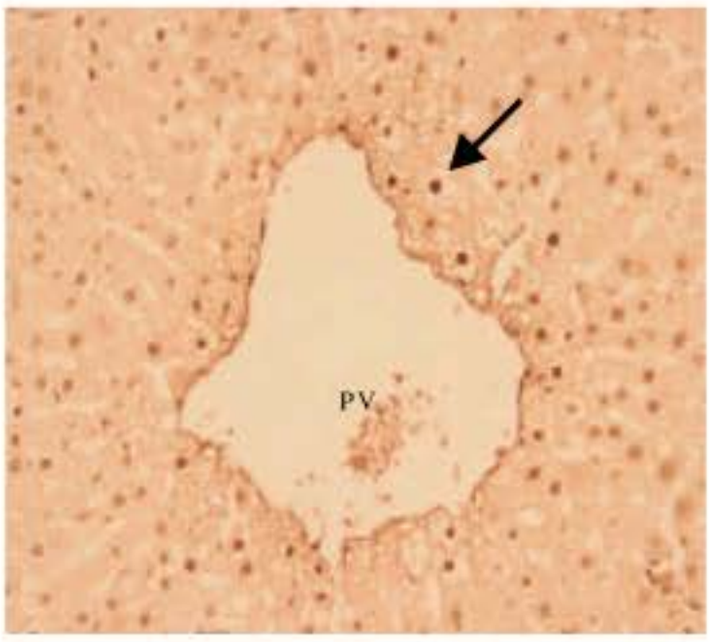

Fig. 7: Photomicrograph shows liver section of 5 mg kg^{-1} diclofenac-treated rat at day 15. Arrow indicates the presence of apoptotic cells around PV. Magnification: 200x

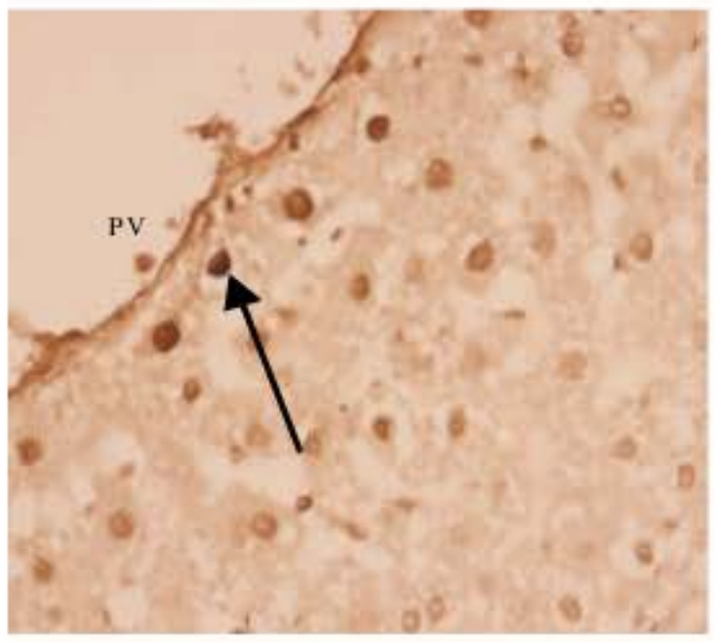

Fig. 9: Photomicrograph shows liver section of 10 mg kg^{-1} ibuprofen-treated rat at day 15. Arrow indicates presence the of apoptotic cells around PV. Magnification: 400x

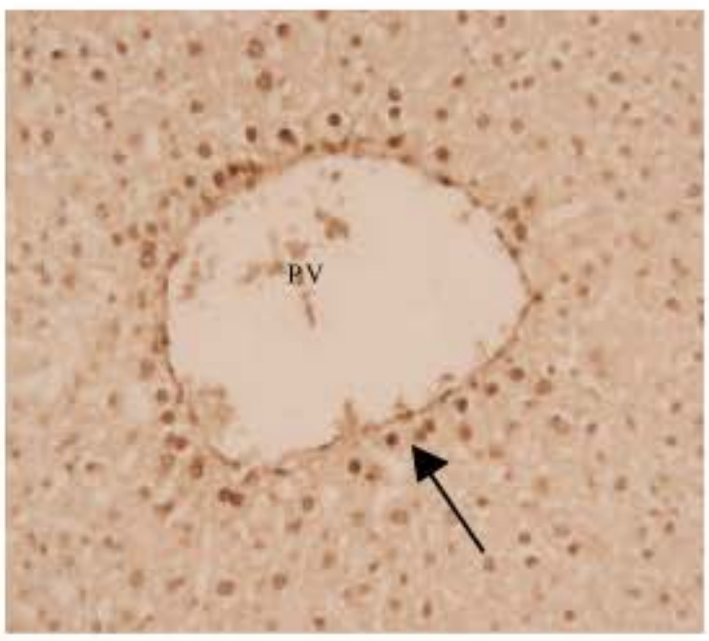

Fig. 8: Photomicrograph shows liver section of 10 mg kg^{-1} diclofenac-treated rat at day 15. Arrow indicates the presence of apoptotic cells around PV. Magnification: 200x

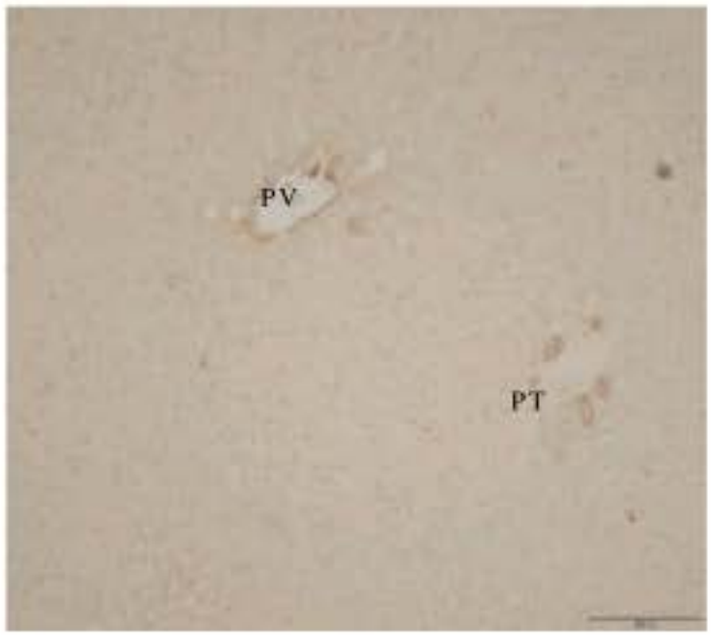

Fig. 10: Photomicrograph shows liver section from saline-treated rat at day 15. Normal architecture of hepatocytes is preserved around PV and PT. Magnification: 100x

(Fig. 8) diclofenac-treated groups after 15 days of treatment. However, such observation was not seen around PTs. Specimens obtained from rats treated with lower doses and time points showed similar observation compared to saline with complete absence of apoptotic cells around PV regions (Fig. 10).

In the case of ibuprofen, apoptotic hepatocytes around some PV were only discovered in 10 mg kg^{-1} dosed group on day 15 as indicated in Fig. 9. The 3 (after day 3, 6, 9, 12 and 15), 5 (after day 3, 6, 9, 12 and 15) and 10 mg kg^{-1} ibuprofen-treated rats (3, 6, 9, 12) showed similar observation to saline as can be seen in Fig. 10.

DISCUSSION

The H and E stained liver sections revealed significant hepatocellular changes in 3, 5 and 10 mg kg^{-1} diclofenac treated rats after day 15. The changes include presence of lymphocytic and neutrophilic infiltrations and mild focal necrosis, which were mainly observed around PVs. These changes are both frequency and dose-dependent, since significant alterations were only seen on day 15 with prominent changes in 10 mg kg^{-1} diclofenac-dosed rats. This coincides with previously reported studies (Kretz-Rommel and Boelsterli, 1993a, b; Masubuchi *et al*., 1998).

Earlier study with diclofenac have indicated the presence of sinusoidal and PV dilatations, enlarged PT, degeneration of cells and infiltration in PT, hepatocyte necrosis, mild fibrous tissue proliferation, interstitial and PT inflammations (Aydin *et al*., 2003). On the contrary, in the present study, we could only observe mild changes in the liver sections treated with diclofenac which could be attributable to low concentrations of diclofenac used in present study as compared to 50, 150 and 200 mg kg^{-1} (Aydin *et al*., 2003) administered consecutively for five days.

Whilst, with ibuprofen treatment, similar but milder morphological changes were observed in the H and E stained liver sections when compared to diclofenac of the same dosages and also time intervals. The observations include the presence of mild focal lymphocytic and neutrophilic infiltrations. These changes were also more prominent around PVs. Common clinical manifestations of ibuprofen-induced hepatotoxicity include hepatocellular injury, cholestatic and steatosis (Tolman, 1998). Such changes were not observed possibly due to low doses of ibuprofen used in present study.

In TUNEL assay, apoptotic cells were discovered at day 15 in 5 and 10 mg kg^{-1} diclofenac and 10 mg kg^{-1} ibuprofen-treated groups surrounding some PVs. The absence of apoptotic cells in some of the treatment groups indicate that the given amount of drugs did not exceed the threshold to cause apoptosis (Saikumar *et al*., 1999).

Both in H and E stained liver sections and TUNEL assay, the changes were mainly observed in the hepatocytes surrounding PV area, since these hepatocytes receive blood supply which has lower level of essential nutrients and O_2 that makes them susceptible to injury, compared to the hepatocytes closest to the PT which receive blood that is most enriched with oxygen and nutrients; making them less prone to injury (Sturgill and Lambert, 1997).

Prominent changes around hepatocytes around PV could also be due to higher CYP450 enzyme activity around this region (Sturgill and Lambert, 1997). This may lead to the formation of more reactive metabolites around PVs. In the case of diclofenac, CYP450 enzyme system involved are CYP2C9 that yields 4-OH diclofenac and CYP3A4 to 5-OH diclofenac. Recent review indicates that these metabolites can be further oxidized to p-benzoquinone imines; electrophiles that can form covalent adduct with cellular protein macromolecules (Boelsterli, 2003). The formation of these adducts with cellular macromolecules may disrupt some physiological functions performed by these proteins (Boelsterli, 2003) as reported for benoxaprofen (BNP) (Fakurazi, 2001). This NSAID was identified to form a 110 kDa adduct which corresponds to a bile acid transporter protein. The covalent modification of this protein was suspected to cause accumulation of bile salt and bile acid in the hepatocyte, subsequently leading to apoptosis (Fakurazi, 2001).

As for ibuprofen, 2-hydroxy and 2-carboxy ibuprofen were found to have no apparent pharmacological activity (Al-Nasser, 2000). The glucuronidation pathway (Spahn-Langguth and Benet, 1992; Castillo *et al*., 1995) and formation of acyl-CoA thioester (Li *et al*., 2003) were previously identified to play crucial role in the toxicity.

CONCLUSION

This study has shown that, diclofenac is probably more potent in inducing histomorphological changes at low doses. Both the drugs seem to exert time and dose dependent liver morphological alterations to the treated animals.

ACKNOWLEDGMENT

The authors would like to acknowledge the Malaysian Toray Science Foundation (MTSF) (Grant No. 54853) and Ministry of Higher Learning Education of Malaysia for financial assistance (Grant No. 55165).

REFERENCES

Al-Nasser, I.A., 2000. Ibuprofen-induced liver mitochondrial permeability transition. Toxicol. Lett., 111: 213-218.

Aydin, G., A. Gokcimen, E. Cicek and O. Gokalp, 2003. Histopathologic changes in liver and renal tissue induced by different doses of diclofenac sodium in rats. Turk. J. Vet. Ani. Sci., 27: 1131-1140.

Boelsterli, U.A., 2003. Diclofenac-induced liver injury: A paradigm of idiosyncratic drug toxicity. Toxicol. Applied Pharmacol., 192: 307-322.

Bort, R., K. Mace, A. Boobis, M.J. Gomez-Lechon, A. Pfeifer and J. Castell, 1999. Hepatic metabolism of diclofenac: Role of human CYP in the minor oxidative pathways. Biochem. Pharmacol., 58: 787-796.

Campos, C.B.L., G.R. Degasperi, D.S. Pacifico, L.C. Alberici and R.S. Carreira *et al*., 2004. Ibuprofen-induced Walker 256 tumor cell death: Cytochrome c release from functional mitochondria and enhancement by calcineurin inhibition. Biochem. Pharmacol., 68: 2197-2206.

Castillo, M., Y.M.F. Lam, M.A. Dooley, E. Stahl and P.C. Smith, 1995. Disposition and covalent binding of ibuprofen and its acyl glucuronide in the elderly. Clin. Pharmacol. Ther., 57: 636-644.

Fakurazi, S., 2001. The formation of hepatic protein adducts in the livers of rats and mice treated with NSAIDs. Ph.D. Rhesis, Imperial College of Science, UK.

Galati, G., S. Tafazoli, O. Sabzevari, T.M. Chan and P.J. O'Brien, 2002. Idiosyncratic NSAID drug induced oxidative stress. Chem. Biol. Interact., 142: 25-41.

Gomez-Lechon, M.J., X. Ponsoda, E. O'Connor, T. Donato, J.V. Castell and R. Jover, 2003a. Diclofenac induces apoptosis in hepatocytes by alteration of mitochondrial function and generation of ROS. Biochem. Pharmacol., 66: 2155-2167.

Gomez-Lechon, M.J., X. Ponsoda, E. O'Connor, T. Donato, R. Jover and J.V. Castell, 2003b. Diclofenac induces apoptosis in hepatocytes. Toxicol. *In vitro*, 17: 675-680.

Kretz-Rommel, A. and U.A. Boelsterli, 1993a. Diclofenac covalent protein binding is dependent on acyl glucuronide formation and is inversely related to P4590-mediated acute cell injury in cultured rat hepatocytes. Toxicol. Applied Pharmacol., 120: 155-161.

Kretz-Rommel, A. and U.A. Boelsterli, 1993b. Selective protein adducts to membrane protein in cultured rats hepatocytes exposed to diclofenac: Radiochemical and immunochemical analysis. Mol. Pharmacol., 45: 237-244.

Laine, L., 2001. Approaches to non-steroidal anti-inflammatory drug use in the high-risk patient. Gastroenterol, 120: 594-606.

Li, C., M.P. Grillo and L.Z. Benet, 2003. *In vivo* mechanistic studies on the metabolic activation of 2-phenylpropionic acid in rat. J. Pharmacol. Exp. Thr., 305: 250-256.

Masubuchi, Y., H. Saito and T. Horie, 1998. Structural requirements for the hepatotoxicity of non-steroidal anti-inflammatory drugs in isolated rat hepatocytes. J. Pharmcol. Exp. Ther., 287: 208-213.

Masubuchi, Y., S. Nakayama and T. Horie, 2002. Role of mitochondrial permeability transition in diclofenac-induced hepatocyte injury in rats. Heptology, 35: 544-551.

O'Connor, N., P.I. Dargan and A.L. Jones, 2003. Hepatocellular damage from non-steroidal anti-inflammatory drugs. Q. J. Med., 96: 787-791.

Rubeinstein, J.H. and L. Laine, 2004. Systematic review: The hepatotoxicity of non-steroidal anti-inflammatory drugs. Aliment Pharmacol. Ther., 20: 373-380.

Saikumar, P., D. Zheng, V. Mikhailov, M. Denton, J.M. Weinberg and M.A. Venkatachalam, 1999. Apoptosis: Definition, mechanisms and relevance to disease. Am. J. Med., 107: 489-506.

Spahn-Langguth, H. and L.Z. Benet, 1992. Acyl glucuronide revisited: Is the glucuronidation process a toxification as well as a detoxification mechanism? Drug Metab. Rev., 24: 5-48.

Sturgill, M.G. and G.H. Lambert, 1997. Xenobiotic-induced hepatotoxicity: Mechanism of liver injury and methods of monitoring hepatic function. Clin. Chem., 43: 1512-1526.

Teoh, N.C. and G.C. Farrell, 2003. Hepatotoxicity associated with non-steroidal anti-inflammatory drugs. Clin. Liver Dis., 7: 401-413.

Tolman, K.G., 1998. Hepatotoxicity of non-narcotic analgesics. Am. J. Med., 105: 13S-19S.