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Research Article

Acetaminophen Induces Mitochondrial Permeability Transition in Rats Without Causing Necrotic Liver Damage

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Abstract

Mitochondrial Permeability Transition (MPT) is reported as the mechanism of acetaminophen induced hepatic damage, however, rat models are resistant to acetaminophen induced toxicity. The occurrence and degree of mitochondrial permeability transition after treatment with 400 mg kg⁻¹ of acetaminophen in albino Wistar rats were assessed. Animals were randomly distributed into seven groups; control, 12, 24, 36, 48, 60 and 72 h based on varying time (in hour) post acetaminophen prior to sacrifice after treatment. Mitochondrial Membrane Permeability Transition (MMPT) pore opening and mitochondrial cytochrome c release were estimated. Opening of MMPT pore and cytochrome c release were observed in 12, 24, 36 and 72 h, when compared with the control group. Liver function and histological results indicated no liver damage. It is concluded that toxic dose of acetaminophen induced mitochondrial permeability transition in rat hepatic tissues without leading to necrotic damage suggesting that rat hepatic tissues evade damage by mechanisms downstream of MPT.

Key words: Acetaminophen, mitochondrial permeability transition, cytochrome c, necrosis, ALT, ALP

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Acetaminophen (paracetamol, N-acetyl-p-aminophenol; APAP) is a safe and effective analgesic and antipyretic, though its overdose can be hazardous to the liver, being the primary cause of acute liver failure (Bernal, 2003; Larson *et al.*, 2005; McGill *et al.*, 2012). For decades, ample research has been directed into its toxicity in humans and rodents (Davidson and Eastham, 1966; Jaeschke *et al.*, 2012) and conclusions has been drawn by declaring it the best characterized hepato-toxicant to man and some rodents but not to rats (McGill *et al.*, 2012). Different mechanisms of APAP hepato-toxicity have been proposed and amongst these include mitochondrial dysfunction induced toxicity. Mitochondrial permeability transition is characterized by opening of Mitochondrial Membrane Permeability Transition (MMPT) pore and release of mitochondrial proteins such as cytochrome c (Kinnally and Antonsson, 2007). The MMPT pore is a non-selective, high-conductance channel with multiple macromolecular components (Alano *et al.*, 2002; Adegbite *et al.*, 2015; Iyanda-Joel *et al.*, 2015) such as Adenine Nucleotide Translocase (ANT) and cyclophilin D which form at sites where, the inner and outer membranes of the mitochondrion meet (Connern and Halestrap, 1994). Cytochrome c functions in mitochondrial respiration where, it plays an important role in the electron transport chain as a carrier of electron from complex 3 to complex 4 of the mitochondrial respiratory system (Ow *et al.*, 2008). Beyond this activity, cytochrome c is also involved in cell death which could be apoptotic or necrotic (Li *et al.*, 1999; Gao *et al.*, 2001), when released from the mitochondrial inter-membrane space during opening of the MMPT pore, thus, 'a double edged sword of life and death'. Although, MPT was reported to be a likely mechanism in APAP-induced hepatotoxicity and blockade of toxicity both *in vitro* and *in vivo* has been shown by MPT inhibitors (Masubuchi *et al.*, 2005; Kon *et al.*, 2007), it is therefore, pertinent to assess MPT in rat which is an already established model resistant to APAP induced hepatotoxicity.

MATERIALS AND METHODS

Materials: Wistar strain of specific pathogen free albino rats (100-120 g). Mannitol, sucrose, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), Ethylene Glycol Tetra Acetic acid (EGTA), spermine, rotenone, sodium succinate hexahydrate, bovine serum albumin, ethanol, sodium hydroxide (NaOH), copper sulfate pentahydrate (CuSO₄·5H₂O) were products of Sigma-Aldrich Co, USA. Sodium bicarbonate (Na₂CO₃), potassium hydroxide (KOH) and folin

ciocalteau were purchased from BDH chemicals, England. Acetaminophen (paracetamol) was purchased from May and Baker, Plc, Nigeria. Rat cytochrome c enzyme linked immunosorbent assay (ELISA) kit was purchased from Hangzhou East Biopharm Co. Ltd., China. All other chemicals were of analytical grade.

Experimental design: Experimental animals were obtained from the Institute of Medical Research and Training (IMRAT), Nigeria and were kept under standard environmental conditions (25±21 °C; 12/12 h light/dark cycle). The animals were randomly grouped into seven of five animals per group. Each group apart from the control represented post APAP treatment time prior to sacrifice (control, 12, 24, 36, 48, 60 and 72 h). The animals were fed with standard diet and water *ad libitum* and were allowed to acclimatize for three weeks before commencement of experiment. Except for the control which was given normal saline, all animals were treated with 400 mg kg⁻¹ of acetaminophen through the oral route before sacrifice. Test groups were sacrificed after 12, 24, 36, 48, 60 and 72 h following APAP ingestion. The experimental animals were handled and used in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH., 1985). The experimental procedure was approved by the ethics committee of the department of biological sciences, Covenant University, Nigeria.

Blood sample and tissue preparation: Animals were sedated with diethylether and blood samples collected via cardiac puncture into lithium heparin bottles. Plasma was obtained by centrifugation of the blood at 4000 rpm (Model SM-80-2 Surgifield medical, England) for 10 min. Plasma samples were stored at -20 °C in eppendorff tubes until required for assay. Livers were excised, trimmed and collected into beakers; homogenizing buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 M KOH and 1 mM EGTA, pH 7.4) was immediately added for suspension and making of a 10% homogenate using a potter glass homogenizer. These were carried out on ice.

Mitochondria extraction: Mitochondria were isolated essentially by modified established methods (Bababunmi *et al.*, 1979; Lapidus and Sokolove, 1993). The homogenate supplemented with a protease inhibitor cocktail (Abcam plc, UK) was subjected to centrifugation in a high speed refrigerated centrifuge (SM-18B, Surgifield medical, England) twice at 2300 rpm to sediment the cell debris and nuclear fraction, the supernatant were then centrifuged at 13000 rpm to sediment the mitochondria while, the

supernatant being the post mitochondrial fraction was immediately refrigerated as the cytosolic fraction. Mitochondria were washed with a washing buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH and 0.5% BSA, pH 7.4), suspended in swelling buffer (210 mM mannitol, 70 mM sucrose and 5 mM HEPES-KOH, pH 7.4) and immediately dispensed in 2 mL eppendorf tubes. Isolated mitochondria were used within 6 h of isolation. Protein content of cytosolic and mitochondrial fractions was determined by folin c method, using bovine serum albumin as standard (Lowry *et al.*, 1951).

Mitochondria swelling assay: Mitochondria (1 mg of protein/mL) in the swelling buffer were pre-incubated in a 1 cm light path glass cuvette in the presence of 0.8 μ M rotenone and 5 mM sodium succinate. Mitochondrial membrane permeability transition pore opening was then quantified as changes in absorbance at 540 nm. Mitochondria with absorbance ranging between 0.02 and 0.08 were intact, thus, MMPT pore opening was considered at absorbance values greater than 0.08.

Percentage (%) induction of MMPT pore opening was calculated using the equation:

$$\frac{\text{Change in absorbance} - 0.08}{\text{Change in absorbance}} \times 100$$

Cytochrome c release: The 1 mg mL⁻¹ protein of isolated mitochondria and cytosol were assayed for cytochrome c using cytochrome c ELISA kit (Hangzhou East Biopharm Co. Ltd.) following the manufacturer's instruction. Values were obtained by an ELISA plate reader (Model SM-3 Shanghai Jihui Scientific, China) at 450 nm.

Liver function tests: Alanine aminotransferase (ALT) and alkaline phosphatase (ALP) assays were carried out using colorimetric assay kits (Randox Lab. Ltd., UK) following the manufacturer's instruction.

Total cholesterol: Total cholesterol assay was carried out using colorimetric assay kits (Randox Lab. Ltd., UK) following the manufacturer's instruction.

Histological studies: Histological examinations were carried out by established methods (Adebayo *et al.*, 2014). Liver tissues were fixed in formalin for 72 h and sliced into a thickness of 2.1 mm. The tissues were dehydrated with alcohol of graded concentrations. They were further treated with paraffin wax and cast into blocks; sections of the tissues were

then cut on a microtome to 5 μ m. These were later attached to a slide and allowed to dry. The sample slides were subsequently stained with haematoxylin-eosin and examined under a light microscope; photomicrographs of the samples were recorded.

Statistical analysis: Statistical package for the social sciences (SPSS) version 15.0 was used for the statistical analysis (SPSS Inc. Chicago Illinois, USA). Group comparison was done using one way analysis of variance (ANOVA). Least significant different test was used to analyze significance of difference between different groups. The $p < 0.05$ was considered statistically significant.

RESULTS

Liver function test: Activities of plasma alanine amino transferase (ALT) (μ L⁻¹) for the groups were 10.54 ± 3.86 , 20.07 ± 4.52 , 18.5 ± 4.82 , 24.9 ± 2.27 , 21.73 ± 1.63 , 10.1 ± 1.79 and 5.96 ± 0.44 for control, 12, 24, 36, 48, 60 and 72 h, respectively. When compared with the control, all test groups had higher ALT activity although not significant ($p > 0.05$) except for 72 h group which had a significantly ($p < 0.05$) low activity. These levels were not high enough to infer liver damage. Aspartate amino transferase (AST) activity observed were 68.25 ± 5.29 , 54.44 ± 8.33 , 55.31 ± 6.76 , 60.12 ± 11.37 , 73.75 ± 7.75 , 69.00 ± 7.95 and 79.31 ± 3.72 for control, 12, 24, 36, 48, 60 and 72 h, respectively. There was no significant difference ($p > 0.05$) among the groups and with the control. Activities of alkaline phosphatase (ALP) (μ L⁻¹) were 499.56 ± 39.9 , 583.74 ± 78.3 , 643.08 ± 100.77 , 905.28 ± 157.3 , 466.44 ± 88.32 , 468.28 ± 156.79 and 544.64 ± 78.669 for 0, 12, 24, 36, 48, 60 and 72 h, respectively. Activities in 12, 24, 36 and 72 h were higher than the control but only 36 h was significant ($p < 0.05$). This result is certainly in line with the findings that acetaminophen is not toxic to rat livers whereas, 400 mg kg⁻¹ dose of acetaminophen is highly hazardous to mouse liver (Fig. 1).

Mitochondrial permeability transition

Mitochondrial membrane permeability transition pore opening: Isolated mitochondria were observed to swell and their MMPT pore opened in test groups which included 12, 24, 36 and 72 h, while MMPT pore opening were not observed in the control, 48 and 60 groups. Percentage induction of MMPT pore opening were 15, 128, 74 and 29% for 12, 24, 36 and 72 h, respectively. It is observed that the 24 h group had the highest pore opening. This was 8 fold higher than the

12 h group which had lowest pore opening. Pore opening decreased from 24 h as the time of post APAP treatment progressed (Fig. 2).

Mitochondrial cytochrome c release: Values of cytosolic cytochrome c (ng mL⁻¹) observed were 2.02±0.50, 6.70±0.54, 7.80±1.72, 6.50±0.60, 3.60±0.43, 2.34±0.67 and 5.52±0.25

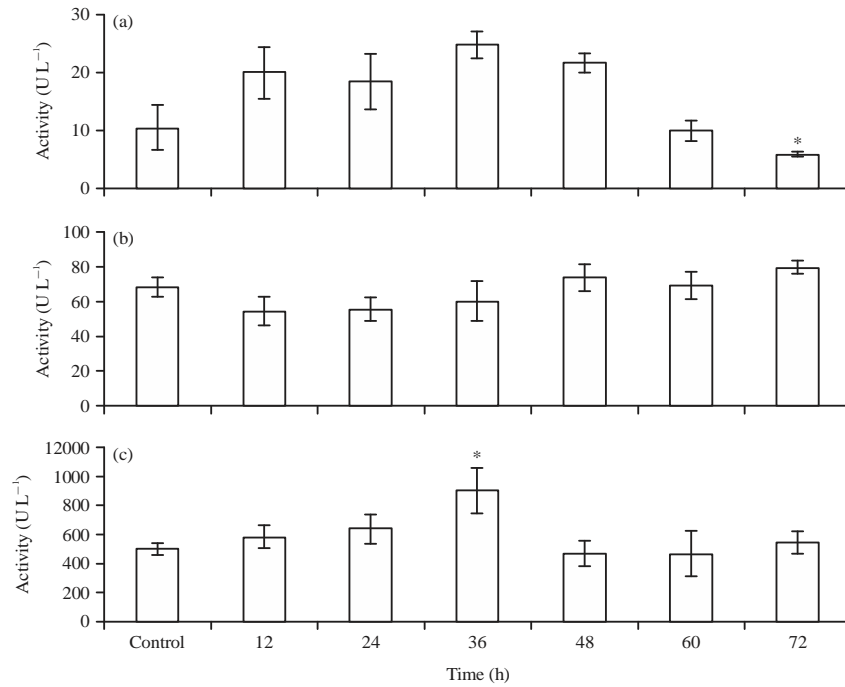


Fig. 1(a-c): Liver function test, (a) Graph showing plasma ALT activity in APAP treated rats, (b) Graph showing plasma AST activity in APAP treated rats and (c) Graph showing plasma ALP activity in APAP treated rats, values are presented as mean ± SEM, *p<0.05 when compared with the control

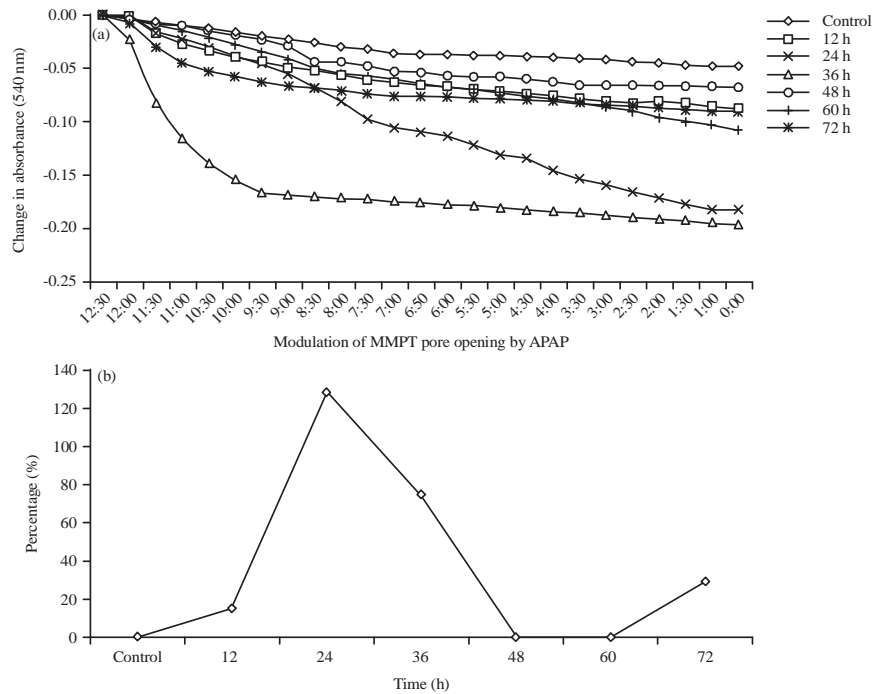


Fig. 2(a-c): Continue

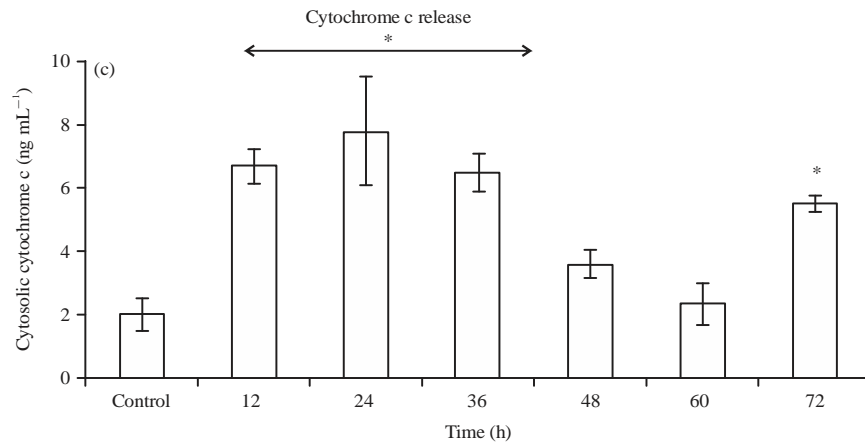


Fig. 2(a-c): Mitochondrial permeability transition, (a) Induction of MMPT pore opening in APAP treated rats, (b) Graph showing percentage induction of MMPT pore opening in APAP treated rats and (c) Graph showing cytochrome c release in APAP treated rats, values represent Mean \pm SEM, * $p < 0.05$ when compared with the control

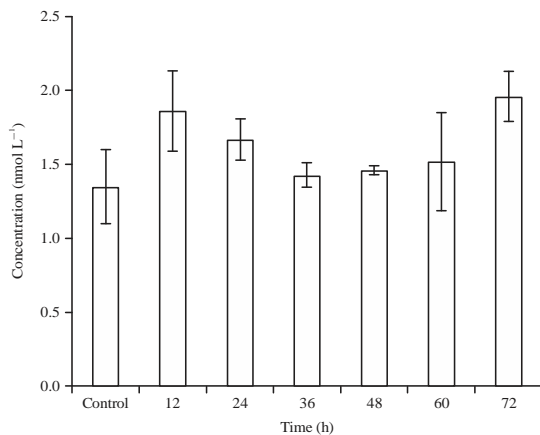


Fig. 3: Graph showing plasma total cholesterol in APAP treated rats, values represent Mean \pm SEM, * $p < 0.05$ when compared with the control

for control, 12, 24, 36, 48, 60 and 72 h, respectively. Cytochrome c was observed to be significantly high ($p < 0.05$) in the 12, 24, 36 and 72 h groups when compared with the control. However, no significant difference ($p > 0.05$) was observed in the 48 and 60 h groups. Comparison among test groups showed significant decrease in the 60 h group, when compared with the 12 and 24 h groups. It is observed a hyperbolic progress of cytochrome c release from the 12 h group to the 72 h group, although cytochrome c release was not extremely high in all treated groups.

Total cholesterol: Plasma levels of total cholesterol were 1.35 ± 0.25 , 1.86 ± 0.27 , 1.67 ± 0.14 , 1.43 ± 0.08 , 1.46 ± 0.03 , 1.52 ± 0.33 and 1.96 ± 0.14 for control, 12, 24, 36, 48, 60 and 72 h, respectively. We observed no significant difference

($p > 0.05$) in the levels of total cholesterol of the test groups when compared with both the control and among the groups (Fig. 3).

Histopathological studies: Microvesicular steatosis was observed in all groups including the control group. We also observed varying levels of periportal infiltration of inflammatory cells and disseminated infiltration of zone 2 by inflammatory cells in all test groups, however, these were not significantly different (Fig. 4).

DISCUSSION

The data revealed a pattern of permeability transition that occurs in rat hepatic mitochondria after APAP treatment. The 400 mg kg^{-1} of APAP was not toxic to livers of our rat model, as shown by results obtained from ALT, ALP, total cholesterol and histological studies. The MMPT pore opening which is associated with the release of cytochrome c was found to have occurred and the degree of pore opening and its associated cytochrome c release were highest in 24 h post APAP treatment. Although, MPT was reported to be promoted by oxidative stress (Chen *et al.*, 2008) and in turn associated with a very large increase in oxidative stress (Reid *et al.*, 2005), some researchers also reported that no relevant oxidative stress was observed in rats after APAP overdose (McGill *et al.*, 2012). This therefore, suggests that MPT observed in our result was independent of oxidative stress. Because APAP possesses weak anti-inflammatory properties (Bessemers and Vermeulen, 2001), we observed the presence of inflammatory cells from histological studies. However, the role of inflammatory cells in APAP toxicity is still being broadly studied. We suggest that

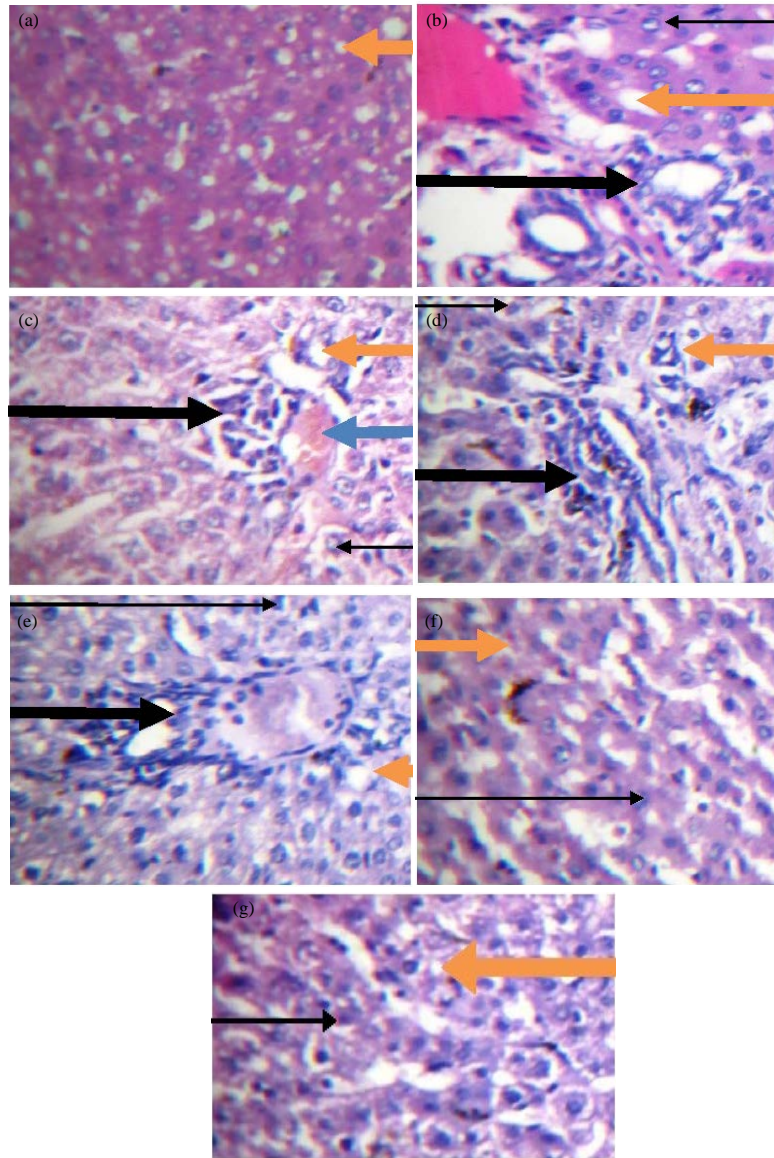


Fig. 4(a-g): Histology result of liver sections examined, (a) Microvesicular steatosis in the control group (orange arrow), (b) Periportal infiltration by inflammatory cells (black arrow), disseminated infiltration of zone 2 by inflammatory cells (slender arrows) and Microvesicular steatosis (orange arrow) in the 12 h group, (c) Sections show congestion of vessels (blue arrow), periportal and perivascular infiltration by inflammatory cells (black arrow), disseminated infiltration of zone 2 by inflammatory cells (slender arrows) and Microvesicular steatosis (orange arrow) in the 24 h group, (d) Periportal infiltration by inflammatory cells (black arrow) and disseminated infiltration of zone 2 by inflammatory cells (slender arrows) and Microvesicular steatosis (orange arrow) in the 36 h group, (e) Mild periportal infiltration by inflammatory cells (black arrow), disseminated infiltration of zone 2 by inflammatory cells (slender arrow) and marked disseminated Microvesicular steatosis (orange arrow) in the 48 h group, (f) Disseminated infiltration of zone 2 by inflammatory cells (slender arrows) and Microvesicular steatosis (orange arrow) in the 60 h group and (g) Disseminated infiltration of zone 2 by inflammatory cells (slender arrows) and Microvesicular steatosis (orange arrow)

inflammatory responses observed led to the initiation of MPT and the translocation of cytochrome c from mitochondrial

inter-membrane space to the cytosol through the activation of death receptors since these cells release cytokines that

mediates cell death in surrounding tissues (Shen and Pervaiz, 2006). These inflammatory cells may have been sequestered into liver tissues in such an increased amount as a result of N-acetyl-p-benzoquinone imine (NAPQI); a metabolite of acetaminophen. The NAPQI is formed from the metabolism of APAP by cytochrome p450 2E1 (CYP2E1) drug metabolizing enzyme (Cover *et al.*, 2005). Since, CYP2E1 is also mitochondrial in origin (Knockaert *et al.*, 2011), NAPQI might have directly caused the release of cytochrome c from mitochondria. The increased levels of MMPT pore opening and cytochrome c release in the 24 h group correlates with the report that toxic response of hepatic tissues occurred between the 6th and 24th hour post-APAP toxic dose (McGill *et al.*, 2012). Since there were no necrotic lesions observed in the livers examined, then MPT observed here would have resulted in some alternative downstream effects in rat tissues other than necrosis. It was reported that the relative amount of ATP present in the cytosol appears to be an important deciding factor as to whether hepatic cells die by necrosis or apoptosis (Kon *et al.*, 2004). Although, we do not have sufficient data here to infer apoptosis, a non toxic cell death as the alternative effect of MPT but we understand that opening of the MMPT pore leads further to the release of mitochondrial pro-apoptotic proteins such as Smac/Diablo, HtrA2/Omi, apoptosis inducing factor and endonuclease G (Verhagen *et al.*, 2000; Li *et al.*, 2001; Gao *et al.*, 2001; Wang and Lin, 2013) and some authors have shown that pore opening and the release of cytochrome c without observing morphological features of apoptosis such as cell shrinkage, membrane bound fragments and nuclear aggregation (Elmore, 2007) may connote necrosis (Soeda *et al.*, 2001); which we did not observe. However, we observed hepatic steatosis in all groups instead but as the liver is the primary organ of lipid metabolism, it is often associated with steatosis (Ahmed and Byrne, 2010). It is also worthy of note that eukaryotic cells scavenge and eliminate compromised mitochondria and other organelles by autophagy. This serves as a protective measure from indiscriminate cell death by necrosis or apoptosis (Rodriguez-Enriquez *et al.*, 2004). Rat hepatic cells might have also employed autophagy as a means of getting rid of mitochondria that have undergone permeability transition, thereby avoiding either types of cell death. The decrease in MPT in groups after 24 h may be due to the decreasing concentrations of APAP which resulted from hepatic metabolism. This implies that pore opening and cytochrome c release in hepatic tissues were caused by APAP and not mere basal metabolic activities. Much evidence suggests that cytochrome c release, a consequence of MMPT pore opening

plays a central role as an initiator of death machinery in cases of general cellular damage (Green and Reed, 1998; Qu and Qing, 2004).

CONCLUSION

Mitochondrial Permeability Transition (MPT) is regarded as the mechanism of APAP induced hepatic tissue damage in mouse. It also occurs in rats with no obvious damage to hepatic tissues. This may therefore, suggest that rat hepatic tissues evade necrotic damage by undergoing apoptosis or autophagy when exposed to toxic doses of APAP.

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