Original
ISSN 2521-8492

Programmed Cell Death Induced by Hydrogen Peroxide

Shams Mahdi[1*], Entisar Raheem Al-Kennany[2]

[1]Central Public Health Lab, Public Health Department, Ministry of Health, Baghdad, Iraq.
[2]College of Dentistry, Al-Iraqia University, Baghdad, Iraq.
*Correspondence to: Shams Mahdi (E-mail: shamsmahdimd@gmail.com)
(Submitted: 20 July 2024 – Revised version received: 05 August 2024 – Accepted: 26 August 2024 – Published online: 26 September 2024)

Abstract
Objective: By studying hydrogen peroxide induced apoptosis, this study aims to provide an understanding for the mechanism of various liver diseases and thus drug development.
Methods: Oxidative stress was experimentally induced in rats through daily oral administration of 1% hydrogen peroxide (H_2O_2) for 15 and 30 days, leading to liver injury characterized by cell death of hepatocytes, particularly in the centrilobular region.
Results: This study aimed to investigate the role of apoptosis in the pathogenesis of H_2O_2-induced hepatic injury. Apoptotic hepatocytes and Kupffer cells were identified and quantified using light microcopy. Liver sections revealed characteristic apoptotic changes, including a honeycomb appearance, cellular shrinkage, nuclear condensation and fragmentation, apoptotic body formation and phagocytosis of apoptotic cells by neighboring cells. The number of apoptotic cells was positively correlated with the duration of H_2O_2 exposure (15 and 30 days). Ultrastructural analysis showed liver injury marked by mitochondrial swelling, cristae lysis, fragmentation of the endoplasmic reticulum and disruption of cytoplasmic structures. Additionally, rats treated with H_2O_2 exhibited a significant reduction in hepatic glutathione (GSH) levels compared to control.
Conclusion: The findings of this study suggest that apoptosis plays a significant role in the pathological process underlying liver injury induced by oxidative stress mediated by H_2O_2.
Keywords: Apoptosis, necrosis, kupffer cell, liver, H_2O_2, electron microscope, GSH

Introduction

Hydrogen peroxide (H_2O_2) and its reactive derivatives, such as hydroxyl radicals and superoxide anions, are key contributors to oxidative stress, driving lipid peroxidation, protein degradation and cell apoptosis.[1,2] While reactive oxygen species (ROS) are primarily known for inducing cellular damage, they also play critical roles in cell signaling, regulating processes like proliferation and apoptosis through reversible protein modifications.[3] Apoptosis or programmed cell death PCD, is essential for removing damaged or unnecessary cells, maintaining homeostasis, and reducing inflammation.[4–6] Triggers of apoptosis include developmental signals, oxidative stress, and cytokines.[7,8] In liver disease, ROS are implicated in both cell death and tissue injury, though the mechanisms remain unclear. Apoptotic cell death is marked by cellular shrinkage and chromatin condensation, with organelles as mitochondria, lysosomes, and the endoplasmic reticulum involved in damage sensing and signal integration.[9] Lysosomal enzymes, like cathepsins D and B, are translocated early in apoptosis preceding cytochrome C release and caspase activation, with caspase-3 playing a central role in H_2O_2- induced apoptosis.[10–12]

The liver an ideal system for studying apoptosis, as its occurrence is typically rare under normal conditions but increases with exposure to hepatotoxic agents.[13] This study investigated liver injury induced by daily oral administration of 1% H_2O_2 in rats, focusing on the role of apoptosis in hepatocyte damage. Apoptotic changes were examined using light and electron microscopy, with the goal of advancing knowledge on liver disease mechanisms as non-alcoholic fatty liver disease and hepatocellular carcinoma, drug development, liver transplantation and regenerative medicine.

Materials and Methods

Animal subjects: twenty old male albino rats, 30 days old each, were obtained from College of Veterinary Medicine, University of Mosul. The animals were housed and bred and divided into 2 groups; group 1 served as the control group, while group 2 was treated with 1% hydrogen peroxide (H_2O_2) administered orally daily for 30 days.

Experimental procedure: at days 15 and 30 post- H_2O_2 treatment, the animals were sacrificed under ether anesthesia. The livers of both control and treated animals were examined. Liver tissue was perfused via the portal vein with 1.5% glutaraldehyde in a 0.062 mol/L cacodylate buffer (pH 7.4) containing 1% sucrose. Tissue blocks were further processed by immersion in 0.1 mol/L phosphate-buffered 1% osmium tetroxide (pH 7.4) for 2 hours, followed by dehydration and embedding in epoxy resin. Semi-thin sections (0.5 mm) were stained with toluidine blue for evaluation by light microscopy. Thin plastic sections were stained with 1% toluidine blue in 1% borax at 60o for 1–2 minutes, as described by Robert in 1978.[11]

Counting apoptotic cells: apoptotic cells were identified following the method outlined by Atroshi et al.[13] Random areas of liver tissue were selected for cell counting, with the number of apoptotic cells counted per mm^2 using an ocular lens with a standard square grid under oil immersion. The area of the grid was calculated using a stage micrometer, measuring 0.0049 mm^2. The total number of cells counted within this area was divided by the area of the square (0.0049 mm^2) to estimate the number of apoptotic hepatocytes per mm^2. Counting was performed in the centrilobular zone of the liver.

Determination of glutathione (GSH) levels: glutathione content in hepatocyte was assessed in-situ using perfused livers from rats treated with 1% H_2O_2 for 15 and 30 days. Freshly isolated hepatocytes were harvested by

centrifugation at 2000g in a refrigerated centrifuge, then washed and suspended in phosphate- buffered saline (PBS). Cells were lysed through repeated freezing- thaw cycles. The levels of reduced (GSH) and oxidized glutathione (GSSG) were measured in the clear supernatant obtained after centrifugation at 22000g for 15 minutes using high- performance liquid chromatography.[14,15]

Statistical analysis: statistical analyses were performed using Student's *t*-test for independent samples. A *p*-value of <0.05 was considered statistically significant. Data are presented as mean ± standard deviation (SD).

Results

Glutathione content: Table 1 shows reduced liver tissue GSH levels 15- and 30-days post-1% H_2O_2 treatment. The GSH content decreased to 2.21 ± 0.010 mmoL/g and 2.01 ± 0.02 mmoL/g, respectively, compared to the control group.

Apoptotic and necrotic cells: Table 2 demonstrates an increase in apoptotic cell count after H_2O_2 administration. Apoptotic cell count rose to 87.25 ± 55.41 and 104.2 ± 52.33 at days 15, and 30 respectively. Necrotic cells also increased, with 25.23 ± 41.02 and 27.52 ± 32.31 at day 15 and 30 respectively

Microscopic lesions: at day 15 post- H_2O_2 administration, vacuolar degeneration of hepatocytes was observed, particularly in the midzonal region of the liver lobule. The hepatocytes displayed pale, foam-like cytoplasm and detachment from adjacent cells giving a honeycomb appearance (Figure 1).

By day 30, apoptotic foci formed a circular arrangement around the centrilobular zone, consisting of hepatocytes with nuclear fragmentation, chromatin condensation, and vacuolar changes (Figure 2 left). Cells exhibit shrunken morphology with fragmented nuclear remnants that were often phagocytosed by adjacent cells. Figure 2 right is showing toluidine blue staining of apoptotic bodies.

Table 1. **Effect of oral administration of 1% H_2O_2 on tissue GSH of hepatocytes in rat liver**

Samples	GSH (mmol/g wet tissue)	
	Day 15 post 1% H_2O_2	Day 30 post 1% H_2O_2
Control	3.32 ± 0.05	3.45 ± 0.04
H_2O_2 treated	2.21 ± 0.01	2.01 ± 0.02

-The value are given as a total glutathione intracellular GSH7GSSG.
-Value expressed as means ± SD significantly at $P < 0.05$.
-No. of rats/group = 10.

Table 2. **Effect of oral administration of 1% H_2O_2 on number of apoptotic and necrotic hepatocytes**

Types	Control/mm²		H_2O_2 / mm²	
	Day 15 post 1% H_2O_2	Day 30 post 1% H_2O_2	Day 15 post 1% H_2O_2	Day 30 post 1% H_2O_2
Apoptosis	10.4 ± 8.21	18.91 ± 9.28	87.25 ± 55.41	104.2 ± 5 2.33
Necrosis	5.2 ± 1.2	7.3 ± 2.3	25.23 ± 41.02	27.52 ± 32.31

-Values expressed as means SD significantly at $P < 0.05$. No. of rats/group = 10.

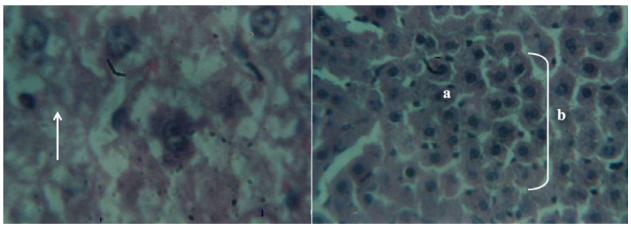

Fig. 1 **Left: light micrograph of rat liver section treated with 1% H_2O_2, showed vacillation of cytoplasm (arrow). Right: (a) cells losing their contact (b) honeycomb-shape. (H & E). 1000X.**

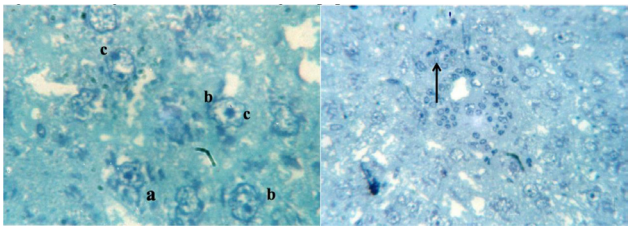

Fig. 2 **Plastic section of liver rat treated with 1% H_2O_2, showing Left: (a) apoptotic hepatocytes, (b) blebbing of plasma membrane, (c) shrinkage, condensation of nuclear chromatin. *I.E.* Resin. 1000X. Right: apoptotic bodies in centrilobular area (arrow). T.B. Resin. 1000X. T.B. toluidine blue.**

Other toluidine blue-stained semi-thin sections further revealed apoptotic hepatocytes, and nuclear remnants, accompanied by cytoplasmic hyper eosinophilia and nuclear hypertrophy (Figure 3).

Additionally, Kupffer cells became hypertrophic with irregular plasma membranes, suggestive of apoptosis, while lipid- laden Ito cells were distended (Figure 4).

Ultrastructural changes: ultrastructural analysis of liver sections revealed significant mitochondrial rarefaction and lysis of cristae, with moderate swelling. Fragmentation and lysis of the endoplasmic reticulum, cytoplasmic disruption, and cell membrane blebbing were evident. Space between the nuclear membrane and nuclear material was occasionally noted (Figure 5).

Results of this study also showed some areas of necrosis characterized by pyknosis, fragmentation of nuclei (karyorrhexis) in addition to some hepatocyte vacuolation, acidophilic changes associated with infiltration of mononuclear inflammatory cells.

Discussion

Both necrosis and apoptosis can be triggered by various drugs and toxins.[16,17] This study aimed to investigate the role of apoptosis in hydrogen peroxide (H_2O_2)- induced liver injury. Our findings provide clear evidence of apoptosis in hepatocytes within the necrotic (midzonal) zone of the liver, marking the first time this process has been confirmed in H_2O_2-induced liver damage. The identification of apoptosis was based on morphological changes observed through both light and electron microscopy, including cell shrinkage, chromatin condensation, apoptotic body formation, and phagocytosis by neighboring cells, which aligns with findings of Hockenberry's and Azizova's.[18,19] These features are distinct from other forms of cell death and suggest that apoptosis plays a significant role

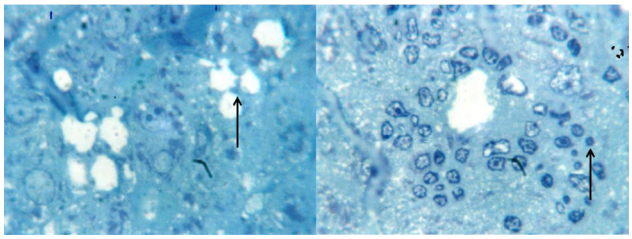

Fig. 3 **Plastic section of liver rat treated with 1% H_2O_2, revealed Left: hepatic Ito cells become distended with lipid (arrows). Right: apoptotic bodies engulphed by neighboring hepatocytes in centrilobular area (arrows). T.B. Resin. 1000X.**

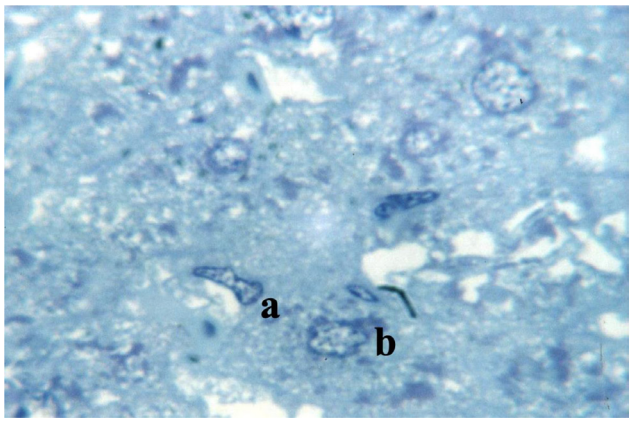

Fig. 4 **Plastic section of liver rat treated with 1% H_2O_2, showing (a) apoptotic Kupffer cells, and (b) apoptotic hepatocytes. T.B. Resin. 1000X.**

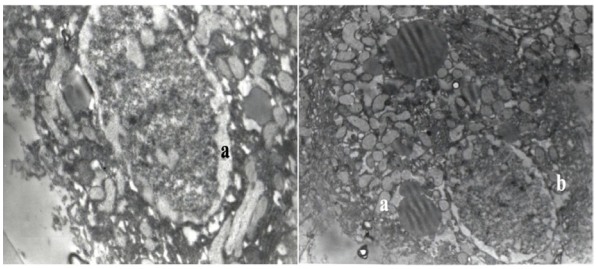

Fig. 4 **Electron micrograph of rat liver section treated with 1% H_2O_2, showing blebbing 1% H_2O_2 increase space between nuclear membrane (a). Right: (a) lysis of cristae, (b) lysis of endoplasmic reticulum.**

in the progression of liver lesions. A hallmark of apoptosis, cell shrinkage, was noted early in the process. This shrinkage is a result of cellular dehydration and loss of intracellular water, leading to cytoplasmic condensation;[20] and the loss of phospholipid asymmetry in the plasma membrane, which exposes phosphatidylserine on the cell surface, marking the cell for phagocytosis[21] The loss of cell-to-cell contact, as cells detached and became rounded, marked the progression of the programmed cell death. Apoptotic cells were also found in the sinusoidal lumen, similar to findings in sodium fluoride and lead nitrate exposures.[21,22] ROS generated during oxidative stress are known to cause various types of cellular injury, including hepatocyte damage.[23] Caspase activation, a central element in apoptosis, can be triggered by both cell surface and intracellular sensors, with mitochondrial damage acting as a key signal amplifier,[24] non-caspase protease such as cathepsins, calpains, and the proteosome complex also play crucial role and mediating apoptosis. Cathepsins are thought to promote apoptosis by cleaving cytosolic substrates, such as caspases, or by directly damaging the nucleus and mitochondrial or lysosomal membranes, leading to the release of. Pro-apoptotic factors.[25–28] On the other hand, apoptotic cell counts showed substantial hepatocyte loss, affecting up to half of the liver lobule through both apoptosis and necrosis (table 2). Apoptosis is considered to be more efficient in removing damaged cells, minimizing the release of inflammatory mediators that can cause further tissue damage. After 15 days of H_2O_2 administration, apoptotic cells were sporadic, while focal apoptosis in Apoptotic cells were found sporadically throughout the lobule at 15 days post- H_2O_2 administration, while focal apoptosis became more pronounced in the centrilobular area after 30 days, suggesting a time-dependent effect. Under oxidative stress, as seen in this study, hepatocytes experience a depletion of GSH which plays a key role in maintaining redox balance. When cells are deprived of GSH, they are more susceptible to oxidative stress and less capable of neutralizing ROS produced by cellular mechanism.[29,30] Prolonged GSH depletion promotes apoptosis, while short-term depletion can have varied effect.[31,32] Additionally, Kupffer cells may contribute to mitochondrial damage, further influencing hepatocyte apoptosis.[33]

Conclusion

Overall, apoptotic pathways in hepatocytes involve a complex series of molecular and biochemical events that result in the orderly disintegration of the cell. These characteristic changes, detectable by microscopy and cytometry, serve as essential markers for identifying apoptosis.

Conflict of Interest

Nil.

Financial Support and Sponsorship

Nil.

References

1. Aramouni K, Assaf R, Shaito A, Fardoun M, Al-Asmakh M, Sahebkar A, Eid AH. Biochemical and cellular basis of oxidative stress: Implications for disease onset. Journal of Cellular Physiology. 2023 Sep;238(9):1951–63.
2. Wang B, Wang Y, Zhang J, Hu C, Jiang J, Li Y, Peng Z. ROS-induced lipid peroxidation modulates cell death outcome: Mechanisms behind apoptosis, autophagy, and ferroptosis. Archives of toxicology. 2023 Jun;97(6):1439–51. Majno G, Joris I. Apoptosis, Oncosis and Necrosis. Am J Pathol, 1995; 146:3–15.
3. Yuan J, Ofengeim D. A guide to cell death pathways. Nature Reviews Molecular Cell Biology. 2024 May;25(5):379–95. Cook, PR, Principles of structure and function. Wiley - Liss, New York, 2002.
4. Obeng E. Apoptosis (programmed cell death) and its signals-A review. Brazilian Journal of Biology. 2020 Oct 23;81(4):1133–43.
5. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science, 1995; 1456–1462.

6. Guo J, Yang WT, Mai FY, Liang JR, Luo J, Zhou MC, Yu DD, Wang YL, Li CG. Unravelling oncosis: Morphological and molecular insights into a unique cell death pathway. Frontiers in Immunology. 2024 Aug 29;15:1450998.
7. Guicciardi ME, Deussing J, Miyoshi H, Bronk SF, Svingen PA, Peters C, Kaufmann SH, Gores GJ. Cathepsin B contributes to TNF - alph -mediated hepatocytes apoptosis by promoting mitochondrial release of cytochrome C. J Clin Invest, 2000; 106:1127–1137.
8. Kashyap D, Garg VK, Goel N. Intrinsic and extrinsic pathways of apoptosis: Role in cancer development and prognosis. Advances in protein chemistry and structural biology. 2021 Jan 1;125:73–120.
9. Mahapatra KK, Mishra SR, Behera BP, Patil S, Gewirtz DA, Bhutia SK. The lysosome as an imperative regulator of autophagy and cell death. Cellular and Molecular Life Sciences. 2021 Dec 1:1–5.
10. Kerro JF. History of the events leading to the formation of the apoptosis concept. Toxicology, 2002; 181–182.
11. Robert RS. Fish pathology. Baillier Tindall, London, 2007.
12. Bhadra K. A mini review on molecules inducing caspase-independent cell death: A new route to cancer therapy. Molecules. 2022 Sep 28;27(19):6401.
13. Atroshi F, Biese I, Saloniemi H, Ali-Vehsa T, Saaris, Rizzo A, Veijalanin P. Significance of apoptosis and its relationship to antioxidants after ochratoxin administration in mice. J Pharm Pharmacut sci, 2000; 3:281–291.
14. Reed D. Glutathione : Toxicological implication. Annu Rev Pharamcol Toxcol, 1990; 30:603–631.
15. Sun X, Berger RS, Heinrich P, Marchiq I, Pouyssegur J, Renner K, Oefner PJ, Dettmer K. Optimized protocol for the in situ derivatization of glutathione with N-ethylmaleimide in cultured cells and the simultaneous determination of glutathione/glutathione disulfide ratio by HPLC-UV-QTOF-MS. Metabolites. 2020 Jul 17;10(7):292.
16. Corcoran GB, Fi L, Jones DP, Moslen MT, Nicotera P, Obcrhammer FA, Buttyan B. Contemporary issues in toxicology, Apoptosis : Molecular control point in toxicity. Toxicol Appl Pharmacol, 1994; 128:169–181.
17. Shojaie L, Iorga A, Dara L. Cell death in liver diseases: A review. International journal of Molecular Sciences. 2020 Dec 18;21(24):9682.
18. Hokenbery D. Defining apoptosis. AM J Pathol, 1995; 146:16–19.
19. Azizova F, Ubaydullayeva M. Cytological picture of apoptosis in liver cells. Universum: медицина и фармакология. 2023(9 (102)):10–3.
20. Romero-Perez PS, Dorone Y, Flores E, Sukenik S, Boeynaems S. When phased without water: Biophysics of cellular desiccation, from biomolecules to condensates. Chemical reviews. 2023 May 3;123(14):9010–35.
21. Shashi A, Thapar SP. Histopathology of fluoride - induced hepatotoxicicty in rabbits, Fluoride 2000, 34:34–42.
22. Dini L, Pagliara P, Carla EC. Phagocytosis of apoptotic cells by liver: Morphological study. Miscrosc Res Techn, 2002; 57:530–540.
23. Sharma P, Nandave M, Nandave D, Yadav S, Vargas-De-La-Cruz C, Singh S, Tandon R, Ramniwas S, Behl T. Reactive oxygen species (ROS)-mediated oxidative stress in chronic liver diseases and its mitigation by medicinal plants. American Journal of Translational Research. 2023;15(11):6321.
24. Heilig R, Lee J, Tait SW. Mitochondrial DNA in cell death and inflammation. Biochemical Society Transactions. 2023 Feb 27;51(1):457–72.
25. Frolova AS, Chepikova OE, Deviataikina AS, Solonkina AD, Zamyatnin Jr AA. New perspectives on the role of nuclear proteases in cell death pathways. Biology. 2023 May 31;12(6):797.
26. Schott P, Van Griekinge W, Van de Craen M, Van Loo G, Desmedt M, Grooten J, Cornlissen M, De Ridder L, Vandekerckhove J, Fires W, Vandenabeele P, Beyaer R. Cathepsin B- mediated activation of the pro inflammatory caspase -11. Biochem Biophys Res Commun, 1998; 251:379–387.
27. Cirman T, Oresic K, Mazovee GD, Tirk V, Reed SC, Myers RM, Salvesen GS, Turk B. Selective disruption of lysomes in Hella cell triggers apoptosis mediated by cleavage of Bid by multiple papain -like lysosomal cathapsins. J Biol Chem, 2004; 279:3578–3587.
28. Brunk UT, Neuzil J, Eaton JW. Lysosomal involvement in apoptosis. RedoxRep, 2001; 6:91–97.
29. Ghibell L, Fanellic C, Rotilio G, Lafavia E, Coppdas S, Colussi C, Civitareale, Ciriolo MR. Rescue of cells from apoptosis by inhibition of active GSH extrusion. Faseb J, 1999; 13:95–102.
30. Milosevic N, Naier P. Lead stimulate intercellular signaling between hepatocytes and Kupffer cells. Eur J Pharmacol, 2000; 401:317–328.
31. Martinez-Jaramillo E, Jamali F, Abdalbari FH, Abdulkarim B, Jean-Claude BJ, Telleria CM, Sabri S. Pro-Oxidant Auranofin and Glutathione-Depleting Combination Unveils Synergistic Lethality in Glioblastoma Cells with Aberrant Epidermal Growth Factor Receptor Expression. Cancers. 2024 Jun 25;16(13):2319.
32. Haouzi D, Lekehal M, Tinel M, Vadrot N, Caussanel L, Letteron P, Moreau A, Feldmann G, Fau D, Pessayre D. Prolonged, but not acute glutathione depletion promotes fas - mediated mitochondrial permeability transition and apoptosis in mice. Hepatology, 2001; 33:1181–1188.
33. Kurosa L, Miura S, Fukumura, Youei Y, Saito H, Tada S, Suematsu M, Tsuchiya M. No mediates Kupffer cells induced reduction of mitochondrial energization in syngenic hepatoma cells: A comparison with oxidative burst. Cancer Res, 1993; 53:2676–2682.