Original Article

The Possible Protective Effect of Apple Cider Vinegar on Mercuric Chloride-Induced Changes on Rat Hepatocytes: Immunohistochemical and Ultrastructure Study

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Abstract

Introduction: Mercuric chloride is a toxic form of mercury capable for induction of oxidative liver damage. Apple cider vinegar (ACV) is a powerful antioxidant agent being used in salad dressings. Our study aimed to assess the beneficial effect of ACV against mercuric chloride-induced hepatic cell damage through an ultrastructural and immunohistochemical study. **Materials and Methods:** Forty Wistar rats used divided into four groups (10 rats each); control; Group A (ACV): Rats received 2 ml/kg ACV; Group B ($HgCl_2$): Rats received 1 mg/kg $HgCl_2$, and Group C (ACV + $HgCl_2$): Rats received 2 ml/kg ACV 30 min before giving 1 mg/kg $HgCl_2$. Doses given orally by intragastric tube for 30 days. **Results:** Toluidine blue results of $HgCl_2$ group revealed hepatocytes with irregular boundaries, eccentric deeply stained nuclei, and large cytoplasmic vacuoles. Electron microscopic results showed dilated rough endoplasmic reticulum, and smooth endoplasmic reticulum, cytoplasmic vacuolations, areas of cytoplasmic rarefaction, degenerated mitochondria, nuclear membrane irregularities, and dilated bile canaliculi with lost microvilli. Moreover, there was significantly increased expression of HSP60 and number of hepatocytes with proliferating cell nuclear antigen-positive nuclei. ACV + $HgCl_2$ group showed improvement of the previous changes. **Conclusion:** ACV could be promising for attenuation of liver cell damages induced by several toxins through its powerful antioxidant properties.

Keywords: Apple cider vinegar, hepatocytes, immunohistochemistry, mercuric chloride, ultrastructure

Introduction

The liver is a large organ of the human body and a primary site of metabolism and excretion of toxins and drugs; so it is a crucial organ for safety health and well-being.[1]

Mercuric chloride ($HgCl_2$) is the chemical compound of mercury and chlorine, has been used in the treatment of syphilis, but it is no longer used because of its mercury toxicity.[2] Mercury is a global component in the environment; individuals exposed to it either by inhalation, ingestion by food chain or through absorption from the skin.[3] Mercury also considered as a major pollutant present in the soil, water in addition to air.[4] Furthermore, trace amounts of mercury are added to skin-whitening cosmetics in addition to its usage as antiseptic as well as disinfectant.[5]

Mercuric chloride is a toxic form of mercury since it easily binds to tissue proteins forming complexes that inhibit the different enzymes involved in multiple biochemical reactions with subsequent tissue damage.[6] Moreover, it is known as a pro-oxidant element inducing tissue oxidative stress with subsequent damage of DNA, lipids and proteins activating many tissue pathological processes.[4] As the liver is considered to be the chief site for mercury metabolism and accumulation; it can cause severe hepatic damage with marked biochemical and morphological cell changes.[7]

The current medicine has a slight effect in relieving hepatic disorders, so looking for plant-based preparations for treating

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such disorders is required.[1] Apple cider vinegar (ACV) is a strong antioxidant agent with free radical scavenging properties. It is used in salad dressings, vinaigrettes, cooking as well as in food preservatives.[8] "Vinegar is the best edible" is a prophetic hadith told by Prophet Muhammad (peace be on him); indicating that prophetic medicine strongly recommended eating vinegar.[8] Recently, ACV proved to have protective effects against different toxins through reducing lipid peroxidation besides suppressing tissue oxidative damage.[9] In the liver, it partially stabilized the hepatic enzyme activity in addition to the improvement of nonenzymatic antioxidant level.[10]

Hence, through the previous data, the current study aims to assess the beneficial antioxidant effect of ACV against $HgCl_2$-induced hepatic cell damage through ultrastructural and immunohistochemical study.

Materials and Methods

Animals and groups

Forty adult male Wistar albino rats obtained from the animal housing, Tanta University, Egypt. Their weights 150–200 g, and housed in plastic cages using air conditioned room (22°C ± 1°C) with a qualified humidity (60% ± 5%). Rats fed a standard laboratory diet with water *ad libitum*. The use of the experimental animals in the present research carried out in accordance with the guidelines in Tanta University and with the approval of the University Animal Experiment Committee with the registered number (33994/8/20) and didn't contain any studies that involves human.

Four groups used in the current work (10 rats each); control group: further subdivided into two subgroups (5 rats each); Subgroup a: rats kept without treatments and Subgroup b: rats received distilled water in a dose corresponding to its experimental groups; Group A (ACV group): rats received ACV (Egyptian Spanish Company for Essential Oils, Egypt) in a dose of 2 ml/kg dissolved in distilled water in accordance to Omar *et al*. (2015); Group B ($HgCl_2$ group): rats received $HgCl_2$ (Sigma-Aldrich, Egypt) dissolved in distilled water in a dose of 1 mg/kg according to Uzunhisarcikli *et al*. (2016); Group C (ACV + $HgCl_2$ group): rats received ACV 30 min before $HgCl_2$ in a dose of 2 ml/kg and 1 mg/kg, respectively. Doses were given orally through an intragastric tube for 30 days. At the end of the experiment, rats anesthetized through i.p., injection of pentobarbital in a dose of 60 mg/kg, and liver specimens obtained for ultrastructural and immunohistochemical study.

Processing for ultrastructural study

The specimens obtained from the liver processed according to Graham and Orenstein (2007). Shortly, specimens cut into small pieces, fixed in 2.5% glutaraldehyde, followed by fixation in 1% osmium tetroxide at 4°C, then dehydrated and embedded in epoxy resin. After that, semithin sections obtained and stained with toluidine blue for light microscopic examination. Whereas, the ultrathin sections cut into 50 nm using LKB Bromma 8800 ultramicrotome, then picked up on 200 mesh copper grids stained with 2% uranyl acetate and lead citrate. Sections then examined by JEOL, Germany transmission electron microscopy at the Electron Microscopic (EM) Unit, Faculty of Medicine, Tanta University, Egypt.

Proliferating cell nuclear antigen and heat shock protein 60 immunohistochemistry

In accordance with Yan *et al*. (2009) and Salama *et al*. (2013); specimens embedded in paraffin after fixation in formalin, then deparaffinized and rehydrated in descending grades of alcohol. Sections then placed in 0.3% hydrogen peroxide/methanol for about 20 min and washed with phosphate-buffered saline (PBS) to block the endogenous peroxidase activity. Afterward, sections treated by a serum-free protein blocking solution for 20 min at room temperature so that the nonspecific protein binding sites blocked. Sections then incubated with anti-proliferating cell nuclear antigen (PCNA) antibody (1:200) (Santa Cruz Biotechnology Inc., California, USA) and anti-heat shock protein 60 (HSP60) antibody (1:100) (Abcam, Cambridge, United States), overnight at 4°C. Then, slides washed in PBS buffer and incubated with biotinylated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc.) for 20 min at 37°C. At last, 1–2 drops of diaminobenzidine (DAB) and chromogen added to 1 ml of DAB substrate to be applied to the sections 5–10 min. Finally, sections counterstained with Mayer's hematoxylin, then were dehydrated, cleared, and examined using a light microscope (Olympus, Optical Co., LTD, Tokyo, Japan). For PCNA: positive nuclear reaction seen, while HSP60 presented brown cytoplasmic reaction. Regarding negative control; the primary antibody replaced by PBS.

Statistical analysis

Number of hepatocytes with proliferating cell nuclear antigen-positive immunostaining

Through using a light microscope (Olympus, Japan) at ×400, the number of hepatocytes with positive nuclear staining for PCNA counted in 10 arbitrarily nonoverlapping selected fields in each slide of the different experimental groups. Then expressed as the number of PCNA-positive cells/mm^2.

Estimation of the color intensity of hepatocytes' heat shock protein 60 cytoplasmic immunostaining

Ten dissimilar images (×400) from each group used by ImageJ software (The National Institute of Health, Bethesda, Maryland, USA) to estimate the color intensity of hepatocytes' HSP60 cytoplasmic immunostaining which was reported as the mean of measured blue nuclei minus mean of the cytoplasmic background.

Statistically significant differences between the different experimental groups of the present research assessed through Minitab Statistical Software for Windows (version 16.1, Minitab Inc., State College, PA, USA). Then, variances of data analyzed by either two-tailed Student's *t*-test or the Mann–Whitney's U-test after valuation by *F*-test. The *P* value considered significant when <0.05.

Results

Toluidine blue results

Toluidine blue-stained sections of the control as well as ACV groups showed polyhedral liver hepatocytes with large central rounded vesicular nuclei and prominent nucleoli in addition to some cells with double nuclei. $HgCl_2$ group revealed cells with degenerative changes through which hepatocytes appeared with irregular boundaries besides eccentric, deeply stained nuclei of many cells, and vesicular nuclei of a few of them. In addition, most of the hepatocytes noticed to have large vacuoles disseminated throughout the cytoplasm, and others appeared with smaller ones in addition to either central or peripheral nuclei. Regarding ACV and $HgCl_2$ group, markedly reduced pathological cell changes seen to be nearly similar to the control group [Figure 1].

Electron microscopic results

EM examination of the control and ACV groups; normal hepatocytes with its cytoplasm containing a large number of mitochondria; besides, rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), lipid droplets, glycogen granules, peroxisomes, and lysosomes. In addition, every hepatocyte displayed a vesicular nucleus with prominent nucleoli. Furthermore, hepatocytes separated from each other by narrow bile canaliculi with microvilli projecting into its lumen and along the sides of the bile canaliculi hepatocytes attached to each other by desmosomes [Figure 2].

Considering $HgCl_2$ group; the cytoplasm contained multiple lysosomes and peroxisomes, dilated RER, and SER and cytoplasmic vacuolations with areas of cytoplasmic rarefaction. In addition, there were polymorphic mitochondria; by which some were degenerated with destroyed cristae while others were filamentous, and electron-dense accompanied by nuclear membrane irregularities and indentation as well. As regards bile canaliculi, they were markedly dilated with lost microvilli [Figure 3].

Moreover, ACV and $HgCl_2$ groups exposed the majority of hepatocytes with a normal EM picture that was similar to the control group [Figure 4].

Proliferating cell nuclear antigen results

Few hepatocytes with positive PCNA immunohistochemical reaction in control & ACV groups seen. While in $HgCl_2$ group, a significant decrease in the number of hepatocytes with positive nuclei for PCNA remarkably seen. In ACV & $HgCl_2$ group, nearly normal picture observed [Figures 5 and 6].

Heat shock protein 60 results

Control group and ACV revealed nonsignificant differences from each other by which hepatocytes showed mild HSP60 cytoplasmic immunoreaction. Hepatocytes in $HgCl_2$ group demonstrated a significant increase in the cytoplasmic immunoreaction for HSP60 when compared to control group. Adversely, in ACV and $HgCl_2$ group a significant decrease in HSP60 mean color intensity was observed in comparison to $HgCl_2$ group to be semblance to the control group [Figures 7 and 8].

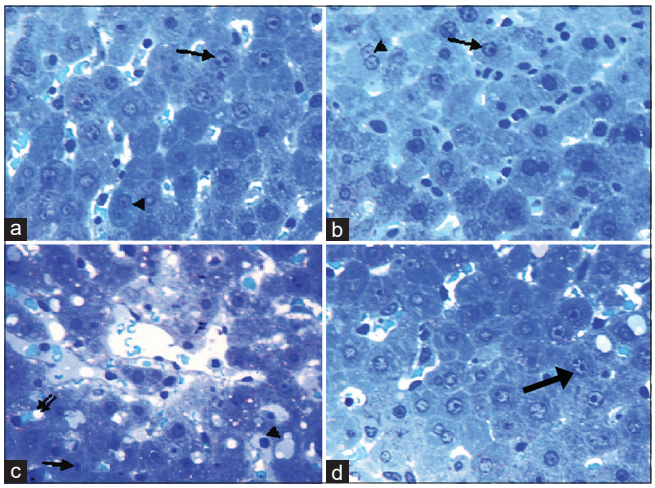

Figure 1: (a) Control: Polyhedral liver hepatocytes with large central rounded vesicular nuclei and prominent nucleoli (→) and some cells with binary nuclei (►). (b) Apple cider vinegar group: Hepatocytes with large central rounded vesicular nuclei and prominent nucleoli (→) and other cells with two nuclei (►). (c) $HgCl_2$ group: Hepatocytes with irregular boundaries besides eccentric, deeply stained nuclei of many cells (→), small (two arrows), and large cytoplasmic vacuoles (►). (d) Apple cider vinegar and $HgCl_2$ group: Polyhedral hepatocytes with large central rounded vesicular nuclei and prominent nucleoli (→) (Toluidine blue ×1000)

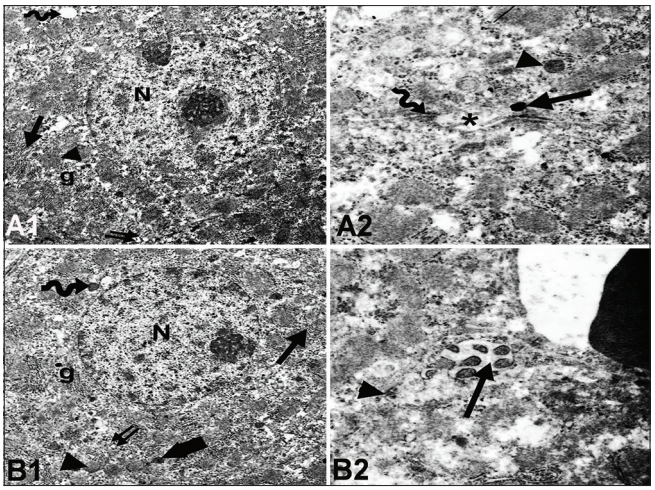

Figure 2: (A1) Control group showed hepatocytes' cytoplasm containing mitochondria (►), rough endoplasmic reticulum (→), smooth endoplasmic reticulum (two arrows), lipid droplets (wavy arrow), glycogen granules (g), vesicular nucleus with prominent nucleolus (N) (× 2000). (A2) control group showed peroxisomes (►) and lysosomes (→), narrow bile canaliculi with microvilli projecting into its lumen (*), and desmosomes (wavy arrow) (× 5000). (B1) Apple cider vinegar group showed mitochondria (►), rough endoplasmic reticulum (→), smooth endoplasmic reticulum (two arrows), glycogen granules (g), peroxisomes (thick arrow) and lysosomes (wavy arrow), vesicular nucleus with prominent nucleolus (N) (× 2000); (B2) Apple cider vinegar group showed narrow bile canaliculi with microvilli projecting into its lumen (→), and desmosomes (►) (×5000)

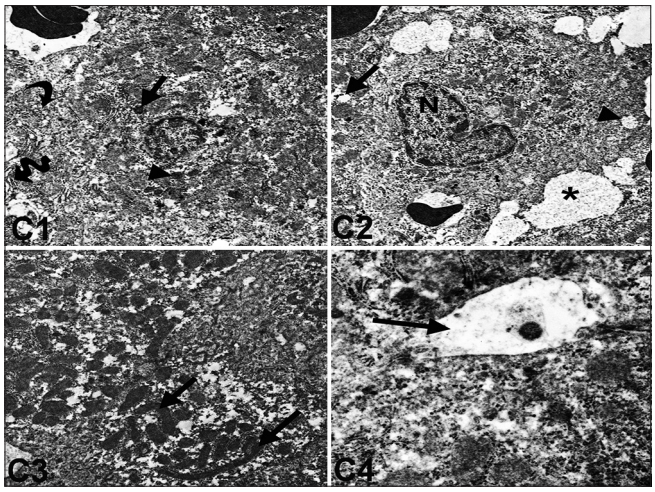

Figure 3: Electron microscopic of $HgCl_2$ group; (C1) revealed multiple lysosomes (→), and peroxisomes (►), dilated rough endoplasmic reticulum (wavy arrow), and smooth endoplasmic reticulum (curved arrow) (×2000). (C2) areas of cytoplasmic rarefaction (*), and vacuolations (→), degenerated mitochondria with destroyed cristae (►), nuclear membrane irregularities and indentation (N) (×2000). (C3) electron-dense filamentous mitochondria (→) (×2000). (C4) bile canaliculi markedly dilated with lost microvilli (→) (×5000)

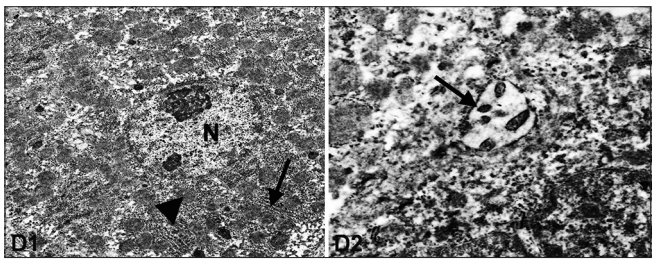

Figure 4: Apple cider vinegar and $HgCl_2$ group: (D1) Euchromatic nucleus and prominent nucleolus (N), rough endoplasmic reticulum (→), mitochondria (►) (×2000). (D2) Bile duct with microvilli (→) (×5000)

Discussion

Exposure of the liver to different toxins can affect its function causing the liberation of free radicals in the body.[11] Powerful antioxidants are needed to scavenge radicals' activity, so protecting liver cells from injury and damage.[12] ACV is a type of vinegar made from apple must through crushing apples and squeezing out the liquid with the addition of bacteria and yeast starting the alcoholic fermentation procedure, so sugars will turn into alcohol, then the alcohol is transformed into vinegar through acetic acid-forming bacteria that is called acetobacter.[13] It is a strong and powerful detoxifying agent through its antioxidant as well as radical scavenging properties.[9]

Our study showed that $HgCl_2$-induced liver cell injury manifested by degenerative cell changes as demonstrated by light and EM examination, in addition to a significant decrease in the number of hepatocyte with PCNA positive nuclei as well as increased expression of its cytoplasmic HSP60. On the other hand, the administration of ACV 30 min before $HgCl_2$ revealed a reversal of the previous findings as proved by our study results.

In the current research, the administration of $HgCl_2$-induced diverse degenerative cell changes in the form of irregular cell boundaries, eccentric, deeply stained nuclei, and large cytoplasmic vacuoles in addition to degenerative changes among the different cell organelles. This could be attributed to the toxic effects of $HgCl_2$ through the generation of free radicals inducing oxidative cell injury as well as the excessive release of reactive oxygen species followed by increased lipid peroxidation inside the cell.[14,15] Therefore, damage to DNA, oxidation of different proteins, as well as the formation of nitric oxide and peroxidation of cell constituents will ensue impairment of the antioxidant system[16,17] previously mentioned that; Hg_2 has a great affinity for thiol-containing

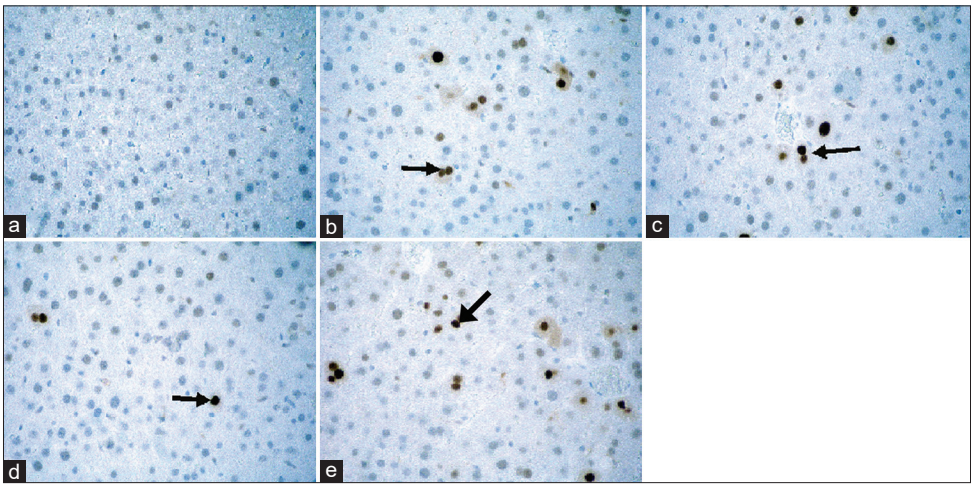

Figure 5: (a) Negative control with no PCNA immunoreaction. (b) Control: Expressed scarce hepatocytes with positive PCNA immunoreaction (→). (c) Apple cider vinegar group: Showed few hepatocytes with positive PCNA immunoreaction (→). (d) $HgCl_2$ group with decreased number of hepatocytes with positive nuclei for PCNA (→). (e) Apple cider vinegar + $HgCl_2$ group showed few hepatocytes with positive PCNA immunoreaction (→) (PCNA immunostaining ×400)

molecules such as glutathione, forming a complex that prevents Hg_2 from binding to cellular proteins hence, causing tissue injury.

Conversely, the administration of ACV markedly attenuates the degenerative cell changes induced by $HgCl_2$. This might be due to its antioxidant properties in addition to its free radical scavenging effects, so decreasing lipid peroxidation, as well as DNA damage, and suppressing the $HgCl_2$-induced oxidative hepatic cell injury.[10] Recently Ho *et al*. (2017) proved that ACV contains many bioactive substances, including polyphenol, carotenoids, and vitamins, especially Vitamin C and E; all of which could give the powerful antioxidant properties of ACV.

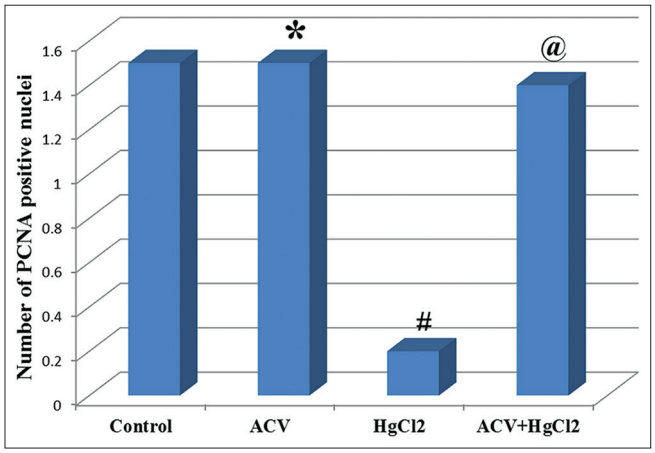

Figure 6: Number of hepatocytes with PCNA-positive nuclei of the different experimental groups, expressed as mean ± standard error of mean (n = 10 rats/group). Control: Control group; Apple cider vinegar (ACV): Apple cider vinegar group, $HgCl_2$: Mercuric chloride group, and apple cider vinegar (ACV) + $HgCl_2$: Apple cider vinegar and mercuric chloride groups. $^{*}P > 0.05$ nonsignificant relative to control; $^{\#}P < 0.05$ significant relative to control; and $^{@}P < 0.05$ significant relative to $HgCl_2$ group

In the present work, PCNA immunohistochemical results showed a significant decrease in the number of hepatocytes with positive nuclei for PCNA in $HgCl_2$ group. It was proved that mercury inhibits cell proliferation and growth through its interference with the cell cycle as it blocks the S-phase, decreasing the DNA replication.[18] Moreover, $HgCl_2$-induced decrease in the cell proliferation through the induction of autophagic cell death in hepatocytes preferring apoptosis rather than inducing changes in the cell cycle.[19] Oppositely, the administration of ACV showed a significant increase in the number of hepatocytes with PCNA positive nuclei; and this could belong to the different constituents of ACV especially Vitamin A, B_6, C, E, thiamin, riboflavin, niacin, beta-carotenes besides, lycopene which improves the cell survival.[20]

HSPs are highly preserved sequence of genes whose expression induced by heat shock.[21] HSP60 is a member of HSPs playing an important role in many basic processes of the cell.[22] It acts as a molecular chaperone that stabilizes the trafficking of nascent peptides during normal growth.[23] Under stress conditions (e.g., oxidative stress), its expression increased to protect the cell through stabilizing the unfolded as well as the misfolded peptides, giving the cell a time to repair or re-synthesize its damaged proteins.[24]

The present work revealed significantly increased HSP60 expression in the cytoplasm of hepatocytes regarding $HgCl_2$ group. It is strongly correlated with $HgCl_2$-induced oxidative injury as it leads to destabilization of the intracellular proteins as well as cell organelles, especially mitochondria.[25] Belles *et al*., (1999) previously mentioned that HSP60 is present in the mitochondrial matrix as it controls the import and folding of oxidative enzymes; so its production underexposure of hepatocytes to oxidative stress will be increased to improve the cell survival. On the contrary, our study showed that ACV significantly decreased the hepatocytes' cytoplasmic expression of HSP60. The mechanism of which that ACV

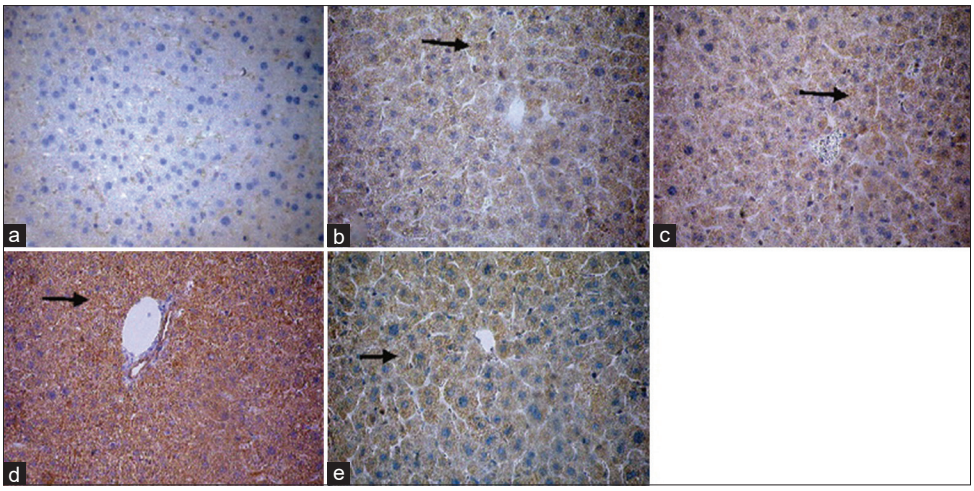

Figure 7: (a) Negative control showed no HSP60 reaction. (b) Control: Revealed hepatocytes with mild HSP60 cytoplasmic immunoreaction (→). (c) Apple cider vinegar group showed hepatocytes with mild cytoplasmic immunoreaction for HSP60 (→). (d) $HgCl_2$ group demonstrated hepatocytes with strong HSP60 cytoplasmic immunoreaction (→). (e) Apple cider vinegar + $HgCl_2$ group with mild HSP60 immunoreaction (→) (HSP60 immunostaining ×400)

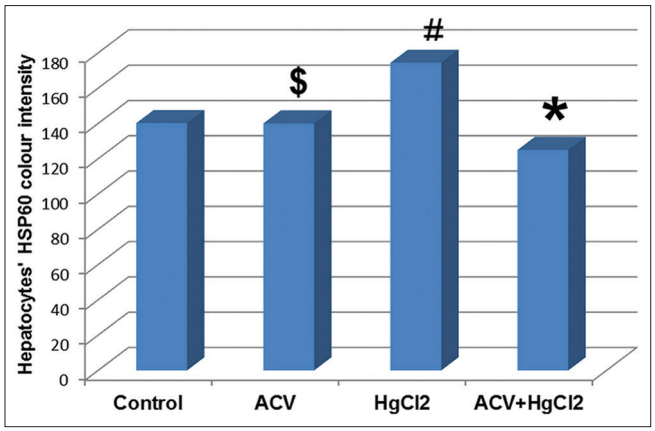

Figure 8: Hepatocytes' HSP60 cytoplasmic immunostaining color intensity of the research experimental groups. The data were expressed as mean ± standard error of mean (10 rats per each group). Control was the control group; Apple cider vinegar (ACV) was an apple cider vinegar group, $HgCl_2$ was mercuric chloride group, and apple cider vinegar (ACV) + $HgCl_2$ were apple cider vinegar and mercuric chloride groups. $^{\$}P > 0.05$ nonsignificant relative to control; $^{\#}P < 0.05$ significant relative to control; and $^{*}P < 0.05$ significant relative to $HgCl_2$ group

with its major constituents as flavonoids and polyphenols provide several pharmacological actions, including antioxidant properties; so could reverse the oxidative stress on hepatocytes, consequently decreasing the cellular levels of HSP60.[26]

Conclusion

ACV could attenuate $HgCl_2$-induced ultrastructural and immunohistochemical changes in rat hepatocytes through its strong antioxidant properties. Hence, ACV could be promising for attenuation of liver cell damages induced by various toxins.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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