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Original Article

HEPATOCYTE APOPTOSIS INDUCTION BY ACETAMINOPHEN THROUGH MODULATION OF CASPASE/BAX PATHWAY IN MICE

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ABSTRACT

Objective: Acetaminophen (APAP) overdose contributes to liver damage through modulation of pro-apoptotic processing. This study evaluated the involvement of caspase/Bax factors in APAP hepatotoxicity *in vivo* and *in vitro.*

Methods: The involvement of caspase/Bax factors in APAP hepatotoxicity was evaluated in BALB/c mice and on isolated primary mouse hepatocytes. *In vitro* MTT assay was carried out on primary cultured mouse hepatocytes treated with APAP (2.5, 5, 10 mmol) and Annexin V/PI staining was employed to cell suspension for imaging under fluorescence microscopy. In addition, caspase-3 concentrations were determined in cell lysates. *In vivo*, mice were treated with a toxic dose of APAP (700 mg/kg) and immunodetection of Bax was made by Western Blot. Vitamin C (Vit C) was used as a hepato-protectant due to its known antioxidant activities.

Results: *In vitro* dose-dependent increase in mitochondrial electron transport capacity was evident in isolated mouse primary hepatocytes incubated with the high dose of APAP (10 mmol) compared to both nontreated cells and cells pre-treated with Vitamin C (Vit C) (0.5 mmol) (p<0.05). Apoptosis was confirmed in hepatocytes through Annexin V staining after APAP treatment and the signal was reduced when hepatocytes were pre-treated with Vit C. In addition, caspase-3 concentration was decreased in cells pretreated with Vit C prior to APAP exposure. *In vivo*, Bax immunodetection utilizing western blotting was increased in mice treated with the toxic dose of APAP (700 mg/kg) and attenuated through pre-treatment with Vit C.

Conclusion: Modulation of apoptotic caspase/Bax pathway is present in hepatocytes undergoing APAP-induced toxicity.

Keywords: Liver injury, Hepatoprotection, Hepatotoxicity, Cell proliferation, Programmed cell death, Mitochondrial stress

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INTRODUCTION

The exact mechanism of toxicity behind Acetaminophen (APAP, also known as N-acetyl-para-aminophenol or paracetamol) is not fully elucidated despite its popular worldwide use as a safe and effective analgesic and antipyretic at therapeutic doses. Around 25% of administered APAP dose undergoes the first-pass metabolism primarily by conjugation with glucuronic acid and sulphate, thereafter, directly excreted in the urine [1]. Overdosing with APAP results in a fully saturated conjugative pathway shifting APAP metabolism into the cytochrome P450 (CYP450) pathway, therefore, leading to increased production of the toxic metabolite, namely, N-acetyl-p-benzoquinone imine (NAPQI), which reduces glutathione (GSH) levels until depleted allowing disruptive binding to mitochondrial proteins [2]. It has been widely reported that NAPQI-protein adduct formation leads to mitochondrial oxidative stress that generates superoxide anion O_2^- and other reactive oxygen species (ROS) and nitrogen-oxygen species (NOS) [3]. However, before NAPQI manages to induce cell death, initial mitochondrial metabolic activation has been reported to be critical as it needs to be amplified and propagated in order to initiate the cellular injury [4].

Since apoptosis is carefully programmed and balanced in a physiological context, the failure of this regulation results in critical pathological conditions [5]. In apoptosis, the integrity of mitochondrial architecture is lost causing depletion of ATP and translocation of Bax, a pro-apoptotic Bcl-2 protein, from the cytosol to the mitochondrion that causes pore formation in the outer mitochondrial membrane. This, in turn, releases pro-apoptotic cytochrome c from the mitochondrial intermembrane space and activates effector caspase-3 [6]. Moreover, once the cell undergoes apoptosis, an inner leaflet restricted lipid component called phosphatidylserine (PS) is translocated to the outer membrane scrambling the plasma membrane lipid asymmetry, thus, indicating apoptosis [7].

Since the toxicity of APAP has been widely described as dose-dependent and several stages might be involved in its toxicity, the aim of the present study was to investigate the involvement of caspase/Bax pathway as a possible mechanism engaged in the activation of liver apoptosis during APAP hepatotoxicity. In addition, the extent of tackling APAP-induced caspase/Bax pathway activation by vitamin C (Vit C); as an efficient antioxidant known for its ROS scavenging and hepatoprotective effects, was approached [8].

MATERIALS AND METHODS

Materials

Acetaminophen (APAP), sodium chloride (NaCl), hydrochloric acid (HCl), L-ascorbic acid, and Dulbecco's Modified Eagle Media (DMEM) (with L-glutamine) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Isoflurane was supplied from Hikma Pharmaceuticals (Amman, Jordan). Hank's Balanced Salt Solution (HBSS) Ca^{2+} and Mg^{2+}free, HBSS with Ca^{2+} and Mg^{2+} and collagenase II were purchased from Gibco (Gaithersburg, MD, USA). Fetal bovine serum (FBS), and trypsin were both obtained from Biowest (Nuaillé, France). 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) was purchased from Alfa Aesar (Massachusetts, USA). While streptomycin/penicillin was purchased from Caisson Labs (Utah, USA).

Cell Titer 96 Non-Radioactive Cell Proliferation Assay kit was purchased from Promega (Wisconsin, USA). Mouse Casp3 (caspase-3) enzyme-linked immunosorbent assay (ELISA) kit was purchased from MyBioSource (San Diego, USA). For western blotting, Mini-PROTEAN TGX Precast Gels, Clarity Western ECL Substrate, 10x Tris/Glycine/SDS (TGS) buffer and 2x Laemmle were all obtained

from Biorad (California, USA). Gang-Nam prestained protein ladder was provided from iNtRON Biotechnology (Gyeonggi, South Korea). Tris Base, 2-mercaptoethanol and Tween 20 were obtained from Santa Cruz Biotechnology (Texas, USA) while methanol was supplied from Tedia company (Ohio, USA). Non-fat milk was purchased from Laita Diary Company (Brest, France). Nitrocellulose transfer membrane, Anti-Bax antibody and Goat Anti-Rabbit IGg HandL-HRP were obtained from Abcam (Cambridge, UK).

Preparation of buffers and culture medium

All perfusion buffers were freshly prepared using sterile techniques in a sterile class 2 biosafety cabinet (Topair systems, New York, USA), according to Al Shaker *et al.* (8). Perfusion buffer I was prepared by the addition of 0.5 mmol EDTA, 25 mmol HEPES and 0.5 mmol Tris base to HBBS without $Ca^{2+}$and Mg^{2+}. Perfusion buffer II was prepared by adding 25 mmol HEPES and 0.5 mmol Tris base to HBSS with $Ca^{2+}$and Mg^{2+}. Perfusion buffer II with collagenase II was prepared by dissolving 1000 U/ml collagenase II in perfusion buffer II. Buffers were kept warm at 37 °C in a water bath and used within 30 min from preparation.

Plating medium was prepared by the addition of pre-warmed (37 °C) 10% FBS, 100 U/ml penicillin, 100 mg/ml of streptomycin and 10 mmol HEPES buffer to DMEM medium. Liver collection medium was prepared by suspending 25 mmol HEPES in cold DMEM.

Animal handling

The study protocol was reviewed by the Scientific Committee of the Department of Pharmacology and Biomedical Sciences and approved by the Scientific Ethical Committee of the Faculty of Pharmacy and Medical Sciences, University of Petra, Amman, Jordan (Approval number: 5-2016/2017). Adult male BALB/c mice with an average weight range of 22±3 g were housed at the Animal House of the University of Petra. Mice were kept in air-conditioned environment under controlled temperatures (22-24 °C), humidity (55-65%), and photoperiod cycles (12 hr light/12 hr dark). All experiments were achieved in accordance with University of Petra Institutional Guidelines on Animal Use which adopt the guidelines of the Federation of European Laboratory Animal Science Association (FELASA).

Mouse liver cannulation and perfusion

An adult male BALB/c mouse was anaesthetized by isoflurane and the liver was cannulated surgically through the portal vein as described by Al Shaker *et al.* (9); with few modifications where the pre-warmed perfusion buffer I was infused for approximately 20 min at a flow rate of 3 ml/min until the liver had blanched to a light-brown colour. The perfusion was later switched to perfusion buffer II containing collagenase II at a flow rate of 4 ml/min for 15 min. The liver was then dissected and soaked in a cooled liver collection medium in order to be transferred to the cell culture hood.

Culture of primary mouse hepatocytes

Hepatocytes were cultured in a 96-well plate and allowed to recover and grow in a humidified sterile CO_2 incubator overnight prior the day of experimentation. After 16 h of incubation, the 96-well plate was divided into 9 groups. Group 1 as blank (media only), group 2 as a negative control (untreated healthy cells), group 3 Vit C 0.5 mmol, and groups 4, 5, and 6 treated with APAP 2.5, 5 and 10 mmol respectively. Groups 7, 8, 9 were pretreated with 0.5 mmol Vit C for 4 h prior the addition of APAP at concentrations of 2.5, 5, and 10 mmol, respectively. Treatment of hepatocytes and further mentioned assays were determined within the first 24 h from plating to assure minimal decline of CYP450 activity.

Determination of mitochondrial electron transport capacity in isolated hepatocytes

Apoptotic bodies are intact cells and maintain viable mitochondria, which provides cells with the needed ATP for energy-dependent cell death namely, apoptosis [11]; therefore, MTT assay was approached for that detection [12, 13]. Cultured and treated primary hepatocytes were subject to mitochondrial electron transport capacity determinations assessed by conducting an MTT assay using CellTiter 96 Non-Radioactive Cell Proliferation Assay kit, as instructed by the manufacturer (Promega, Wisconsin, USA). Optical density (OD) measurements were performed using Promega's Glomax multi detection system (Wisconsin, USA).

Annexin V/Propidium Iodide double staining

Freshly isolated mouse hepatocytes were prepared as described earlier and incubated with APAP (10 mmol), Vit C (0.5 mmol), a combination of both APAP and Vit C for 3 h in a humidified CO_2 incubator under 37˚C. Following treatment, hepatocytes were resuspended in binding buffer, Annexin V and Propidium Iodide (PI) dye solutions. After 5 min of incubation in the dark, samples were investigated under fluorescence microscopy using fluorescence green and Texas red filters as per the manufacturer's instructions (Abcam, Cambridge, UK).

Caspase-3 concentration assay

Caspase-3 concentrations were determined in cell lysate samples of cultured primary mouse hepatocyte using Mouse Casp3 (Caspase-3) ELISA kit (MyBioSource, San Diego, USA). Collection of cell lysates was proceeded by the addition of 0.25% Trypsin to the treated hepatocytes cell pellets with further sonication for 10 seconds using Qsonica probe sonicator (Newtown, CT. USA), followed by centrifugation at 1500 rpm for 10 min at 4 °C. The lysed supernatant was collected and tested immediately according to the manufacturer's protocol. Optical density (OD) was read at 450 nm using an ELISA reader (Promega's Glomax multi detection system, Wisconsin, USA).

***In vivo* immunodetection of bax by western blot**

BALB/c mice were randomized into 4 groups (n=8 per group) and acclimatized for ten days before *in vivo* experiment. Mice were treated as following; group 1 served as a control, group 2 administered APAP (700 mg/kg) suspended in 0.5% CMC solution, group 3 received Vit C (100 mg/kg) [14], while group 4 was pretreated with Vit C followed by APAP. All mice received either a daily single oral dose of 0.5% CMC or Vit C dissolved in CMC (100 mg/kg) for three days prior APAP administration. Mice were fasted overnight with free access to water, and on the fourth-day groups 2 and 4 received APAP (700 mg/kg) concomitantly with their corresponding treatments. Six hours post-APAP administration, blood was withdrawn from the orbital sinus under anaesthesia and serum was separated and stored at-20˚C for further analysis. Mice were euthanized by cervical dislocation after blood sampling.

Semi-quantitative determinations of Bax levels were performed by western blot analysis according to standard protocols. Briefly, previously stored serum samples obtained from BALB/c mice were mixed with loading dye buffer and heated for 10 min at 80˚C in a mini heating dry bath (Major science, California, USA). Samples and a reference protein ladder were loaded on precast denaturing 10% SDS-PAGE gels with a final protein concentration of 10 µl/ml and run in Biorad Mini-PROTEAN Tetra System (California, USA) under standard running conditions. After electrophoresis, the proteins were blotted on nitrocellulose transfer membrane using wet buffer method. Blots were pre-blocked with 5% non-fat milk in Tris-Tween-Buffer-Saline (TTBS) and probed with primary antibody Bax in milk at a 1:1000 ratio and secondary antibody anti-bax peroxidase-conjugated in milk (ratio of 1:10000) Blotted membranes were exposed to enhanced chemiluminescence (ECL) substrate, it was visualized on LI-COR C-DiGit Chemiluminescence Western Blot Scanner (LI-COR Biotechnology, NE, USA).

Statistical analysis

One-way analysis of variance (ANOVA) was performed to detect statistical significance between tested groups using SPSS software, version 22 (IBM Corporation, NY, USA). Values with p-value less than 0.05 were considered significant. Values are presented as mean±standard error of the mean (SEM).

RESULTS AND DISCUSSION

In the presented work, mice were selected as the animal model of choice to reveal the mechanism by which APAP induces its hepatotoxicity. Mice were previously found more sensitive to APAP

toxicity than rats, accounted to alterations in APAP metabolism and protein binding, in addition to differences in mitochondrial dysfunction and oxidative stress between species [15, 16]. However, it has been reported that APAP is capable of inducing hepatotoxicity in rats in a similar manner to that induced in mice when a three-fold higher dose is employed [17, 18].

APAP Induction of mitochondrial-mediated hepatotoxicity

A dose-dependent increase in mitochondrial electron transport capacity was noted in APAP-treated hepatocytes as illustrated in fig. 1. Incubating hepatocytes with 2.5 and 5 mmol APAP caused an insignificant increase in the mitochondrial electron transport capacity in comparison to untreated cells (p>0.05). However, a significant amplification of mitochondrial electron transport capacity was observed in hepatocytes treated with 10 mmol APAP therefore, this dose was used in the subsequent studies (p<0.05). The capability of APAP in inducing mitochondrial electron transport capacity in a dose-dependent manner. A significant increase in mitochondrial electron transport capacity was indicated when isolated hepatocytes were challenged with 10 mmol APAP compared to lower concentrations (fig. 1). The mitochondrial apoptotic pathway has been considered as a vital mean of initiating cell survival [19], as it initiates the production of pro-apoptotic proteins in response to the induced insult, activating caspase activity and apoptosis [20]. Despite to CYP450 enzyme activation, which is a potential source of ROS and oxidative stress, the mitochondria stays the major source of ROS formation after toxic stimuli, in addition, its abnormal metabolic activity in apoptosis increased NAPDH oxidase production [21]. Furthermore, mitochondrial activity is necessary in ATP-dependent cell death, including apoptosis [22, 23].

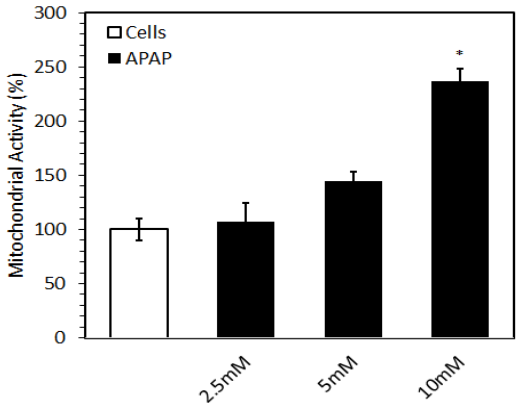

Fig. 1: Percentage of mitochondrial electron transport capacity of hepatocytes exposed to different doses of APAP (2.5, 5 and 10 mmol) over a period of 2 h, Each data point represents the mean±SEM (n=8 wells). 10 mmol APAP had significantly increased the mitochondrial electron transport capacity of hepatocytes. *p<0.05

The Impact of pretreating intoxicated hepatocytes with Vit C

As illustrated in fig. 2, the mitochondrial electron transport capacity in hepatocytes solely treated with Vit C was not changed (p>0.05). However, pretreating hepatocytes with Vit C prior to APAP intoxication reduced the APAP-induced dose-dependent induction of mitochondrial electron transport capacity. Such effect was found significant at the dose of 10 mmol APAP (p<0.05). Nevertheless, such a reduction in activity was not detected in isolated hepatocytes treated with lower doses of APAP (p>0.05). It has been reported that Vit C could ameliorates the hepatotoxic effects of high doses of APAP on rodents through normalizing GSH levels, superoxide dismutase (SOD), and malondialdehyde (MDA) [24–26]. In addition to its antioxidative property by acting as an important free radical scavenger in the extracellular fluids, Vit C has been reported to inhibit the ability of ROS from binding to the mitochondrial membrane and limiting its disruption [27,28]. Thereafter, the antioxidant Vit C was selected to inhibit the cytotoxic effects of APAP and test its influence on the insult *in vivo* and *in vitro.* Vit C was capable of inhibiting the cellular insult caused by APAP. The presented data revealed that pretreatment with antioxidant Vit C effectively lowered APAP-induced escalation in mitochondrial electron transport capacity. These results are in agreement with other studies which showed that APAP toxicity could be ameliorated by hepatoprotective scavenging effects [24, 29, 31].

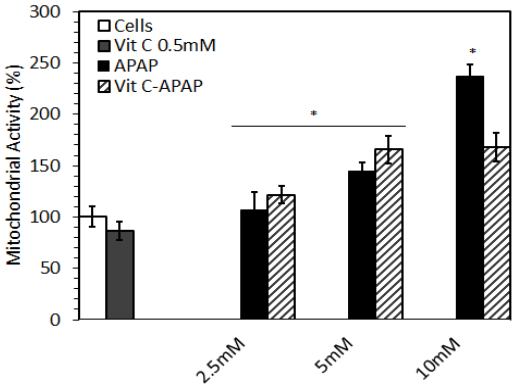

Fig. 2: The hepatoprotection produced by Vit C (0.5 mmol) on cells treated with different doses of APAP (2.5 mmol, 5 mmol and 10 mmol). The data shows that Vit C significantly reduced APAP-induced increase in cell mitochondrial electron transport capacity in 10 mmol APAP-treated hepatocytes. *p<0.05

Induction of apoptosis in hepatocytes

Untreated hepatocytes did not show any noticeable binding to Annexin V or PI. On the other hand, Annexin+ve/PI–ve cells, representing apoptotic cells, were observed after APAP treatment as shown in table I and fig. 3. However, fewer cells were seen positive to both Annexin V and PI, indicating an intermediate or late stages of apoptosis or were undergoing subsequent secondary necrosis. Isolated primary hepatocytes incubated with Vit C prior to APAP presented the absence of necrotic cells and a significant decrease in apoptotic bodies. Annexin staining of APAP-treated hepatocytes suggested that the major mode of cell death post APAP cellular toxicity is apoptosis rather than necrosis (fig. 3). This result agrees with other published reports that demonstrated the occurrence of apoptosis in more than 60% of cells exposed to APAP [22,32]. The observation of some necrotic cells agrees with a previously published study that mentioned the switch in cell death to necrosis at a dose of 10 mmol APAP and above [3], thus, proving that the cellular decline is initiated through apoptosis and further progression is dose and time-dependent. It was recently reported that Vit C defeats ROS generation that is required for physiological caspase-dependent apoptosis thereby reducing the possible cellular damage [33]. The current study showed that Vit C protected the cellular architecture of tested hepatocytes, as observations shifted towards a reduction in Annexin V+ve/PI–ve cells when compared to APAP-treated cells.

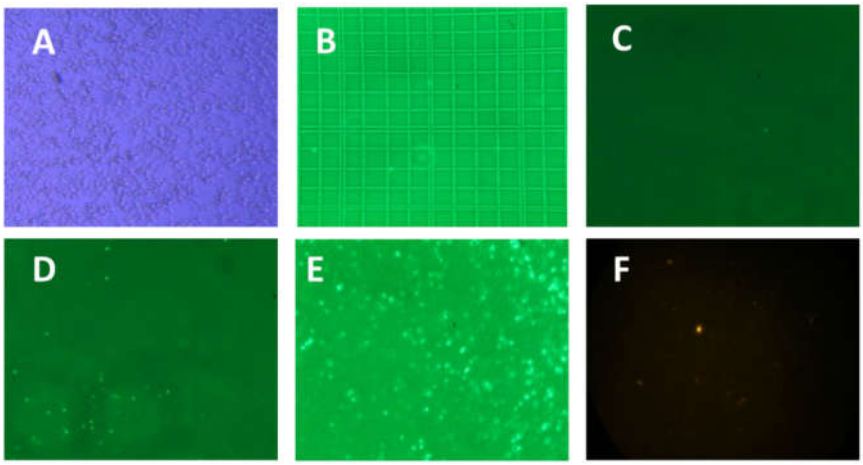

Fig. 3: Representative images of isolated primary mouse hepatocyte stained with Annexin V and PI. (A) Untreated cells visualized on the normal light bright field; (B) Untreated hepatocytes visualized on fluorescent Green Filter; (C) Cells treated with Vit C (Green Filter) showing normal intensity of Annexin V staining; (D) APAP treated hepatocytes (Green Filter) showing the high intensity of Annexin V staining; (E) Vit C-APAP treated hepatocytes (Green Filter) indicating reduced intensity compared to APAP alone; (F) PI staining of APAP treated hepatocytes visualized on Texas Red Filter. All images were captured as original objective 40x

Table 1: Scoring of Annexin V and Propidium Iodide (PI) staining of primary isolated mouse hepatocytes treated with Vit C or APAP with and without pretreatment with Vit C

Treatment	Stains	
	Annexin V	PI
Control	-	-
Vit C	-	-
APAP (10 mmol)	++	+
Vit C–APAP (10 mmol)	+	-

Apoptotic cells take in Annexin V stain; necrotic cells take in Annexin V and PI stain. (-)<5%; (+) 5-20%; (++) 20-40%; (+++) 40-60%; (++++) 60-100% of hepatocytes seen under fluorescent microscopy

Activation of caspase-3 in hepatocytes

The results demonstrate that hepatocytes treated with 10 mmol APAP exhibited high caspase-3 concentrations that were significantly decreased when hepatocytes were pretreated with 0.5 mmol Vit C prior to APAP treatment. Vit C treatment significantly suppressed caspase-3 concentration in comparison to control hepatocytes. However, all three groups presented caspase-3 activities below levels of the untreated hepatocytes, as shown in fig. 4. However, incubating the cells with Vit C alone or prior to APAP intoxication highly reduced caspase-3 concentration in the cells, leaving the impact of Vit C to greater importance in caspase-dependent studies. In addition, Vit C remarkably decreased caspase-3 activity in APAP treated cells. The major two key caspase cascades are caspase-9/-3 and caspase-8/-3 cascades. The activation of both caspase-9 and-8 cascades yields to the expression of executioner caspase-3; however, caspase-9 is associated with increased mitochondrial electron transport capacity due to the triggering of its dysfunction unlike caspase-8, which is associated with death receptor signal transduction [34]. In the current study, it was found that the addition of APAP to isolated lysed mouse hepatocytes did not trigger the increase in caspase-3 cellular content (fig. 4).

Modulation of bax protein serum content by vitamin C

As presented in fig. 5, and compared to the control group, the serum Bax level was considerably increased in mice intoxicated with 700 mg/kg APAP. However, such detection was reversed in animals pretreated with 100 mg/kg Vit C prior to APAP administration. Bax is a key member of the Bcl-2 protein family, which plays a leading role in the mitochondrial cell death pathway, in addition to maintaining the mitochondrial membrane integrity. Thus, inhibiting the release of the apoptogenic cytochrome c [5]. In this study, western blot analysis indicated a trendlike increase in Bax levels in APAP-treated mice. That, comes in line with several studies that reported up to 4-fold increase in bax levels in comparison to untreated counterparts as early as 1 hour after APAP treatment, with a further increase during the next several

hours [35–37]. The pre-treatment with Vit C attenuated APAP-induced increases of Bax serum levels and that comes in line with the literature, as hepatocytes pretreated with antioxidants prior to APAP result in an enhancement of the anti-apoptotic Bcl-2 level and decrease Bax level, which confirms the hepatoprotective effect of antioxidant [37].

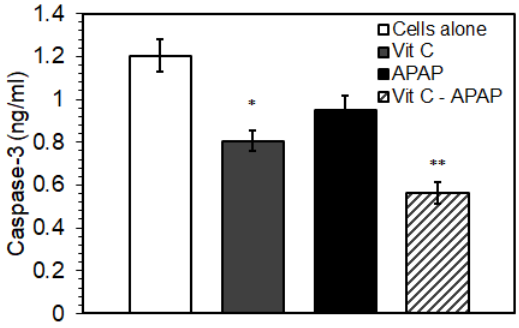

Fig. 4: Caspase-3 concentration of isolated mouse hepatocytes as determined by ELISA. Cells were either untreated, treated with 0.5 mmol Vit C only, treated with 10 mmol APAP alone, or treated with 10 mmol APAP after pretreatment with 0.5 mmol Vit C. Data are expressed as units for caspase-3 activity, and represent means±SEM, n=6. In comparison to APAP (p<0.05). In comparison to Cells alone (*p<0.05)**

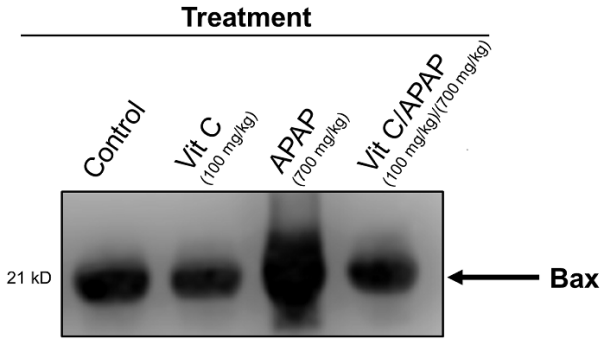

Fig. 5: Serum bax levels determined by Western Blotting in APAP-induced toxicity and effects of Vit C pretreatment. Mice groups were either untreated, treated with 700 mg/kg APAP alone or 100 mg/kg Vit C, and treated with 700 mg/kg APAP after pre-treated with 100 mg/kg Vit C

CONCLUSION

Despite the knowledge provided in the literature about APAP and its toxicity, a mechanistic approach is still warranted to understand the influence of APAP on death pathways, cell survival and liver integrity. This study confirms the development of hepatocellular injury in mice after a high dose of APAP through the ability of APAP in triggering the mitochondrial electron transport capacity of hepatocytes. Apoptosis was found to be the initial cause of the hepatic injury induced by APAP as evident by the morphological evaluation of the APAP-treated cells. These findings were supported by caspase-3 enzyme activity elevation in APAP-treated cells, and increased Bax protein serum levels as detected in serum of APAP-intoxicated mice. When mice were pretreated or the isolated hepatocytes were pre-incubated with Vit C, reduced Bax serum levels and cellular caspase 3 concentrations were evident, respectively. Such actions might suggest the strong capability of Vit C in preventing intoxicated hepatocytes from entering the apoptotic stage.

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AUTHORS CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation and data collection were performed by Rasha Abu-Ajamieh and Nidal Qinna. All authors contributed to the data analysis. The first draft of the manuscript was written by Rasha Abu-Ajamieh and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

No potential conflict of interest was reported by the authors.

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