Vitamin E is a potent regulator of inflammatory cytokines released during hepatic toxicity in rats

Mona A. El Shemy[1,*], Ayman Samir Farid[1]

1 Department of Clinical Pathology, Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh 13736, Qalyubia, Egypt

[*]**Corresponding:** Mona Abd El-Zaher El Shemy (DVM, MVSc, PhD)
E. mail: Mona.Alshemy@fvtm.bu.edu.eg

A B S T R A C T

Paracetamol is a the most utilized analgesic and antipyretic drug, but it may induce hepatotoxicity at increased doses. So, we design this experiment to study the hepatotoxic effect of paracetamol and to detect the role of vitamin E in the alleviation of this toxic effect.

Forty Wistar male rats were classified into 4 groups (10 rats per group); Control (distilled water), Vitamin E (100 mg/kg b. wt. orally for four weeks), Paracetamol (1000 mg/kg b. wt. orally for 3 consecutive days), Vitamin E plus Paracetamol (100 mg vitamin E/kg b. wt. orally for four weeks followed by a dose of paracetamol 1000 mg/kg b. wt. orally daily for 3 consecutive days). Blood and hepatic specimens were collected at the end of treatment. Erythrogram, leukogram, liver enzymes, MDA and expressions of IL-1β, IL4, MCP-1 and TGF mRNA were determined. Also, tissue sections from liver was examined histopathologically.

Paracetamol caused damage of liver tissue indicated by significant increase in the serum ALT and AST activities with a significant decrease in the levels of serum total protein and albumin. Moreover, paracetamol produced a significant elevation in the level of liver malondialdehyde and significant upregulations in the pro-inflammatory biomarkers expression in the hepatic homogenate. The hepatic tissue revealed severe inflammation and hepatic damage. However, vitamin E alleviated the oxidative stress and the hepatotoxicity induced by paracetamol.

Our experiment revealed that Paracetamol is a hepatotoxic drug in rats. Meanwhile, vitamin E reverses that harmful hepatotoxic effects produced by Paracetamol.

Keywords: paracetamol, vitamin E, oxidative stress, liver damage, pro-inflammatory and anti-inflammatory cytokines.

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1. INTRODUCTION

Liver is a main organ in the body that has a vital role in the detoxification of any harmful substance. It regulates numerous metabolic functions. Drug-induced hepatotoxicity as a result of the dangerous side effects to the prescribed drugs is considered a major cause of hepatic damage (Olsen and Whalen 2009). Although the wide use of paracetamol as a safe medication (Lesko and Mitchell 1999), it may cause severe hepatic necrosis and fatal hepatic failure when given in high doses (Rajanayagam et al., 2015). The mechanisms underlying paracetamol-induced hepatotoxicity is regarding mainly to its oxidative stress (Abd El-Ghffar et al., 2017). The liver metabolized paracetamol and gives highly reactive metabolites. When the production rate of these metabolites exceeds the detoxification rate by glutathione, it causes oxidation to the tissue macromolecules and induce lipid peroxidation, glutathione depletion and damage to hepatocytes (Ibrahim et al., 2017). These damaged hepatocytes activate the immune system resulting in the synthesis of pro-inflammatory cytokines (Abd El-Ghffar et al., 2017).

Vitamin E is an important antioxidant and anti-inflammatory substance that has an essential role in alleviation the oxidative damage in animals and humans (Al Rasheed et al., 2012). Vitamin E is a lipid-soluble chain-breaking antioxidant which prevents lipid peroxidation of polyunsaturated fatty acids by scavenging of various free radicals (ROS), thus it helps to preserve the integrity of biological membranes, lipoproteins and lipid stores against oxidative stress (Regoli and Giuliani 2014; Fritsche et al., 2017).

Therefore, we tried to investigate the possible impact of vitamin E supplementation as protective therapy for *in vivo* paracetamol-induced hepatic toxicity in rats.

2.MATERIALS AND METHODS

2.1. Experimental animals:

Forty male adult Wister rats (125 g) were taken from the Experimental Animal Unit, Faculty of Veterinary Medicine, Benha University, Egypt. Animals were allocated in separate clean metal cages and fed on balanced commercial diet with fresh and clean water. Animals were acclimatized before the experiment. The nutritional and environmental condition for animals were adjusted through the experimental period. The guidelines of the Ethics Committee of the Faculty of Veterinary Medicine, Benha University are considered.

2.2. Chemicals

Vitamin E was provided as oily preparation (Alpha Tocopherol; Sigma Chemical Co., St. Louis, MO, USA) and administrated orally 1000 mg/kg b.w once daily through a stomach tube according to Abraham (2004). Paracetamol (Sanofi-Aventis Egypt, Cairo, Egypt). Paracetamol was provided as tablet 500 mg/tablet (El-Naser Co., Cairo, Egypt) and was administrated orally 1000 mg/kg b.w by a stomach tube according to Al Rasheed et al., (2012).

2.3.Sampling:

The samples of blood were collected from the retro-orbital venous plexus after that specimens of liver were obtained by scarification. Blood was taken into 2 tubes: the first plain centrifuge tube to separate serum for biochemical analysis (ALT, AST, total proteins, and albumin) and the second one on EDTA tube for hematological examination.

Liver samples were removed after scarification and placed on ice and then stored at –80°C until examination. Liver homogenate (prepared as 1 g of hepatic tissue) was obtained from all rats then washed and homogenized in ice-cold 1.15% solution of potassium chloride in 50 mmol in buffer solution of potassium phosphate (pH 7.4) to obtain a liver homogenate of 10% (W/V; Weight of liver tissue, g per Volume of the buffer, mL). Homogenization of the lver tissue was performed by using a sonicator (4710 Ultrasonics Homogenizer, Cole-Parmer Instrument Co., USA). Centrifuge the homogenate.

2.4. Haematological Examination

The haematological studies including erythrogram and leukogram counts were determined according to Thrall et al. (2012).

2.4.1 Determination of serum AST, ALT, Albumin and Total protein

Serum AST, ALT, albumin and total protein levels were examined by the kits from Diamond Diagnostics, Egypt in accordance to their instructions of manufacturer.

2.4.2. Determination of lipid peroxidation by-products

Lipid peroxidation by-products in hepatic tissue homogenate were estimated in accordance with Ohkawa et al., (1979), based on the reaction of the thiobarbituric acid with malondialdehyde in acidic media at 95^{o}C for 45 min to form thiobarbituric acid-reactive substances (TBARS). The formed pink-colored substance was extracted by the spectrophotometer at the absorbance 535 nm (Model, JASCO 7800, UV/VIS, Japan). The MDA level was appeared in nmol/g tissue.

2.5. Analysis of mRNA expression of hepatic IL-1B, IL-4, MCP-1 and TGF genes using real time-PCR

To understand perfectly the protective role of vitamin E administration on hepatic inflammation caused by paracetamol over dosage, expression of various cytokines were analyzed by real time-PCR using sense and anti-sense primers in the experiment as previously described (Farid et al., 2010) using the following primers sets: IL-1β (GenBank ID: M98820.1), sense (5′-CAC CTC TCA AGC AGA GCA CAG-3′) and anti-sense (5′-GGG TTC CAT GGT GAA GTC AAC-3′); IL-4, sense (5-CAG GGT GCT TCG CAA ATT TTA C-3′) and anti-sense (5- ACCG AGA ACC CCA GAC TTG TT-3′); MCP-1, sense (5′- ATG CAG TTA ATG CCC CAC TC-3′) and anti-sense (5′- TTC CTT ATT GGG GTC AGC AC-3′) ; TGF-β1 sense (5′-CGT GGA AAT CAA TGG GAT CAG-3′) and anti-sense (5′-CAG GAA GGG TCG GTT CAT GT-3′); and 18S rRNA (GenBank ID: NR_046237.1) as a housekeeping gene, sense (5′-GAG GTG AAA TTC TTG GAC CGG-3′) and anti-sense (5′-CGA ACC TCC GAC TTT CGT TCT-3′).

The extraction of total cellular RNA from the hepatic tissue was done by using RNeasy Mini kit (Qiagen, Valencia, CA, USA). Real-time PCR was estimated using a Power SYBR Green RNA-to-C_t 1-step kit (Applied Biosystems, Foster City, CA, USA).

The real-time-PCR cycling program consisted of reverse transcription at 48°C for 30 min, initial PCR activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for

1 min. Real-time-PCR assay was done by 7300 real-time-PCR system (Applied Biosystems).

Thermal cycling and fluorescence detection were done by using a 7300 real-time-PCR system (Applied Biosystems, Foster City, CA, USA).

Differences in the gene expression were calculated from the obtained values of C_t provided by real-time PCR instrumentation by the comparative CT method to a reference (housekeeping) gene (18S rRNA) (Schmittgen and Livak 2008).

2.6. Histopathological examination:

Specimens were taken from liver of all groups, fixed in 10% buffered neutral formalin for 24 hours. After proper fixation, the specimens were washed in running tape water, dehydrated in different grades of ethyl alcohol, cleared in xylol and embedded in paraffin, then blocked and sectioned as 5 µm thickness. Then stained by hematoxylin and eosin after (Bancroft and Cook 1994) and examined microscopically.

2.7. Experimental design

Forty male rats were randomly separated into 4 groups (10 animals per group). All animals were treated orally by a stomach tube as follows: the first one (control group) obtained distilled water; the second group allowed vitamin E (100 mg/kg b.wt) (Al Rasheed et al. 2012) for four weeks. The third group received paracetamol (1000 mg/kg b.wt) daily once for three consecutive days according to Abraham (2004). The fourth group received vitamin E (100 mg/kg b.wt) for four weeks then given paracetamol (1000 mg/kg b.wt) daily once for 3 following days at the end of the study. Blood for serum samples was taken from the retroorbital venous plexus and hepatic samples were collected from the rats from all group at the end of the study for estimation of the function of liver, the oxidative parameters, gene expression and the histopathological changes.

2.8.Statistical Analysis:

Statistical analysis was done by using the statistical software package SPSS for Windows (Version 16.0; SPSS Inc., Chicago, Ill). The significance of differences between more than two groups was estimated by one-way ANOVA. If there were significant differences between individual groups by one-way ANOVA, these differences were estimated by LSD test. Results are obtained as the mean ± SEM. A *P*-value of less than 0.05 was considered significant (Kinnear and Gray 2006).

3. RESULTS

3.1. Hematological Results:

Significant reductions in RBCs, hemoglobin concentration, hematocrit value with increase in white blood cells, granulocytes and lymphocytes counts was recorded in paracetamol-treated group compared with control, vitamin E and vitamin E-protected groups (Table 1).

3.2.Serum AST, ALT, albumin, total proteins and malondialdehyde levels

Table 2 showed significant increase in serum AST, ALT and liver malondialdehyde levels with significant reductions in serum albumin and total proteins levels in paracetamol-treated group when compared with control group. Moreover, it revealed non-significant changes of serum AST, ALT, total protein, albumin, and hepatic malondialdehyde in vitamin E group when compared with control group. Meanwhile, a significant decrease of serum AST, ALT and malondialdehyde levels with significant increase in serum albumin and total proteins levels were

found in vitamin E protected group compared to paracetamol group. Serum ALT, AST and malondialdehyde in vitamin E protected group showed non-significant difference compared to control and vitamin E and paracetamol groups.

3.3.Liver tissue IL-1β, IL4, MCP-1 and TGF mRNA expression:

Figure 1 showed the intensity of hepatic IL-1*β*, IL-4, MCP-1 and TGF-*β*1 mRNA normalized to 18s rRNA in the Vitamin E, Paracetamol and Vitamin E+ paracetamol-treated rats compared to the control one.

Paracetamol administration produced a significant increase in the hepatic IL-1, MCP-1 and TGF expression in relation to control group, and this up-regulation was ameliorated by vitamin E as protection in regard to IL-1 and MCP-1. Meanwhile, Significant up-regulations for hepatic IL-4 by vitamin E was reported in comparison with control, and this effect became lessen in group received paracetamol and vitamin E as protection, however; its level still elevated than control group. Paracetamol and vitamin E protected groups showed also significant up-regulation in the hepatic TGF-*β* when compared with control and vitamin E groups. Vitamin E group showed a significant up-regulation in MCP-1 expression with a non-significant difference in the hepatic IL-1 and TGF expression when compared with control group.

3.4. Histopathological examination of hepatic tissue

Microscopically, the liver of control rats and rats that received vitamin E only showing normal histological structure of the hepatic tissue (Figure 2.A and B). But, in group, that receiving paracetamol only, showing severe degenerative changes in hepatocytes which manifested by neutrophil areas of coagulated necrosis in the hepatic parenchyma. the necrotic areas characterized by maintaining of tissues architecture but loss of cellular details (Figure 2.C). Moreover, focal mononuclear cellular infiltration particularly areas were also deleted (Figure 2.D). The group protected by Vitamin E showing mild degenerative changes in the hepatocytes in the form of vacuolar and hepatic degeneration (Figure 2.E).

Table (1):

parameter	Control	Vitamin E	Paracetamol	Vit.E+ Paracetamol
RBCs (10^6/µL)	08.68 ± 0.34^{b}	08.52 ± 0.22^{b}	06.79 ± 0.47^{a}	08.46 ± 0.12^{b}
Hb (g/dL)	18.33 ± 1.2^{b}	18.36 ± 0.14^{b}	13.80 ± 0.93^{a}	17.70 ± 0.45^{b}
Hct (%)	46.78 ± 0.60^{b}	46.56 ± 0.38^{b}	39.04 ± 2.40^{a}	47.28 ± 0.61^{b}
WBCs (10^3/µL)	08.46 ± 0.13^{a}	08.50 ± 0.05^{a}	10.53 ± 0.31^{b}	08.20 ± 0.40^{a}
Lymphocyte (10^3/µL)	06.11 ± 0.008^{a}	06.24 ± 0.10^{a}	07.74 ± 0.05^{b}	06.23 ± 0.14^{a}
Granulocytes (10^3/µL)	01.63 ± 0.07^{a}	01.65 ± 0.09^{a}	01.72 ± 0.03^{b}	01.70 ± 0.01^{a}
Monocyte (10^3/µL)	00.72 ± 0.06^{a}	00.61 ± 0.06^{a}	00.73 ± 0.14^{a}	00.87 ± 0.01^{a}

Table (2):

Parameter	Control	Vitamin E	Paracetamol	VitE+ paracetamol
ALT (U/L)	24.89 ± 0.88[a]	24.41 ± 0.49[a]	104.1 ± 0.44[b]	25.54 ± 0.14[a]
AST(U/L)	44.17 ± 0.52[a]	44.81 ± 2.90[a]	171.06 ± 8.7[b]	55.87 ± 5.20[a]
TP (g/dl)	07.90 ± 1.06[b]	07.07 ± 0.75[b]	03.40 ± 1.40[a]	06.30 ± 0.55[b]
Albumin (g/dl)	03.80 ± 0.01[c]	03.70 ± 0.02[b,c]	02.17 ± 0.05[a]	03.60 ± 0.05[b]
MDA(nmol/g)	34.72 ± 0.90[a]	33.91 ± 0.88[a]	56.85 ± 2.60[b]	35.01 ± 0.19[a]

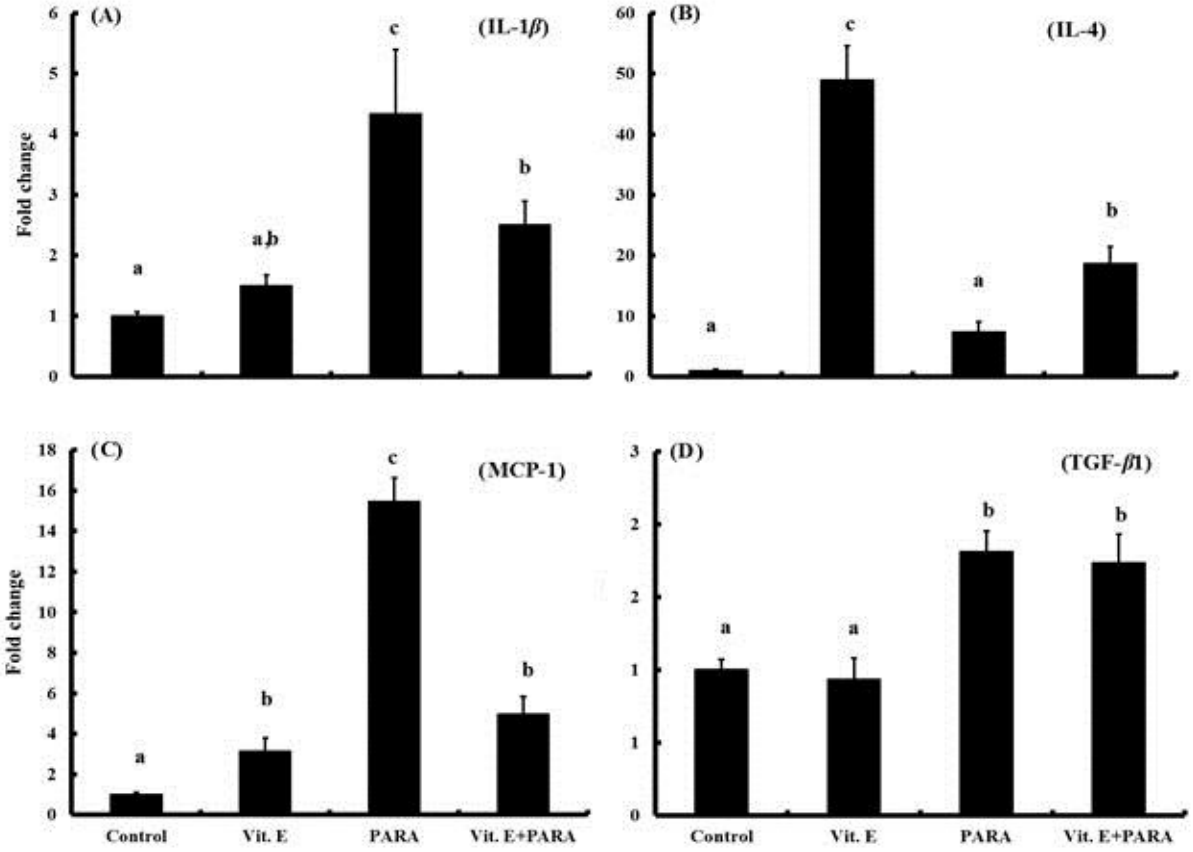

Figure 1:

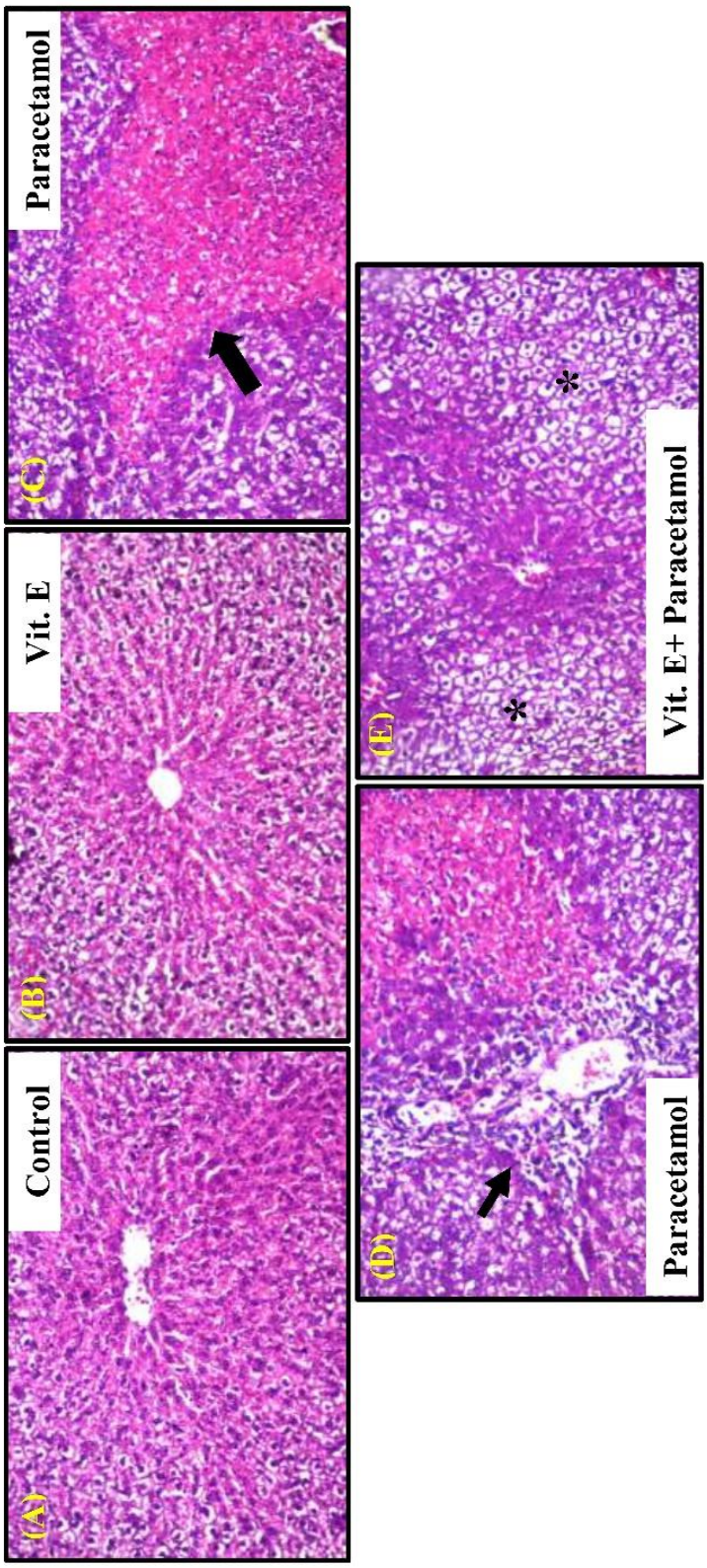
Control
(A)
Vit. E
(B)
Paracetamol
(C)
Paracetamol
(D)
Vit. E+ Paracetamol
(E)

Figure 2.

Tables and Figures Legend

Table 1: RBCs count, Hb concentration, Hct % and WBCs count in Control, Vitamin E, Paracetamol and Vitamin E protected groups (Mean± S.E.M) (*n* = 3-4).

Means with different superscripts within the same row were significantly different.

Table 2. ALT, AST, Total Protein, Albumin and MDA in Control, Vitamin E, Paracetamol and Vitamin E protected groups (Mean± S.E.M) (*n* = 3-4).

Means with different superscripts with in the same row were significantly different.

Figure 1. mRNA expression of hepatic IL-1, IL-4, MCP-1, and TGF-β1genes. Total RNA was prepared from hepatic tissues of different experimental groups. The expression levels were evaluated by real-time PCR.

Means with different superscripts with in the same row were significantly different. Bars represent means ± S.E.M. (*n* = 3-4).

Figure 2. Histopathological examination of rat liver sections of different experimental groups. Liver sections of rat tissue were deparaffinized, stained with hematoxylin-eosin (HE), and examined under light microscopy. Wide bold arrow indicates wide focal areas of coagulative necrosis was detected in the hepatic parenchyma (C). Narrow bold arrow indicates with inflammatory cells infiltration in the centrilobular area surrounding the central vein (D). Bold stars indicate ballooning degeneration in the hepatocytes (E). Liver section of control and vitamin E groups with no abnormalities (A and B; respectively). 40 X magnification.

4. DISCUSSION

Paracetamol is the main often drug involved in drug-induced hepatotoxicity (Lee, 2003). Paracetamol is metabolized into sulfate and glucuronide. Liver is the main site of detoxification and is highly susceptible to these agents toxicity (Ibrahim et al., 2017)**.** In therapeutic uses, the produced substances are conjugated with glutathione. In drug overdoses, these toxic metabolites cause depletion of glutathione stores that induce liver cell damage (Renton, 2000). Most of paracetamol molecules are oxidized to reactive N-acetyl-P-benoquinemine by P450 enzyme and form semiquinone radical. This radical can yield to macromolecules in the cellular membrane and causes lipid peroxidation and hepatic damage (Sini et al., 2017).

In our experiment, paracetamol administration induced marked decreases in RBCs count, hemoglobin concentration, and PCV. This anemia might be due to the hepatic damage caused by paracetamol (Senthilkumar et al., 2014) or might be attributed the decrease of the erythropoiesis rate or the damage of the mature RBCs. It is also suggested that paracetamol inhibits erythropoietin release from the kidney

(Samuel et al., 2015). These effects of paracetamol on RBCs, Hb concentration and hematocrit are matched with Oyedeji et al., (2013); Reddy et al., (2017); Sini et al., (2017).

Furthermore, paracetamol produced a significant leukocytosis, lymphocytosis and granulocytosis compared with control group which is possibly due to immune system stimulation and an interleukin-1 mediated elevation due to colony stimulating factor. The results of paracetamol elevation of WBCs count matched with Samuel et al. (2015) who reported a significant increase in WBCs count in paracetamol group which may be because of the body defense mechanism in a trial for the body protection from being susceptible to infections following the liver damage as evidenced by histopathology.

In our study, hepatic damage was occurred by taking of paracetamol (1000 mg/kg b. wt for three days). This is evidenced by significant elevation in serum AST and ALT in paracetamol group in comparison to the control group. The elevation of the level of ALT and AST is an indicator of hepatocellular damage (Johnkennedy et al., 2010). The elevation in AST and ALT levels after paracetamol administration is consistent with the results reported by Shenoy et al. (2002); Ita et al. (2009); Reddy et al. (2017); Sini et al. (2017). Hepatic damage is evidenced also by significant decreases in serum albumin and total proteins in paracetamol group when compared to control group that matches with Sini et al. (2017) and Reddy et al. (2017). Moreover, paracetamol administration elevated the lipid peroxidation as supported by the significant increase in liver MDA level which confirms the presence of hepatic damage as a result of promoted lipid peroxidation during the damage of tissue and the failure of antioxidant defense mechanism to inhibit the formation of free radicals. This matches with Hinson et al. (2010) and Ibrahim et al. (2017) who reported that lipid peroxidation has been produced due to liver damage by paracetamol administration.

Meanwhile, the administration of vitamin E produced a significant decrease in paracetamol-induced increase of serum AST and ALT levels and a significant elevation in the total serum protein and albumin levels. This revealed that vitamin E ameliorates the hazards effects of paracetamol on the liver. These results matched with Abdel-Azeem et al. (2013). Moreover, serum albumin level, despite its elevation, was still significantly decrease than the control one. Moreover, the levels of AST, ALT and total protein in serum reached to non-significant levels in relation to control group and vitamin E control group. This ensured that vitamin E stopped the liver cell damage, but liver cell function was not returned completely. Furthermore, our study noted the powerful anti-oxidant effect of vitamin E through a significant decrease in paracetamol-induced increase in serum MDA level which is in accordance with Abd El-Ghffar et al. (2017) and Palipoch et al. (2014) and might be because the ability of vitamin E to remove free radicals and maintaining the cell membrane stability.

The mechanism by which hepatic injury occurred as a result of paracetamol over dose administration in rats is mostly induced by various pro-inflammatory cytokines

associated with hepatic toxicity. This is supported by the infiltration of the inflammatory cells in the centrilobular area surrounding the central vein. This result ascertains that cytokines are produced by non-parenchymal liver cells, especially the intrahepatic macrophages (Kupffer cells) or extrahepatic macrophages in hepatic tissues (Krenkel et al., 2014). This notion is supported by the up-regulation of hepatic IL-1β mRNA expression as a results of paracetamol administration. Several investigators have reported that paracetamol-induced hepato-toxicity resulted in elevation of the inflammatory cytokines. IL-1β is elevated early in the toxicity with acetaminophen and may be essential in the iNOS induction (James et al., 2003) the later induce liver damage related to inflammation (Gardner et al., 1998).

The beta chemokines are exclusively chemotactic for mononuclear cells; the prototype of this group is MCP-1 which is monocyte and lymphocyte chemoattractants (Fisher et al., 1999). MCP-1 in our study showed significant increases after paracetamol toxicity. Both IL-1 and TNF-α are potent inducers of MCP-1 (Khoruts et al., 1991). Although the MCP-1 role in the inflammatory process of liver is unknown, this chemokine may induce the delaying of the proliferation of the hepatocyte (Leifeld et al., 2003). It also maintains the monocytes migration into tissues and their subsequent differentiation into macrophages (Simpson et al., 2003). MCP-1 has a major stimulatory function in the inflammatory infiltrate, and also it has immunomodulatory effects, including enhanced the expression of adhesion molecules in monocytes and induction the synthesis of a pro-inflammatory cytokine, thus modulating the inflammatory cascade in the liver tissue (Jiang et al., 1992). Increased the levels of TGF-β have been supposed to prevent the hepatic cells proliferation and also, to act as an apoptosis inducer and a negative feedback mechanism (McMahon et al., 1986; Dooley et al., 2000). Thus, its up-regulation may explain multiple wide focal areas of coagulative necrosis in the hepatic parenchyma as a result of paracetamol over dose (Figure 2C).

The effects of paracetamol on IL-4 and TGF-β were explained by Krenkel et al., (2014) who concluded that paracetamol had stimulated Kupffer cells which then released several cytokines and signaling molecules including superoxide and nitric oxide. Meanwhile, the activated Kupffer cells released anti-inflammatory cytokines as a compensatory mechanism of the hepatic cells to paracetamol.

5.CONCLUSION

In conclusion, over doses of paracetamol induce injurious hepatotoxic effect, inflammatory effect and oxidative stress. Vitamin E co-administration reduces the releasing of inflammatory mediators and remove the paracetamol – induced oxidative stress.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interest

All authors declare that they have no competing interest.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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