Research Article

Study on the Effect of Necrostatin-1on APAP-Induced Hepatotoxicity in Adult Albino Rats

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Abstract
Drug induced hepatotoxicity is a common cause of acute liver injury. Acetaminophen (paracetamol, N acetyl p-acetaminophen, APAP) is a widely used analgesic and antipyretic drug. However, its overdose is one of the main cause of acute liver failure. The current study is an attempt to investigate the different mechanisms of APAP-induced hepatoxicity including inflammatory response, oxidative stress and different cell death pathways and the ability of necrostatin-1 to antagonize the APAP-induced hepatoxicity. **Materials and methods:** thirty adult male albino rats were used and divided into 3 equal groups: control groups, paracetamol treated group (APAP group): each rat received paracetamol (APAP) dissolved in saline at a dose level of 2 gm/kg body weight given as a single dose orally by oral gavage, paracetamol + necrostatin-1(RIPK1 inhibitor) (APAP+Necrostatin-1 group): each rat of this group received pretreatment of necrostatin-1 at a dose level of 1.8 mg/kg taken as a single IP injection1 hour before receiving APAP as the previous regimen. An overnight fast and blood samples from jugular vein were obtained. Blood samples were collected for the assay of serum liver enzymes, serum lipid profile, serum tumor necrosis factor alpha (TNF-α), serum interleukin-33 (IL-33) and serum reduced glutathione (GSH). Histological examination of the liver, and immune-staining for caspase-3 was also done. **Results and conclusion:** The liver is a particular target for drug toxicity because of its role in clearing and metabolizing chemicals. The pathophysiology and intrinsic mechanisms underlying paracetamol (APAP) induced hepatotoxicity are different because aetiologies and mechanisms vary between oxidative stress, inflammation and cell death including apoptosis, necrosis and necroptosis. Necrostatin-1 is proved to have antioxidant, anti-inflammatory, anti-apoptotic and anti-necroptotic effect that antagonize APAP-injurious effect.
Keywords: Hepatotoxicity, Paracetamol, Necrostatin-1

Introduction
Liver disease is an important clinical issue worldwide with a variety of underlying causes. Hepatotoxicity is a clinical term that represents liver injury due to chemical, herbal or dietary supplement leading to different mechanisms of hepatic function impairment (Paech et al., 2017).

Hepatotoxins are synthetic or naturally occurring compounds that cause different forms of liver injury through their direct or indirect effect on liver cells. Depending on the toxin, its ability to induce liver toxicity may be predictable or unpredictable (idiosyncratic) (Senior, 2008).

Acetaminophen (paracetamol, N acetyl p-acetaminophen, APAP) is a widely used analgesic and antipyretic drug. Its overdose is the number one cause of acute liver failure and remains a major problem. APAP is safe at the therapeutic dose, but at higher doses, it produces hepatic degeneration that is presented biochemically by increased serum alanine transaminase (ALT), aspartate transaminase (AST), mild hyperbilirubinemia, increased prothrombin time and glycogen depletion (da Rocha et al., 2017).

Therapeutic doses of APAP are mainly sulfated or glucuronidated and then excreted in urine, but only small amount is metabolized by

cytochrome P450 leading to the formation of a small amount of reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). At APAP over-dose sulfation and glucuronidation are saturated and there is increased NAPQI formation (Woolbright and Jaeschke, 2017).

All these events trigger cell necrosis with the release of cellular contents and activation of pro-inflammatory cytokines such as IL-1B, IL6, IL10, and TNF-α (Du et al., 2017).

Recently, it has been reported that apoptosis and necroptosis have a crucial role in the pathophysiology of hepatotoxicity induced by different mechanisms. So, the direction to use anti-apoptotic and anti-necroptotic agents as hepatoprotective agents is a matter of interest (Yuan et al., 2017).

Nec-1, the 5-(1H-indol-3-ylmethyl)-2-thiohydantoin 1, is a potent specific inhibitor of necroptosis. Necroptosis, which happens as a result of death receptor signaling of the RIPK1/RIPK3/MLKL necrosome, is involved in the pathogenesis of hepatotoxicity. The kinase activity of RIP1 has been reported to be critical for mediating programmed necrosis, the caspase-independent necrotic cell death pathway. Nec-1has the ability to inhibit RIPK1 and then inhibit RIP-mediated necroptosis(Ning et al., 2018).

The current study is an attempt to investigate the different mechanisms of APAP-induced hepatoxicity including inflammatory response, oxidative stress and different cell death pathways and the ability of necrostatin-1 to antagonize the APAP-induced hepatoxicity.

Materials and methods

I -Animals:

Thirty adult male albino rats of local strain weighing between 150-250 grams were used throughout the present study. Rats were housed at room temperature with normal day/night cycles. The rats were fed a standard diet of commercial rat chow and tap water and left to acclimatize to the environment for two weeks prior to inclusion in the experiment. Principles of laboratory animal care were followed, and the experimental procedures used in this study were approved by the Animal Care and Use Committee of Faculty of Medicine, Minia University, Egypt.

II- Experimental Groups:

The rats were randomly divided into the following groups:

1- Control groups:

consisted of 10 rats, each rat received dimethyl sulfoxide (DMSO) vehicle taken as a single IP injection.

2- Paracetamol treated group (APAP group):

consisted of 10 male rats, each rat received paracetamol (APAP) dissolved in saline at a dose level of 2 gm/kg body weight given as a single dose orally by oral gavage ***(McGill et al.., 2012).***

3- Group treated by paracetamol + necrostatin-1 (RIPK1 inhibitor) (APAP+Necrostatin-1 group):

consisted of 10 Male rats, each rat of this group received pretreatment of necrostatin-1 dissolved in 2% DMSO at a dose level of 1.8 mg/kg taken as a single IP injection1 hour before receiving APAP as the previous regimen (Kim and Lee, 2017).

At the end of all experiments, all rats were sacrificed by decapitation after an overnight fast and blood samples from jugular vein were obtained. Blood samples were collected in tubes and left to clot at room temperature then centrifuged at 3000 rpm for 15 min in a cooling centrifuge. The supernatant serum was then withdrawn into labeled eppendorf tubes and stored at -20° C till the time of assay of serum liver enzymes including alanine transaminase (ALT) and aspartate transaminase (AST), serum lipid profile including total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL), serum tumor necrosis factor alpha (TNF-α), serum interleukin-33 (IL-33) and serum reduced glutathione (GSH).

III) Histological procedures:

Parts of rat livers which were fixed in 10% neutral buffered formalin solutions were processed for preparation of paraffin blocks. Six-micrometer sections were cut, mounted on glass slides, deparaffinized with xylene, rehydrated and used for Hematoxylin and eosin (H&E) staining using standard techniques (Bancroft and Gamble, 2008).

- **Immunohistochemical staining:**

Other sections were immune-stained for caspase-3 according to the manufacture's guidelines. Briefly, after de-waxing and rehydration, sections were incubated with trypsin then washed with PBS. The non-specific binding sites were blocked in normal goat serum (1:50). Then sections were incubated for 30 min with the primary; caspase-3 (1:500). Then washed with PBS and incubated with the secondary antibody (Vector laboratory 1:2000). Sites of peroxidase binding were detected using chromogenic 3,3'-diaminobenzidine (DAB) tetra hydrochloride substrate. Tissue sections were lastly counterstained with hematoxylin (Côté, 1993).

Positive reactions were brown nuclear/cytoplasmic or both for anti-Caspase-3 antibody.

Results

I. Effect of Paracetamol treatment on the different measured parameters:

The data demonstrated that serum alanine transaminase (ALT) and aspartate transaminase (AST), the liver injury markers, were significantly increased in the APAP-treated group compared to the control group (Figure 1).

The serum total cholesterol level (TC), triglycerides (TG) and low density lipoprotein (LDL) were significantly increased in the APAP-treated group compared to the control group, while serum high density lipoprotein (HDL) level was decreased in APAP-treated group compared to control group (table 1).

The data demonstrated that tumor necrosis factor alpha (TNF-α), interleukin-33 (IL-33), inflammatory markers were significantly increased in the APAP-treated group compared to the control group, and the serum reduced glutathione was significantly decreased in the APAP-treated group compared to the control group (table 2, figure 2).

Liver sections of control group displayed normal lobular architecture with no detectable immunolabelling for activated caspase-3 (apoptotic marker). APAP group showed obvious high immunolabelling for activated caspase-3 (table 3).

II. Effect of necrostatin-1 pretreatment, before Paracetamol treatment, on the different measured parameters:

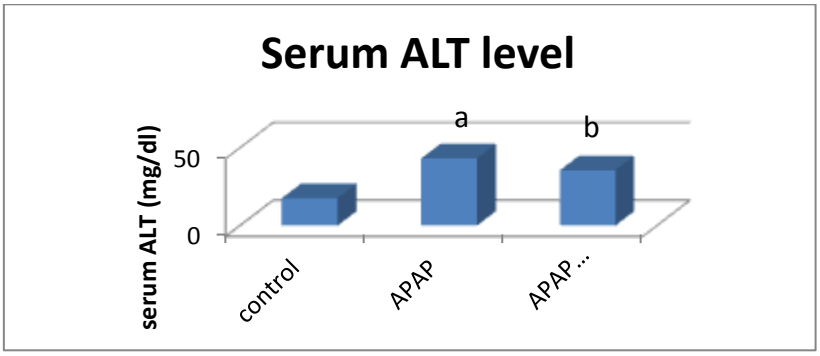
The elevation in liver enzymes induced by APAP was significantly decreased by administration of necrostatin-1as compared to the APAP group (Figure 1).

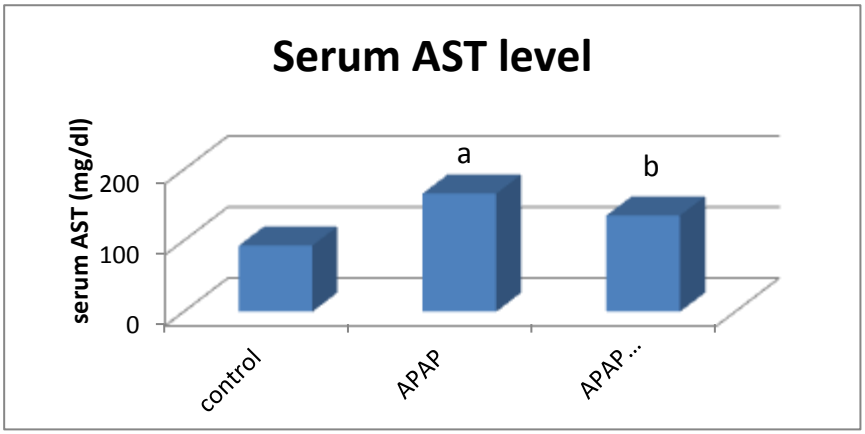
Administration of necrostatin-1 reduced this deterioration in lipid profile as indicated by the significantly lower TC, TG and LDL levels and significantly higher HDL level compared to the APAP group (table 1).

Pretreatment with necrostatin-1significantly reduced these inflammatory markers, TNF-α and IL-33, and significantly increased the serum level of GSH compared to the APAP group (table 2, figure 2).

Regarding the Immune-stained sections for activated caspase-3, pre-treatment of rats with necrostatin-1 in (APAP+necrostatin-1) groups showed few scattered immunolabeled cells. The immunolabeling was mainly confined to hepatocytes and some endothelial cells. The expression was mostly cytoplasmic (table 3).

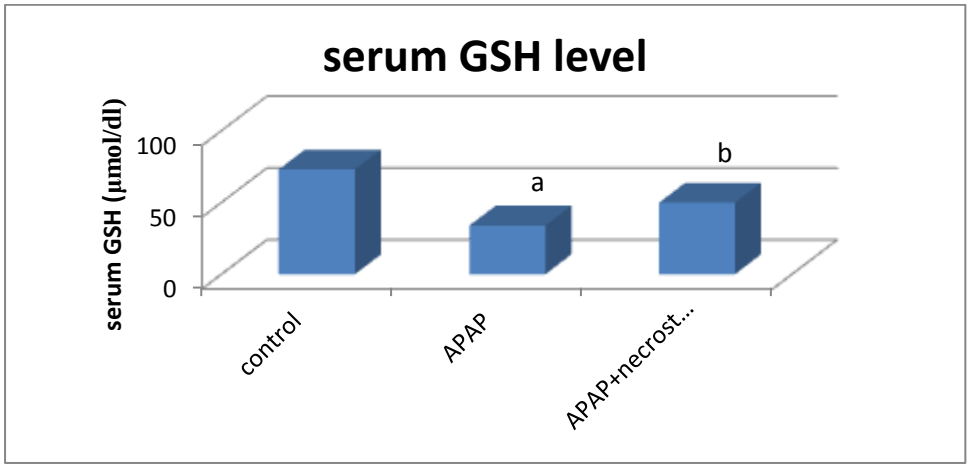
Figure (1): Effects of different lines of treatment on serum Alanine transaminase (ALT) & Aspartate transaminase (AST):





a: significant difference from the control group, b: significant from APAP group. Significant: P<0.05).

Figure (2): Effects of different lines of treatment on serum reduced glutathione (GSH).



a: significant difference from the control group, b: significant from APAP group. Significant: P < 0.05.

Table (1): Effects of different lines of treatment on serum lipid profile:

Groups (*n* = 10) / Parameters	Control	APAP treated	*APAP + necrostatin- 1*
Total cholesterol (mg/dl)	126.22± 11.49	186.13± 9.39 [a]	154.54± 11.5 [a, b]
Serum triglycerides(mg/dl)	61.89± 5.1	136.88± 9.19 [a]	106.32± 4.49 [a, b]
Serum HDL cholesterol (mg/dl)	45.63± 4.3	30.79± 2.74[a]	37.4± 1.95 [a, b]
Serum LDL cholesterol (mg/dl)	68.23± 4.2	127.96± 10.11[a]	90.88± 7.27 [a,b]

Data represent mean ± S.D. *n*: number of rats in each group. a: significant difference from the control group, b: significant difference from the APAP group Significant: P < 0.05.HDL: high density lipoprotein, LDL: low density lipoprotein

Table (2): Effects of different lines of treatment on some serum inflammatory markers:

Groups (*n* = 10) / Parameters	Control	APAP Treated	*APAP + necrostatin-1*
Serum TNF-α (ng/L)	17.08± 3.83	43.64 ± 4.66 [a]	34.59± 3.13[a, b]
Serum IL-33 (ng/L)	40.95± 5.15	97.55± 14.82 [a]	79.19± 8.83[a, b]

Data represent mean ± S.D. *n*: number of rats in each group. a: significant difference from the control group, b: significant difference from the APAP group. Significant: P < 0.05. TNF-α: tumor necrosis factor alpha, IL-33: interleukin-33)

Table (3): Effects of different lines of treatment on scoring of active caspase-3:

Groups (*n* = 10) / Parameters	Control	APAP treated	*APAP + necrostatin- 1*
Caspase-3	0.67± 0.08	8.33± 1.63 [a]	1.83± 0.37 [a, b]

Data represent mean ± S.D. *n*: number of rats in each group. a: significant difference from the control group, b: significant difference from the APAP group. Significant: P < 0.05.

Discussion

Drug-induced hepatotoxicity is one of the most common causes of acute liver failure and liver transplantation is the only definitive curative treatment, but lack of donors limits the transplantation. So, an intensive search for therapeutic strategies has been done to prevent the onset of acute liver failure (Kemelo et al., 2014).

Acetaminophen (N-acetyl-p-aminophenol [APAP]) is the most widely used antipyretic and analgesic drug, which is considered to be safe at the recommended therapeutic concentrations. An overdose of APAP can cause severe liver damage resulting in acute liver failure

In the present study there was significant increase in the serum level of ALT and AST along with disturbed hepatic architecture as seen in the histological images, indicating the liver injury induced by APAP in the APAP-treated group compared to the control group.

These results come in line with previous studies as (Alanazi et al., 2017).

In the APAP-treated group, there was a significant change in the lipid profile in the form of significant increase in serum cholesterol, low density lipoprotein (LDL) and triglycerides levels, and significant decrease in serum high density lipoprotein (HDL) level.

These results can be explained by the negative impact of APAP on the liver mentioned above, since the formation and clearance of lipoproteins occur in the liver. Liver receives cholesterol and fatty acids from the diet and peripheral tissues and converts them into lipoprotein complexes, eventually, releases into the blood circulation. The liver diseases disrupt plasma lipids through different ways as the plasma triglyceride and cholesterol are reduced in chronic liver disease as cirrhosis and fibrosis due to the lower biosynthetic capacity of lipoprotein, but the reverse occurs in acute liver diseases due to failure of clearance capacity (Mandal et al., 2013).

In the present study there was a significant increase in the hepatic immuno-expression of caspase-3, indicating the liver injury induced by APAP in the APAP-treated group as compared to the control group. This result comes in line with previous studies as (Jaeschke et al., 2018) that has reported that high doses of APAP lead to apoptosis and significantly increased caspase-3 levels.

Necrostatin-1 (Nec-1) is a potent and selective inhibitor of necroptosis, a mechanism of programmed cell death separate from apoptosis. Necrostatin-1 functions as an allosteric inhibitor of the death domain receptor interacting protein kinase-1 (RIPK1) in the necroptosis pathway (Cho et al., 2011).

In the present study, pre-treatment with Nec-1 before receiving APAP significantly decreased the serum level of the liver injury markers, ALT and AST, compared to the APAP-treated group, indicating the protective effects induced by Nec-1 on the APAP-induced acute liver injury. These results come in line with previous studies as (Fan et al., 2016) who has reported the protective effects of Nec-1 in different liver disease conditions.

For studying the mechanisms involved in (Nec-1)-induced liver protection, some oxidative, inflammatory, and apoptotic markers were measured in the (Nec-1)-treated group.

In the present study, there was significant increase in the serum level of antioxidant enzyme, GSH, in the (Nec-1)-treated group compared to the APAP-treated group. This result comes in line with (Ning et al., 2018),who has demonstrated the hepato-protective effect of Nec-1 through the suppression of necroptosis, reduction of inflammation and oxidative stress.

Nec-1has no direct antioxidant activity. It inhibits RIPK1 and necrosome formation and so, it decreases intracellular ROS production, which eventually prevents RIPK-dependent necrosis. Nec-1 prevents glutamate-induced toxicity in liver. Glutamate inhibits cysteine transport and depletes intracellular GSH levels (Xu et al., 2007).

In order to study the effect of Nec-1 on the inflammatory responses, the serum level of the inflammatory cytokines, TNF-α and IL-33, were measured in the (Nec-1)-treated group. Our study demonstrated that Nec-1 attenuates APAP-induced inflammatory responses, since there was significant decrease in the serum levels of TNF-α and IL-33when compared to the APAP-treated group, indicating the anti-inflammatory effect of Nec-1.These findings come in line with previous studies as (Sawai, 2014) who has reported the strong relationship between necroptosis and release of cytokines, the anti-inflammatory effect of Nec-1 and its inhibitory effect on TNF-α induced necroptosis.

Most of the mechanistic understanding of necroptosis derives from studies on tumor necrosis factor alpha receptor 1 (TNFR1) signaling. When TNF-α binds to the receptor, if caspase 8 compromises, the signals may switch to the formation microfilament-like necrosome complex, which contains RIP1 and RIP3. The pro-necrotic protein, mixed lineage domain like

(MLKL) is subsequently phosphorylated by RIP1/RIP3oligomerizes and forms pores on plasma membrane. The MLKL pores lead to expulsion of cellular contents into extracellular

space and recruitment of immune cells to the damaged tissues, finally eliciting immune responses leading to release of more cytokines including TNF-α and IL-33 (Liao et al., 2017).

Some studies have demonstrated the direct role of necroptosis in IL-33 release. They have shown that full-length, bioactive IL-33 is released during necroptotic, but not apoptotic cell death. These findings differentiated this necroptotic release from the apoptotic modify-cation, in which IL-33 is cleaved intracellularly by apoptotic caspases to an inactive product. Altogether, these data support a model where IL-33, similar to the other nuclear IL-1 family members, functions as damage-associated molecular pattern (DAMP) to modulate immune response(Shlomovitz et al., 2019).

IL-33 is suspected to be a necroptotic DAMP, RIPK3-induced necroptosis increases IL-33 gene expression. Nec-1 down-regulates liver IL-33 protein expression and attenuates hepatocellular damage and serum IL-33 (Yang et al., 2018).

Concerning the effect of Nec-1 administration on the lipid profile, our study showed that Nec-1 attenuates APAP-induced dyslipidemia by inducing significant decrease in the serum cholesterol, low density lipoprotein (LDL) and triglycerides levels, and significant increase in serum high density lipoprotein (HDL) level in the (Nec-1)-treated group compared to the APAP-treated group. Similar data were obtained in previous studies as (Zhe-Wei et al., 2018), that reported the benefits of targeting necroptosis to treat dyslipidemia associated with different disease conditions.

Some studies have demonstrated that inhibition of RIPK1 and RIPK3, the upstream regulators of MLKL, can improve hepatic insulin sensitivity. Nec-1(RIPK1 inhibitor) administration enhances insulin sensitivity, but not increased insulin secretion. Normal insulin action is to mediate suppression of free fatty acid release from visceral adipose tissue. It was known that there are three major components of the dyslipidemia of insulin resistance are increased triglyceride levels, decreased HDL cholesterol, and changes in the composition of LDL cholesterol. So, Nec-1 improves insulin sensitivity and decreases levels of TGs and LDL (Xu et al., 2019).

Some studies have shown the potential relationship between the impairment in cholesterol homeostasis and the induction of necroptosis. They have also shown that exogenously added 24(S)-hydroxycholesterol (24S-OHC) creates intracellular lipid droplets at an early stage due to the esterification of 24S-OHC by acyl-CoA: cholesterolacyltransferase 1 (ACAT1). 24S-OHC was found to induce caspase-independent cell death (necroptosis) and this was partially but significantly inhibited by Nec-1(Funakoshi et al., 2016).

In the present study, we found significant decrease in the hepatic immuno-expression of caspase-3, in the (Nec-1)-treated group compared to the APAP-treated group. This result comes in line with (Polito et al., 2016), who has reported that the caspase activation is slower and lower in the presence of Nec-1, demonstrating that the necroptosis blockage can also delay the apoptotic pathway and suggesting that apoptosis and necroptosis share some molecular mechanisms during the first phase of the two processes.

Some studies have shown that inhibition of apoptosis, caspases blockers or in case of ATP depletion (as caspases are dependent on level of ATP) might increase necroptosis as a feedback mechanism. But, Nec-1 (inhibitor of necroptosis) leads to decreased apoptotic activation through reducing the caspase-3 cleavage. Nec-1 has an anti-apoptotic effect not only through inhibiting the casapase-3 cleavage, but also by up-regulation of (B-cell lymphoma 2) Bcl-2, the anti-apoptotic protein (Chang et al., 2014)

Conclusion

The liver is a particular target for drug toxicity because of its role in clearing and metabolizing chemicals. The pathophysiology and intrinsic mechanisms underlying paracetamol (APAP)

induced hepatotoxicity are different because aetiologies and mechanisms vary between oxidetive stress, inflammation and cell death including apoptosis, necrosis and necroptosis. Necrostatin-1 is proved to have antioxidant, anti-inflammatory, anti-apoptotic and anti-necroptotic effect that antagonize APAP-injurious effect.

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