ABSTRACT
Lipopolysaccharide is one component of the gram-negative bacterial cell wall and its massive release within the circulation resulted in hepatotoxicity through inducing oxidative stress after a short period of time from the exposure. **Aim:** This study aimed to investigate the anti-oxidant effect of alpha lipoic acid (ALA) against liver oxidative stress induced by bacterial lipopolysaccharides (LPS). **Materials and methods:** The experiments were performed on forty adult male albino rats for a month. Each group consisted of ten rats and was distributed as follows: (Group I): normal control saline, (Group II): LPS intoxicated group, (Group III): orally pre-treated with ALA, and (Group IV): orally pretreated with ALA before injection with LPS. **Results:** LPS injection amplified liberating pro-inflammatory cytokines (TNF-α, IL-1β & IL-6) (II- 10 anti-inflammatory cytokine) as well as lipid peroxidation (MDA) and hydrogen peroxide (H$_2$O$_2$) as liver oxidative stress biomarkers. On the other hand, liver reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) levels were decreased. Liver function enzymes ALT, AST, ALP, GGT, beside total cholesterol, and triglyceride were significantly increased above normal rates. ALA was able to limit bacterial poison and its oxidative side effect on the whole body. **Conclusion:** Precautions, by taking ALA for a month before LPS injection was found to improve inflammations, antioxidant activities, and liver architecture.

**Keywords:** Lipopolysaccharide, liver, oxidative stress, alpha-lipoic acid.

1. Introduction
Liver is the main organ of the body that is responsible for the maintenance of most metabolic functions, as well as, elimination and detoxification of exogenous and endogenous substances as xenobiotic, drugs, viral infections and chronic alcoholism (Leise et al., 2014). Severe or acute hepatotoxicity may be produced due to the massive release of endotoxin or LPS into systemic circulation after killing bacteria (Pilkhwal et al., 2010). Lipopolysaccharide (LPS) is one constituent of the gram-negative bacterial wall, which is released via the destruction of bacterial cell wall and it acts as a potent bacterial product which is used for the initiation of host inflammation (Hosseini et al., 2018). Endo-toxemia can rise levels of inflammatory cytokines and reactive oxygen species (ROS), which in turn has a harmful effect on vascular endothelium and leads to impairment of tissue respiration and in addition to the probability of causing acute whole-body inflammation (Zhu & Lei, 2011).

Oxidative stress is a disproportion between free radicals and antioxidants of cells. Diabetes, hypertension and consequent cardiovascular disease are occur mainly due to changes in reduction-oxidation homeostasis is resulted in the generation of excess free oxygen radicals. Loss of redox homeostasis contributes to pro-inflammatory and pro-fibrotic pathways that stimulate damages in metabolic cell signaling (Whaley-Connell et al., 2019). Furthermore, LPS induced elevation in lipid peroxidation is an index of oxidative stress that depends on both time and dose (Garofalo et al., 2019). For the oxidative stress involved in injury after LPS intoxication, nutritional antioxidants can improve the effectiveness of treatment rules planned to recover the endotoxemia induced by LPS (Fenn et al., 2012).
Antioxidant is a vital substance which has the ability to protect the body from damage caused by free radical induced by oxidative stress (Ozsoy et al., 2008). Alpha-lipoic acid (ALA) is a natural compound synthesized in plants, animals and also humans and is critical for aerobic metabolism. Yeast, liver, kidney, spinach, broccoli and potatoes are good sources of ALA which changes quickly in various tissues to its redox couple, dihydrolipoic acid (DHLA) (Gorąca et al., 2011). ALA has the ability to regenerate other antioxidants and for this reason it is called an antioxidant of antioxidants (Bilska et al., 2008). It was reported that treatment with ALA can prevent many pathologic conditions mediated by oxidative stress (Kwiecień et al., 2013) and reduce apoptosis in liver cells (Kaya-Dagistanli, 2013).

2. Materials and Methods

2.1 Animals

Forty adult male Sprague Dawely white albino rats (Rattus norvegicus) (weighing 120-140 g) were obtained from the Experimental Animal Care Center, Faculty of Agriculture, Minia University, El-Minia, EGYPT. All Rats were maintained according to all ethics of (National Research Council, 2011). All animals were weighed, housed in an isolated polypropylene cage and fed on commercial rodent pellets and water under good hygienic laboratory conditions.

2.2. Drugs

Lipopolysaccharides, from Escherichia coli serotype O127:B8, purchased from Sigma–Aldrich (USA), ALA purchased from EVA Pharm Co., Egypt were used as the main drugs in this study.

2.3. Experimental Design

Animals were divided into four groups. Each of them consisted of ten rats as following:

Group I: It is the normal control group and was administrated with normal saline for 30 days.

Group II: It is the disease control group and was administrated with normal saline for 30 days and LPS (1mg/kg, i.p.) was administered on the 30th day (Mokhtari-Zaer et al., 2020).

Group III: This group was administered orally with (60 mg ALA /kg b. w.) for 30 days (El-Feki et al., 2016).

Group IV: It is the positive control group and was controlled orally with (60 mg ALA /kg b. w.) for 30 days and then LPS (1mg/kg, i. p.) was administered on the 30th day.

All rats were sacrificed at 4 and 24 hours (i.e. five rats per hour), after the last administration doses in all treated group. Serum and liver samples were obtained for performing immunological, biochemical and histopathological studies.

2.3.1. Preparation of tissue homogenate

After dissection, a part of liver tissue was perfused with a phosphate buffer saline (PBS) solution, with pH = 7.4 containing 0.16 mg/ml heparin to remove any red blood cells and clots. The tissue was homogenized in 5 – 10 ml cold buffer (50 mM potassium phosphates, pH 7.5) per gram tissue. Tissue homogenate was centrifuged at 4000 r.p.m for 15 minutes. The supernatant was removed and stored at -80 ° for antioxidants assay.

2.3.2. Immunological analyses

Boster’s rat TNF-α ELISA Kit (EK0526) is a sandwich enzyme-linked immune-soronet assay technology, through which, monoclonal antibody from mouse specific for TNF- α was pre-coated onto well plates, then standards and test samples were added to the wells; a biotinylated detection polyclonal antibody from goat specific for TNF-α was added subsequently, followed by washing with PBS. Avidin-Biotin Peroxidase Complex was added and unbound conjugates were washed away with PBS buffer. HRP substrate TMB was used to visualize HRP enzymatic reaction. Like TNF-α ELISA Kit, Boster’s rat IL-1β (EK0393), IL-6 (EK0412) and IL-10 (EK0418) ELISA Kits were based on standard sandwich ELISA Kits.
2.3.3. Antioxidants assay
In liver homogenate, lipid peroxidation level was determined by measuring thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et al., (1979). Hydrogen peroxide ($H_2O_2$) concentration was measured according to the method of Aebi (1984), while liver reduced glutathione level (GSH) was determined according to the method of Beutier et al., (1963). In addition, liver catalase activity was assayed following the method of Aebi (1984), and Superoxide dismutase (SOD) activity was measured according to the method of Nishikimi et al., (1972).

2.3.4. Biochemical analyses
Liver functions kits, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total cholesterol and serum triglyceride were bought from Bio-Systems chemical company, Egypt and were used for biomedical analysis.

2.4. Statistical analysis
All data were coded, charted, and analyzed statistically using version 20 SPSS program software. Analysis were done for parametric quantitative variables using one way ANOVA test and post Hoc Tukey's correction between groups. The level of significance was taken at ($P$ value $\leq 0.05$).

3. Results

3.1. Serum pro and anti-inflammatory cytokines
LPS injection significantly increased levels of serum TNF-$\alpha$ (166.8 pg/ ml : 71.6 pg/ ml), IL-1$\beta$ (122.12 pg/ ml : 80.79 pg/ ml) and IL-6 (1411 pg/ ml: 64.4 pg/ ml) at four and twenty four hour respectively, compared to saline treated group, while rats treated with ALA for a month before LPS administration had a significant effect on limiting inflammatory cytokines and augmented the levels of anti-inflammatory cytokine IL-10 with time (358.4 pg/ ml : 32.2 pg/ ml compared to (141.8 pg/ ml : 25 pg/ ml) in LPS group) (Figure. 1, 2, 3, and 4).

![Fig. 1: Concentrations of serum TNF-α in normal saline group, LPS, ALA and their combinations four and twenty-four hours.](image)

![Fig. 2: Concentrations of serum IL-1β in normal saline group, LPS, ALA and their combinations at four and twenty-four hours.](image)
Fig. 3: Concentrations of serum IL-6 in normal saline group, LPS, ALA and their combinations at four and twenty four hours.

Fig. 4: Concentrations of serum IL-10 in normal saline group, LPS, ALA and their combinations at four and twenty four hours.

3.2. Antioxidant activity

Figures (5 and 6) described that LPS significantly ($p \leq 0.05$) amplified levels of liver lipid peroxidation (MDA) from (4.76 nm/1 g and 4.87 nm/1 g) in control to (6.78 nm/g and 5.88 nm/g) respectively. Levels of hydrogen peroxide reached to (1.89 nm/g and 2.01 nm/g) in LPS group compared to (0.35 nm/g and 0.49 nm/g) at saline group with time. Treatment with ALA (60 mg ALA/kg b. w.) was able to reduce levels of MDA to (5.66nm/g and 5.17nm/g) and $\text{H}_2\text{O}_2$ to (0.67nm/g and 0.88nm/g).

Fig. 5: Levels of MDA in in normal saline group, LPS, ALA and their combinations at four and twenty-four hours.
Endogenous antioxidant activities of GSH, SOD and catalase content went down due to LPS intoxication when compared to control rats and their activities decreased from (11.29 mg/g, 11.22 mg/g), (2.09 U/g, 2.36 U/g) and (1.82 U/L, 1.83 U/L) to (6.98 mg/g, 5.1 mg/g), (0.72 U/g, 0.87 U/g) and (0.73 U/L, 1.28 U/L). The antioxidant activity of ALA improved levels and activity of liver GSH, SOD and catalase (Figure 7, 8 and 9).

![Fig. 6: Levels of H2O2 in normal saline group, LPS, ALA and their combinations at four and twenty-four hours.](image)

![Fig. 7: Levels of GSH in normal saline group, LPS, ALA and their combinations at four and twenty-four hours.](image)

![Fig. 8: Levels of SOD in normal saline group, LPS, ALA and their combinations at four and twenty-four hours.](image)
3.3. Liver function tests

Rats challenged with LPS showed a significant increase in serum ALT, AST, ALP, GGT, Total cholesterol and triglyceride levels ($p < 0.001$) at different times compared with the control group, while, the levels of all those enzymes decreased significantly ($p < 0.001$) on supplementation with ALA daily for one month before LPS injection.

Table 1: Effect of lipopolysaccharides (LPS), alpha lipoic acid (ALA) and their combination (LPS&ALA) on serum biochemical parameters in male albino rats at four and twenty four hours.

| Parameter             | Groups | Saline       | LPS          | ALA          | ALA& LPS     |
|-----------------------|--------|--------------|--------------|--------------|--------------|
|                       |        | 4h           | 24h          | 4h           | 24h          |
| ALT                   |        | 37.6 ± 2.07  | 60.2 ± 2.38  | 32.4 ± 2.41  | 53.2 ± 3.49  |
|                       |        | 37.2 ± 2.58  | 87.2 ± 8.01  | 31.8 ± 1.48  | 57.4 ± 5.59  |
| AST                   |        | 132.8 ± 1.48 | 185.6 ± 7.82 | 131.2 ± 3.03 | 157.4 ± 5.41 |
|                       |        | 131.8 ± 1.48 | 254.6 ± 6.11 | 129.8 ± 3.96 | 138.8 ± 5.71 |
| ALP                   |        | 189.2 ± 2.38 | 247.4 ± 10.81| 187 ± 1      | 215 ± 5.14   |
|                       |        | 187 ± 2.91   | 377.2 ± 8.28 | 187.6 ± 1.14 | 298 ± 4.3    |
| GGT                   |        | 3.4 ± 0.36   | 17.31 ± 2.75 | 3.36 ± 0.37  | 6.27 ± 1.72  |
|                       |        | 3.44 ± 0.32  | 11.8 ± 0.68  | 3.4 ± 0.35   | 4.43 ± 0.86  |
| Total cholesterol     |        | 63.6 ± 3.57  | 75.6 ± 2.7   | 63.2 ± 3.96  | 64 ± 3.87    |
|                       |        | 63.8 ± 2.77  | 109.2 ± 3.76 | 63.8 ± 3.56  | 76.6 ± 4.82  |
| Triglyceride          |        | 66.4 ± 3.04  | 89.4 ± 6.38  | 66.6 ± 3.57  | 103.4 ± 3.57 |
|                       |        | 66.6 ± 3.57  | 88 ± 2.12    | 66.6 ± 3.57  | 76.2 ± 2.68  |

Values are expressed as means ± SD (n= 5).
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs LPS
# $p < 0.05$, ## $p < 0.05$ and ### $p < 0.001$ vs saline.

3.4. Histopathological examination

Fixed liver tissue samples were embedded after dehydration in paraffin wax, sectioned at 5micro and stained with hematoxylin and eosin.
Fig. 10: A photomicrograph of a cross sections in the liver of a rat administered with saline (control group 1), showing a central vein (thick arrow), arranged strands of normal and polygonal hepatocytes, normal blood sinusoids and kupffer cells (down thin arrow). (2), Rats administered orally with alpha lipoic acid showing nearly normal liver morphology, with dilated central vein (thick arrow) surrounded by arranged strands of slightly shrinked hepatocytes, normal blood sinusoids and normal kupffer cells(down thin arrow).(3) 4h rat group, injected intraperitoneally with a single dose of LPS, showing highly dilated and ruptured central vein (thick arrow), congested with hemolysed blood infiltrated with leucocytes (Double arrow), and increased number of activated kupffer cells (Plus) with shrinking in hepatocytes in some areas. (4) Liver of 24h LPS group, showing congested central vein which with hemolyzed blood (thick arrow), infiltrated with leucocytes (double arrow), lined with fibroblasts, highly vacuolated hepatocytes associated with necrosis (curved arrow) and loss of typical hepatic cords organization and obliteration of blood sinusoids.(5) 4h rat group, pretreated orally with ALA, then, injected intraperitoneal with a single dose of LPS, showing normal central vein (thick arrow) surrounded by arranged strands of polygonal hepatocytes less activated kupffer cells (plus). (6) The liver of 24h rat group, pretreated orally with ALA, then, injected with LPS, showing moderately dilated central vein (thick arrow), infiltrated with few leucocyte (double arrows), sinusoidal dilation (lighting blot), and there is activated kupffer cells in some areas (plus).

4. Discussion

Liver injury is an important manifestation of sepsis/septic shock induced by LPS intoxication. LPS is responsible for causing inflammation leading to septic shock or organ failure (Depboylu et al., 2013). Circulating LPS binds to toll-like receptor-4 (TLR-4) on hepatic phagocytes and macrophages, leading to their stimulation. Activated macrophages subsequently release pro-inflammatory cytokines as well as reactive oxygen species (ROS) and reactive nitric species (RNS) (Zhu & Lei, 2011).

In our study, LPS intoxication resulted in a significant increase in the level of serum TNF-α, and these results came in a good agreement with Tateda et al., (1996) who reported that the level of TNF-α was increased one hour after LPS infusion, peaked at two hours and its level was significantly increased when compared to the control group. Moreover, Kupffer cells activation during endotoxemia leads to the secretion of a wide variety of pro-inflammatory cytokines, especially TNF-α. Endotoxemia increases the levels of pro-inflammatory cytokines also from neutrophils and leads to damaging of hepatocytes Bharrhan et al., (2010).

Serum TNF-α is a pro-inflammatory cytokines its peak time is two hours and still detected with time till twenty-four hour was able to initiate other inflammatory cytokines (El-Feki et al., 2016). TNF-α initiates IL-1β due to LPS-induced tolerance of macrophages that can lead to histone modification at the promoter regions of the genes encoding the pro-inflammatory cytokines IL-1β LPS, also collaborates with caspase-11 that leads to activation of caspase-11 independent of TLR4. Activated Caspase-11 cleaves Gasdermin D (Gsdmd) forming trans-membrane pores that ultimately cause cell
lysis and released of active Gsdmd N-terminal fragment support secretion of IL-1α and IL-1β and then triggers cell pyroptosis (El Gazzar et al., 2009; Qiu et al., 2020).

LPS injection significantly stimulated the release of other pro-inflammatory cytokines in serum such as IL-6 in which its peak time was four hours and still detectable till twenty-four hours. Increased levels of serum IL-6 reflect the relationship between the host inflammatory responses with the severity of the disease and served as a marker of prognosis in sepsis (Kim & Deutschman, 2000).

Existing of TNF-α and IL-1β as a result of LPS injection can stimulate IL-6 release. TNF-α, IL-1β and IL-6 also induce activation of neutrophils, enhances the adhesion of leucocytes with endothelial cells, stimulates chemokines secretion, macrophage chemotactic protein-1 and vascular cell adhesion molecule-1, which are the key to overexcited infection and resulting in damaged liver (Wang & Jiang 2013; Panesar et al., 1999). IL-6 may adjust liver fibrosis and inflammation via the stimulation of STAT3 in hepatic stellate cells and Kupffer cells, separately (Miller et al., 2011).

Treatment with ALA (60 mg / kg b. w.) daily for one month prior to the LPS intoxication had the ability to reduce the increase in serum inflammatory cytokines due to is ability to promote the animal health and its ability to initiate the anti-inflammatory cytokines specially IL-10 levels that resulted in increasing the survival rate by more than 80% and decreasing TNF-α production in some experimental animals (Tanaka et al., 2015).

IL -10 was able to limit the production of TNF-α by macrophages and endogenous IL-10 clearly protects the liver against hepatotoxicity induced by Gal/LPS (Louis et al., 1997). In the liver, Kupffer cells produce IL-10 in response to LPS stimulation and down regulate the release of TNF-α, IL-1β and IL-6. Endotoxin administration is an extensively studied model of IL-10 induction from monocytes and macrophages (Fiorentino et al., 1991).

LPS intoxication significantly increased lipid peroxidation which is a key of oxidative damage as a result of the imbalance between antioxidants and ROS or intensification of free radicals (Bharrhan et al., 2010). Breakdown yields of lipid hydroperoxides such as MDA was able to lead to a chaotic cross-linkage with protein and nucleic acids, causing oxidation to proteins and damaged DNA (Bertók, 2005). In the present study, pre-treatment with ALA for a month before LPS intoxication significantly decreased the formation of MDA in liver which may be partly due to the ability of ALA to scavenge free radicals.

LPS also, increased levels of H2O2, and this result came in agreement with Lv et al., (2017) who reported that LPS injection resulted in activation of peroxiredoxin gene, which is responsible for a significant increased the ROS content in macrophage and reduced the antioxidant capacity of tissues, increased its oxidative capacity and oxidative stress. ALA was able to reduce singlet oxygen and hydroxyl radicals and its functions in improvement of oxidative stress severity and the anti-oxidative impact by ALA in 4h and 24h after LPS may be symbolic that it can decompose O2− and H2O2 and this result came in a crement with (Cimen et al., 2019).

Losses the protection of antioxidant against LPS intoxication are an acute step in inducing hepatic injury, as well as changes in liver architectures, endogenous antioxidant enzymes levels, and non-enzymatic antioxidants (Xia et al., 2009). Our results showed a significant reduction in the activities of reduced GSH levels, SOD and catalase activities along with in the liver of rats intoxicated with LPS. This might end with a loaded anions of superoxide and wasteful decontamination of H2O2 which forms OH− ions, augmenting lipid membrane oxidation, thereby, leading to oxidative injury in various tissues (Tiwari et al., 2018).

In this study, ALA-treated rats showed an enhancement in the activity of reduced GSH, SOD and catalase when compared to LPS-challenged group. Similarly, Akpinar et al., (2008) found that, ALA contributes to antioxidant defense by increasing catalase activity. While Mahdavi & Safa (2019) illustrated that ALA may be beneficial for Huntington disease (HD) patients by increasing the activity of antioxidant enzymes.

Serum ALT, AST, ALP and GGT are normally found at high concentrations within the liver cells under normal conditions and those enzymes are released into the circulation during hepatocyte necrosis or membrane damage and identified by elevated serum enzyme levels (Akpinar et al., 2008). In the present study, LPS intoxication resulted in a state of inflammation and oxidative damage at liver cells that was able to elevate levels of all these marker enzymes indicating liver damage induced by endotoxin. Pre-treatment with ALA before LPS injection ameliorated the toxic effects of LPS and the above markers, decreased towards the normal level. The results obtained in this study are in a harmony
with Heibashy et al., (2013) who confirmed that treatment with ALA prior to LPS challenge was significantly able to decrease liver damage.

The antioxidant activities of both ALA and DHLA are sufficient to protect cell membrane of liver through limiting inflammatory cytokines, initiating IL- 10, reducing ROS and promoting antioxidant activity of cells.

It was observed that serum total cholesterol was significantly increased, peaked at twenty-four hours, in LPS injected rats when compared to control and these results came in accordance with Feingold et al., (1992) who reported that serum cholesterol level was increased and continued for at least twenty-four hours after LPS administration, while, Hussein et al., (2015) observed that there were not any changes in serum total cholesterol concentration in two and five hours rats intoxicated with LPS. The detectable increase of serum total cholesterol at twenty-four hours may be due to the effect of LPS on hepatic HMG-CoA reductase activity, mass, and mRNA levels. HMG-CoA reductase is the rate controlling enzyme of mevalonate pathway that produces cholesterol. Mmaximal activity of HMG-CoA reductase in the liver was increased in the low and high dose of LPS intoxicated animals; thus, LPS administration increases HMG-CoA reductase and mRNA levels in the liver (Feingold et al., 1992).

Pre-treated groups with ALA before LPS intoxication significantly decreased concentration of cholesterol and these results agreed with Hussein et al., (2015) who reported that ALA had the potential to improve dyslipidemia and oxidative stress and may exert some protective effect on atherosclerotic vascular change in hyper-lipidemic rats.

LPS injection besides increasing total cholesterol were sufficient to elevate serum triglyceride (TG) that peaked at four hours, when compared to control group and these results came in accordance with Feingold et al., (1992) who reported that high dose of LPS resulted in hypertriglyceridemia by decreasing hepatic lipoprotein catabolism. LPS intoxication may produce hypertriglyceridemia by increasing hepatic lipoprotein secretion, reducing lipoprotein clearance and decreasing lipoprotein lipase activity. Moreover, it was proposed that, hypertriglyceridemia was be due to the catabolic effects of a circulating factor on fat cells that directly cause cachexia (Anderson & Borlak 2008).

Pre-treatment with ALA before LPS intoxication significantly limited this increase of serum triglyceride (TG), and this result was in a harmony with Hussein et al., (2015) who reported that administration ALA to rats fed on high fat diet caused a significant decrease in elevated serum triacylglycerol concentration and such decrease in serum triacylglycerol was explained by Bennani-Kabchi et al., (2000) who related them to the increase rate of lipolysis by the increase of plasma lipase activity.

5. Conclusion

Lipopolysaccharides extracted from gram negative bacteria are adequate to increased inflammation and oxidative stress when released in to circulation and affect badly functions of liver. The anti-inflammatory and antioxidant activity of lipoic acid is sufficient to improve the body cells against free radicals released during stress and limit bacterial toxins.

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