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L.carnitine can attenuate cadmium induced hepatotoxicity in rat: emphasis on TLR4-NFκB axis.

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Abstract: Cadmium (Cd), an environmental health hazard, is a causative agent of many pathological conditions especially liver diseases. The possible ameliorative role of L. Carnitine on cadmium- provoked liver injury was discussed in this study. Twenty-four male rats were daily challenged for 30 days with $CdCl_2$ (cadmium chloride) 5mg/kg/day and/or L. Carnitine 100 mg/kg/day. L. Carnitine administration halted the alteration of liver function biomarkers induced by cadmium which appeared as decreased AST, ALT and ALP levels. L. Carnitine prohibited $CdCl_2$-generated oxidative stress via Nrf2/HO.1 pathway by increasing TAC and decreasing MDA and NO. L. Carnitine also restrained inflammatory insult created by $CdCl_2$ by suppressing TLR4/NFκB pathway through reducing the inflammatory mediators including TNF-α, IL-1β and IL-18. Finally, our biochemical data were confirmed by the improvement of the liver histology by L. Carnitine. These results revealed that TLR4/NFκB axis implicated in L. Carnitine protection against $CdCl_2$-triggered hepatotoxicity accompanied by inhibition of oxidative stress and this may provide new ideas in order to treat cadmium-related diseases.

Keywords: L. Carnitine; Cadmium Chloride; Hepatotoxicity.

1. INTRODUCTION

Liver diseases are considered as a crucial health problem causing about 2 million deaths around the world [1]. Cadmium is a toxic compound to which man can be exposed that has a long half-life within the human body. Once absorbed, it accumulates in the body organs especially in the liver [2, 3]. Humans are intoxicated by cadmium via industrially contaminated food, cigarette smoking and inhalation of contaminated air thus it promotes tissue injury by causing inflammation and oxidative stress [3].

In the same context, it induced liver damage mechanistically by two pathways, Primary injury is triggered by Cd binding to sulfhydryl groups of molecules in mitochondria causing oxidative stress but secondary injury is a result of stimulation of Kupffer cells and boosting of inflammatory mediators [4] thus oxidative stress and inflammation are regarded as the main causative agents of cadmium-induced liver injury [5].

The transcription factor called Nuclear factor erythroid 2-related factor 2 (Nrf2) creates genes responsible for anti-oxidative stress reaction and drug detoxification resulting in the regulation of cellular defense against oxidative damage [6] so its pathway is considered as a pharmacological target to mitigate many liver diseases [1] particularly Cd toxicity which causes hepatic damage in rats via activation of oxidative stress and inhibition of Nrf2 pathway [7].

One of the most crucial mechanisms responsible for the pathogenesis of several diseases is TLR4 /NF-κB pathway [8] it was confirmed that Cd intoxication triggered TLR4/NF-κB pathway [9]. NF-κB activation causes

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up-regulation of some pro-inflammatory cytokines so cadmium-induced liver inflammation is accompanied by excessive output of pro-inflammatory mediators such as TNF-α, IL-1β and IL-18 [2, 10].

Recently, agents with anti-oxidative and anti-inflammatory properties have been used to counteract oxidative stress and inflammation which cause several health complications.

L-carnitine is a non-essential amino acid derivative naturally present and widely distributed in nature [11] it halted muscle injury, diminished cellular damage and decreases free radical generation. In addition, it decreases hypoxia-induced cellular and biochemical alterations via enhancement of blood flow and oxygen supply to the muscle tissue [12] L-carnitine has a hepato-protective effect related to its capability to detoxify free radicals and to motivate the antioxidant systems [11, 13] notably, it exerts antioxidant action via activating Nrf2 signaling pathway [14]. Moreover, it has an anti-inflammatory effect by down-regulation of TNF-α and NF-kβ [15] in addition; Acetyl-L-carnitine can exert protective effect against neuro-inflammation caused by lipopolysaccharide via inhibition of TLR4/NFκB pathway [16]

Herein, we assess the hepato-protective role of L-carnitine on a rat model of cadmium-caused hepatic damage and explain possible mechanisms by which L-carnitine exerts its action.

2. METHODS

2.1. Animals

We used adult male Wister albino rats (150-200 g) body weights in our study. Source of animals is the breeding colony of Egyptian Drug Authority (EDA) and kept in its animal house. During our study, rats given food and water ad libitum. Animals required adaption period for a 2-weeks prior to starting of the experiment and we must provide 40%–60% relative humidity, 21–24°C and a 12-h light–dark cycle for animals. The ethical standards for laboratory animal research were followed the protocols were confirmed by the EDA's standard operating procedures.

We obtained (Approval number: NODCAR/I/10/2023) in handling the experimental animals and corresponds to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Reagents and chemicals

Sigma-Aldrich Co. is the source of the used Cadmium chloride which dissolved in distilled water and administered per os with the dose (5 mg/kg/day) daily for 30 consecutive days as used by **Yang et al [17]**. L. Carnitine was purchased from Mepaco Pharmaceutical Co., Ltd. Egypt and was dissolved also in distilled water and given daily with a dose (100 mg/kg; orally) **Ahmed et al [18].**

2.3. Experimental design

The schematic representation of the study methodology is illustrated in **Figure 1**.

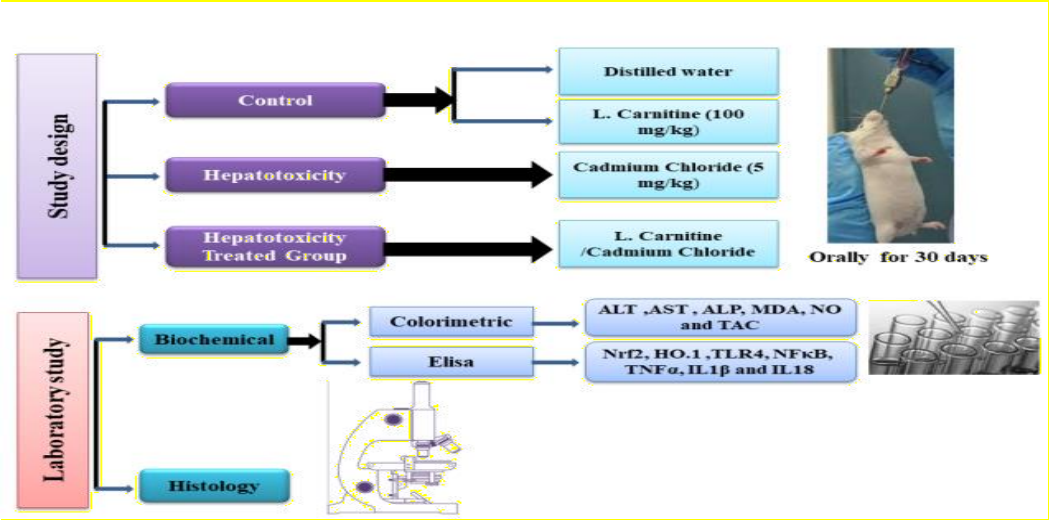

Figure 1: Methodological Overview: Study Design, Biochemical Analysis and Histological Examination

Our experiment performed using four groups of animals (6 in each group). **Group I**: animals given distilled water for 30 days and was used as normal control animals.

Group II: contained rats which administered L. Carnitine 100 mg/kg; per os for 30 days. **Group III**: rats were challenged with cadmium chloride 5 mg/kg/day; orally for 30 consecutive days. **Group IV**: rats were co-treated with L. Carnitine along with cadmium as previously described.

On the 31st day, Blood samples were collected when the rats were under anesthesia and subsequently centrifuged for 15 min at 4000 to collect serum for assessment of liver function biomarkers. After that, rats were euthanized and livers were removed, cleaned, cleansed with ice-cold saline then weighed. For histopathological examination, 10% formalin solution used for keeping different parts of the livers from each treated group. Another part was homogenized in phosphate buffer saline for the evaluation of oxidative and inflammatory biomarkers.

2.4. Parameters to be estimated

2.4.1. Determination of hepatic function indices

Serum content of hepatic function biomarkers was assessed using colorimetric method as follow:, alanine aminotransferase (ALT; Bio Diagnostic CO., Egypt),aspartate aminotransferase (AST; Bio diagnostic, Egypt) and alkaline phosphatase (ALP, Bio-med diagnostic, Egypt) along the same lines as the manufacturers' guidelines.

2.4.2. Assessment of oxidative stress markers

Liver homogenate of the different treated groups was used for estimation of Nrf2 using (MyBiosourse, Rat Nuclear Factor Erythroid 2Related Factor 2 {NFE2R2} Elisa kit. Cat. N. MBS752046), HO.1 estimated by (Rat Heme Oxygenase 1 Elisa Kit. Cat. N. MBS764989), total antioxidant capacity was assessed by (Bio diagnostic, TAC colorimetric kit, Egypt. Cat. NO. TA 25 12), MDA content determined using (Bio diagnostic, MDA colorimetric kit Egypt. Cat. NO. MDA 25 28) and finally, evaluation of nitric oxide occurred using (Bio diagnostic, NO kit Egypt. Cat. NO. 25 33).

2.4.3. Determination of TLR4 and NFκB

(CUSABIO®, Elisa Kit China, Cat. NO. CSB-E12108m) was used for the determination of NFκB and (Elabscience Biotechnology Inc. USA, Cat. NO. E-EL-R1121) was used for estimation of TLR4 in agreement with manufacturer's instructions.

2.4.4. Pro-inflammatory biomarkers determination

TNF-α, IL1β and IL18 expressions were measured in liver homogenate using (CUSABIO®, China Elisa Kit. Cat. NO. CSB-E11987r), (MyBioSource® rat IL1β Elisa Kit. Cat. NO. # MBS825017) and (Elabscience Biotechnology Inc., USA kit Cat. NO.E-EL-M0858) respectively, were used corresponding to the manufacturer's guidelines.

2.4.5. Histopathological examination

Hepatic tissue samples were fixed in 10% formalin for twenty-four hours. As the method explained by **Bancroft and Gamble [19]**, Histopathological samples were prepared and subsequently examined using a light microscope.

2.5. Statistical analysis

Graph Pad Prism software version 5 (Graph Pad Software Inc,. San Diego, USA) was used to carry out statistical analysis .Our results were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and data in form of means ± SE. For all statistical tests, the level of significance was provided at $p < 0.05$.

3. RESULTS

3.1. L.Carnitine administration Improves Serum hepatic enzymes impairment in Cadmium-challenged rats

As shown in **Figure 2,** cadmium intoxication provoked marked alterations in hepatic function biomarkers. This was demonstrated by a significant elevation of ALT, AST and ALP by nearly 3-folds, 199% and 98.65% respectively, in comparison to control animals. The administration of L.Carnitine to cadmium-intoxicated animals counteracted these aberrations in form of a considerable decrease in serum hepatic enzymes by 50% for ALT, 52% for AST and 31% for ALP versus cadmium treated animals. These findings revealed the capability of L .Carnitine for rescuing hepatic injury rendered by cadmium in rats.

3.2. Giving L. Carnitine to intoxicated rats minimizes Cadmium-Induced oxidative stress in their liver

Cadmium triggered oxidative damage and this shown by significant decline in liver content of Nrf2 and its downstream HO.1 and TAC by 70.9 %, 62.6 % and 56.3% respectively, alongside a significant increase in MDA and NO by 145.3 % and nearly 3-folds respectively, in comparison to control animals.

On the other hand, Administration of L. Carnitine for cadmium-intoxicated animals halted this oxidative stress, as demonstrated by a significant elevation in Nrf2, HO.1 and TAC by 156%, 85.4 % and 95.3% respectively, with significant reduction in MDA and NO by 44.4% and 58.9 % respectively, relative to cadmium treated rats as demonstrated in **Figure 3**. These data revealed the involvement of oxidative stress suppression by L. Carnitine in the attenuation of cadmium induced hepatotoxicity in rats.

3.3. L. Carnitine co-treatment curtails Cadmium-Induced Pro-Inflammatory events and TLR4/NF-κB Pathway Activation in liver of rats

We found a significant elevation of hepatic TNF-α, IL1β and IL18 protein expression by 158.9%, 287.8% and 65.9 % respectively, as a result of cadmium administration in comparison to control animals as shown in **Figure 4** which proved cadmium ability to activate the pro-inflammatory responses in the animal's liver. This was affirmed by the significantly up-regulated expression of hepatic TLR4 and its downstream NFκB by 92.0% and nearly 4-folds % respectively, versus to control animals.

Administration of L. Carnitine to cadmium-intoxicated rats ameliorated these inflammatory responses as demonstrated by a significant decrease of TNF-α, IL1β and IL18 by 50.6%, 60.5% and 29.7 % respectively,. In the same context, the protein expression of hepatic TLR4 and NFκB were significantly down-regulated by 31.5 % and 41.1%, respectively as compared to cadmium treated rats. These findings clarify the role of inflammatory and TLR4/ NFκB pathway inhibition by L. Carnitine in the improvement of the hepatic injury provoked by cadmium in rats.

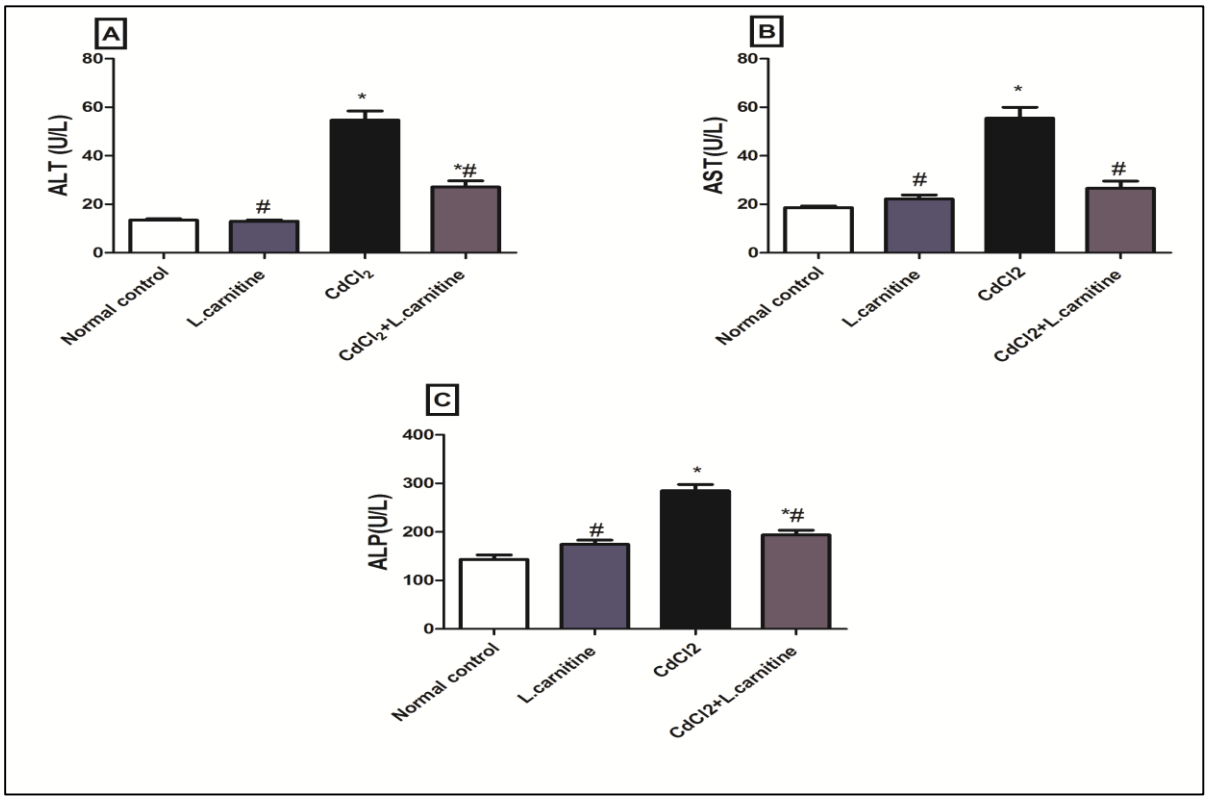

Figure 2: Effect of L. carnitine administration on (A) ALT, (B) AST and (C) ALP levels in cadmium treated rats. Values are presented as mean ± SE. * or # statistically significant from the control or cadmium group, respectively, using one way analysis of variance followed by Tukey–Kramer as a post hoc test at $p < 0.05$.

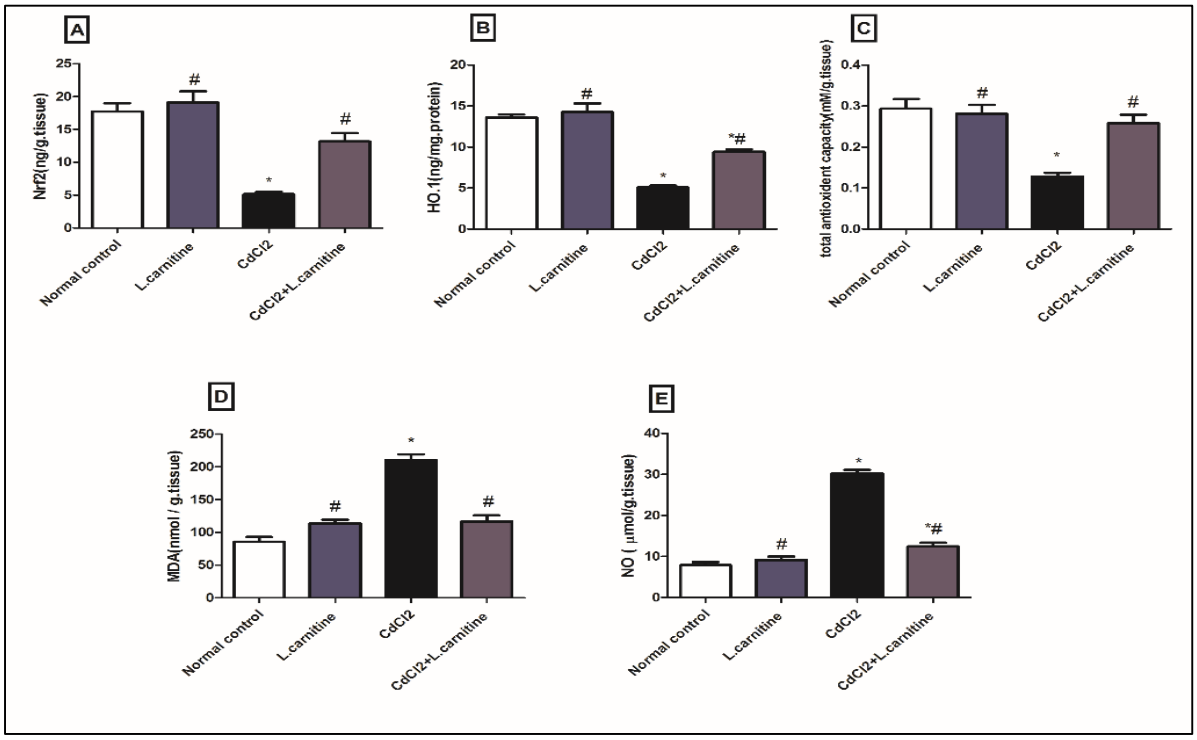

Figure 3: Effect of L. carnitine administration on (A) Nrf2, (B) HO.1, (C) Total antioxidant capacity, (D) MDA and (E) NO levels in cadmium treated rats. Values are presented as mean ± SE. * or # statistically significant from the control or cadmium group, respectively, using one way analysis of variance followed by Tukey–Kramer as a post hoc test at $p < 0.05$.

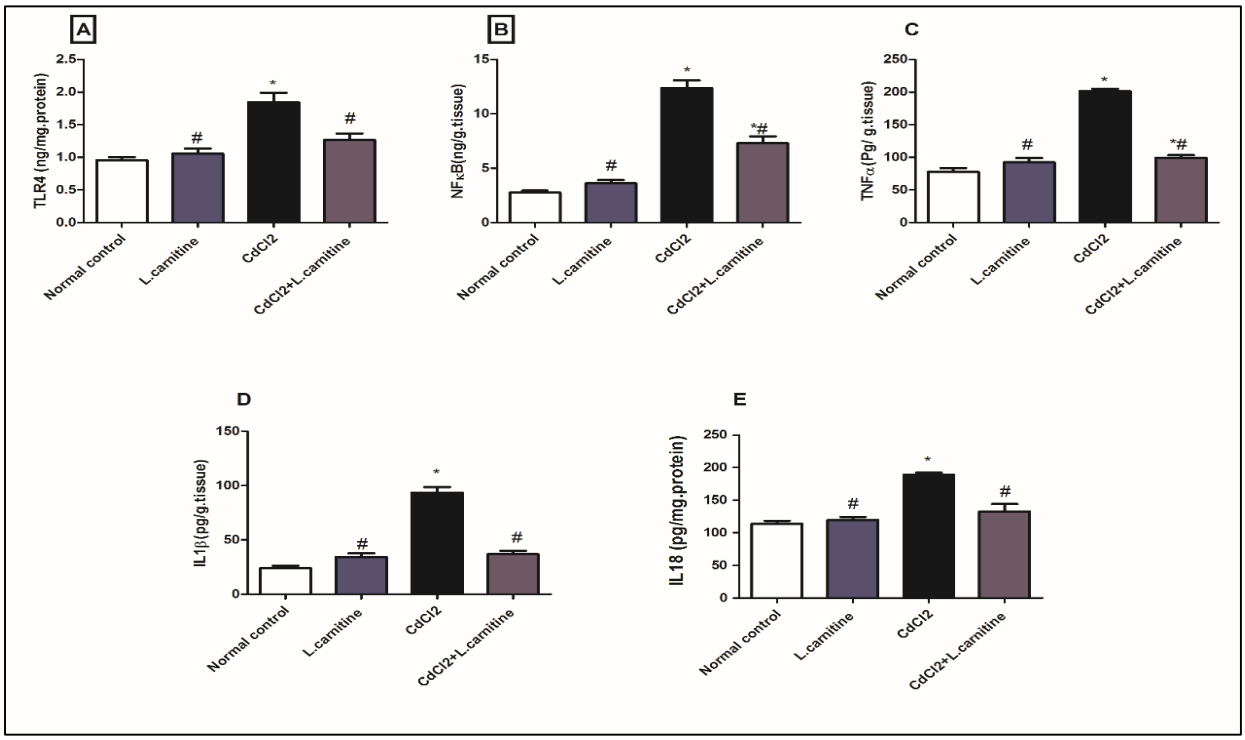

Figure 4: Effect of L. carnitine administration on (A) TLR4, (B) NFκB (C) TNF α (D) IL1β and (E) IL18 levels in cadmium treated rats. Values are presented as mean ± SE. * or # statistically significant from the control or cadmium group, respectively, using oneway analysis of variance followed by Tukey–Kramer as a post hoc test at $p < 0.05$.

3.4. L. Carnitine Administration Attenuates Cadmium-Induced Histological Lesions in liver of Rats

Histologic image of control rats **(Figure 5A)** demonstrated normal liver architecture with no histological abnormalities as well as a typical central vein and hepatocyte arrangement. Hepatocyte nuclei were visible within cells as dark red structures whereas the cytoplasm was colored red.

Hepatic strands that go from the lobule's edge to the central vein appear normally as well as normal sinusoids and usual portal region. Bi-nucleated cells and kupffer cells were found in large numbers**.** Similar to control rat, L. Carnitine treated rats as appeared in **(Figure 5B)** showed normal hepatic histology with hepatic cords which positioned appropriately around the sinusoids. Hepatocytes and portal components seemed to be in good shape. But in cadmium treated animals **(Figure 5C and D)**, in liver sections there was degenerated hepatocytes, lobular disorganization, nuclear disintegration and fatty degeneration, as well as disarrangement of normal hepatic cells. The portal region was revealed to have lymphocyte infiltration. On the other hand, Administration of L. Carnitine for cadmium intoxicated animals **(Figure 5E)** revealed signs of improvement appeared as normal hepatocytes with active euchromatic nuclei which looked to be in excellent health and no inflammatory cell infiltrations were seen.

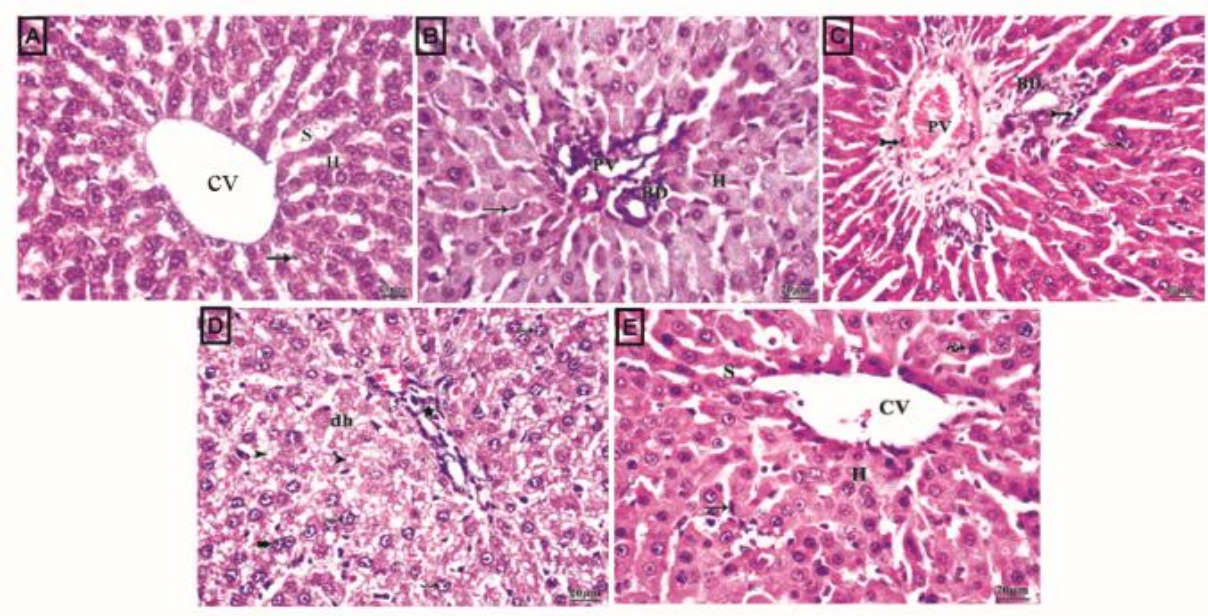

Figure 5: Photomicrographs of hepatic tissue of (A) control rat and (B) L. carnitine treated rat showing with normal histological structure showing central vein (CV), portal vein (PV), bile duct (BD), hepatocytes (H) and kupffer cells (arrow), (C and D) cadmium treated rat showing mononuclear cell invasion (Bifid arrow), pyknotic cell (Wavy arrow), degenerated hepatocyte (dh), lymphatic infiltration (Star), cytoplasmic vacuole (arrow head) and binucleated cell (bold arrow). and (E) L. carnitine + cadmium treated rat showing central vein (CV), hepatocytes (H) ,pyknotic cell (Wavy arrow) and kupffer cells (arrow).

4. DISCUSSION

Cadmium is one of the more toxic environmental pollutants [20] liver is the main target organ for Cd toxicity resulted in hepatocellular injury by oxidative stress which considered the main mechanism of cadmium induced hepatic damage [21,22]. Once Cd caused oxidative stress and inflammation, it affected the permeability barrier of plasma membranes [23, 24]**.** Transaminases such as ALT and AST are important liver injury markers therefore increased ALT and AST levels indicated liver damage [25] also to evaluate hepatic cells function, ALP level is assessed as any increase in ALP activity is associated with the biliary pressure [26]**.** The results of our study revealed that cadmium

increased serum ALT, AST and ALP levels [27, 28] and this related to the ability of cadmium to affect membranes integrity, increasing cell-membrane permeability and cellular leakage resulting in the release of some ALT and AST into the blood confirming liver tissue damage [17, 25, 26]. On contrast, the significant decrease in ALT, AST and ALP levels in the Cd and L. Carnitine co-treated group means that L. Carnitine ameliorated the Cd-induced liver toxicity as previously proved by **Abu-El-Zahab et al ,Abdel-Emam et al [29,30].**

Oxidative stress occurs when generation of reactive oxygen species (ROS) rises above cellular antioxidant capacity resulted in pathogenesis of several health complications such as liver diseases [31, 32]**.** Oxidative stress caused by Cadmium is related to its ability to bind to sulfhydryl groups on critical molecules in mitochondria [4]**.**

Nrf-2 is a key transcription factor that has ability to defeat oxidative stress via regulating the expression of antioxidant and detoxification enzymes as HO-1 [32] so Nrf2 stimulation could mitigate drug-induced liver injury in mice therefore Nrf2 activators have clinical importance as they activate Nrf2 thus enhance the expression of cyto-protective genes for treating liver diseases [33]**.**

Related to these facts, we found that cadmium caused down-regulation of Nrf2 and HO-1 protein expression causing oxidative stress action correlated to weakened Nrf2 signaling [7, 34-36]**.**

MDA is end product of lipid peroxidation so its level represents the degree of lipid peroxidation [37] accordingly; it is used as an indicator of oxidative stress-induced liver impairment in cadmium challenged rats [20]. Additionally, the overproduction of NO induces nitrosative stress through peroxynitrite formation which also damages the tissues [38] besides; The TAC reflected the total antioxidant ability in the whole body which contributed to its ability to reduce Fe3+ to Fe2+ [39]. In our study, cadmium increased MDA content and nitric oxide level along with a decreased level of total antioxidant capacity. This result was early mentioned by **Agır and Eraslan, Sanjeev et al, Moradkhani et al, Omidifar et al, Bhardwaj and panchal [40-44].** In the same context, L. Carnitine amended this cadmium-induced oxidative stress by promoting expression of Nrf2 and HO-1 which is a vital detoxifying enzyme [36] and this ameliorative effect appeared as an augmented level of total antioxidant capacity along with a considerable reduction of MDA and nitric oxide levels and this coincides with previously concluded data [45-51] .

So briefly, The main mechanisms by which L-carnitine exerts its protective effect to combat hepatic injury could be achieved through stimulation of endogenous anti-oxidative molecules [47], Elimination of free radicals, regulation of nitric oxide, activation of anti-oxidative damage enzymes, as well as chelation of ferrous ions which promotes oxidative stress [52,53]**.**

Finally , our findings that L. Carnitine plays a synergistic role to restrict Cd-mediated liver injury are similar to those documented by **Abu-El-Zahab et al** [9] who proved that L-carnitine modulated Cd-induced liver toxicity in mice by reducing Cd-triggered oxidative stress and Cd-mediated ALT and AST levels alteration and by increasing non-enzymatic antioxidant activities .

Cadmium induces hepatic injury through induction of inflammation which regarded as one of the main causes of liver injury [54, 55]**.** Toll like receptors (TLRs) are important regulators of inflammation and are considered as an effective link between inflammation and fibrosis in chronic liver injury [56]. TLR4 is stimulated either by exogenous ligands as lipopolysaccharide or by endogenous ligands; damage-associated molecular pattern molecules from injured cells triggering tissue damage of liver by different causes as when it activated, several pro-inflammatory cytokines are released due to its capability to mediate IκB phosphorylation resulting in NF-κB activation [57-59]**.**

Nuclear factor-κB (NFκB) is a transcription factor that performs a vital role in stimulating genes responsible for many pathological disorders and also responds to cell injury and infection [60] .NF-kB signaling pathway is the most important pathway involved in liver inflammation so actually, NF-kB activation is connected to hepatocyte injury and liver fibrosis [61]**.** Once NF-κB activated either by oxidative stress or by TLR4 stimulation [31, 59 ,62] it controls the pro-inflammatory response via promoting the expression of some pro-inflammatory cytokines such as TNF-α, IL-1β and IL-18 [2,10, 63] with great concern given to TNF-α which is an important downstream cytokine of the NF-κB stimulation [64] additionally **,** these inflammatory mediators bind to the pattern recognition receptors such as TLR 4 on hepatocytes resulting in cell damage [54].

Notably, it is known that Cd toxicity activated the TLR4/NF-κB signaling pathway resulting in cell damage [8].

With regard to these data, we found that cadmium activated TLR4 in rat liver cells and promoted the expression of NF-κB. This comes to an agreement with already proved results [8, 23, 65] and also enhanced the synthesis of TNF-α, IL-1β, and IL-18 and this similar to early obtained data [23,55,66,67] particularly TNF-α which is released from non-parenchymal cells and it responsible for many complications observed with cadmium-induced hepatotoxicity[68].

On contrast, L-carnitine can inhibit the TLR-4/NF-κB inflammatory pathway [16], we found that L-carnitine treatment corrected inflammatory responses via decreasing TLR4 activation and down-regulation of NF-κB protein expression and this in coincidence with **Morid et al, Sun et al** [69, 70] together with reduction of the hepatic levels of TNF-α, IL-1β and IL-18 in consensus with **Morid et al, Emran et al**, **Zheng et al** [69,71,72]. So it is clear that this anti-inflammatory effect is explained by the ability of L-carnitine to prevent NF-κB movement from cytoplasm to nucleus and prevent the production of TNF-α and interleukins [15, 47, 74] leading to improvement of all parameters related to oxidative stress and inflammation [71]

In this experiment, the liver of Cd-challenged animals showed many histological lesions as degenerated hepatocytes, lobular disorganization, nuclear disintegration and fatty degeneration, disarrangement of normal hepatic cells and lymphocyte infiltration .by using these results to evaluate the organ damage [7], Cd provoked deleterious pathological effects in rat liver similar to early observations [41, 43, 74] this abnormal effect may be correlated to the oxidative damage which seen in cadmium toxicity [22, 75, 76].

Otherwise, all these histopathological alterations induced by cadmium were abrogated by L-carnitine in the form of restoration of normal histological architecture of the liver and enhanced antioxidant enzymes. These findings concurred with **Hassan et al , Rashad et al** [48,77] who found that L-carnitine decreased oxidative stress and restored liver morphological alterations.

5. Conclusion:

Cadmium induced inflammatory disorder by NFκB/TLR4 pathway furthermore generated oxidative damage by impairment of Nrf2, HO.1 and degradation of non-enzymatic antioxidants and these are considered the key mechanisms participates in cadmium-induced hepatic damage thus the ameliorative effect of l.carnitine is because of its capacity to overcome these mechanistic pathways.

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Ethical Statement: The ethical standards for laboratory animal research were followed the protocols were confirmed by the EDA's standard operating procedures. We obtained (Approval number: NODCAR/I/10/2023) in handling the experimental animals and corresponds to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Author Contribution: Alzahraa A. Elhemiely: Conceptualization; methodology; validation; formal analysis. The author revised the results and accepted the final form of the manuscript.

List of Abbreviations: CdCl2 : cadmium chloride, AST: aspartate aminotransferase , ALT: alanine aminotransferase; ALP: alkaline phosphatase; Nrf2: Nuclear factor erythroid 2-related factor 2; HO.1: Hemeoxygenase.1; TAC: total anti-oxidant capacity; MDA: Malonaldahyde; NO: nitric oxide; TLR4: Toll like receptors 4; NFκB; Nuclear factor kappa B; TNF-α: Tumer necrosis factor alpha; IL-1β: interleukin 1 beta; IL-18: interleukin 18; ROS: reactive oxygen species.

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