MOLECULAR MECHANISMS OF CLOZAPINE-INDUCED PANCREATIC DAMAGE AND ITS MODULATION BY L-CARNITINE IN A RAT MODEL

Dalia Ibrahim Ahmed Mesallam[1], Eman Ahmed Alaa El-Din[1], Mai Said Ibrahim[1], Samaa Salah Abd El-Fatah[2], Elham Elshawadfy Megahed[1]

[1]*Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Zagazig University, Egypt*
[2]*Human Anatomy and Embryology Department, Faculty of Medicine, Zagazig University, Egypt*

ABSTRACT

Background: Clozapine (CLZ) has been considered the mainstay drug in treatment of resistant schizophrenia. Diabetes mellitus has befallen during clozapine therapy. L-carnitine (LC) has protective effects against many health hazards. **Aim of the work:** This experiment aimed to examine the molecular mechanisms of pancreatic insult caused by CLZ in rats and the possible ameliorating effect of LC against that toxicity. **Material and Methods:** Thirty-five adult male albino rats were allocated into five groups equally: control groups (negative and vehicle), LC-treated group received 350 mg/kg/day LC, CLZ-treated group gavaged 25 mg/kg/day CLZ and the combined group gavaged LC+CLZ in the same previous doses. All treatments were given orally for 4 weeks. **Results:** CLZ-treatment triggered a significant rise in the mean values of body weight (BW) and serum of fasting blood glucose (FBG), amylase, insulin, Triglyceride Glucose (TyG) Index and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). Also, a significant upsurge in lipid peroxidation and pro-inflammatory (Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-kB)) accompanying by a significant decrease of catalase (CAT), Hemo oxygenase-1 (HO-1) enzyme and nuclear factor erytheroid 2-related factor (Nrf2) activities and anti-inflammatory (IL-10), and decreased expression of Peroxisome proliferator activated receptor alfa (PPAR-α) in pancreatic tissues has occurred. There was histological and immunohistochemical evidence of pancreatic tissue injury with increased collagen fibers. The previous abnormalities were reversed when LC was given in the combined group. **Conclusion:** LC can ameliorate the pancreatic oxidant, inflammatory, and apoptotic impacts induced by CLZ.

Keywords: *Pancreas, L-carnitine, Clozapine, Insulin, PPAR-α*

Corresponding author: Dr. Dalia Ibrahim Ahmed Mesallam
Email: diamaae@yahoo.com
ORCID: 0000-0003-2731-9487

INTRODUCTION

Schizophrenia is a chronic disease that affects thinking, emotion, and behavior. It usually begins early at ages between 15 and 35 years causing constant disability and high mortalities *(Gong et al., 2021)*. Schizophrenia is fairly common, can affect 1.1% of the population, and 30% of them develop treatment-resistant according to the Egyptian National Institute of Mental Health *(Zhao et al., 2021)*.

Antipsychotic medications can be classified into typical or first-generation drugs and atypical or second-generation drugs *(Veselinović et al., 2019; de Bartolomeis et al., 2022)*. Clozapine (CLZ), (a tricyclic dibenzodiazepine), is an atypical antipsychotic drug. It has blocking activities on dopaminergic (D4), noradrenergic, serotonergic, histamine, and cholinergic (M2) receptors *(Gammon et al., 2021)*.

Clozapine is exceedingly effective in treatment-resistant schizophrenia (TRS) *(Boydstun et al., 2023)*. It reduces symptoms of psychosis by 40%, hospital entry and deaths and suicidal attempts compared to other antipsychotics *(Patel et al., 2019; Fenton and Kang, 2023; Casetta et al., 2024)*.

It was first introduced in 1961 where agranulocytosis was the main side effect *(Ellington, 2023)*. CLZ has serious metabolic side effects counting weight gain, metabolic syndrome and dyslipidemia *(Dionisie, 2024)*. L-Carnitine, essential amino acid (β-hydroxy-trimenthlyl-aminobutyric acid), is synthesized

from lysine and methionine. It has important functions in metabolic activities *(Rusdiyana et al., 2023)*. It exhibits anti-inflammatory and anti-apoptotic effects. LC has protective effects on different organs like brain, heart and etc… *(Keshani et al., 2024)*.

Xiang et al. (2013) and *Sadighara et al. (2017)* testified the pancreatic protective role of LC through improvement of oxidative stress, inflammatory cytokines, and mitochondrial β-cells dysfunction.

THE AIM OF THE WORK

This research aimed to examine the molecular mechanisms of pancreatic insult caused by CLZ in rats and the possible ameliorating role of LC in reducing pancreatic oxidative overload, inflammation and apoptosis induced by CLZ.

MATERIAL AND METHODS

Material

Leponex tablets: each contains 25 mg CLZ, from Novartis pharmaceutical company.

L-Carnitine capsules: each contains 350 mg LC from Sigma Aldrich Co. branch in Cairo, Egypt.

Rats and experimental design:

According to mean amylase was 967 ± 190 vs 739 ± 150 in experimental group vs protected group. At 80% power and 95% CI, sample size was calculated to be 35 rats using open Epi program in Community Medicine Department. Thirty-five adult male albino rats weighing (175-210 gm) were gained from Faculty of Medicine animal house, Zagazig University. Before starting experiment, seven days adaptation period with no treatment for accommodation with new environment was done, to assess physical state and eliminate diseased ones, Nutriment and water was given to all animals equally. Guidelines of Institutional Animal Care and Use Committee Zagazig University, Egypt were followed in this study, (Ethical approval number **ZU-IACUC/3/F/76/2023).**

Rats were alienated into five groups (7 rats/each). All medications were given orally for 4 weeks.

-Group I (negative control): no treatment was given (to assess the basic values of the parameters studied in this research).

-Group II (vehicle control): distilled water was given (1 mL/day) as a solvent of CLZ and LC.

-Group III (LC treated): LC was given daily at dose of 350 mg/kg/day *(Abd-Elrazek and Ahmed-Farid, 2018)*, dissolved in distilled water.

-Group IV (CLZ treated): CLZ was given at dose of 25 mg/kg/day (1/10 of LD_{50}) *(Wang et al., 2008)*, dissolved in distilled water.

-Group V (LC + CLZ- treated group): This group was given daily CLZ and LC at the same previous doses (LC treatment was given two hours before CLZ treatment).

Methods:

I. Measurement Body weight:

Body weights (BW) were measured at the commencement and the termination of experiment to calculate the gain in BW (BW gain = final weighing- starting weighing).

II. Biochemical studies:

II-1- Pancreatic Function Tests:

II-1-1-Fasting blood glucose (FBG) and Insulin:

After overnight fasting, blood samples were collected to reduce variation in blood sugar *(Ayala et al., 2010)*. The animals were sedated with ether to decrease anxiety *(Akbarzadeh et al., 2007)*. FBG was assessed by using oxidase technique *(Tietz, 1995)*. Serum insulin was assessed using a sensitive rat radioimmunoassay kit according to the manual instructions.

II-1-2- Homeostatic Model Assessment of Insulin Resistance (HOMA-IR):

HOMA IR is a measure of insulin resistance *(Wallace et al., 2004)*. A normal range for HOMA-IR is 0.7 to 2.0, with values >2.0 represents clinically significant insulin resistance *(Cacho et al., 2008)*. The formula used for calculation: [fasting glucose (mg/dl) x fasting insulin (mlU/ml) /405] *(Matthews et al., 1985)*.

II-1- 3- Triglyceride Glucose Index (TyG index).

Triglyceride glucose index is a screening method for insulin resistance *(Simental-Mendía et al., 2008)*. Individuals with an index of 4.49 or greater are more susceptible to insulin resistance. The formula used for calculation: TyG=ln [Fasting triglyceride

(mg/dl) x Fasting glucose (mg/dl)] / 2 (*Salazar et al. (2017)*.

II-1- 4-Serum Amylase:

Serum amylase was measured spectrophotometrically at 340 nm according to *Whitlow et al. (1979)*.

II-2-Oxidative stress parameters in pancreatic tissue:

Pancreatic tissue levels of malondialdehyde (MDA) and CAT were colorimetrically according to *Ohkawa et al. (1979)* and *Hugo and Lester (1984)*, respectively, using kits delivered from Bio-diagnostic Company; Dokki, Giza, Egypt.

The pancreatic tissue levels of Nrf2 and HO-1 were also estimated colori-metrically according to *Khodir et al. (2017)* and *Atef et al. (2017)* correspondingly, using the ELISA kits (Ray Biotech, CUSABIO, United States) following the manufacturer's directions.

II-3-Inflammatory biomarkers in Pancreatic tissue:

The pancreatic tissue levels of the proinflammatory NF-kB and IL-10 were assayed colori-metrically according to *Wang et al. (2011)* and *Kamel et al. (2012)*, respectively, using the commercial ELISA Kit (Ray Biotech, CUSABIO, United States) according to the manufacturer's instructions.

III- RT-PCR for PPAR-α gene expression in pancreatic tissue:

According to *Alam et al. (2019)* and *Khamis et al. (2020)*, total RNA from pancreatic tissues was extracted using Trizol reagent based on the manufacturer's guidelines (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA concentration was determined spectrophotometrically. Then, reverse transcription of RNA to complementary DNA was performed. RT-PCR was performed using SYBR Green (Qiagen).

The primers used were: PPAR-α forward, 5'-GTCCTCTGGTTGTCCCCTTG-3' and PPAR-α reverse, 5'-GTCAGTTCACAGGGAAGGCA -3' and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5′-GCATCTTCTTGTGCAGTGCC -3′ and GAPDH reverse, 5′-TACGGCCAAATCCGTTCACA-3′.

GAPDH was used as internal control to normalize the amount of target gene in the reactions where gene expression was calculated according to ΔCT technique (**Livak and Schmittgen, 2001).**

IV- Histopathological and immunohisto-chemical studies:

The pancreas was fixed in 10% formalin then 5μm paraffin sections were prepared and stained with hematoxylin and eosin. Masson–Trichrome stain to assess the presence of collagen fibers in pancreatic tissues was done (*Bancroft and Layton 2013*).

Immunohistochemistry staining was performed on paraffin slices utilizing the marked streptavidin-biotin immune peroxidase method (*Janardhan et al., 2018*). For Caspase- 3, a rabbit monoclonal antibody was used (1:1000) (EPR18297, ab184787, Waltham, MA, USA). Xylene deparaffinized, alcohols of decreasing concentration rehydrated, and phosphate buffer solution (PBS) washed the segments. The segments were also treated with H2O2 at a concentration of 3% and then rinsed with PBS so the main antibody was administered. Wash with water and incubate with the enzyme conjugate and 3, 3-diaminobenzidine tetrahydrochloride then add the biotinylated secondary antibody. Mayer's Hematoxylin was used to counterstain segments. Antiserum was replaced with PBS to provide negative controls (DAB Substrate Kit, Thermo Fischer Scientific, Rockford, IL, USA).

V- Morphometric evaluation:

The following parameters were assessed with ImageJ software, version K 1.45: The mean area % of collagen fibers in sections stained with Masson trichrome (examined at a 400 magnification). The mean area % of pancreatic sections stained with caspase-3 immunohistochemically (at X400 magnification). Ten non-overlapping fields for each parameter for each specimen (drawn from five distinct rats in each group).

STATISTICAL ANALYSIS:

Data were collected and presented using (SPSS) Statistical Package of Social Science, software version 20 (2011; SPSS Inc). Statistical methods were performed; student T-test for two group comparison, One-way analysis of variance followed by post-hoc test (least significant difference for multiple group comparison).

RESULTS

The results displayed no statistically significant difference between (using student T-test) the control groups (negative and vehicle) concerning all assessed parameters, so the negative control one was used in the statistical contrast to other treated ones.

I. Body weight (BW)

Treatment with CLZ for 4 weeks caused a significant rise in the BW gain, (P<0.01). Co-administration of LC with CLZ significantly reduced the BW gain (P<0.01; **Table 1**).

II. Biochemical studies

II-1- Pancreatic Function Tests:

II-1-1 –Fasting blood glucose (FBG) and insulin levels:

Clozapine treatment produced a significant raised in mean values of FBG (312.80±61.47) and insulin (11.85±1.34) as compared to negative control (FBG=100.96±5.76; insulin= 7.00±0.81), and LC-treated (FBG= 94.31±4.27; insulin=6.28±1.11), groups (P<0.001). LC co-treatment with CLZ relatively reversed these changes (FBG= 135.42±15.36; insulin=8.33±1.11) **(Figure 1)**.

II-1-2 Homeostatic Model Assessment of Insulin Resistance Level (HOMA-IR):

HOMA-IR mean values significant rise in rats treated with CLZ (9.30±2.78) as compared to their corresponding values in negative control (1.73±0.19), and LC-treated (1.45±0.24) groups (P<0.001). Administration of LC with CLZ produced a significant (P<0.001) diminution in HOMA-IR mean values (3.20±0.41) as compared to their values in CLZ-treated group (9.30±2.78) **(Figure 1)**.

II-1-3- Triglyceride Glucose Index Level (TyG index):

TyG index mean value significant rise in rats treated by CLZ (5.32±0.08) as compared to negative control (4.27±0.01) and LC-treated (4.22±0.02) ones (P<0.001). Administration of LC with CLZ produced a significant decline in TyG index (4.50±0.02) as compared to CLZ alone (5.32±0.08) (P<0.001) **(Figure 1)**.

II-1-4 - Amylase enzyme level:

Animals treated with CLZ led to a significant (p<0.001) rise in the serum amylase levels (972.42±65.56) as compared to negative control (213.28±1.97) and LC-treated (210.28±2.56) ones. Though, co-treatment of LC with CLZ amended serum amylase levels (264.14±34.70) **(Figure 2)**.

II-2-Oxidative stress parameters in pancreatic tissue:

Rats treated with CLZ developed a significant upsurge in MDA (10.15±1.05) as compared with control (0.48±0.001) and LC-treated (0.47±0.002) groups (P<0.001) and associated with a significant antioxidant's exhaustion (CAT=0.72±0.04, Nrf2=0.56±0.003 and HO-1=0.89±0.003) in pancreatic tissues when compared with control (CAT=6.64±0.31, Nrf2=3.63±0.41 and HO-1=3.65±0.55) and LC-treated (CAT=6.85±0.03, Nrf2=3.63±0.94 and HO-1=3.59±0.81) groups (P<0.001). Temporarily, LC significantly repressed pancreatic lipid peroxidation (1.25±0.55) and restored its antioxidant defense mechanisms (CAT=5.35±0.95, Nrf2=3.01±0.38 and HO-1=2.89 ±0.49) **(Figure 3)**.

II-3-Inflammatory biomarkers in Pancreatic tissue:

Rats treated with CLZ significantly showed an elevation in pro-inflammatory (NF-kB) cytokines (9.58±0.78) and reduction in anti-inflammatory (IL-10) (165.57±17.42) in pancreatic tissues when compared with control (NF-KB=0.77±0.003 and IL-10=926.71±53.66) and LC-treated (NF-KB=0.69±0.09 and IL-10=938.00±33.21) groups (P<0.001). On the other hand, LC intake resulted in a noticeable diminution in pancreatic NF-kB (1.12±0.05) and upsurge in IL-10 (903.00±55.03) **(Figure 4)**.

III- Toxicogenomic studies in pancreatic tissue

- RT-PCR for PPAR-α gene expression:

Animals treated by CLZ developed a highly significant lessening in PPAR-α mean value (0.14±0.017) as compared to their corresponding values in control (1.86±0.021), and LC-treated (1.66±0.08) groups (P<0.001). Administration of LC with CLZ caused a very highly significant rise in PPAR- α mean values (1.28±0.035) as compared to their values in CLZ-treated (0.14±0.017) animals (P < 0.001), a significant decline as compared with control one (1.86±0.021) (P<0.05) **(Figure 5)**.

Using the combined results from all tested rats, serum amylase correlated positively with

tissue oxidative (MDA; r=0.99; P<0.001) and inflammatory biomarkers (NF-KB; r=0.98; P<0.0001), while correlated negatively with tissue antioxidant (CAT; r = −0.923; P<0.001; NRf2; r= -0.904; P<0.001 and HO-1; r = −0.882; P<0.001) and anti-inflammatory parameters (IL-10; r = −0.978; P<0.001) and pancreatic PPAR-**α**; r = −0.905; P<0.001.

IV- Histopathological and immunohisto-chemistry results

Pancreatic sections from negative control, vehicle and LC-treated groups stained by hematoxylin and eosin displayed normal histological architectures of islets cells, pancreatic exocrine cells, and other stromal tissues. They revealed numerous large oval pale cells with a few rounded acidophilic cells surrounded by small capillaries and located in between exocrine acini's **(Figure 6a and b)**, Sections from CLZ-treated group revealed destruction of the islets of Langerhans with necrotic changes, cytoplasmolysis and pyknotic nuclei**.** Some areas in the exocrine part revealed dissociated and vacuolated acinar cells. The ducts were dilated with stratification of their lining epithelium. There were hemorrhages; congestion with thick-walled blood vessels and extravasated intense eosinophils infiltrates **(Figure 6c, d and e)**.

The combined group (LC and CLZ-treated) showed moderate improvement as maintaining the majority of pancreatic parenchyma but, the islets cells were smaller in number compared with control group with few pyknotic cells **(Figure 6f)**.

The Masson's trichrome stain displayed normal collagen distribution in control and LC-treated rats **(Figure 7a and b)**. Rats treated with CLZ showed significant collagen accumulation around the wall of blood vessels and in between acini in different studied areas **(Figure 7c)**. Co-administration of LC with CLZ resulted in lower collagen deposition **(Figure 6d)**. This was proved statistically by comparing the area% of Masson's trichrome staining among various groups, with no significant difference between control and LC groups, a significant rise in CLZ group, and a significant reduction when LC co-administered with CLZ **(Figure 6e)**.

The pancreatic sections stained against Caspase-3 immunohistochemistry of control and LC-treated groups showed non-observable stained islets cells (weak staining) **(Figure 8a, b)**. In rats administered CLZ, numerous islet cells exhibited widely dispersed nuclear and cytoplasmic staining **(Figure 8c)**. In contrast to the CLZ-treated group, co-treatment of LC with CLZ showed significantly reduced immunolabeling in islet cells **(Figure 8d)**. Statistics indicated that the CLZ-treated group had a considerably larger area percentage of immunopositive expressions of pancreatic caspase-3 than the control group did. When comparing the LC+CLZ-treated group to the CLZ-treated group, there was a significant drop in the percentage of caspase-3 area % **(Figure 8e)**.

Table (1): Initial and final body weight among different studied groups using ANOVA and post-hoc test.

Body weight	-ve Control	L-Carnitine	Clozapine	L-Carnitine+Clozapine	p-value
			$\overline{X}$± SD		
Initial body weight (gm)	182.00±3.21	183.71±4.07	180.85±3.97	182.85±3.28	>0.05
Final body weight (gm)	242.60± 5.71	245.4± 4.11	350.43±5.16[a]	260.30±4.71[a,b]	<0.001**

*N.B All values are expressed as mean±SD. (SD: standard deviation), Number of rats in each group=7 rats, -ve: negative non significant (P >0.05) , **: statistically highly significant (p<0.001),* [a] *P < .01 vs control.* [b] *P < .01 vs CLZ group.*
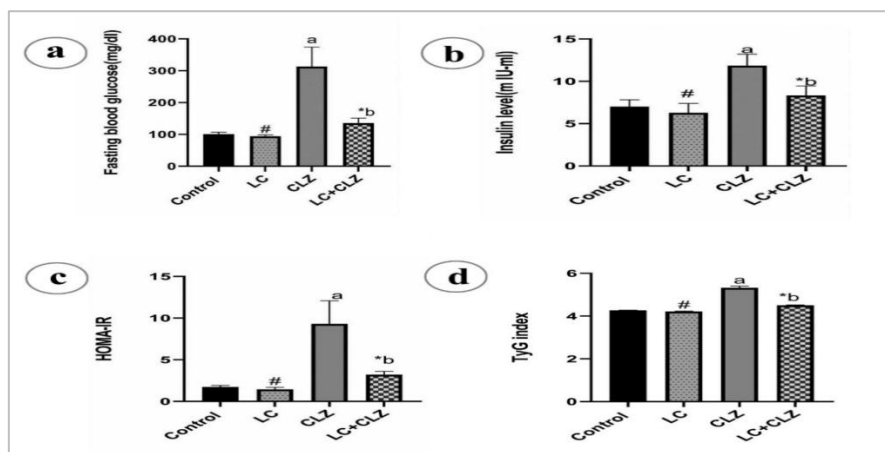
Figure (1): a) Fasting blood glucose mg/dl, b) Insulin level (mIU-ml), c) HOMA-IR, d) TYG index in different studied groups. #p>0.05, *p<0.05, a p<0.001 compared to negative control group, b p<0.001 compared to CLZ group. HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; TyG index: Triglyceride Glucose Index. LC : L-Carnitine, CLZ: Clozapine.
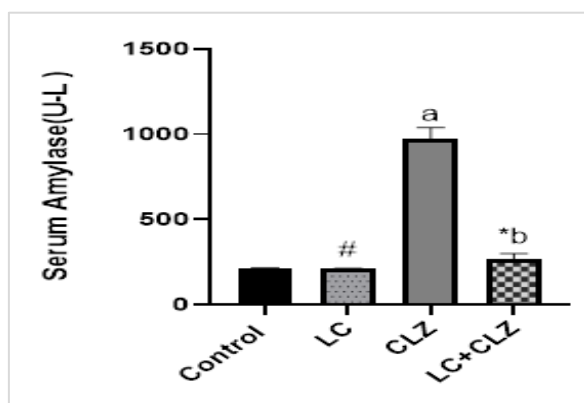

Figure (2): Serum Amylase (U-L) in different studied groups. #p>0.05, *p<0.05, ap<0.001 compared to negative control group, bp<0.001 compared to CLZ group. LC: L-Carnitine, CLZ: Clozapine.

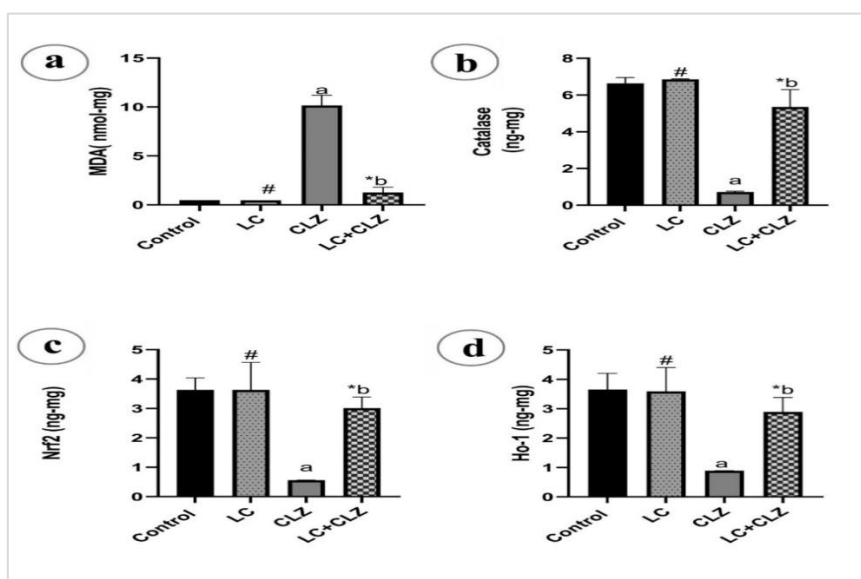*Figure (3): a) MDA (nmol-mg), b) Catalase (ng-mg), c) Nrf2 (ng-mg), d) HO-1 (ng-mg) in different studied groups. #p>0.05, *p<0.05, a p<0.001 compared to negative control group, b p<0.001 compared to CLZ group. MDA: Malondialdehyde; Nrf2: Nuclear factor erythroid 2-related factor; HO-1: Hemo oxygenase-1. LC: L-Carnitine, CLZ: Clozapine.*
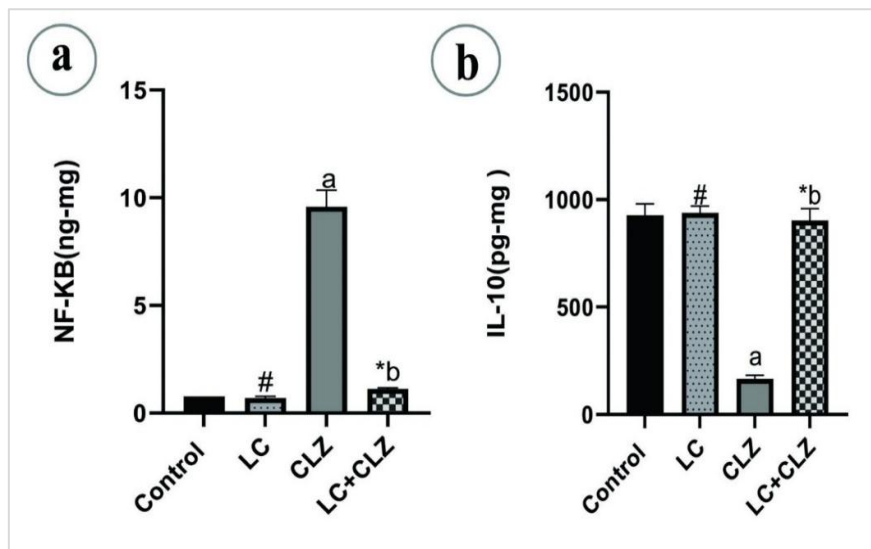
*Figure (4): a) NF- kB (ng-mg), b) IL-10 (pg-mg) in different studied groups. #p>0.05, *p<0.05, a p<0.001 compared to negative control group, b p<0.001 compared to CLZ group. NF-kB: Nuclear Factor Kappa-light-chain-enhancer of activated B cells; IL-10: Interleukin 10. LC: L-Carnitine, CLZ: Clozapine.*
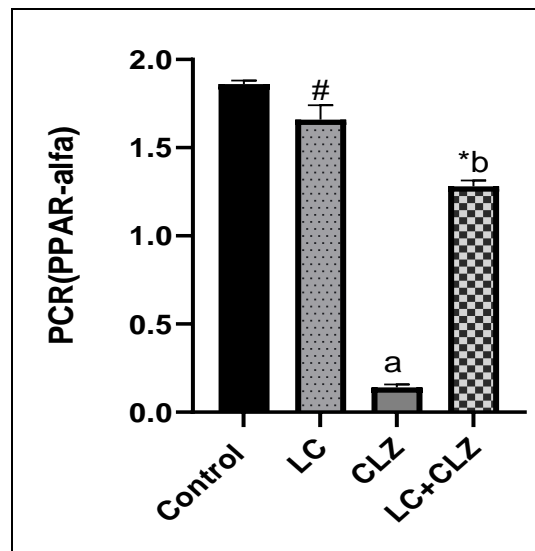

*Figure (5): PCR (Pancreatic PPAR-alfa) in different studied groups. PPAR-alfa: Peroxisome proliferator activated receptor alfa. #p>0.05, *p<0.05, a p<0.001 compared to negative control group, b p<0.001 compared to CLZ group. LC: L-Carnitine, CLZ: Clozapine.*
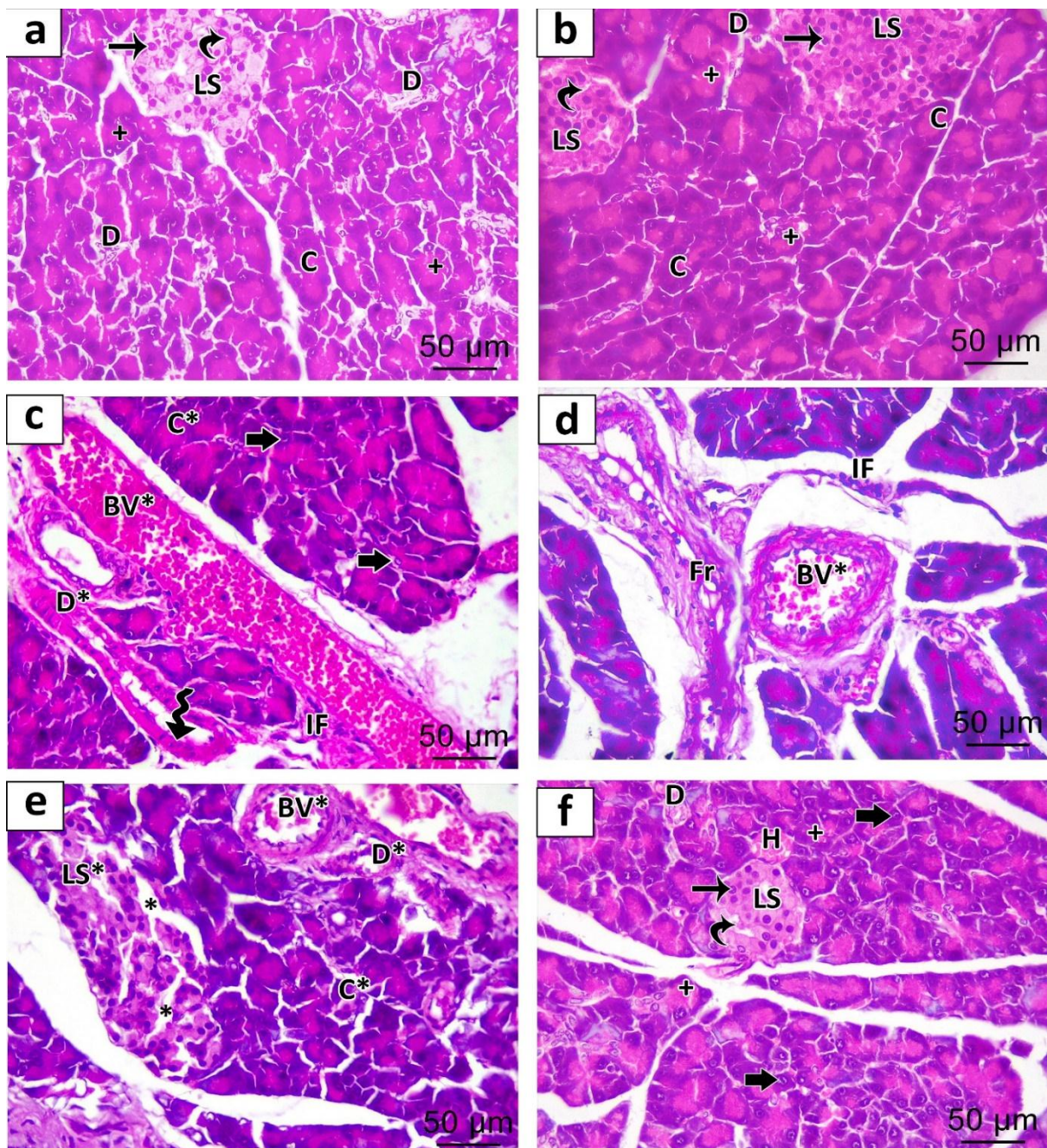
Figure (6): *Photomicrographs of the rat pancreas in the (a) control and (b)LC-treated groups showing the normal parenchymatous structure of the pancreas with exocrine and endocrine parts. The pancreatic acini (C) appear with apical acidophilic granules (+). The duct (D) shows deeply stained cytoplasm and darkly stained nuclei. The islets of Langerhans (LS) reveal β-cells (arrow) around multiple fenestrated capillaries (curved arrow). CLZ-treated group (c, d, e) presenting disorganized islets (LS*) with empty spaces between cell clusters (an asterisk). The disorganized structure of acini(c*) with vacuolated cytoplasm of its cells (thick arrow). The disorganized duct (D*) shows epithelium lining proliferation (zigzag arrow). The dilated congested blood vessel (BV*) with thickened walls, fibrosis (Fr) and infiltration (IF) also appeared. The animals administered LC with CLZ(f) showed moderate improvement; The acinar cell cytoplasm (C) appears with dark-stained nuclei and apical acidophilic granules (+). The duct (D) shows deeply stained cytoplasm and darkly stained nuclei. The islets of Langerhans (LS) reveal β-cells (arrow) around multiple fenestrated capillaries (curved arrow). Vacuolated cytoplasm of acinar cells (thick arrow) and small area of hemorrhage(H) are still noticed.*   ***Scale bar = 50 μm, X400***
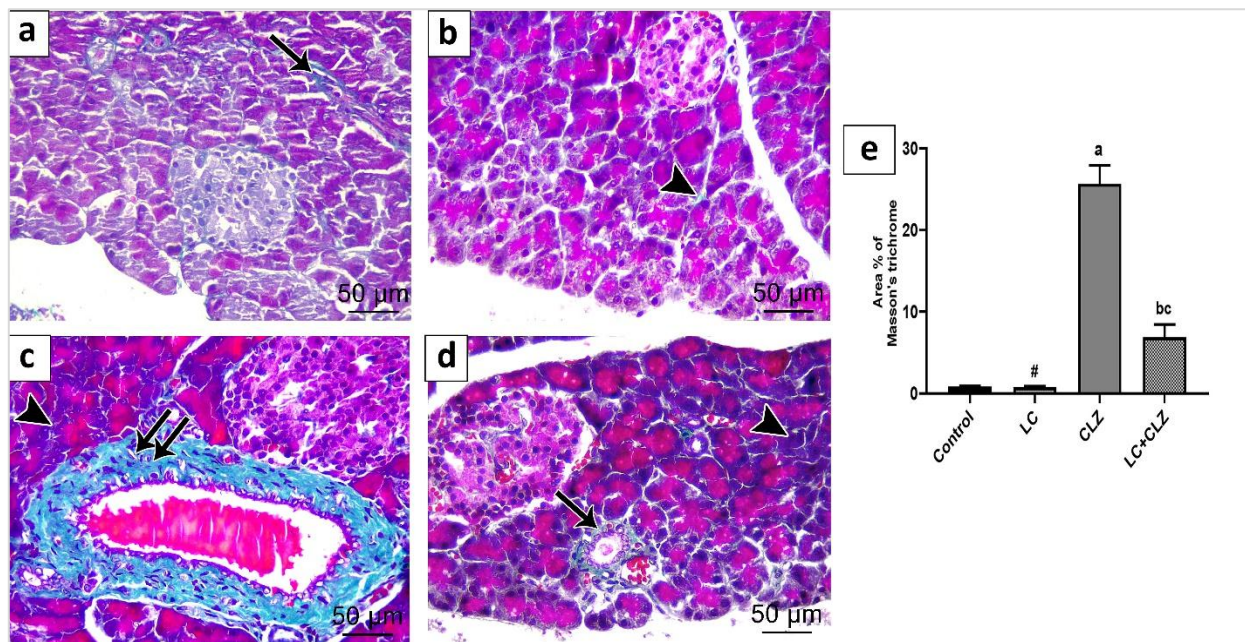
Figure (7): *Representative Images showing Masson's trichrome stained rat pancreas in the sections in different experimental groups: (a) control, (b) LC, (c) CLZ (d)LC+CLZ-treated groups. Arrows indicate marked collagen distribution around the wall of blood vessels and arrowheads in between acini in different experimental groups. (e) Intensity of pancreatic Masson's trichrome (% area). Data are expressed as means ± SD. #p>0.05, a p<0.001 compared to negative control group, b p<0.001 compared to CLZ group. LC: L-Carnitine, CLZ: Clozapine.* **Scale bar = 50 μm, X400**

Figure (8): *Representative Images showing caspase-3 immuno-positive cell expression: (a) control, (b) LC, (c) CLZ (d) LC+CLZ-treated groups. Arrowheads indicate a positive immune reaction in the cytoplasm and nuclei of different studied groups. (e) Immunostaining intensity of pancreatic caspase -3 (% area). Data are expressed as means ± SD. #p>0.05, a p<0.001 compared to negative control group, b p<0.001 compared to CLZ group. LC: L-Carnitine, CLZ: Clozapine* **Scale bar = 50 μm, X400**
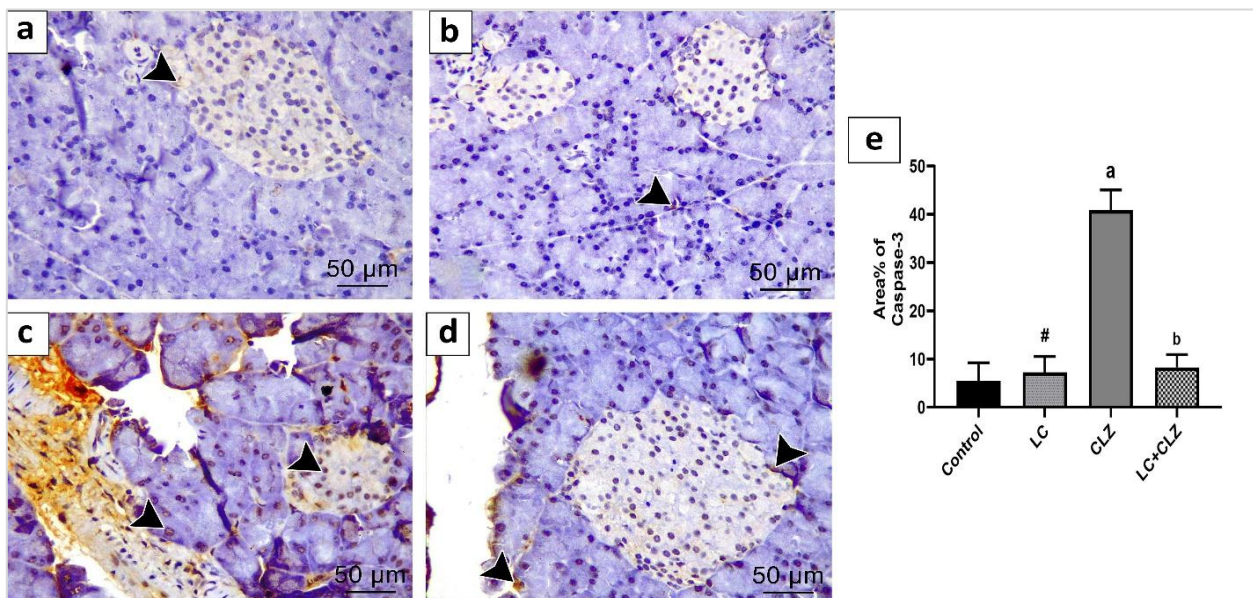
DISCUSSION

Clozapine treatment is accompanying with numerous grave side effects as agranulocytosis, dyslipidemia, metabolic syndrome, weight gain, glucose intolerance and type II diabetes mellitus *(Abdelrahim, 2013)*. LC is an innocuous and effective ancillary that can shield pancreas from numerous threats *(Sadighara et al., 2017)*. It is essential in the long-chain fatty acids transportation across the mitochondrial membrane especially in the cells that need high energy. Also, the latter has many protective properties against oxidation, inflammation and apoptosis *(Mansour et al., 2021)*.

This research aimed to examine the molecular mechanisms of pancreatic insult caused by CLZ in rats and the possible ameliorating role of LC in reducing pancreatic oxidative overload, inflammation and apoptosis induced by CLZ.

In the existing experiment, there was a significant rise in the rats' BW treated with CLZ when compared to control rats. The former results matched with *George et al. (2021)* who reported that, rats treated with CLZ developed a significant rise in rats BW compared to control. Also, *Ferreira et al. (2020)* reported that CLZ induced BW gain explained by creation of pro-inflammatory cytokines in insulin-responsive cells leading to insulin resistance. CLZ has blocking effects on multiple receptors that linked to BW gain *(Yuen et al., 2021)*.

Co-administration of LC with CLZ induced decrease in rat's body weight. This result was supported by *Wang et al. (2021)* who found that, LC helps body mass loss by increasing catabolism. Similarly, *Esmail et al. (2021)* reported that oral intake of LC results in decrease in whole-body weight of rats on high fat diet. LC enhances the structure of the entire body by normalization of food intake and decreasing visceral fat accumulation.

The pancreatic function parameters studied in this research showed increased FBG, serum insulin, HOMA-IR, TyG index and serum amylase in CLZ-treated group.

These results matched with *Chang et al. (2021)* who revealed that continuous CLZ administration in mice on high fat diet worsen hyperglycemia. In addition, *Yuen et al. (2021)* found that CLZ is associated with side effects including metabolic syndrome, diabetes mellitus type II.

Also, *Hidayah et al. (2022)* revealed that CLZ can increase fasting blood glucose. This is due insulin resistance, impairment of cholinergic-stimulated insulin excretion by blocking muscarinic (M2) receptors leading to hyperglycemia, diabetes and inadequate compensation by the beta-cells.

Similarly, *Skokou et al. (2022)* start that insulin levels were correlated positively to serum CLZ level. Hyperinsulinemia was present in 30–60% of cases.

Insulin hormone adjusts glucose levels in the blood stream and regulates storage of glucose in the hepatic, muscular and adipose tissues *(Rahman et al., 2021)*. The degree of glucose-carbohydrate consumption is regulated by the degree of pancreatic insulin secretion. Generally, the diffusion of glucose into the cells is imperfect except for hepatic and cerebral cells and could be augmented significantly by insulin *(Nakrani et al., 2021)*.

Insulin resistance is clinically defined as failure of glucose homeostasis by insulin. It has an indispensable role in the metabolic disorders development and related to an augmented cardio-vascular disease risk and diabetes mellitus type II *(Tahapary et al., 2022)*. HOMA-IR is a measure of insulin resistance and is also a good indicator of beta cell function *(Cacho et al., 2008)*. TyG index is a screening method for insulin resistance *(Tahapary et al., 2022)*.

According to *El-Seweidy et al. (2014)*, significant upsurge in FBG, serum insulin, and HOMA-IR in the rats treated with CLZ were reported as compared with the rats' control.

Milano et al. (2022) also concluded that, long-term antipsychotic led to adverse metabolic effects as dyslipidemia and hypertriglyceridemia. CLZ doubled triglyceride levels, increased cholesterol levels and endothelial dysfunction. Entirely of these fundamentals are linked to metabolic disorder and are probable causes for pancreatitis.

According to *Zhao et al. (2021)*, the serum amylase level of rats augmented by the CLZ. Pancreatitis is one of the CLZ side effects, usually manifests by increase serum amylase. Amylase, a digestive enzyme, produced by the pancreas and salivary glands and present at very small levels in other tissues *(Akinfemiwa et al., 2022)*. Its chief role is altering complex carbohydrates to simple sugars by hydrolyze the glyosidic bonds in the starch *(Azzopardi et al., 2016)*.

Lally et al. (2018), in their systematic review, reported that the commencement of the acute pancreatitis was after 4 weeks from CLZ intake.

Pancreatic function parameters impairment by CLZ were confirmed by the histopathological findings, including destruction of the islets of Langerhans with necrotic changes, some areas in the exocrine part revealed dissociated cells and vacuolated acinar cells. There were hemorrhages, congestion and inflammatory cells infiltrates. In addition, increased collagen deposition around the wall of blood vessels and in between acini were detected by using Masson's trichrome stain. These findings suggested that CLZ induced cellular toxicity.

The current results were consistent with *Abdelrahim (2013)*, who found that, the CLZ treated rats revealed relatively larger islets of Langerhans that had irregular outline comparing with the control one that revealed a regularly rounded islet. In addition, pancreatic interlobular ducts in rats treated with CLZ looked wider and had irregular outline comparing with the control one that revealed smaller regularly rounded interlobular ducts.

On co-administration of LC with CLZ showed improvement in pancreatic function parameters when compared to CLZ group, indicating the LC potential role in moderation of pancreatic toxicity. In addition, there was partial improvement of histopathological changes including collagen deposition on combined group (LC with CLZ)

The results of the present study were supported by *Masoumi-Ardakani et al. (2020)* study who showed that, LC supplementation decreased fasting blood glucose in Streptozotocin-induced diabetes in rats. Similarly, *Ezz et al. (2023)* found that, LC was effective in averting diabetes by lowering blood glucose, BW, and elevated lipids. *El-Sheikh et al. (2019)* recorded in their study that, LC was an auspicious line as supplementation to anti-diabetic drugs to moderate insulin resistance, diabetic control.

In addition, *Fathizadeh et al. (2019)* reported that, LC reduced FBG, insulin, HOMA-IR, and HbA1c levels in their meta-analysis of randomized controlled trials on humans. Besides, *Karalis et al. (2020)* strongly suggested that, LC caused significant decrease in fasting blood glucose and triglyceride after administration for three months in patients with type II diabetes.

According to *Ibrahim et al. (2019)*, treatment with LC at different doses in rats with acute pancreatitis decreased level of serum amylase due to its powerful antioxidant effects. Moreover, *Masoumi-Ardakani et al. (2020)* reported that, LC treatment at 300 mg/kg/day improved pathological changes including lost cells and necrotic changes. According to *Talpur et al. (2019)*, LC treatment in diabetic rats protected pancreatic β-cell by increasing the pancreatic islets of Langerhans size.

Several mechanisms might clarify CLZ-induced pancreatic toxicity; the study results proved that CLZ-induced oxidative stress as imitated by a significant upsurge in MDA, and a diminution in antioxidant defense status including CAT, Nrf2 and HO-1 enzymes in pancreatic tissue and that agreed with results found by *Heiser et al. (2010); Abdel-Wahab and Metwally (2014)*.

The results of current work agreed with *Bakhshii et al. (2021)* who revealed that CLZ is associated with elevated ROS, mitochondrial dysfunction, rised MDA level, GSH exhaustion in rat cardiomyocytes.

Malondialdehyde is one of the key oxidation products poly-unsaturated fatty acids. Raised MDA level is an imperative lipid peroxidation indicator and involved in ROS-induced cell damage *(Almatroodi et al., 2020)*.

Catalase localized in peroxisomes, catalyzes the decomposition of hydrogen peroxide to H_2O and O_2 thus shielding the cells from oxidative damage *(Ramesh et al., 2008)*.

Nrf2 is presented impounded as an inactive complex in the cell cytoplasm. Its linking

with repressor protein-Keap1 averts their translocation into the nucleus. Once enthused by oxidative stress, it quickly detaches from its repressor protein-Keap1 and translocate into the nucleus facilitating the cytoprotective genes transcription such as HO-1 (*Kang et al., 2015*). So, transcription of HO-1 is controlled by ROS signaling through Nrf2 activation (*Habtemariam, 2019*).

Zhang et al. (2021) in their study on Zebrafish Embryos found that, CLZ caused rise of oxidative biomarker including ROS and MDA levels, and decline the CAT and SOD activities. Oxidation and inflammation considered as an imperative role in a CLZ induced hazardous effects.

Ronaldson et al. (2015) revealed that CLZ induced cardiotoxicity is mediated through conversion of CLZ in cardiac cells to nitrenium ion (an active metabolite) that activates cellular injury and ROS and MDA production.

Also, *Pourahmad et al. (2020)* found that, CLZ caused cell death in human lymphocytes through ROS production, mitochondrial and lysosomal harm.

In the current research, co-treatment of LC and CLZ revealed a significant diminution in MDA and rise in CAT, Nrf2 and HO-1 enzyme in pancreatic tissue compared to CLZ group. The former results were reinforced by *Ezz et al. (2023)* study which showed that, LC decreased MDA enzyme activity and increased SOD and CAT activity. LC prevented the hyperglycemia induced downregulation of Nrf2

Also, *Talpur et al. (2019)* reported that LC treatment is a forceful anti-oxidative regimen capable of increasing plasma anti-oxidant levels. *Salama et al. (2021)* found that, treatment with 100 mg/kg/day LC orally reimbursed serum GSH and MDA to standard levels in rats with acute lung injuries. According to *Majidi et al. (2021)*, Co-administration of LC has augmenting the antioxidants levels (enzymatic and non-enzymatic), and dropping oxidative stress in diabetic rats.

Guerreiro et al. (2019) reported that, LC averts oxidative stress, adjusts nitric oxide level and cellular respiration. Additionally, these properties are accredited to its anti-oxidative and free radical scavenging activities. It also protects cellular DNA and membranes against destruction caused by free oxygen radicals.

Administration of CLZ in the current study showed a disturbance in pancreatic tissue inflammatory markers by a significant rise in NF-κB and decrease in IL-10 when compared to the control. *Abdel-Wahab and Metwally (2014)* observed that, rats treated with CLZ showed over expression of myocardial NF-κB p65 protein synthesis.

The results of the existing study were agreed with *de Leon et al. (2020)*, who found that, most cases of CLZ elicited inflammation during titration and could manifest serositis, pneumonitis, hepatitis, pancreatitis, myocarditis, nephritis, and skin disorders. Also, *Ratcliff et al. (2022)* reported CLZ induced pancreatitis supporting the potentially systemic nature of the CLZ triggered inflammatory response. Pancreatitis is mediated by life threatening immune sensitization mechanism.

Nuclear Factor (κB) is involved in many cellular processes as cell proliferation, apoptosis and neural development. Also, response to infection and inflammation are functions of NF-κB. Malfunctioning of the NF-κB results in chronic inflammatory conditions like tumors and neuro-degenerative disorders. The pathway regulation is highly important in treatment of these conditions (*Biswas and Bagchi, 2016*).

Inflammatory cytokines are signaling molecules that start inflammatory response. They have strong effects on numerous cells that produce inflammatory mediators, such as TNF-α, IL-1β, IL-6, IL-10, COX-2, and iNOS (*Scarpioni et al., 2016*).

The results displayed a highly significant lessening in NF-κB and upsurge in IL-10 mean values in combined (LC and CLZ) group as compared with CLZ group.

The former results were reinforced by *Li et al. (2019)* who stated that, LC repressed the activation of NF-κB by raising cytoplasmic expression of IκB-α and declining nuclear expression of NF-κB p65. Also, LC reduced DNA-binding activity of nuclear NF-κB p65 suggesting its inflammation inhibitory effect.

Additionally*, Emran et al. (2021)* found that, improved oxidative stress and inflammation via downregulating TNF-α and IL-1β in rats with myocardial infarction. *Yahyapoor et al. (2022)* reported that, LC maintains acetylated CoA to free CoA ratio within the mitochondria. LC blocked inflammation and abridged CRP, IL-6, and TNF-α.

In the existing study, the rats received CLZ developed a highly significant lessening in PPAR-α gene expression level in pancreatic tissues in comparison to control rats. The former results were consistent with *Fernø et al. (2009)*, who reported that, PPAR-α gene in rat's liver demonstrated a marked down regulation after CLZ administration.

Pancreatic peroxisome proliferator-activated receptors, a ligand-activated nuclear receptor, have a transcriptional activity and regulate multiple physiological functions and control the expression of genes involved in lipid/glucose metabolism, insulin sensitivity and mitochondrial biosynthesis. Inflammatory responses, oxidative stress, apoptosis, and cell proliferation are functions of PPAR-α *(Fan and Evans, 2015; Maciejewska-Skrendo et al., 2019)*.

Moreover, *Berawi et al. (2020)* reported that, PPAR-α gene expression limits the initial inflammatory mediators as NF-κB and cytokines, so decreased its expression leads to dyslipidemia, atherosclerosis, and diabetes mellitus.

Souza-Tavares et al. (2023) found that PPAR-α plays an essential role in glucose metabolism. *Venezia et al. (2021)* also concluded that, PPARs are vital regulators for nutrient homeostasis. PPAR signaling Modulation can impair pancreatic organogenesis during development and increase obesity.

Co-administration of LC with CLZ showed a significant upsurge in PPAR-α gene expression level in pancreas when compared to CLZ group. The former results were supported by *Li et al. (2023)* who found that, LC augmented the number of mitochondria and boosted the protein expression of mitochondrial biogenesis regulatory factors as PGC1, PPAR-α, and ERR-α.

According to *Yousefinejad et al. (2019)*, LC could cause an increase in the level of prostacyclin (PGI2) production and PPAR-α activity in the rat's tubular cells. Also, LC and genistein administration increased the hepatic PPAR-α and CPT-1 gene expression, and had synergistic effects in rats with nephrotic syndrome.

The pancreas of CLZ group in the current study showed diffusely distributed cytoplasmic labeling for caspase-3 in large number of islets cells. These results were reinforced by *Abdel-Wahab and Metwally (2014)*, who found that, sections from rats' heart treated with different doses of CLZ for 3 weeks displayed augmented positive caspase-3 immunoreactivity with the utmost effect with 25 mg/kg/d CLZ.

Caspase-3 is an apoptotic marker. It is necessary for the cleavage of many proteins and DNA fragmentation *(Slee et al., 2001)*. The presence of activated caspase-3 is a precise implement for recognizing apoptotic cells in tissue sections, even earlier all the morphological features of apoptosis happen *(Duan et al., 2003)*.

The co-treatment of LC with CLZ resulted in a weak immunolabelling response, indicating an improvement in the histopathological alterations caused by CLZ.

Abd Elkader et al. (2023) reported that, combining LC and Thiamethoxam dramatically reduced caspase-3 and glial fibrillary acidic protein (GFAP) expression in brains of male rats. The neuro-protective effects of LC were linked to its antiapoptotic and anti-inflammatory properties.

Hassan et al. (2023) stated that, LC reestablished oxidative balance in rat's kidney tissue and antagonized caspase-3 facilitated apoptotic cell death. LC administration conquers caspase-3 activity and augments total antioxidant capacity.

The current experiment proved that both oxidative and inflammatory process subsidize to CLZ caused pancreatic insult. This was verified by the positive correlation between serum amylase and tissue MDA (oxidative biomarker) and tissue NF-Kβ (inflammatory parameter) and negative correlation to antioxidant (CAT, NRf2 and HO-1), the anti-inflammatory cytokine (IL-10) and PPAR-**α**.

CONCLUSION

Clozapine has toxic pancreatic effects in rats which are characterized by elevation in pancreatic functions parameters, and alterations in the histopathological and immunohistochemical properties of the organ. The mechanism of CLZ-induced pancreatic insult occurs through the initiation of oxidative stress, inflammation, and apoptosis in rat pancreas tissue. The LC serves as moderating factor for CLZ oxidant, inflammatory, and apoptotic impacts.

RECOMMENDATIONS

Depending on the results of this study, the following guidelines are recommended: close monitoring of potential pancreatic toxicity in patients treated with CLZ is required with periodical check and investigations; clinicians should be highly alert when patients on CLZ therapy display any signs of pancreatic toxicity because CLZ-related pancreatitis may be often unrecognized as the clinical presentation is highly variable; and further studies are needed to Identify other mechanisms of CLZ-induced pancreatic toxicity and identify benefits of LC as antioxidant, and anti-inflammatory, antiapoptotic and health promotors.

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