Role of beta1-adrenergic Blockade in Alleviation of Clozapine-induced Cardiotoxic effect in rats via modulation of cardiac cell death, macrophages, and gap junction intercellular communication proteins

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Abstract

Background: Clozapine (CLZ) is an effective choice for the treatment of resistant schizophrenia; however, there is a risk of clozapine-induced cardiotoxicity. Connexin-43 (CX43) and Vimentin are intracellular structural-functional proteins involved in cardiac pathophysiology.

Objectives: The current investigation aimed to assess the cardioprotective mechanisms of beta1-adrenergic blockade by atenolol (ATN) against clozapine (CLZ) induced cardiotoxicity, focusing on the implications for gap junction and intermediate filament proteins.

Materials and methods: Thirty-two male rats were divided into four equal groups: control, clozapine-induced myocarditis (dose 25 mg/kg/day) i.p., myocarditis, and treated with oral ATN with dosage of 5 and 10 mg/kg), for 3 weeks. Biomarkers of cardiac injury, oxidative stress, inflammation (IL1β & TNFα), apoptosis, the levels of vimentin & CX43 expression & CD86 were determined via chemical, ELISA, RT-PCR, histopathological, and immunohistochemical investigations.

Results: Clozapine-treated rats exhibited increased cardiac injury biomarkers, cardiac oxidative stress indices (NO and TBARS), proinflammatory cytokines, and increased expression of caspase-3, vimentin, CX43, and CD86, along with suppression of antioxidants (GSH and GSH-Px). Atenolol showed dose-dependent suppression of the biochemical and histopathological disturbances related to clozapine-induced cardiotoxicity and significant downregulation the expression of vimentin and CX43.

Conclusion: The cardioprotective novel role of ATN in clozapine-induced myocarditis is linked to its selective β1-adrenergic blockade and its ability to repair myocardial proteins vimentin & CX43 by reversing the inflammatory response, oxidative damage, and cellular apoptosis.

Keywords: Atenolol; Cardioprotective; Clozapine; Intermediate filament proteins; Macrophage.

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Introduction

Although it's considerable efficacy in treating psychotic diseases, particularly those that are resistant to conventional antipsychotic medications, and its comparative safety, Clozapine (CLZ), as a typical antipsychotic medication, has certain side effects which restrict its clinical usage **(Liu et al., 2025)**. Earlier preclinical and clinical trials highlighted the risk of the serious and potentially fatal cardiotoxicity of clozapine which may appear involving pericarditis, myocarditis **(Markovic et al., 2011, Vickers et al., 2022)**, heart failure, and cardiomyopathy **(Yuen et al., 2018)** even in young individuals on clozapine therapy **(Ronaldson et al., 2012, Abdel-Wahab et al., 2014)**.

Studies that clarify the exact pathophysiological mechanisms of clozapine-induced cardiotoxicity are inadequate and these mechanisms are still poorly understood. Studies noted increased sympathetic activity with elevated catecholamines in the blood and urine of patients with clozapine treatment associated with perturbed sympathetic regulation **(Yuen et al., 2018, Sara et al., 2013)**. This increase in circulating norepinephrine and epinephrine was related to induced neurotransmitter spillover **(Sara et al., 2013)**. Cardiotoxicity induced by clozapine also has associated rise in cell apoptosis, oxidative and nitrosative stresses, and proinflammatory cytokines **(Abdel-Wahab and Metwally, 2015)**. Early diagnosis in addition to intervention with of β-adrenergic blockers alone or with angiotensin-converting enzyme inhibitors may attenuate the cardiotoxic effects of clozapine improve the electrocardiographic and echocardiographic indices as well as cardiac hemodynamic stabilization **(Daniel et al., 2023)** . The possible protective effect of non-selective β-blocker propranolol and its possible mechanisms of action had been explored in our earlier investigation **(Abdel-Wahab et al., 2021)** but the exact mechanisms of the possible protective role of selective β1-adrenoceptor antagonists in the alleviations of cardiotoxic effects of clozapine have not been fully elucidated yet.

Vimentin and connexin-43 are intracellular structural cytoskeletal proteins that play multiple roles in regulating tissue homeostasis. They are vital structural proteins found in different types of cells. vimentin is a type III insoluble intermediate filament protein **(Wu and Wang, 2019, Ostrowska-Podhorodecka et al., 2022)**. vimentin plays a central role in the integrity and physiology of the cell, maintaining the mechanical protection, and intercellular signal transduction. vimentin is a mainly cytoplasmic protein, however extracellular circulating form of vimentin or cell surface vimentin is also present associated with cell activation or inflammation **(Ostrowska-Podhorodecka et al., 2022)**. vimentin is also included in some inflammatory responses **(Ramos et al., 2020)** and some consequences of cell injury including apoptosis signaling **(Chen et al., 2023)** . Connexins are another group of proteins, from which CX43 is a member that plays a role in cellular communication through channels, or via gap junctions which connect cells. Moreover, Cx43 transfers several important signaling molecules **(Wu and Wang, 2019)**. They are pivotal for various physiological cellular processes that maintain cellular homeostasis and can influence pathological contexts. **(Zhang et al., 2024)**. Gap junctions have a vital role in heart function by permitting the electrical conduction and the ions exchange, that is essential for intercellular communication **(Wu and Wang, 2019)**. Therefore, we postulated that malfunction of CX43 and/or vimentin in the myocardium is linked to clozapine-induced cardiotoxicity.

Therefore, the study aimed to assess the protective role of ATN as a β1-cardioselective beta antagonist against

clozapine-induced myocarditis and cardiac apoptosis in rat model and to examine the implications of macrophage infiltration, proinflammatory cytokines, and the cytoskeletal vimentin and CX43 proteins, in the underlying process.

Materials and methods

Animals

Thirty two rats were randomly categorized into four equal groups (8 each). clozapine was supplemented to three groups of rats at 0.1 ml dose of 25 mg/kg per day intraperitoneally (i.p.) only or with ATN (5 or 10 mg/kg daily, p.o) for three weeks. The fourth group of rats (controls) received 0.9% saline. The doses of clozapine implemented to induce myocardial toxic effects and ATN were according to prior research **(Wang et al., 2008, Abdel-Wahab et al., 2021)** . After three weeks, the animals underwent anesthesia (Ketamine/Xylazine from Sigma Aldrich, USA of dose at 45 mg/kg and 5 mg/kg i.p. respectively). After cardiac puncture samples of blood were obtained, centrifuged 3000 rpm to get the serum (at temperature of 25 ˚C for 15 min) to get the serum. The animals were sacrificed (decapitation) and heart was removed, saline -washed, blotted, and divided into two halves. The right half of the dissected heart tissue was homogenized in PBS (pH 7.4). The homogeneous tissues were then centrifuged at 3000 rpm (temperature at 4 ˚C for 30 min). The supernatants were stored (-80 °C).

Materials

Clozapine and Atenolol were purchased from (Sigma-Aldrich Chemical Co., USA). clozapine was dissolved in phosphate-buffered saline (PBS) **(Abdel-Wahab et al., 2021)**. Thiobarbituric acid reactive substances, reduced glutathione (GSH), Ellman's reagent, Griss reagent & bovine serum albumin were purchased from Sigma (Sigma-Aldrich, USA).

Histopathological and immunohistochemical analysis

The ventricular part of the left half of the heart was fixed (10% neutral buffered formalin). The fixed cardiac samples were dehydrated using an ascending grade of the ethanol, cleared in xylol, and embedded in paraffin wax. The blocks were cut into sections (4–6 μm thick), stained with Harri's Hematoxylin and Eosin (H&E) for examination by light microscope. The sections were assessed for typical histopathological evidence of cardiotoxicity induced by clozapine. Other paraffin sections were incubated, stained using monoclonal primary antibodies against CD 68 macrophages, caspase-3, vimentin, and CX43 (Abcam, USA) at a 1:100 dilution. Avidin–biotin complex staining (ABC kit from Vector laboratories Inc.) was used to detect the binding of antibodies and immunoreactivity product was labeled using diaminobenzidine chromogen and combined with counter-staining with Mayer's Hematoxylin.

Biochemical assays

Measurement of serum creatine kinase CK-MB: Determined using a commercial CK assay kits supplied by Stanbio Laboratories (Texas, USA) according to the operator's instructions. The rate of NADH production, estimated at 340 nm, is directly proportional to CK-MB's actual activity **(Abdel-Wahab et al., 2021**).

Measurement of serum LDH activity Was detected by LDH assay kits (Randox Laboratories Inc). according to the manufacturer's guidelines. The reaction kinetics was measured via a spectrophotometer (340 nm).

Measurement of serum and cardiac Troponin-I (cTnI) Assessed by rat troponin I ELISA kit (Abcam co., UK) according to the guidelines manufacture. The absorbance was measured via spectrophotometer (450 nm). Their concentrations were estimated as previously reported **(Adamcova et al., 2016)**.

Measurement of lipid peroxidation in tissue homogenate: Detected according to the previous method of Ohkawa et al. **(Ohkawa et al., 1979)**.

Measurement of nitric oxide (NO) in tissue homogenate: Determined by total nitrite assay, an indicator of NO development, according to the method previously described **(Green et al., 1982)**. The nitrite concentration was determined spectrophotometrically by application of the Griss reagent.

Measurement of GSH level and GSH-Px activity: Ellman's reagent was utilized to measure the GSH content by the previous explained method **(Griffith, 1980)**. The activity of GSH-Px was evaluated according to the prior method explained **(Paglia and Valentine, 1967)**.

Cytokine determination: The cardiac tissue homogenates of IL-1β and TNFα were assessed using IL-1β and TNFα ELISA kits , R&D Systems. (MN Co., USA), as described by the manufacturer's guidelines.

Measurement of total protein: The total protein concentration in the homogenates was detected as previously mentioned **(Lowry et al., 1951)**. The absorbance was evaluated via a spectrophotometer at 750 nm.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) of vimentin and CX43 genes

The alterations in the vimentin and CX43 gene expression in the rat cardiac tissue were assessed via quantitative RT-PCR reactions, with β-actin was used as the endogenous reference control. Quantitative RT-PCR reactions were performed with Invitrogen SYBR green qPCR master mix kits and the real-time PCR machine (Eppendorf MastercyclerRealPlex2). Total RNA from the rat ventricular tissues specimens was isolated using a TRIzol extraction kit (Invitrogen, USA), was purified by an RNeasy purification kit (Qiagen, Germany) following the kit instructions, and was quantitated using a spectrophotometer at 260 nm. Reverse transcriptase-PCR was done using the specific primers sequences for vimentin, and CX43. A reaction mixture comprising of 10 mM dNTP mix and Random Hexamer solution (50 ng/ml) were mixed with the isolated RNA 1 g for each plus distilled water, afterward incubated (5 min at 65 °C) and then for 2 min (4 °C). A reaction mix has $MgCl_2$ (25 mM), 10x RT buffer, 0.1 M DTT, and RNaseOUT. The mix was incubated (25 °C for 2 min). Superscript II RT (50 units) was subsequently added, and the resulting mixture was incubated (10 min/25 °C), for 50 min at 42 °C, and for 15 min at 70 °C and subsequently cooled. RNAse was added, the mixture was incubated (20 min at 37 °C). The amplification of reverse transcribed cDNA was performed by using Thermo Scientific SYBR Green qPCR master mix (USA) and the primers sequences outlined in (**Table.1)**. The $2^{-\Delta\Delta Ct}$ equation was applied to evaluate the cycle threshold (Ct) data for target and housekeeping genes, and the expression level was normalized to β-actin.

Table 1. The primers sequences used to detect mRNA expression.

Gene	Sequence (5`– 3`)
Vimentin	F: 5′-GCACCCTGCAGTCATTCAGA-3' R: 5′-GCAAGGATTCCACTTTACGTTCA-3'
Connexin-43 (CX43)	F: 5′-ACAAGGTCCAAGCCTACTCCA-3′ R: 5′-CCCCAGGAGCAGGATTCTGA-3′
B-actin	F: 5'-AGGAGTACGATGAGTCCGGC-3' R: 5'-CGCAGCTCACTAACAGTCCG-3'

Statistical analysis

All data and results were represented as the mean ± SEM & the values obtained in various studied groups were examined via one-way analysis of variances (ANOVA), Tuky's test was used as a post-hoc multiple comparisons test when statistically significant differences were found. Statistical significance value was defined at the $p < 0.05$.

Results

Impact on serum biomarkers of cardiac injury

The levels of CK–MB in serum (**Fig. 1A**), serum LDH (**Fig. 1B**), and cTnI (**Fig. 1C**) were significantly increased in the clozapine-treated group ($p < 0.001$) compared to controls. The treatment of rats with ATN at doses of 5 and 10 mg/kg resulted in a significant reduction in the mean levels of CK–MB ($p < 0.01$) at both doses. the decrease in LDH levels was dose-dependent, with $p < 0.05$ for the 5 mg/kg dose and $p < 0.01$ for the 10 mg/kg dose. The level of cTnI was also significantly less ($p < 0.05$) in the group receiving the 10 mg/kg dose of ATN when compared to the clozapine-treated rats.

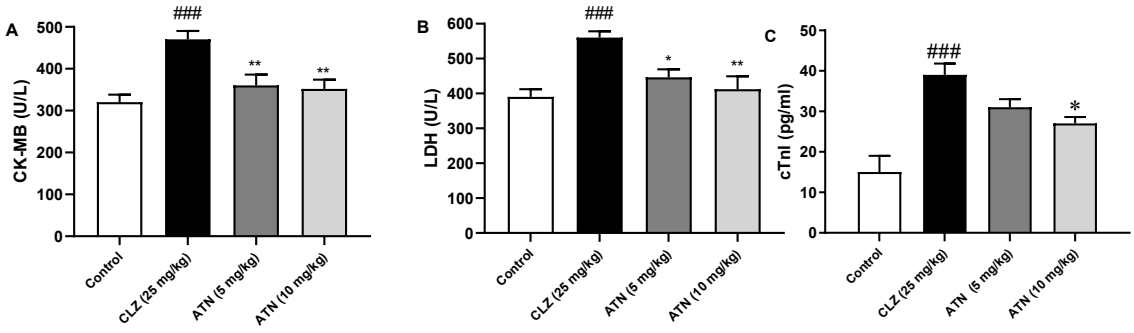

Fig. 1. Serum levels of creatine-phosphokinase (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin-I (cTnI) (A,B, and C respectively) in the four groups.

Results represented as mean ± SEM (n = 8 each); ### $p < 0.001$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. clozapine-treated group.

Impact on cardiac histopathological changes

To further examine the histopathological cellular alterations in myocardial cells following treatment with clozapine, which led to clozapine-induced cardiotoxicity, and to assess the effects of concomitant supplementation with ATN (5 or 10 mg/kg) over a period of three weeks, a histopathological analysis was conducted. The cardiac tissues from the control rats displayed a normal architecture of the cardiac muscle (**Fig. 2A**). In contrast, histological sections from the clozapine-treated group exhibited degeneration and disorganization of the myocardial tissue (**Fig. 2B**). On the other hand, animals that received both clozapine and ATN at both doses demonstrated significant improvements in all histopathological abnormalities, with the most notable improvement observed in the group treated with 10 mg/kg ATN compared to the cardiac tissue from animals treated with clozapine only (**Fig. 2C** and **2D**).

Impact on cardiac oxidative stress

As displayed in (**Table.2)** the results indicated that clozapine treatment significantly elevated the mean levels of cardiac lipid peroxidation marker TBARS and cardiac total nitrite compared to the levels estimated in control rats ($p < 0.001$ and $p < 0.01$ respectively). When compared to rats given clozapine alone, both groups treated with clozapine plus ATN showed decline in the levels of myocardial TBARS ($p < 0.05$) at both tested dose levels (5 and 10 mg/kg) of

ATN. A significant decrease was noticed in total nitrite levels in treated group with an ATN (10 mg/kg) ($p < 0.05$) when compared with clozapine-treated rats.
The GSH levels in the cardiac tissues showed significantly lower values ($p < 0.01$) in the clozapine-treated group when compared with controls. Animals treated with clozapine plus ATN at both tested doses didn't cause marked changes in the cardiac GSH levels compared with the group treated with clozapine alone.

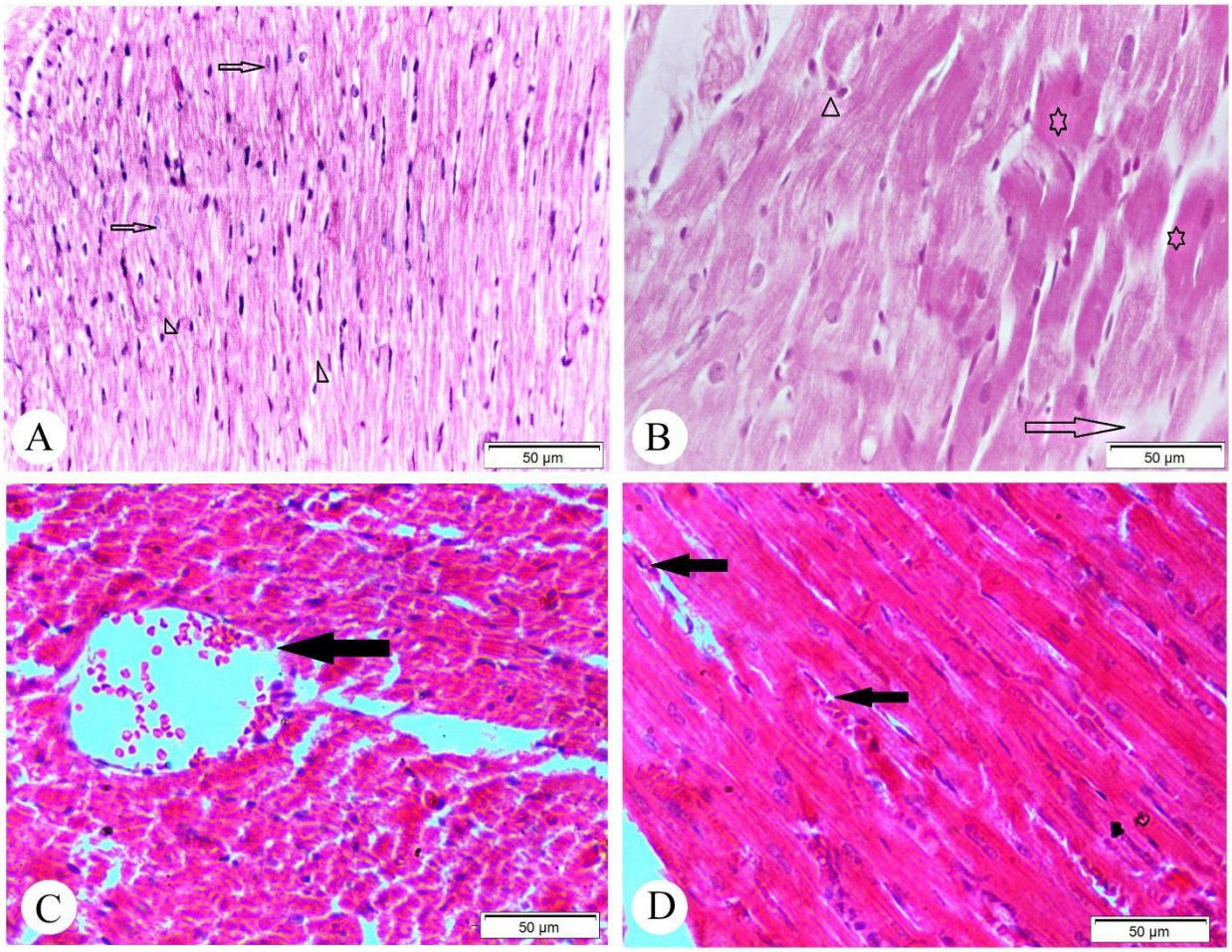

Fig. 2. The left ventricular H&E-stained sections (×400) (A) Normal myocardial architecture was observed in controls. (B) Degeneration and disorganization were detected in the myocardial tissue in animals after administration of clozapine (arrows). Less degeneration was shown in the myocardial tissue of groups treated by clozapine and β- blocker atenolol (ATN) in (C) 5 mg/kg with congestion of blood vessels (Black arrow) and (D) 10 mg/kg, some inflammatory cells were observed (Black arrow).

The activity of GSH-Px showed a significant suppression in group treated with clozapine ($p < 0.01$) compared to the controls, indicating that clozapine diminished the levels of antioxidative protection. In contrast, treatment with ATN at both doses did not show any significant impact on GSH-Px activity in comparison to the group treated with clozapine (**Table.2**).

Table 2. Effect of clozapine alone and in combination with atenolol (ATN) on myocardial thibarbituric acid reactive substances (TBARS), nitrite, reduced glutathione (GSH), and glutathione peroxidase (GSH-Px) activity in different groups.

Treatment (mg/kg/d)	TBARS (µmol/g protein)	Nitrite (µmol/g protein)	GSH (nmol/g protein)	GSH-Px (IU/g protein)
Control	327.56 ± 14.13	4.76 ± 2.16	29.54 ± 2.87	31.47 ± 2.65
clozapine	448.33 ± 13.67	16.35 ± 2.32 [b]	14.54 2.66 [b]	18.77 ± 3.14 [b]
clozapine + ATN (5)	372.46 ± 22.87	8.77 ± 2.31	16.68 ± 2.94	22.64 ± 1.56
clozapine + ATN (10)	365.16 ± 19.36	5.84 ± 2.13 [c]	18.25 ± 3.67	25.43 ± 1.47

Results are represented as mean ± SEM (n = 8). [a]$p < 0.001$ vs. control group. [b]$p < 0.01$ vs. control group. [c]$p < 0.05$ vs. clozapine-treated group.

Impact on cardiac pro-inflammatory cytokines

Measurement of the cardiac levels of the proinflammatory cytokine IL-1β showed a significant ($p < 0.001$) increase in the clozapine-treated rats compared with the levels of control rats. Rats in groups treated with clozapine and ATN demonstrated suppression in the mean level of cardiac IL-1β; with ATN in a 5 mg/kg dose ($p < 0.05$), and more with ATN in a dose of 10 mg ($p < 0.01$) relative to the level in the group that was treated with clozapine (**Fig. 3A**).

The results showed a significant ($p < 0.01$) elevation in the cardiac mean value of TNFα in the clozapine-treated rats compared to the level estimated in the controls. The rats treated with clozapine with ATN had a significant ($p < 0.01$) suppression in the cardiac mean levels of TNFα with a dose of 10 compared with TNFα mean serum levels observed in rats that was treated with clozapine only (**Fig. 3B**).

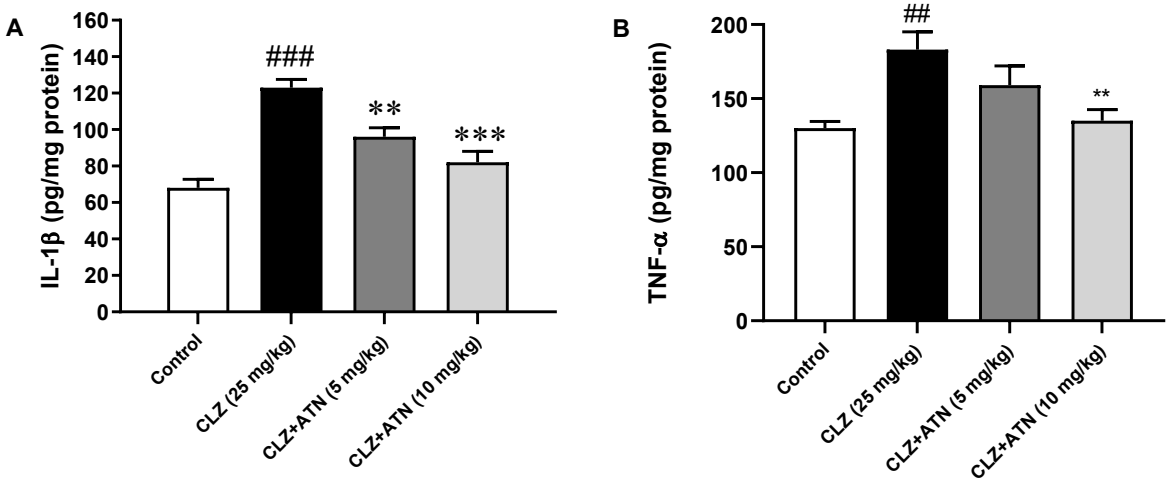

Fig. 3. Cardiac tissue level of the interleukin-1β (IL-1β) and of tumor necrosis factor alpha (TNFα) (A and B respectively) in the different groups. Results in each group represent mean ± SEM (number = 8); ### $p < 0.001$, ##$p < 0.01$ vs. control group; [***]$p < 0.001$, [**]$p < 0.01$ vs. clozapine-treated group.

Impact of ATN on clozapine-induced alterations in cardiac CD68 macrophages

In animals of the control group, a negative CD68 immunostaining was detected (**Fig. 4A**). The clozapine-treated animals showed a highly positive CD68 immunostaining between the cardiac muscle (**Fig. 4B**). The groups treated with clozapine plus ATN 5 mg/kg revealed a less localized positive CD68 immunostaining between the cardiac muscle (**Fig. 4C**). The animal groups treated with clozapine and ATN at 10 mg showed a mild positive immunostaining for CD68 between the cardiac muscle (**Fig. 4D**).

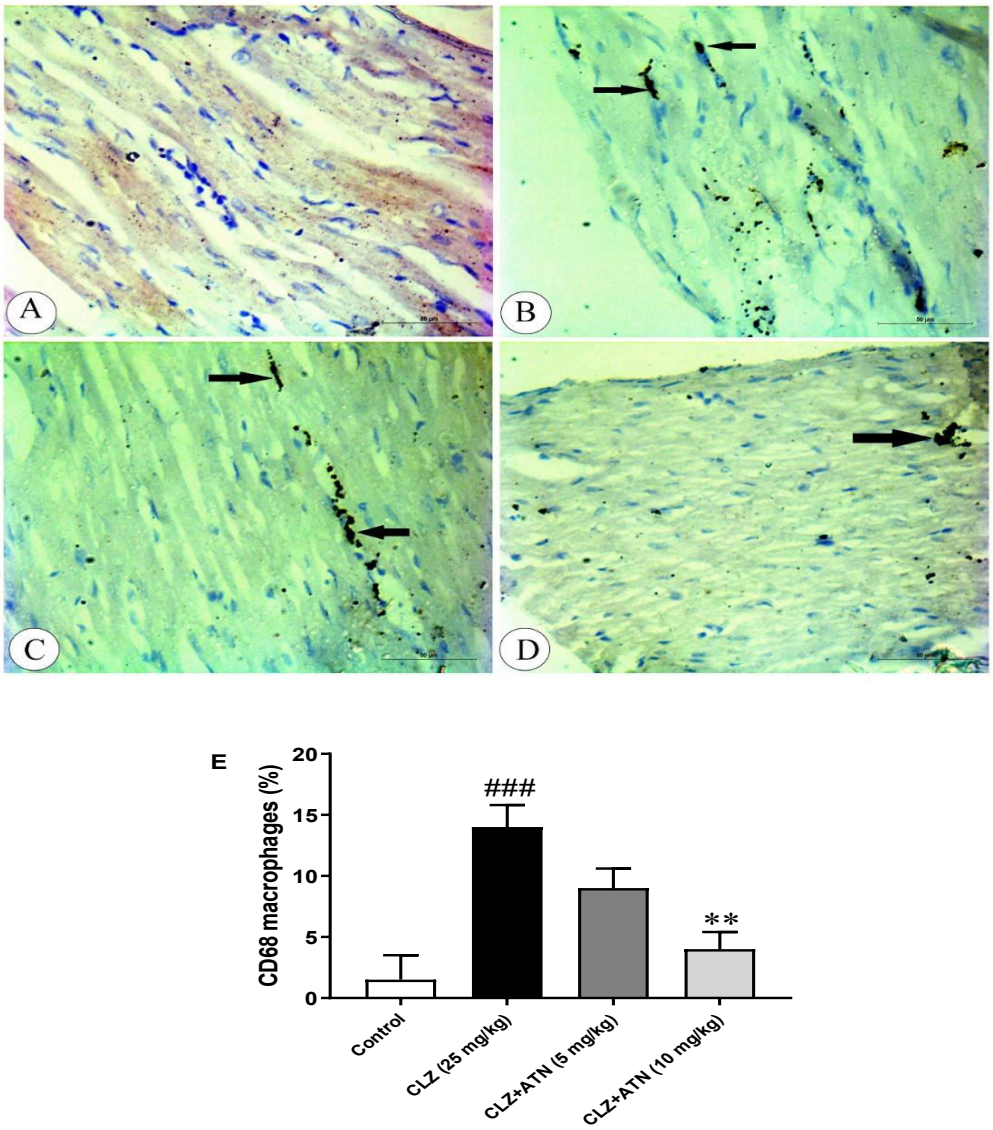

Fig. 4. CD 68 macrophage immunoreactivity in the studied groups showed in different sections of cardiac muscles (A) The control group, demonstrating negative CD68 immunostaining between the cardiac fibers. (B) The clozapine-treated group, showed a highly positive CD68 immunostaining in between the cardiac muscle (arrow). (C) Clozapine + ATN (5 mg/kg), group showing few CD68 positive cells (arrow). between the muscle fibers. (D) Clozapine + ATN (10 mg/kg) showing few CD68 positive cells between the muscle fibers (arrow). (E) extent of CD68 macrophages accumulation. Results in each group are expressed as mean ± SEM (n = 8); ### $p < 0.001$ vs. control; $^{**}p < 0.001$ vs. clozapine-treated group.

Impact on cardiac immunostaining of caspase-3

In the controls, a negative immunostaining expression of caspase-3 was observed within the sarcoplasm of the cardiac muscle (**Fig. 5A**). High caspase-3 immunopositive expression in the cardiac muscle sarcoplasm was found in the clozapine-treated (**Fig. 5B**). In contrast, treatment with clozapine and ATN at a dosage of 5 mg/kg, demonstrated moderate immunopositive expression of caspase-3 within the cardiac sarcoplasm part (**Fig. 5C**). Meanwhile, treatment with clozapine and ATN at a dosage of 10 mg/kg, displayed mild immunopositive caspase-3 expression of in the sarcoplasm (**Fig. 5D**).

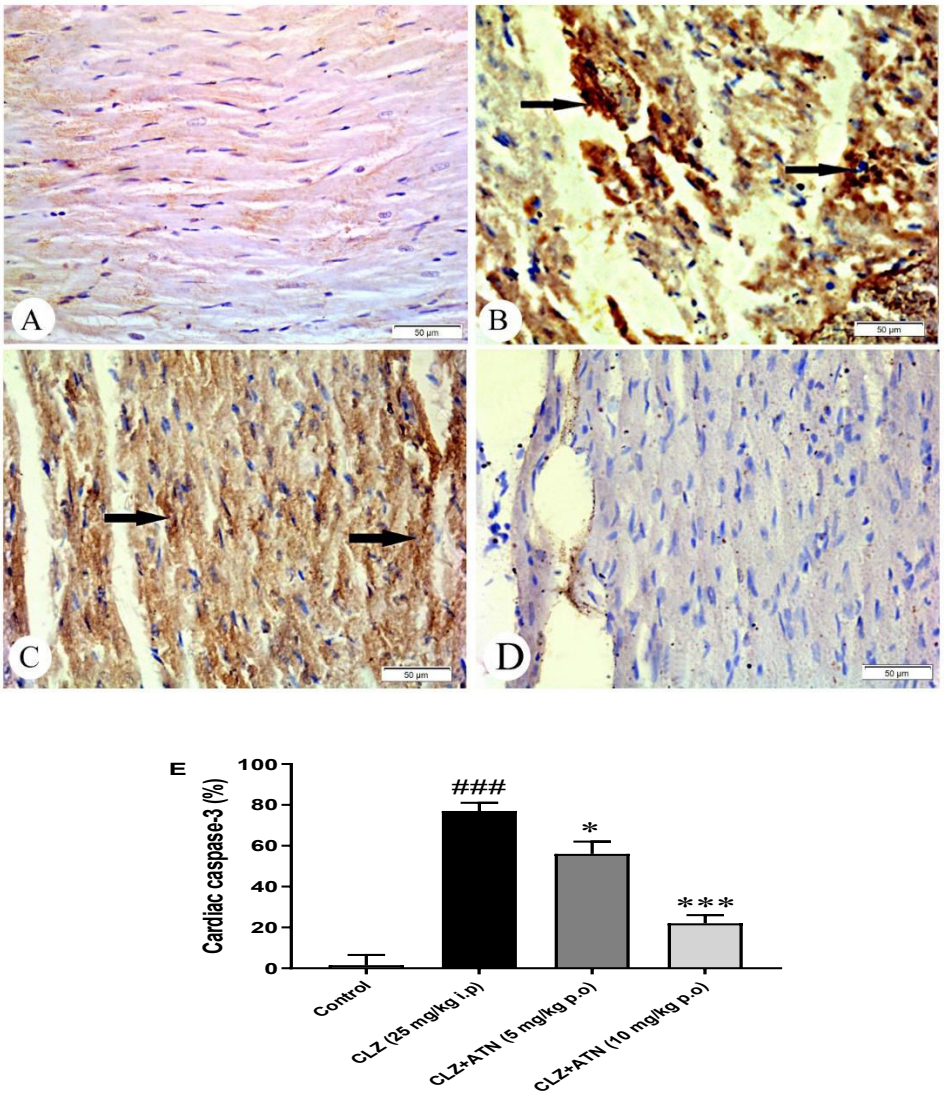

Fig 5. The immunostaining of the caspase-3 in myocardial sections. (A) A heart muscle section of controls, showing a negative immunostaining for caspase-3 in the sarcoplasm. (B). A heart muscle section of clozapine-treated, demonstrating a strong positive mmunostaining for caspase-3 i in the sarcoplasm (arrowhead). (C). A heart muscle tissue section of clozapine + ATN (5 mg/kg), demonstrating moderate positive myocardial immunostaining for caspase-3 in the sarcoplasm (arrowhead). (D). A heart muscle section of clozapine + ATN (10 mg/kg,), demonstrating mild positive caspase-3 immunostaining within the sarcoplasm (arrowhead). (E) Caspase-3 expression extent. Results are expressed as the mean ± SEM (number = 8 each); ### $p < 0.001$ vs. control; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. clozapine-treated group.

Impact on cardiac vimentin protein

Fig.6 displays the immunohistochemical analysis of cardiac sections' vimentin protein expression. Controls exhibited limited immunopositive expression of vimentin mainly in the interstitial fibroblasts, endothelial lining of blood capillaries and endomysium (**Fig. 6A**). clozapine-treated animal group showed a strong positive immunoreactivity of vimentin in the tissue fibroblast (**Fig. 6B**). Rats administered clozapine and ATN (dose 5 mg/kg) had moderately positive immunoreactivity for vimentin in the fibroblast section (**Fig. 6C**). Additionally, rats given clozapine and ATN (dose 10 mg/kg), exhibited mild positive immunoreactivity for vimentin in the cardiac fibroblast (**Fig. 6D**).

The findings of RT-PCR mRNA levels of expression are consistent with the detected immunohistochemistry data. The cardiac vimentin gene expression elevated in the clozapine-treated in comparison to the controls ($p < 0.001$). Supplementation of ATN at a dose of 5 significantly decreased cardiac vimentin expression ($p < 0.05$) whereas ATN supplementation at a 10 mg/kg dose was more potent in decreasing vimentin expression ($p < 0.001$) when compared to vimentin mRNA expression levels in clozapine-treated animals (**Fig. 6F**).

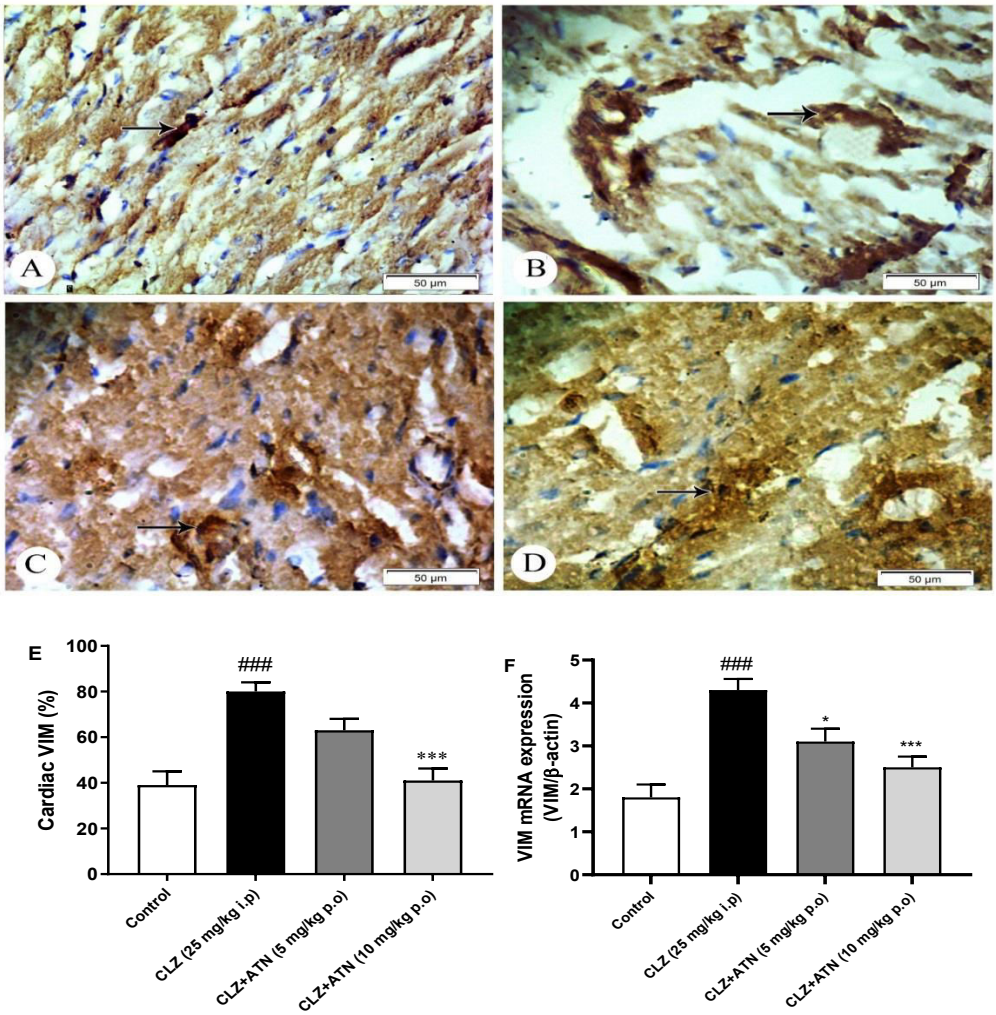

Fig. 6. Myocardial vimentin immunostaining in cardiac muscle sections. (A) The control group revealed limited immunoreactivity of protein vimentin in the endothelial cells and endomysium (arrowhead). (B). A clozapine-treated, revealing a highly positive

immunoreactivity in the cardiac fibroblast (arrowhead). (C). Animal group treated with clozapine + ATN (5 mg/kg), revealing moderate positive vimentin immunoreactivity within the fibroblast (arrowhead). (D). section from clozapine + ATN (10 mg/kg) treatment, revealing mild positive vimentin immunoreactivity in the cardiac fibroblast (arrowhead). (E) Vimentin expression extent (F) levels of mRNA transcript of vimentin in all studied groups. Results in each group expressed as mean ± SEM (number = 8); ### $p < 0.001$ vs. control group; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. clozapine-treated group.

Impact on cardiac CX43

Controls displayed a normal CX43 protein distribution at the intercalated disc between muscle fibers (**Fig. 7A**). Based on the results, the clozapine-treated group's CX43 protein expression was considerably increased in myocardial fibers that have disordered distribution (**Fig. 7B**). In the group supplemented with clozapine plus ATN 5 mg/kg, a less focal rise in CX43 protein expression within the myocardial tissue fibers was noticed in comparison with clozapine-treated group (**Fig. 7C**). In addition, the group supplemented with clozapine and ATN of 10 showed a strong suppression in the expression extent of Cx43 protein in comparison to clozapine-treated (**Fig. 7D).**

The findings of RT-PCR mRNA expression displayed a significant higher expression levels in the heart CX43 gene in the clozapine-treated rats relative to controls ($p < 0.001$). ATN resulted in a significant decrease in the cardiac CX43 expression at a dose of 5 ($p < 0.05$) and a marked ($p < 0.001$) decline in CX43 expression at a dose of 10 (**Fig. 7F**).

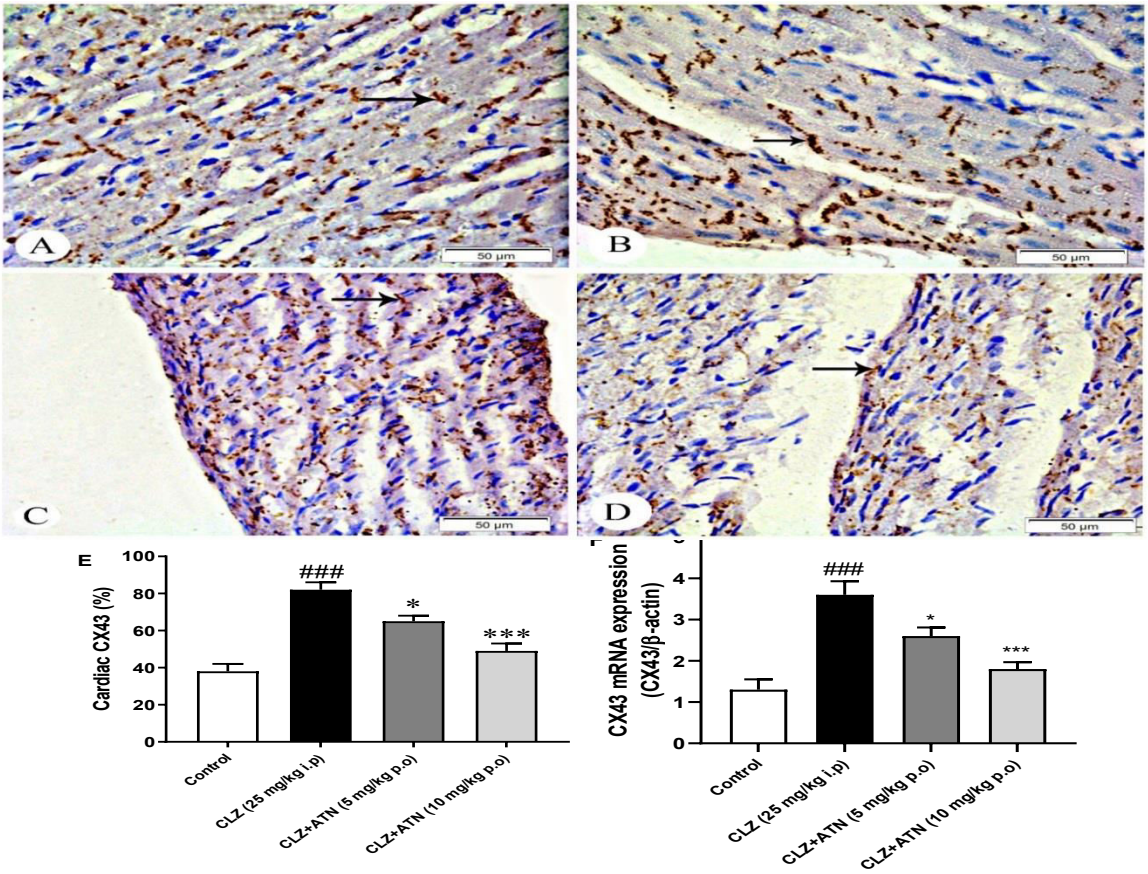

Fig. 7. Connexin-43 (Cx43) immunostaining section in the heart. (A) A section in the heart muscle of the controls, exhibiting the normal distribution of the Cx43 at the intercalated discs (see arrowhead). (B) A section in the heart of the group treated with clozapine, exhibiting a

rise in the Cx43 expression level and disordered distribution of it (arrowhead). (C) A section in the heart muscle of clozapine + ATN (5) exhibiting a focal rise in Cx43 expression level in-between the cardiac muscle fibers (arrowhead). (D) A section in the heart muscle of clozapine + ATN (10), exhibiting a decline in the expression level of Cx43 compared to the group on clozapine (arrowhead). (E) Cx43 expression extent (F) mRNA transcript levels of Cx43 in all studied groups. Results in each group are expressed as mean value ± SEM (n = 8); ### $p < 0.001$ vs. controls; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. clozapine-treated group.

Discussion

Clozapine-induced cardiotoxic effects is a serious health concern and the can result in disability among psychotic disease patients on clozapine therapy. The typical manifestations were persistent systolic heart failure, which could cause comorbidities, arrhythmias, and abrupt death **(Vickers et al., 2022)**. Hence, identifying the underlying process is essential to prevent the development of adverse myocardial remodeling, avoid endomyocardial biopsy, improve the quality of life, and reduce mortality. The present study demonstrated that the clozapine group showed significant cardiomyocyte injury evidenced by a considerable rise in the serum values of CK-MB, cTnI, and LDH compared to controls. The rise in the blood levels of those biomarkers is regarded as a reliable and significant signal of either acute or late myocardium damage **(Omran et al., 2022)**. The toxic impact of clozapine on cardiac tissue was supported by the histological alteration in the cardiac tissue sections (H&E-stained) that demonstrated degeneration and disorganization of the myocardial tissue. Similar histopathological alterations were reported in prior researches **(Abdel-Wahab and Metwally, 2014a, Abdel-Wahab et al., 2021)**.

The pathophysiologic mechanisms by which clozapine causes cardiotoxicity are still not clear enough. Earlier studies showed that myocarditis after clozapine is frequently associated with a hypercatecholaminergic state, that correlates with the extent of the myocardial tachycardia and inflammation caused by the drug **(Wang et al., 2008, Higgins et al., 2019)**.

The persistent tachycardia observed after clozapine treatment can lead to left ventricular dysfunction that results in cardiomyopathies **(Ronaldson, 2016)**. β-adrenergic receptors blocking medications have been demonstrated to alleviate and reverse cardiomyopathy produced by clozapine **(Wang et al., 2008, Ronaldson, 2016, Abdel-Wahab et al., 2021)**. These earlier results are consistent with the findings of the current investigation, which found that rats treated with ATN and clozapine showed a significant decline in the levels of cardiac biomarkers as well as the histopathological signs of myocardial damage brought on by this drug. The protective role of ATN can be linked to its capability to block the impacts of increased secretion of catecholamines induced by CLZ, which cause tachycardia by activating the myocardial β1-adrenergic receptors which is the predominant adrenergic receptor in cardiac function and raises myocardial tissue oxygen consumption by enhancing cardiac work **(Braunwald, 2000, Ronaldson, 2016, Higgins et al., 2019)**.

The hypercatecholaminergic state enhanced by clozapine can result in myocardial tissue ischemia and the production of some reactive oxygen species molecules (ROS) that attacks cellular vital components, elevates lipid peroxidation, and impairs the myocardial cellular membrane integrity **(Su et al., 2019, Mohammadi et al., 2021)**. Higher lipid peroxidation by clozapine was proved in our findings as indicated by elevated myocardial TBARS levels. These findings

match with those of previous research **(Abdel-Wahab and Metwally, 2014b)**. Moreover, there was a significant higher level in the cardiac tissue nitrite levels, a stable byproduct and biomarker of NO, in the clozapine-group. This agrees with the former studies **(Abdel-Wahab and Metwally, 2014b, El Shehaby et al., 2020)**. It has been shown that clozapine increases inducible NO synthase (iNOS) production levels. The released NO from cardiac tissue cells can increase lipid peroxidation, and enhanced generation of ROS in addition to reactive nitrogen species molecules (RNS) **(Nair et al., 2019b)**.

Alternatively, clozapine impairs the physiological form of NO synthase, and limits the release of the NO that induces vasodilation. This effect contributes to vasoconstriction and triggering cardiovascular diseases **(Nair et al., 2019a)**. Additionally, the buildup of ROS and RNS is linked to pathogenic cytotoxic changes, leading to the inactivation of the endogenous antioxidant enzyme defenses. These consequences were observed in our findings in clozapine-treated rats in the form of a decline in cardiac tissue GSH levels and decreased GSH-Px activity. The elevated oxidative stress and the consequent inhibition of antioxidants are significant changes that provoke a series of interactions leading to cardiac disorders after clozapine treatment **(D'Oria et al., 2020)**.

N-oxidation of clozapine resulted in increased production of chemically unstable reactive molecules of nitrogen species that enhances myocardial cell damage by disturbing mitochondria structural and functional integrity **(Arzuk et al., 2021)**. The chemically reactive molecules also interact with several proteins in cardiac tissues, causing direct toxic effects that activate an immune response. This response attracts inflammatory infiltrates to cardiac myocytes, initiating myocarditis **(Bellissima et al., 2024)**.

Clozapine therapy has been associated with immunomodulation along with elevated proinflammatory cytokines. This indicates the role the systemic inflammatory response in the pathophysiology of myocarditis **(Leung et al., 2023)**. The present results demonstrated increased the macrophages buildup and IL-1β and tumor necrosis factor mean values in the cardiac tissues of the clozapine-treated animals. Additionally, increased oxidative stress damage may reduce the of anti-inflammatory cytokines (IL-8 and IL-10) production **(Jia et al., 2019)**, and hence increasing inflammation in heart tissues.

The present study showed that co-supplementation of ATN with clozapine lowered the elevation in lipid peroxidation and oxidative stress parameters in the heart tissues of rats treated with ATN compared to clozapine treatment. Previous studies showed that ATN has no direct antioxidant properties **(Tamuli et al., 2015, Sorriento et al., 2018)**, hence the decrease in lipid peroxidation in animals treated with clozapine plus ATN may be related to an indirect effect of ATN through blocking cardiac β1-adrenergic receptors, decreasing oxygen demand, myocardial ischemia and ROS production **(Braunwald, 2000, Higgins et al., 2019)**.

Furthermore, groups treated by ATN had a significant decline in the heart tissue level of nitrite relative to clozapine alone-treated rats. Evidence suggests the interaction of sympathethoadrenal and renin-angiotensin systems and oxidative/nitrative stress pathways, these factors could potentiate each other and be implicated in numerous cardiovascular diseases **(Zucker et al., 2004)**.

Renal sympathetic activation via the β1-adrenergic receptor mediates the release of renin and controls the levels of angiotensin-II levels. Angiotensin-II had been shown to boost NO release in the

cardiac cells and stimulate the production of superoxide radical **(Miller and Arnold, 2019)**. Theses superoxide radical rapidly combines with NO, leading to protein oxidation, lipid peroxidation, cellular injury, and inflammation which are associated with clozapine-induced cardiotoxicity **(D'Oria et al., 2020)**.

The hemodynamic effects of reducing B1-adrenergic activation that decrease myocardial workload caused by salt and water retention and as well as vasoconstriction, by blocking the sympathetic-driven release of renin **(Klapholz, 2009)**. These consequences provide protection to the heart against ischemia and subsequently lessen ROS as well as RNS generation stimulated by clozapine-induced catecholamine overactivation.

The current study reported that the cardioprotective impact of blocking beta 1 receptor by ATN against clozapine cardiotoxic effects with insignificant changes in the levels of cardiac GSH and cardiac GSH-Px, supporting the previous study that showed the absence of significant effects for ATN on endogenous GSH and antioxidant enzymes **(Chen et al., 1995)**. Besides, results showed a considerable decline in the cardiac IL-1β and tumor necrosis factor levels, macrophage buildup, especially with the high ATN dose. The capability of ATN to reduce the cardiac levels of the proinflammatory TNFα and IL-1β could be partly due to the capability of ATN to counteract the excessive sympathetic impact on the heart caused by clozapine-induced hypercatecholaminergic state.

The increased generation of ROS and RNS provoked by clozapine and their deposition in the cardiac tissues stimulate the mitochondrial apoptotic cellular signaling pathway, activate some cellular apoptotic proteins, and stimulate caspase-3 causing cellular apoptosis **(Wu et al., 2016, Redza-Dutordoir and Averill-Bates, 2016, Elmorshdy Elsaeed Mohammed Elmorshdy et al., 2023)**. The immunohistochemical analysis found a considerable rise in the positive stained cardiac tissue and the expression patterns of the caspase-3 in the group treated with CLZ, confirming clozapine-induced cardiac cell death. On the other hand, animals treated with ATN exhibited a significant decline in caspase-3 positively stained cells and a reduction in caspase-3 levels of expression relative to the clozapine-group. These results displayed the anti-apoptotic impact of ATN against clozapine-enhanced cardiomyocyte apoptosis.

The results indicated that clozapine-treated rats displayed significant elevation in both mRNA and protein expression levels of both CX43 and vimentin. These results reflect alterations in the gap junction that could affect ionic conductance across membranes of cardiomyocytes leading to arrhythmia seen in patients receiving clozapine **(Dhein and Salameh, 2021)**. A redox imbalance damage cellular molecules and structural cell proteins, including vimentin and CX43. This damage can result in the alteration and weakening of gap junctions and intermediate filaments (IF) **(Sarrouilhe et al., 2017)**. Furthermore, raised levels of pro-inflammatory cytokines due to clozapine promote the expression of caspase-3 **(Jang et al., 2021)**. The rise in mRNA expression of vimentin and CX43 that was seen in the clozapine-treated group may signify a compensatory mechanism to stimulate cellular proliferation and replacement to repair the damaged proteins **(Battaglia et al., 2018, Greer et al., 2017)**.

Also, the rise in protein expression levels of CX43 may be related to an enhanced ROS and RNS production, which may lead to protein changes that stimulate CX43 gene expression **(Moldogazieva et al., 2018)**.

This study showed that ATN significantly reduced the elevation in

vimentin, Cx43 mRNA gene, and protein expression induced by clozapine treatment with more observed effects in the large-dose group (10 mg/kg). We suggest that this reduction might be due to its β1-adrenergic antagonistic effect that counteracts the effect of clozapine-induced rise of catecholamines. This effect helps decrease cardiac ischemia and redox imbalance, ultimately reducing the cytotoxic effects on vimentin and CX43 proteins. Additionally, the reduction in ROS and RNS production diminishes capsase-3 generation, which in turn decrease its damaging impact on vimentin protein. The regulation of these proteins may in part contributes to the potential benefits of β1-adrenergic blockade therapy. Further studies are needed to test the regulatory effects of β1-adrenergic signaling on these cardiac proteins and to explore the potential mechanisms through which β1-blockade might facilitate their restoration.

Conclusion

This study is the first to demonstrate that selective β1-adrenergic receptor blockade by ATN, has a protective role in alleviating clozapine-induced myocardial toxic effects in rat model. This protective effect occurred via a considerable reduction in clozapine-induced rise in lipid peroxidation and NO levels, macrophage accumulation, pro-inflammatory cytokines (TNFα and IL-1β) levels, and the apoptotic biomarker (caspase-3). This effect is associated with a decline in the mRNA and levels of protein expression of the cardiac proteins vimentin and CX43 which are increased by clozapine. The cardioprotective effects against clozapine-induced apoptosis, oxidative stress & the inflammatory response is related to its selective β1-adrenergic blocking effects. Additionally, it helps preserve vimentin and CX43, crucial cardiac proteins vital for the normal functional and structural properties of the cardiomyocyte. Therefore, the B1 adrenergic receptor may be a target to attenuate clozapine-induced cardiotoxic adverse effects which might have therapeutic benefits among patients with psychiatric disorders. Further investigations are needed to test the effectiveness of selective β1 antagonists as a treatment for other types of cardiotoxicities.

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