Contents lists available at ScienceDirect

Toxicology Reports

Clozapine-induced cardiotoxicity in rats: Involvement of tumour necrosis factor alpha, NF-κβ and caspase-3

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A B S T R A C T

Clozapine, an ideal antipsychotic drug for the treatment of resistant schizophrenia, is considered the most underutilised treatment for schizophrenia. However, safety concerns have been raised about clozapine-induced cardiotoxicity, which may lead to sudden death, particularly in young patients. The exact mechanism of clozapine cardiotoxicity has not yet been thoroughly studied. This study aimed to investigate the possible mechanisms of clozapine-induced cardiotoxicity in a rat model. Young male Wistar rats were treated with clozapine (10, 15 and 25 mg/kg/day, i.p.) for 21 days. Haemodynamic and echocardiographic studies were performed for assessment of cardiac functions. Heart sections were studied histopathologically and immunohistochemically. Serum and cardiac markers of cardiotoxicity, oxidative stress, inflammation and apoptosis were evaluated. Heart sections of CLZ-treated animals showed increased cardiac inflammation that correlated with the clozapine dose. Serum levels of CK-MB and LDH levels increased, as did cardiac levels of TNF-α, MDA, NO, myeloperoxidase (MPO), 8-OHdG, caspase-3 and NF-κB p65. In contrast, GSH levels and GSH-Px activity decreased. Furthermore, immunohistochemical examination of the heart sections showed positive immunostaining for both 3-nitrotyrosine and caspase-3 in all clozapine-treated groups. Clozapine, particularly in relatively high doses, has a clear cardiotoxic effect. This cardiotoxicity is accompanied by increased myocardial oxidative stress, inflammatory cytokines, DNA damage and apoptosis with attenuation in antioxidant defences, thus explaining the previously reported myocarditis and pericarditis during clozapine therapy in clinical studies.

1. Introduction

Clozapine, a tricyclic dibenzodiazepine, is an atypical antipsychotic drug that is very efficacious in treating psychosis, particularly in patients refractory to other agents [1]. It has a strong antagonistic activity on D4-dopaminergic receptors [2] serotonergic, noradrenergic [3], histamine [4] and cholinergic M2 receptors [5]. It differs from traditional antipsychotic drugs in that it has relatively weak D2-receptor activity and few extrapyramidal side effects, and it is effective in treating resistant schizophrenia [6].

Clozapine appears to be particularly beneficial in patients with schizophrenia who are suicidal and those with substance use disorder [7]. However, some adverse effects of clozapine have limited its clinical use [8]. A

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common and serious adverse effect requiring regular monitoring is cardiotoxicity [7]. Several cases showing clozapine-induced myocarditis (including deaths) have been reported internationally, 85% of which developed in the first 2 months of therapy [8]. Most of the patients in the reported cases were under 50 years of age. Clinical studies showed potentially fatal myocarditis, pericarditis, heart failure and eventually death associated with clozapine treatment [9].

The mechanism of clozapine-induced cardiotoxicity is not yet clearly understood. Previous studies showed the presence of cardiac and peripheral blood eosinophilia associated with clozapine cardiotoxicity, indicating a possible IgE-mediated hypersensitivity reaction [10]. In addition, clozapine treatment has been associated with increased levels of the catecholamines, norepinephrine and epinephrine [11]. Hyper-catecholaminergic states can significantly exacerbate myocarditis in both animals and patients [11,12]. Moreover, clozapine-induced myocarditis has been associated with an increased release of inflammatory cytokines [13].

Numerous reports have shown an increase in the level of reactive oxygen species (ROS) in the myocardium during the development of myocarditis and heart failure in experimental animals and in patients [14]. Myocardial ischaemia can lead to cell injury with the release of ROS [15]. Cell injury in the ischaemic area also causes infiltration of neutrophils, which produce ROS and cytokines. Certain cytokines, such as tumour necrosis factor-α (TNF-α), trigger mitochondrial release of ROS [16]. In addition, an increase in ROS has been detected in various animal models of heart failure [17,18]. An increase in oxidative stress, which may result from increased production of ROS, a relative deficit in the endogenous antioxidant defences, or both, can cause myocarditis, contractile dysfunction and cardiomyopathy [17].

Therefore, this study aimed to investigate the possible mechanisms of clozapine-induced cardiotoxicity and the role of oxidative stress and proinflammatory cytokines in that process. This study also investigated whether clozapine-induced cardiotoxicity is associated with cardiac DNA damage and possible apoptosis in a rat model.

2. Materials and methods

2.1. Chemicals

Clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo [b,e] [1,4] diazepine) (Sigma–Aldrich, Bayouni Trading Co. Ltd., Al-Khobar, Saudi Arabia) was dissolved in 0.1 M HCl and pH-balanced in phosphate-buffered saline (PBS) (Sigma–Aldrich, Bayouni Trading Co. Ltd., Al-Khobar, Saudi Arabia). This solution was administered intraperitoneally (i.p.) daily in 0.1-ml doses. All other chemicals used in this study were of analytical grade.

2.2. Animals

The animals used in this study were young male Wistar rats, 3–4 weeks of age and 120–150 g in body weight, from the animal facility of King Saud University, Riyadh,

Saudi Arabia. Animals were housed in groups of 10 rats in standard clear polycarbonate cages, with food and water available ad libitum. Animals were kept on a 12-h light–dark schedule (6:00 am–6:00 pm), and all experimental testing was conducted during the light phase, between 9:00 am and 12:00 pm. All experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). The Institutional Animal Use and Care Committee approved the experimental protocol. All efforts were made to minimise animal suffering and to reduce the number of animals used.

2.3. Experimental protocol

The animals were randomly divided into four groups. Clozapine was administered in doses of 10 ($n = 10$), 15 ($n = 10$) and 25 ($n = 13$) mg/kg/day i.p. for 21 days in three groups. The fourth group ($n = 10$), the control group, was treated with saline. The moderate to high doses of clozapine were based on previous reports [12]. The animal's body weight (BW) was measured before and after the study period. At the end of the study period (21 days), rats were anaesthetised with 2% halothane in O_2 and subjected to echocardiographic study followed by haemodynamic measurements. At the end of haemodynamic measurements, blood samples were drawn by cardiac puncture. Hearts were excised, washed with ice-cold saline, blotted with a piece of filter paper, and weighed immediately (HW), and the ratio to BW (HW/BW) was calculated. Hearts were then divided midventricularly into two halves, with one half immediately snap-frozen in liquid nitrogen for subsequent biochemical assays. Ventricles of the second half were used for histological and immunohistochemical studies.

2.4. Cardiac function assessments

Left ventricular (LV) function analysis was performed via echocardiography and haemodynamic measurement. Two-dimensional echocardiographic studies were performed under 0.5% halothane anaesthesia using an echocardiographic machine equipped with a 7.5-MHz transducer (SSD-5500; Aloka, Tokyo, Japan). M-mode tracings were recorded from the epicardial surface of the right ventricle; the short-axis view of the left ventricle was recorded to measure the LV dimension in diastole (LVDd) and LV dimension in systole (LVDs). LV fractional shortening (FS) and ejection fraction (EF) were calculated and expressed as percentages.

After the instrumentation, the concentration of halothane was reduced to 0.5% to record steady-state haemodynamic data. Haemodynamic parameters such as the mean blood pressure (MBP), peak LV pressure (LVP), LV end-diastolic pressure (LVEDP), and the rate of intraventricular pressure were recorded as previously described [19].

The study was performed in a blinded manner.

2.5. Histopathological studies

Slices from ventricles of each heart were fixed in a 10% neutral formalin solution, then embedded in

paraffin, sectioned at a thickness of 5 μm and stained with haematoxylin and eosin (H/E), and examined by light microscopy. The ventricle specimens were evaluated for typical histopathological features associated with clozapine-induced cardiotoxicity (including inflammation, myocyte vacuolar degradation, necrosis of myofibers, and interstitial fibrosis).

2.6. Biochemical studies

Heart tissue was homogenised (Biohom homogeniser) in 20-mM phosphate buffer (pH 7.4) containing 0.5 mM butylated hydroxytoluene to prevent sample oxidation. The homogenates were centrifuged at 3000 rpm at 4 °C for 15 min. Serum and the supernatant of the homogenate were used for biochemical assays.

2.6.1. Determination of CK-MB and LDH activities

Creatinine kinase (CK-MB) activity was estimated in serum according to the method of Bishop et al. [20] using diagnostic kit (Stanbio Laboratory, TX, USA). The increase in absorbance at 340 nm is measured spectrophotometrically to calculate CK-MB level as (U/L). LDH activity was determined using diagnostic kit provided from Biogamma (Rome, Italy). The increase in absorbance is measured spectrophotometrically at 340 nm at 1 min intervals for 3 min. Serum total LDH activity was calculated as (U/L) according to the method of Whitaker [21].

2.6.2. Determination of TNF-α

TNF-α in the cardiac homogenate was assayed using enzyme-linked immunosorbent assay (ELISA) using a microplate reader (Spectra III Classic, Tecan, Salzburg, Austria) as previously described [22].

2.6.3. Determination of lipid peroxidation

Lipid peroxidation was determined in the cardiac homogenates because thiobarbituric acid reactive species (TBARS; referred to as malondialdehyde, MDA) are considered markers of oxidative stress. The colour intensity is measured spectrophotometrically at 532 nm. Concentration of TBARS was calculated for each sample after reference to the standard curve.

2.6.4. Determination of myocardial total nitrite/nitrate

Nitrate and nitrite are assayed calorimetrically as indicators of NO in the tissue because the half-life of NO is too short and it is proportionately converted into nitrite and nitrate. Then the total nitrite is then measured by Griess reaction, according to the method described by Green et al. [23].

2.6.5. Determination of reduced glutathione

Reduced glutathione (GSH) was determined according to the method described before by Beutler et al. [24]. The procedure is based on the reduction of 2-nitrobenzoic acid by glutathione to produce a yellow compound which was measured spectrophotometrically at 405 nm.

2.6.6. Determination of glutathione peroxidase

Glutathione peroxidase (GSH-Px) activity was determined spectrophotometrically by the method of [25].

2.6.7. Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured as an index of neutrophil accumulation. Tissue MPO activity was assessed using a commercial assay kit (Hycult Biotech Inc., Burlington, CA).

2.6.8. Determination of 8-hydroxy-2-deoxyguanosine

8-OHdG is produced by the oxidative damage of DNA by reactive oxygen and nitrogen species and serves as an established marker of oxidative stress. Cayman's 8-hydroxy-2′-deoxyguanosine assay kit purchased from Cayman's Chemical Co. (USA) was used. It is a competitive assay that can be used for the quantification of 8-OHdG in serum and tissue homogenate. It recognises both free 8-OHdG and DNA-incorporated 8-OHdG. This assay depends on the competition between 8-OHdG and 8-OHdG-acetylcholinesterase (AChE) conjugate (8-OHdGTracer) for a limited amount of 8-OHdG monoclonal antibody. All procedures were carried out in accordance with the manufacturer's instructions.

2.6.9. Determination of total protein

Total protein concentration was also determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemicals, Texas, USA).

2.7. Western blotting for NF-κB p65 cardiac protein expression

Briefly, 50 μg from each sample homogenate was denatured by boiling for 5 min in 2% SDS and 5% 2-mercaptoethanol and loaded into separate lanes of a 12% SDS-PAGE gel. The samples were separated electrophoretically at 100 V for 2 h. The separated proteins were electrically transferred onto PVDF membranes using a T-77 ECL semi-dry transfer unit (Bioscience, Washington, USA) for 2 h. The membrane was blocked in TBS buffer containing 0.05% Tween and 5% non-fat milk for one hour. The membranes were then incubated with either mouse monoclonal anti-NF-κB p65 or mouse monoclonal anti-actin (Santa Cruz Biotechnology, Inc.). Polyclonal goat anti-mouse immunoglobulin conjugated to alkaline phosphatase (Sigma–Aldrich, Chicago, USA) diluted 1:5000 in the 10×-diluted blocking buffer served as secondary antibody. Protein bands were detected by adding alkaline phosphatase buffer (100 mM Tris pH 9.5; 100 mM NaCl; 5 mM MgCl$_2$) containing the substrate, 6.6 μl NBT/ml and 3.3 μl BCIP/ml (from stock of 50 mg/ml nitro blue tetrazolium (NBT) and 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 70% formamide). Colour reactions were stopped by rinsing with stop buffer (10 mM Tris–Cl, pH 6.0, 5 mM EDTA). Relative intensities of protein bands were analysed by scanner and quantified by AIDA Image Analyzer software.

Table 1
Changes in heart weight (HW), body weight (BW) and percent mortality of rats after treatment with clozapine in doses of 10, 15, 25 mg/kg/d for 21 days.

Treatment (mg/kg)	n	BW (g)	HW (g)	HW/BW $\times 10^{-3}$	% Mortality
Saline	10	133.6 ± 2.2	1.43 ± 0.06	6.12 ± 0.30	0.0
Clozapine (10)	10	137.4 ± 3.4	1.56 ± 0.04	6.75 ± 0.25	0.0
Clozapine (15)	10	140.4 ± 2.1[a]	1.63 ± 0.07 [a]	6.78 ± 0.11[a]	0.0
Clozapine (25)	13	143.7 ± 2.8[a]	1.83 ± 0.09[b]	7.50 ± 0.16[b]	23.07

Results represent mean ± SEM.
  [a] $p < 0.05$ vs. saline-treated rats.
  [b] $p < 0.01$ vs. saline-treated rats.

2.8. Immunohistochemistry

In brief, the sections were de-paraffinised in xylene and rehydrated through graded alcohols, then boiled in 0.01 M citrate buffer (pH 6.0) for 10 min. Hydrogen peroxide (0.3%) was added to block any endogenous peroxidase activity. To block nonspecific binding, the sections were incubated with a goat-serum blocking solution composed of 10% normal goat serum in phosphate-buffered saline, pH 7.4 and 0.05% sodium azide. The sections were incubated with anti-caspase-3 (at 1:100 dilution) and anti-3-nitrotyrosine (at 1:400 dilution) antibodies, respectively, used at 4 °C overnight. Polydetector secondary antibody was used to avoid contaminating endogenous biotin or streptavidin (Bio SB, Santa Barbara, CA). After washing, the antigen–antibody complex was applied and stained with diaminobenzidine (Bio SB). Counterstaining was performed lightly with haematoxylin. None-immune serum was used instead of the first antibody as a negative control. All the control slides yielded negative results. One pathologist, who was unaware of the fate of the tissue site [26], performed the evaluation of the immunostained slides.

2.9. Statistical analysis

InStat version 2.0 (GraphPad Prism 5, ISI Software, Philadelphia, PA, USA, 1993) was used to compute statistical data. All experimental results are expressed as the mean ± SEM. Comparisons between experimental and control groups were performed by one-way analysis of variance (ANOVA) followed by Bonferroni's test for post hoc comparison when appropriate. A value of $p < 0.05$ was considered significant.

3. Results

3.1. General toxicity

The general appearance and BW of animals were recorded during the time course of the study and HW at the end of the study. In the control group and groups treated with clozapine dose 10 mg/kg there was no significant changes in BW and HW. The BW, HW and the HW/BW ratio were significantly increased during the experiment in groups treated with clozapine at doses of 15 and 25 mg/kg compared with the control values (Table 1).

3.2. Effect of clozapine on myocardial functions

Results of changes in haemodynamic and echocardiographic functional parameters are shown in Table 2. Treatment of animals with clozapine in the tested doses for 21 days resulted in left ventricular remodelling and systolic dysfunction in these animals. These changes appeared as increases in LVEDP and LVDS and decreases in LVP, FS and EF. These effects were significant in moderate to large doses (15 and 25 mg/kg) of clozapine.

3.3. Clozapine induced cardiomyopathy

Histopathological studies of cardiac sections of both control and clozapine-treated animals showed evidence of myocarditis and myocardial cellular infiltration in cardiac sections of clozapine-treated rats compared to control rats. These changes took the form of focal subendocardial fibrosis with marked interstitial oedema and perinuclear vacuolation. Myocarditis increased with increasing clozapine doses, with the highest incidence induced by treatment at 25 mg/kg. Inflammatory lesions were found in both the left and right ventricles, primarily in the myocardium below the endocardium of the left ventricle, in the posterior papillary muscle of the left ventricle and in the septum, consistent with myocarditis (Fig. 1A–D).

3.4. Effect of clozapine on cardiac biochemical parameters

3.4.1. Effect on serum CK-MB, LDH levels

Results from measurement of serum CK-MB and LDH showed significant changes in their levels among the tested groups [$F(3,39) = 7.059$, $p = 0.0007$] and [$F(3,39) = 6.517$, $p = 0.0012$], respectively. Serum CK-MB significantly increased with the 15 mg/kg dose ($p < 0.05$) and with the 25 mg/kg dose ($p < 0.01$) compared with control (Fig. 2A). In addition, the serum LDH level significantly increased ($p < 0.05$) with the 10-mg/kg dose and ($p < 0.01$) with the 15 and 25 mg/kg doses of clozapine (Fig. 2B).

3.4.2. Effect on cardiac MPO activity and THF-α level

Cardiac levels of TNF-α changed significantly after treatment with clozapine [$F(3,39) = 6.511$, $p = 0.0012$]. Clozapine treatment significantly increased TNF-α level ($p < 0.05$) at the 15 mg/kg/d dose and ($p < 0.01$) at the 25 mg/kg dose when compared to control (Fig. 3A). Cardiac MPO activity measurement showed increases in its concentration in clozapine-treated animals at the significance level of

Table 2
Changes in haemodynamic and echocardiographic functional parameters after 21 days of treatment with clozapine in doses of 10, 15, 25 mg/kg/d in rats.

Functional parameters	Control	Clozapine (10)	Clozapine (15)	Clozapine (25)
HR (beat/min)	355 ± 8.3	361 ± 7.6	377 ± 6.6[a]	386 ± 6.4[b]
MBP (mmHg)	94 ± 8.2	96 ± 5.3	93 ± 6.4	98 ± 6.5
LVP (mmHg)	112 ± 4.0	109 ± 3.3	104 ± 3.4[a]	94 ± 3.7[b]
LVEDP (mmHg)	4.52 ± 2.5	6.74 ± 2.4	11.93 ± 2.3[a]	14.76 ± 3.1[a]
LVDd (mm)	6.8 ± 1.4	7.3 ± 1.6	7.8 ± 1.5	8.4 ± 1.9
LVDs (mm)	3.5 ± 0.5	5.5 ± 0.7[a]	6.2 ± 0.9[a]	7.5 ± 1.2[b]
FS (%)	38.5 ± 2.3	28.4 ± 3.3[a]	26.6 ± 2.6[b]	19.4 ± 2.8[c]
EF (%)	72.4 ± 5.5	62.4 ± 7.3	55.6 ± 6.5[a]	38.8 ± 7.3[b]

Results are presented as the mean ± SEM ($n = 10$). HR, heart rate; MBP, mean blood pressure; LVP, left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; LVDd, left ventricular dimension in diastole; LVDs, left ventricular dimension in systole; FS, fractional shortening; EF, ejection fraction.

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$ vs control.

$p < 0.01$ with doses of 10 and 15 mg/kg and at $p < 0.001$ with the dose of 25 mg/kg/d (Fig. 3B).

3.4.3. Effect on cardiac oxidative parameters

Results obtained from the effects of clozapine on cardiac levels of MDA, NO, GSH and GSH-Px activity are shown in Table 3. Clozapine treatment significantly affected myocardial lipid peroxidation and cardiac levels of MDA [$F(3,39) = 7.158, p = 0.0007$]. Post hoc analysis indicated that clozapine treatment significantly increased cardiac MDA levels at doses of 15 mg/kg ($p < 0.05$) and 25 mg/kg ($p < 0.01$) relative to control. In addition, regarding myocardial NO level, there was a significant difference between treated groups [$F(3,39) = 7.374, p = 0.0006$]. Clozapine treatment significantly increased cardiac NO levels at doses of 15 mg/kg ($p < 0.05$) and 25 mg/kg ($p < 0.01$) relative to controls.

Moreover, clozapine treatment decreased the myocardial GSH level [$F(3,39) = 3.512, p = 0.0248$], which was significant relative to controls for the 25-mg/kg dose.
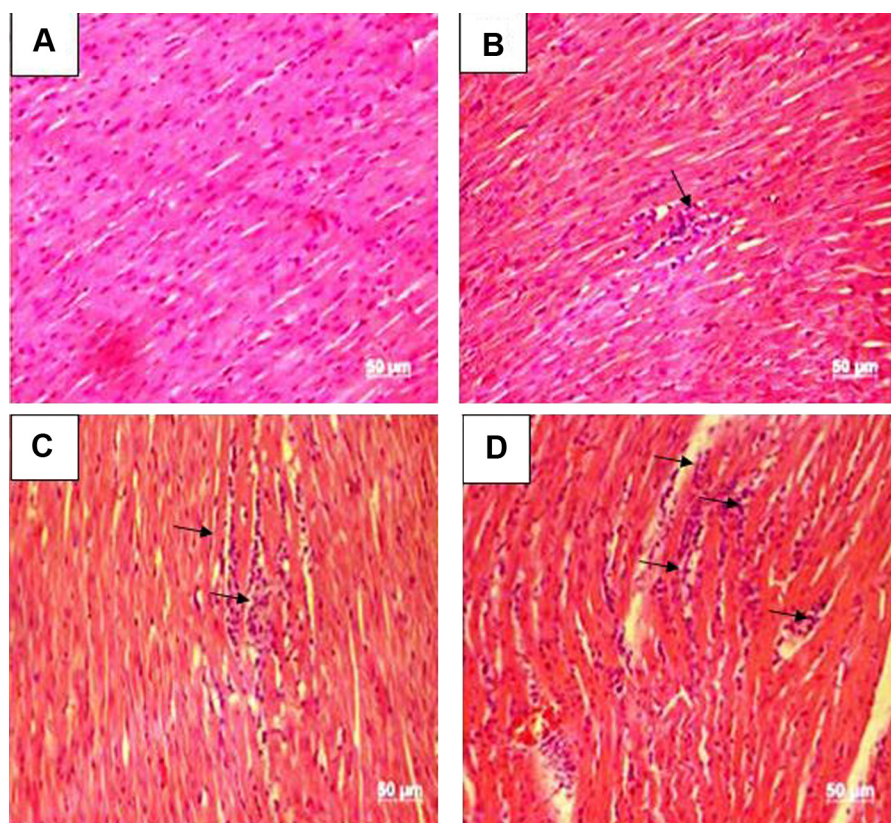
Fig. 1. Representative H&E-stained sections (40×) of the left ventricle from saline- and clozapine-treated rats. (A) There is no myocardial inflammation after administration of saline for 21 days. (B) There are inflammatory lesions in the myocardium after administration of 10-mg/kg/d clozapine for 21 days. (C) There are larger inflammatory lesions in the myocardium after administration of 15-mg/kg/d clozapine for 21 days. (D) There are extensive inflammatory lesions in the myocardium after administration of 25 mg/kg/d clozapine for 21 days.

Table 3

Effect of clozapine in doses of 10, 15 and 25 mg/kg/day for 21 days on myocardial malondialdehyde (MDA), total nitrate/nitrite, intracellular reduced glutathione (GSH) levels and glutathione peroxidase (GSH-Px) activity in rats.

Treatment (mg/kg/d)	MDA (μmol/g protein)	Total nitrate/nitrite (μmol/g protein)	GSH (nmol/g protein)	GSH-Px (IU/g protein)
Control	315.62 ± 15.34	3.33 ± 1.14	33.46 ± 3.52	25.45 ± 3.24
Clozapine (10)	332.26 ± 17.25	3.64 ± 2.38	30.45 ± 3.33	18.34 ± 2.48
Clozapine (15)	387.63 ± 13.26[a]	10.34 ± 1.23[a]	23.46 ± 3.25	14.67 ± 2.66[a]
Clozapine (25)	412.52 ± 21.32[b]	11.24 ± 1.12[b]	19.85 ± 3.23[b]	12.45 ± 2.13[b]

Results in each group represent mean ± SEM ($n = 10$).

[a] $p < 0.05$ vs. control rats.

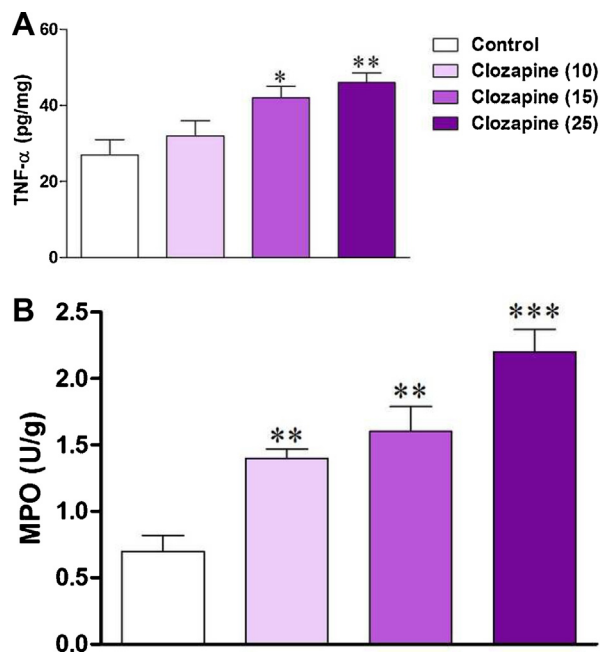[b] $p < 0.01$ vs. control rats.
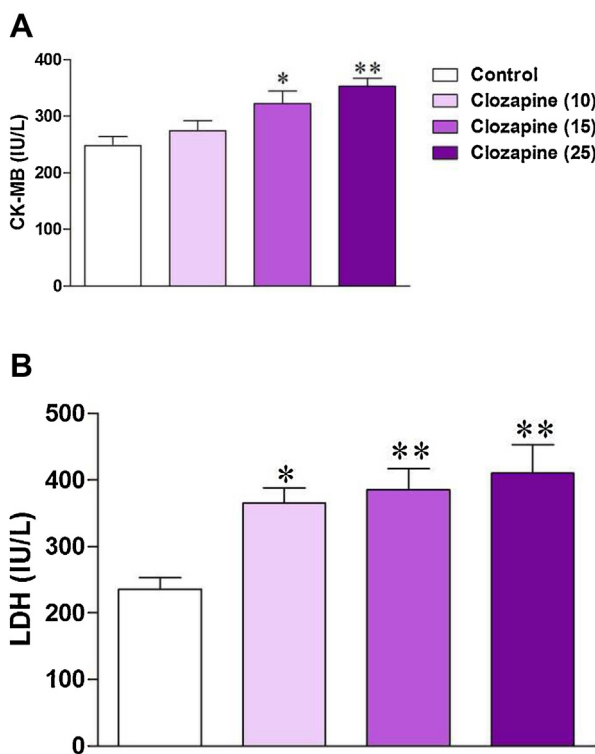
Fig. 2. Effect of clozapine treatment in doses of 10, 15 and 25 mg/kg/d, i.p., for 21 days on serum levels of (A) creatinine phosphokinase isoenzyme (CK-MB) and (B) lactate dehydrogenase (LDH) in rats. Results in each group represent mean ± SEM ($n = 10$). $^*p < 0.05$ vs. control. $^{**}p < 0.01$ vs. control.


Fig. 3. Effect of clozapine treatment in doses of 10, 15 and 25 mg/kg/d, i.p., for 21 days on (A) cardiac levels of tumour necrosis factor alpha (TNF-α) and (B) cardiac myeloperoxidase (MPO) activity in rats. Results in each group represent mean ± SEM ($n = 10$). $^*p < 0.05$ vs. control. $^{**}p < 0.01$ vs. control. $^{***}p < 0.001$ vs. control.

Furthermore, clozapine treatment attenuated the GSH-Px activity [$F(3,39) = 4.586$, $p = 0.0081$], which was significant relative to controls at significance level $p < 0.05$ for the dose of 15 mg/kg and $p < 0.01$ for the dose 25 mg/kg.

3.4.4. Effect on serum and cardiac 8-OHdG levels

8-Hydroxy-2′-deoxyguanosine (8-OHdG) is a product of oxidatively damaged DNA and is formed by hydroxy radicals and singlet oxygen. Measurement of 8-OHdG levels revealed significant changes among clozapine-treated groups [$F(3,39) = 8.850$, $p = 0.0002$] and [$F(3,39) = 6.512$, $p = 0.0012$] in serum and cardiac tissues, respectively. After 21 days of clozapine treatment, the serum 8-OHdG levels significantly increased ($p < 0.05$) with the dose of

15 mg/kg and more significantly increased ($p < 0.01$) with the dose of 25 mg/kg (Fig. 4A). In the hearts, 8-OHdG levels significantly increased ($p < 0.05$) with the dose 10 mg/kg and more significantly ($p < 0.01$) increased with the doses 15 and 25 mg/kg compared to control levels (Fig. 4B).

3.5. Effect of CLZ on cardiac NF-κB level

We used Western blotting to estimate the level of NF-κB p65 protein that was synthesised by heart cells in response to clozapine treatment. Clozapine-treated rats exhibited over-expression of NF-κB p65 protein synthesised by the heart. This increase was significant at the levels of $p < 0.05$ with 10 mg/kg, $p < 0.01$ with 15 mg/kg and $p < 0.001$ with 25 mg/kg of clozapine (Fig. 5).
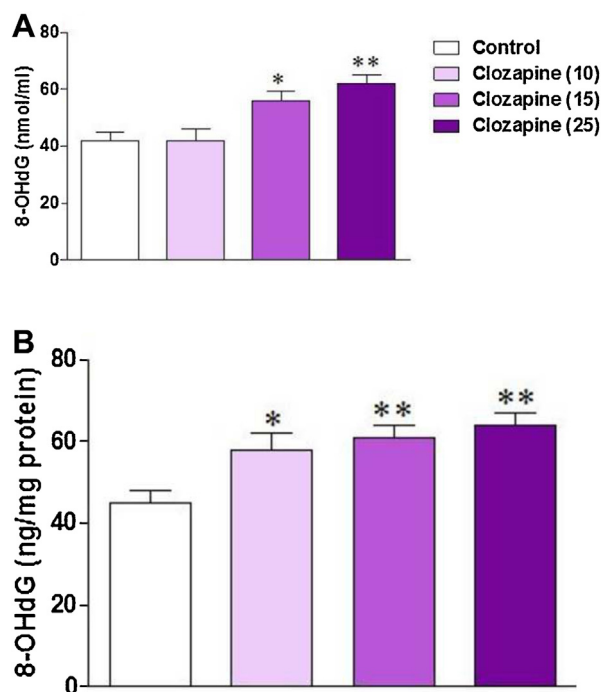
Fig. 4. Effects of clozapine in doses of 10, 15 and 25 mg/kg/d, i.p., for 21 days on the 8-hydroxy-2-deoxyguanosine (8-OHdG) levels in both serum (A) and myocardium (B) of normal rats. Results in each group represent mean ± SEM (n = 10). *$p < 0.05$ vs. control. **$p < 0.01$ vs. control.
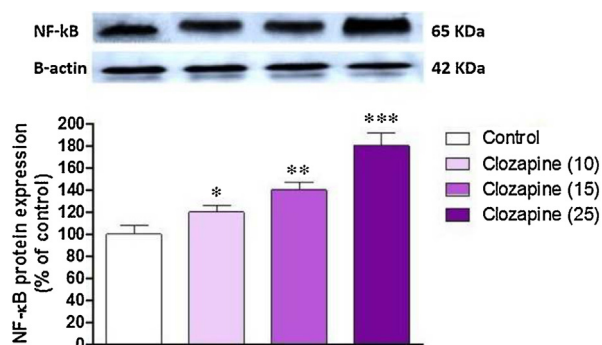
Fig. 5. Effects of clozapine in doses of 10, 15 and 25 mg/kg/d, i.p., for 21 days on cardiac NF-κβ p65 expression. Results in each group represent mean ± SEM (n = 10). *$p < 0.05$ vs. control. **$p < 0.01$ vs. control. ***$p < 0.001$ vs. control.

3.6. Immunohistochemical detection of 3-nitrotyrosine and caspase-3

The control group did not show any immunoreactivity for 3-nitrotyrosine (Fig. 6A), an indicator of peroxynitrite. Administration of clozapine (10, 15, and 25 mg/kg) led to a gradual increase of immunoreactivity of 3-nitrotyrosine, which was evident from the increased intensity of the brown staining of cardiac tissues when compared to the control group (Fig. 6B–D). The control group showed little immunoreactivity for caspase-3 (Fig. 7A). Clozapine-treated groups at all three dose levels showed significant increases in the numbers of cardiac cells

showing immunoreactivity for activated caspase-3 in the cytoplasm (Fig. 7B–D) compared with the control group.

The immunohistochemical staining for caspase-3 was quantified, and the results are summarised in Fig. 7E. This immunohistochemical finding was confirmed by spectrophotometric measurement of caspase-3 activity in cardiac tissues. Caspase-3 activity increased in response to clozapine treatment at the significance level $p < 0.05$ with 10 mg/kg, $p < 0.01$ with 15 mg/kg and $p < 0.001$ with the dose 25 mg/kg after 21 days of treatment (Fig. 7F).

4. Discussion

Approximately 30% of individuals diagnosed with schizophrenia suffer from treatment-resistant or refractory schizophrenia. The gold standard for treatment of refractory schizophrenia is clozapine [8]. However, a significant number of patients cease clozapine therapy. The main cause is drug-induced adverse effects, most notably including myocarditis and cardiomyopathy [7].

The exact mechanisms of clozapine-induced cardiac toxicity are not yet fully understood. Existing evidence points to a multitude of molecular mechanisms involved in clozapine-induced cardiotoxicity. In this study, we investigated possible mechanisms of clozapine cardiotoxicity and the cause of sudden death observed in many patients during the course of clozapine therapy. Because most of the reported cases of clozapine cardiotoxicity were in young patients, we performed this study in young (3–4 weeks old) rats treated with clozapine for 21 days.

In the present study, all animals treated with clozapine appeared sedated, lethargic and sick for at least 1 h after clozapine injection, which may reflect the lethargy reported in some patients that has been related to clozapine cardiotoxicity [27]. Clinically, patients receiving clozapine should be regularly monitored by echocardiography during treatment; FS and EF are considered the standard indicators of LV function used for diagnosis of cardiotoxicity. Because clinical cardiac changes were difficult to interpret by echocardiography in short-term studies like this one, we therefore also measured myocardial functional parameters (LVEDP) by haemodynamic analysis to further strengthen our findings on cardiac changes after clozapine treatment. Clozapine-treated animals showed dose-related decreases in FS and EF but increases in LVEDP, LVDd and LVDs, indicating LV dysfunction consistent with cardiomyopathy. Previous studies showed that the potential cardiotoxicity of clozapine may be in the form of myocarditis and cardiomyopathy [28–30].

In addition, our results showed that treatment with clozapine in the tested doses induced marked dose-related inflammatory and cardiotoxic effects, with the highest incidence in response to 25 mg/kg clozapine. Inflammatory lesions were observed in both the left and right ventricles, mainly in the myocardium below the endocardium of the left ventricle, in the posterior papillary muscle of the left ventricle and in the septum. Although the available clinical studies showed that clozapine cardiotoxicity is sudden and dose independent, our results showed dose related cardiotoxic effect of clozapine. Similar results have been shown in by Wang et al. [12]. These difference in results
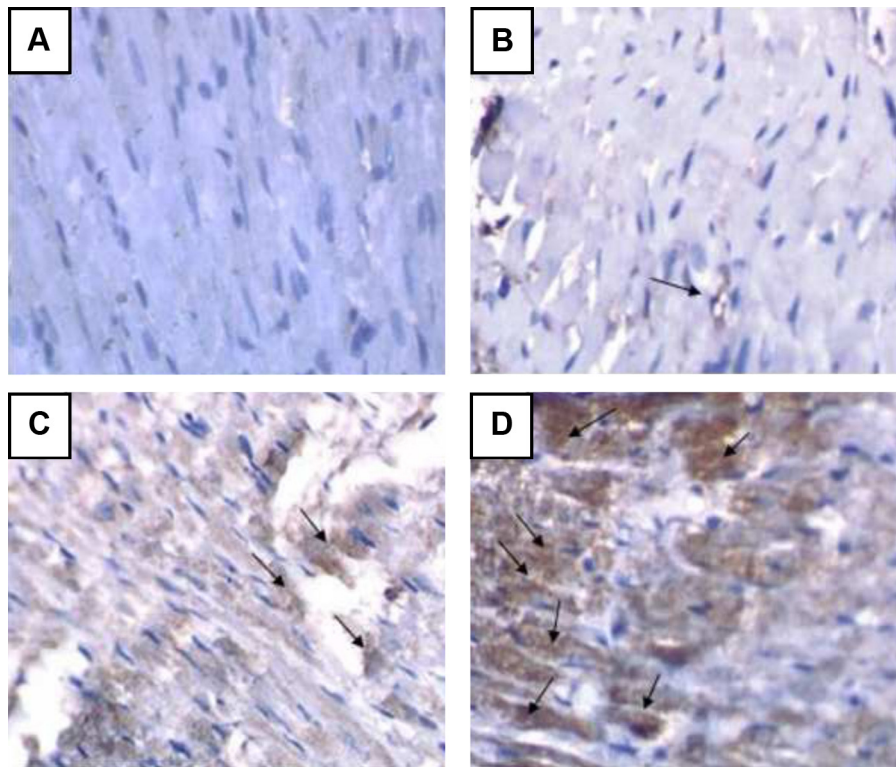
Fig. 6. Immunohistochemical staining of 3-nitrotyrosine in heart. (A) Section from a control rat showing normal architecture of myocardial tissue and absence of 3-nitrotyrosine staining. (B–D) Sections from rats treated with clozapine in doses 10, 15 and 25 mg/kg/d, i.p., for 21 days showing increased massive derangement of myocyte structure and strong 3-nitrotyrosine positive staining with the maximal effect from 25 mg/kg/d of clozapine. Magnification, 400×.

of cardiotoxicity between clinical and animal studies may be attributed to difference in haemodynamics or the rate of formation of clozapine metabolites and free radicals. The cardiotoxic effects were confirmed by elevation in the activities of serum CK-MB and LDH, the two enzymes that are considered important markers of early and late cardiac injury, especially during clinical follow-up of drug-induced cardiotoxicities [31].

Among various hypotheses of clozapine-induced cardiotoxicity, Killian et al. [7] proposed that clozapine-induced myocarditis may result from a type I IgE-mediated acute hypersensitivity reaction. This hypothesis is supported by the onset of clozapine-induced myocarditis, which commonly includes peripheral eosinophilia and eosinophilic myocardial infiltrates [10]. These reports are consistent with our results showing an increase in cardiac MPO level, which is an index of neutrophil migration, in clozapine-treated animals. Activated eosinophils induce tissue injury and necrosis through the production and release of reactive oxygen metabolites and cytotoxic proteins (e.g., proteases and MPO) into the extracellular fluid [32]. One possible explanation of this hypothesis comes from the fact that clozapine undergoes bioactivation in myocardial tissue to a chemically reactive nitrinium ion metabolite, which stimulates cellular injury, lipid peroxidation and free radical production [33]. These results are consistent with our results showing increased cardiac levels of the lipid peroxidation product (MDA) with

clozapine treatment. This nitrinium ion also binds with proteins in the myocardium, leading to formation of an antigenic complex that stimulates the immune response and macrophages [34]. This complex subsequently leads to myocardial cell damage via the release of free radicals and the activation of a variety of proinflammatory cytokines such as TNF-α [35]. The increase of cardiac TNF-α by clozapine is dose-dependent [12]. These findings are consistent with the results of this study. TNF-α is known to be able to attract leukocytes to inflammatory sites, enhancing the generation of reactive species [36]. Moreover, TNF-α seems to be responsible for regulating the production of some mediators that stimulate inflammatory reaction, such as NF-$\kappa\beta$ and COX-2 [37]. The results of this study clearly showed an increase in cardiac NF-$\kappa\beta$ levels in CLZ-treated animals.

Previous studies support the concept of the involvement of TNF-α in clozapine-induced cardiotoxicity, in which clozapine can stimulate in vivo release of TNF-α and various interleukins [12,13]. In addition, clozapine-induced myocarditis in humans is accompanied by the release of proinflammatory cytokines, including TNF-α [38].

Imbalance in the autonomic system with decreased parasympathetic tone and increased adrenergic drive has been hypothesised to explain the electrophysiological effects of clozapine, mainly tachycardia at rest. Persistent inappropriate tachycardia has been demonstrated to induce an impairment of left ventricular function both
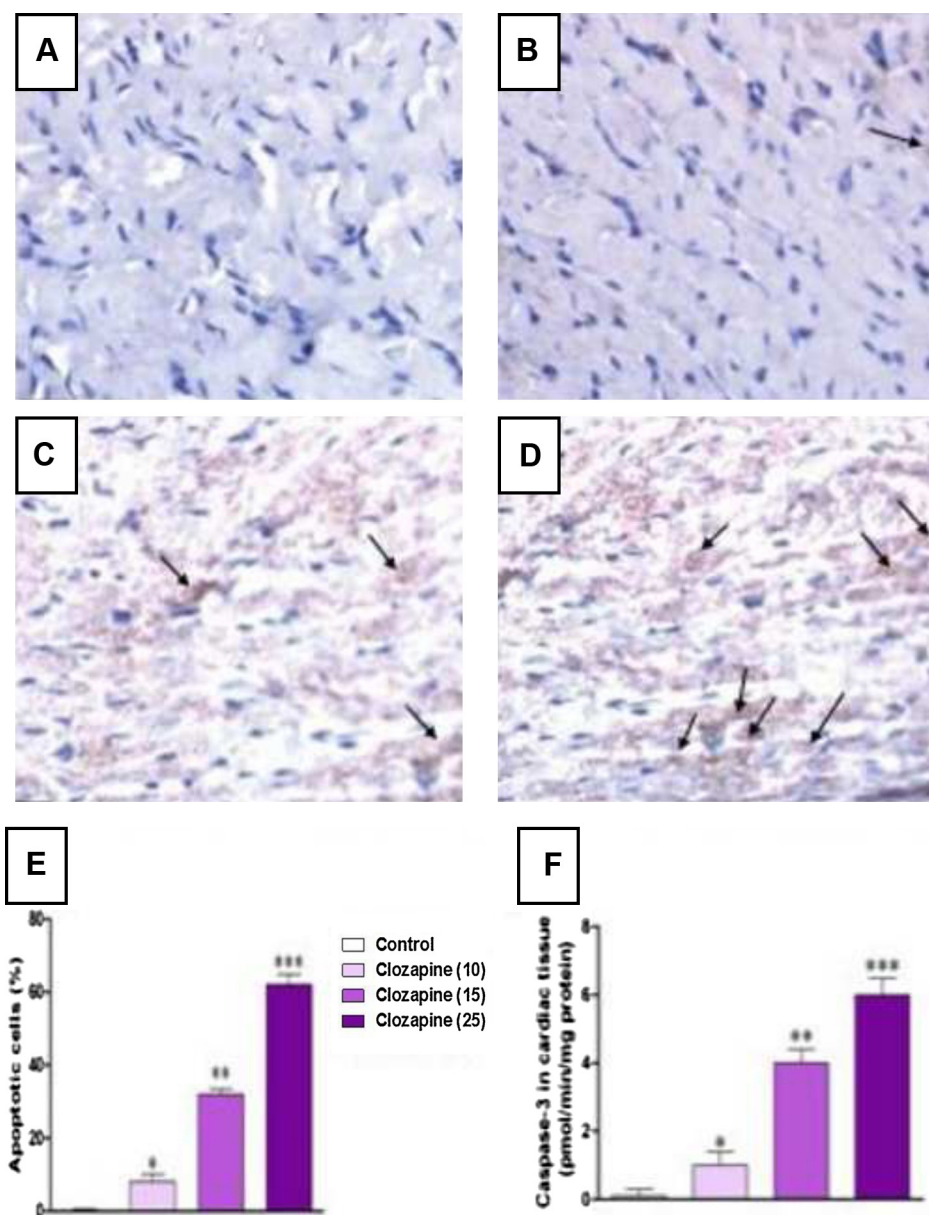
Fig. 7. The immunohistochemical reaction for activated caspase-3 in heart. (A) Section from a control rat showing few immunostained cardiac muscle fibres with caspase-3 staining. (B–D) Sections from rats treated with clozapine in doses of 10, 15 and 25 mg/kg/d, i.p., for 21 days, showing increased positive immunoreactivity for caspase-3 with the maximal effect from 25 mg/kg/d of clozapine. Magnification, 400×. (E) Percentage of active caspase-3-positive cells in cardiac tissues of clozapine-treated rats. (F) Caspase-3 activity in control and clozapine-treated rats, estimated by ELISA assay. Results in each group represent mean ± SEM ($n = 10$). *$p < 0.05$ vs. control. **$p < 0.01$ vs. control. ***$p < 0.001$ vs. control. ****$p < 0.0001$ vs. control.

in animal models and in humans [39]. Clozapine induces a rise in plasma catecholamines that correlates with the degree of myocardial inflammation [40]. Moreover, the histopathology of clozapine-treated mice showed a significant dose-related increase in myocardial inflammation that correlated with plasma catecholamine levels. Propranolol, a beta-adrenergic blocking agent, significantly attenuated these effects [12].

The clozapine-induced increase in serum levels of catecholamines increases the myocardial oxygen demand, both directly and indirectly via direct myocardial stimulation and increasing cardiac loads [41] in addition it decreases myocardial oxygen perfusion [42]. Moreover, increased serum level of catecholamines stimulates renin–angiotensin–aldosterone system leading to further increase in cardiac loads, the fact that explains the protective role of angiotensin converting enzyme inhibitors as captopril against clozapine cardiotoxicity [43]. Increased cardiac loads with decreased perfusion myocardial ischaemia and increased generation of free reactive

oxygen species, leading to increase in myocardial lipid per-oxidation, inflammation and cell injury. These effects were reflected in our results in form of increased myocardial lipid peroxidation product MDA and 8-OHdG, the marker of oxidative DNA damage.

Our results showed that clozapine significantly increased the cardiac level of nitrites, a stable product and indirect marker of NO. In addition, the immunohis-tochemical study showed increased immunoreactivity to 3-nitrotyrosine, the marker of peroxynitrite, in cardiac tissues of clozapine-treated animals. The myocardial cyto-toxicity of peroxynitrite involves direct oxidative injury to cardiac cells and damage to proteins, lipids and DNA [44] and the nitration of tyrosine residues of pro-apoptotic proteins in cardiomyocytes [45].

Previous studies showed increases in cardiac NO lev-els following exposure to clozapine, an effect that can be related to the drug itself or to its metabolite N-desmethylclozapine via its agonistic activity towards M1 receptors on cardiac vagal preganglionic fibres [46]. NO is an immune regulator and an effector molecule that mediates tissue injury. Increased formation of NO may induce negative inotropic effects and become deleteri-ous to the heart. Where, excess amounts of NO produced by inducible nitric oxide synthase (iNOS) appeared to contribute to the progression of myocardial damage in myocarditis [47]. Previous studies have demonstrated that the cytokines released by the surrounding inflammatory cells were responsible for the induction of iNOS in the cells of the cardiovascular system, including the myocytes. TNF-α can trigger iNOS expression in macrophages and cardiac myocytes. The overproduction of NO by proinflammatory cytokines may depress myocyte contractility [48].

Excessive production of ROS and RNS in myocardial cells leads to the exhaustion of cellular GSH stores and dysregulation of antioxidant enzymes as GSH-Px, thereby leading to the impairment of antioxidant defences (Solaini and Harris [15]). These effects were reflected in our results in the form of decreases in the GSH level and GSH-Px activity in cardiac tissues in clozapine-treated animals. Clinical and experimental investigations sug-gested that increased oxidative stress associated with an impaired antioxidant defence status initiates a cascade of reactions responsible for clozapine-induced cardiotoxicity [49].

The increase in free radical formation and the atten-uation of antioxidant defences by clozapine, can lead to oxidative damage to cellular lipids, proteins and DNA [44]. This can explain the observed increase in both serum and cardiac levels of 8-OHdG the biomarker of DNA damage and the increase in the expression of NF-κB p65, the nuclear factor that contributes in inflammatory response and cell apoptosis. Moreover, the results showed increased expression of caspase-3 in cardiac tissues of clozapine-treated animals. Caspase-3 is an important marker of apoptosis, and this finding indicates that the clozapine-induced cardiotoxicity can lead to apoptosis of cardiac cells. This can be attributed to the observed increase in oxidative stress with attenuation of antioxidant defences and the consequent cellular and DNA dam-age. Over the long term, these changes can lead to the

development of myocarditis and cell apoptosis in cardiac muscle and to profound cardiac injury and cardiomyopathy [14].

5. Conclusion and recommendations

In conclusion, clozapine-induced cardiotoxicity is a seri-ous and potentially lethal complication during the course of clozapine therapy for schizophrenia. Increased myocar-dial oxidative stress, inflammatory cytokines, cellular and DNA damage and apoptosis with attenuation in antioxi-dant defences are all contributing factors. This necessitates a high degree of clinical care through the course of cloza-pine therapy. The use of echocardiographic monitoring as a routine periodical check during the course of clozapine therapy, and biohumoral investigation if any signs of car-diotoxicity starts to appear is recommended. Interruption of the clozapine treatment or combination with other drugs that can modulate the above-mentioned pathogenesis in susceptible patients requires further studies.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

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