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Short Communication

Clozapine Disrupts Endothelial Nitric Oxide Signaling and Antioxidant System for its Cardiovascular Complications

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ABSTRACT

Objective: Many drugs in current practice require additional safety labels in order to prevent potential risks to the major organ system. Psychotropic agent clozapine has been reported to produce myocarditis and other cardiac complications on repeated use. Our study aimed to establish the role of clozapine in vascular damage associated with nitric oxide metabolism. **Method:** Isolated aortic strips incubated with clozapine at different dose levels were estimated for nitrite release and antioxidant systems such as glutathione and catalase. Vascular integrity assessment was performed by recording the acetylcholine induced relaxation of phenyephrine pre-contracted aorta. **Result**: From our study, it was found that clozapine depilates the nitric oxide level in the endothelium and enhance the oxidative stress. The aorta fails to relax completely after adding acetylcholine indicates the deranged eNOS signaling in the endothelium. **Conclusion**: From the experimental findings, it was concluded that clozapine could depress the eNOS regulation and thereby perhaps initiates cardiovascular complications through subsequent vascular events.

Key words: Endothelium, Nitric oxide, GSH, Catalase, Oxidation.

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INTRODUCTION

Ample array of drugs have been withdrawn from clinical use due to their broad cardiac events such as hypertension, ventricular arrhythmia, angina etc., accompanied with or without cardiac death. Few such drugs are Rosiglitazone, Valdecoxib, Rofecoxib, Cisapride, Tegaserod etc, Drug induced cardiac effects are often predictable, dose dependent and hence can reduce the incidence of mortality.[1] Most drug candidates require additional safety labels to prevent potential risk like angina, myocarditis, severe hypertension, etc.

Psychotropic agents are also in the figure which was reported with angina, atherosclerosis, cerebrovascular disease, congenital heart defects, coronary artery disease, heart attack, myocarditis, peripheral vascular disease and stroke.[2] Clozapine, a tricyclic dibenzodiazepine derivative one among the most preferred drug due to its greater effectiveness and slumped incidence of extra pyramidal symptoms.[3] But criticism of clozapine still exists since it came to clinical practice for its progression of metabolic syndromes which provoke diabetes and cardiovascular disorders.[4] In a case study, they observed the signs of acute myocardial infarction and diagnosed with myocarditis after clozapine treatment for 7 days at a dose of 200mg/day.[5] It was reported that clozapine escalates oxidative mitochondrial stress in neutrophils which probably contributes to the induction of apoptosis.[6] However, there is only negligible information regarding the action of clozapine in the blood vessels. Moreover none explained about the interaction of clozapine with endothelial nitric oxidation metabolism which is a prime element behind the cardiovascular homeostasis.

Here we present our experimental findings of the effect of clozapine on vascular nitric oxide imbalance and its physiological outcome using *in vitro* aortic ring study.

MATERIALS AND METHODS

Materials

Clozapine, Acetylcholine and Phenylephrine were purchased from Sigma-Aldrich (Milan, Lombardy, Italy). N-(2-Hydroxy ethyl)-piperazine ethane sulfonic acid, N-(1-Naphthyl) ethylenediamine, sulphanilamide, orthophosphoric acid, L-Glutathione, 5,5′-Dithiobis(2-nitrobenzoic acid) and potassium dichromate were purchased from HiMedia Ltd (Mumbai).

Preparation of aortic rings

Fresh thoracic aorta was isolated from healthy wistar rats after sacrificing by carbon dioxide asphyxiation. The aorta was immediately transferred into ice cold Potassium chloride (150mM). It was then cleaned and sliced into small pieces of about 1cm length which was used for our study.[7] All the proceedings were performed in controlled conditions.

Experimental evaluation

Aortic rings weighing 100-120mg were transferred to test tubes containing ice cold N-(2-Hydroxy ethyl)-piperazine ethane sulfonic acid (HEPES) buffer and added with different concentrations (0.5,1.0, 1.5, 2.0 and 3.0µg/mL) of clozapine. Control aorta was added with HEPES buffer and the volume was made up to 10X of tissue weight using HEPES buffer. All the samples were incubated at 37°C for 60min in BOD incubator.

Estimation of nitric oxide release

The amount of nitric oxide released from the aorta was indirectly estimated from its metabolite nitrite using Griess reaction.[8] 10µl of supernatant solution from each group was added with 20µl Griess reagent A and B. Then the volume was made up to 1000µl with ultra-pure water. The

mixture was then incubated at 37°C for 30min before reading the absorbance at 540nm primary filter and 630nm secondary filter using semi auto biochemistry analyzer. (STAT FAX 3300, Awareness Technology Inc, USA).

Vascular reactivity assessment

The thoracic aorta was cut into rings of 3–4 mm length and immediately transferred into Kreb's solution. The rings were suspended on tissue bath (EMKA Bath-2) containing 10mL Kreb's solution bubbled with carbogen (95% O_2 + 5% CO_2) maintained at 37°C.[9] The changes in isometric force were recorded using IOX-2 (Emka Technologies S.A.S, Paris, France). Baseline tension was adjusted to 2g and all the subsequent measurements were generated above the baseline. The aorta was pre-contracted with phenylephrine (1 μM/L). Vascular integrity was assessed by adding acetylcholine (10nM – 1mM) cumulatively and the Ach induced relaxation was expressed as a percentage reduction of phenylephrine induced contraction. The vascular reactivity was again recorded after incubating the aorta with clozapine at three higher concentrations (1.5, 2.0 and 3.0μg/mL) for about 30min.[10]

Estimation of oxidative markers

The oxidative stress in aorta was accessed by quantifying tissue glutathione level and catalase activity after preparing the aortic homogenate. For GSH, 100μl of homogenate after centrifugation with 5% sulfosalicylic acid was added with 800μl Na_2HPO_4 and 100µL Ellman's reagent.[11] The absorbance of the mixture was measured in biochemistry analyzer at 405nm after 5 min. For estimating catalase activity, 100μL homogenate was mixed with 1000μL of hydrogen peroxide and incubated for 5min. 2000μL of dichromate-acetic acid mixture was added to the above solution and kept at 100°C for 10min. The absorbance was measured at 570nm against regent blank after centrifugation (3000 Rpm for 15min).[12]

Statistical analysis

Results were generated from 3 independent experiments (n = 3) with 3 replicates and the data analysis was performed with Instat-Pro. The results were illustrated using Graph-Pad Prism 5 and variations between results were expressed as standard errors mean (SEM). The data were statistically analyzed by one-way ANOVA using Tukey–Kramer multiple comparison test.

RESULTS

Effect of clozapine on aortic nitrite

After incubating aorta with clozapine, no significant reduction of nitrite was found at 0.5µg/mL and there after a dose dependent drop off was observed at each dose level (Figure 1a). Clozapine at the dose of 3.0µg/mL reduces the aortic NO level to about 35% (48.02µM/L) from the untreated aorta (73.3µM/L) illustrates the impairment of NO metabolism which may further leads to subsequent destruction in the cardiovascular system.

Effect of clozapine on vascular reactivity

In normal aorta, acetylcholine relaxed the phenylephrine pre contracted aorta to a maximum of 76.34% at log -4M. This depicts the normal tone and activity of the healthy endothelium. On incubation with clozapine, the response gradually declines to 56.32%, 29.63% and 11.35% for clozapine 1.5, 2.0 and 3.0μg/mL respectively indicates the signs of endothelial disruption which obliviously declines the vascular nitric oxide release. The results were shown in Figure 1b.

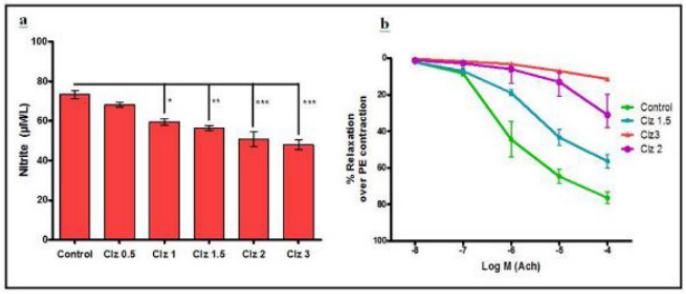

Figure 1a: Showing the nitrate release from aorta incubated at different concentrations of clozapine. b. Vascular reactivity assessment by evaluating the relaxation effect of acetycholine on phenylephrine pre-contracted aorta. Data are shown as mean ± SEM (n=3). Data are shown as mean ± SEM (n=3). **p<0.05,** p<0.01 and ***p<0.001 significantly different from control untreated group. (Clz: Clozapine)*

Table 1: Effect of clozapine on aortic oxidative stress.

Treatment (µg/L)	GSH (pM/mg tissue)	Catalase activity (U/mg tissue)
Control	4.16±0.17	0.63±0.028
Clz 0.5	2.78±0.07*	0.39±0.039*
Clz 1.0	2.45±0.05*	0.37±0.006*
Clz 1.5	2.13±0.05*	0.33±0.002*
Clz 2.0	1.63±0.05*	0.31±0.012*
Clz 3.0	1.02±0.02*	0.11±0.002*

Data are shown as mean ± SEM (n=3). *p<0.05significantly different from control untreated group. (Clz: Clozapine)

Effect of clozapine on vascular oxidation

Endothelial glutathione level and catalase activity of untreated aorta was found to be 4.16 pM and 0.63 U respectively. The sulfhydryl group declined significantly in all the clozapine treated groups in a dose dependent manner. The level was dropped by 24.52% after incubating with clozapine at 3.0μg/mL. In case of catalase activity, an abatement of the enzyme activity at in all dose level was observed. The activity was found to be monstrously diminished to a maximum of 0.11U at highest dose level of 3.0μg/mL. (Table 1)

DISCUSSION

In our laboratory we performed an *in vitro* study to examine the involvement of endothelial damage for cardiac side effects induced by clozapine. Clozapine is the last-line therapy to treat schizophrenia after multiple drug failure in schizophrenic patients. Besides the primary insult agranulocytosis, potential fatal cardiac effects like myocarditis, dilated cardiomyopathy, venous thromboembolism and pericarditis are the most proclaimed adverse effects associated with its use.[13] Acute toxic symptoms were evidenced after attaining a plasma concentration above 2000μg/L.[14] Based on previous reports and statements regarding clozapine, we have randomly selected the dose at a range of 500 to 3000μg/L to understand the physiological changes based on varying dose levels (therapeutic and toxic dose). The incubation time of 1hr was preferred after a preliminary exploration by incubating aorta with L-NAME (standard eNOS inhibitor) at 15, 30 and 60 min. The nitrite level was declined about 60% from after 60min incubation. Endothelial dysfunction is occasionally pervasive throughout the body as patients with known atherosclerosis.[15] This turns out to be the consequence from inadequate levels of NO and predominantly it is the baseline risk factors for

cardiovascular disorders. Patients met with coronary artery bypass grafting (CABG), displayed reduced endothelial function which is principally related to poor NO bioavailability.[16] These findings witnessed NO as a contemporary alternative metric to monitor for secondary prevention. Our finding certainly showcases the declined nitrate release from aorta after incubating with clozapine. The deficiency in NO was further confirmed by vascular reactivity assessment using isolated rat aorta (3 toxic doses selected based on the significance in reducing nitrite level). Parasympathetic stimulation of the endothelium induces NO-dependent vasodilatation by eNOS translocation from the plasma membrane. Ach is known to be the classical neurotransmitter in favoring endothelial mechanotransduction which facilitates flow-mediated dilatation.[17] In our present study, responses to the endothelium-dependent vasodilatation of acetylcholine (-6 to -4 logM) were significantly blunted in aortic rings incubated with the drug. Such vasodilator dysfunction extending into the coronary microcirculation contributes to the ischemic manifestations of coronary artery disease during myocardial ischemia.[18] These findings strongly evidenced that the eNOS signaling is also desired to be disturbed during clozapine treatment.

Impaired NO synthase, decreased L-arg uptake and increased lipid oxidation shows detectable Increase Reactive Oxygen species (ROS) level in the endothelium which promotes endothelial dysfunction. This is attributed to high oxidative stress and inflammation which can be worsen with other conditions (cold, mental stress, anger) etc. A report said that patients with cardiac and systemic glutathione deficiency are closely associated with the impaired functional status and structural abnormalities of the heart.[19] Reduction in tissue and plasma GSH levels is considered to be the risk factors of CVD. Increased oxidative stress also inactivates the endogenous antioxidant enzyme by releasing hydrogen peroxide. One such enzyme activity named catalase (CAT) has its property in blocking the oxidative stress along with enhancing the Super-Oxide Dismutase (SOD) activity. Drugs targeting antioxidant enzymes in endothelial cells perhaps offer a future perspective for the revolution of competent cardio-protective remedies and in contrast, any disturbances in such systems will also provoke the development of deadly disorders. From our experimental findings it is perhaps obvious that clozapine even at therapeutic dose can initiates endothelial destruction as well as it was notified that a fair indication of erratic outcome in the vascular endothelium at toxic dose. These changes in ROS directly relate the activation of inflammatory response which further contributes to vascular abnormality on prolonged exposure. Also, neurohormones including catecholamines and angiotensin II all emerge to induce myocardial changes at least in part via oxidative stress.[20] Together it was capable to wrap up that, the reported cardiovascular effects of clozapine is perhaps due to the initiation of NO imbalance which switch on further cascade events constantly on repeated use and leads to deadly sufferings if used without safety measures.

CONCLUSION

Our study demonstrates that clozapine agitates nitric oxide metabolism by altering the eNOS signaling and accelerates oxidative stress in the endothelium. NO deficiency alters the vascular tone which further promotes the peripheral resistance and may leads to cardiac complications. We have hypothesized that the physiological consequences of clozapine is due to its effects in vascular eNOS coupling which triggers oxidative stress.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

NO: Nitric oxide; **HEPES:** N-(2-Hydroxy ethyl)-piperazine ethane sulfonic acid; **GSH:** Glutathione; **ROS:** Reactive oxygen species.

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