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Cardioprotective effects of animal grade piperazine citrate on isoproterenol induced myocardial infarction in wistar rats: Biochemical and histopathological evaluation

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The present study was designed to investigate the cardioprotective effect of animal grade piperazine citrate on isoproterenol-induced myocardial infarction in rats by studying cardiac marker enzymes and histopathological changes of the cardiac muscle. Isoproterenol administration showed significant (P<0.001) increase in the serum levels of cardiac injury markers (creatine kinase-MB and troponin-I), 358.98 ± 7.68 iu/l and 13.16 ± 0.35 ng/ml compared to the normal control group of 291.58 ± 3.56 iu/l and 9.66 ± 0.20 ng/ml respectively. Pretreatment with 15 and 30 mg/kg body weight of piperazine citrate showed a decrease in the troponin-I levels when compared with the isoproterenol group; 13.16 ± 0.35 to 12.39 ± 0.22 ng/ml in the group that received 15 mg/kg piperazine citrate (p = 0.0881) and 13.16 ± 0.35 to 11.79 ± 0.30 ng/ml (p = 0.0132) in case of the group pretreated with 30 mg/kg piperazine citrate. With regards to CK-MB, the treated groups with piperazine citrate 15 and 30 mg/kg body weight showed a reduction in the values, 340.76 ± 5.10 (p = 0.0763) and 344.17 ± 8.24 iu/l (p = 0.2178) respectively, compared to the isoproterenol group value of 358.98 ± 7.68 iu/l. Histopathological investigation showed that there was no significant architectural changes in the normal control group that received only normal saline. Structural aberrations caused by isoproterenol were also significantly reduced in the piperazine citrate treated groups. Therefore, the results of the present study suggest that low dose piperazine citrate has a significant effect on the protection of the heart.

Key words: Piperazine citrate, myocardial infarction, histopathology, Wistar rat.

INTRODUCTION

Acute myocardial infarction (AMI) amongst other cardiovascular diseases (CVD) remains the major threat...
to human life and is considered one of the leading causes of death globally today and as such currently constitutes a major preoccupation of clinical cardiology (Nestelberger et al., 2019). AMI falls within the range of acute coronary syndromes (ACS), which describes any condition brought on by a sudden reduction or blockage of blood flow to the heart and includes unstable angina (UA), non–ST-elevation myocardial infarction (NSTEMI), and ST-elevation myocardial infarction (STEMI). Pathologically speaking, the term myocardial infarction refers to the death of cardiac myocytes. It occurs when myocardial ischemia, reduction in myocardial oxygen supply, surpasses a critical threshold and in the process overwhelms the inherent mechanisms for myocardial cellular repair. This critical threshold condition if allowed to exist without management for any appreciable length of time would result in irreversible myocardial cell damage or death. A number of factors can lead to critical myocardial ischaemia. These factors include increased myocardial metabolic demand and decreased delivery of oxygen and nutrients to the myocardium via the coronary circulation (Zhou et al., 2018, 2019). Thrombus is the major culprit responsible for the interruption in the myocardial oxygen and nutrients supply, when it is superimposed on an ulcerated or unstable atherosclerotic plaque resulting in coronary occlusion (Cotran et al., 1994). Stenosis (focal or dynamic) also plays a role in limiting the supply of oxygen and nutrients thereby precipitating myocardial infarction (MI). Severe hypertension and cardiac valvular pathologies are some other conditions associated with increased myocardial metabolic demand (Johansson et al., 2017). The death of myocytes is generally confluent; this pattern of injury distinguishes infarction pathologically from other forms of myocardial injury, which tend to destroy myocytes more diffusely (Allan and Wayne, 2002). Since cardiovascular disease is diverse in nature comprising different type of diseases that affect the heart and blood vessels with coronary heart disease (CHD), in particular being the foremost cause of premature death in many countries (Grimes, 2012; Khot et al., 2003; Al-Kateb et al., 1998). According to Manfroi (2002), the prevalence of acute MI as the first manifestation of ischaemic heart diseases is high in approximately 50 to 70% of patients and is a common cause for hospital admission (Manfroi, 2002).

Numerous factors that are linked to the severity of the disease have been identified, such as hyperlipidemia, hypertension, diabetes mellitus, smoking, male gender, and family history of atherosclerotic arterial disease. Other factors that may contribute to the severity of the disease include; age, obesity, heavy alcohol consumption and physical inactivity. The presence of any risk factor is associated with doubling the relative risk of developing atherosclerotic coronary artery disease (Hajar, 2017; Cotran et al., 1994; Pais et al., 1996). Several studies have shown that the incidence of modifiable risk factors including smoking, hypercholesterolemia, obesity, and hypertension in patients presenting with myocardial infarction is up to 90% (Khot et al., 2003; Greenland et al., 2003). A 16-year epidemiologic study (1990-2006) by the National Registry of Myocardial Infarction (NRMI) presented the trends in characteristics and mortality of patients with AMI in the U.S., and calculated the mortality rates for AMI in patients who received guideline-based acute therapy (Rogers et al., 2008).

Isoproterenol has been variously documented to cause myocardial necrosis resulting in MI through production of severe stress in the myocardium when given in high doses (Rona, 1985; Rajadurai and Prince, 2006; Karthikeyan et al., 2007; Patel et al., 2010). Several mechanisms have been proposed to elucidate the isoproterenol induced aberration of myocardial architecture and generation of highly cytotoxic free radicals through auto-oxidation of catecholamines is implicated as one of the significant causative factors (Patel et al., 2010).

Piperazine is an anthelmintic drug clinically employed in human and veterinary medicine against the intestinal roundworms, Ascaris lumbricoides and Enterobius (Oxyuri) vermicularis. Its anthelmintic properties were first reported by Fayard (1949). In the mammalian, piperazine citrate has been shown to produce a slowing of the rate and a reduction in the force of contraction of both the isolated spontaneously beating atrial preparation and the perfused Langendorff heart (Onuaguluchi, 1966). The negative chronotropic and inotropic activity progressed to direct muscle depression on increasing the concentration of piperazine citrate (Mason and Gillian, 1972). Similarly, piperazine citrate decreased both the rate and force of contraction of the isolated frog heart (Onuaguluchi, 1966). In the anaesthetized cat and rat preparations, piperazine citrate caused dose-dependent decreases in the blood pressure and reduced the heart rate (Mason and Gillian, 1972; Ghasi et al., 2009).

Isolated tissue studies showed that piperazine has a direct nonspecific smooth muscle relaxant action. It inhibited contractions of guinea-pig ileum and rabbit duodenum caused by various agonists including barium chloride, histamine, 5HT, and acetylcholine by a direct smooth-muscle depressant action (Onuaguluchi, 1966; Onuaguluchi, 1981, 1984). It also reversed the effect of adrenaline on the guinea-pig vas deferens and oxytocin-induced contractions in the rat uterus. Onuaguluchi and Igbo demonstrated that piperazine citrate reduced in the mortality due to ouabain-induced arrhythmia in the toad and concluded that piperazine citrate could be a potential antiarrhythmic agent (Onuaguluchi and Igbo, 1985). In the human volunteers, orally ingested piperazine citrate caused a reduction in heart rate and prolonged the P-R interval (Onuaguluchi and Ghasi, 2006). Similar results were obtained in the rat model at moderate doses of piperazine citrate. At high doses, however, such as 100 mg/kg piperazine caused serious aberrations (Ghasi and Onuaguluchi, 2007). The pattern of electrocardiographic
changes seen in the rat following subchronic treatment with piperazine citrate indicates that the antiarrhythmic action is due to potassium channel blockade (Ghani et al., 2012) which will explain its non-specific action. It has also been reported that piperazine citrate showed 100% cardioprotection of the rat heart at all doses against BaCl2-induced ventricular fibrillation indicating remarkable prophylactic value (Ghani, 2008).

MATERIALS AND METHODS

Experimental animals

Twenty four albino Wistar rats of either sex and with no signs of ill-health, weighing originally between 120 and 150 g were obtained from the animal house of Department of Pharmacology and Therapeutics, College of Medicine, University of Nigeria, Enugu campus where the research was carried out. The animals were fed with standard poultry super starter mash and had access to water ad libitum.

Experimental design

All the 24 rats were randomized into four (4) groups, consisting of six (6) rats in each group and allowed to acclimatize for one week. At the end of acclimatization, they received treatment as follows:

Group 1 (control): Animals received standard laboratory diet and drinking water ad libitum and served as a control group.

Group 2 (ISO treated): Animals received standard laboratory diet and drinking water and then subcutaneously injected with isoproterenol (85 mg/kg) daily for 2 consecutive days, on the 29th and 30th day at an interval of 24 h and served as isoproterenol group.

Groups 3 (Piperazine + ISO): Animals in this group were pretreated with Piperazine citrate 15 mg/kg o.d for 30 days. After the treatment, isoproterenol (85 mg/kg) was administered subcutaneously (sc.) to the rats at an interval of 24 h for two days, on the 29th and 30th day.

Groups 4 (Piperazine + ISO): Animals in this group were pretreated with Piperazine citrate 30 mg/kg o.d for 30 days. After the treatment, isoproterenol (85 mg/kg) was administered subcutaneously (sc.) to the rats at an interval of 24 h for two days, on the 29th and 30th day.

Induction of experimental myocardial infarction

Isoproterenol was dissolved in sterile water and injected subcutaneously to the rats (85 mg/kg b.w) 24 hourly for 2 consecutive days to induce experimental myocardial infarction. At the end of the experiment (24 h after the second dose or 48 h following the first dose of isoproterenol 85 mg/kg injection) (Daniela et al., 1998), blood was collected from the retro-orbital plexus and serum was separated and used for various biochemical estimations. All the rats were anaesthetized with pentobarbital sodium (50 mg/kg, i.p.). Subsequently, the animals were sacrificed and the hearts harvested for histopathological studies.

Materials and methods for estimation of cardiac marker enzymes

Estimation of serum creatine kinase-MB (CK-MB)

Methods and calculations used for estimation of serum creatine kinase-MB are as outlined in Hans (1974). The creatine kinase (CK) activity is dependent on the ATP/Mg2+ ratio and the optimum molar ratio is 1/1, each should be as specified in the reagent below. The auxiliary and indicator enzymes are pyruvate kinase (PK) and lactate dehydrogenase (LDH).

(i) Creatine + ATP CPK creatine phosphate + ADP

(ii) ADP + phosphoenolpyruvate PK ATP + pyruvate

(iii) Pyruvate + NADH + H+ LDH Lactate + NAD+

A quantity, 0.50 ml of the serum, 0.70 ml of buffer/coenzyme mixture, 0.05 ml of LDH/PK suspension, 0.10 ml of GSH solution was pipetted into a cuvette, mixed and allowed to stand for 15 min at 25°C and 1.75 ml of creatine-glycine buffer was added to it, mixed and the absorbance at 365 nm was measured.

Estimation of serum cardiac troponin-I

A quantity, 100 μl of the sample and 100 μl of MAB-HRP conjugate was pipetted into a burette, mixed and incubated for 6 h at 4°C under gentle shaking. 200 μl of the substrate o-phenylenediamine/H2O2 was added to it, mixed and incubated in the dark for 15 min, then 50 ml of 2 mol/L H2SO4 was again added, mixed and the absorbance at 492 nm was measured.

Histopathology

The heart was quickly dissected out after sacrifice and without delay washed with saline and thereafter fixed in 10% buffered formalin. The fixed tissues were processed in an automatic tissue processor for dehydration, clearing and impregnation and embedded in paraffin. Serial sections (5 μm thick) were cut, using the hertz rotary microtome (Cambridge model). Each section was stained with hematoxylin and eosin (H&E) as described by Baker and Bilverton (1985). The sections were examined under the light microscope (Olympus BX10, Tokyo, Japan) for histopathological changes and photomicrographs (Olympus DP12 camera, Japan) were taken.

Statistical analysis

Data were analysed using the SPSS version 13.0 software. The results were presented as means ± standard error of the mean (SEM). Student’s t-test was performed in order to compare the results from each drug dose with those from control experiment, and a P value of < 0.05 was taken as statistically significant difference. One-way analysis of variance (ANOVA) was then done to estimate the differences between the different doses of the test groups that were pretreated with piperazine, to determine if the effect observed was dose dependent.

RESULTS

Table 1 shows the effect of piperazine citrate treatment on cardiac marker enzymes namely; Troponin-I and Creatine kinase-MB, in normal and isoproterenol-induced myocardial infarction in rats. The activities of these enzymes were increased significantly (P<0.001) in isoproterenol treated rats as compared to normal control group of rats (9.66±0.20 to 13.16±0.35 ng/ml for the troponin-I and 291.6±3.56 to 359.0±7.68 IU/l in the case of creatine kinase). Piperazine citrate 15 mg/kg
pretreatment in isoproterenol treated animals decreased the troponin-I activities from 13.16±0.35 to 12.39±0.22 ng/ml. This decrease was found to be statistically insignificant (p=0.0881). However, the decrease in troponin-I (13.16±0.35 to 11.79±0.30 ng/ml) observed in the 30 mg/kg piperazine citrate treated group was statistically significant compared to isoproterenol-alone treated rats (p=0.0132). For the creatine kinase study, piperazine citrate (15 and 30 mg/kg) pretreatment in isoproterenol treated animals decreased the creatine kinase-MB activities (359.0±7.68 to 340.76±5.10 and 344.17±8.24 IU/L, respectively) when compared to isoproterenol-alone treated rats. The values in each case were found to be statistically insignificant (P=0.2810 and 0.1475 respectively).

Plates 1 to 4 show the histopathological photographs of heart tissues of control rats and those treated with piperazine citrate. Myocardial tissue from normal control animals showed clear integrity of myocardial architecture following histopathological examination. Normal untreated rats (Plate 1) showed normal cardiac fibres without any infarction, and the cells were not infiltrated with inflammatory cells. Histopathological findings confirmed the induction of myocardial infarction by isoproterenol. Heart tissues from isoproterenol treated rats (Plate 2) showed extensive myocardial structure derangement comprising subendocardial necrosis with capillary dilatation, oedema and leukocyte infiltration as compared to the control group. The architectural damage caused by isoproterenol appeared ameliorated following pretreatment with piperazine citrate and this effect seemed to be dose dependent. In rats that received 15 mg/kg piperazine citrate (Plate 3) there was presence of some degeneration of the myocardium with evidence of necrotic areas and marked infiltration of inflammatory cells. Pretreatment with 30 mg/kg piperazine citrate (Plate 4) depicted moderate degeneration of the myocardium and infiltration of inflammatory cells with fairly intact tissue parenchyma and normal myocardial fibres.

**DISCUSSION**

Isoproterenol-induced MI has proved to be a standardized model for the study of the beneficial effects of many drugs on cardiac function (Patel et al., 2010; Madhesh et al., 2011; Shaik et al., 2012). Isoproterenol is a synthetic catecholamine, a sympathomimetic agent and an agonist at β-adrenergic receptor, where it produces severe stress in the myocardium leading to myocardial infarction, at supramaximal doses (Rona, 1959, 1985). Catecholamines are the neurotransmitters that are released following stimulation of the sympathetic system and they help the body in responding to stress and emergencies. They play important role in the regulation of myocardial contractility and metabolism. Excessive exposure of the heart to catecholamines results in cellular damage, seen in clinical conditions such as angina pectoris, acute coronary insufficiency and myocardial infarction. When injected with isoproterenol, animals develop infarct like lesions resembling myofibrillar degeneration (Baroldi, 1974). The myocardial necrosis will then lead to alteration of cardiac function through increase in lipid peroxidation, level of myocardial lipids, activities of the cardiac enzymes and antioxidants (Rajadurai and Prince, 2006; Karthikeyan et al., 2007).

Several theories have been advanced to explain the mechanisms involved in isoproterenol induced myocardial infarction. Being a non-selective β1 and β2 adrenoceptors agonist, isoproterenol activates the adrenergic receptors leading to an increase in the rate and contractility of the myocardium. Therefore, isoproterenol produces relative ischemia due to myocardial hyperactivity (Yeager and Lams, 1981). Increases in intracellular Ca\(^{2+}\) overload (Bloom and Davis, 1972) and cyclic adenosine monophosphate (Bhagat et al., 1978) also result in myocardial hyperactivity and, thus, infarction. Other suggested mechanisms include depletion of high energy phosphate stores and oxidative stress (Singal et al., 1982; Rajadurai and Prince, 2006). Various experimental and clinical studies have shown that enormous amount of reactive oxygen species such as, superoxide, hydrogen peroxide and hydrogen radicals are generated in failing myocardium (Singal et al., 1982; Rajadurai and Prince, 2006).

The oxidation of hydroxyl groups in catecholamines leading to the conversion into quinones and the subsequent formation of adrenochromes most probably

**Table 1.** Effect of piperazine citrate treatment on cardiac marker enzymes in normal and isoproterenol (ISO) induced myocardial infarction in rats.

| Group | Treatment                  | Troponin-I (ng/ml) | P-value     | CK-MB (IU/L) | P-value     |
|-------|---------------------------|--------------------|-------------|--------------|-------------|
| I     | Normal control            | 9.66±0.20          |             | 291.58±3.56  |             |
| IV    | ISO                       | 13.16±0.35         | <0.0001     | 358.98±7.68  | <0.0001     |
| V     | PC(15 mg/kg) + ISO        | 12.39±0.22         | 0.0881      | 340.76±5.10  | 0.0763      |
| VI    | PC(30 mg/kg) + ISO        | 11.79±0.30         | 0.0132      | 344.17±8.24  | 0.2178      |

All values are presented as mean±S.E.M. for nine rats in each group. The isoproterenol group is compared to the normal control group, whereas the piperazine treated groups are compared with the isoproterenol group.
account for the hazardous effects of catecholamines. During this reaction, highly toxic oxygen-derived free radicals are generated which are detrimental to extra- and intracellular enzymes and proteins (Thompson and Hess, 1986). Adrenochrome and other oxidation metabolites of catecholamines can cause cell necrosis and contractile failure in the rat’s heart (Yates et al., 1981).

In the myocardium, diagnostic marker enzymes of myocardial infarction such as CTnI and CK-MB abound and these marker enzymes are released into the extracellular fluid when the myocardium necrosed (O’ Brien et al., 1997; Upaganlawar et al., 2009, Vaibhav et al., 2010). The amount of these cellular enzymes in serum reflects the alterations in plasma membrane integrity and/or permeability (Farvin et al., 2004).

Expectedly in the present study, there was significant elevation in the levels of these marker enzymes in serum of rats that received isoproterenol compared to normal control rats. Piperazine citrate, however, shows a dose dependent cardioprotective activity as the 30 mg/kg body weight of the drug caused a significant reduction in the release of the cardiac marker enzyme, troponin I in ISO-induced myocardial infarction rats compared to the lower dose of 15 mg/kg body weight. Piperazine citrate pretreatment in isoproterenol treated rats resulted in the lowered activity of the marker enzymes in serum. This demonstrated that piperazine citrate could maintain membrane integrity of the myocardium thereby restricting the leakage of these enzymes. This result supports a previous study by Ghasi (2008), showing that piperazine protects the rat heart against sudden cardiac death from barium chloride-induced ventricular fibrillation. The mechanism of action may be due to membrane stabilizing action of piperazine citrate thereby causing negative inotropic and chronotropic activity.

Histopathological examination of myocardial tissue in normal control group of rats showed clear architectural integrity of the myocardial cell membrane, and no infarction or inflammatory cell infiltration was observed. Rats injected with Isoproterenol showed coagulative necrosis with capillary dilatation, edema, separation of cardiac muscle fibers and infiltration of inflammatory cells. The reduction in histological changes such as
Plate 2. Photomicrographs from Heart section of rat in Group IV treated with Isoprotenerol [ISO] only showing marked degeneration and necrosis of myocardial fibres [thick arrows] and presence of inflammatory cellular infiltrates [Ic]. Stain: H&E. Mag: A - x100; B - x400.

Reduced inflammatory cell infiltration and moderate degeneration of the myocardium with fairly intact tissue parenchyma in piperazine citrate treated groups confirms the cardioprotective effect of piperazine citrate.
Plate 3. Photomicrograph from Heart section of rat in Group V treated with 15 mg/kg b.wt. of *Piperazine Citrate* and ISO showing some degeneration of the myocardium with marked infiltration of inflammatory cells [arrows] as shown. Stain: H& E; Mag: x400.

Plate 4. Photomicrographs from heart section of rat in Group VI treated with 30 mg/kg b.wt. of *Piperazine Citrate* and ISO showing fairly intact tissue parenchyma with normal myocardial fibres [MF]. Stain: H& E; Mag: A - x100.
Conclusion

The present study demonstrated that subcutaneous injections of supramaximal dose of isoproterenol produced myocardial infarction in rats as evident by the release of myocyte injury markers in serum. Myocardial lesions were associated with histopathological changes including coagulative necrosis with capillary dilatation, edema, separation of cardiac muscle fibers and infiltration of inflammatory cells. In addition, the present study provided experimental evidence that piperazine citrate reduced the cardiac marker enzyme levels and preserved histo-architecture following isoproterenol administration. These findings therefore suggest that animal grade piperazine citrate has cardioprotection effects against myocardial injury and may be beneficial to animals treated by it.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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