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Dose Response Study on Myocardial Injury Induced by Isoproterenol injection in Male Wistar Rats

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ABSTRACT

Isoproterenol (ISO) is one of the chemicals that are widely used as an infarction-inducing agent. This study aimed to perform a dose response study on myocardial infarction induced by isoproterenol subcutaneous (s.c) injection in male Wistar rats. Rats were divided into five groups: healthy controls with no isoproterenol treatment and groups receiving subcutaneous (s.c) injection of isoproterenol at a dose of 85mg/kg, 120mg/kg, 150mg/kg, and 200mg/kg. Twenty-four hours following the injection, rat's blood was taken through lateral veins to measure the CK-MB and LDH levels, the biomarkers of myocardial infarction. A necropsy was also performed to obtain the heart for histopathological examination. It was found that isoproterenol increased serum CK-MB and LDH levels. Moreover, histopathology examination showed a significant myocardial injury in groups treated with isoproterenol starting at the dose of 85 mg/kg. The severity of myocardial infarction intensified with the increase of dose. Mild to moderate injury was found in group treated with 85 mg/kg and 120 mg/kg, severe damage with 150 mg/kg and very severe damage was found in 200 mg/kg group. It is concluded that the s.c injection of ISO produced myocardial injury after 24 hours in rats in a dose response manner.

1. INTRODUCTION

Ischemic heart disease (IHD) is a condition where the blood supply and oxygen are not sufficient for cardiac muscle need [1]. The obstructive plaque blocking blood flow through the coronary arteries will trigger IHD [2]. This disease manifests as myocardial ischemia (myocardial infarction). Myocardial ischemia is one of the main causes of death globally. Indeed, it has remained as the top ten causes of death for more than two decades. In 2017 alone, myocardial ischemia has claimed 9 million deaths worldwide [3]. The risk factors for ischemic heart disease include high cholesterol, hypertension, diabetes mellitus, obesity, lack of physical activity, smoking, alcohol, stress, old age, diet, family history and post menopause [4].

One of many causes of IHD is atherosclerotic plaque. Atherosclerosis on its way to myocardial ischemia will involve several processes, i.e., endothelial dysfunction, inflammation, vascular proliferation, and matrix changes [5]. Atherosclerosis is initiated by trigger factors that damage the structure and function of vascular endothelium, causing endothelial dysfunction [1], [6]. Endothelial dysfunction causes an increase in the permeability of the endothelial membrane, so that LDL easily enters the tunica intima, eventually LDL accumulates in the tunica intima. The accumulated lipoprotein will undergo oxidation and glycation to form oxidized LDL [4]. Normally, the endothelium has a low affinity for leukocyte adhesion. However, the presence of glycated and oxidized LDL will trigger the release of leukocyte adhesion molecules, thereby facilitating the binding between monocytes and T lymphocytes to endothelial cells that allows monocytes and lymphocytes to enter the tunica intima [1], [4]. In the tunica intima, monocytes turn into macrophages. Macrophages increase the expression of transport receptors on the endothelial surface that facilitate lipoprotein uptake. Then, lipoproteins are stored and will be oxidized by macrophages [4]. After that, macrophages will engulf the oxidized LDL. Macrophages ingest oxidized LDL because there has been a structural change in LDL so that LDL is detected as a foreign body. Eventually, the macrophages will have a fat load, and these are what are referred to as foam cells. Some foam cells in intima lesions will die by the process of apoptosis. Collections of foam cells that have a fat load will become fatty streaks, which are the basis of atherosclerotic plaques [1]. On the other hand, smooth muscle cells, which are normally located in the tunica media, migrate to the tunica intima. This is caused by endothelial dysfunction and the presence of cytokines or chemokines secreted by macrophages, so all of these factors will trigger smooth muscle cell migration to the tunica intima. While in the tunica intima, smooth muscle cells will have the ability to secrete the extracellular matrix (collagen, proteoglycans, and elastin) which are responsible for maintaining the integrity of the arterial walls. Inflammation that occurs in the vascular wall will cause overexpression of extracellular matrix proteins to form a fibrous cap that will cover mature atherosclerotic plaques [4]. Arteries that continue to accumulate plaque will enlarge to prevent luminal stenosis, at this stage there are no symptoms. Luminal stenosis will only occur if plaque growth overrides the expansion of the artery. If the plaque is unstable, it will rupture, causing acute coronary syndrome or myocardial ischemia [4].

Several methods can be used to induce myocardial ischemia, one of which is by using drugs that can trigger myocardial ischemia. Isoproterenol (ISO) is one of the chemicals that are widely used as an infarction-inducing agents since it can create myocardial injury in animal model with similar features with myocardial infarction in humans [7], [8]. The mechanisms of isoproterenol in inducing cardiac necrosis include increased oxygen consumption associated with myocardial hyperfunction due to increased chronotropism and inotropism as well as coronary hypotension [7]. Another mechanism, i.e., increased accumulation of calcium (Ca^{2+}) [8], isoproterenol increases Ca^{2+} overload in the cytosol where the ion is also associated with activation of adenylate cyclase enzyme and decreased ATP levels during isoproterenol administration. Finally, there is an increase in oxidative stress due to several metabolic products derived from isoproterenol and triggers the formation of free radicals [7]. The mechanism of cardiotoxicity and myocardial infarction due to isoproterenol is mainly through the formation of highly cytotoxic free radicals through catecholamine autoxidation [9]. Oxidation of catecholamines forms quinoids which lead to the production of superoxide anions and hydrogen peroxide. In the presence of iron, these two substances will form highly reactive hydroxyl radicals and damage proteins, lipids, DNA and increase the size of myocardial infarction [8]. This study aimed to perform a dose response study on isoproterenol-induced myocardial infarction through serum biomarker and histopathological analysis.

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

Isoproterenol hydrochloride (USP Cat.) was obtained from Sigma Chemical Co., NaCl 0.9 percent, Formaldehyde 10% in Phosphate Buffer Solution, Alcohol 70%, Ether, Aquadest, Hematoxylin and Eosin (H&E).

2.2 Experimental Animals

Experimental animals used in this research is wistar rats (180-300 g). Animals were cared for according to laboratory management standards at the Biopharmaceutical Laboratory at Hasanuddin University with a temperature of 25±2^{o}C and 12-hour day-night cycle. Experimental animals were fed standard pellets and given water *ad libitum*. This research protocol has been approved by the Health Research Ethics Commission Faculty of Medicine – Hasanuddin University Education Hospital – Central General Hospital Dr. Wahidin Sudirohusodo Makassar and has received a recommendation for ethical approval with No. 730/UN4.6.4.5.31/PP36/2021.

2.3 Experimental protocols

Rats were divided into five group: isoproterenol 85 mg/kg, 120 mg/kg, 150 mg/kg, 200 mg/kg and a control without isoproterenol. Isoproterenol was injected subcutaneously. After 24 hours, rat blood was withdrawn from the lateral veins and the hearts were harvested, immediately washed with saline, and fixed in formaldehyde for 48 hours.

2.4 Serum Biochemical Analysis

The blood samples were collected in vacutainer tubes, centrifuged with 300 rpm for 20 minutes. The standard kit was used to determine the level of Creatine-Kinase Myocardial Band (CK-MB) and Lactate Dehydrogenase (LDH) (Human Diagnostic®) in the serum using a Humalyzer 3500 instrument.

2.5 Histopathological Examination

The heart tissue was made in paraffin blocks and cut into several parts with a thickness of 5 µm. Each part was stained with hematoxylin and eosin (H&E). The feature of heart histology was observed by using a microscope. Myocardial injury was categorized as normal if there is no or very minimum damage; mild if the damage is less than 25 percent, moderate if the damage is 25-50 percent, severe if the damage is 50-75 percent; and very severe if the damage is more than 75 percent [10].

3. RESULTS

3.1 Biochemical Parameters

The mean value of CK-MB level of the control rats was 20,6 U/L. While, those given isoproterenol 85 and 120 mg/kg had increased CK-MB value of 135.7 U/L and 120.2 U/L, respectively. Indeed, increasing the dose of ISO to 150 mg/kg led to CK-MB value of 187.1 U/L. Meanwhile, with the 200 mg/kg ISO treatment, the CK-MB value remarkably increased to 1037 U/L. Based on the result, the highest CK-MB value was showed by 200 mg/kg ISO group, which elevated more than 50 times of the normal value.

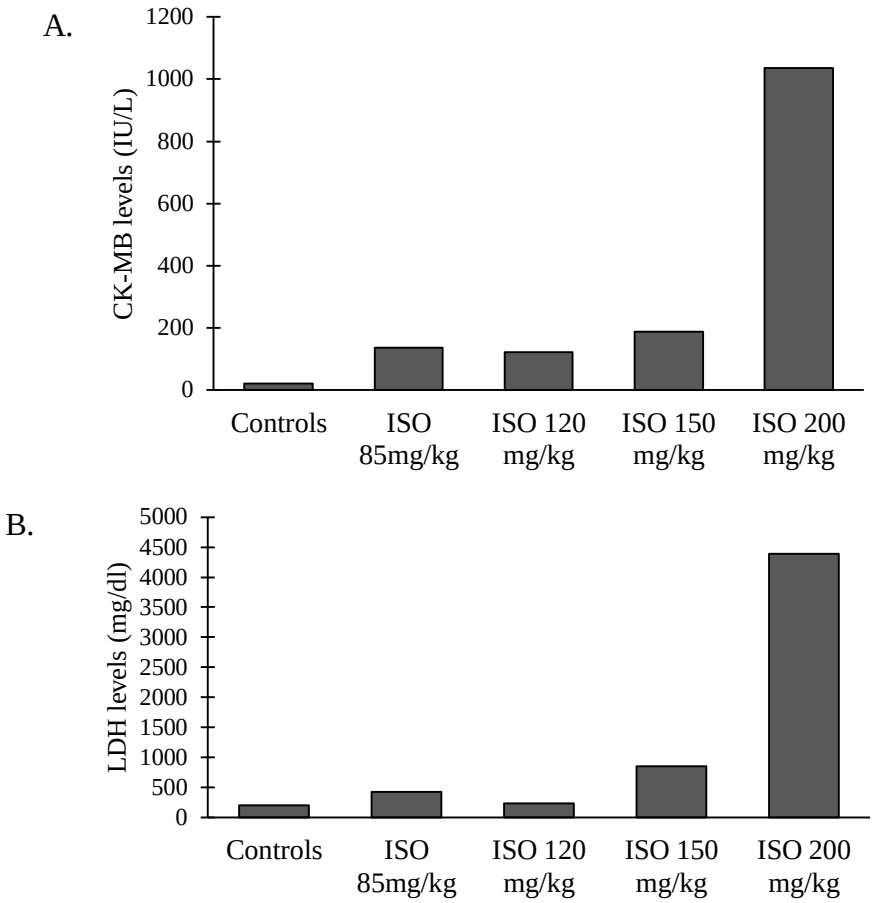

Figure 1. Mean levels of CK-MB (A) and LDH (B) in control rats and those treated with isoproterenol.

The mean value of LDH for the healthy control was 197 mg/dl. The rats injected with isoproterenol 85 mg/kg had LDH value of 429.9 mg/kg, ISO 120 mg/kg had LDH value of 229.8 mg/dl, and ISO 150 mg/kg group had CK-MB value of 850.7 mg/dl. Meanwhile, the 200 mg/kg ISO group had LDH value of 4387 mg/dl. Again, the highest LDH level was achieved by the 200 mg/kg ISO group, which elevated more than 20 times of the normal value.

3.2 Histopathological Parameters

The result showed that the rats that were not given ISO (Figure 2.) did not show any myocardial damage. The doses of ISO 85 mg/kg (Figure 3.) and ISO 120 mg/kg (Figure 4.) on histological examination showed a mild to moderate damage. More severe damage was found at ISO 150 mg/kg (Figure 5.), and very severe damage was found in ISO 200 mg/kg group (Figure 6.). It is apparent that the damage to myocardial structure due to s.c injection of ISO occurred in a dose dependent manner.

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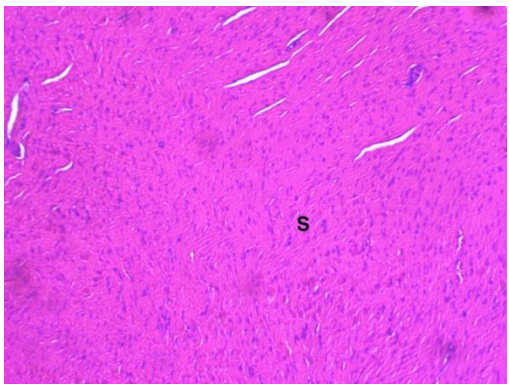

Figure 2. The microphotograph of myocardial tissue of control rat. No abnormality was found.

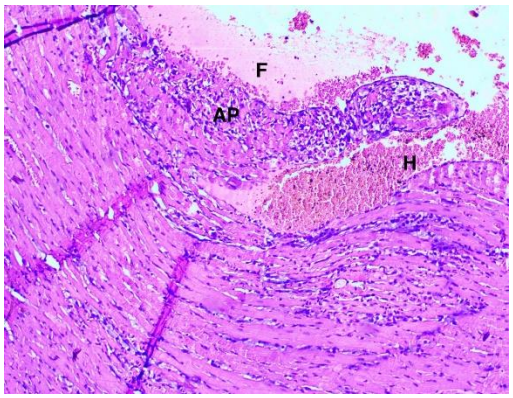

Figure 3. The microphotograph of myocardial tissue of rats treated with ISO 85 mg/kg. Hemorrhage (H), apoptosis (AP), and fibrin (F) were visible with mild to moderate damage.

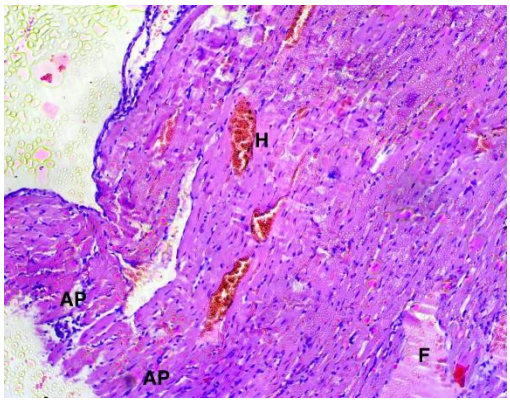

Figure 4. The microphotograph of myocardial tissue of rats treated with ISO 120 mg/kg. Hemorrhage (H), apoptosis (AP), and fibrin (F) were visible with moderate damage.

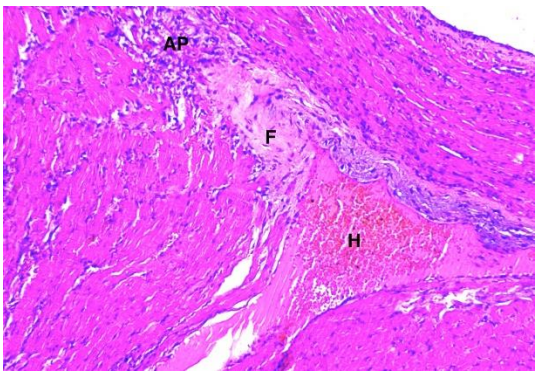

Figure 5. The microphotograph of myocardial tissue of rats treated with ISO 150 mg/kg. Hemorrhage (H), apoptosis (AP), and fibrin (F) were visible with severe damage.

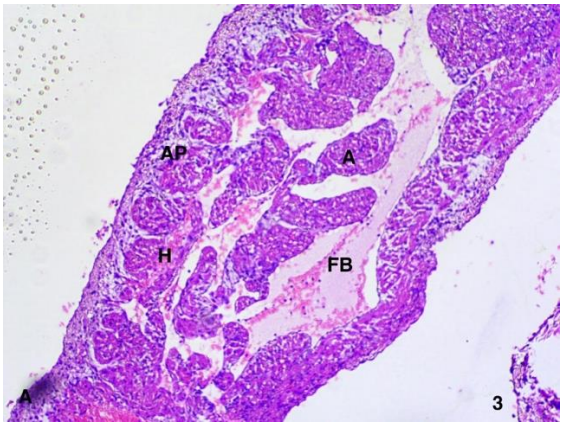

Figure 6. The microphotograph of myocardial tissue of rats treated with ISO 200 mg/kgBW, Hemorrhage (H), atrophy (A), apoptosis (AP), and fibrin (F) were visible with very severe damage.

4. DISCUSSION

The heart has a crucial role for human body. The heart works by pumping blood carrying oxygen and nutrition and removing the cellular metabolism waste, such as carbon dioxide and toxic metabolites. Heart's oxygen demand can increase in certain conditions, such as stress, disease, increased physical activity, and increased metabolic demand [11], [12]. When the heart's oxygen supply did not match with the oxygen demand, the ischemic state may occur [13]. Prolonged ischemia due to organs or tissues can cause organ or tissue injury characterized by cellular necrosis [14]. In the heart, ischemic condition may lead to myocardial infarction. This research was conducted to determine a dose response study on myocardial infarction induced by an inducer, isoproterenol, in male Wistar rats. To evaluate the damage in heart tissue, several cardiac biomarkers, such as CK-MB and LDH, were used as predictors to support histopathological observation. CK-MB is a biomarker of necrosis in cardiac myocytes and widely use to diagnose myocardial infarction [6]. Besides CK-MB levels, LDH is also a good predictor of myocardial infarction. LDH is one of the inflammation mediators and the marker of cell damage that can be used as prognostic and monitoring tools, as well as disease progression. The increasing level of LDH is also found in doxorubicin-treated animals that experience myocardial injury [15], [16].

The rats received isoproterenol 85-120 mg/kg had CK-MB levels up to six times and LDH two times higher than the healthy control group. This indicates that the induction of myocardial ischemia using isoproterenol had a significant effect on CK-MB and LDH levels in experimental animals. Indeed, increasing the dose to 200 mg/kg triggered a remarkable elevation of both biomarkers for up to 50 and 20 times than the normal

level. These results are in line with the other studies that also found the administration of isoproterenol can trigger myocardial ischemia [17], [18].

Histological observation of normal cardiomyocytes showed a transversely shaped-like muscles, with 1-2 nuclei in each cell, and the nucleus was pale and located in the middle. The intercalary discs that cross the muscle fibers was noticeable (Figure 2). Surrounding the cells, endomysium connective tissue was present, where fibroblast nuclei in the endomysium can also be found [19]. When myocardial ischemia occurs, the cardiomyocyte structures was changed, which mostly shown the presence of coagulative necrosis, i.e., cell death due to ischemia which causes denaturation of protein structures (Figure 3). In addition, the neutrophil influx was evident, followed by loss of the nucleus, phagocytosis by macrophages, and formation of granulation tissue at the periphery (Figure 4,5). Other changes that were found including edema, bleeding (hemorrhagic), apoptosis, fibrin, and plasma hypereosinophilia (Figure 6). Isoproterenol-induced myocardial ischemia usually shows cardiac muscle fibers with muscle separation, edema of the intramuscular spaces, and the presence of inflammatory cells [20]. In our study, a very severe damage of the heart cells was found with the highest dose of isoproterenol.

Isoproterenol is a potent synthetic catecholamine, when given at high doses, it produces lesions resembling myocardial infarction on microscope, and it may also lead to sudden death in the experimental animals. High doses of catecholamines cause myocardial necrosis as they promote oxidative reaction, and the oxidative products cause necrosis and contractile failure in the rat's heart [21]. Auto-oxidation of catecholamines also generates highly cytotoxic free radicals that initiate the peroxidation of membrane-bound PUFAs, leading to altered myocardial membrane permeability, intracellular calcium overload, and irreversible tissue damage [21]. The damage of myocardial cells causes the heart membrane to become more permeable and rupture, resulting in a leakage of intracellular enzymes, such as CK-MB and LDH. Therefore, CK-MB and LDH are important biomarkers of myocardial infarction, especially CK-MB, because they are abundant in myocardial tissue and almost absent in other tissues [9].

There are several mechanisms involved in the pathogenesis of isoproterenol. First, isoproterenol induces overstimulation of β-adrenoceptors which causes an increase in oxygen consumption and ultimately results in an imbalance between oxygen supply and oxygen demand of cardiac muscle cells, resulting in necrosis of the heart [10]. The mechanism of myocardial ischemia by isoproterenol also involves a calcium overloading process which would increase the Ca2+ overload in the cytosol [7]. Moreover, various signaling pathways such as NFκB, mitogen-activated protein kinase (MAPK) such as p38 are activated and augment cell death [17].

5. CONCLUSION

It can be concluded that the isoproterenol may induce a myocardial infarction in male rats. The doses used in this study, from 85 to 200 mg/kg, were demonstrated to be able to induce myocardial injury, and the intensity of damage was dependent on the dose used. The higher the dose the higher the biomarker levels and the more intensive the injury found.

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