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Potential of bovine lactoferrin as an inhibitor of cardiomyocyte apoptosis in Dexamethasone-induced hypertensive Sprague dawley rats

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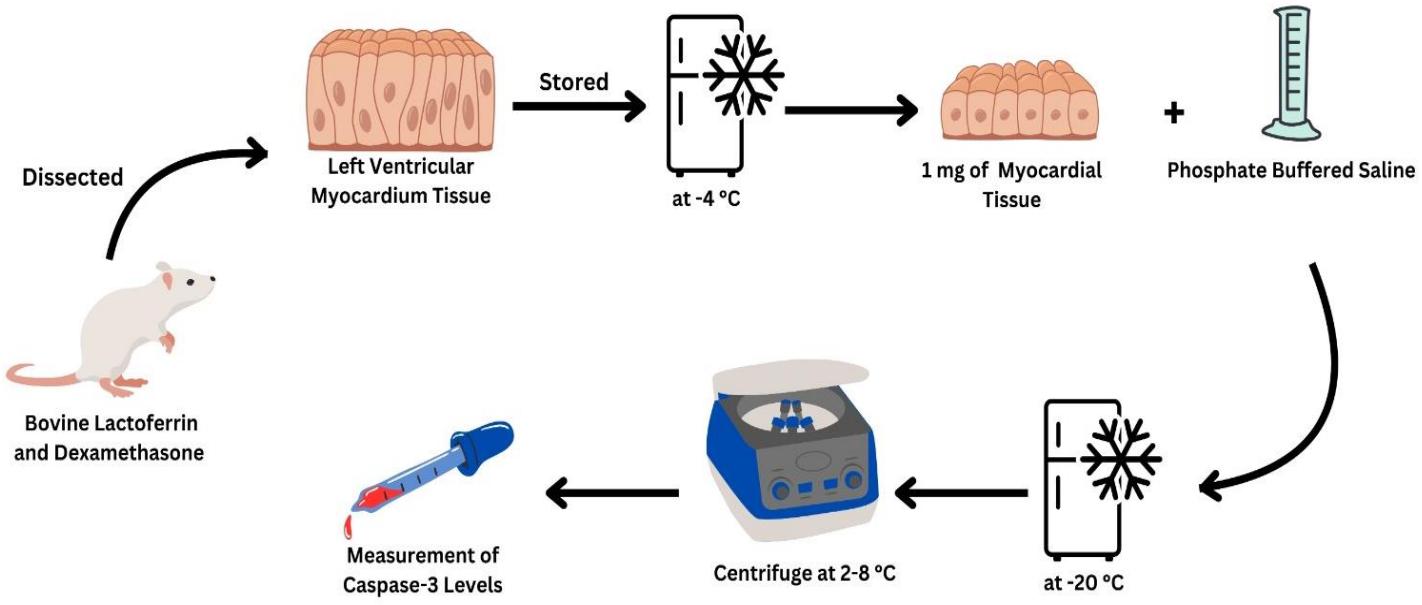
ABSTRACT

Elevated blood pressure, also known as hypertension, significantly increases the risk of developing heart disease. It can damage heart muscle cells and trigger apoptosis (cell death). Antioxidants like lactoferrin can help protect the heart from damage by reducing the generation of reactive oxygen species (ROS), harmful molecules that can damage cells. This study aims to evaluate the efficacy of lactoferrin in alleviating apoptosis in cardiomyocytes by assessing caspase-3 levels. This study randomly assigned 32 rats into 6 groups: control, dexamethasone, positive control, and 3 lactoferrin groups (100, 300, and 500 mg/kg BW/day). All groups except control received dexamethasone for 14 days. On day 14, rats were euthanized, and heart muscle tissue was collected. Caspase-3 levels were measured and analyzed using One-Way ANOVA. The average caspase-3 enzyme levels in the lactoferrin-treated groups with 100, 300, and 500 mg/kg BW/day were 2,605.0, 2,720.9, and 2,518.8 ng/mL, respectively.



A numerical rise in caspase-3 levels was recorded in all three lactoferrin-treated groups compared to the dexamethasone-treated group (negative control), and the difference reached statistical significance (p=0.041). While this study hints at a potential protective effect of lactoferrin against cell death in rat heart muscle cells, more research with larger groups of animals and more extended treatment periods is needed to confirm its effectiveness and determine the ideal dosage.

Keywords: Apoptosis, Cardiomyocytes, Caspase-3, Hypertension, Lactoferrin.

INTRODUCTION

According to the World Health Organization (WHO), hypertension was identified as a significant contributor to cardiovascular disease, affecting a substantial population of approximately 1.13 billion individuals in 2015 [1]. Hypertension is the most prominent risk factor for disease burden in Southeast Asia, East Asia, and Oceania [2]. The Basic Health Research documented the prevalence of hypertension in Indonesia at 34.1%, indicating a substantial number of cases estimated at 63,309,620 [3]. This rise in hypertension is accompanied by an increase in other vascular risk factors, including diabetes, obesity, smoking, insufficient physical activity, and stroke prevalence.

Ezzati et al, [4]. Asserted that elevated blood pressure is a key factor contributing to global morbidity and mortality. Hypertension has been shown to induce damage to target organs, with the heart being particularly susceptible. Increased blood pressure reduces arterial elasticity and can damage the wall [5]. This damage facilitates the accumulation of cholesterol and fat on these lesions, ultimately leading to the blockage of blood vessels. This process forms the basis for much of the organ damage attributed to hypertension [6].

In heart conditions such as coronary artery disease or a heart attack (myocardial infarction), the heart muscle may undergo remodeling due to the sustained damage it experiences. This remodeling can result in alterations in the size, shape, and function of the heart chambers and the thickness and elasticity of the heart walls. The phrase 'remodeling' was initially introduced to describe the response of healthy heart tissue to localized damage and the progression from an acute heart attack to long-term heart failure [7, 8]. Two types of remodeling are associated with hypertension: vascular remodeling and cardiac remodeling [9-11].Vascular remodeling pertains to structural changes in the blood vessels, particularly the arteries, in response to chronically high blood pressure. Conversely, cardiac remodeling involves structural changes within the heart muscle [12].

Cardiac remodeling occurs due to irreversible damage to heart muscle cells, known as cardiomyocytes, and has been demonstrated to be associated with cardiomyocyte apoptosis [13]. Cardiomyocytes are crucial in the heart's ability to contract and pump blood. In chronic conditions such as hypertension, the heart undergoes structural and functional changes to cope with the increased workload [14, 15]. The apoptosis of cardiomyocytes during cardiac remodeling has significant consequences for the overall function of the heart. The loss of functional heart muscle cells can impede the heart's capacity to contract effectively and pump blood, resulting in reduced cardiac output and an elevated risk of heart failure [16]. Oxidative stress, induced by the generation of reactive oxygen species (ROS), is a contributing factor to apoptosis in individuals with hypertension [17]. Studies have consistently shown an elevated production of ROS in both experimental models and human hypertension [18]. The term ROS encompasses O2-derived free radicals, including superoxide anion ($O2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), peroxyl ($RO2^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals, as well as nonradical species derived from O2, such as hydrogen peroxide (H2O2) [19]. Reactive oxygen species play a vital role in the pathophysiology of hypertension. This is supported by evidence showing increased oxidative stress in hypertension and the efficacy of antioxidants or agents inhibiting NAD (P) H oxidase-driven ROS generation in reducing or even preventing elevated blood pressure in hypertensive animals [18]. Antioxidants, compounds capable of neutralizing ROS, are considered a promising therapeutic option due to their mechanism of action. For example, lactoferrin is a glycoprotein that enhances antioxidant function in the human body [20]. However, as of now, there is no research confirming the potential of bovine lactoferrin as an inhibitor of hypertension-induced cardiomyocyte apoptosis. Therefore, this study aims to examine the effects of lactoferrin on hypertension-induced cardiomyocyte apoptosis by testing the activity of caspase-3, a quantitative enzyme involved in the apoptosis process.

MATERIALS AND METHODS

Research Design

This research employed an experimental design with animal subjects. It was conducted at the Animal House of the Faculty of Medicine and Health Sciences, the Pharmacology Laboratory of the Faculty of Medicine and Health Sciences at Universitas Katolik Indonesia Atma Jaya, and the Integrated Laboratory of the Faculty of Medicine, Universitas Indonesia. The research occurred from May to December 2021, and ethical clearance was obtained under reference number 225/UN21.FK/EC/2021.

Subjects

Thirty-two Sprague Dawley rats, obtained from the Indonesian Food and Drug Authority, were used as subjects in this study. They were male, one and a half months old, and weighed 200-250 g. All rats were acclimatized for 7 days in cages with a standard diet for rats and distilled water as their drink. The mouse subjects were categorized into 6 groups, as explained in Table 1.

Table 1: Group tests of rats

Group Type	Treatment Details				Total Subject
	Standard Food and Drink	**Dexamethasone (Injection)**	**Amlodipine (Oral)**	**Bovine Lactoferrin (Oral)**	
Placebo Control Group	Yes	No	No	No	8
Negative Control	Yes	Yes (day 0 to 14) Dose = 0.1 mg/kg BW/day	No	No	8
Positive Control	Yes	Yes (day 0 to 14) Dose = 0.1 mg/kg BW/day	Yes (day 8 to 14) Dose = 500 mg/kg BW/day	No	4
Test Group I	Yes	Yes (day 0 to 14) Dose = 0.1 mg/kg BW/day	No	Yes (day 8 to 14) Dose = 100 mg/kg BW/day	4
Test Group II	Yes	Yes (day 0 to 14) Dose = 0.1 mg/kg BW/day	No	Yes (day 8 to 14) Dose = 300 mg/kg BW/day	4
Test Group III	Yes	Yes (day 0 to 14)Dose = 0.1 mg/kg BW/day	No	Yes (day 8 to 14) Dose = 500 mg/kg BW/day	4

Intervension Substances

Pure bovine lactoferrin (99%) was obtained from Xi'an Ruisaen Biotechnology. The powdered preparation was emulsified with distilled water and formulated into various doses according to the test groups (100, 300, and 500 mg/kg BW). Different doses of bovine lactoferrin were administered orally to the rats using a feeding tube. Additionally, dexamethasone was obtained from Phapros, Tbk. A 1 mL syringe was used to draw the liquid product, which was injected subcutaneously into the rats at the prescribed dosage. Amlodipine was obtained from Phapros, Tbk. A 10 mg tablet of amlodipine was dissolved in water to prepare a 500 mg/kg concentration. This solution was then administered orally to the rats using a probe. Amlodipine was utilized as a positive control in the study due to its established efficacy in lowering blood pressure and improving blood flow. It serves as a standard reference against which the effects of other interventions could be compared.

Sample Preparation of Tissues

Rats were dissected to obtain the left ventricular myocardium tissue, which was then placed in organ tubes and stored at a temperature of -4 °C. A total of 1 mg of mouse myocardial tissue was rinsed with 1x Phosphate Buffered Saline (PBS), followed by homogenization in 1 mL of 1x PBS in a homogenizer and stored overnight at a temperature of -20 °C in a freezer. Subsequently, two freeze-thaw cycles were conducted to disrupt the cell membranes. The resulting homogenate was then centrifuged for 5 minutes at a speed of 5000xg at a temperature of 2-8 °C in a centrifuge.

Measurement of Caspase-3 Levels

The Cusabio Rat Caspase 3 ELISA Kit, obtained from Cusabio Technology LLC, was used for the assay. A 100 µL standard solution was added to each microwell, covered, and incubated for 2 hours at 37 °C. The liquid was then discarded, and 100 µL of biotin-antibody (1´) was added to each well, followed by incubation for 1 hour at 37 °C. Each well underwent three washes with 200 µL of wash buffer. Next, 100 µL of HRP-avidin (1´) was added to each well and incubated again for 1 hour at 37 °C. Subsequently, the wells were rinsed with 200 µL of wash buffer, and 90 µL of tetramethylbenzidine (TMB) substrate was added to each well. The microwell plate was incubated for 15-30 minutes at 37 °C, and then 50 µL of stop solution was added. The final step involved reading the microplate at 450 nm for 5 minutes.

Statistical Analysis

Caspase-3 level analysis was conducted to compare the test groups. Data analysis began by testing the normality of the data employing the Shapiro-Wilk normality test due to the small sample size, which was below 30 data points. If the data distribution was normal, the overall data was tested using the One-Way ANOVA test. If the data distribution was non-normal, the Kruskal-Wallis test was used. Statistical analyses were conducted utilizing IBM SPSS Statistics 25.0 software. A significance level of $p < 0.05$ was considered statistically significant.

RESULTS

The caspase-3 levels of the control group without treatment, positive control, and negative control were compared using an independent-sample t-test to ensure the validity and reliability of the caspase-3 data. Table 2 shows the caspase-3 enzyme levels in the control groups, indicating that the positive control at day 14 obtained the highest content of caspase-3 enzyme levels. Surprisingly, this group at day 7 showed the lowest caspase-3 enzyme levels. Furthermore, Table 3 presents paired sample t-tests for all control groups. The results of this test demonstrated that the control group without treatment, positive control, and negative control functioned as expected and can be used for comparison with the various test groups.

Note: Total rats used in day 7 and 14 were four for placebo and negative controls, while for positive control were two rats for day 7 and 14.

Table 2: Comparison of caspase-3 enzyme levels in control groups

Group	Day	Mean Caspase-3 Level (ng/mL)
Placebo Control Group	7	2,549.250
	14	2,576.250
Positive Control	7	2,402.625
	14	2,891.125
Negative Control	7	2,438.000
	14	2,438.000

Note: Total rats used in day 7 and 14 were four for placebo and negative controls, while for positive control were two rats for day 7 and 14.

Table 3: Significance value of paired-sample t-test in control groups

Pair	Significance Value
Placebo Control Day 7 – Negative Control Day 7	0.049*
Placebo Control Day 14 – Negative Control Day 14	0.047*
Placebo Control Day 14 – Positive Control Day 14	0.029*
Negative Control Day 14 – Positive Control Day 14	0.044*

Note: *means that the capcase-3 level between groups is significantly different; Total rats used in day 7 and 14 were four for placebo and negative controls, while for positive control only in day 14 with a total of two rats.

Table 4: One-way ANOVA test for caspase-3 enzyme levels among test groups compared to positive control

Group Test	Mean Caspase-3 Level (ng/mL)	Significance Value
Positive Control Day 14	2,891.125	0.937
Test Group I Day 14	2,605.000	
Test Group II Day 14	2,720.875	
Test Group III Day 14	2,518.750	

Table 4 demonstrates no significant difference in caspase-3 enzyme levels among the test groups administered bovine lactoferrin at 100, 300, and 500 mg/kg/day compared to the positive control on the 14th day (p = 0.937). Therefore, bovine lactoferrin may yield comparable results to amlodipine in increasing caspase-3 levels, as indicated by the absence of significant differences observed in the data.

Table 5: One-way ANOVA test for caspase-3 enzyme levels among test groups compared to negative control

Group Test	Mean Caspase-3 Level (ng/mL)	Significance Value
Negative Control Day 14	2,438.000	0.041
Test Group I Day 14	2,605.000	
Test Group II Day 14	2,720.875	
Test Group III Day 14	2,518.750	

Table 5 demonstrates the significant difference (p = 0.041) in caspase-3 enzyme levels between the test groups and the negative control group. This indicates that the utilization of bovine lactoferrin has demonstrated a substantial positive influence on the level of caspase-3.

DISCUSSION

In this study, the measurement of the caspase-3 enzyme was conducted on Sprague Dawley rat cardiomyocyte tissue induced with hypertension using a dexamethasone preparation. The research findings indicate that dexamethasone can induce cardiomyocyte apoptosis in experimental animals. This is evidenced by the testing of caspase-3 enzyme levels on the 14th day, particularly in the positive control group. Dexamethasone is among the compounds that induce hypertension by stimulating mineralocorticoid receptors, leading to sodium and fluid retention in the kidneys. Additionally, increased activity of the Renin-Angiotensin-Aldosterone System (RAAS), cardiac hypercontractility, and endothelial dysfunction may contribute to the hypertensive effect [25, 26]. Furthermore, the occurrence of hypertension is further influenced by decreased nitric oxide levels and increased oxidants [27]. It is this hypertensive effect that ultimately induces cardiomyocyte apoptosis [28].

Given its demonstrated anti-apoptotic effects, this study proposes bovine lactoferrin as an antioxidant compound. In a study by Xue et al, [29]. Lactoferrin administration inhibited chondrocyte apoptosis. Apoptosis is a programmed cell death activity to maintain homeostasis between cell formation and cell death. Detecting apoptosis in cells can be achieved through morphology and biochemical methods [21, 22]. One example of a biochemical method is the analysis of DNA fragmentation, changes in the cell plasma membrane and organelles, and the measurement of the caspase-3 enzyme [23]. The levels of caspase-3 enzyme can be used to detect cardiomyocyte apoptosis, as this enzyme is a key executor of the apoptosis process in general [23, 24]. The mechanism involves the induction of CREB1 phosphorylation, which has proven anti-apoptotic effects, as evidenced by a significant decrease in caspase-3 levels in the lactoferrin intervention group compared to the control group. Beyond its anti-apoptotic properties, lactoferrin also exhibits cellular and tissue repair effects. Experimental studies in mice with osteoarthritis (OA) have shown that lactoferrin induces cartilage tissue repair [29].

Furthermore, research by Fernández-Musoles et al,. Has demonstrated that lactoferrin possesses anti-hypertensive effects by inhibiting the action of angiotensin I on its receptors. This leads to a reduction in the conversion of angiotensin me to angiotensin II, consequently diminishing the vasoconstrictor effects of angiotensin II. Supporting this, Safaeian et al, [27]. Provided evidence that the administration of bovine lactoferrin significantly reduces blood pressure in rats induced with hypertension using dexamethasone for 14 days.

Based on the data obtained, the researchers concluded that administering dexamethasone for 14 days at a dose of 0.1 mg/kg/day is sufficient to induce hypertension in the test rats. Drawing on the study by Wang et al, [27]. And various existing theories, hypertension

is known to trigger cardiomyocyte apoptosis. This conclusion is substantiated by the results of our study, revealing a significant difference in mean caspase-3 levels between the untreated control and the negative control, indicating a significant occurrence of apoptosis in the group receiving dexamethasone for 14 days. This finding is further supported by the positive control group (dexamethasone for 14 days and amlodipine for 7 days), which exhibits significantly different mean levels of caspase-3 enzyme compared to the negative control group (dexamethasone for 14 days). Therefore, the possibility that still exists at present is that the dosage of bovine lactoferrin or the duration of exposure has not yet reached the threshold to induce anti-apoptotic effects on the cardiomyocytes of the test rats.

The study has some limitations. The lack of a notable decrease in caspase-3 enzyme levels in the test groups may be due to the absence of induced hypertension, a limitation as blood pressure was not directly measured. Individual characteristics among the test rats could also affect the results. Additionally, the duration of bovine lactoferrin exposure is a crucial factor for establishing its anti-apoptotic effects.

CONCLUSION

This study demonstrates that administering bovine lactoferrin at 100, 300, and 500 mg/kg/day has an anti-apoptotic effect on cardiomyocytes in experimental rats. The effect is significant in the negative control, suggesting that the treatments may increase the significance of caspase-3. These findings suggest that lactoferrin has the potential as an antioxidant agent to reduce oxidative stress levels. However, further studies are needed to enhance our understanding of lactoferrin's potential as an antioxidant in vivo.

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