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Antihypertensive and renal protective effect of bovine lactoferrin in dexamethasone-induced hypertension rats

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ABSTRACT: Current antihypertensive drugs are still limited to be used as a monotherapy and their effectiveness in preventing end-organ damage is insufficient, therefore complementary therapy is needed to reduce end-organ damage and make hypertension treatment more effective. In this study, we are investigating the potential of bovine lactoferrin (BLf) as an antihypertensive and renal protective agent. 30 rats were randomly divided into 6 groups: BLf 100mg/kg, BLf 300 mg/kg, BLf 500 mg/kg, Dexamethasone, Amlodipine 1 mg/kg, and Control. Dexamethasone, Amlodipine 1mg/kg, and all BLf groups were given subcutaneous dexamethasone injections from day 1 to 14. Starting from day 8 to 14 Amlodipine 1 mg/kg and BLf groups received oral BLf or amlodipine according to mentioned doses. The results of this study demonstrated that BLf at all doses was effective in lowering systolic blood pressure, increasing serum nitric oxide (NO) level, and improving kidney function but ineffective in lowering diastolic blood pressure. BLf has the potential to be utilized as a complementary antihypertensive therapy and renal protective agent in hypertension.

KEYWORDS: bovine lactoferrin; nitric oxide; systolic blood pressure; diastolic blood pressure; renal protective; dexamethasone

1. INTRODUCTION

Hypertension is a significant challenge for global public health due to its high prevalence across the globe. It is estimated that in 2000 there were 972 million adults who had hypertension, this figure is expected to rise to 1.56 billion in 2025. Hypertension increases the risk of cardiovascular disease complications and chronic kidney disease; however, it is treatable with lifestyle changes and pharmacological therapy [1]. Most antihypertensive drugs that are currently available cannot be used as monotherapy due to limited efficacy, thus research and development of complementary drug is essential [2].

Current evidence suggests that higher dairy consumption is associated with a lower risk of cardiometabolic diseases [3]. The proteins in milk are thought to have blood pressure lowering effect [4]. Previous in vivo study suggested that one of the proteins in milk, lactoferrin had antihypertensive effect [5]. Lactoferrin is an 80 kDa protein that is produced by the mucosal epithelial cells and neutrophils of various mammals such as bovine, goat, horse, and human [6]. It is known for its antimicrobial, anti-cancer, antioxidant, immunomodulatory [6], and antiviral properties [7]. The antihypertensive mechanism of lactoferrin is not yet fully elucidated, however previous study suggested that its antihypertensive effect might be mediated by nitric oxide (NO) [5]. This hypothesis was based on a study demonstrating the hypotensive effect of bovine lactoferrin was attenuated by NO synthase inhibitor [8].

Chronic administration of glucocorticoids beyond physiological level caused hypertension both in humans and animals [9]. Previous studies showed that dexamethasone administration was associated with hypertension and diminishing NO levels [10,11]. Inhibition of NO production was linked to increased glomerular injury and renal hemodynamics impairment [12]. In another study, the administration of L-

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arginine (the precursor of NO) disrupted renal hemodynamic and renal excretory functions alterations in rats treated with NO inhibitor [13]. These findings suggest that NO has renoprotective effect.

This study aims to elucidate the antihypertensive mechanism of bovine lactoferrin and evaluate possible renoprotective effect of bovine lactoferrin (BLf).

2. RESULTS

2.1. Effects of bovine lactoferrin on systolic and diastolic blood pressure

As shown in Fig. 1a,b, the Dexamethasone group had significantly higher systolic and diastolic blood pressure compared to the Control group(*P<0.05). It was found that BLf at all 3 doses significantly lowered SBP (#P<0.05, Fig. 1a). Surprisingly, All BLf doses failed to reduce DBP significantly (Fig. 1b), only Amlodipine 1mg/kg was able to decrease DBP significantly (#P<0.05, Fig. 1b).

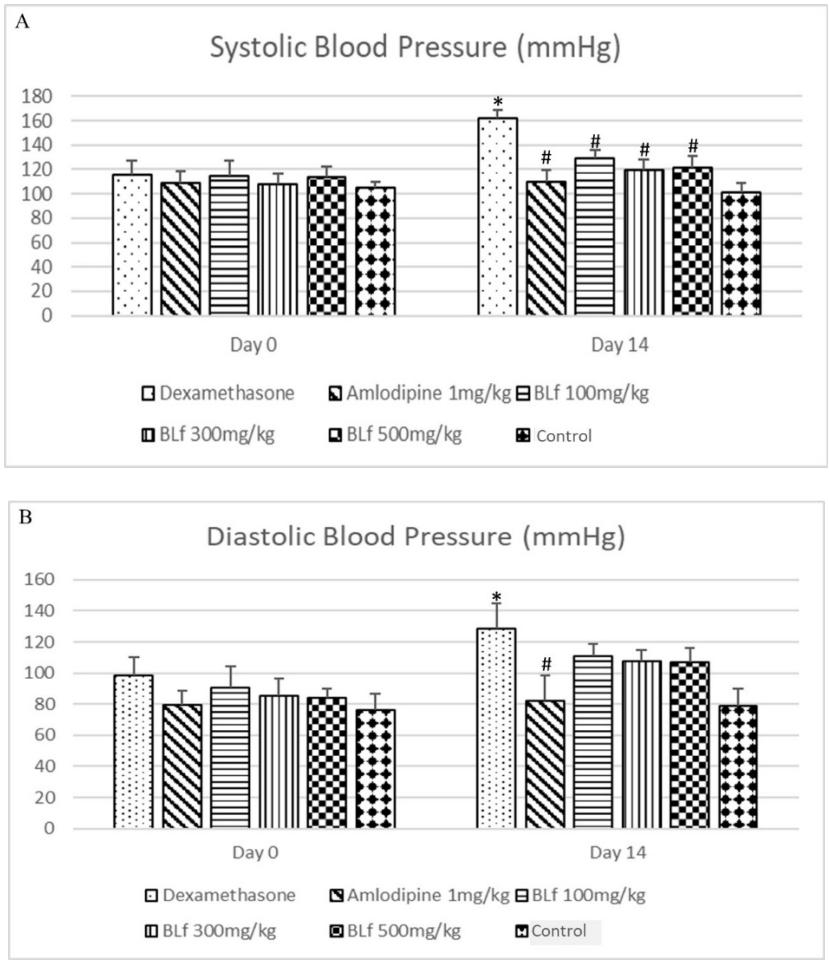

Figure 1. Effects of BLf in dexamethasone-induced hypertension rats on systolic (a) and diastolic (b) blood pressure. Values are mean±SEM (n=5), *P<0.05 compared to Control group, #P<0.05 compared to Dexamethasone group. Group comparison was done using One-Way ANOVA, followed by Tukey post hoc test.

2.2. Effects of bovine lactoferrin on heart rate

Fig. 2 revealed that the administration of dexamethasone did not significantly change HR. Blf administration at all doses and Amlodipine 1mg/kg did not have significant effect on HR.

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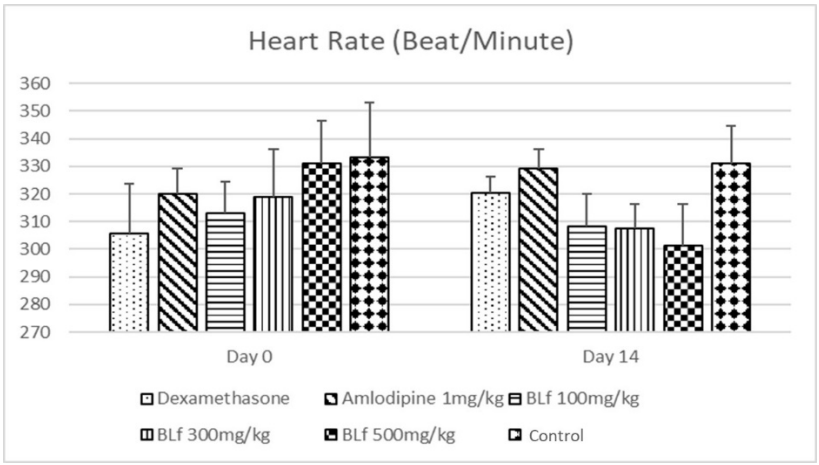

Figure 2. Effects of BLf in dexamethasone-induced hypertension rats on heart rate. Values are mean±SEM (n=5), *P<0.05 compared to Control group, #P<0.05 compared to Dexamethasone group. Group comparison was done using One-Way ANOVA, followed by Tukey post hoc test.

2.3. Effects of bovine lactoferrin on nitric oxide

As depicted in Fig. 3, compared to the Control group, Dexamethasone group had significantly lower serum NO (*P<0.05,). All Blf groups had significantly higher serum NO compared to Dexamethasone group (#P<0.05, Fig. 3).

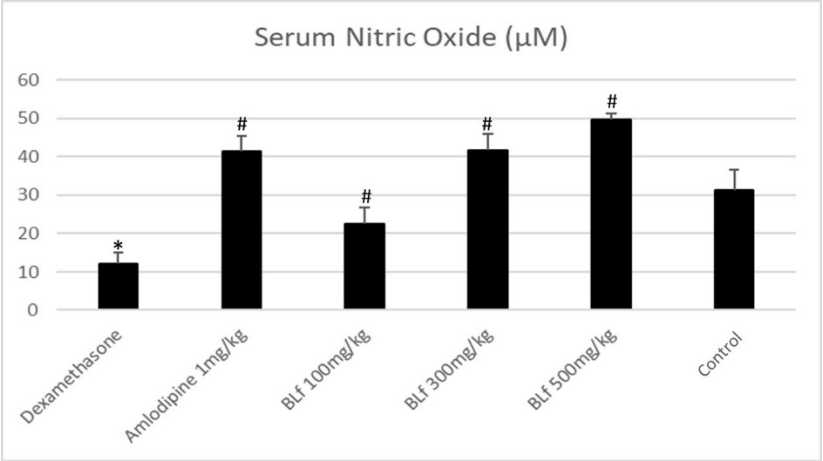

Figure 3. Effects of BLf in dexamethasone-induced hypertension rats on serum nitric oxide. Values are mean±SEM (n=5), *P<0.05 compared to Control group, #P<0.05 compared to Dexamethasone group. Group comparison was done using One-Way ANOVA, followed by Tukey post hoc test.

2.4. Effects of bovine lactoferrin on serum urea and creatinine

In Fig. 4a,b, it can be seen that Dexamethasone group had significantly higher both serum urea and creatinine compared to Control group (*P<0.05, Fig. 4a,b). Amlodipine 1mg/kg group significantly prevented increase in serum urea (#P<0.05, Fig. 4a); however, its administration failed to prevent increase in serum creatinine (Fig. 4b). BLf 100, 300, 500 mg/kg had significantly lower serum urea and creatinine compared to Dexamethasone group (#P<0.05, Fig. 4a,b).

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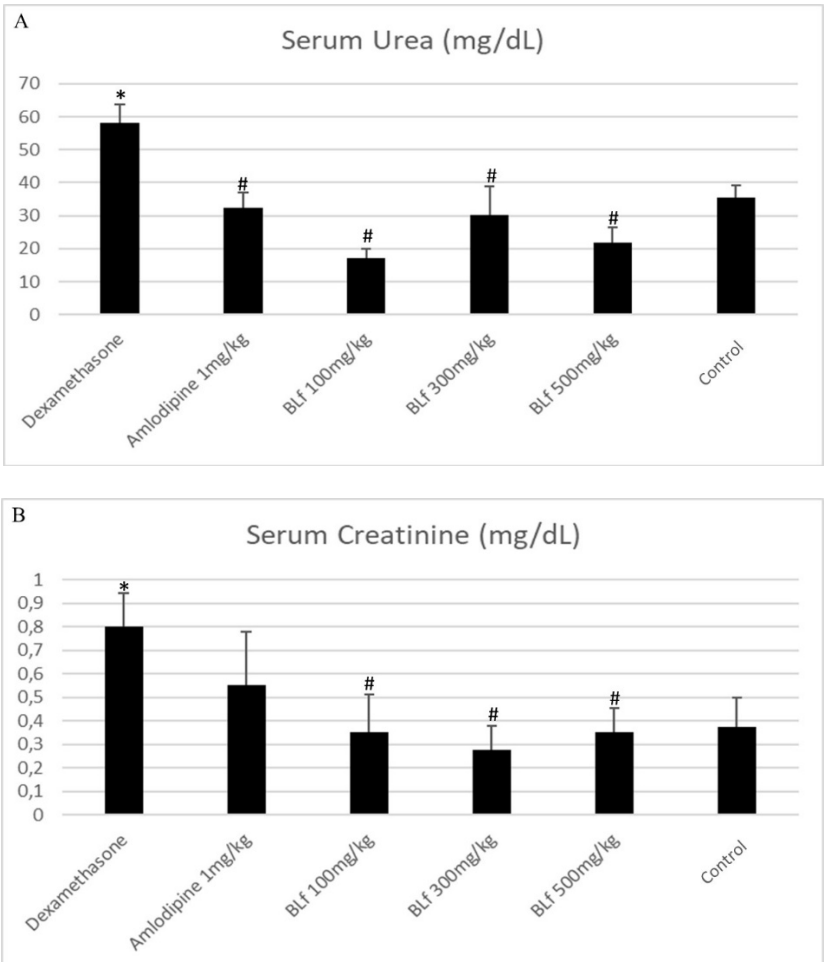

Figure 4. Effects of BLf in dexamethasone-induced hypertension rats on serum urea (a) and serum creatinine (b). Values are mean±SEM (n=5), *P<0.05 compared to Control group, #P<0.05 compared to Dexamethasone group. Group comparison was done using One-Way ANOVA, followed by Tukey post hoc test.

2.4. Effects of bovine lactoferrin on kidney histopathology

Kidney histopathological examinations are shown in Fig. 5. Dense tufts of capillaries and Bowman's space can be seen clearly in the Control group (Fig. 5a). Histological appearance of Dexamethasone group presented glomerular atrophy, karyolysis cells, pyknotic cells, swelling cells, and tubular dilatation (Fig. 5b). A dilated tubule and mesangial cell karyolysis can be seen in the histological slide of Blf 100mg/kg group, indicating renal damage (Fig. 5c). The BLf 300mg/kg also showed some tubular dilatations and pyknotic nuclei that indicate renal damage (Fig. 5d). The histological appearance in the Blf 500mg/kg group showed tubular dilatations, karyolytic necrotic cells, and pyknotic nuclei (Fig. 5e). All histological appearances of BLf groups showed renal damage to some extent; however, the damage was much milder compared to Dexamethasone group. This evidence was consistent with the serum biochemical analysis that showed all BLf groups had significantly lower serum urea and creatinine compared to Dexamethasone group. Meanwhile, the histological slide of Amlodipine 1mg/kg also showed necrotic cells, swelling cells, and pyknotic nuclei indicating renal damage (Fig. 5f).

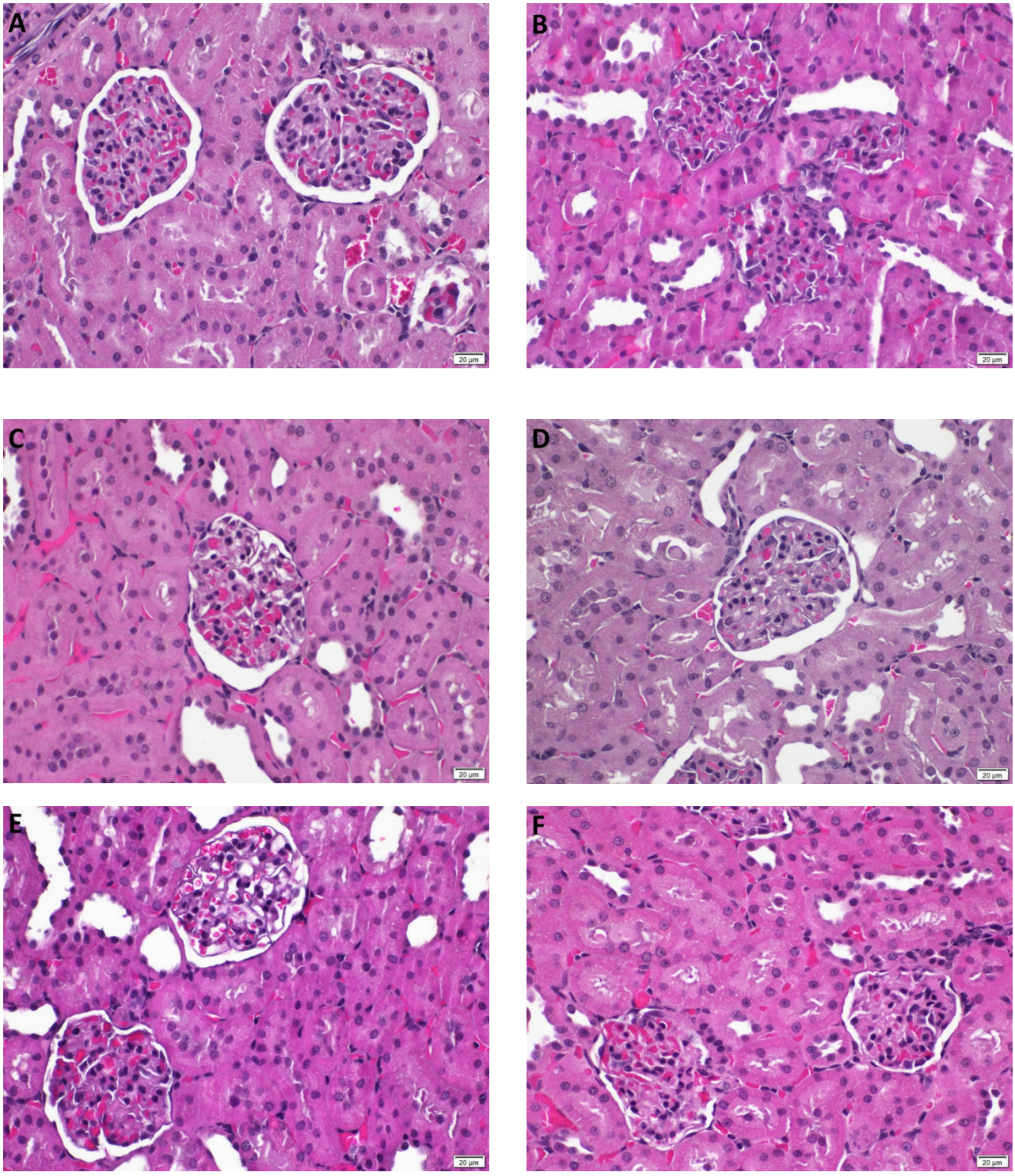

Figure 5. Effects of BLf in dexamethasone-induced hypertension rats on kidney histology (H&E x400). (a) Control, (b) Dexamethasone, (c) BLf 100mg/kg, (d) BLf 300mg/kg, (e) BLf 500mg/kg, (f) Amlodipine 1mg/kg.

3. DISCUSSION

The present study demonstrated that BLf was effective in lowering SBP; but not DBP. Previous study by Safaeian et al demonstrated that the administration of Blf at 30, 100, 300 mg/kg prevented the development of systolic hypertension in dexamethasone-induced hypertension rats [5]. Another study by Hayashida et al also showed that Blf had hypotensive effect [8]. The findings of our study were consistent with previous evidence regarding the antihypertensive effect of Blf.

We also found that serum NO increased in all BLf groups. NO which is a potent vasodilator plays a key role in maintaining vascular tone [14]. Deficiency of NO causes dysregulation in vascular tone, thus

contributing to the development of hypertension [15-17]. Dexamethasone-induced hypertension is associated with overproduction of reactive oxygen species (ROS) and the presence of antioxidants prevented oxidative stress and blood pressure from increasing. İt is suggested that the antihypertensive effect of Blf might be due to antioxidant properties of Blf since ROS is associated with the reduction of NO bioavailability [5]. Another study showed that the hypotensive effect of Blf was attenuated by the administration NO synthase inhibitor [8]. This evidence suggests that the hypotensive effect of Blf is mediated by increased NO bioavailability. We hypothesize that BLf likely stimulates NO production and prevents the interaction between ROS and NO, thus increasing NO bioavailability and subsequently decreasing blood pressure. However, the exact mechanism of increasing NO bioavailability remains to be elucidated.

In the present study, we also found that BLf administration did not affect HR. This finding is consistent with previous study that showed intravenous injection of BLf did not have significant effect on HR [8]. Based on the present and previous study, it can be concluded that the antihypertensive effect of BLf is unlike beta-blockers that decrease HR and cardiac output [17].

Currently available antihypertensive drugs such as diuretics, angiotensin-converting enzyme inhibitors, Ang II type 1 receptor blockers, beta-blockers, calcium channel blockers, and mineralocorticoids receptor antagonists are effective in curing hypertension; however, their ability to prevent end-organ damage is still limited [18]. This limitation suggests that there is an urgent need to find novel therapy that can reduce organ damage in hypertensive patients.

Renal failure is one of the most common end-organ damage caused by hypertension [19]. There is increasing amount of evidence that NO deficiency is linked to renal dysfunction [20,21]. It is believed that renal injury in hypertension is due to susceptibility of the glomerular capillary to barotrauma [21]. It has been suggested that NO-mediated vasodilatation might have a critical role in providing protective decompression, therefore preventing glomerular hypertension [22].

The result of serum biochemical analysis showed lower serum urea and creatinine level in BLf groups compared to Dexamethasone group. Serum urea and creatinine are markers to assess kidney function, high levels of these markers are associated with renal failure [23]. Our findings indicated that BLf had renal protective effect. This renal protective effect was also confirmed by histopathological appearance that showed less damage in all BLf groups compared to Dexamethasone group. This renal protective effect is likely mediated by NO through the mechanism explained previously.

Previous toxicity study has demonstrated that BLf administration up to 2000 mg/kg/day for 13 weeks did not cause any adverse reactions or animal deaths [24]. This result suggests that BLf is relatively safe. The result of this study and previous studies suggests that BLf is safe and has the potential to be utilized as a complementary therapy, especially to prevent renal damage in hypertensive patients. Further study is warranted to evaluate its biological relevance in humans.

4. CONCLUSION

In conclusion, BLf showed SBP lowering effect and renal protective effect that is likely mediated by NO. However, it was not effective in lowering DBP and affecting HR.

5. MATERIALS AND METHODS

5.1. Materials and chemicals

BLf was purchased from Xi'an Ruisaen Biotechnology Co., Ltd, China (Batch number: RSN201119 with >95% purity). Dexamethasone was purchased from Phapros, Indonesia. Amlodipine was purchased from Ifars Pharmaceutical Laboratories, Indonesia. VCl3 was purchased from Sigma-Aldrich Co., USA. N-naphthylethylenediamine and sulfanilamide were purchased from Merck KGaA, Germany. All other chemicals were of analytical grade.

5.2. Experimental animals

30 male Sprague Dawley rats aged 5 weeks old (150 ±10 g) were obtained from Badan Pengawas Obat dan Makanan (National Agency of Drug and Food Control of Indonesia). The sample size was determined by using resource equation approach as described in the literatures [25,26]. Rats were housed in standard laboratory with 12 h light/ dark cycles and room temperature (±23°C). Rats were acclimatized to the laboratory for 1 week and another 2 weeks to adapt to noninvasive tail-cuff method (described in section 5.3.1.). All rats were allowed to free access food and water. Animals experiment was done in accordance with internationally accepted guidelines for animal experimentation. This study was approved by the Ethics

Committee of School of Medicine and Health Sciences of Atma Jaya Catholic University of Indonesia (No: 11/02/KEP-FKIKUAJ/2021). The weight of all rats was measured again prior to experimentation.

30 rats were divided to 6 groups:

(1) Control (no treatment, standard diet from day 1-14)

(2) Dexamethasone (Subcutaneous dexamethasone 0.1 mg/kg from day 1-14)

(3) BLf 100 mg/kg (Subcutaneous dexamethasone 0.1 mg/kg from day 1-14, oral Blf 100 mg/kg from day 8-14)

(4) BLf 300 mg/ kg (Subcutaneous dexamethasone 0.1 mg/kg from day 1-14, oral Blf 300 mg/kg from day 8-14)

(5) BLf 500 mg/kg (Subcutaneous dexamethasone 0.1 mg/kg from day 1-14, oral Blf 500 mg/kg from day 8-14)

(6) Amlodipine 1 mg/kg (Subcutaneous dexamethasone 0.1 mg/kg from day 1-14, oral amlodipine 1 mg/kg from day 8-14)

Previous study has evaluated the antihypertensive effect of Blf at 30, 100, 300 mg/kg [5], in this study we are adding the Blf 500 mg/ kg to evaluate the effect of Blf at higher dose. Amlodipine which is a calcium channel blocker was chosen as reference drug since it was one of the first-line treatments for hypertension [27]. Amlodipine dose was based on conversion from human dose [28]. Dexamethasone 0,1 mg/kg was administered subcutaneously to all groups for hypertension induction [29]. All animals were sacrificed on day 14 under ketamine and xylazine anesthesia. Blood samples were immediately collected in tubes and kept at 4°C for 30 minutes. Subsequently, the tubes were centrifuged at 1500 G for 10 minutes to obtain the serum. Both kidneys were extracted, washed, and fixed in 10% formaldehyde for histopathological examination.

5.3. Methods

5.3.1. Systolic, diastolic blood pressure, and heart rate measurement

Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), and Heart Rate (HR) were recorded on day 0 and day 14 using LE 5002 Non-Invasive Blood Pressure Meter Panlab Harvard Apparatus. SBP, DBP, and HR measurements were done in accordance with the manufacturer's protocol. The rat was restrained in an acrylic restrainer and heated within 30°C Panlab Heater for 15 minutes before measurement. SBP, DBP, and HR values were obtained by averaging 3 successful measurements.

5.3.2. Serum nitric oxide analysis

Serum NO was measured indirectly by measuring nitrite and nitrate (Decomposition products of NO) using Griess reaction [30]. Serum samples were deproteinized by adding acetonitrile 1:1 (v:v) [31].The mixture was mixed thoroughly using vortex and centrifuged for 20 minutes. The supernatant was obtained and placed in a new tube followed by the addition of VCl3 solution (400 mg VCl3 prepared in 50 ml HCl 1 M) for the reduction of nitrate to nitrite. Subsequently, Griess reagent (Sulfanilamide 2% prepared in 3M HCl, 0,1% N-naphthylethylenediamine prepared in distilled water) was added [32]. After 30 minutes of incubation, the absorbance of the sample was measured at 540 nm using a spectrophotometer (Shimadzu UV-1800). Serum nitrite concentration was obtained by using a nitrite standard curve.

5.3.3. Serum urea and creatinine analysis

Serum urea and creatinine were measured to evaluate renal function. Serum samples were assayed using commercial kits from DiaSys Diagnostic System GmbH, Germany (Creatinine FS, code 17119910021, Urea FS, code 131019910021). The procedure was done in accordance with the manufacturer's protocol.

5.3.4. Kidney histopathological Studies

Histopathological slides were made with standard methods: dehydration, embedding, and sectioning. Slides were stained using hematoxylin–eosin (HE Staining). Tissue sections were assessed under a light microscope (Olympus CX 33) independently by 2 reviewers in a blinded manner.

5.4. Statistical analysis

Statistical analysis was performed using Stata 13 software. Data normality was tested using Shapiro-Wilk test, meanwhile homogeneity of variance was tested using Levene's test. Data are represented in figures as mean ± SE and were analyzed using One-Way ANOVA followed by Tukey post hoc test. Results will be considered statistically significant when the p-value was less than 0.05. MS Excel 2013 was used to generate figures.

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Author contributions: Concept – E.D., D.N., I.V., L.H.; Design – E.D., D.N., L.H.; Supervision – D.N., T.D., D.H., L.H.; Resources – E.D., D.N., T.D., L.H.; Materials – E.D., T.D.; Data Collection and/or Processing – E.D., D.N., T.D.; Analysis and/or Interpretation – E.D., D.N., T.D., D.H., I.V., L.H.; Literature Search – E.D.; Writing – E.D.; Critical Reviews – E.D., D.N., T.D., D.H., I.V., L.H.

Conflict of interest statement: The authors declared no conflict of interest.

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