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Blood Pressure Lowering Effect and Antioxidative Activity of Casein Derived from Goat Milk Yogurt in DOCA-salt Hypertensive Rats

Masdiana, C. Padaga[1,*], Aulanni'am Aulanni'am[2], Hidayat Sujuti[3], Widodo[4]

[1]Biomedical Science Graduate Program, Faculty of Medicine, Brawijaya University, Malang, 65145, Indonesia.
[2]Biochemistry laboratory, Faculty of life Sciences, Brawijaya University, Malang, 65145, Indonesia.
[3]Biochemistry Laboratory, Faculty of Medicine, Brawijaya University, Malang, 65145, Indonesia.
[4]Department of Biology, Faculty of life Sciences, Brawijaya University, Malang, 65145, Indonesia.

Abstract: There is an increasing amount of evidence that oxidative stress related to hypertension and damages the function of aorta and kidney. It is a well established fact that food protein, especially milk casein generates bioactive peptides with antihypertensive and antioxidative activities. The present study sought to investigate blood pressure lowering effect and antioxidative activity of the casein derived from goat milk yogurt in Deoxycorticosterone acetate (DOCA)–salt induced hypertensive rats (*Rattus norvegicus*). Casein were separated from the goat milk yogurtby centrifugation.The rats were orally administrated with casein in daily dose of 300 and 600 mg/kg body weight for 4 weeks. Systole blood pressure (SBP), renal and aorta total malondialdehyde (MDA) and histopathology of kidney and aorta abdominalwere examined. Mean of SBP were lowered in the hypertensive rats following casein therapy from their respective values observed at the time of administration. Renal and aorta injury was observed in DOCA-salt hypertensive group rats compared to normotensive group rats, as renal and aorta MDA significantly increased ($P < 0.05$). In contrast , treatment of DOCA-salt hypertensive rats with different dose of casein significantly reduced the total renal and aorta MDA, as well as repair kidney damage, suppressed smooth muscle cell proliferation and lessen aorta wall thickening compared to controls. This is the first report that demonstrated blood pressure lowering and antioxidative effectsof casein derived from goat milk yogurt in DOCA–salt model of hypertension.
Keywords: Goat milk,DOCA–salt, casein, antihypertensive, antioxidative.

Introduction

Hypertension is a major cause of mortality because of its association with cardiovascular disease, cerebrovascular disease and renal disease. It has a prevalence of 26.4% in the adult population, totaling nearly one billion individuals and has been estimated to increase up to 29% (1.5 billion) by the year 2025[1].

The renin-angiotensin aldosterone system (RAAS) is a hormonal cascade that has funtions in pathogenesis of cardiovascular diseases. Angiotensin II, a potent vasoconstrictor is the primary active product

of the RAAS that play the role in the development of hypertension[2]. Hypertension also has been associated with stress oxidative which is resulted from an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system[3]. In this case, ROS production by NADPH oxidase is increased causes vascular disease and dysfunction. ROS production in other organs, particularly the kidney likely contribute to blood pressure regulation[4]. DOCA salt induced is an endocrine hypertension model that progress quickly severe hypertension and oxidative stress[5]thus allowing an understanding progressionof the disease and potential use of the biopeptides as therapeutic agent.

Milknaturallycontains proteinsas precursor of bioactive peptides that havea variety ofbiological functions[6]. Proteins in milkarein the form ofinactivecompounds,proteolysisprocessesare neededto release thepeptidefrom theprecursorproteinto producebioactivepeptidecompounds[7].It is a well established fact that milk caseins, may act as a precursors of biologically active peptides with health beneficial effects[89].

Goat milkis knownto haveseveraladvantages overcow's milkand other dairy products[10]. Proteins derived from goat milk are important sources of peptides with antihypertensive and antioxidative activity[11 12 13]. The bioactive peptides can inhibit the angiotensin converting enzyme (ACE) that have an important role in converting angiotensin-I to angiotensin-II by blocking the active site of the enzyme[14]. Angiotensin-II is both a potent vasoconstrictor and stimulator for the synthesis and release of aldosterone which subsequently increases blood pressure by promoting sodium retention in the distal tubules[215].Given the above considerations, the inhibition of ACE could be useful in the treatment of hypertension[16]. Antioxidative peptidesshowedthe activityof scavenger against superoxide anion, radicalhydroxyland2,2-diphenyl-1-picrylhydrazyl (DPPH)[17]. Hence, antioxidants is considered a useful therapeutic approach in the treatment of high blood presure [4].

To understand dose dependent effects of fermented goat milk caseintreatment on systole blood pressure (SBP), malondialdehyde (MDA) level and histopathology of kidneyand abdominal aorta, we used DOCA-salt induced hypertension rats(*Rattus norvegicus*) strains Wistar as animal model.Goat milk fermented with yogurt starter containing three species of BAL namely *Lactobacillus bulgaricus*, *L. acidophilus* and *Streptococcus thermophilus*. Casein was separated from the yogurt by centrifugation. The current study demonstrated the decrease of SBP, reduction of MDA level in the aorta and kidney and also repairing kidney and aorta damage after a single oral administrationof casein -fermented goat milk derived to DOCA-salt induced hypertension rats .

Experimental

Chemicals

yogurt starter *L. bulgaricus*, *S. thermophilus* and *L.acidophilus* (*Yógourmet* , Lyo-SAN INC: 500 Aeroparc, C.P. 589, Lachute, QC. Canada, J8H, 464), *Deoxycorticosterone acetate* (DOCA) *salt* (Sigma, Pcode 1001376001, USA), NaCl, corn oil (Sigma, Pcode 1000925370 C8726-500 ml),

Preparation of goat milk yogurt and casein.

Raw goat milk (Etawa crossbred) for yoghurt production was collectedfrom a local farm (Batu, Indonesia). The starter yogurt was purchased from *Yógourmet* (Lyo-SAN INC: 500 Aeroparc, C.P. 589, Lachute, QC. Canada, J8H, 464) containing *L. bulgaricus*, *S. thermophilus* and *L.acidophilus*.

Goat milk yoghurt (GMY) was prepared using the method of Posecion*et al*., (2005) with some modifications[18]. In brief, raw milk was pasteurized at 72°C for 15 s and cooled to the incubation temperature (43°C) by immersing the stockpot in cold water. The pasteurized milkwas further aseptically inoculated with an aliquot of the starter culture (3%) followed by incubation at 43 °C, until the required pH of yogurt 4.5±0.1 was reached. Cooling to 4ºC was done to halt further acidification. Casein was prepared by centrifugation of yogurt at 12.000rpm for 10 min at 5ºC`[19]. The casein was freeze-dried and kept at -20 ºC until used.

Animal model

All procedures were carried out in accordance with conventional guidelines for experimentation with animals. Twelve-week old male rats (*Rattus norvegicus*) strains Wistar were used. The rats were divided into 4 groups, i.e normotensive-NTN (A), hypertensive-HTN (B), HTN + casein 300 mg/kg (C), HTN + casein 600 mg/kg (D), and were housed in groups of four per cage, as a number for replication, in a regulated environment with a 12 h light/dark cycle. Hypertensive rats were prepared by induction ofdeoxycorticosterone asetat (DOCA), twice a weekfor 5weeks(10 injections). DOCAwas injectedsubcutaneouslyin thecervical spinewitha dose of20mg/kgfor5times, followed by a dose of10mg/ kgfor5times and were given 1% NaCl in drinking water . DOCAwas dissolvedin0.5mlcornoil[20 21]. The treated hypertensive rats received, by oral administration, using a canula, a daily dose of casein in daily dose of 300 and 600 mg/kg body weight dissolved in reverse osmosis water for 4 consecutive weeks. The control group received a normal diet.

Measurement of blood pressure

Systolic blood pressure (SBP) was measured in awake rats using the tail-cuff method witha photoelectric sensor (blood pressure analyzer, IITC, Model 179, Woodland Hills-USA) as previously described[22]. SBP was measured weekly beforeDOCA induction, post induction and also up to 4 weeks post-administration of casein. The average of at least three readings, taken in a quiescent state.

Malondialdehyde (MDA) level and

Malondialdehyde (MDA) levelwhich is a marker of lipid peroxidationwas measured using the TBA reaction method in kydney and aorta tissue homogenates from treated and untreated DOCA-salt rats and normotensive rats using a prior adopted method[23].

Histopathology of kidneyand abdominal aorta.

All rats were deeply anesthetizedwith Chloroform 10%. Kidneys and abdominal aorta were fixed in cold 10% buffered formalin, the tissues were then transversally trimmed and submitted to a routine process for paraffin embedding. The renal, as well as, abdominal aorta sections were prepared, deparaffinized, and stained with hematoxylin and eosin (H&E) for histology analysis.

Statistical Analysis

The results of systole blood pressure (SBP) and Malondialdehyde (MDA) were expressed as means ± standard deviation (SD). Differences between trial groups were statistically analysed using analysis of variance (ANOVA), followed by the post hoc Tukey testfor determining significant difference at $p < 0.05$.

Results and Discussion

Effect of casein on Systolic Blood Pressure (SBP)

Induction of DOCA-salt 2 times weekly for 5 weeks at the dose of 20mg/kg bb (5x injections) followed by 10mg/kg bb (5x injections) produced a gradual elevation of systolic BP (Figure 1). The mean systolic BP of DOCA-salt induced rats (HTN) was significantly higher(201,25 ± 2,06 mmHg) in comparison to normotensive rats (NTN) in basal conditions (108,00 ± 2,94). In contrast, the SBP of HTN rats treated with casein significantly decreased after the administration of casein in a daily dose of 300 mg/kgbw and 600 mg/kgbw. The final SBP at the end of experiment (week 10) were 144,00 ± 2,45 mmHg and 152,75 ± 2,36 mmHg, respectively. Treatment of HTN rats with casein at the dose of 600 mg/kgbw gave better antihypertensive effect. In this study, casein derived from goat milk yogurt showed blood pressure lowering effect and caused major changes of the SBP in the treated DOCA-salt hypertensive rats for more than 50 mmHg. Geerling *et al*., (2007) have observed antihypertensive effect of goat milk casein hydrolysate in spontaneously hypertensive rats[11]. In contrast to the present study, the blood pressure lowering effect of the goat milk casein was lower (15 mmHg). This effect mainly due to activity of angiotensin converting enzyme

inhibitor (ACEI) peptides in casein[24]. In our study, induction of the DOCA-salt lead to reabsorption of sodium and water that increased circulating blood volume and resulted in elevated of blood pressure[2025].

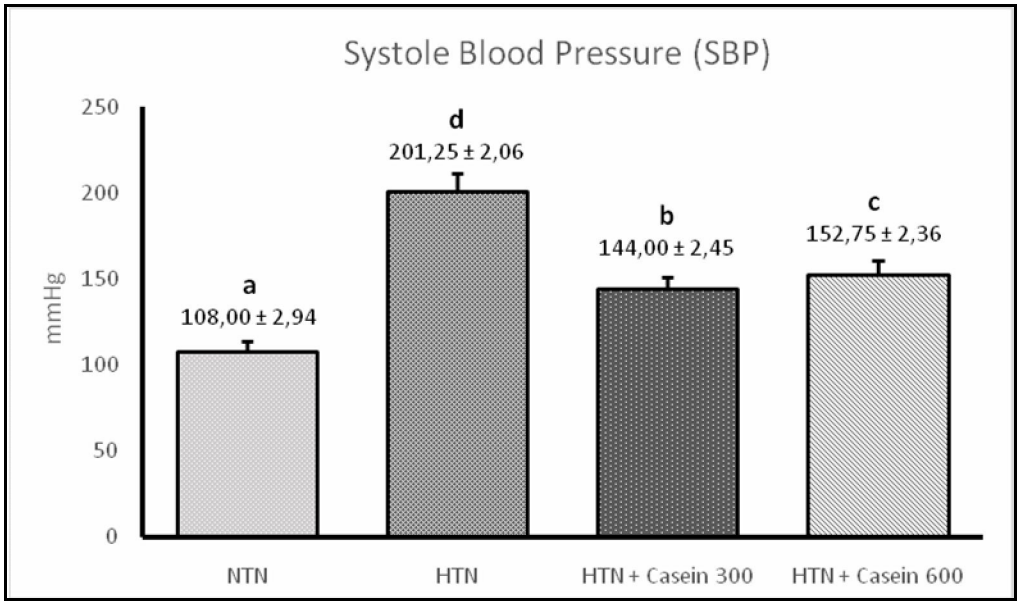

Figure 1. Bar graph showing systole blood pressure (SBP) in normotensive Wistar rats (NTN), DOCA-salt induced hypertensive rats (HTN), 300 mg casein - treated HTN (HTN+casein 300), 600 mg casein - treated HTN (HTN+casein 600). Systolic bloodpressure was determined by indirect tail-cuff method. Values not sharing a common superscript indicate statistical significance at $p<0.05$.

Effect of casein on malondialdehyde (MDA) level of kidney and aorta abdominal

The serum levels of MDA as the markers of oxidative stress in kidney and abdominal aorta of the normotensive (NTN), the hypertensive (HTN) and the casein - treated hypertensive rats were shown in Table 1 and 2. Induction of normotensive rats with DOCA-salt for 5 weeks resulted in increased of MDA level. The MDA level of DOCA-salt hypertensive rats was significantly differ ($P<0.05$) compared to normotensive rats. There was considerably increased of MDA in kidney up to 109,03% and 92,45% in abdominal aorta of DOCA-salt hypertensive rats. These finding was evidence of renal injury and aorta damages related to stress oxidative, thus indicating an increase in the oxidative stress levels in the hypertensive rats as compared to those in the normotensive group.Elevation of blood pressure in the DOCA-salt modelmay activate oxidative stress through an unregulated NADPH oxidase[26]. Oral administration of 300 and 600 mg/kg bw of casein for a period of four consecutive weeks to the hypertensive rats significantly reduced MDA level ($p<0.05$). However, there was not significant different effect of the dose of casein to the renal and aorta MDA level of the treated hypertensive rats. Eventhough, casein at the dose of 600 mg/kg bw gave better effect in reducing renal MDA level (41,21%) as compared to the dose of 300 mg/kg bw (33,42%). Several studies have reported the antioxidative activity of food peptides[272829]. To our knowledge, this is the first study reporting the fermentation of goat milk to release bioactive peptide with antioxidative activity that may reduce the effect of oxidative stress in DOCA-salt hypertensive rats. This study also demonstrated that bringing of the blood pressure to the normotensive state using casein containing antihypertensive peptides not only decreased the blood pressure but that also attenuated organ damages.

Table 1. Effect of casein derived from goat milk yogurt on the levels of Malondialdehyde (MDA) in kidney of DOCA-salt hypertensive rats (mean±SD) (n=4)

Groups	MDA Level (mean+SD) µg/mL	Increase in MDA level (%) compared to normotensive	Reduce in MDA level (%) compared to hypertensive rats
Normotensive (NTN)	$0,332 \pm 0,066^{a}$	-	-
Hypertensive (HTN)	$0,694 \pm 0,073^{c}$	109,03	-
HTN + casein 300	$0,462 \pm 0,024^{b}$	-	33,42
HTN + casein 600	$0,408 \pm 0,049^{ab}$	-	41,21

Values not sharing a common superscript differ significantly at P<0.05.

Table 2. Effect of casein derived from goat milk yogurt on the levels of Malondialdehyde (MDA) in abdominal aorta of DOCA-salt hypertensive rats (mean±SD) (n=4)

Groups	MDA Level (mean+SD) µg/mL	Increase in MDA level (%) compared to normotensive	Reduce in MDA level (%) compared to hypertensive rats
Normotensive (NTN)	$0,318 \pm 0,087^{a}$	-	-
Hypertensive (HTN)	$0,612 \pm 0,097^{b}$	92,45	-
HTN + casein 300	$0,439 \pm 0,081^{a}$	-	28,26
HTN + casein 600	$0,428 \pm 0,046^{a}$	-	30,06

Values not sharing a common superscript differ significantly at P<0.05

Effect of casein on histopathology of kidney and aorta abdominal

Histological analysis of the kidney and aorta abdominal in normotensive rats was showing normal glomeruli and tubules, and histopathological changes was observed in the architecture of kidney and aorta abdominal of all DOCA-salt hypertensive group (Figure 2 and 3).The kidneys of the normotensive rats exhibited normal glomerulus surrounded by the Bowman's capsule, proximal and distal convoluted tubules without any changes (Fig 2A). On the contrary, the kidneys of DOCA-salt induced hypertensive rats showed necrotic changes of glomeruli and the proximal convoluted tubule with karyolysis of nuclei (Fig 2B).

This result in agreement with previous research that reported renal injury in DOCA-salt hypertensive rats showing by segmented glomerulo nephritics in glomeruli, swelling tubules and thickening of the small blood vessels wall[26].A consistent line of evidence has shown that induction of DOCA and a high-salt diet cause quick and severe hypertension and consequently contributes to stress oxidative and organ damages[530].

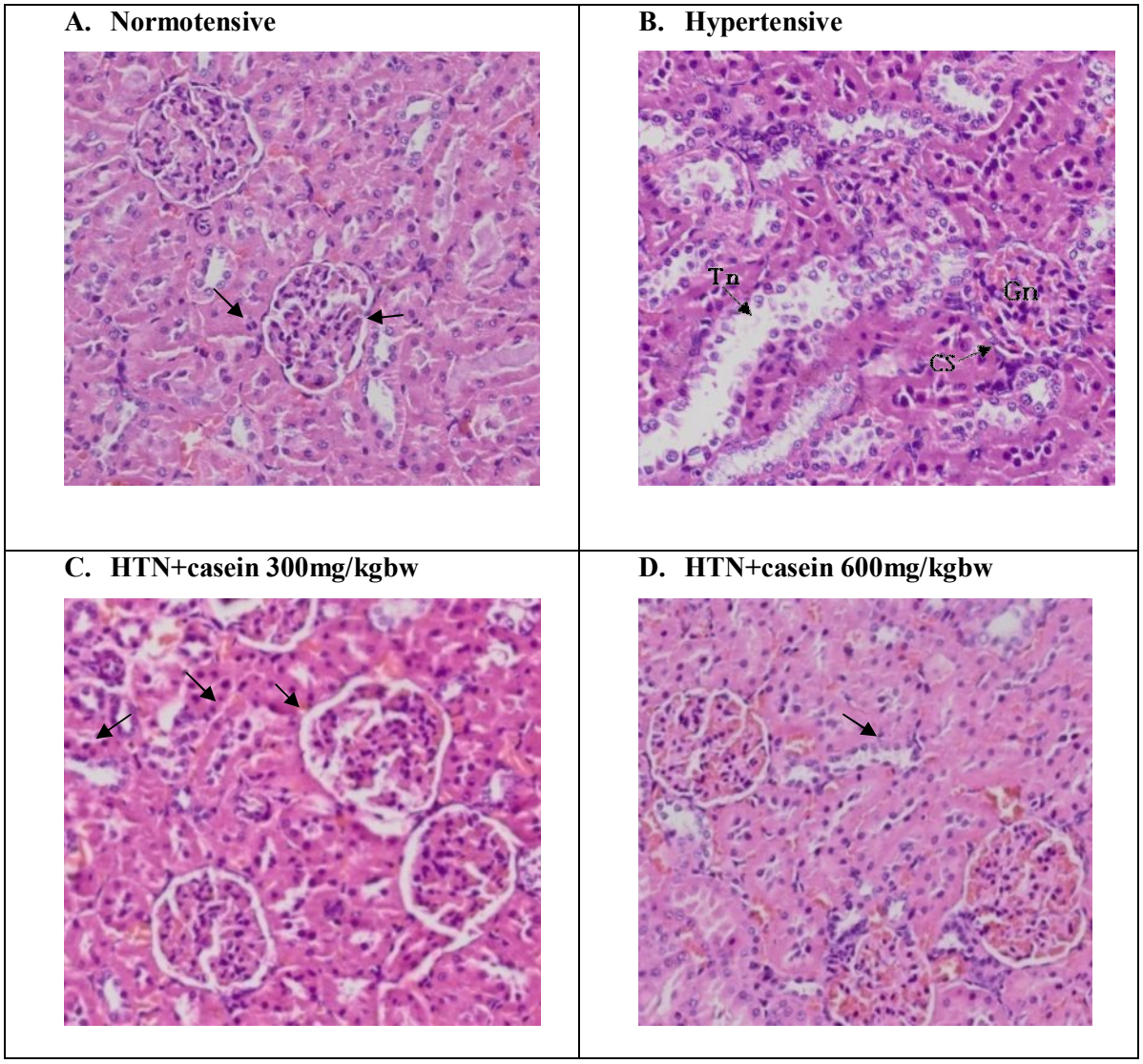

Figure 2. Histopathological changes in the kidney (H&E 40x).A: Normotensive-rat kidney showing normal glomeruli and tubules; B: DOCA-salt hypertensive (HTN) kidney showing congestive area of the glomerulus and necrotic proximal tubule with karyolysis of nuclei could be well observed; C: DOCA-salt + casein (300 mg/kg bw) treated kidney showing near normal architecture of the glomerulus and fewer necrotic tubules; D: DOCA-salt + casein (600 mg/kg bw) treated kidney showing near normal architecture of the glomerulus and mild swelling of tubulus; (G) Glomerulus; (GC) Congestive Glomerulus; (T) Tubulus;(Tn) necrotic tubulus; (Cs) capsular space.

Treatment of the hypertensive rats with 300 and 600 mg/kg bw of casein for a period of four consecutive weeks showed features of healing effect of necrotic kidney. Fewer necrotic area of glomerulus and mild swelling in tubules was decrease (Fig 2C&D). Dose dependant effect was also observed in recovery of kidney damage as evidenced microscopically. Tubular injuries were not observed in the hypertensive rats treated with 600 mg/kg bw of casein. This result suggested that antioxidative peptides-containing caseinmay play an important role in reducing structural changes in kidney.

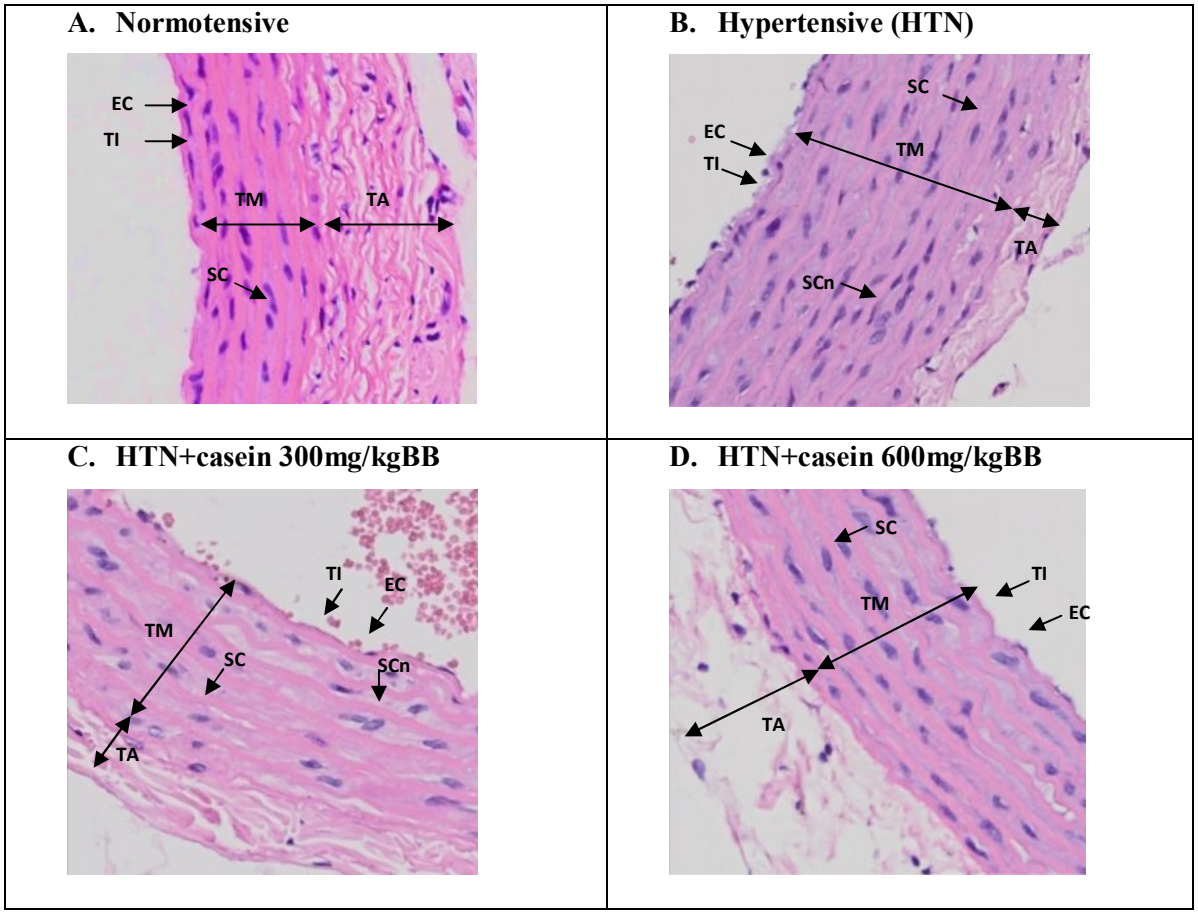

Figure 3 .Histopathological changes in the aorta (HE, 40x).Normotensive-rat Aorta (A); DOCA-salt hypertensive (HTN) aorta (B); HTN + Casein (300 mg/kg bw) treated aorta(C);; HTN + Casein (600 mg/kg bw) treated aorta(D); (EC) Endotel cell; (SC) Smooth Muscle cell; (SCn) Necrotic Smooth Muscle cell; (TI) *Tunica Intima;* (TM) *Tunica Media;* (TA) *Tunica Adventitia.*

Hypertension is associated with structural and functional alterations of blood vessels. Figure 3 revealed that the aorta abnominal of normotensive rats (A) showed no histological changes, while in the hypertensive rats (B), there were thickening of tunica media (TM) and alteration of tunica intima (TI), increased numbers of smooth muscle cells (S) as well as the evidence of necrotic smooth muscle cells (Sa).Disruption of tunica intima, marked hypertrophy and thickening with proliferation of myocytes in tunica media was reported in histological studies with rats submitted to DOCA-salt hypertension[21]. The tunica media thickness (TM) and amount of necrotic smooth muscle cells (Sa) were substantially changed by administration of casein for 4 weeks, in the present study(Fig 3C and 3D). These finding suggested a significant effect of this treatment on the development of hypertension.Although many evidence clearly indicated the antioxidative activity of milk biopeptides[31], few study had evaluated the antihypertensive and antioxidant effects exerted by casein bioactive peptides derived from fermented goat milk in DOCA-salt hypertensive model.

Conclusion

Assessment ofthe antihypertension and antioxidative properties of fermented goat milk yogurt-derived casein in the present study showed asignificant effect in reducing systole blood pressure, attenuating renal and abdominal aorta damages in DOCA-salt hypertensive model. These findings have suggested that the casein with high bioactive peptides contentmight be considered as potential antihypertension and antioxidants that resistant to digestive proteases. However, further investigation is needed to evaluate bioavailability and efficacy of the bioactive peptides after GI digestion, whichwas significant for the preparation of nutraceutical food component.

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