Activity of Caprine CSN1S2 Protein Reducing the COX-2 and IL-17 Expression of Aorta Tissue in Type 2 Diabetes Mellitus Rat

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Abstract. Type 2 diabetes mellitus (T2DM) is a degenerative disease that leads to increased inflammation and cyclooxygenase protein production, which causes tissue abnormalities. The aim of this study was to determine the effect of caprine CSN1S2 protein against abnormal metabolic pathways in the aorta of DM rats. The twenty-four-animal model was control, diabetes and treatment groups. Histopathological evaluation of the aortic tissue by hematoxylin eosin staining. The expression of cyclooxygenase and inflammatory cytokine was measured by western blotting. In the DM750 groups, the amount of discontinued-endothelial was significantly more reduced than in the other groups. The amount of macrophages in the DM1500- group decreased more than in the DM and DM375 groups. The amount of foam cells in the DM750 and DM1500 groups decreased more than in the DM group and was close to all control groups. The expressions of COX-2 and IL-17 were effectively reduced and vice versa the expression of IL-10 was increased in DM750 compared with the other groups. Meanwhile, COX-1 expression did not change in all groups. This study indicates that caprine CSN1S2 protein at a dose of 750 mg/kg BW has a significant effect on controlling, protecting, and repairing abnormalities in the aortic tissue of T2DM rats.

Keywords: *aorta; caprine CSN1S2 protein; cyclooxygenase; type 2 diabetes mellitus; inflammation.*

1   Introduction

Type-2 diabetes mellitus (T2DM) is a disease that has an impact on physiological abnormalities as a sign of hyperlipidemia [1]. The risk of T2DM is increased when LDL levels in the body are elevated. This leads to the occurrence of hyperlipidemia [2,3]. Hyperglycemia followed by hyperlipidemia increases the interaction between free fats and free glucose, which produces oxidized LDL (ox-LDL). It also causes an immune reaction when entering the vessel walls [4,5]. Immune cells that respond to ox-LDL are activated and cause inflammation of the aortic tissue [6]. The inflammation of aortic tissue induces the aorta to enhance the production of cyclooxygenase 2 (COX-2) protein [7,8].

Cyclooxygenase 2 is a protein that has a role as an indicator of cell damage [9]. COX-2 generally exists in very small amounts in tissues. COX-2 is an activator of prostaglandin E_2 (PGE$_2$) [8]. Physiologically, PGE$_2$ plays a role in stimulating inflammation in tissues and specifically affects cellular damage in Langerhans islets [10]. Increased COX-2 production in the target tissue is a sign of abnormality that leads to tissue damage.

Vascular tissue is one of the target tissues that are susceptible to damage due to increased production of COX-2 [10]. A recent study revealed that elevated COX-2 expression levels in the aorta indicate a pathological condition and can lead to atherosclerosis [11]. Another study showed that COX-2 activity is associated with activity of the immune system in the aortic wall layer [12]. The influence of COX-2 on immune cell activity causes the tissue damage to become more severe [13]. Aortic tissue damage is not only indicated by protein levels that indicate a pathological condition. It is also based on other parameters, such as deterioration of the discontinued endothelium, immune cell activity in the tunica intima and sub tunica intima, and foamy cell formation [14-19].

Based on a previous study, CSN1S2 derived from caprine milk is a protein that has a biological function as bioactive compound. It was found that caprine CSN1S2 protein had a positive influence on the ileum microstructure in the rat model of rheumatoid arthritis (RA) [20]. It also has an immunomodulatory role when inflammatory activity occurs in RA [21]. The result of two other studies proved the role of caprine CSN1S2 protein as a selective inhibitor of receptors of advanced glucose end products (RAGE) [22] and immunosuppression compounds of pro-inflammatory cytokines in RA [21]. Caprine CSN1S2 protein acts as an antioxidant by reducing the oxidation effect on osteoblast when oxidant compounds are feasible [23]. In spite of the various potentials of caprine CSN1S2 protein discovered in previous studies, the effects of caprine CSN1S2 protein in vascular diabetes still remains unclear. This study explored

the effect of caprine CSN1S2 protein on controlling abnormal mechanisms in T2DM rats based on histological data, the COX-1, COX-2, IL-10 and IL-17 expression.

2   Materials and Methods

2.1   Isolation of Caprine CSN1S2 Protein

Bioactive caprine CSN1S2 protein isolated from Ethawah breed goat milk was obtained from UPT Ethawah breed goats, Singosari, East Java, Indonesia. The caprine CSN1S2 protein isolation procedure was taken from a previous study [24] with some modification. The purity of the caprine CSN1S2 protein was measured using a Nanodrop spectrophotometer.

2.2   Animal Study

The animal models used were twenty-four male rats (*Rattus novergicus*), Wistar strain, 8 weeks old. These animals were purchased from Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta, Indonesia. The twenty-four animals model was divided into 8 groups, with three repeated, consisting of control (C), control rats treated with caprine CSN1S2 protein at doses of 375 mg/kg BW (CM375), 750 mg/kg BW (CM750), and 1500 mg/kg BW (CM1500), diabetes mellitus (DM), diabetes rats treated with caprine CSN1S2 protein at doses of 375 mg/kg BW (DM375), 750 mg/kg BW (DM750), and 1500 mg/kg BW (DM1500). The T2DM rats were fed normal hyper-cholesterol rat food for two months and then induced with a single dose of streptozotocin (25 mg/kg BW) (Sigma-Aldrich). The treatment with caprine CSN1S2 protein was conducted for 28 days by oral administration. After the treatment, the experimental animals were dissected, organs and aorta were collected and stored in 10 mM PBS and 4% paraformaldehyde. This study was approved by the research ethics committee of Brawijaya University with ethical certificate number 417-KEP-UB.

2.3   Histopathological Analysis

Hematoxylin-eosin (HE) staining was conducted for histopathological analysis according to previous studies [20,21] with some modifications. The histopathological observations of the specimens used an Olympus BX-53 microscope with 600x magnification.

2.4   Western Blotting

Sample protein of aorta tissue from all groups was isolated by the procedure described in previous studies [20,25] with some adaptation, after which

separation was done using 15% of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To identify protein and cytokine expression in the aortas from all rat groups, western blotting analysis was conducted using several primary antibodies, such as rabbit anti-rat PTGS1, rabbit anti-rat PTGS2 (1:1000 Proteintech Group, Inc.) mouse-anti-interleukin (IL)-10 (1:1 500, Bioss, Inc.), and rat-anti-IL-17 (1:1 500, Santa Cruz Biotechnology, Inc.). Protein separation from SDS-PAGE was transferred to polyvinylidene fluoride membrane, followed by washing using phosphate buffer saline Tween-20 and incubation with secondary antibodies labeled AP (1:2500) in thermomorphic biphasic solvent for 1 hour at room temperature. The membrane was then exposed to the targeted protein with a western-blue-substrate solution of nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indolyl phosphate (Gathering MD, USA). The specific protein density was measured by Bio-Rad Quantity One software.

3   Result

3.1   Effect of CSN1S2 protein on Histopathology of Aorta Tissue

Histological observation of the aorta in the control rats showed formation of intima, media, and adventitia (Figure. 1(a)). In contrast, the DMT2 group showed tunica intima, tunica media, and tunica adventitia. The tunica intima had endothelial discontinued cells; foamy cell formation was accompanied by an increase of the number of monocytes in the media layer (Figure 1(e)).

The control rats treated with CSN1S2 proteins from Ethawah breed goat milk had a surface similar to that of the control aorta, but measurement of the tunica intima of the CM750 group ($0.597 \pm 0.067\mu m$) (Figure 2(d)) showed that this had decreased compared to the other groups. In the DM375 group, the formation of foamy cells was less than in the DM group. In the DM375 group, a lower number of macrophages in the intima and media layer were identified. In the DM750 and DM1500 groups, the histological structures of the intima, media, and adventitia was similar to those of the control groups.

3.2   Effect of Caprine CSN1S2 Protein on Aortic Wall Thickness

The measurement of the aorta layer thickness showed a difference in the intima and media layer between the control, T2DM model, CSN1S2 protein control and DM-treated with CSN1S2 groups. The tunica intima layer in the CM750 ($0.597 \pm 0.067\mu m$) group was thinner than in the other groups (Figure 1(c)). The tunica media lining in the control group ($5.287 \pm 0.124\mu m$) and DM group ($5.398 \pm 0.879\mu m$) had a similar thickness. The control groups treated with CSN1S2 CM750 ($6.757 \pm 0.747\mu m$) and CM1500 ($6.881 \pm 0.013\mu m$) showed

significant differences with the control and the DM groups (Figure 2(e)). The increase of the aortic wall thickness in the treatment group showed the effect of the caprine CSN1S2 protein in the metabolic process, which caused an elevated volume of the tunica media layer. The obtained results showed that the aorta structure of the rats treated with caprine CSN1S2 had increased thickness of the intima and media layer correlating with the caprine CSN1S2 dose.
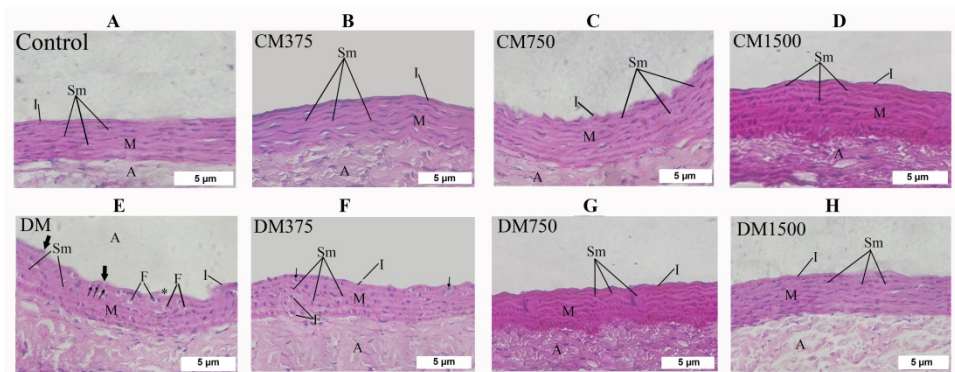

Figure 1 Photomicrograph of rat aorta hematoxylin-eosin staining with scale 600x. The aorta structure of the control rats had a tunica intima (I) with complete endothelium, tunica media (M) and tunica adventitia (A); (a) Control (C), (b) control rats treated with caprine CSN1S2 protein at doses of 375 mg/kg BW (CM375), (c) 750 mg/kg BW (CM750), (d) 1500 mg/kg BW (CM1500), (e) diabetes mellitus (DM), (f) diabetes rats treated with caprine CSN1S2 protein at doses of 375 mg/kg BW (DM375), (g) 750 mg/kg BW (DM750), and (h) 1500 mg/kg BW (DM1500).

3.3 Abnormal Cell Amount

The calculation of discontinuous endothelial cells in treatment groups CM750 (5.66 ± 3.05ab), CM1500 (6.00 ± 2ab), and DM1500 showed a higher number of control endothelial cells, but there was no statistically significant difference with the control group (Figure 2(a)). In the T2DM model group, the number of damaged cells was significant; it was increased compared to the other groups (65.66 ± 23.86c). The damage was influenced by the existence of tissue damage caused by COX-2 in endothelial cells.

Endothelial damage is caused by increased oxidant activity in cell environments, such as ox-LDL. The DM375 treatment group showed a significant decrease of discontinuous endothelial cells (33.66 ± 5.85b $p < 0.05$) as an indicator of improvement of aortic tissue in this group (Figure 2(a)).
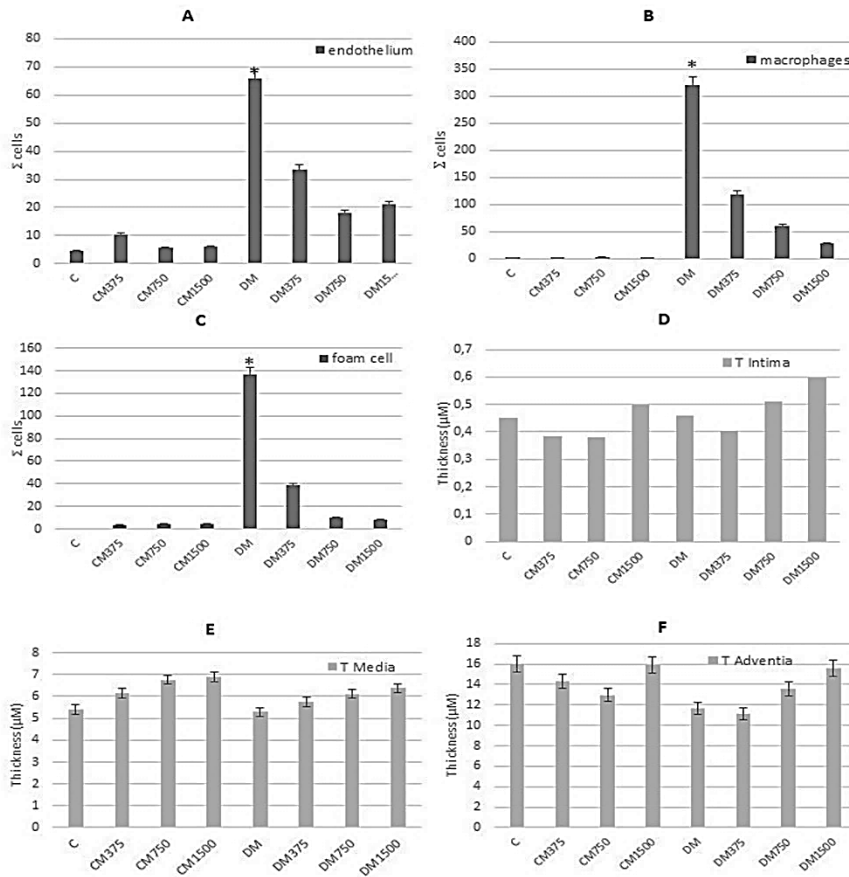
Figure 2 Abnormality cell in HE slides of aorta tissues. (a) The number of discontinued endothelial cells for several groups; (b) The number of macrophages in the aorta layer; (c) The amount of foamy cell formation for several groups; (d) Tunica intima thickness measurement; (e) Tunica media thickness measurement; (f) Tunica adventitia thickness measurement.

The macrophage count in the control group showed a significant difference ($0.333 \pm 0.57a$ $p < 0.05$) compared to the T2DM and T2DM-treated model groups. The control group treated with caprine CSN1S2 showed no significant difference compared to the control group at all doses. The T2DM group showed a significant elevation of the number of infiltrating macrophages in the aortic wall ($319.00 \pm 17.52e$ $p < 0.05$). The increased number of macrophages in aortic tissue is an indication of increased immune cell activity in the aortic wall. The DM375 ($120.00 \pm 11.53d$ $p < 0.05$), DM750 ($59.66 \pm 12.05c$, $p < 0.05$), and DM1500 (30.00 ± 11.4 b, $p < 0.05$) groups each showed a significant reduction of the number of macrophages infiltrating into the aortic layer (Figure 2(b)).

The calculation of foamy cells in the control and treated groups at various doses showed that the amount of foamy cell formation did not differ significantly between groups. However, the amount of foamy cells in the T2DM model group did increase significantly (139.00 ± 19c, $p < 0.05$) compared to the control groups (Figure 2(c)). This condition indicates that macrophage activity acts as an ox-LDL receptor scavenger in the aortic layer [26]. The DM375 group showed a significant decrease in the number of foamy cells formed (39.00 ± 9b, $p < 0.05$) compared to the T2DM model group (Figure 2(c)), but it was still higher than in the control group. For the DM750 and DM1500 groups the results showed a significant decrease in the number of foamy cells, which was similar in the control group (Figure 2(c)).

3.4   Cyclooxygenase Expression and Inflammation Effect

Western blotting analysis was done to measure the protein expression level in the aortic tissue. The protein measured consisted of cyclooxygenase 1 (72kDa), Cyclooxygenase 2 (69kDa), IL-17 (18kDa) and IL-10 (15kDa) (Figure 3(a)). The measurements of the COX-1 expression level in the different groups showed no significant differences based on the statistical calculations ($p < 0.05$).

The expression level of COX-2 in the DMT2 model group (0.742 ± 0.09 INT/mm^2) was significantly higher than in the other groups ($p < 0.05$) (Figure 3(b)). In the DM750 group (0.313 ± 0.025 INT/mm^2) and the DM1500 group, (0.283 ± 0.03 INT/mm^2), the COX-2 expression level was significantly reduced compared to the control group (Figure 3(b)). This result proves that caprine CSN1S2 protein functioned as a suppressor of COX-2 production.

IL-17 expression in the control group was 0.243 ± 0.06 INT/mm^2. It was low for the control groups treated with caprine CSN1S2 protein. For the CM375 group it was 0.276 ± 0.03 INT/mm^2, for the CM750 group it was 0.317 ± 0.04 INT/mm^2, for the CM1500 group it was 0.315 ± 0.05 INT/mm^2. This proves that the target tissue was stable and under control, especially regarding inflammatory activity in the target tissue. The DM model group showed significant elevation of IL-17 expression levels ($0.701 + 0.04$ INT/mm^2), compared to all other groups ($p < 0.05$). In the DM750 group ($0.303 + 0.04$ INT/mm^2), and the DM1500 group ($0.299 + 0.05$ INT/mm^2), the expression levels of IL-17 were down-regulated, similar to the control group (Figure 3(c)).

The IL-10 expression levels in the control groups treated with caprine CSN1S2, such as the CM375 group (0.291 ± 0.04 INT/mm^2), the CM750 group (0.354 ± 0.07 INT/mm^2), and the CM1500 group (0.324 ± 0.04 INT/mm^2) had no significant difference with the control group (0.275 ± 0.05 INT/mm^2). The IL-

10 production at certain levels requires the tissue to maintain homeostasis and also anti-inflammatory cytokines. [27]. The IL-10 expression level in the T2DM rat model group (0.261 ± 0.04 INT/mm^2) did not differ from the control group.
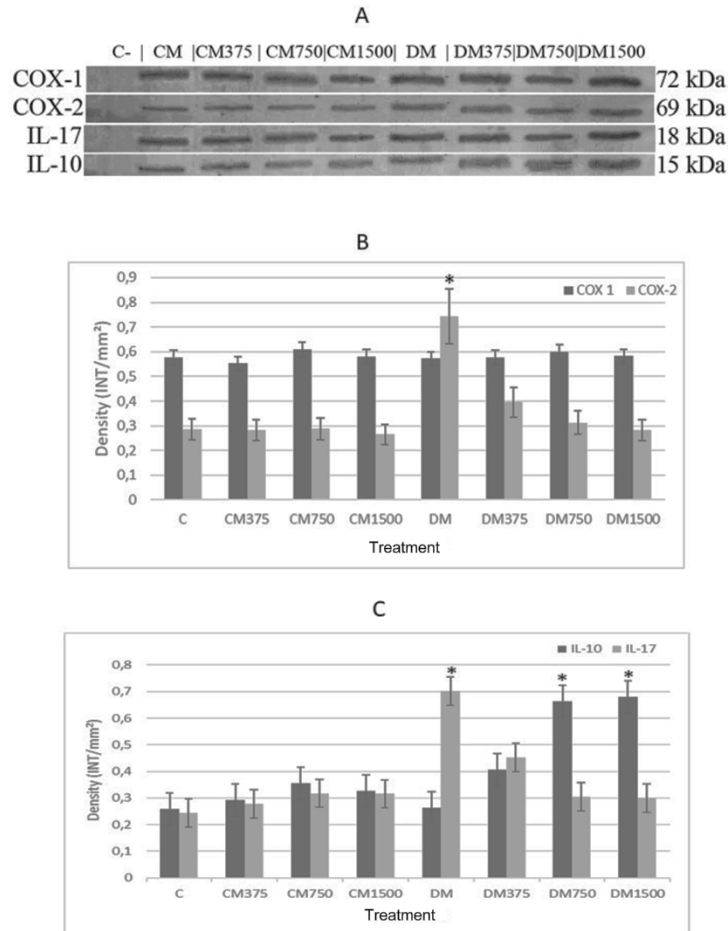

Figure 3 Protein expression in aortic tissue. (a) Protein expression profile using western blotting method; (b) Measurement of COX-1 and COX-2 expression in aortic tissue; (c) Measurement of inflammatory cytokine expression. Control (C), control rats treated with caprine CSN1S2 protein at doses of 375 mg/kg BW (CM375), 750 mg/kg BW (CM750), and 1500 mg/kg BW (CM1500), diabetes mellitus (DM), diabetes rats treated with caprine CSN1S2 protein at doses of 375 mg/kg BW (DM375), 750 mg/kg BW (DM750), and 1500 mg/kg BW (DM1500).

Meanwhile, the IL-10 expression in the T2DM rat group decreased compared to the control group (Figure 3(c)). It was also shown that after treatment with a

higher dose of CSN1S2, the IL-10 level increased as well. In the DM750 group it was $0.662 + 0.08$ INT/mm^2 and in the DM1500 group it was $0.679 + 0.09$ INT/mm^2. These groups had a higher elevation of IL-10 expression than the other groups ($p < 0.05$). Appearance of a large amount of IL-10 indicates that improvements are made in the target tissue (Figure 3(c)). In this case, it indicated improvement of the aortic tissue

4   Discussion

Our investigation revealed that there was a change in the aortic characteristic structure between the control and the T2DM model group. Histological observation of the diabetic aorta showed the presence of damage in the endothelium structure compared to the control group. The aortic histology of theT2DM rat model also showed a number of foamy cells and macrophage infiltration in the tunica intima and tunica media layer. The result revealed abnormalities in the T2DM model. The onset DM with blood glucose 600 mg/dl had a cholesterol level of 400 mg/dl according to the animal model and became hyperlipidemic (data not shown). This level is an indicator of elevated lipid levels in the circulatory system [2]. Hyperlipidemia followed by hyperglycemia (T2DM) causes an elevated risk of reaction of glucose with LDL molecules producing ox-LDL [28-30]. The ox-LDL activity in the aorta wall induces immune responsive cells such as monocytes and macrophages to act as scavenger receptors to phagocytes of the ox-LDL and the formation of foamy cells [18,27,29]. Abnormalities were seen from the elevated number of macrophages that penetrated into the tunica intima and tunica media. Furthermore, abnormalities were also revealed by increased IL-17 and COX-2 expression levels. IL-17 has an effect on endothelial dysfunction in the vascular system [31]. This impact was identified by foamy cell formation and significantly higher endothelial damage than in the control groups.

The effectiveness of treatment with caprine CSN1S2 protein was observed in the T2DM group treated with a dose of 375 mg/kg BW. In this model, a repaired histological structure of the aorta was found. The structure of the tunica intima was found to be improved based on the reduction of discontinued endothelial cells. Caprine CSN1S2 protein activities also reduce the number of macrophages and foamy cells in the tunica intima and media layers, which were significantly lower than in the T2DM model group. Recently, a study revealed that caprine CSN1S2 protein plays a role as a selective inhibitor receptor of advanced glucose end products [2,9]. According to these studies, the inhibition of the AGEs-RAGE binding process in the target cells reduces the downstream protein cascade metabolism pathway, as shown in Figure 4.

The result supports positive effects of the CSN1S2 protein of Ethawah breed goat milk, as shown by the elevated COX-2 expression level in IL-10 and the decreased COX-2 expression level in IL-17 compared to the T2DM group. The positive effects of the CSN1S2 protein from Ethawah breed goat milk on these parameters have a biological immunomodulatory function [20]. The increased IL-10 expression level proved the role of CSN1S2 in activating the immune system to produce anti-inflammatory cytokines [27]. Furthermore, the CSN1S2 protein of Ethawah breed goat milk had the effect of down-regulating the COX-2 expression and inhibiting the PGE_2 production by blocking protein cascade activity. This process was impacted by the role of PGE_2 as an inflammatory agent with an autocrine mechanism that increases the inflammatory effect on the tissue target that was blocked [8,30].
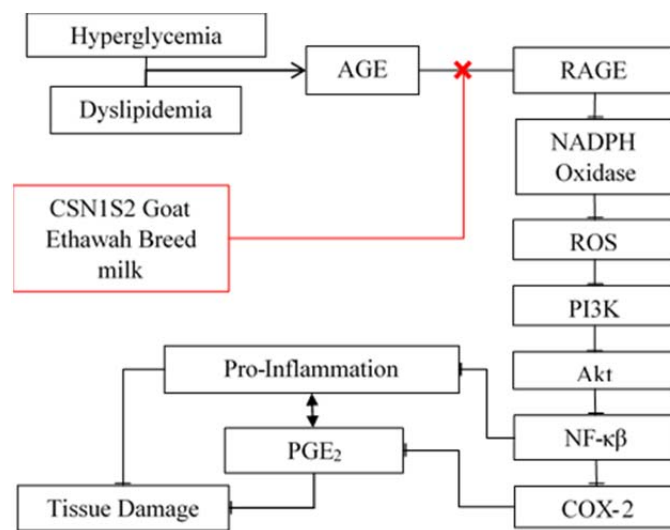

Figure 4 Protein signaling pathway of caprine CSN1S2 protein inhibits AGEs-RAGE binding and activation of COX-2 and pro-inflammation in vascular tissue.

The dose of CSN1S2 significantly impacted tissue repair under DM. This was proved by the histopathology analysis of the DM750 and DM1500 groups, which had an intact surface appearance that was similar to that of the control group. This was proved by the numbers of macrophages and foamy cells in the aorta wall, which were significantly lower compared to the DM and DM375 groups. This was proven by the decreased levels of IL-17 and COX-2 followed by an elevated expression level of IL-10 in the DM375 group. The decrease of the IL-17 level was positively affected by the inflamed area in the aorta tissue [32]. This was supported by the increased levels of IL-10 expression in the groups treated with CSN1S2 at all doses. An elevated level of IL-10 in the DM aortic tissue has an atheroprotective effect in the early stages of atherosclerosis

[27]. These results suggest that proper doses of CSN1S2 protein have a positive effect, not only as immunomodulator and immunosuppressor against inflammatory conditions but also by suppressing abnormal mechanisms in the aortic tissue.

Different results were shown in the measurement of COX-1 expression levels in the aortic tissues. The COX-1 expression levels were stable for all treatments. It was indicated that both the control group and the DM group were not affected by the regulation of the COX-1 produced. Similar results were also visible in the measurements of COX-1 expression levels in the groups treated with CSN1S2, both in the control and the DM group for various doses of CSN1S2. Based on previous studies, COX-1 in vascular tissue acts as a 'housekeeping' protein that plays a role in regulating tissue homeostasis [33,34]. COX-1 has many vital roles in cell metabolism reactions, thus the presence of COX-1 at an optimal level is needed [35]. However, treatment with caprine CSN1S2 induced cellular homeostatic balance in the target tissue.

Our results suggest that treatment with CSN1S2 protein at a dose of 750 mg/kg BW had a positive impact on suppressing immune cells that activate COX-2 and IL-17, which cause cellular damage in aorta tissue, leading to atherosclerosis. Other factors showed that CSN1S2 has no risk of inhibiting or blocking the cellular mechanisms that maintain homeostatic balance. The results of this study are evidence of the role of CSN1S2 protein in improving the aortic structure and the aortic immune response to COX-2 expression from hyperglycemia leading to an indication of atherosclerosis.

5   Conclusions

Treatment with bioactive CSN1S2 protein from Ethawah goat breed milk has a positive role in reducing and repairing damaged aortic tissue, which leads to an indication of atherosclerosis and suppresses inflammation caused by immune response activity. The effect was specifically significant at a dose of 750 mg/kg BW.

Acknowledgements

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