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Anti-oxidative and anti-inflammatory activity of *Achatina fulica* mucus in streptozocin-nicotinamide-induced diabetic kidney disease: an animal model study

Wachid Putranto[1,2*], Gigih Fitriawan[1,2], Ratih Tri Kusuma Dewi[1,2], Aryo Suseno[1,2], Arief Nurudhin[1,3], Yulyani Werdiningsih[1,3], Santy Ayu Puspita Perdhana[1,2], Nurhasan Agung Prabowo[1,4], Yeremia Suryo Pratama[1,4]

[1]Faculty of Medicine, Sebelas Maret University, Surakarta, Indonesia
[2]Division of Nephrology, Department of Internal Medicine, Dr. Moewardi General Hospital, Surakarta, Indonesia
[3]Division of Rheumatology, Department of Internal Medicine, Dr. Moewardi General Hospital, Surakarta, Indonesia
[4]Department of Internal Medicine, Faculty of Medicine, Sebelas Maret University Hospital, Sukoharjo, Indonesia

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ABSTRACT

Introduction: Diabetic kidney disease (DKD) progression resulted in increased intrarenal oxidative stress and increased inflammatory resulting in further renal fibrosis. *Achatina fulica* mucus was regarded to exerts anti-oxidative and anti-inflammatory effect.

Objectives: This study aims to observe the effect of administration of *A. fulica* mucus on oxidative stress and inflammation biomarkers in DKD-induced rats

Methods and Materials: In this study, we used 32 males white Wistar rats divided into four groups; a control, and other three different groups induced with 45 mg/kg streptozocin (STZ) and 110 mg/kg nicotinamide (NA) intra-peritoneally. *Achatina fulica* mucus was administered orally in the last groups; 3.5 mL/d (S1), and 7 mL/d (S2). Post-test measurement of inflammatory and oxidative biomarker was used to determine the outcome.

Results: The study resulted in reduction of malondialdehyde (MDA), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), high sensitivity C-reactive protein (hs-CRP), vascular endothelial growth factor (VEGF), and interleukin-1β (IL-1β) in *A. fulica* mucus administration in our STZ-NA induced rats, with higher dose of the mucus further reduce the inflammatory and oxidative stress biomarkers.

Conclusion: Current study showed the potential of *A. fulica* mucus usage in future management of inflammation and oxidative stress in diabetes and DKD.

Implication for health policy/practice/research/medical education:
Achatina fulica mucus has the potential to be developed as an adjuvant therapy for patients with diabetic kidney disease; however, additional clinical research is required.

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Original Article

Introduction

Diabetic kidney disease (DKD) or known as diabetic nephropathy is one of the microvascular complications of diabetes mellitus (DM) that occurs due to chronic hyperglycemia conditions. Cytokine activation, further glycosylation products, and hyperglycemia might contribute to renal hyperfiltration and lesions (1). This is the main cause of end-stage renal disease (ESRD) worldwide. Individuals with ESRD and DM have poorer survival rates than ESRD patients without DM (2).

In DKD there is an increased activity of intrarenal oxidative stress resulting from increment of reactive oxygen species (ROS) and inadequate antioxidant mechanisms (3). In addition to oxidative stress activation, the complicated pathophysiology of DKD revealed that inflammatory signaling pathway also contributed

*Corresponding author: Wachid Putranto,
Email: wachidputranto@staff.uns.ac.id

(4). These pathways resulted in progression of renal fibrosis, an integral part of DKD's pathophysiology (3). Multiple indicators showing increased oxidative stress and inflammation have been found to be enhanced in DKD patients. One of oxidative stress biomarker, malondialdehyde (MDA) is a lipid oxidation product that can reflect the level of ROS formation and oxidative damage (5), and showed to be increased in diabetes and DKD (6). Besides MDA, it is known that there is an increase in the production of several growth factors, including transforming growth factor-beta (TGF-β), a profibrotic agent that plays a role in the occurrence of renal fibrosis in DKD patients (7). Additional growth factors have been shown to be elevated in DKD patients, such as tumor necrosis factor-α (TNF-α) (8) and vascular endothelial growth factor (VEGF) (9). Other prominent marker of inflammation, such as high sensitivity C-reactive protein (hs-CRP) (10) and interleukin-1β (IL-1β) (11), were also reported.

Current management of DKD only slows the progression of the disease but has not been able to stop the process of the microvascular complication of DM. *Achatina fulica* mucus is known to contain acharan sulfate which have antioxidant and anti-inflammatory functions (12). Previous researches suggested that *A. fulica* mucus could be used to suppress oxidative stress and inflammation (13), potentially alleviating inflammatory activity in DKD.

Objectives

This study aims to observe the effect of administration of *A. fulica* mucus on oxidative stress and inflammation biomarkers in DKD-induced rats.

Materials and Methods

Study design

This study is an experimental study with male Wistar rats (*Rattus norvegicus*), using a post-test only control group design. The research was conducted at the laboratory of the center for food and nutrition studies, inter-university center, Gajah Mada University Yogyakarta from September to October 2022. Data processing and statistical analysis were carried out at Moewardi general hospital Surakarta.

Animals

This study includes 32 male white rats of the Wistar strain (*Rattus norvegicus*), 3-4 months old, weighing 150-300 g, healthy and have never been subjected to any treatment were conducted in this study. Rats are purchased from Veterinary Faculty of Gajah Mada University. Rats are maintained under controlled temperature conditions of 25 °C, humidity of 55% with a light/dark cycle of 12 hours. All rats were fed using standard food and drink *ad libitum* mineral water.

Substances

The streptozocin (STZ) (item no 13104, ≥95% purity, molecular weight (MW): 265.2) was pre-ordered at Cayman Chemical Co. (Ann Arbor, Michigan, USA) and nicotinamide (NA) (item no 24317-72, ≥98.5% purity, MW: 122.12) was pre-ordered at Nacalai Tesque (Nakagyo-ku, Kyoto, Japan), and both were purchased from PT Kairos Jaya Sejahtera (Sleman, Special Region of Yogyakarta, Indonesia).

2-Deoxy-2-(3-methylnitrosourea)-1-D-glucopyranose or STZ is a white powder containing the natural chemical glucosamine-nitrosourea, which is produced by *Streptomyces achromogenes* and has a broad-spectrum antibacterial activity (14). STZ has a molecular weight of 265 g/mol. STZ was found to be diabetogenic in 1963, and has been used to cause diabetes in laboratory animals (15).

Streptozotocin can be given as a single high-dose injection (>60 mg/kg) resulting in severe pancreatic cell destruction and type 1 DM in animal models, or medium-dose injection (40-55 mg/kg) resulting in partial insulin secretion abnormalities like type 2 DM (16). Higher doses in induced rats lead to moderate β cells destruction, and remaining Langerhans cells will enlarge and secrete less insulin. Mesangial matrix growth, glomerulosclerosis, and TGF-β expression, essential to DKD development also occurred in STZ administration of 60 mg/kg doses in Wistar rats. Proteinuria as hallmark of DKD, was observed at least 3 weeks after the STZ induction (16).

Nicotinamide (NA) can prevent DNA methylation, via its activity as an inhibitor of poly ADP ribose polymerase. Giving NA prior to STZ induction could moderate STZ's damage to pancreatic cells; this is based on the hypothesis that STZ produces DNA damage, which triggers DNA repair processes requiring significant amounts of nicotinamide adenine dinucleotide. This moderation of the damage extends to the intraperitoneal injection of nicotinamide at a higher dose (>200 mg/kg) 15 minutes before injecting STZ, which allowed the animal models to have lower mortality with increased glucose level and impaired glucose tolerance. To prevent early mortality due to insulin release from overly injured pancreatic islets following STZ injection, nicotinamide must be given before the STZ injection. Ideal composition of intraperitoneal STZ and nicotinamide administration was found on STZ dosage range 45-65 mg/kg body weight and nicotinamide dosage range 100–120 mg/kg, because of a greater rise in blood glucose than in higher doses of nicotinamide (17).

Achatina fulica mucus preparation

The snails (*A. fulica*) were purchased from snail collector at Sumberlawang Market, Sragen, Indonesia. To prevent bacterial contamination, snail shells were sterilized first with 70% alcohol. Snail mucus is obtained by breaking the snail shell. The tip of the pipette was utilized to remove the slime from snail meat. The mucus then stored in a sterile container with controlled temperature of 150 °C and not exposed to direct sunlight. The slime was then mixed with ethanol and centrifuged in the laboratory of the center for food and nutrition studies of Gajah Mada University in Yogyakarta, Indonesia.

The oxidative and inflammatory biomarker

The biomarkers measured in this study are MDA, TGF-β, TNF-α, VEGF, hs-CRP, and IL-1β levels after the intervention. MDA is the result of lipid peroxidation and is conducted as a biomarker of oxidative stress due to inflammation (5). TGF-β is a profibrotic growth factor that is involved in many cellular processes and has been identified as a key cytokine in the development of diabetic nephropathy (7). TNF-α, in conjunction with its TNFR2 inflammatory pathway, plays a role in the progression of DKD (8). VEGF also played a vital role in the progression and occurrence of DKD in DM patients (9). Other inflammatory markers, hs-CRP (10) and IL-1β (11), were also found to be elevated on DKD. All of the biomarkers were measured using the enzyme-linked immunosorbent assay (ELISA) method and interpreted by a competent laboratory analyst in the laboratory of the center for food and nutrition studies, inter-university center, Gajah Mada university, Yogyakarta.

Experimental design

The rats were divided into 4 groups with each group consisting of 8 rats with sim-Simple random sampling as follows:

- Negative control (coded Control): 0.5 mL of NaCl 0,9% intraperitoneal + 0.5 mL/d pure H2O orally for 2 weeks
- Positive control (coded STZ-NA): 45 mg/kg STZ and 110 mg/kg NA intraperitoneal + 0.5 mL/d pure H2O orally for 2 weeks
- Snail mucus group 1 (coded STZ-NA+S1): 45 mg/kg STZ and 110 mg/kg NA intraperitoneal + 3.5 mL/d snail mucus orally for 2 weeks
- Snail mucus group 2 (coded STZ-NA+S2): 45 mg/kg STZ and 110 mg/kg NA intraperitoneal + 7 mL/d snail mucus orally for 2 weeks

Before the induction, all rats were acclimatized for 7 days. STZ was first dissolved in buffer citrate (volume of 0.1 mL/kg, sodium citrate 5 mmol/L, pH 4.5), and NA was dissolved in 0.1 mL/kg of NaCl 0.9%. DKD was induced by administering NA intraperitoneal, followed by STZ intraperitoneal 15-minutes apart. After the administration, all of the rats were kept for 3 weeks for the nephropathy to occur. Diabetes was confirmed by high glucose values (over 250 mg/dL) in the blood of twelve-hour-fasted rats, measured using an Accu-Chek Instant digital glucometer (Roche, Warsaw, Poland). DKD on the rats would be observed in 3 weeks after the induction, as shown in previous studies (16,17) The rats were weighed before the induction, and each week after induction until the end of the study, to see the dynamics of their weight. Blood samples would be collected 24 hours after the last snail mucus administration, with a volume of 2 mL of blood taken through the retroorbital plexus. The biomarkers would be measured from these blood samples.

Statistical analysis

The body weight, glucose level, and biomarkers level were recorded and analyzed with SPSS for Windows Release 25.0. A one-way ANOVA test was performed to determine whether there were differences in body weight, glucose level, and each of the biomarker levels, with a P value of <0.05 considered significant. A post-hoc test using Tukey will be performed to determine the mean differences among the groups in each parameter measured, with a P value of <0.05 considered significant.

Results

Body weight and glucose level after induction of STZ-NA

Figure 1 shows the significant reduction compared to control in mean body weight of the diabetic rat models after only one week of induction with STZ-NA. The non-diabetic rats' mean body weight steadily increases in the control group on each measurement. *Achatina fulica* mucus administration at 3 weeks post-induction showed increments in mean body weight in both STZ-NA+S1 and STZ-NA+S2 at the measurement at 5 weeks post-induction. Both dose of *A. fulica* mucus does not differ significantly on body weight.

Figure 2 shows that all of the STZ-NA-induced rats were successfully introduced to diabetes by passing the cut-off (>250 mg/dL) at the measurement of glucose level at one-week post-induction of STZ-NA. The administration of *A. fulica* mucus showed a significant reduction in glucose levels in the STZ-NA+S1 and STZ-NA+S2 groups compared to the non-treated STZ-NA group at 5 weeks post-induction. Higher dose of *A. fulica* mucus shows more reduction of glucose level.

Level of inflammatory parameter

Table 1 shows the significant mean differences of the inflammatory parameters level among the groups,

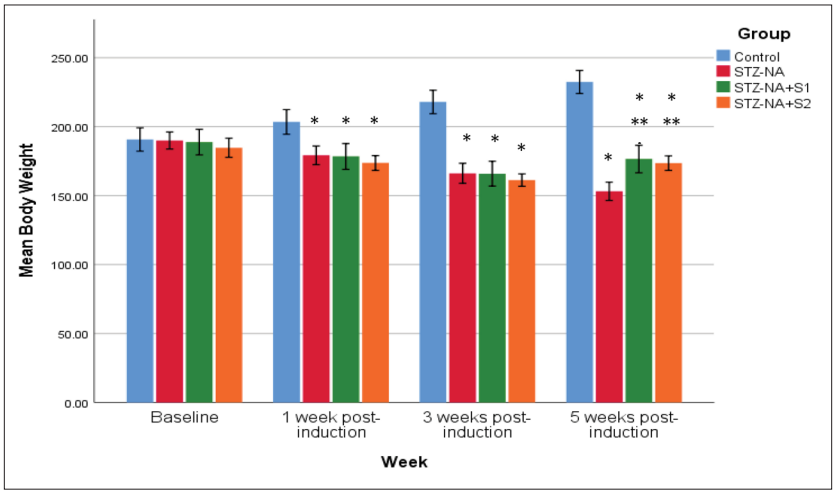

Figure 1. The effect STZ-NA induction on body weight of the diabetic rats. Snail mucus was administered on 3 weeks post-induction of STZ-NA. *P<0.001 versus control; **P<0.001 versus STZ-NA.

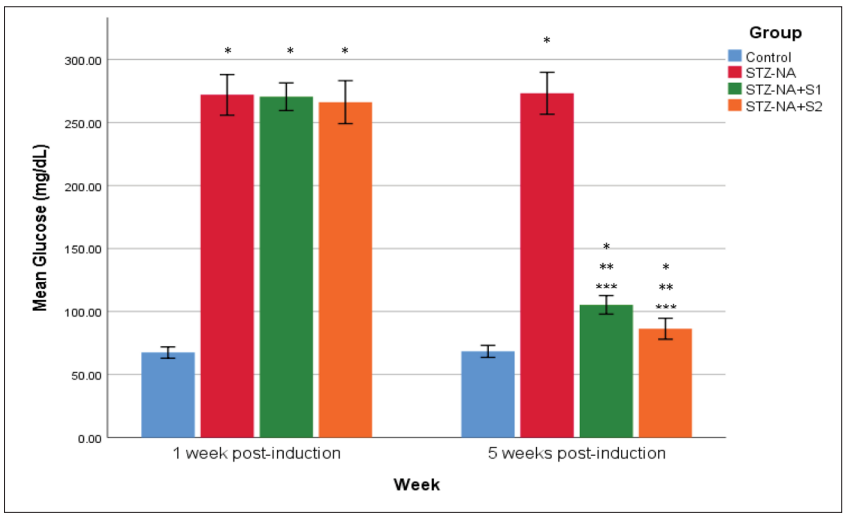

Figure 2. The effect STZ-NA induction on glucose level of the rats. Snail mucus was administered on 3 weeks post-induction of STZ-NA. *P<0.001 versus control; **P<0.001 versus STZ-NA; ***P<0.001 versus 1 week's post-induction.

with all of STZ-NA induced rats showed higher level than control group. This showed overall elevated inflammatory activities in all DKD mice. Higher dose of mucus in STZ-NA induced rats showed lower mean of inflammatory parameters on measurement compared to the lower dose group.

Figure 3 displays the administration of *A. fulica* mucus showed significant reduction of mean MDA, TGF-β, TNF-α, hs-CRP, VEGF, and IL-1β on both administered groups compared to STZ-NA group. Higher dose of the mucus further significantly reduced the mean of these parameter.

Discussion

Chronic inflammation is integral in all CKD patients, including DKD ones. Chronic inflammatory biomarkers have been found to increased alongside the deterioration in renal function. All of the inflammatory markers used in our study; MDA, TGF-β (7), TNF-α (8), VEGF (9), hs-CRP (10), IL-1β (11) have been found to be increased in the setting of DKD and associated with progression of renal fibrosis (4). In diabetes, abundance of inflammatory cytokines may attract infiltrating macrophages, which may cause local damage and promote the release of additional inflammatory cytokines (18), also increase the production of reactive oxygen/nitrogen species (19).

Despite significant advances in renal replacement therapy, the mortality rate for patients with ESRD, remained high with cardiovascular disease continues to be the leading cause of morbidity and mortality in these patients. This tendency also extends to DKD (20), with various pathways, including dysregulation of blood

Table 1. The mean, standard deviation, and ANOVA analysis of the effect of snail mucus on inflammatory parameter levels among the groups

Parameter	Groups (Mean ± SD)				*P* value
	Control	STZ-NA	STZ-NA+S1	STZ-NA+S2	
MDA	1.29 ± 0.11	9.78 ± 0.56	3.26 ± 0.24	2.33 ± 0.16	<0.001*
TGF-β	4.72 ± 0.56	32.40 ± 1.46	13.92 ± 1.01	8.14 ± 0.80	<0.001*
TNF-α	6.19 ± 0.56	20.28 ± 0.64	10.22 ± 0.59	8.28 ± 0.32	<0.001*
hs-CRP	3.06 ± 0.76	14.04 ± 0.08	6.93 ± 0.29	4.71 ± 0.51	<0.001*
VEGF	23.19 ± 0.99	37.17 ± 2.19	30.95 ± 0.59	27.03 ± 1.33	<0.001*
IL-1β	36.55 ± 0.57	176.62 ± 7.81	65.67 ± 1.25	50.18 ± 3.29	<0.001*

MDA, malondialdehyde; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; hs-CRP, high sensitivity C-reactive protein; VEGF, vascular endothelial growth factor; IL-1β, interleukin 1-β.
Note: *ANOVA analysis showed significant if $P<0.05$.

pressure, retention of uremic toxins, anemia, and altered mineral metabolism, in addition to cumulative vascular damage caused by diabetes. Diabetes increased the risks for mortality and ESRD, but the progression of the kidney disease did not take account of the diabetes status, further highlighting the importance of intrinsic renal pathology (20). Systemic inflammation also has been shown to precedes the progression of microalbuminuria in diabetic patients (21). As an inherent component of the pathology of DKD, attenuating inflammation has become the focus of the recent studies regarding DKD treatment approaches (22). The target of the inflammatory attenuation must be

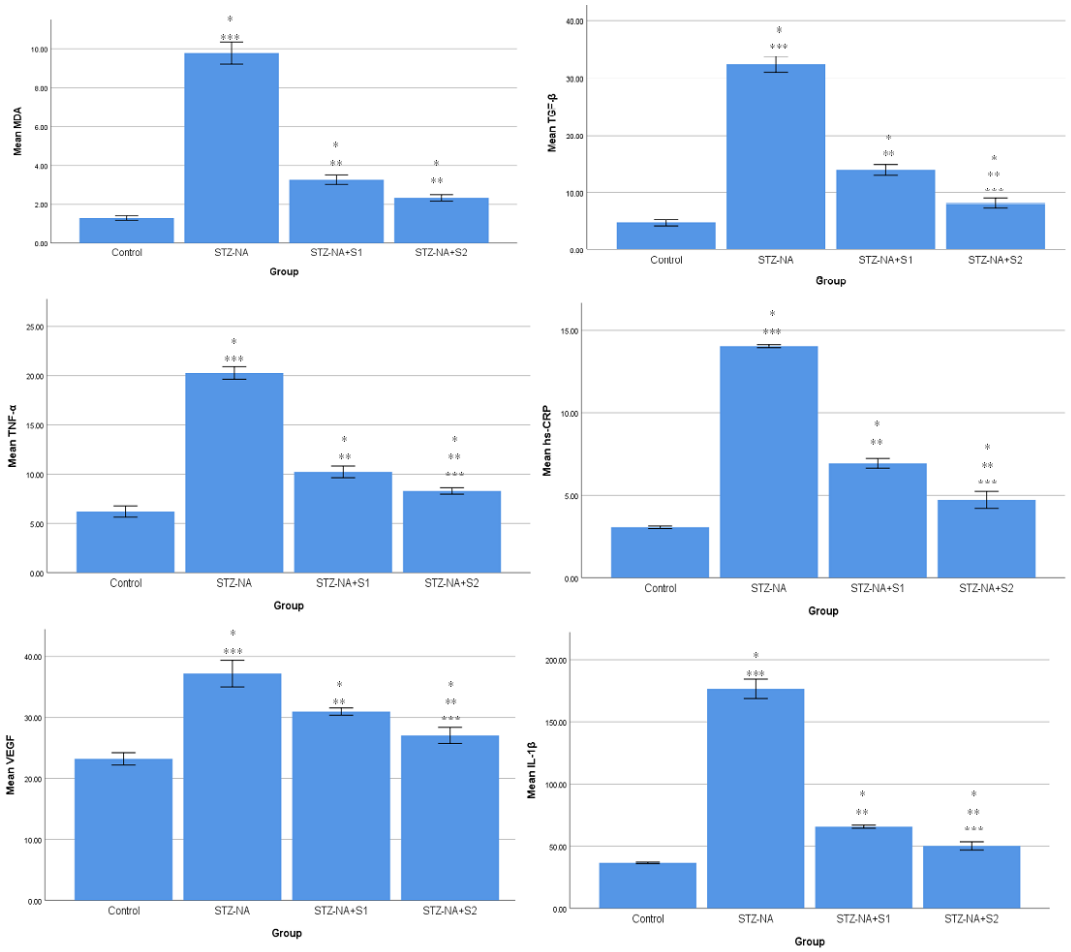

Figure 3. Tukey post-hoc analysis of the effect of snail mucus on MDA, TGF-β, TNF-α, hs-CRP, VEGF, and IL-1β levels among the groups. *$P<0.001$ versus control; **$P<0.001$ versus STZ-NA; ***$P<0.001$ versus STZ-NA+S1.

precisely directed, as simply attenuating inflammation with nonsteroidal anti-inflammatory medications in DKD, via its effect on renal prostaglandin signaling, could predispose further renal injury (22). The proposed targets of the inflammation parameter in our study are the oxidative stress and inflammatory cytokines that contributes to the progression of renal fibrosis, a central pathological process in DKD.

Achatina fulica mucus was shown in various studies to exert wound-healing capabilities (23), especially due to its abundance of glycosaminoglycans (GAGs), the novel acharan sulfate. This GAG had activity and structure similar to heparan sulfate, without its anticoagulant property (24). Heparan sulfate had a well-known anti-inflammation and immune modulatory efficacy (12,25). Due to its similarities, acharan sulfate also have significant anti-oxidative, immunomodulatory, and antimitogenic properties in various studies (12,13).

The induction of DKD in our animal model using STZ-NA showed excellent result, as shown with hyperglycemia and reduced body weight in induced rats. This was achieved in 3 weeks after the induction, as already mentioned in previous studies (16,17) The constant reduction of body weight in our study was consistent in STZ-NA induced rats with the administration of *A. fulica* mucus showed efficacy in restoring body weights of the rats in subsequent measurement, comparable to high-fat diet as shown in previous studies (26). This was hypothesized as the consequences of the improvement of heparan sulfate status after the mucus administration. Heparan sulfate had important activity in improving the insulin sensitivity and glucose homeostasis (25). The depletion of heparan sulfate, as shown in DKD, also contributes to increased oxidative stress and beta islets failure (27). The hypothesis was similar activity of acharan sulfate to heparan sulfate enabled the improvement of body weight via improvement in insulin sensitivity in our animal models.

As previously described in previous studies (13), *achatina fulica* mucus exerts anti-oxidative, and anti-inflammation properties, as shown in the marked improvements in our biomarkers; MDA, TGF-β, TNF-α, hs-CRP, VEGF, and IL-1β in our animal models. The description of the mucus effect on these biomarkers in diabetic rat models was scarce in the literature, but the effect was described in other models. The antioxidant property of *A. fulica* mucus (13) was evidenced by the reduction of MDA level in STZ-NA induced rats, and higher dose further reduce this. As a lipid oxidation product that could reflect the level of ROS formation and oxidative damage (5), this improvement was essential due to significance of oxidative damage in progression of DKD (22). The reduction of inflammatory cytokines in our study was greater at dose of 7 mL/d effectiveness than a dose of 3.5 mL/d. These improvements of inflammatory biomarkers; TGF-β, TNF-α, hs-CRP, VEGF, and IL-1β in STZ-NA induced rats after mucus administration was thought due to the mucus' anti-inflammatory properties (12), and acharan sulfate's molecular similarity to heparan sulfate (25). Heparan sulfate also essential in regulating angiogenesis, whereas its dysfunction was essential in microvascular complication and profibrotic activity in DKD progression (28).

The principal role of heparan sulfate in modulating the permeability of glomerular basement membrane (GBM) was essential in modulating the proteinuria, the hallmark of DKD. The hyperglycemia in diabetes upregulates the heparanase, heparan sulfate degrading-enzyme, in the glomerular epithelial cell (29). This GAG regulates the density of the basement membrane and glomerular cell, prevent coagulation of the vessel walls, and modulates intimal smooth muscle cell (30). Heparan sulfate role also extends to modulates the permeability selectivity of the GBM, due to its abundance, thus the loss of this GAG predisposes the nephropathy and proteinuria in DKD (29). These were essential in reducing the progression of DKD's nephropathy, as the reduction of proteinuria was traditionally viewed as better prognosis indicator (22).

The primary limitation of our study is that albuminuria or kidney biopsies were not performed on our experimental animals to confirm the diagnosis of DKD. According to several previous studies, STZ-NA induction will result in albuminuria and severe kidney damage within three weeks (16,17). Several biomarker parameters, particularly TGF-β, have increased in STZ-NA groups, indicating that the molecular signaling leading to renal fibrosis has increased. Further studies incorporating histopathological analysis are required to confirm our finding on cellular and tissue-level.

The results showed that *A. fulica* mucus administration contributed to restoring body weight, reducing oxidative stress, and reducing inflammatory cytokines in STZ-NA-induced DKD rats. This was thought to be attributed to acharan sulfate's molecular similarity to heparan sulfate. Regarding its potential efficacy in the current approach of inflammation-targeted therapy in DKD, further in-depth studies are required.

Conclusion

This study found that the administration *A. fulica* mucus significantly reduce MDA, TGF-β, TNF-α, hs-CRP, VEGF, and IL-1β in STZ-NA induced diabetic rats in our study. This efficacy was dose-dependent, with a dose of 7 mL/d provides better effectiveness than a dose of 3.5 mL/d. Thus, current study showed the potential of *Achatina fulica* mucus usage in future management of

inflammation and oxidative stress in diabetes and DKD.

Limitations of the study

The most significant limitation of our investigation is that albuminuria or kidney biopsies were not conducted on our experimental animals to confirm the diagnosis of DKD. Further investigations involving histopathological analysis are necessary to corroborate our cellular and tissue-level findings.

Authors' contribution

Conceptualization: Wachid Putranto, Gigih Fitriawan, Ratih Tri Kusuma Dewi, Aryo Suseno, Arief Nurudhin, and Yulyani Werdiningsih.
Data curation: Wachid Putranto, Gigih Fitriawan, Santy Ayu Puspita Perdhana, Nurhasan Agung Prabowo, and Yeremia Suryo Pratama.
Formal analysis: Wachid Putranto, Gigih Fitriawan, Ratih Tri Kusuma Dewi, Aryo Suseno, and Nurhasan Agung Prabowo.
Funding acquisition: Wachid Putranto, Gigih Fitriawan, Ratih Tri Kusuma Dewi, Aryo Suseno, Arief Nurudhin, Yulyani Werdiningsih, Santy Ayu Puspita Perdhana, and Nurhasan Agung Prabowo.
Methodology: Wachid Putranto, Gigih Fitriawan, Ratih Tri Kusuma Dewi, Arief Nurudhin, and Nurhasan Agung Prabowo.
Investigation: Wachid Putranto, Gigih Fitriawan, Ratih Tri Kusuma Dewi, Arief Nurudhin, and Santy Ayu Puspita Perdhana.
Project administration: Wachid Putranto, Gigih Fitriawan, Aryo Suseno, Yulyani Werdiningsih, and Nurhasan Agung Prabowo.
Resources: Wachid Putranto, Gigih Fitriawan, Aryo Suseno, Yulyani Werdiningsih, and Nurhasan Agung Prabowo.
Supervision: Wachid Putranto, Gigih Fitriawan, Ratih Tri Kusuma Dewi, Arief Nurudhin, and Santy Ayu Puspita Perdhana.
Validation: Wachid Putranto, Gigih Fitriawan, Aryo Suseno, Yulyani Werdiningsih, and Santy Ayu Puspita Perdhana.
Visualization: Wachid Putranto, Gigih Fitriawan, Arief Nurudhin, and Nurhasan Agung Prabowo.
Writing–original draft: Wachid Putranto, Gigih Fitriawan, Ratih Tri Kusuma Dewi, Aryo Suseno, Arief Nurudhin, and Yulyani Werdiningsih.
Writing–review and editing: Wachid Putranto, Gigih Fitriawan, Santy Ayu Puspita Perdhana, Nurhasan Agung Prabowo, and Yeremia Suryo Pratama.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

The research and the protocol of this study was in accordance with the guidelines of animal studies and was approved by Health Research Ethics Committee in Moewardi General Hospital with the number: 1.132/VII/HREC/2022, in accordance with ARRIVE guidelines. Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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