Snail mucus suppresses anti dsDNA antibodies in lupus mice

A Nurudhin1, N A Prabowo1,2, A Ramadhani1,2

1 Faculty of Medicine, Sebelas Maret University, Sutami No 36 Street, Surakarta, Indonesia
2 Sebelas Maret University Hospital, Ahmad Yani No 200 Street, Sukoharjo, Indonesia
*Corresponding author: dr.nurhasan21@staff.uns.ac.id

Abstract. Lupus is closely related to weather changes. Changes in extreme weather due to climate changes can trigger lupus flares and cause death. Therefore, many studies are trying to find a drug that can cure lupus. Snail mucus has the properties of suppressing the immune system. The aim of this study is to see how snail mucus affects the levels of anti-dsDNA antibodies in lupus model mice. Experimental study uses a posttest-only group design. The control group was mice with 0.5 cc intraperitoneal (IP) saline. The lupus nephritis group was mice with pristane 0.5 cc IP, and the therapy group was mice with 0.5 cc IP pristane and 0.5 cc snail mucus per day at the 4th month of treatment for 14 days and after that the mice blood serum was taken and examined in the laboratory. The dsDNA antibody examination used the ELISA method. Statistical test with Anova followed by post hoc test. P is significant if it is less than 0.05. There was an increase in the levels of antibody dsDNA (p = 0.016) in the lupus group (75.13 ± 22.5 mg/dL) compare with the control group (56.96 ± 11.77 mg/dL), but there was a decrease in anti-dsDNA antibodies in the mice with snail mucus (56.01 ± 8.29 mg/dL; p = 0.02), and the methyl prednisolone group (53.47 ± 13.53 mg/dL; p = 0.009) when compared to the lupus group. Snail mucus suppresses anti dsDNA antibody levels in lupus mice.

1. Introduction
Lupus is a multisystem illness characterized by the production of antibodies and the deposition of complement immune complexes. Lupus is an autoimmune condition that is chronic and inflammatory, tissue injury as a result [1 - 4]. It has been demonstrated that the combination of hereditary and environmental variables has a key role in the etiopathogenesis of SLE (Systemic Lupus Erythematosus) [5]. Recent studies have discovered a seasonal variation trend in SLE manifestations, which can be explained by a deficiency of vitamin D, sensitivity to ultraviolet (UV) light, and the presence of infectious agents [6]. These findings are unsurprising, due to a hereditary tendency to photosensitivity, individuals with SLE prefer to avoid extensive sun exposure. Low vitamin D levels were identified in individuals with systemic lupus erythematosus, and an inverse association between plasma vitamin D levels and disease activity was discovered by Ben-Zvi et al. [7]. Melatonin levels were also shown to be lower in SLE patients than in healthy controls, with disease activity and melatonin levels having an inverse relationship. Campillo et al. looked into the effects of melatonin on SLE cells, showing that melatonin has significant anti-inflammatory properties in these cells. Melatonin tends to exert its effects in two distinct ways: by inhibiting Th1 cells and inducing T-reg cells, both of these things help to create an anti-inflammatory environment [8]. The etiologies of SLE have been linked to microorganism infections. The human herpesvirus EBV has been related to the etiopathogenesis of SLE. The human splicesome and EBV nuclear antigen-1 have a molecular resemblance, which induces autoantibodies in approximately 40% of SLE patients [9], was found to be responsible for this phenomenon. On the other
hand, it has been reported that EBV infections are substantially more prevalent during the winter [10]. UV-A, UV-B, and UV-C are the three types of UV radiation. Because most UV-C radiation is absorbed by the ozone layer, the bulk of sunlight that reaches the planet is UV-A and UV-B. Despite these consequences, UV radiation causes keratinocyte apoptosis, resulting in their accumulation. Photosensitivity in SLE is characterized by the aggregation of apoptotic cells. After being exposed to light radiation, 93 percent of Patients with SLE exhibited clinical and histological signs of photosensitivity, according to Sanders et al. These findings back up the theory that UV radiation causes cutaneous lupus to worsen. UV radiation is higher during the summer, which is consistent with seasonal variation in SLE cutaneous symptoms previously recorded [10].

The inflammatory disease systemic lupus erythematosus affects several organ systems. During the relapse and flare periods, the clinical symptoms vary considerably and fluctuate [11]. It's an autoimmune disorder marked by illness flare-ups and the buildup of tissue damage. Patients with SLE have lived longer as a consequence of the development of more precise immunosuppressive and antibacterial drugs, supplemental care for organ failure and treatment-related comorbidities, and better awareness of the condition among physicians and patients during the last several decades. The average 5-year survival rate improved steadily from approximately 50% in the 1960s to more than 90% in the 1990s, according to information from the majority of well-established lupus clinics throughout the world [12]. The global prevalence of SLE is estimated to be between 4.3 and 150 per 100,000 people, with women having a greater frequency. SLE patients have a mortality rate that is two to five times higher than the general population. SLE not only raises mortality rates, but it also lowers quality of life and puts a strain on finances. Nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, antimalarial medications, and immunosuppressive agents are the most often utilized treatments for SLE. While these medicines may help prevent active aggravation of SLE, they also have the potential to cause significant side effects and increase the risk of serious infections. As a result, many SLE patients seek help from alternative treatments [11].

Snail mucus is one treatment option for SLE. Achatina Fulica is a land snail that secretes a glycoprotein secretion that has been shown to have biological effects, including antibacterial properties against gram-positive and negative bacteria [13]. A unique glycosaminoglycan (AF-GAG) was isolated and described from the soft body of the snail Achatina fulica. The primary components of AF-GAG, which had a molecular weight of 118 kDa, were iduronic acid (IdoA) and N-acetylg glucosamine (GlcNAc). This GAG has a highly consistent and homogenous structure, unlike mammalian heparin and heparan sulfate [14]. Achasin and mytimacin-AF proteins are found in the mucin fraction. Snail mucus from the Achatina Fulica species contains achasin and glycosaminoglycan proteins in the form of heparan sulfate, acharan sulfate, heparin, and hyaluronic acid. Heparan sulfate is well known for its growth factor binding and storage properties. Additionally, it stimulates the migration of inflammatory cells to the wound region immediately after damage, one of which is lymphocytes. Achasin in snail mucus also has antimicrobial properties and has the effect of inhibiting inflammation [15].

Antibodies against double-stranded DNA (dsDNA) are diagnostic and prognostic indicators of SLE, and their presence has been associated with lupus nephritis. These antibodies are sometimes stored in the glomeruli and can be eluted from SLE patients' and lupus mice's kidneys [16]. The aim of this study is to see how snail mucus affects the levels of anti-dsDNA antibodies in lupus model mice.

2. Material and methods

2.1. Study design
This is an experiment with mice as the experimental animals and only a post-test control group.

2.2. Population and study setting
The research subjects were male mice of the subspecies Mus musculus Balb/C strain aged 3-4 months, bodyweight 20-30 grams, obtained from the Faculty of Veterinary Medicine, Gajah Mada University. The food ingredients for mice are used as standard BR I mice feed. The group was divided into 4, namely
the control group (NaCl 0.9% 0.5 cc intraperitoneally on the first day of treatment). The lupus group (Pristan 0.5 cc on the first day of treatment), the lupus group with standard therapy (Pristan 0.5 cc on the first day of treatment and the dose of methylprednisolone 0.5 mg per kg orally per day at month 4 for one month), the lupus group with snail mucus therapy (Pristan 0.5 cc on the first day of treatment, 0.5 cc of oral snail mucus at month 4 for one month). Mice were sacrificed at the end of the 5th month. Before being sacrificed, the blood serum was taken from the mice for ELISA examination.

2.3. Variables and Data Collection
Induction of lupus in mice using Pristan, according to the past study [2, 4]. Following the instructions of testing instruments (Sigma Aldrich), an ELISA dsDNA antibody test was performed. The Double Antigen Sandwich Technique is used in this package. The Double Antigen Sandwich theory is focused on characteristics of the studied antigen with more than two valances that can simultaneously distinguish coated antigen and detection antigen. The following is the basic procedure: 1.) To make immobilized antibodies, attach the antigen and solid phase carriers. Uncombined antigens and impurities should be washed away. Irrelevant proteins are used to seal the remaining binding sites. 2.) Add immobilized antigens to the test subject for a touch reaction. Combine antibodies and antigens on carriers into the antibodies complex after a while. Uncombined antigens and impurities should be washed away. 3.) Add antigens to the immune complexes to combine with the antibodies. Wash the antigens that have not been mixed thoroughly. The amount of enzyme on the carrier is now proportional to the amount of the measured material in the samples. 4.) Mark the avidins with horseradish peroxidase and incorporate them with the antigens. Thoroughly wash out the enzyme markers that have been added. The amount of enzyme on the carrier is now proportional to the amount of the measured material in the samples. 5.) Add coloring substrates and calculate the concentration of specimens.

2.4. Data analysis
Analysis of research data using a different test, The ANOVA test is used first, followed by the LSD post hoc test, with a meaningful result if the p value is less than 0.05.

2.5. Ethical clearance
The ethical committee of the Universitas Sebelas Maret Faculty of Medicine approved this study with ethical clearance number 059 /UN27.06.6.1/ KEPK/EC/2020.

3. Results
There was an increase in the levels of antibody dsDNA (p = 0.016) in the lupus group (75.13 + 22.5mg/dL) compare with the control group (56.96 ± 11.77 mg/dL), but there was a decrease in the levels of anti-dsDNA antibodies in the snail mucus mice (56.01 ± 8.29 mg/dL; p = 0.02), and the methyl prednisolone group (53.47 ± 13.53 mg/dL; p = 0.009) when compared to the lupus group. Description of the results of this study is depicted in the boxplot below.

![Boxplot of snail mucus decreased the level of dsDNA antibodies in lupus mice](image-url)

**Figure 1.** Boxplot of snail mucus decreased the level of dsDNA antibodies in lupus mice
4. Discussion
This study uses Pristan as a method for making lupus in mice, and this is like previous studies [2, 4]. Mineral oil contains a lot of pristane, which is an isoprenoid alkane. Injecting pristane intraperitoneally is the standard method for receiving ascitic fluid enriched in monoclonal antibodies. BALB/c mice produce anti-ribonucleoprotein, anti-DNA, and anti-histone autoantibodies after receiving pristane injections. In pristane-treated mice, IC accumulation in the kidneys causes severe nephritis [17]. The bulk of the histopathological characteristics associated with SLE were caused by single pristane injections into several mice strains, and it is one of the few animal models of SLE that expresses Signature genes for type I interferon (IFN), as identified in SLE patients. Despite its fabricated roots, the induced SLE model is very useful for identifying the role of a single gene or component in the pathogenesis of SLE, as backcrossing into spontaneous SLE strains takes a long time and effort [18].

SLE is an autoimmune illness characterized by the production of a wide range of autoantibodies as a result of polyclonal B cell activation, apoptotic pathway dysfunction, or idiotypic network dysregulation. Antibodies against double-stranded DNA (anti-dsDNA) are thought to be a particular SLE marker [19]. Because of their high frequency (between 70% and 98%), sensitivity, and specificity, the presence of these autoantibodies may be used to practically diagnose SLE (57.3 percent and 97.4 percent, respectively) [19]. They are exceedingly unlikely to be detected in other pathological disorders or in stable subjects (less than 0.5 percent) [20]. Additionally, Anti-dsDNA antibodies were discovered in SLE patients several years before illness manifestation, indicating that they are implicated in the progression of a clinically evident disease. Anti-dsDNA antibodies are pathogenic, according to several lines of evidence. These autoantibodies, in particular, have been linked to kidney involvement, as evidenced by their deposition in the glomeruli, the subepithelial and subendothelial spaces, the basement membrane, the mesangium, and the tubules in SLE patients with active nephritis. Additionally, anti-dsDNA complexed with DNA can be used to evaluate dendritic cell activity, B and T cells are activated, and proinflammatory cytokines are released as a result. Increases in anti-dsDNA antibodies serum levels have been shown in the literature to predict disease relapse, especially in the case of renal disease exacerbation [19].

The use of snail mucus was discovered in this research to reduce dsDNA antibody levels in lupus mice. Snail mucus contains glycosaminoglycan. The glycosaminoglycan (GAG) chains' complex structure and heterogeneity are essential in a variety of biological functions associated with proteoglycans. Using multidimensional and immunohistochemical protein recognition technologies, Gesteira et al. discovered numerous proteoglycans in the gastropod Achatina Fulica organs, including perlecain proteoglycans, aggrecan, and heparan sulfate [21]. A new glycosaminoglycan, acharan sulfate from Achatina fulica, inhibits vascular endothelial growth factor (VEGF) activity without affecting its development, according to Ajoy Kumar's study. In endothelial vascular cells, acharan sulfate inhibits VEGF mitogenic activity. Numerous biological roles for Acaharan sulfate have been suggested in snails, but since GAG was discovered only recently, it was only tested because of its structural resemblance to heparin and heparan sulfate, as well as its biological role [22].

Heparan sulfate protects chemokines, cytokines, and growth factors from enzymatic proteolysis by forming chemokine gradients in the vascular endothelium and controlling their production by physical sequestration in the matrix [23]. Direct signaling through TLR-4, phagocytosis, and controlling the interactions of inflammatory mediators with their receptors are some of the other functions of heparan sulfate. Alternative complement activation pathways are also regulated by heparan sulfate. Heparan sulfate's structural heterogeneity enables it to perform a variety of functions, such as inhibiting the complement cascade by binding factor H to its surface, speeding up the inactivation of C3b, or activating the cascade by binding the stabilizing factor properdin to apoptotic cells [23].

Heparan sulfate also aids angiogenesis by inhibiting VEGF and reducing fibroblast growth factor mitogenic activity (FGF) [24]. Heparan sulfate administration may be able to replace the glomerular sulfate membrane filtration that has been compromised by immune complexes in lupus nephritis. Hepatic sulfate also has the ability to speed up the maturation of immature dendritic cells [25]. Heparan sulfate and heparin can bind to complement proteins, causing the complement cascade to be inactivated.
These associations can be used to establish Heparan sulfate/heparin-based complement interventions. Heparan sulfate modulates cell activity by interactions complement factors, chemokines, cytokines, growth factors, adhesion molecules, matrix proteins, and other proteins. Yu et al. and Sahu et al. published two systematic studies on heparin's ability to bind to proteins. The complement cascade is inhibited by the majority of these interactions, which have a regulatory role [26].

This study uses methylprednisolone as a standard drug for lupus. The use of methylprednisolone can suppress anti-dsDNA antibodies. Methylprednisolone is a glucocorticoid (GCs) medication. The first mechanism by which GCs function is by interfering with the transcription of pro-inflammatory molecules in the genome. The process is initiated by the binding of GCs to the cytosolic-GC receptor (cGR). The GC-cGR complex joins the nucleus and acts as a transcription factor [27]. The genomic pathway is what it is called. The GC-cGR complex's first reaction transrepression occurs in the nucleus, which entails inhibiting genes involved in the synthesis of cytokines and other proteins during the inflammatory process, resulting in an anti-inflammatory effect. A second phase known as transactivation begins as the intranuclear concentration of GCs rises. While this pathway stimulates the expression of various inhibitory genes, it is largely responsible for the activation of gluconeogenesis, insulin tolerance, skin atrophy, and inhibition of bone development, all of which are well-known GC side effects. Low dosages of prednisone (7.5 mg/day) have been linked to a 50 percent increase in cGR saturation. The receptor gets rapidly saturated from 50% to close to 100% at moderate doses (>7.5 mg/day to 30 mg/day of prednisone), maintaining a less linear connection with the daily dosage. Prednisone doses of 30–40 mg/day are thought to be sufficient almost thoroughly to saturate cGR. Higher dosages, up to 100 mg per day, are used, transactivation is the main response, resulting in the development of unintended reactions with no substantial increase in anti-inflammatory effects [27]. The limitation in this study is that research is still being carried out on experimental animals, where we need to be carried out further clinical trials to determine the truth of the effect of snail mucus.

5. Conclusion
Snail mucus decreased the level of anti-ds-DNA antibody in lupus mice. Further research needs to be done to determine the main mechanism of the decrease in the level of anti-ds-DNA antibody in the administration of snail mucus.

References
[1] Nurudhin A, Widyastuti R, Prabowo N A, Adnan Z A, Werdiningsih Y 2021 The effect of Mmorongaoleifera leaf extract on mean platelet volume and neutrophil to lymphocyte ratio in lupus Bangladesh J Med Sci 20(1) 68–73
[2] Prabowo N A, Nurudhin A, Novia SA 2020 Correlation Between Renal Activity Index and C3 Complement Expression in Mouse Lupus Nephritis Model 4th Int Conf Sustain Innov 2020 Health Sci Nurs 2021 75–9
[3] Nurudhin A, Prabowo N A, Yulyani, Adnan Z A, Adil 2020 Effect of moringa oleifera leaf extract on high sensitivity C-Reactive protein, ESR And MEX SLEDAI score in lupus patients Int J Hum Heal Sci 4(4) 291
[4] Prabowo N A, Adnan Z A, Nurudhin A, Werdiningsih Y, Prasetyo K 2021 Mesenchymal stem cell conditioned medium as good as methyl prednisolone in decreasing levels of interleukin 10 and the degree of pulmonary vasculitis in lupus mice Bangladesh J Med Sci 20(2) 426–30
[5] Kamen D L 2014 Environmental influences on systemic lupus erythematosus expression Rheum Dis Clin North Am 40(3) 401–412
[6] Duarte-Garcia A, Fang H, To C H, Magder L S, Petri M 2012 Seasonal variation in the activity of systemic lupus erythematosus J Rheumatol 39(7) 1392–8
[7] Ben-Zvi I, Aranow C, Mackay M, Stanevsky A, Kamen D L, Marinescu L M, et al. 2010 The impact of vitamin D on dendritic cell function in patients with systemic lupus erythematosus PLoS One 5(2)
[8] Medrano-Campillo P, Sarmiento-Soto H, Álvarez-Sánchez N, Álvarez-Rios A I, Guerrero J M,
Rodriguez-Prieto I, et al. 2015 Evaluation of the immunomodulatory effect of melatonin on the T-cell response in peripheral blood from systemic lupus erythematosus patients J Pineal Res 58(2) 219–26
[9] Caza T, Oaks Z, Perl A 2014 Interplay of infections, autoimmunity, and immunosuppression in systemic lupus erythematosus Int Rev Immunol 33(4) 330–63
[10] Watad A, Azrieanl S, Bragazzi N L, Sharif K, David P, Katz I, et al. 2017 Seasonality and autoimmune diseases: The contribution of the four seasons to the mosaic of autoimmunity J Autoimmun 82 13–30
[11] Ma Y C, Lin C C, Li CI, Chiang J H, Li T C, Lin J G 2016 Traditional Chinese medicine therapy improves the survival of systemic lupus erythematosus patients Semin Arthritis Rheum 45(5)596–603
[12] Mak A, Cheung M W L, Chiew H J, Liu Y, Ho R C 2012 Global trend of survival and damage of systemic lupus erythematosus: meta-analysis and meta-regression of observational studies from the 1950s to 2000s Semin Arthritis Rheum 41(6)830–9
[13] Liu J, Zhou L, He Z, Gao N, Shang F, Xu J, et al. 2017 Structural analysis and biological activity of a highly regular glycosaminoglycan from Achatina fulica Carbohydr Polym 18 433–41
[14] El-Gendy K S, Gad A F, Radwan M A 2021 Physiological and behavioral responses of land molluscs as biomarkers for pollution impact assessment A review Environ Res 193 110558
[15] Riyani N J 2017 Pengaruh lendir bekicot (Achatina Fulica) terhadap jumlah limfosit pada proses penyembuhan luka soket gigi pasca pencabutan gigi tikus wistar (Rattus norvegicus) [Thesis] (Malang: Kedokteran Gigi Universitas Brawijaya)
[16] Malkiel S, Atisha-fregoso Y, Diamond B 2020 Anti-DNA antibodies Second Edition. Systemic Lupus Erythematosus (USA: Elsevier Inc Academic Press) pp 231–235
[17] Li Wei, Titov A A, and More L 2017 An update on lupus animal models Curr Opin Rheum 29(5) 434–441
[18] Gunawan M, Her Z, Liu M, Tan SY, Chan X Y, Tan W W S, et al. 2017 A novel human systemic lupus erythematosus model in humanised mice Sci Rep 7(1) 1–12
[19] Conti F, Cecarelli F, Perricone C, Massaro L, Marocchi E, Miranda F, et al. 2015 Systemic lupus erythematosus with and without Anti-dsDNA Antibodies: Analysis from a Large Monocentric Cohort Mediators Inflamm 2015 328078
[20] Cozzani E, Drosera M, Gasparini G, Parodi A 2015 Serology of lupus erythematosus: Correlation between immunopathological features and clinical aspects Autoimmune Dis 2014 321359
[21] Gesteira T F, Coulson-Thomas V J, Ogata F T, Farias E H C, Cavalheiro R P, De Lima M A, et al. 2011 A novel approach for the characterisation of proteoglycans and biosynthetic enzymes in a snail model Biochim Biophys Acta - Proteins Proteomics 1814 1862–9
[22] Vieira T C R G, Costa-Filho A, Salgado N C, Allodi S, Valente A P, Nasciutti L E, et al. 2004 Acharan sulfate, the new glycosaminoglycan from Achatina fulica Bowdich 1822: Structural heterogeneity, metabolic labeling and localization in the body, mucus and the organic shell matrix Eur J Biochem 271(4) 845–54
[23] Collins L E, Troeberg L 2019 Heparan sulfate as a regulator of inflammation and immunity J Leukoc Biol 105(1) 81–92
[24] Pacicca D M, Patel N, Lee C, Salisbury K, Lehmann W, Carvalho R, et al. 2003 Expression of angiogenic factors during distraction osteogenesis. Bone 33(6) 889–98
[25] Kim R, Emi M, Tanabe K, Arihiro K 2007 Potential functional role of plasmacytoid dendritic cells in cancer immunity J Immunology 121(2)149–57
[26] Zaferani A, Talsma D, Richter M K S, Daha M R, Navis G J, Seelen M A, et al. 2014 Heparin/heparan sulphate interactions with complement - A possible target for reduction of renal function loss? Nephrol Dial Transplant 29(3) 515–22
[27] Porta S, Danza A, Arias Saavedra M, Carlomagno A, Goizueta M C, Vivero F, et al. 2020 Glucocorticoids in Systemic Lupus Erythematosus J Clin Med 9(9) 2709.
Acknowledgement
We want to express our deepest gratitude to the histology laboratory where this research was conducted, the research assistants, and clinical pathology laboratory officers of the Faculty of Medicine, Sebelas Maret University Surakarta.