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Simvastatin: a new therapeutic strategy for Chronic Kidney Disease (CKD)

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ABSTRACT

Background: Chronic kidney disease (CKD) is a progressive disease whose prevalence increases annually. Studies in recent years report that statins (hydroxy-3-nethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors) affect preventing the progression of kidney damage, in addition to their ability to prevent cardiovascular risk in patients with CKD. This study aims to determine the effect of statins on kidney function and renal fibrosis in chronic kidney disease.

Methods: This research is pure experimental (randomized post-test only control group design). The research sample consisted of 20 white mice (*Mus musculus* L.), Swiss line, aged 3-4 months, and weight 30-40 grams. Samples were randomly grouped into four treatment groups: control (K, n = 5), treatment with simvastatin at a dose of 5.2 mg/kgBW (P1), treatment with simvastatin at a dose of 10.4 mg/kgBW (P2), and treatment with simvastatin at a dose of 20.8 mg/kgBW (P3). Interstitial fibrosis was assessed using Picrosirus Red staining. Data analysis used One-Way ANOVA and Tukey's post hoc tests with a significance value of p<0.05 in SPSS version 25.0 for Windows.

Results: The results showed that the control group (K) was the group with the highest serum creatinine level (1.07 ± 0.43) compared to the other treatment groups (p <0.05). Administration of simvastatin at doses of 5.2 mg/kgBW, 10.4 mg/kgBW, and 20.8 mg/kg BW in CKD mice resulted in lower interstitial fibrosis than controls. There was a significant difference between renal tissue fibrosis in the simvastatin group at a dose of 5.2 mg/kgBW (P1) with a dose of 10.4 mg/kgBW (P2) and a dose of 20.8 mg/kgBW (P3).

Conclusion: Simvastatin can improve kidney function and reduce the degree of renal fibrosis in animal models of CKD. Thus, it can be a therapeutic strategy for CKD.

Keywords: Simvastatin, Fibrosis, Kidney Function, Mice, Chronic Kidney Disease.
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INTRODUCTION

Chronic kidney disease (CKD) is a progressive disease whose prevalence continues to increase yearly. The prevalence of CKD in the United States reaches 15% or around 37 million people.[1] The prevalence in Asia is reported to vary, namely 10.6% in Nepal, 16.8% in China, 23.3% in Pakistan, and 30.9% in Iran.[2–4] This increase in prevalence has a direct impact on increasing medical costs and decreasing the quality of life of patients. The total cost of treatment for patients undergoing dialysis is reported to range from 7.9 million United States Dollars (USD) to 62 million USD.[5]

Research in recent years reports that statins (hydroxy-3-nethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors) are reported to affect preventing the progression of kidney damage, in addition to their ability to prevent cardiovascular risk in patients with CKD. Previous studies reported that statins significantly improved fibrosis in the glomerulus (glomerulosclerosis) in an animal model of subtotal nephrectomy but not significantly in an animal model of ischemic reperfusion injury.[6,7] These results are similar to other studies which found that statins can improve glomerulosclerosis.[8,9] Statins have also been reported to improve tubular atrophy and interstitial fibrosis, although the mechanisms for repairing fibrosis are still not fully understood.[9,10]

The pathway of fibrosis inhibition by statins remains unclear. Several studies reported that statins were able to inhibit fibrosis through the inhibitory effect of the homeobox protein Hox-A13 (HOXA13)–uterine sensitization-associated gene-1 (USAG-1) axis, the angiotensin II/Smad pathway, nuclear factor-kappa B (NF-κB), and MAPK.[10–14] Statins can inhibit RhoA and Rac-1 proteins which play a role in inflammatory processes and oxidative stress.[15] However, other studies report different results. Statins were reported to have no significant effect on inhibition of the SAPK/JNK MAPK pathway or p38 MAPK, NF-Kβ, expression of inflammatory markers such as vascular cell adhesion molecule-1 (VCAM-I), and 8- hydroxy-2'-deoxyguanosine (8-OHdG) which is an important biomarker of oxidative stress.[8,16] Statins are even reported to be unable to improve kidney function and calcification in atherosclerotic plaques and are nephrotoxic because they trigger subacute tubulointerstitial nephritis.[17,18]

Based on data from previous studies, it is known that giving statins is one of the efforts to prevent the progression of kidney damage. This is important to study

considering the high prevalence, incidence, risk of mortality and morbidity, increased financial burden and decreased quality of life due to CKD. Although there have been several results of previous studies, several studies are still fragmented, and there is still controversy. Therefore, research using experimental animals is one way to obtain more complete data, especially regarding the mechanism of this inhibition. This is important to prevent further kidney damage, slow the progression toward end-stage renal disease (ESRD), and prevent cardiovascular risk.

METHODS

Research design

This analytical study uses a pure experimental design (randomized post-test only control group design). The sample size was determined using the degree of freedom of analysis of variance (ANOVA). Sampling using a simple random sampling technique.

Experimental animals

The experimental animals in this study were 20 Swiss strain white mice (*Mus musculus* L.) obtained from the Animal Laboratory Unit, Department of Pharmacology, Faculty of Medicine, Udayana University, Denpasar. The inclusion criteria in this study were: male mice aged 3-4 months, weighing 30-40 grams. The exclusion criteria in this study were sick or dead mice during acclimatization. The dropout criteria in this study were mice that died after undergoing subtotal nephrectomy surgery. Animals were randomly divided into 4 groups, namely the control group (K, n=5), the treatment group with a dose of simvastatin 5.2 mg/kgBW (P1, n=5), the treatment group with a dose of simvastatin 10.4 mg/kgBW (P2, n = 5), and the treatment group with a dose of simvastatin 20.8 mg/kgBW (P3, n = 5). A subtotal nephrectomy procedure was performed in group K, and 1% CMC solution per sonde was administered. In group P, a subtotal nephrectomy procedure was performed, and simvastatin was given in 1% CMC solution with graded doses of 5.2 mg/kgBW (P1), 10.4 mg/kgBW (P2), and 20.8 mg/kgBW (P3) per sonde once a day for 14 days.

Simvastatin

Simvastatin (Sigma-Aldrich®) was dissolved in 1% CMC solution and administered per sonde once daily with graded doses of 5.2 mg/kg, 10.4 mg/kg, and 20.8 mg/kg for 14 days. This dose refers to the median dose of simvastatin in humans, which is 40 mg/day (the dose range in humans is 10-80 mg/day) converted to mouse doses. This dose selection is by previous studies.[7]

Animal Model

The subtotal nephrectomy procedure was performed through an incision in the flank region of the mice to perform a unilateral nephrectomy on the right kidney. The following day ablation was performed on the superior and inferior poles of the left kidney (2/3 of the kidney).[7,19] Prior to incision, the experimental animals were given an anesthetic combination of ketamine and xylazine 90 mg/kgBW (K) and 10 mg/kbBW (X), which were injected intraperitoneally (i.p). The incision wound was sutured using silk thread 3.0 and antiseptic was administered at the incision site after the surgical procedure.[6]

Housing

Mice were maintained based on the treatment group in standard cages, temperature 22+20C, humidity 50±5%, and 12 hours light-dark cycle at the Animal Research Laboratory, Faculty of Medicine and Health Sciences, Warmadewa University. All mice were first acclimatized for 7 days. The mice were caged based on the treatment group in cages measuring 40 cm x 30 cm x 15 cm. The top was covered with woven wire and given husks as a base. Experimental animals are given access to drinking water (tap water) and food (standard mouse feed) containing 20-25% protein, 10-12% fat, 45-55% starch, 4% crude fiber, 5-6% ash, vitamins A (15,000-20,000 IU/Kg) vitamin D (5,000 IU/Kg), alpha-tocopherol 50 mg/Kg, linoleic acid (5-10 g/Kg), thiamine (15-20 mg/Kg), riboflavin 8 mg/Kg Kg), pantothenate 20 mg/Kg, vitamin B12 30 ug/Kg, biotin 80-200 ug/Kg, pyridoxine 5 mg/Kg, inositol 10-1000 mg/Kg and kaolin 20 g/Kg.

Termination

Termination was carried out after 2 weeks from the first day of subtotal nephrectomy surgery. Termination using ketamine 300 mg/kg BW (3 times the anesthetic dose) intraperitoneally.[6] The carcass is tightly wrapped in black plastic and incinerated after taking the organs.

Sampling

Blood samples were taken through the right eye's retroorbital (orbital vein). Followed by a laparotomy procedure by opening the abdomen to the chest, intracardiac perfusion was carried out by flowing 0.9% NaCl at the apex of the heart. Perfusion is carried out for 15-20 minutes until, macroscopically, all organs appear pale in color. The remaining kidney is then taken. The kidney was cut longitudinally for histopathological examination. The kidney section was put into a 10% BF solution without removing the kidney capsule.

Creatinine Serum Examination

Blood samples were centrifuged at 10,000 rpm for 10 minutes to obtain serum. The serum was stored at -200C before further analysis. Serum creatinine levels (DiaSys, Holzheim, Germany) were obtained by spectrophotometry (Thermo Scientific™ Genesys™ UV-visible-spectrophotometers) at a wavelength of 510 nm.[6]

Interstitial Fibrosis Examination

Interstitial fibrosis area fractional examination was assessed using picrosirus red/Direct Red 80 staining (Sigma-Aldrich® Cat. No. 365548). Paraffin blocks were cut to a thickness of 4 μm on uncoated slides. The staining procedure begins by placing the slide at room temperature for 30-60 minutes. Prepare a 1% picric acid solution (1 gram of picric acid dissolved in 100 mL of distilled water) and a solution of picrosirius red (1 mL of Sirius red in 9 mL of 1% picric acid). Continue the deparaffinization procedure in xylene I, II, and III solutions for 5 minutes each, then in 100% I, 100% II, 90%, 80%, and 70% alcohol solutions. The next slide was put in distilled water for 10 minutes to ensure the tissue was clean. Drop the picrosirius red solution on the slide and let it stand for 1 hour. After 1 hour, proceed with the dehydration procedure, namely immersing the slides in 100% alcohol I, II, and II for 3 minutes, then in xylene I, II, and III solutions for 5 minutes. In the final

stage, the slide was covered with Canada Balsam and cover glass and allowed to stand for 1 day.[20]

Observations were made on each slide using a light microscope connected via Optilab. Ten visual fields per slide will be observed at 400X magnification. Furthermore, Image J software calculated the fibrosis area fraction and presented it as a percent fraction area.[7] In this study, the observations were made by one person. The researcher was assisted by an assistant to code the slides being assessed so that the observer did not know the group of each photographed slide (blinding). Coding has been done since the paraffin block was cut and attached to the uncoated slide. This aims to reduce interobserver bias that may occur during observations.

Data analysis

The data normality test used the Shapiro-Wilk test (95% confidence interval (CI), a = 5%) because the number of samples in this study was ≤ 50. The homogeneity test used the Levene test. The analysis was continued with a parametric test and all research data were presented as mean ± standard deviation (SD). The parametric test is the one-way ANOVA test followed by Tukey's post hoc test. Data were analyzed using SPSS version 25.0 for Windows.

RESULTS

Characteristics of Experimental Animals

The average body weight of mice at the beginning of the treatment was 33.80 ± 1.64 grams in group K, 33.00 ± 2.23 grams in group P1, 34.00 ± 1.22 grams in group P2, and 34.20 ± 0.83 grams in P3 group. The analysis results showed no significant difference in the weight of the mice at the start of the study between the four treatment groups (p>0.05). Different results were found at the end of the study. The average weight of mice at the end of the treatment was 32.00 ± 1.22 in group K, 33.80 ± 1.48 grams in group P1, 34.80 ± 1.30 grams in group P2, and 32.80 ± 0.83 grams in the P3 group. At the end of the study, there was a significant difference in the weight of mice between the K group and the P2 group (p <0.05). A comparison of the mean weight values of mice at the beginning and end of treatment is shown in Table 1.

Table 1. Differences in weight of treatment and control mice

Groups	n	Average weight (grams)	
		Early treatment (Mean±SD)	End treatment Mean±SD
K	5	33.80±1,64	32.00±1.22
P1	5	33.00±2.23	33.80±1.48
P2	5	34.00±1.22	34.80±1.30
P3	5	34.20±0.83	32.80±0.83

K (subtotal nephrectomy); P1 (subtotal nephrectomy + simvastatin 5,2 mg/kgBW); P2 (subtotal nephrectomy + simvastatin 10,4 mg/kgBW); and P3 (subtotal nephrectomy + simvastatin 20,8 mg/kgBW)

Table 2. Creatinine serum level of treatment and control mice

Variable	Groups	n	Mean±SD	p
Creatinine Serum (mg/dL)	K	5	1.07±0.48	<0.05*
	P1	5	0.79±0.21	
	P2	5	0.69±0.12	
	P3	5	0.47±0.10$^{\#*\Delta}$	

One way ANOVA; $^{\#}p$<0,05 *vs* K; $^{*}p$<0,05 *vs* P1; $^{\Delta}p$<0,05 *vs* P2; K (subtotal nephrectomy); P1 (subtotal nephrectomy + simvastatin 5,2 mg/kgBW); P2 (subtotal nephrectomy + simvastatin 10,4 mg/kgBW); and P3 (subtotal nephrectomy + simvastatin 20,8 mg/kgBW); *Statistically significant if p-value less than 0.05.

Table 3. Differences in creatinine serum level of treatment and control mice

Creatinine Level (mg/dL)	Mean Difference	CI 95%		p
		Minimum	Maximum	
K vs. P1	-0.12	-0.44	0.19	0.708
K vs P2	-0.19	-0.51	0.12	0.315
K vs P3	-0.57	-0.89	-0.25	0.001*
P1 vs P2	-0.07	0.89	-0.39	0.891
P1 vs P3	-0.45	0.01	-0.77	0.005*
P2 vs P3	-0.37	-0.69	-0.05	0.020*

Post hoc Tukey; K (subtotal nephrectomy); P1 (subtotal nephrectomy + simvastatin 5,2 mg/kgBW); P2 (subtotal nephrectomy + simvastatin 10,4 mg/kgBW); and P3 (subtotal nephrectomy + simvastatin 20,8 mg/kgBW); *Statistically significant if p-value less than 0.05; CI: Confidence Interval.

Table 4. Interstitial fibrosis of treatment and control mice

Variable	Groups	n	Mean±SD	p
Interstitial fibrosis (%)	K	5	12.62±3.90	<0.05*
	P1	5	8.62±2.43	
	P2	5	4.14±1.66$^{\#*}$	
	P3	5	2.61±0.63$^{\#*}$	

One-Way ANOVA; $^{\#}p$<0,05 *vs* K; $^{*}p$<0,05 *vs* P1; K (subtotal nephrectomy); P1 (subtotal nephrectomy + simvastatin 5,2 mg/kgBW); P2 (subtotal nephrectomy + simvastatin 10,4 mg/kgBW); P3 (subtotal nephrectomy + simvastatin 20,8 mg/kgBW); *Statistically significant if p-value less than 0.05.

Serum Creatinine

The results of serum creatinine levels showed that the K group had the highest serum creatinine level (1.07 ± 0.43 mg/dL) compared to the other treatment groups. The average serum creatinine level in the P1 group was 0.79 ± 0.21 mg/dL; in the P2 group, it was 0.69 ± 0.12 mg/dL; in the P3 group, it was 0.47 ± 0.10 mg/dL. This result was stated to be statistically significant with p <0.05. Tukey's post hoc test showed a significant difference between serum creatinine levels in the P3 group compared to P2, P1, and K (Tables 2 dan 3). Significant results of decreased serum creatinine levels were only shown in the P3 group (dose 20.8 mg/KgBW).

Table 5. Differences in interstitial fibrosis of treatment and control mice

Interstitial Fibrosis (%)	Mean Difference	CI 95%		p
		Minimum	Maximum	
K vs. P1	0.16	-0.08	0.41	0.256
K vs P2	0.49	0.24	0.73	0.000*
K vs P3	0.67	0.43	0.92	0.000*
P1 vs P2	0.32	0.07	0.57	0.008*
P1 vs P3	0.51	0.26	0.75	0.000*
P2 vs P3	0.18	-0.05	0.43	0.170

Post-hoc Tukey; K (subtotal nephrectomy); P1 (subtotal nephrectomy + simvastatin 5,2 mg/kgBW); P2 (subtotal nephrectomy + simvastatin 10,4 mg/kgBW); P3 (subtotal nephrectomy + simvastatin 20,8 mg/kgBW); *Statistically significant if p-value less than 0.05; CI: Confidence Interval.

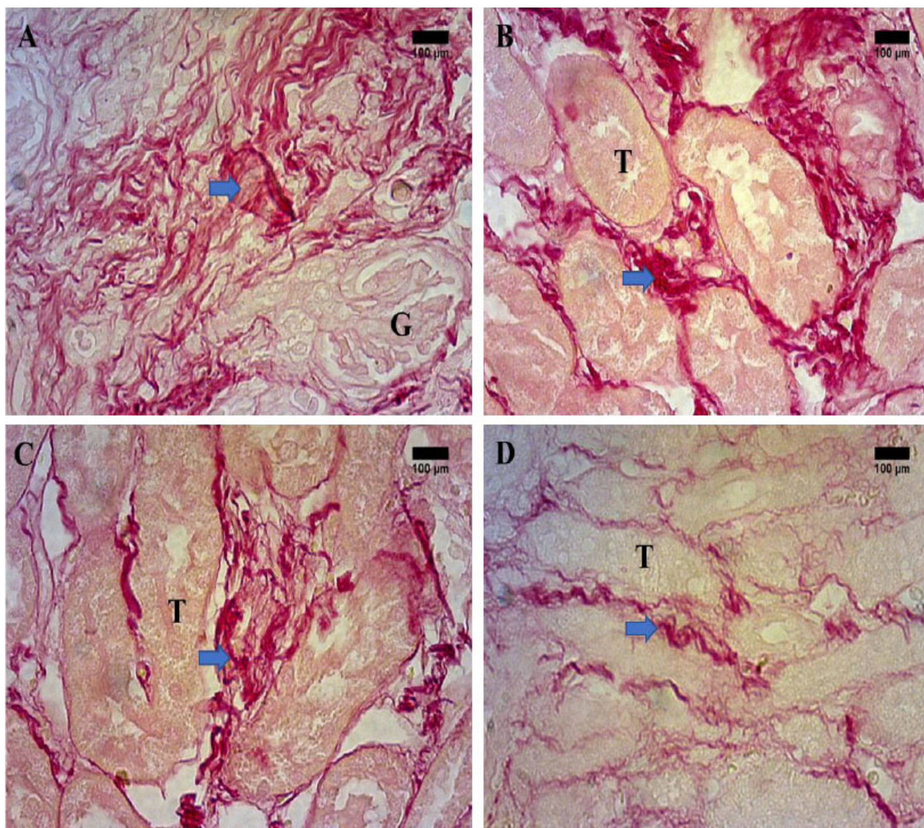

Figure 1. Overview of renal interstitial fibrosis in treated and control mice. Renal interstitial fibrosis appears red (blue arrow). Histological preparations were viewed using a light microscope at a magnification of 400 times. (A) K (control); (B) P1 (subtotal nephrectomy + simvastatin 5.2 mg/kgBB); (C) P2 (subtotal nephrectomy+ simvastatin 10.4 mg/kgBB); and (D) P3 (subtotal nephrectomy+ simvastatin 20.8 mg/kgBB). T (renal tubule) and G (glomerulus)

Still, at a dose of 5.2 mg/KgBW and 10.4 mg/KgBW, there was also a decrease in serum creatinine levels included in the normal values level.

Interstitial fibrosis of the kidney tissue

Interstitial fibrosis appears red in the interstitial areas of the kidney (Figure 1). The analysis found that the highest interstitial fibrosis occurred in group K (12.62 ± 3.90) and the lowest in group P3 (2.61 ± 0.63). The One-Way ANOVA test found a significant difference in interstitial fibrosis between the treatment groups ($p<0.05$). This significant difference was found between the K group and the P2 and P3 groups and between the P2 and P3 groups and the P1 group ($p<0.05$) (Table 4).

These results indicate that administration of simvastatin at doses of 5.2 mg/kg, 10.4 mg/kg, and 20.8 mg/kg in mice with CKD resulted in lower interstitial fibrosis of kidney tissue than controls.

DISCUSSION

This study used male mice with an average body weight between 33.00 grams to 34.20 grams at the start of the treatment. The results showed no significant difference in the weight of the mice at the beginning of the study between the four treatment groups ($p>0.05$). Different results were found at the end of the study. At the end of the study, it was found that there was a significant difference in the weight of mice between the K group and the P2 group ($p <0.05$). In the treatment group that received simvastatin at a dose of 5.2 mg/kg and 10.4 mg/kg, there was an increase in body weight, but not at a dose of 20.80 mg/kg. These results are consistent with early studies, which found differences in the weight of mice at the end of the study between the treatment groups.[7]

The increase or decrease in weight that occurred in this study was probably caused by the stress factor experienced by the experimental animals. Stress is known to affect psychosocial behavior and change responses to food. This is because the body's system that regulates the stress response, namely the hypothalamic-pituitary-adrenal axis, also regulates the response to food. Stress can increase or decrease appetite. The effects are influenced by many factors, such as glucocorticoid hormone levels (influenced by the severity of stress experienced). These interactions between glucocorticoids and neuropeptides play a role in eating behavior such as neuropeptide Y (NPY), alpha-melanocyte stimulating hormone (a-MSH), agouti-related protein (AgRP), melanocortins and their receptors such as CRH, urocortin, and peripheral signals (such as leptin, insulin, and ghrelin).[21] Changes in eating behavior due to stress experienced in mice depend on gender and the length of the stress period experienced.[22]

The study showed that the K group had the highest serum creatinine level (1.07 ± 0.43 mg/dL) compared to the other treatment groups. These results

indicate that the surgical procedure of subtotal nephrectomy can lead to impaired kidney function, characterized by increased serum creatinine levels. This finding is similar to other studies that reported that the subtotal nephrectomy procedure caused a decrease in kidney function and damage to kidney structures such as glomeruli, tubules, and renal interstitials.[23,24] The subtotal nephrectomy model can cause a reduction in kidney function characterized by increased serum creatinine and proteinuria as well as decreased hemoglobin levels which are general features of CKD.[7,25] Although it is known that this model can trigger kidney damage that mimics kidney damage in humans, the effect on biochemical parameters, morphology, and other markers of kidney damage is very dependent on the variety of methods and animal strains used in a study.[26]

Fibrosis is a pathological condition resulting from an imbalance in the synthesis and degradation of the extracellular matrix, which results from an imperfect wound-healing process.[27] Fibrosis in the kidney can occur in the glomeruli, tubules, and interstitial, as well as the renal blood vessels.[28,29] Fibrosis in the glomerulus and tubulointerstitial is a common feature of CKD which will lead to end-stage renal failure.[30] Interstitial fibrosis is characterized by the widening distance between the tubular basement membrane and peritubular capillaries due to collagen deposition, which is generally dominated by type 1 collagen.[31] This deposition is triggered by continuous myofibroblast activation.[32,33]

This study indicates that the subtotal nephrectomy procedure can trigger interstitial fibrosis. This was suggested by the highest fibrosis found in group K, namely 12.62 ± 3.90. The analysis showed a significant difference in interstitial fibrosis between the treatment groups ($p<0.05$). These results are from previous studies that have been conducted using the same animal model.[7] Many factors can trigger the occurrence of fibrosis in the kidney. Chronic inflammation and pathological cell death are the main factors underlying this condition.[31,34-37]

Particularly in tubulointerstitial fibrosis, inflammatory and fibrotic processes can be triggered by the activation of tubular epithelial cells due to direct damage to the tubules or indirect effects triggered by injury to the glomerulus.[34] Several pathways are believed to be involved in this fibrotic process. These pathways include TGF-β/SMAD, Wnt/β-catenin, Jagged/Notch, EGF-R, and JAK/STAT.[31]

Administration of simvastatin in the P1, P2, and P3 groups showed a decrease in the degree of interstitial fibrosis with a mean value of 8.62 ± 2.43% in the P1 group, 4.14 ± 1.66% in P2, and 2.61 ± 0.63% in P3. These results further analysis showed significant differences between the K and P2 and P3 groups. These findings indicate that the administration of simvastatin reduces the degree of renal interstitial fibrosis, especially in animal models of chronic renal failure, in line with increasing doses. Simvastatin doses of 10.4 mg/kgBW and 20.8 mg/kgBW show better results than those of 5.2 mg/kgBW.

The limitation of this study is that it has not been able to know the exact molecular mechanism of how simvastatin can improve this fibrosis. Therefore, further studies are still needed regarding the possible underlying pathways or mechanisms, whether through repair of inflammation (anti-inflammatory effect), inhibition of free radicals (antioxidant effect), or repair of endothelial cells, epithelial cells, and myofibroblast cells as extracellular matrix producers.

CONCLUSION

Based on the data from this study, it can be concluded that simvastatin can improve kidney function and reduce the degree of renal fibrosis in animal models of CKD. Therefore, it is necessary to carry out further studies to assess acute, subacute, and chronic toxicity to obtain the pharmacokinetic profile data.

ETHICAL CLEARANCE

This research obtained ethical permission from the Research Ethics Committee of Udayanan University Number: 2200/UN14.2.2.VII.14/LT/2022.

CONFLICT OF INTERESTS

There is no conflict of interest in this research.

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AUTHOR CONTRIBUTIONS

Putu Nita Cahyawati is responsible for compiling research proposals, conducting research, and writing manuscripts. I Nyoman Mantik Astawa, Bagus Komang Satriyasa, and I Made Bakta provided input regarding research methodology, presentation of results, and discussion in preparing proposals and writing manuscripts.

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