Research Article

Effect of Simvastatin on Eosinophilic Inflammation of Bladder Tissue in Interstitial Cystitis Rat Model

Efek Simvastatin terhadap Inflamasi Eosinofilik Jaringan Vesika Urinaria pada Tikus Model Sistitis Interstisial

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ABSTRACT

In the urogenital system, simvastatin is associated with interstitial cystitis adverse effects, but the exact mechanism is not yet clearly defined. This study aims to determine the effect of simvastatin on eosinophilic inflammation of bladder tissue in vivo. Laboratory experimental research design with the post-test only control group using 24 female Wistar rats aged 8-10 weeks were randomly divided into simvastatin 50mg/kg BW (n=12) or placebo carboxymethylcellulose 0.5% (n=12). All groups received treatment through oral gavage for thirty days. After that, each group was divided equally into three subgroups: control rat, day 0 Interstitial Cystitis (IC) rat (IC0), and day 3 IC rat (IC3). Control or IC0 rats each received intravesical instillation of buffered saline or protamine sulfate (PS), respectively, and were terminated immediately less than 3 hours after instillation. The IC3 rats received intravesical PS instillation and were terminated three days post-instillation. The bladder tissue was made in Hematoxylin-Eosin histology preparations. As in previous studies, the results showed successful desquamation of the urothelium after PS instillation. Tissue eosinophil counts were significantly higher in the simvastatin group than in the placebo group in the IC3 model (15.50±5.92 vs. 4.00±2.83, p=0.013). It can be concluded that the mechanism of the adverse effect of simvastatin on bladder tissue is through increased tissue inflammation mediated by eosinophils along with urothelial layer destruction by the protamine sulfate.

Keywords: Bladder, eosinophil, interstitial cystitis, protamine sulfate, simvastatin

ABSTRAK

Pada sistem urogenital, simvastatin dikaitkan dengan efek samping sistitis interstisial, namun belum diketahui dengan jelas mekanisme yang memperantarainya. Penelitian ini bertujuan untuk mengetahui efek simvastatin terhadap inflamasi eosinofilik jaringan vesika urinaria secara *in vivo*. Desain penelitian eksperimental laboratorik dengan *post-test only control group design* menggunakan 24 tikus galur Wistar betina usia 8-10 minggu yang dibagi ke dalam dua kelompok secara acak: simvastatin 50mg/kgBB (n=12), dan plasebo *carboxymethylcellulose* 0,5% (n=12). Semua kelompok mendapatkan perlakuan melalui sonde oral selama tiga puluh hari. Setelah itu, setiap kelompok dibagi rata menjadi tiga subkelompok: tikus kontrol, tikus Sistitis Interstisial (SI) hari ke-0 (SI0), dan tikus SI hari ke-3 (SI3). Tikus kontrol atau tikus SI hari ke-0 masing-masing mendapatkan instilasi buffered saline atau protamin sulfat (PS) intravesika, secara berturut-turut, dan segera diterminasi kurang dari 3 jam paska instilasi. Tikus SI hari ke-3 mendapatkan instilasi PS intravesika dan diterminasi 3 hari paska instilasi. Jaringan vesika urinaria yang diambil dibuat dalam preparat histologi Hematoksilin-Eosin. Hasil penelitian menunjukkan deskuamasi urotelium yang berhasil dilakukan paska instilasi PS sebagaimana studi terdahulu. Hitung eosinofil jaringan signifikan lebih tinggi pada kelompok yang mendapatkan simvastatin dibandingkan kelompok plasebo pada model SI3 (15,50±5,92 vs. 4,00±2,83; p=0,013). Dapat disimpulkan bahwa mekanisme efek samping simvastatin terhadap jaringan vesika urinaria melalui peningkatan inflamasi jaringan yang dimediasi oleh eosinofil bersamaan dengan destruksi lapisan urotelial oleh protamin sulfat.

Kata Kunci: Eosinofil, protamin sulfat, simvastatin, sistitis interstisial, vesika urinaria

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INTRODUCTION

Statins are widely used for lowering LDL cholesterol and preventing cardiovascular disease (1). Statin use increases at age 40 years and patients at high risk of atherosclerotic Cardio Vascular Disease (2). Thus, this drug has urgency in the management of emergency diseases in the cardiovascular system.

The widespread use of statins is still constrained by the resulting adverse effect, affecting drug adherence rates (3). The side effects of these statins can reach 30%, of which the most common are statin-associated muscle symptoms (SAMSs) (4,5). In addition, the side effects that have been proven are new-onset type 2 diabetes mellitus, neurocognitive disorder, hepatotoxicity, renal toxicity, and others (6). However, the exact mechanism that causes these side effects is not known.

In the urogenital system, adverse effects of statin use are associated with interstitial cystitis. This disease presents dysuria, urinary frequency, and urinary urgency, which affects the patient's quality of life (7). In a case-control population study, it was found that there was a significant relationship between routine statin use and the incidence of interstitial cystitis with an odds ratio of 1.58. The exact mechanism is not known, so that further research is needed (8).

Interstitial cystitis is a complex disease and may involve a variety of unknown etiologies. One of the main processes in the pathogenesis of interstitial cystitis is bladder urothelium barrier dysfunction, which can be caused by failure of urothelial cytodifferentiation, chronic inflammation in the sub urothelial tissue, increased apoptotic cells, and decreased proliferative cells. This dysfunction results in the leakage of water, urine, potassium, and other toxic urinary substances into the underlying tissue, triggering symptoms of urgency, frequency, and dysuria (9). Another process that is thought to play a role in the pathogenesis of interstitial cystitis is bladder hypersensitivity, as evidenced by the discovery of eosinophilic cell infiltration in the sub urothelial, detrusor, and adventitia layer of the urinary bladder in H_2O_2 induced-interstitial cystitis-rat models (10). In addition, another clinical study also found an increase in urinary leukotriene E4 and eosinophil protein X excretion in patients with interstitial cystitis (11).

Based on the previously mentioned pathogenesis, we hypothesized that statins exert a side effect of interstitial cystitis through their pro-inflammatory mechanisms, particularly those mediated by eosinophils. Some case reports found the association between statin use and eosinophilia or hypereosinophilic tissue inflammation (12-15). Therefore, this study aims to determine the effect of simvastatin on eosinophilic inflammation of bladder tissue in vivo.

METHOD

This study used an experimental laboratory design with a post-test control group design. This study was conducted on female Wistar strain rats, aged 8-10 weeks, weighing 100-150 grams. The exclusion criteria are rats under stress, illness, or injury, and obese rats. The flowchart of the study is shown in Figure 1.

Animal Preparation

This study used a total of 24 rat samples which were initially subjected to acclimatization for ten days. After that, randomization was carried out using a simple random sampling method and divided equally into two groups, namely group C who only received a placebo carboxymethylcellulose (CMC) 0.5% (n=12) and group S who received simvastatin 50mg/kg BW (n=12). All rats were kept in open, humid, well-ventilated cages and life/light cycle 12 hours/12 hours. Each cage consists of four to five rats. All rats received standard AD2 feed and free access to tap water ad libitum.

Simvastatin Treatment

Simvastatin was prepared from generic tablet form (Kimia Farma, Indonesia). The drug dosage was based on a previous study on simvastatin 50mg/KgBW, a hypocholesterolemic dose in rats (16,17). Based on
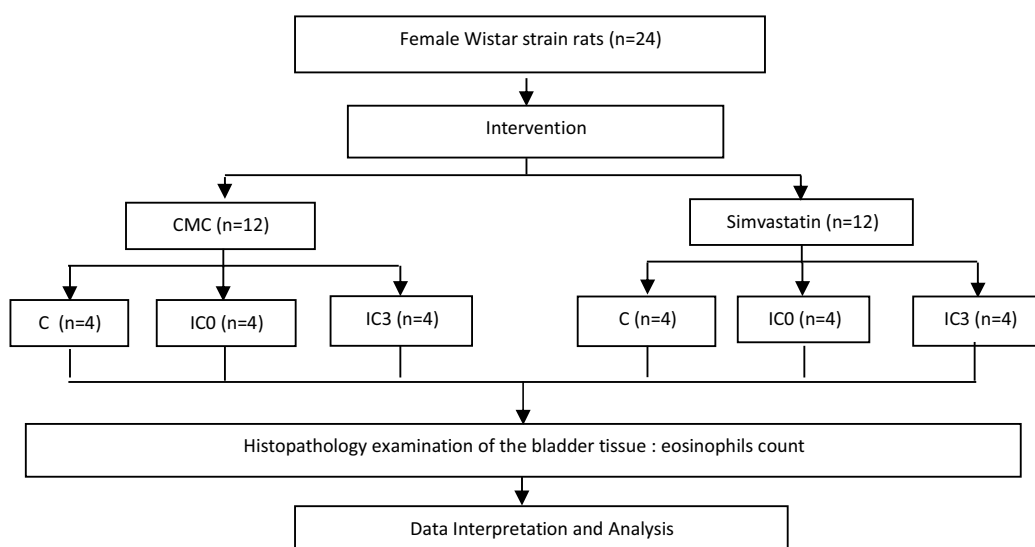
Figure 1. Study flowchart
Note: CMC, Carboxymethylcellulose; C, Control Rat;, IC0, Interstitial Cystitis Rat Day 0; IC3, Interstitial Cystitis Rat Day 3

previous studies, that statin could induce cellular senescence within 20 days in vitro (18), in this study, simvastatin was given for 30 days. The simvastatin tablets were turned into suspension form with 0.5% carboxymethylcellulose (CMC) as the solvent. Simvastatin suspension or 0.5% CMC was administered by oral gavage once daily in the afternoon and was adjusted according to body weight each week.

Induction of Interstitial Cystitis

After completing simvastatin or placebo administration, each group of rats was further divided into three subgroups: control rats, Interstitial Cystitis (IC) day-0 rats, and IC day-3 rats. The IC and control groups were induced by intravesical instillation of protamine sulfate and buffered saline, respectively, based on previous studies (19,20). Protamine sulfate (Sigma Aldrich, Japan) was dissolved in buffered saline with a concentration of 10mg/ml, then put into an instillation tube in the form of a 1 ml spoit mounted to a sterile 22/24G vein catheter. Anesthetic experimental animals using ketamine injection 10% (60mg/Kg) intraperitoneally. The rats were positioned dorsally recumbent and mildly massaged in the lower abdominal region to induce micturition. After identifying the external urethral ostium, the lubricated distal end of the instillation tube was inserted as deep as 3 mm in a cephalocaudal position parallel to the urethra, then rotating the proximal end of the instillation tube vertically about 180 degrees. After that, the distal end of the instillation tube was inserted 7mm deep into the bladder. An amount of 0.6ml of protamine sulfate or buffered saline was instilled with bolus for 30-45 seconds and was maintained in the bladder for 15 minutes while rotating the rats to homogenize the contact of instillation solution to the entire lumen of the bladder. Finally, the instillation tube is slowly pulled out from the urethra.

Tissue Preparation and Eosinophils Count

Tissue collection time was adjusted according to the rat model group. In the control and IC day-0 rat model, the animals were sacrificed less than 3 hours after the instillation procedure. As for the IC day-3 rat model, the animals have sacrificed three days after the instillation procedure. Initially, all rats were killed through the cervical dislocation technique then the bladder organs were excised. The tissue samples were fixed in 10% neutral formaldehyde solution overnight and then made in paraffin blocks according to standard procedures. The paraffin blocks were cut using a microtome with a thickness of 5μm, followed by floating in a warm water container. After that, the specimens were placed on a slide and glued with a thin layer of albumen. The slides were then processed using the hematoxylin-eosin staining procedure.

The slides were examined using a Leica ICC50E microscope with a 400X total magnification lens. Each slide was measured for eosinophil cells count in the entire bladder tissue by a pathologist. The location at which the eosinophil was found was also described.

Statistical Analysis

All collected data were analyzed by SPSS version 16.0 with a 95% confidence interval (α=0.05). Data for the eosinophil count is expressed in terms of mean ± standard deviation, or median ± standard error of mean if not normally distributed. Independent Sample T-test or Mann Whitney method compared the eosinophil count between treatment groups in the same mouse model. A p-

value ≤ of 0.05 was considered significant.

RESULTS

In this study, the desquamation of the bladder urothelium was successfully obtained following the protamine sulfate instillation procedure, as shown in Figure 1. The results of the eosinophil cell count in the treatment group can be seen in Table 1. The tissue eosinophil count in the simvastatin group was always higher than the placebo group, both in the control, IC0, and IC3 rat model. However, a significant difference was only observed in the IC3 rat model (15.50±5.92 vs 4.00±2.83; p=0.013). In the control rat model, the eosinophil was predominantly found at the deeper layer of the bladder. Conversely, in the interstitial cystitis rat model, the eosinophil was predominantly found more closely to the urothelial layer than the deeper layer. The representative image of the bladder tissue in the all-treatment groups are shown in Figure 2.
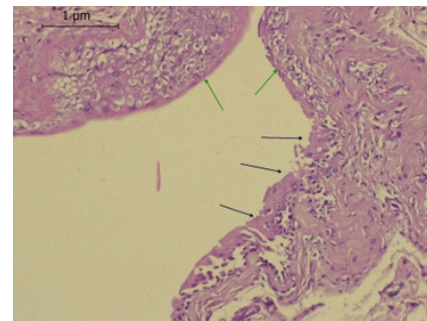
Figure 2. A representative image of the bladder urothelial desquamation (blue arrow) was formed after the activation of protamine sulfate in the C-IC0 group (Magnification 400x). The normal urothelial layer was shown with an intact umbrella cells layer (green arrow)

**Table 1. Eosinophil cells count in all treatment groups**

Treatment Group	Eosinophil Counts		Description
	Mean (SD)	P value#	
C-C	3.50 (9.26)*		Predominantly found at detrusor and adventitia layer, but also found at sub urothelial layer
		0.468$	
S-C	8.25 (4.113)		Predominantly found at adventitia layer, followed by detrusor layer and sub urothelial layer
C-IC0	2.25 (1.708)		Only found at sub urothelial layer
		0.504	
S-IC0	3.75 (3.862)		Predominantly found at detrusor and sub urothelial layer, but also found at adventitia layer
C-IC3	4.00 (2.828)		Predominantly found at sub urothelial layer, followed by detrusor and adventitia layer
		0.013	
S-IC3	15.50 (5.916)		Predominantly found at sub urothelial layer, followed by detrusor and adventitia layer.

Note: C-C, Carboxymethylcellulose-Control Rat; C-IC0, Carboxymethylcellulose -Interstitial Cystitis Rat Day 0; C-IC3, Carboxymethylcellulose-Interstitial Cystitis Rat Day 3; S-C, Simvastatin-Control Rat; S-IC0, Simvastatin-Interstitial Cystitis Rat Day 0; S-IC3, Simvastatin- Interstitial Cystitis Rat Day 3.
* Data were not normally distributed expressed in median (standard error)
#T-independent test
$ Mann-Whitney test
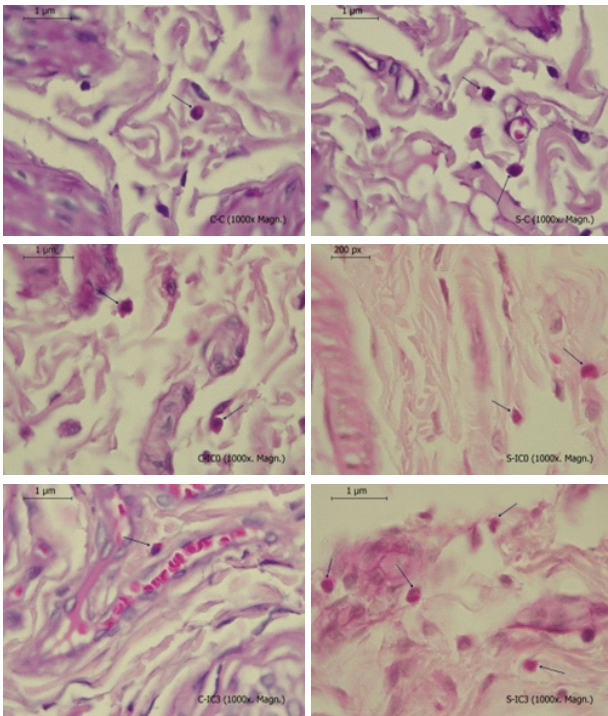
Figure 2. Representative image of the bladder tissue in the all-treatment groups (magnification 1000x). The eosinophils are presented as cell with eosinophilic granules in their cytoplasmic (blue arrow), predominantly in the suburothelial layer

Note: C-C, Carboxymethylcellulose-Control Rat; C-IC0, Carboxymethylcellulose -Interstitial Cystitis Rat Day 0; C-IC3, Carboxymethylcellulose Interstitial Cystitis Rat Day 3; S-C, Simvastatin-Control Rat; S-IC0, Simvastatin-Interstitial Cystitis Rat Day 0; S-IC3, Simvastatin- Interstitial Cystitis Rat Day 3.

DISCUSSION

The tissue eosinophil count in the simvastatin group was always higher than in the placebo group, particularly during the subacute phase of protamine sulfate instillation. Several studies have confirmed the role of eosinophil in interstitial cystitis, either in a clinical or animal study (10,11). This finding may be due to the urothelial disruption caused by protamine sulfate, causing urine leakage to the underlying suburothelial layer. As seen in the results of this study, although tissue eosinophil levels in the simvastatin group were consistently higher, it was only significant after three days after urothelial damage due to protamine sulfate instillation. As is well known, the uroepithelium is an epithelial tissue that lines the distal part of the urinary tract, including the renal pelvis, ureters, urinary bladder, proximal urethra, and ducts of the prostate gland. Functionally, it forms a barrier that accommodates significant changes in urine volume while maintaining no exchange of substances between the urine and the vascular supply (19). To carry out this function, a distinctive structure covers the outermost layer of the urinary bladder, namely the umbrella cell. The barrier function of umbrella cells is supported by several factors, namely the presence of very high resistance tight junctions, glycan components in the apical membrane, apical membranes that have a dense lipoprotein composition, and the ability of umbrella cells to modify their apical surfaces through exocytosis and endocytosis mechanisms (20–23).

Besides the barrier function, the uroepithelium of the bladder can also modulate the movement of ions, solutes, and water across the mucosal surface of the bladder (24). Another function of the uroepithelium is mechanoreceptor-like function. They can release various mediators and neurotransmitters, including ATP, adenosine, and ACh, from their serosal surface, allowing the epithelium to transmit information about the state of the mucosa and bladder lumen to the underlying tissues, namely neural tissue, myofibroblasts, and detrusor muscle (25). Given the vital function of this urothelium, disruption and dysfunction of these structures are most likely to contribute to the mechanism of simvastatin's effect on increasing tissue eosinophils.

Simvastatin can induce urothelial dysfunction in several ways. First, statin may initially interfere with the regeneration process after urothelial destruction by protamine sulfate, provide long-lasting urinary leakage, and increase the eosinophilic infiltration of the tissue (26). In the normal rat, the urothelial regenerates for about one day after the protamine sulfate instillation to reserve the homeostatic function and prevent further urinary leakage (27). In vitro studies have also shown that statins have anti-proliferative and pro-apoptotic features (28). This effect may reduce the proliferative ability of the urothelium, followed by urothelial barrier disruption (9,29).

The second mechanism that statins can trigger urothelial dysfunction is because simvastatin may aggravate the inflammation process, particularly the eosinophils in the suburothelial tissue, independently from the urothelial damage process. Statins can induce cellular senescence by inhibiting DNA repair genes' action, including XRCC4, XRCC6, and Apex1 (28). Furthermore, statins can activate the phosphatidylinositol 3-kinase-Akt (PI3/Akt) pathway to initiate senescence-associated secretory phenotype (SASP) through the mechanistic target of rapamycin (mTOR) pathway (30,31). This SASP can produce pro-inflammatory cytokines, aggravate inflammation, and trigger chronic inflammation in tissues (32).

Finally, It can be concluded that the mechanism of the side effect of simvastatin on bladder tissue is through increased tissue inflammation mediated by eosinophils. This effect may be present along with urothelial layer destruction by the protamine sulfate, similar to the known pathogenesis of interstitial cystitis.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

ETHICAL CLEARANCE

The Health Research Ethics Committee of the Faculty of Medicine, Hasanuddin University, has confirmed the proposal and research protocol (No. 375/UN4.6.4.5.31/PP36/2020).

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