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Effect of Simvastatin to Bladder Detrusor Senescence Acitivity in Protamin Sulfate-Induced Intersititial Cystitis Rat Model

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Abstract:** Statin can increase the risk of interstitial cystitis. Recently, the exact mechanism is yet known. We hypothesize that cellular senescence, particularly in the detrusor muscle cells contributes to the mechanism. In this study, we aim to investigate the effect of simvastatin on bladder detrusor senescence activity in the protamine sulfate-induced interstitial cystitis rat model. Thirty-seven female Wistar rats aged 6-8 weeks old were included and divided into three groups. They were treated with simvastatin 10 mg/kg BW (n=12), simvastatin 50 mg/kg BW (n=13), and the solvent carboxymethylcellulose (CMC) 0.5% (n=12) by oral gavage for 30 days. Each group was then equally subdivided into three groups: control, IC day-0, and IC day-3. The IC rat group was induced by protamine sulfate, while the control group was treated with buffered saline intravesical treatment. In less than three hours after intravesical treatment, all animals in the control and IC day-0 group were sacrificed to collect their bladder tissue. Otherwise, animals in the IC day-3 group were sacrificed three days after intravesical treatment. All collected tissue was measured for senescence marke β-Galactosidase 1 (GLB1) expression by immunohistochemistry procedure. In the control group, the GLB1 expression in rat treated with simvastatin 50 mg/kg BW was 20.12 ± 5.77 % and highest than the rat treated with other treatment, although the difference not significant (p=0.812). In the IC day-0 and IC day-3 groups, the GLB1 expression in rat treated with simvastatin 10 mg/kg BW and CMC 0.5% were higher than in the control group, otherwise, the GLB1 expression in rat treated with simvastatin 50 mg/kg BW was lower than in the control group, although not significant (p>0.05). We conclude that a higher dose of simvastatin can increase the bladder detrusor senescence expression along with their apoptotic activity in the presence of urinary leakage.

Keywords: Bladder pain syndrome, myocyte, interstitial cystitis, senescence, statin

1. INTRODUCTION

Interstitial cystitis (IC) is a chronic inflammatory disease of the bladder with a lower urinary tract symptom (LUTS) characterized by the Hunner's ulcer on cystoscopy examination.**(1)** The symptoms were felt for more than six weeks, with no infection or other identifiable cause.**(2)** The prevalence of IC is about 500 per 100,000 population, which affects women more than men.**(3)** Also, there is no universal treatment guideline for IC worldwide.**(4)**

The main pathogenesis of IC is related to urothelial barrier dysfunction, urothelial hypersensitivity, bladder wall hypersensitivity, and central sensitization.**(3–5)** Bladder wall which mainly consists of detrusor myocyte bundle, become hypersensitive in both rat model and clinical patients with interstitial cystitis.**(6,7)** Bladder wall thickening is another dysfunction that can be found in the IC.**(8)** This suggests that detrusor myocyte dysfunction have a crucial role in the pathogenesis of IC.

Statins are widely used to lower LDL cholesterol and the prevention of primary and secondary cardiovascular disease.**(9)** Recently, statin has been found to have the pleiotropic effect, where it has an anti-inflammatory, antiatherogenic, antifibrosis, and even anti-cancer profile. Conversely, this pleiotropic effect may induce its adverse effect.**(10)** Huang *et al* (2015) was found that the use of statin can increase the risk of IC, but the exact mechanism yet understood until now.**(11)** We hypotesized that bladder detrusor senescence underlies this effect, whereas statins can induce senescence and apoptotic activity in macrophage stem cells by upregulating p16, p53, and Caspase 3,8, and 9 gene expression.**(12)**

Senescence is the stable growth arrest that limits the replication of damaged and old cells.**(13)** Senescence has many properties, one of them is senescence-associated secretory phenotype (SASP), while these cells undergo morphology changes, chromatin remodeling, and metabolic reprogramming and secrete a complex pro-inflammatory factors.**(13)** This SASP can secrete abundant of IL-1β, IL-2, IL-6, TNF-α, proangiogenic factor, and pro-apoptotic factor (14), that alike with BPS/IC behave. **(4)** Therefore, in this study, we aim to investigate the effect of simvastatin to bladder detrusor senescence expression in the interstitial cystitis rat model.

2. MATERIALS AND METHODS

Laboratory experimental with a post-test only control group design was used in this study. Female Wistar rats, aged 8-10 weeks, and weighing 100-150 grams were included. The exclusion criteria were rats under stress, illness or injury, and obesity. This study followed the Helsinki Declaration guideline and was approved by the Ethical Committee of Health Research Medical Faculty of Hasanuddin University with registered number 375/UN4.6.4.5.31/PP36/2020.

A. Animal Preparation

This study used a total of 37 rats that were initially subjected to acclimatization for 10 days. After that, all rats were randomized by simple random sampling method and were divided equally into three groups, namely group S10 who treated with simvastatin 10 mg/kg BW (n = 12), group S50 who treated with simvastatin 50 mg/kg BW (n = 13), and group C who only treated with the solvent carboxymethylcellulose (CMC) 0.5% (n = 12). All rats were kept in open, humid, well-ventilated cages, and 12 hours life / light cycle. Each cage consists of four to five rats. All rats received standard AD2 feed and free access to tap water.

B. Simvastatin Treatment

The choice of drug dosage is based on the previous study **(15,16)**. All groups were treated for 30 days. Initially, a simvastatin suspension was prepared with 0.5% CMC solvent. Simvastatin suspension or 0.5% CMC solvent was administered by oral gavage. The suspension volume was adjusted according to body weight each week. The duration of administration between simvastatin doses is every 24 hours during the day or afternoon.

C. Induction of Interstitial Cystitis

After completing simvastatin or placebo administration, each group of rats was further divided into three subgroups, namely control rats, IC day-0 rats, and IC day-3 rats. The IC group was induced by intravesical instillation of protamine sulfate, while the control group was given intravesical instillation of buffered saline. The intravesical instillation procedure in this study was following previous studies **(17,18)**. Protamine sulfate (Sigma Aldrich, Japan) was dissolved in buffered saline with a concentration of 10 mg/ml, then put into an instillation tube in the form of a 22 / 24G vein catheter mounted on a tuberculin syringe. The rats were anesthetized with 10% ketamine intraperitoneally injection 10% (60 mg / Kg). The rats were positioned dorsally recumbent and gently massaged in the lower abdominal region to induce micturition. After identification of 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 rotate the proximal end of the instillation tube vertically about 180 degrees. After that, the distal end of the instillation tube was inserted 7 mm deep into the bladder. Amount of 0.6 ml of protamine sulfate or buffered saline was instilled with bolus for 30-45 seconds and was maintained in the bladder for 15 minutes. The rats were rotated 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.

D. Tissue Preparation and Immunohistochemical Analysis

Time for tissue collection was adjusted according to the rat model group. In the control and IC day 0 rat model, the rat was sacrificed less than 3 hours after the instillation procedure. As for the IC day-3 rat model, the rat has sacrificed three days after the instillation procedure. Initially, all rats were killed through the cervical dislocation technique then the bladder organs were taken. The tissue samples were fixed in 10% formaldehyde solution overnight and then turn 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 immunohistochemical procedure.

Initially, deparaffinization and hydration were performed using a 60^0C hot plate, followed by antigen retrieval using the Heat Induced Epitope Retrieval (HIER) method. Furthermore, blocking was done with 0.1-0.5% serum albumin bovine. Then the GLB1 antibody was added to the slide at a 1: 100 dilution. After repeated rinsing and blocking processes, Goat Anti-Rabbit IgG H&L (HRP) secondary antibody was added to the slide. After that, repeated rinsing was carried out until it was adequate and the preparations were immersed in chromogen DAB for 10-15 minutes. Counterstain was performed using Mayer's Hematoxylin stain for 1–5 minutes. Furthermore, the slide was dehydrated with ethanol until finally covered with a coverslip and ready to be observed.

The slides were examined using an Olympus CX22LED microscope with 40X magnification objective lens. Each slide was photographed using an Optilab microscope camera to randomly obtain five different images in the detrusor muscle layer. The percentage of immunopositive cells was calculated using the color spectrum analysis method with the help of the ImageJ NIH application according to previous studies **(19)**. Briefly, the detrusor muscle bundle was separated from other surrounding tissue in each image. Then, color deconvolution was performed using H DAB to separate the color spectrum in the main image. Furthermore, filtering was carried out using a 5.00 Gaussian Blur and adjusting the threshold in such a way that it covers the region of interest (ROI) of the colored area without including debris or artifacts. The total area was calculated by setting the adjusted threshold in such a way as to cover the entire detrusor muscle layer in each image, whether stained or not. The percentage of immunopositive cells in each image was then calculated using a formula :

% Immunopostivity = ROI Stain/ ROI Total x 100 %

E. Statistical Analysis

Data were analyzed with SPSS 17.0 with the use of 95% confidence interval ($\alpha = 0.05$). Data for the percentage of GLB1 expression presented in mean ± standard deviation. To compare GLB1 expression between treatment groups in the same mouse model, the One Way ANOVA or Kruskal Wallis method was used. As for comparing the GLB1 expression between several models of treated mice in one particular treatment, the Independent-T test or the Mann-Whitney U test method was used. A p-value ≤ of 0.05 was considered significant.

3. RESULTS

Expression of GLB1 on the detrusor muscle layer in various group treatment is indicated in Fig 1. It was shown that GLB1 expression predominantly appeared on the nucleus of the detrusor muscle cells, not on the cytoplasm.

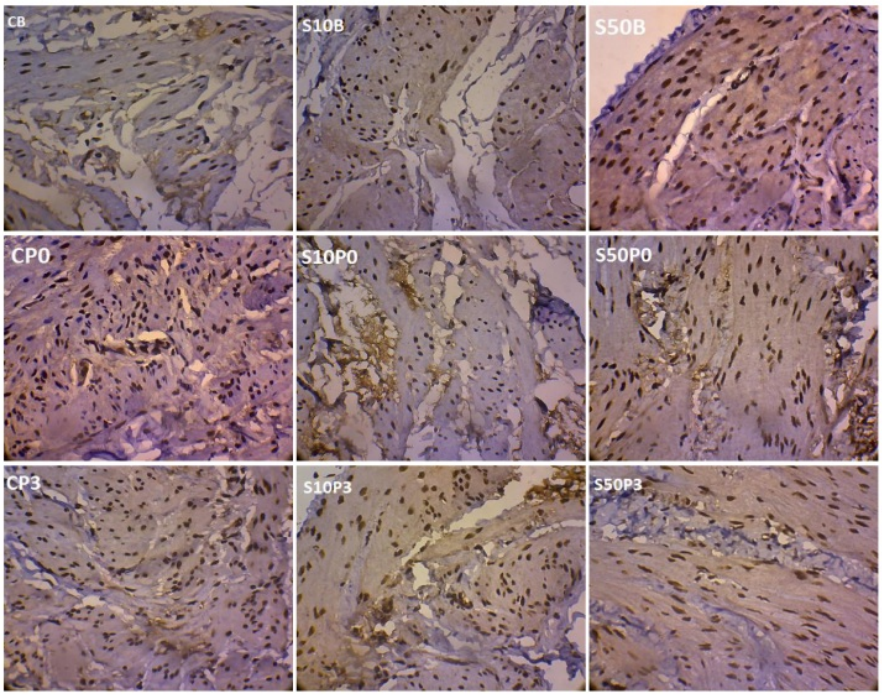

Fig 1 Representative images of detrusor muscle GLB1 expression in control, IC day 0, and IC day 3 rat model according to simvastatin or placebo treatment. The senescence marker was brownish precipitate, which was predominantly expressed in the nucleus.

CB, CMC 0.5 % treatment in control rat model, CP0, CMC 0.5 % treatment in IC rat model day 0, CP3, CMC 0.5 % treatment in IC rat model day 3, S10B, simvastatin 10 mg/BW treatment in control rat model,S10P0, simvastatin 10 mg/BW treatment in IC day 0 rat model, S10P3, simvastatin 10 mg/BW treatment in IC day 3 rat model, S50B, simvastatin 50 mg/BW treatment in control rat model, S50P0, simvastatin 50 mg/BW treatment in IC day 0 rat model, S50P3, simvastatin 50 mg/BW treatment in IC day 3 rat model

Measurement of GLB1 expression on the detrusor muscle layer in various group treatment is indicated in Table I. In the placebo-treated mice, GLB1 expression on the detrusor muscle layer in the IC day 0 rat model was 16.33 ± 4.21%, where this value in the control mouse model was 15.06 ± 2.57% ($p = 0.626$). Furthermore, the expression of GLB1 in the detrusor muscle layer in the IC day 3 rat model was 18.29 ± 3.56%, which was not significantly different from the IC day 0 rat model ($p = 0.503$).

Table I Percentage Expression of GLB1 on The Detrusor Muscle Layer

	Control Rat Model	IC Day 0 Rat Model	IC Day 3 Rat Model	p-1*	p-2*
C	15.06 ± 2.57	16.33 ± 4.21	18.29 ± 3.56	0.626	0.503
S10	14.79 ± 2.21	20.73 ± 6.57	22.07 ± 4.73	0.248 **	0.753
S50	20.12 ± 5.77	18.64 ± 4.17	15.70 ± 3.65	0.693	0.296
	p = 0.812***	p = 0.584***	p = 0.792***		

** Independent-T test*
*** Mann-Whitney U test*
**** One-Way ANOVA*
p-1, significance value difference between IC day 0 and control rat model
p-2, significance value difference between IC day 3 and IC day 0 rat model

In rat treated with simvastatin 10 mg/kg BW, the expression of GLB1 in the detrusor muscle layer in the IC day 0 rat model was 20.73 ± 6.57%, where this value in the control mouse model was 14.79 ± 2.21% (p = 0.248). Furthermore, the expression of GLB1 in the detrusor muscle layer in the IC day 3 rat model was 22.07 ± 4.73%, where this value was not significantly different from the IC day 0 rat model (p = 0.753).

In rat treated with simvastatin 50 mg/kg BW, the expression of GLB1 in the detrusor muscle layer in the IC day 0 rat model was 18.64 ± 4.17%, where this value in the control mouse model was 20.12 ± 5.77% (p = 0.693). Furthermore, the expression of GLB1 in the detrusor muscle layer in the IC day 3 rat model was 15.70 ± 3.65%, where this value was not significantly different from the IC day 0rat model (p = 0.296).

On the other hands, in a control mouse model that received placebo, simvastatin 10 mg/kg BW, and simvastatin 50 mg/kg BW, it was found that detrusor muscle GLB1 expression values were 15.06 ± 2.57%, 14.79 ± 2.21%, and 20.12 ± 5.77% respectively, where the difference was not significant (p = 0.116). Likewise in IC day 0 rat model who received placebo, simvastatin 10 mg / kg BW, and simvastatin 50 mg / kg BW, the detrusor muscle GLB1 expression values were found to be 16.33 ± 4.21%, 20.73 ± 6.57%, and 18.64 ± 4.17% respectively, where the difference was not significant (p = 0.502). The same thing was found in IC day 3 rat model who received placebo, simvastatin 10 mg/kg, and simvastatin 50 mg/kg BW, where the detrusor muscle GLB1 expression values were 18.29 ± 3.56%, 22.07 ± 4.73%, and 15.70 ± 3.65% respectively, where the difference was not significant (p = 0.105). As shown in Fig 2, exposure to protamine sulfate or simvastatin did not significantly affect senescence activity in the detrusor muscle layer both acutely and subacutely.

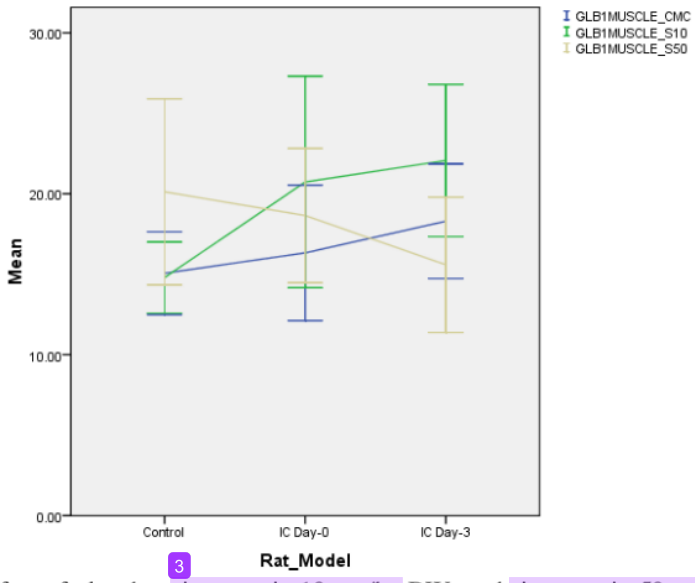

Fig 2 The effect of placebo, simvastatin 10 mg/kg BW, and simvastatin 50 mg/kg BW on GLB1 expression in control, IC day 0, and IC day 3 rat model. Values are expressed as mean ± SD of 4-5 mice in each group.

4. DISCUSSION

In this study, we found simvastatin 50 mg/kg BW can increased the bladder detrusor senescence expression in control rat group. This consistent with previous study from Izadpanah *et al* (2015) which found that statin can induce senescence of the macrophage stem cells by induction of the DNA damage response **(12)**. The bladder wall consists of detrusor smooth muscle cells, fibroblast, interstitial cells of Cajal (ICC), and telocyte (TCs).**(20,21)** Dysfunction of these cells can contribute to bladder wall hyperactivity, a hallmark in BPS/IC pathogenesis.**(22,23)** Little known in the underlying mechanism in the dysfunction of these cells, although there is cellular senescence in these cell types from different organs.**(24–26)**
Bladder detrusor myocytes are characterized by α-smooth muscle actin (α-SMA), the contractile protein like other smooth muscle cell behaves. **(27–29)** Fetal alveolar smooth muscle (ASM) cells that were exposed to moderate hyperoxia with 40% O2 exhibited elevated senescence markers and can excrete various profibrotic and proinflammatory factor, thus able to make naïve ASM to hyper contraction. **(24)** SAMP8 mouse strain, the senescence-accelerated mouse model, exhibited increased micturition frequency and excitatory contractility by mild sensitization to Acetylcholine (ACh), high sensitization of detrusor to ATP, high sensitization of the urethra to noradrenaline (NA), fewer neuronal nitric oxide synthase (NOS) nerves, and vimentin-positive ICs. **(30)**
Another important finding in our study is the bladder detrusor senescence expression only decrease in simvastatin 50 mg/kg BW group treatment after protamine sulfate intravesical treatment acutely and subacutely. Protamin sulfate can alter the umbrella cells of the bladder urothelial, thus promote urinary leakage to the underlying tissue **(17)**. This indicates that this

senescent cell has a high response to the senescence clearance mechanism in presence of urinary leakage, either by apoptosis or the immune system **(31)**. The apoptotic activity may underlie the senescence clearance mechanism rather than the immune system in this study because the statin can decrease the differentiation ability of macrophage stem cells **(12)**. This effect didn't show in simvastatin 10 mg/kgBB and placebo groups. Further study is needed to investigate this finding.

The limitation of this study is only used one senescence marker. Several markers such as p16, p53, p21, or Ki-67 will confirm the presence of senescence in this study. Also, we did not analyze the other markers of interstitial cystitis such as eosinophilic infiltration, mast cell infiltration, and various proinflammatory cytokines. However, to our knowledge, this study is the first to demonstrate that the bladder detrusor myocyte senescence may present in interstitial cystitis related to simvastatin treatment. Further research is needed to confirm the results of this study.

5. CONCLUSIONS

Bladder detrusor myocyte senescence can increase by the simvastatin treatment, while a higher dose of simvastatin may increase its apoptotic activity. This effect may appear when urinary leakage is present, rather than the effect of simvastatin alone. Further studies with other senescence markers along with interstitial cystitis markers are needed to confirm this finding.

ACKNOWLEDGMENT

Nothing to declare

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