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Effect of Rosuvastatin on Rabbit Valve Interstitial Cell Differentiation Induced by Transforming Growth Factor-B1 based on A – Smooth Muscle Actin Expression

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ABSTRACT

Objective: This study aims to show thatRosuvastatin's inhibitory ability on how myofibroblasts are formed from valve interstitial cells.

Methods: The design of this study is an in vitro laboratory experiment using a control group only after the test. A New Zealand rabbit (Oryctolagus cuniculus) male 12 weeks old heart valve was extracted, and interstitial valve cells were isolated. Valve interstitial cells were induced by fibrosis by administering TGF-β1 5 ng/ml and then treated with rosuvastatin at 10 µM/L, 25 µM/L, dan 50 µM/L.

Results: On immunohistochemical staining of TGF-β1-induced valve interstitial cells, the expression of α-SMA 23474 (18452 - 25671) showed myofibroblast differentiation. Administration of Rosuvastatin at doses of 10 µM/L, 25 µM/L, and 50 µM/L significantly decreased α-SMA expression in TGF-β1-induced valve interstitial cells (4.975 (4.08-13.4), 3.955 (2.88-6.82), 3.9 (2.36- 5.01) ($p<0.01$)). Comparing a rosuvastatin dose of 10 µM/L with 25 µM/L and a dose of 10 µM/L with 50 µM/L found a significant difference in the decrease in α-SMA expression ($p<0.001$). Comparing the rosuvastatin dose of 25 µM/L with 50 µM/L did not find a significant difference in the decrease in α-SMA expression ($p=0.74$).

Conclusion: Rosuvastatin can significantly inhibit myofibroblast differentiation in interstitial valve cells induced byTGF-β1.

Keywords: Valve interstitial cells, myofibroblasts, α-Smooth Muscle Actin, Rosuvastatin

1. INTRODUCTION

Acute rheumatic fever can undergo an acute resolution phase by developing into rheumatic heart disease due to an abnormal immune response caused by an infection when group A-hemolytic Streptococci are present. Acute rheumatic fever's pathogenesis is influenced by Group A-hemolytic Streptococci infection via the M protein antigen. In order to create antibodies that can detect epitopes and activate T cells, the M protein interacts with activated B lymphocytes. The presence of activated T cells can enter into the valve causing the release of cytokines and an inflammatory process occurs. High levels of Transforming Growth Factor (TGF) have been found to influence the development of valvular fibrosis through activating the mitogen-activated protein kinase (MAPK) and extracellular signaling-regulated kinase (ERK) pathways, among the numerous cytokines implicated in the inflammatory process. 1/2)[1,2].The current strategy for rheumatic heart disease secondary prevention is the administration of long-term antibiotics whose duration of administration depends on the complications of carditis in the acute phase of rheumatic fever with the longest duration can reach a lifetime. Prophylactic administration in the long term can lead to low compliance and the potential for antibiotic resistance. Long-term administration of antibiotics can cause their effectiveness to be low so that alternative drugs are needed as secondary prophylaxis [3,4,5]. Intervention in the early stages of the inflammatory phase utilizing TGF-β1and antifibrotic drugs offers fresh hope for preventing the development from acute rheumatic fever to valve abnormalities in rheumatic heart disease. Numerous cell types have been examined to see whether statins can reduce fibrogenesis by blocking the TGF-β1pathway, including lung fibroblasts, renal tubular cells, hepatocytes, and ventricular fibroblasts.6-9 One of the statins that have a strong binding interaction and a longer duration of inhibition of HMG-CoA reductase is rosuvastatin. Rosuvastatin has an inhibitory concentration in

inhibiting HMG-CoA reductase activity 50% lower than other statins and has the most effective effect on reducing LDL-C.[6,7,8,9,10] In several studies rosuvastatin affects suppressing the process of fibrogenesis through a TGF-β1/Smad pathway inhibitor, PPARγ activation which is protective against fibrosis and HMG-CoA reductase activity[11,12,13,14]

2. METHOD

Study Design

This research was carried out as a post-test in vitro control group study at the Airlangga University's Institute of Tropical Diseases in Surabaya, Indonesia. One of the treatments was rosuvastatin 10 µM/L, another was rosuvastatin 25 µM/L, and a third was rosuvastatin 50 µM/L. In this investigation, TGF-β1induced valve interstitial cells (VIC) served as the experimental subject. The valves of VIC cultures' experimental unit were removeda 12–13-week–old, 2.5–3 kg New Zealand rabbit (Oryctolagus cuniculus). The models were then split into three groups: a control group and groups receiving rosuvastatin 10 µM/L, rosuvastatin 25 µM/L, and rosuvastatin 50 µM/L. Six replications make increase the Higgins and Klinbaum formula sample size.

VIC's isolation, culture, and characteristics

With a few changes to the choice of animal models, Linn et alearlier .'s instructions for the isolation of rabbit VIC were followed[15]. Lethal injections containing phenytoin (125 mg/kg intraperitoneally) and midazolam (5 mg/kg) were administered to rabbits that met the usual inclusion criteria. After being surgically removed from the rabbit, the leaflets were subsequently digested with the enzyme collagenase II to separate them from the myocytes and valve endothelial cells, and then centrifuged and seeded with cell suspensions. Following centrifugation at 100g and transfer to culture medium, cells adhered.

Alpha Modified Eagle Medium (α-MEM) with 10% fetal supplemental bovine serum (FBS), 1% penicillin/streptomycin solution, 37°C with 95% humidity, and 5% CO2 was used to grow crushed VIC cells. Cells from sections were transplanted to six uncoated wells with α-MEM and 10% FBS at a density of 2600 cells/dish after reaching 80% confluence. The experiment is incubated for one to seven days after the medium has been changed to fresh α-MEM containing 10% FBS during a 24-hour incubation period. The expression of vimentin was studied to characterize VIC. Cells were washed in phosphate-buffered saline and then fixed for 15 minutes with 3% formaldehyde (PBS). A 15-minute application of 1% serum diluted in PBS was used to lessen non-specific binding, followed by another PBS wash. With the addition of Thermo Fisher Scientific's FITC-labeled anti-vimentin, a second PBS wash was completed (Schwerte, Germany). Fluorescent microscopy was used to demonstrate vimentin expression.

Initiative and Protocol

After cell growth in multiwell plates using Alpha Modified Eagle Medium (α-MEM) + 10% FBS attained 80–90% confluency. Next, 50,000 cells/cm3 VIC passage <3 cells were planted per cubic centimeter and cultured on medium + 1% low serum FBS (serum deprivation for 24 hours (Monzack et al. 2009; Zhang et al. 2018). Group P1 was pretreated with a rosuvastatin dose of 10 µM/L Group P2 received pretreatment with rosuvastatin dose of 25 µM/L, then a 72-hour TGF-β1 incubation at a concentration of 5 ng/ml. Group C underwent a pretreatment of 50 µM/Lrosuvastatin, then a 72-hour TGF-β1incubation at a concentration of 5 ng/ml. Group C underwent a pretreatment of 50 µM/L rosuvastatin, followed by a 72-hour incubation with TGF-1 5 ng/ml as a control. Group P4 got just TGF-β15 ng/ml. Fixing the myofibroblasts in 2% paraformaldehyde/PBS and 1% Triton X-100/PBS for 30 minutes allowed us to characterize them. The cell is treated with a mouse monoclonal antibody against the FITC label for α-SMA after being blocked with 2% BSA/PBS before being washed once more with PBS. Fluorescence microscopy evidence of the expression of α-SMA

Analytical Statistics

The SPSS program version 26.0 for Windows will be used to modify, code, and input the primary data that was acquired (SPSS Inc, Chicago, IL). Displaying each group's mean, standard deviation, and box-plot diagrams serves as a descriptive analysis. An unpaired T-test for the same or different variations was used in the unpaired

numerical comparative statistical analysis of the two groups, The Mann-Whitney test was employed if the distribution was not normal. If p <0.05, the difference is deemed to be significant. One-way Analysis of Variance (ANOVA) was used for unpaired numerical comparative statistical analysis between more than two groups, however Kruskal-Wallis with post hoc Mann-Whitney was used for non-normal distributions. If p <0.05, the difference is considered significant..

3. RESULTS

Interstitial Cell of the Valve Isolation and Expression

Use of the previously mentioned technique to isolate rabbit valve interstitial cells (Oryctolagus cuniculus). After being surgically removed, rabbit leaflets were then digested with the collagenase enzyme, isolated from myocytes, and valvular endothelial cells, before being centrifuged and seeded with cell suspensions. Observations showed positive results after 72 hours after culture by analyzing cell characteristics and immunocytochemistry. The outcomes of this study's cultural isolation procedure revealed that cells that could be seen under a light microscope had a spindle-shaped form and a normal cell membrane. Through immunocytochemical analysis, the vimentin-positive markers were used to characterize the valve interstitial cells. Vimentin had a positive expression, as determined by an immunocytochemical examination (more than 95% of a cell's luminosity in a given field of vision). Results from immunocytochemical labeling indicated that vimentin, a marker of valve interstitial cells, was positive.

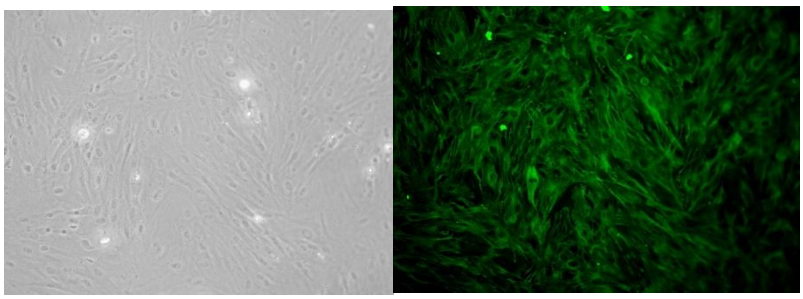

Figure 1: displays a valve interstitial cell (VIC) with a positive vimentin marker.
Phase-contrast microscopy picture on the left, vimentin fluorescent labeling on the right.

Expression of Myofibroblast Markers

After the VICs were exposed to TGF-β1, The VICs changed into myofibroblasts, which are distinguished by their irregularly shaped cells and cytoplasm that is packed with stress fibers. When compared to controls, immunocytochemical labeling following exposure to TGF-β1revealed a significantly higher level of α-SMAexpression (fluorescence over 95% of cells are illuminated in the area of vision). This demonstrates how valve interstitial cells are differentiated into myofibroblasts.

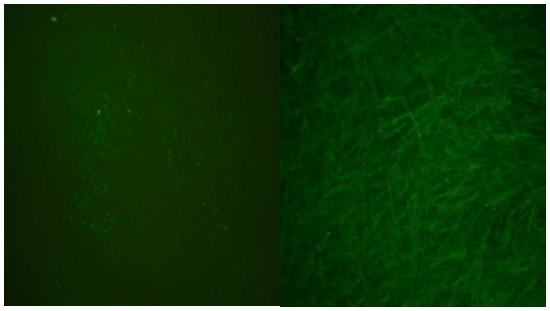

Figure 2: shows the morphology of myofibroblast cells and the expression of α-SMA on immunocytochemistry.
Left: Before TGF-β1exposure, Right: After TGF-β1 exposure

Analysis of Expression and Quantity of α-SMA in Group Markers Treatment and Control

The immunocytochemical examination of α-SMA markers was carried out in the treatment and control groups. Information on the levels of α-SMA expression in TGF-β1-exposed valve interstitial cells treated with rosuvastatin at different doses (10, 25, and 50 µM/L), as well as in the control group, used the Kolmogorov-Smirnov test to determine whether the data were normal; the findings showed that the data distribution was aberrant, and the measurements of concentration and distribution were indicated by median and percentile. The treatment group with the highest α-SMA expression was the rosuvastatin 10 µM/L group with a mean of 4.975 (4.08 – 13.4). The α-SMA expression in the rosuvastatin 25 µM/L group was 3.955 (2.88 – 6.82). The rosuvastatin 50 µM/L group had the lowest α-SMA expression at 3.900 (2.36 - 5.01), whereas the control group had the greatest α-SMA expression at 31.480. (21.46-45.09).

Statistical tests were not suitable for using ANOVA, so the analysis was performed using Kruskal-Wallis, which showed $p<0.001$, indicating a difference in at least the two treatment groups. Differences between treatment groups then were analyzed significantly in the Kruskal-Wallis test, The post hoc Mann-Whitney test was used to examine the data and discovered a significant difference between the treatment groups and the control group. Three different doses of rosuvastatin were compared to the control group: 10 µM/L, 25 µM/L, and 50 µM/L ($p<0.001$). According to the findings of statistical testing, It is possible to significantly block the differentiation of rabbit valve interstitial cells into myofibroblasts based on the expression of -SMA by administering rosuvastatin at dosages of 10, 25, and 50 µM/L.

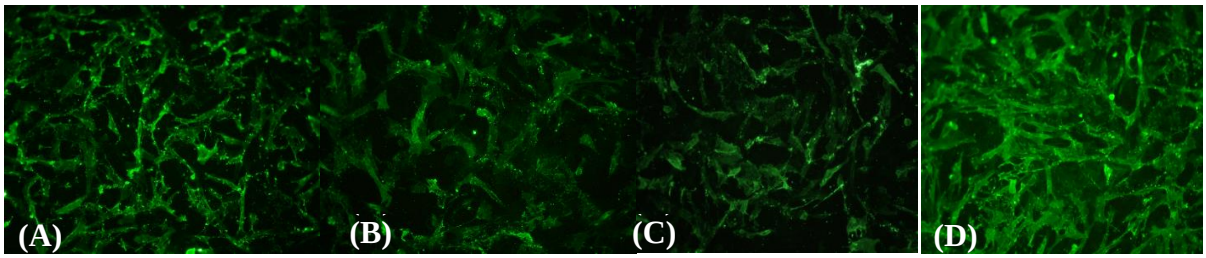

Figure 3. The expression of α-SMA on immunocytochemistry was used to identify myofibroblasts in various treatment groups.
(A) Rosuvastatin 10 µM/L, (B) Rosuvastatin 25 µM/L, (C) Rosvastatin 50 µM/L, and (D) Control group

Analysis of the Significance of Various Rosuvastatin Doses in Inhibiting Myofibroblastic Differentiation Based on α -SMA Expression

Analysis of the significance of the various doses was performed with Kruskal-Wallis. In this test, the control group was not included. The results showed $p<0.001$, This suggests that there are differences between the two treatment groups at the very least. The Kruskal-Wallis test identified a significant difference between the treatment rosuvastatin groups; this difference was further examined by the post hoc Mann-Whitney test, and a significant difference was discovered between the 10 µM/L vs 25 µM/L and 10 µM/L vs 50 µM/L groups ($p<0.001$), but not between the 25 µM/L and 50 µM/L groups ($p=0.74$).

4. DISCUSSION

Valve interstitial cells from the rabbit valve Oryctolagus cuniculus were employed in this investigation. The success of valve isolation with the method we described earlier, was proven by cell morphology on a phase contrast microscope which could be seen in a spindle-shaped pattern and positive luminescence of vimentin markers as a marker of valve interstitial cells. Comparatively to valve interstitial cells that were not exposed to TGF-β1, exposure of valve interstitial cells to TGF-β1 resulted in differentiation of cells into myofibroblasts, which are characterized by a greatly higher expression of α-SMA. The findings of this study are in line with those of earlier studies by Lijnen et al., which also demonstrated the ability of TGF-β1 exposure to cause fibrosis in ventricular fibroblast cells through the mechanisms of fibroblast-mediated collagen synthesis, myofibroblast-mediated collagen synthesis, and collagen differentiation into myofibroblasts[16].One of the

primary profibrotic cytokines, transforming growth factor-β1(TGF-β1), plays a significant role in the pathophysiology of rheumatic heart disease. The elevation of α-smooth muscle actin (α-SMA) can be functionally increased by this cytokine's ability to drive myofibroblastic development. TGF-β1 activation initiates a signaling cascade through the Mitogen-Activated Protein Kinase (MAPK) pathway, the c-Jun N-terminal kinase (JNK) pathway, and the intracellular protein receptors Smad 2/3[17].

After differentiation into myofibroblasts, myofibroblast cells contain actin stress fibers due to the upregulation of α-SMA in their cytoplasm. The presence of these stress fibers gives rise to contractile properties in myofibroblasts.[18] In our study, After exposure to TGF-β1, it is evident that valve interstitial cells are undergoing a process of differentiation into myofibroblasts. These changes were analyzed using a phase contrast microscope which showed large cell morphology, irregular cell membranes, and cytoplasm filled with stress fibers. The characterization of these cells was then confirmed by immunocytochemistry in the presence of α-SMA luminescence. To date, we have not found any studies using rabbits' valve interstitial cells for the manufacture of TGF-β1 and α-SMA based in vitro fibrosis models with a single induction and marker
Rosuvastatin has shown an effect in inhibiting fibrosis in some conditions, such as kidney, cardiac fibroblasts, ventricles, and cells aortic endothelium. Research previously obtained Rosuvastatin can reduce the manifestation of TGF-β1 and α-SMA. Our research demonstrated that Rosuvastatin could reduce the expression of α-SMA. Reduced expression of α-SMA in TGF-β1-induced VIC shows that Rosuvastatin inhibits myofibroblast differentiation. Mechanisms involving inhibition of myofibroblast differentiation based on a study previously could be via repression of TGF-β1-MAPK-ERK pathway, inhibition of HMG-CoA reductase involving inhibition mevalonate synthesis, and increase in secretion Peroxisome Proliferator-Activated γ (PPAR γ).[11-14] These various inhibitory pathways are the basis that rosuvastatin can be used as an anti-fibrotic drug. The results of this study that rosuvastatin starting at similar to earlier research, a dosage of 10 µM/L can block the differentiation of VIC cells brought on by TGF-β1. This provides new evidence that rosuvastatin can inhibit the myofibroblastic differentiation process in VIC cells which in this study used rabbit heart valves.

In our study, there was a significant difference between rosuvastatin doses 10 µM/L vs 25 µM/L and 10 µM/L vs 50 µM/L. However, No discernible change existed between the rosuvastatin dosagesof 25 µM/L and 50 µM/L. Administration of the drug with the minimum dose possible but has an optimal effect is expected to reduce excessive side effects. Our results showed that the optimal dose of rosuvastatin in vitro was 25 µM/L in inhibiting valve interstitial cells' development into myofibroblasts.

5. CONCLUSION

Myofibroblast differentiation in rabbit VIC caused by TGF-β1is inhibited by rosuvastatin. At rosuvastatin dosages, α-SMA expression was significantly decreased. of 10 µM/L, 25 µM/L, and 50 µM/L. The optimal in vitro dose of rosuvastatin in inhibiting myofibroblast differentiation of valve interstitial cells was 25 µM/L

6. ACKNOWLEDGMENT

Not relevant.

7. CONFLICT OF INTEREST

None declared.

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