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Inhibition of Pathological Myofibroblast Differentiation of Valvular Interstitial Cell by Atorvastatin, Olmesartan, and Resveratrol in Experimental Heart Valve Model "Contemporary Model with Rabbit Valve"

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Abstract: *Introduction*. Recent studies revealed that differentiation of valvular interstitial cell into myofibroblasts played an important role in pathological valve remodeling in rheumatic valvular disease. *Objective*. To investigate effects of atorvastatin, olmesartan, and resveratrol on Transforming Growth Factor β1-induced fibrosis. *Methods*. Valvular interstitial cell was isolated from 12-weeks male New Zealand rabbit (*Oryctolagus cuniculus*). Culture cells was divided into 4 groups, control group, group I (0.5 mg/mL Atorvastatin), group II (100 nmol/L Olmesartan), group III (50 μM/L Resveratrol) and group IV (combinations). All group were exposed to 100 nM Transforming Growth Factor β1 for 24 hours. *Results*. Immunochemical staining demonstrated that cells were completely differentiated into myofibroblasts with mean expression of α-smooth muscle actin 24522.64±4566.994. Atorvastatin, olmesartan, resveratrol, and its combination significantly reduced α-smooth muscle actin expression (6823±1735.3, 6942.7±2455.9, 14176.2±3343.3, 5051.8±1612.2 respectively (p<0.001). *Conclusion*. Our data showed atorvastatin, olmesartan, resveratrol, and its combination significantly reduce Transforming Growth Factor β1-induced valvular fibrosis.

Keywords: Myofibroblast; Atorvastatin; Olmesartan; Resveratrol; Valvular Interstitial Cell

1. Introduction

Valvular fibrosis is a histological hallmark of rheumatic heart disease, a terminal form of progressing and unresolved [1]. Valvular interstitial cell differentiation acts as the initial insult in fibrogenesis. Its differentiation into myofibroblast in response to profibrotic cytokines such as transforming growth factor- β 1 (TGF- β 1), potentiate its fibrosis properties [2]. Cardiac myofibroblasts are characterized by expression of the contractile protein α-smooth muscle actin (α -SMA) [3]. Therefore, preventing VIC differentiation into myofibroblasts could be a potential strategy in treating valvular fibrosis.

Transforming growth factor- β1 (TGF - β1) is a key pro-fibrotic cytokine in rheumatic heart disease [4]. It can stimulate the proliferation of CFs and the differentiation of CFs into myofibroblasts, marked by the expression of α-smooth muscle actin (α-SMA) and the secretion of ECM proteins, such as collagen I and collagen III [5–7]. The primary TGF- β1 signaling mechanism is the highly conserved small-mothers-against-decapentaplegic (Smad) pathway [8]. Smads are divided into the following three major groups: 1) receptor-regulated Smads (R-Smads: Smad2 and Smad3), 2) common mediator Smad (co-Smad: Smad4), and 3) inhibitory Smads (I-Smads: Smad6 and Smad7) [9]. Activation of TGF-receptors and R-Smad proteins through phosphorylation results in formation of R-Smad-coSmad complex. Upon translocation to the nucleus, interacts with other transcription factors, and regulates target gene expression [10]. Smad7 overexpression has been found

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to impede TGF- β1-induced signal transduction [11,12], whereas Smad2/3 activation has been demonstrated to be a critical mediator of TGF- β1-induced myofibroblast differentiation [13,14].

TGF1 can also trigger non-canonical signaling cascades such as mitogen-activated protein kinase (MAPK) signaling pathways including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK) [15]. A growing body of evidence suggests that MAPK signaling is vital in ECM production and VIC proliferation [16,17]. As a result, pharmacological treatments in these signaling pathways could be considered a promising therapeutic option for cardiac fibrosis [6]. Therefore, in the present study, we tested the hypotheses that: TGF- β1-induced VIC differentiation based on αSMA expression was significantly reduced by atorvastatin, olmesartan, resveratrol, and its combination.

2. Material and Methods

2.1. Animals

All experiments were carried out in accordance with the Animal Experimentation Guidelines of Sankyo Co., Twelve-week-old *Oryctolagus cuniculus* rabbit were purchased from Stem cell laboratories (Surabaya, Indonesia), and maintained in a room under a temperature controlled at 23721C and a 12-h light–dark lighting cycle. The animals were allowed a standard pellet chow and water ad libitum [18].

2.2. Material

Low-glucose DMEM, fetal bovine serum (FBS), Trypsin and HBSS were from Gibco Pasadena, CA (USA). Qiliqiangxin extracts were provided by Shijiazhuang Yiling Pharmaceutical Co., Shijiazhuang Ltd (China); Olmesartan (OLM) was purchased from Shanghai Sankyo Pharmaceutical Co., Shanghai Ltd. (China); Ang II was purchased from Sigma-Aldrich Co., St. Louis, Missouri LLC. (USA); Rabbit anti-rat aSMA polyclonal antibody was from Bioworld Technology, Nanjing Inc. (China); Rabbit anti-rat TGF- β1 polyclonal antibody was from Santa Cruz Dallas, Texas (USA).

2.3. Valvular Interstitial Cell Isolation and Treatment

Valvular interstitial cell were isolated, as described by Lin et al [19]. In summary, rabbit's valvular tissue was resected, chopped, and immersed in 0.08 percent trypsin. Following 4 or 5 rounds of digestion, the isolated cells were collected, centrifuged, and resuspended in Dulbecco modified Eagle medium (DMEM)/F12 containing 10% fetal calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin in a humidified incubator with 5 percent CO2 and 95% air. Nonadherent cells and debris were aspirated, and fresh medium was added. This differential plating method produced pure cultures of first passage valvular interstitial cells. When the cells were virtually confluent, they were trypsin zed with 0.125 % trypsin and passaged. Valvular interstitial cell isolation was confirmed by positive expression of vimentin and negative for α SMA in immunoassays [20]. After the cells were 80–90% confluent, the medium was replaced by serum-free DMEM the day before pretreated with atorvastatin (effective intervention dose, 0.5 mg/ml), olmesartan (100 nanomol/l) for 30 min, resveratrol (50 μM/L) and exposed to TGF β1 (100 nM) for 24 hrs [21]. Valvular interstitial cell in the second and third passages were used in this study.

2.4. Immunofluorescence quantification and analyses

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PBS was used to wash the cells in the treatment groups before they were fixed with 4 percent paraformaldehyde and permeabilized with 0.1 percent Triton X-100. After 30 minutes of incubation with BSA, the cells were treated with the primary antibody specific for SMA overnight at 4°C. The cells were then treated for 1 hour at room temperature in the dark with Fluorescein isothiocyanate (FITC) secondary antibody, followed by 30 minutes with DAPI. The cells were observed using a fluorescent microscope. The fluorescence intensity of cells was analyzed and quantified using a particular measurement included into imaging software. The intensity of fluorescence could be compared between sections of the same set of stress fibers and between cells of various treatments. The mean fluorescence intensities from ten randomly selected locations, each having a standard 50-lm^2 circular region, were used to analyze the data [21].

2.5. Statistical analysis

All data were presented as means ± SEM (Standard Error of the Mean). The statistical significance was assessed by using one-way ANOVA for multiple-group comparisons. $P < 0.05$ was considered statistically significant.

3. Result

3.1. Effect of TGF- β1 -Induced Differentiation of VIC to Myofibroblasts

Immunochemical staining of control TGF-β1 induced VIC demonstrated that 80% of the cells were αSMA positive with well-organized -SMA filaments in its cytoplasm (Figure 1A), exhibiting nearly complete differentiation of VIC to myofibroblasts with mean expression of αSMA24522.64 ± 4566.994 (Figure 1).

Figure 1. A-B) Myofibroblast Morphology and α-SMA expression in TGF-β1 Induced VIC; C) Myofibroblast Morphology and α-SMA expression in non-TGF-β1 Groups

Figure 2. A-B) Expression of αSMA Atorvastatin Groups; C-D) Expression of αSMA Olmesartan Groups; E-F) Expression of αSMA Resveratrol Groups; G-H) Expression of αSMA Combination Groups; I-J) Expression of αSMA Control Groups

Figure 3. Comparison of Atorvastatin, Olmesartan, Resveratrol, and Combination Groups

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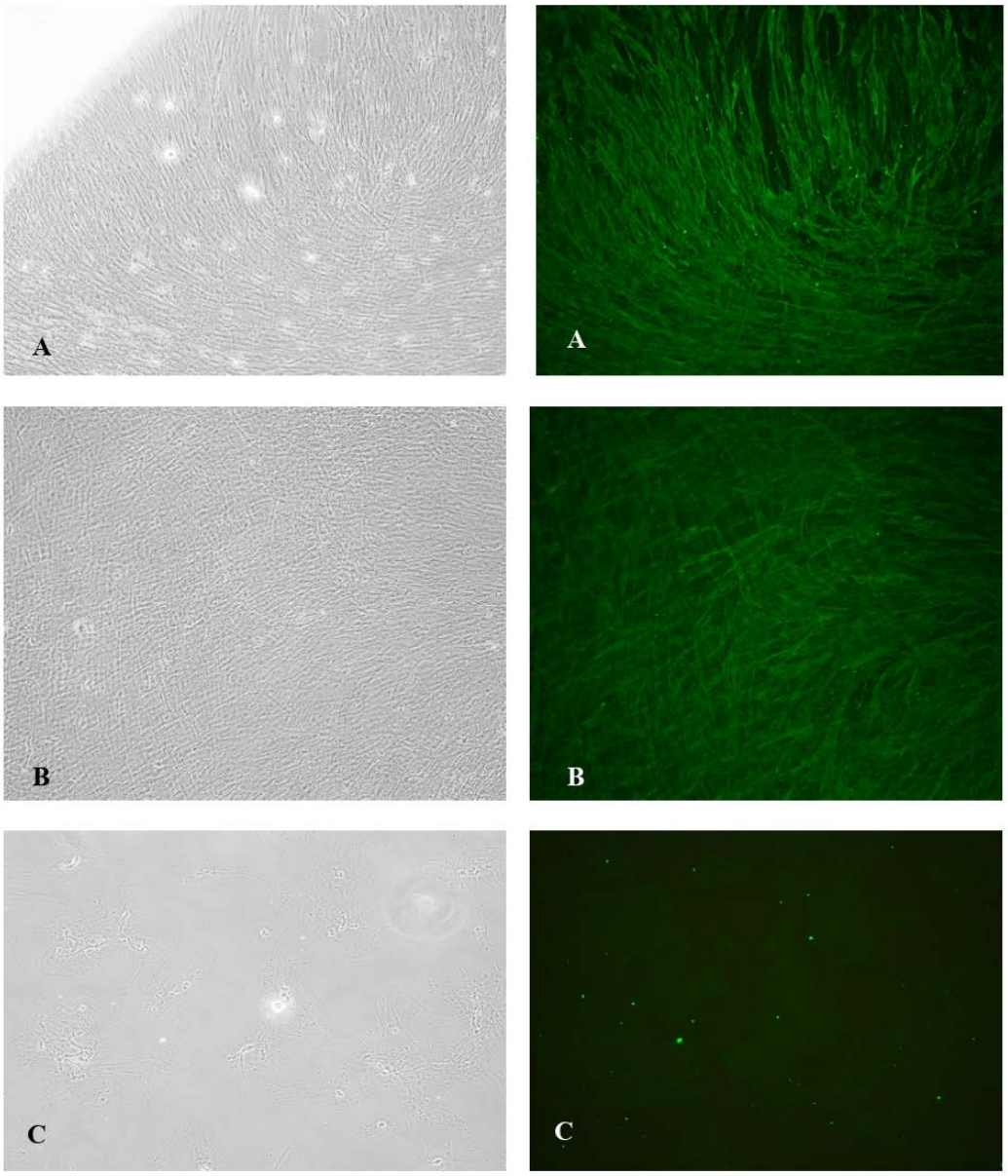

Figure 1. A-B) Myofibroblast Morphology and α-SMA expression in TGF-β1 Induced VIC; C) Myofibroblast Morphology and α-SMA expression in non-TGF-β1 Groups.

3.2. Effect of Atorvastatin, Olmesartan, Resveratrol, and Its Combination in Myofibroblast Differentiation inhibition

In comparison to control TGF- β1 -Induced VIC, atorvastatin, olmesartan, resveratrol, and its combination significantly reduced α-SMA expression compared to control. These intervention groups contain only a few of α-SMA expression, with mean expression of atorvastatin, olmesartan, resveratrol, and combination groups were 6823 ± 1735.3, 6942.7

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± 2455.9, 14176.2 ± 3343.3, 5051.8 ± 1612.2 respectively ($p < .0001$). Combination of atorvastatin-olmesartan-resveratrol exhibit the most potent myofibroblast differentiation inhibition (Figure 2 and 3). Post hoc analysis using Mann-Whitney test, showed no difference between atorvastatin and olmesartan groups despite its significancy.

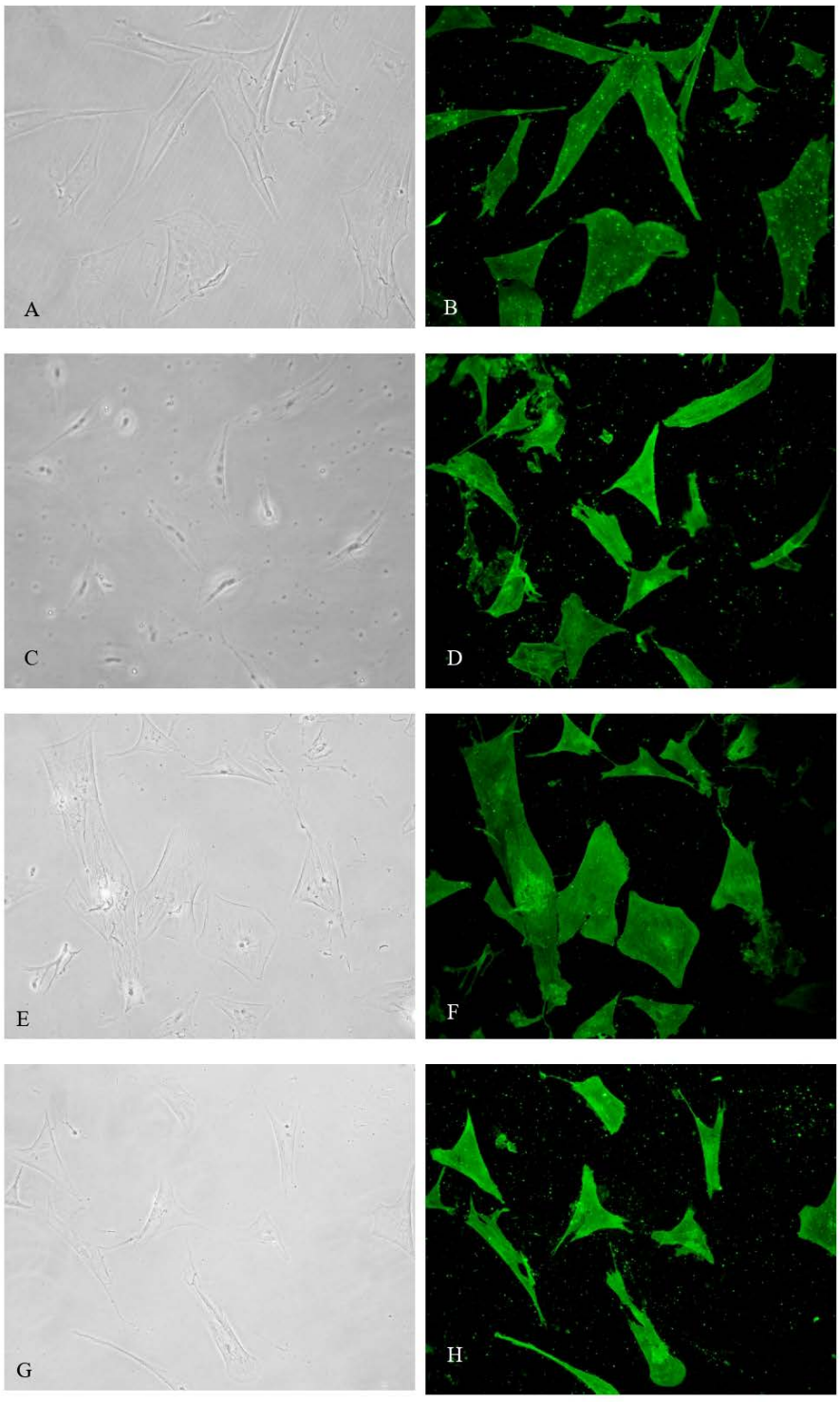

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Figure 2. A-B) Expression of αSMA Atorvastatin Groups; C-D) Expression of αSMA Olmesartan Groups; E-F) Expression of αSMA Resveratrol Groups; G-H) Expression of αSMA Combination Groups; I-J) Expression of αSMA Control Groups

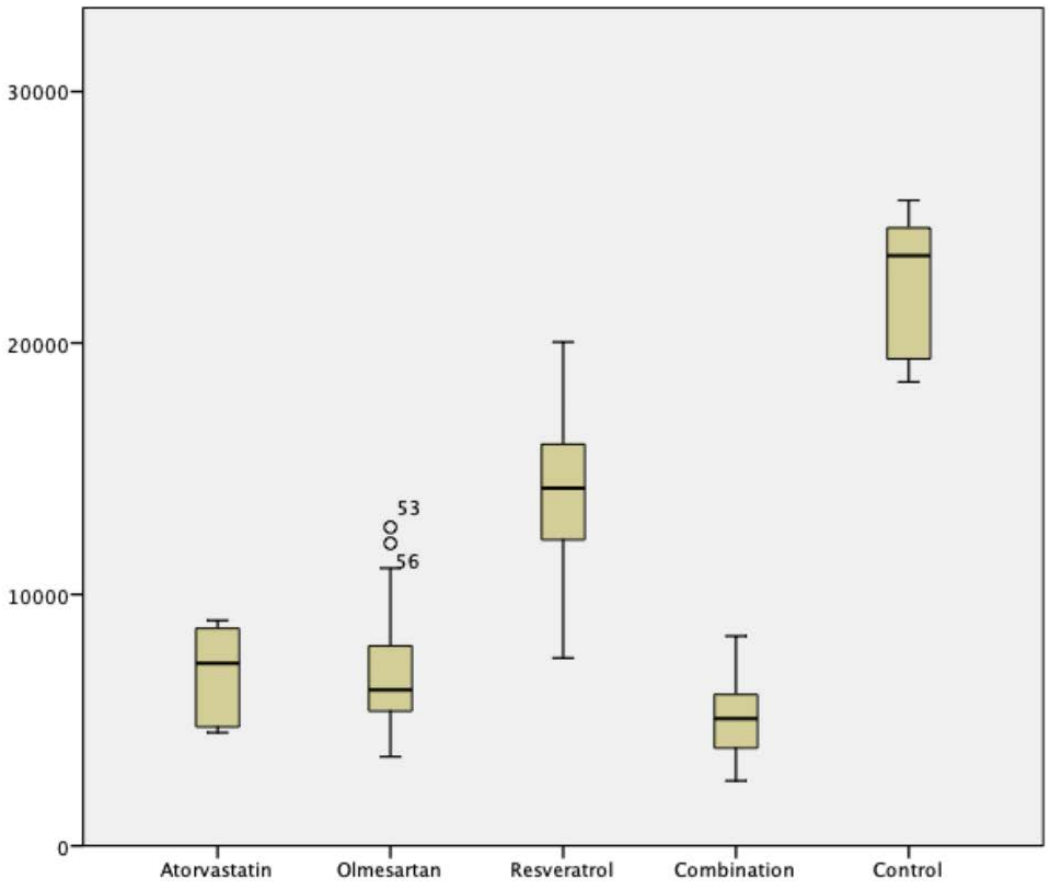

Figure 3. Comparison of Atorvastatin, Olmesartan, Resveratrol, and Combination Groups

4. Discussion

The major finding of this study was that (i) Atorvastatin inhibited TGF- β1 induced valvular interstitial cell differentiation (ii) Olmesartan inhibited TGF- β1 induced valvular interstitial cell differentiation (iii) Resveratrol inhibited TGF- β1 induced valvular interstitial cell differentiation and (iv) Combination of atorvastatin, olmesartan, and resveratrol inhibited TGF- β1 induced valvular interstitial cell differentiation. VICs, as the most common cell type in cardiac valves, contribute to valvular remodeling via a variety of mechanisms. In physiological condition, VIC perform a normal valvular repertoire and homeostasis function. However, during pathological condition, VICs can differentiate into myofibroblast in response to TGF-β1 resulting in valvular fibrosis, calcification, and stenosis [22–24].

Fibroblast proliferation and differentiation is a key mechanism in fibrogenesis. The expression of -SMA indicates the transition from valvular interstitial cells to myofibroblasts, followed by excessive production of inflammatory mediators, growth factors, and synthesis of extracellular matrix of collagen and fibronectin [25]. A number of research [25–28] have found that statins reduce left ventricular hypertrophy and cardiac fibrosis in

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hypertension and coronary artery disease. However, statin benefits in valvular interstitial cell fibrosis models has remained elusive [29].

A growing amount of data suggests that atorvastatin stimulates PPAR-γ via a p38-MAPK pathway by inducing synthesis of 15-deoxy-delta-12,14-PGJ2 (15DPGJ2), an endogenous PPAR-γ ligand [30–34]. Angiotensin II activation via AT1R directly stimulates immune cells via NF-κB activation, resulting in the overexpression of various inflammatory mediators such as MCP-1, RANTES, IL-6, ICAM1, and VCAM1 [35]. These findings have led the investigation of several angiotensin II pathway inhibitors and blockers, including as ACE and AT1R, in animals and other experimental models. An earlier studies revealed that a large dose of angiotensin II causes aortic valve leaflet thickening, endothelial lining discontinuities, and an increase in myofibroblasts in the ApoE-/- animal model of atherosclerosis and olmesartan, an AT1R blocker, administration attenuate these process [36]. Olmesartan has also been demonstrated to diminish macrophage accumulation, osteopontin and ACE overexpression, and decrement in myofibroblasts differentiation in hyper cholesterol fed rabbit's aortic valves [37,38].

Resveratrol (RES; trans-3,4′,5-trihydroxystilbene), a phytoalexin abundant in grape skins, has been recognized as a bioactive element in red wine, has been widely studied for its antifibrosis effect [39]. Study by Olson et al. showed the dual action of resveratrol in inhibiting fibrosis by direct inhibition of MEK activation and attenuation of ERK1/2 signalling and inhibition angiotensin II induced phosphatidylinositide 3-kinase (PI3-kinase)/Akt pathway [40]. Our findings of resveratrol potency also supported similar with study by Zhang Y et al. In their study resveratrol decreased the levels of miR-17, miR-34a, and miR-181a in TGF-β1-treated CFs. Smad7 mRNA and protein levels were reduced when miR-17 was overexpressed, but elevated when miR-17 was silenced. Furthermore, inhibiting miR-17 or overexpression of Smad7 reduced TGF-β1-induced CF proliferation and collagen secretion [41].

In current study the combination of atorvastatin, olmesartan, and resveratrol exhibit the most potent inhibition of myofibroblast differentiation. These combination was selectively selected due to their mechanism of inhibiting myofibrobast differentiation at various stages. Atorvastatin inhibit in distal signalling pathway of SMAD and MAPK. Olmesartan act as competitive inhibitor of angiotensin II at ATII type I receptor and inhibit downstream Ras and JAK/STAT signalling. Resveratrol increased PPARγ levels and act as inhibitor and negative regulator to TNFα, IL-1, and NF-κB signalling pathway.

4.1. Limitations

There are various limitations to this study. One of them is the inability of comparing in vivo and in vitro cells since there are various cell phenotypic and behavior after separation from the organism, including the response to pharmacological stimulation. Although rabbit is the closest phylogenetic relative to humans (next to primates), it was unknown whether cells triggered with TGFB in the rabbit heart would trigger the same protein phosphorylation as human [20].

5. Conclusion

These data suggest that atorvastatin, olmesartan, and resveratrol are likely exerting beneficial effects in inhibit pathological myofibroblast differentiation of valvular interstitial cell in experimental heart valve model by interacting with multiple signalling pathways.

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7. Conflict of interest

The authors declare no conflict of interest regarding this research and article

8. Funding

This study was supported in part by the Universitas Airlangga research grant (grant number: 205/UN3/PT/2021). Funder has no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

9. Availability of data and materials

The data supporting the results of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest regarding this research and article

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Highlights

- Atorvastatin: olmesartan, and resveratrol can inhibit pathological myofibroblast differentiation of valvular interstitial cell.
- Atorvastatin inhibits in distal signaling pathway of SMAD and MAPK.
- Olmesartan acts as competitive inhibitor of angiotensin II at ATII type I receptor and inhibit downstream Ras and JAK/STAT signaling.
- Resveratrol increases PPARγ levels and act as inhibitor and negative regulator to TNFα, IL-1, and NF-κB signaling pathway.

Ethical Approval: All animal procedures comply with the ARRIVE guidelines and carried out in accordance with the laws, regulations, and administrative provisions of the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and the Law of Republic Indonesia Number 18 Year 2009 and Chapter 66 Law of Republic Indonesia Number 18 Year 2009 regarding the protection of animals for experimental and other scientific purposes. This research is approved by the Ethical Committee for Animal Experiments at the Universitas Airlangga, Indonesia (Protocol Number: No.76/EC/KEPK/FKUA, date of approval 8 April 2021).

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Author contribution

- Denny Suwanto: Conceptualization, Writing - Original Draft, Investigation.
- Achmad Lefi: Supervision, Writing – Reviewing.
- Budi Baktijasa Dharmadjati: Supervision, Writing - Reviewing.
- Yudi Her Oktaviono: Supervision, Reviewing
- Agus Subagjo: Supervision, Reviewing
- Oryza Sativa: Data curation, Resources, Investigation.
- Dita Aulia Rachmi: Project administration, Visualization
- Ricardo Adrian Nugraha: Project administration, Visualization

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