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Original Article

Immunohistochemical evidence for involvement of inflammatory cytokines in anti-arrhythmic effects of rosuvastatin in male rats

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Abstract

Introduction: Considering the cardioprotective and anti-inflammatory properties of statins, the aim of the present experiment was to investigate the possible involvement of inflammatory cytokines in anti-arrhythmic effects of rosuvastatin in both *in vitro* and *in vivo* experiments in rats.

Methods: Three weeks after oral administration of either of rosuvastatin or vehicle, the atria were removed and after incubation with ouabain, time of onset of arrhythmia and asystole were recorded. We also used immunohistochemistry technique to identify the differentially expressed proteins interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α in atria.

Results: Rosuvastatin significantly postponed the onset of arrhythmia compared to vehicle-treated group. Injection of ouabain increased the atrial expression of IL-1β, IL-6 and TNF-α proteins, while pretreatment of rats with rosuvastatin could significantly attenuate them.

Conclusion: Our data suggest that rosuvastatin exerts anti-arrhythmic properties at least in part through modulation of inflammatory cytokines.

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Keywords:
Rosuvastatin;
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Introduction

Since most anti-arrhythmic drugs possess serious adverse effects, finding safer anti-arrhythmic agents with fewer side effects is very important. Statins are the main class of drugs, which used for management of hypercholesterolemia (Gagné et al., 2002). Studies have revealed that statins possess various additional beneficial cardioprotective properties against stroke, angina and thrombosis (Sever et al., 2003, Law et al., 2003, Wilson et al., 1998, Furie, 2012). Pleiotropic effects of statins on arrhythmia have also been reported (Turagam et al., 2015) based on the mechanisms including the improvement of heart rate variability (Pehlivanidis et al., 2001), the reduction in corrected QT duration and QT variability (Vrtovec et

al., 2005) and the inhibition of ventricular late potentials (Kayikcioglu et al., 2003). Statins also diminish the risk of ventricular tachyarrhythmia and sudden cardiac death both in coronary artery disease and non-ischemic cardiomyopathy (Buber et al., 2012, Dickinson et al., 2007, Bourne et al., 2010). In addition, statin therapy possesses a beneficial effect on aortic arterial stiffness (Upala et al., 2017) and could diminish the overall mortality, significantly (Nunes, 2017). Frequent experiments report that statins at high doses also have anti-inflammatory effects and improve endothelial function, which can stabilize and even regress plaques (Jain and Ridker, 2005, Weitz-Schmidt, 2002, Diomede et al., 2001, Libby and Aikawa, 2003, Lima et al., 2004). On the other hand, evidence shows that inflammatory cytokines have a key role in occurrence of various cardiac arrhythmias (Marcus et al., 2010, Engelmann and Svendsen, 2005, Duncan et al., 2010).

Ouabain is a cardiac glycoside, which has positive inotropic effects and can improve the heart failure treatment through inhibition of the Na^+/K^+-ATPase and Na^+/Ca^{2+} exchanger and subsequent increase of intracellular calcium and myocardial contraction (Yu and Choi, 1997). Nevertheless, higher doses of ouabain represents an animal model for induction of cardiac arrhythmias. On the other hand, it has been reported that ouabain induces the production of proinflammatory cytokines (Matsumori et al., 2000).

Since a majority of hyperlipidemic patients suffer from comorbid cardiovascular disorders including cardiac arrhythmias and considering the cardioprotective properties of statins, we previously reported that atorvastatin exerts marked anti-arrhythmic properties through diminution of inflammatory cytokines levels in atria (Najjari et al., 2018). Subsequently, in the present experiment, we aimed to detect the atrial immunostaining reactions of inflammatory cytokines in possible anti-arrhythmic properties of another drug of statin family, rosuvastatin in ouabain-induced arrhythmia in isolated rat atria.

Materials and methods

Animals

Male Wistar albino rats weighting 200-230g were used. Rats were kept at the temperature of 22±2°C, humidity 80%, 12-h light/dark cycle (light on at 7am) and had *ad libitum* access to food and water. All experiments were conducted in Tehran University of Medical Sciences in accordance with the recommendations of the ethics committee on animal experimentation of the medical school (number IR.TUMS.MEDICINE.REC.1396.4132), which conforms to the provisions of the Declaration of Helsinki.

Chemicals

Salts for preparing physiological salt solution were purchased from Merck (Germany). Rosuvastatin, ouabain and 4′,6-diamidino-2-phenylindole (DAPI) were prepared from Sigma-Aldrich (USA) and immunohistochemical antibodies against interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α were purchased from Abcam (USA).

Experimental groups

In vitro experiment

For isolated atria recording, 12 rats divided into two equal groups received either of 10mg/kg rosuvastatin dissolved in 1% carboxymethyl cellulose (CMC) in normal saline as vehicle or only 1% CMC in normal saline (Wallace et al., 2000), orally once daily for 3 weeks. Rosuvastatin or CMC were administered by oral gavage in volume of 1ml/kg, once daily. In this procedure, a stainless steel bulb tipped gavage needle was attached to a syringe and used to deliver the compound into the stomach.

In vivo experiment

For immunohistochemical studies, 12 new rats different from the aforementioned rats were divided into three equal groups. The first and second groups received vehicle (1% CMC), while the third group was pretreated with rosuvastatin (10mg/kg in 1% CMC). Rosuvastatin or CMC were administered by oral gavage in volume of 1ml/kg, once daily for 3 weeks. From the days 19-21, the first group was injected by normal saline, whereas the second and third groups received injection of ouabain (0.56mg/kg) (Rodrigues-Mascarenhas et al., 2006). All injections were in volume of 1ml/kg once daily for three consecutive days, intraperitoneally.

Recording of isolated atria

After anesthesia with ketamine (80mg/kg, Alfasan, Netherlands) and diazepam (2mg/kg, Caspian Tamin, Iran), the atria was rapidly removed and immersed in a tissue bath containing 20ml of carbogenated (95% O_2 and 5% CO_2) physiological slat solution at 37°C

and pH 7.4 (Ghebleh Zadeh et al., 2018). The composition of the solution was as follows (mM): NaCl 112.0, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.5, KH_2PO_4 0.5, $NaHCO_3$ 25.0, glucose 10.0 and EDTA 0.004 (Merck, Germany). After 30min equilibration under a preload tension of 1000mg, we recorded the onset time of arrhythmia (bigeminy) and asystole following incubation with ouabain (40µM) (Ghebleh Zadeh et al., 2018) as well as atrial beating rate and contractile force using isometric force transducer of PowerLab system (ADInstrument, Australia) and LabChart software.

Immunohistochemistry

Immunohistochemical study was carried out based on previous report (Cai and Wang, 2017). Briefly, paraffin sections of atrial tissue samples (5 micron thickness) were prepared. After dewaxed, tissue sections were washed in phosphate buffered saline (PBS, PH 7.4) for four times, followed by antigen retrieval by adding HCl 2 N (normal) for 30min. A primary antibody against IL-1β, IL-6 or TNF-α (1:100 dilution) was added. After PBS wash, secondary antibody (Ab6785, Abcam, USA. 1:150 dilution) was added. Then, the fluorescent stain DAPI was added and examined under the fluorescent microscope (Olympus, Japan, 400X). The expression level was observed and analyzed in Image-pro plus system.

Statistical analysis

GraphPad Prism 5 software (San Diego, CA) was used for statistical analyses. Results are shown as mean±SEM. We used unpaired Student's t-test to compare the onset time of arrhythmia and asystole and atrial beating rate and contractile force between treatment and control groups. We also used paired Student's t-test to determine the effects of ouabain on atrial beating rate and contractile force within groups. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was carried out to compare the atrial expression of inflammatory cytokines between groups. *P*-values less than 0.05 were considered statistically significant.

Results

Recording of isolated atria

Pretreatment of rats with rosuvastatin postponed the time of onset of arrhythmia (4.67min) compared to control vehicle-treated group (2.18min, $P \leq 0.01$). Although the time of onset of asystole in rosuvastatin-treated group (9.27min) seems longer than control (6.95min), no statistically significant difference was detected between treatment and control groups ($P > 0.05$, Fig. 1A). The difference in atrial beating rate between treatment (303.62 beat/min before, and significant ($P > 0.05$, Fig. 1B). Similarly, there was no statistically significant difference between atrial

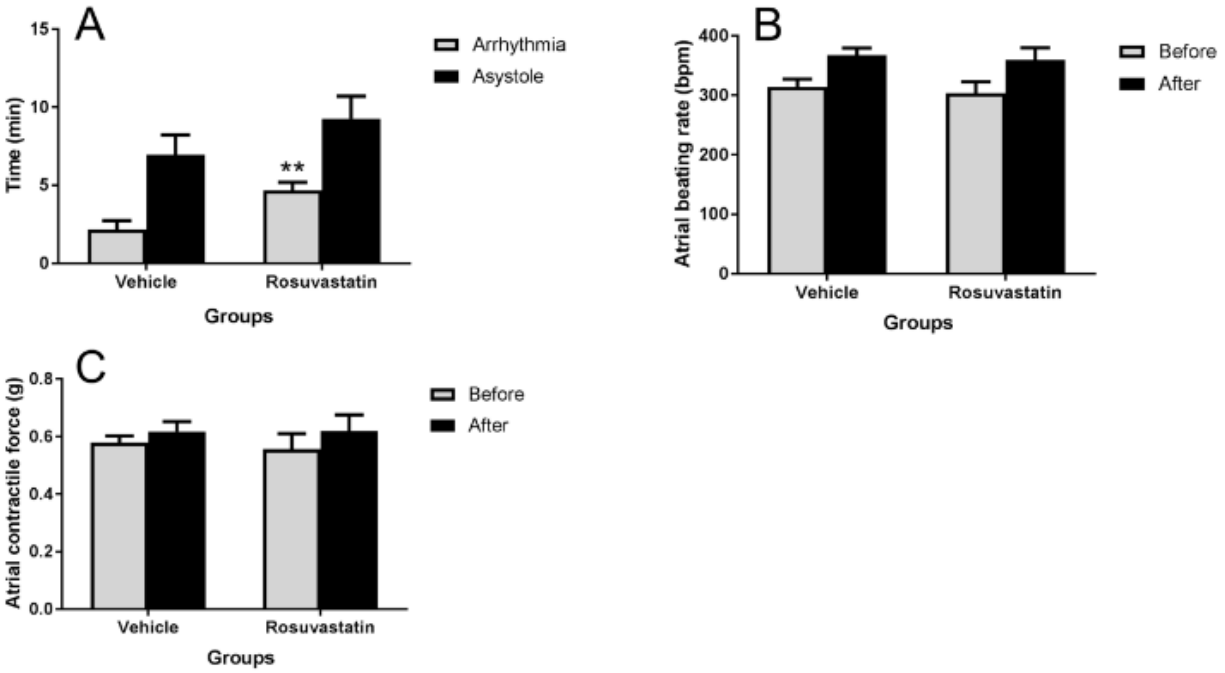

Fig.1. Time of onset of arrhythmia and asystole (A), atrial beating rate (B) and atrial contractile force (C) after ouabain incubation in vehicle and rosuvastatin-treated groups. Data are shown as mean±SEM. Six rats were used in each group. $^{**}P \leq 0.01$ compared to arrhythmia of vehicle-treated group.

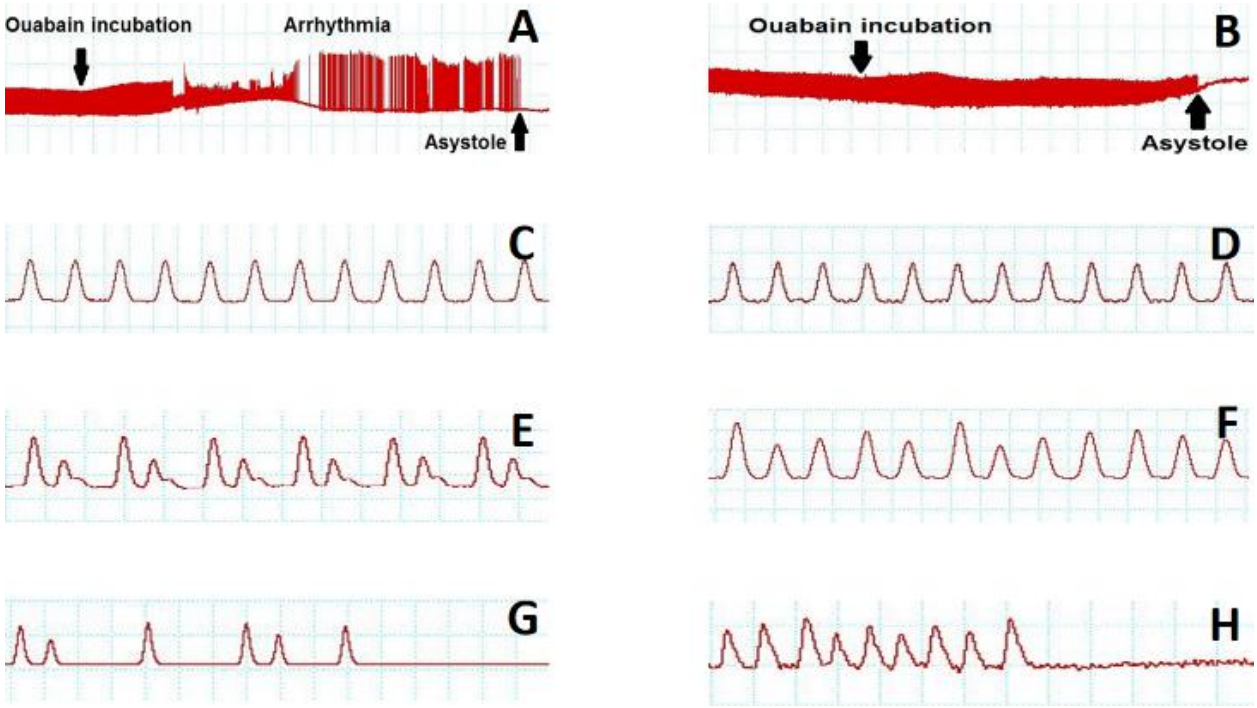

Fig.2. Chronotropic and inotropic pattern in vehicle and rosuvastatin-treated groups. In control group, ouabain-induced arrhythmia was strong (A), while the intensity of arrhythmia in rosuvastatin-treated group was diminished (B). The atrial beatings and contractile force prior incubation of ouabain were similar in both groups (C, D). Bigeminy arrhythmias (twin spikes with strong force), which is the typical manifestation of ouabain-induced arrhythmia were seen in control group (E), while in rosuvastatin-treated group, some small irregularities were recorded (F). The onset time of asystole in rosuvastatin-treated group (H) was later than control group (G).

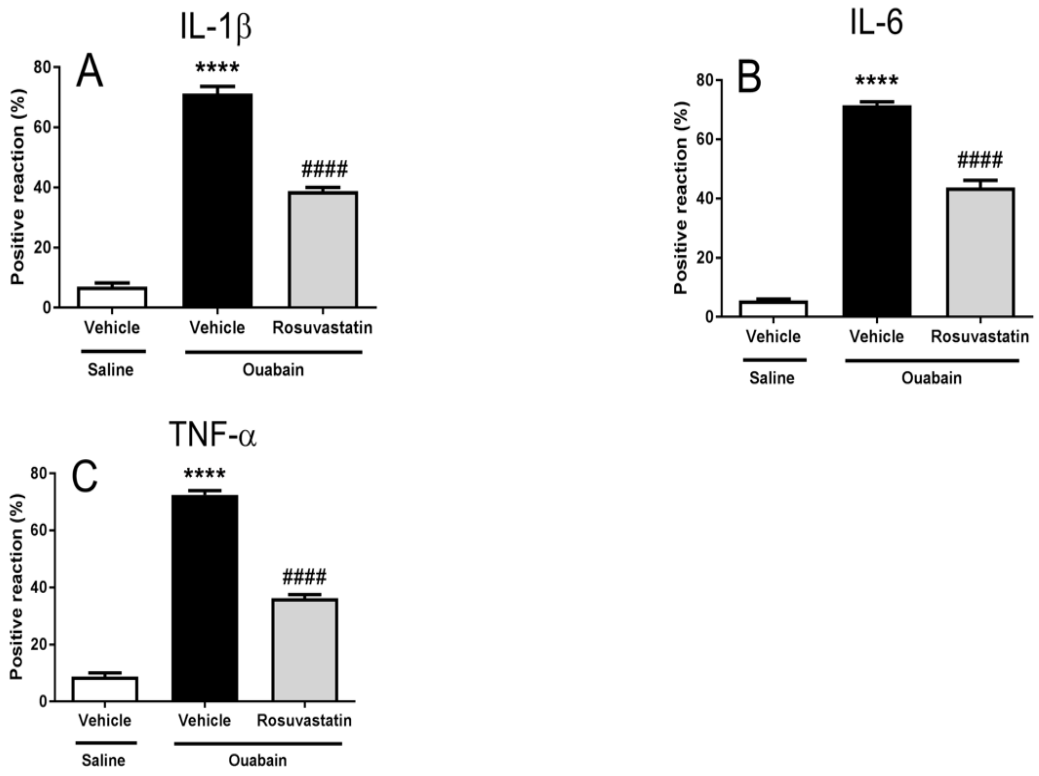

Fig.3. Atrial levels of IL-1β (A), IL-6 (B) and TNF-α (C) in saline and ouabain-injected vehicle and rosuvastatin-treated groups. Data are shown as mean±SEM. Four rats were used in each group. $^{****}P \leq 0.0001$ compared to saline-injected vehicle-treated group; $^{\#\#\#\#}P \leq 0.0001$ compared to ouabain-injected vehicle-treated group.

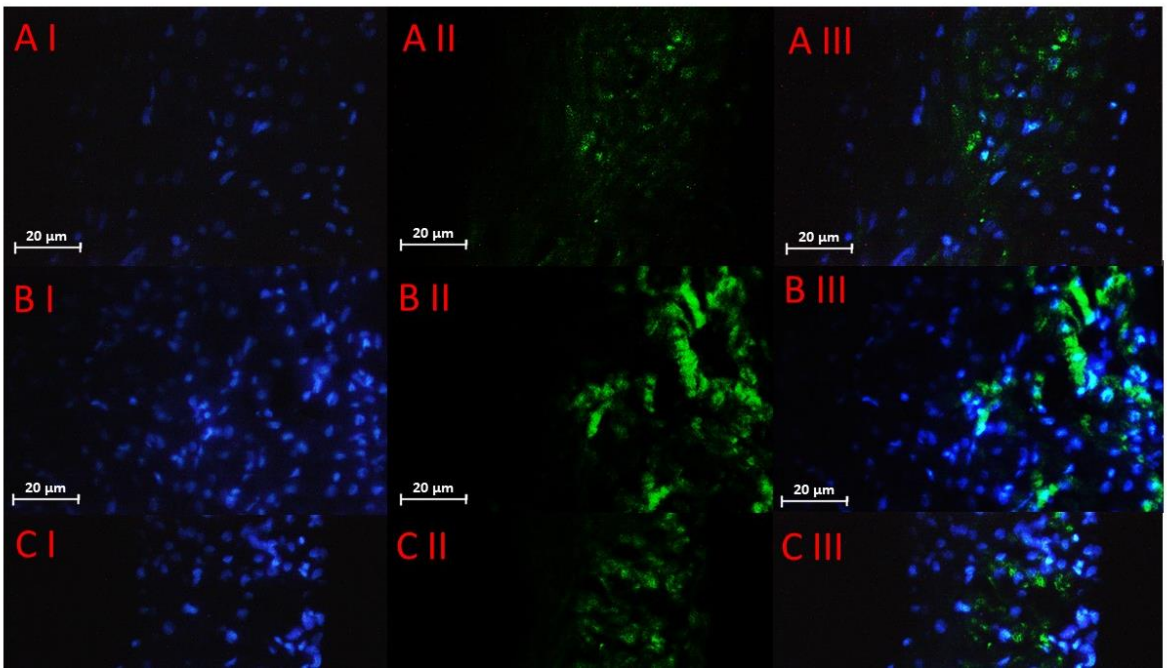

Fig.4. Representative immunostaining of IL-1β in atrial tissue arrays of saline and ouabain-injected vehicle and rosuvastatin-treated groups. A: vehicle+ saline, positive reaction 10%. B: vehicle+ ouabain, positive reaction 75%. C: rosuvastatin+ ouabain, positive reaction 40%. I: nuclei stained by DAPI; II: primary antibody to IL-1β; III: merge. Magnification 400X.

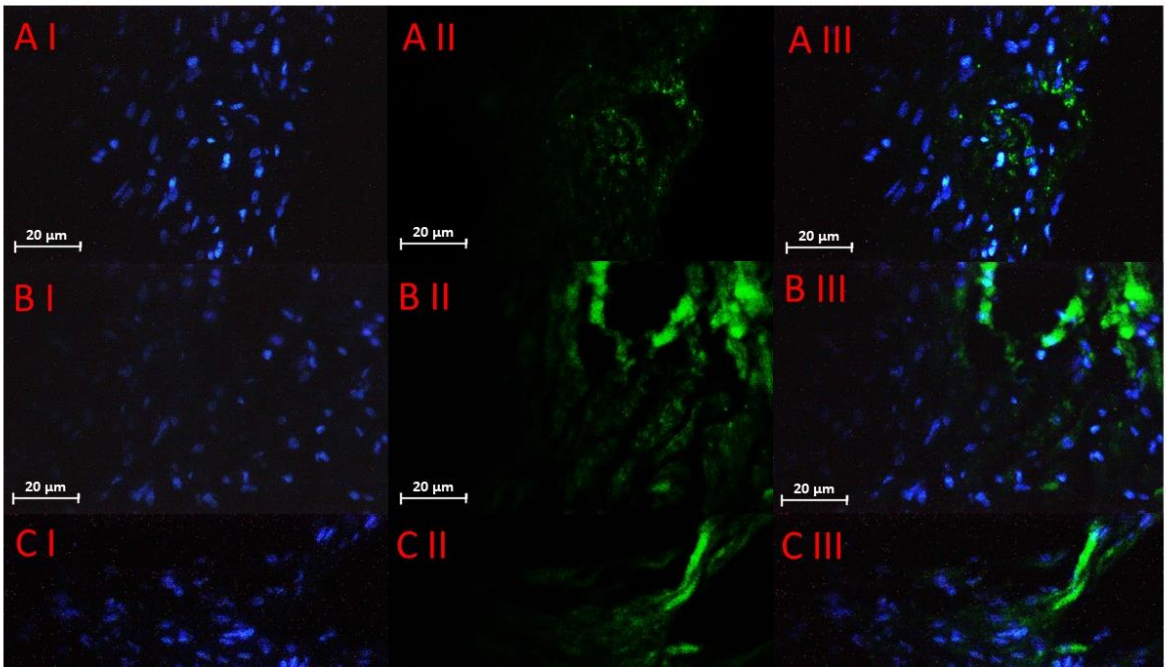

Fig.5. Representative immunostaining of IL-6 in atrial tissue arrays of saline and ouabain-injected vehicle and rosuvastatin-treated groups. A: vehicle+ saline, positive reaction 7%. B: vehicle+ ouabain, positive reaction 75%. C: rosuvastatin+ ouabain, positive reaction 40%. I: nuclei stained by DAPI; II: primary antibody to IL-6; III: merge. Magnification 400X.

contractile force of control (0.57g before, and 0.61g after ouabain incubation) and treatment (0.55g before, and 0.61g after ouabain incubation) groups (*P*>0.05, Fig.1C). Figure 2 compares the chronotropic and inotropic recording pattern in vehicle and rosuvastatin-treated groups. In control group, incubation of ouabain showed dramatic arrhythmias (Fig. 2A), while pretreatment of rats with rosuvastatin 359.63 beat/min after ouabain incubation) and control (314.55 beat/min before, and 367.82 beat/min after

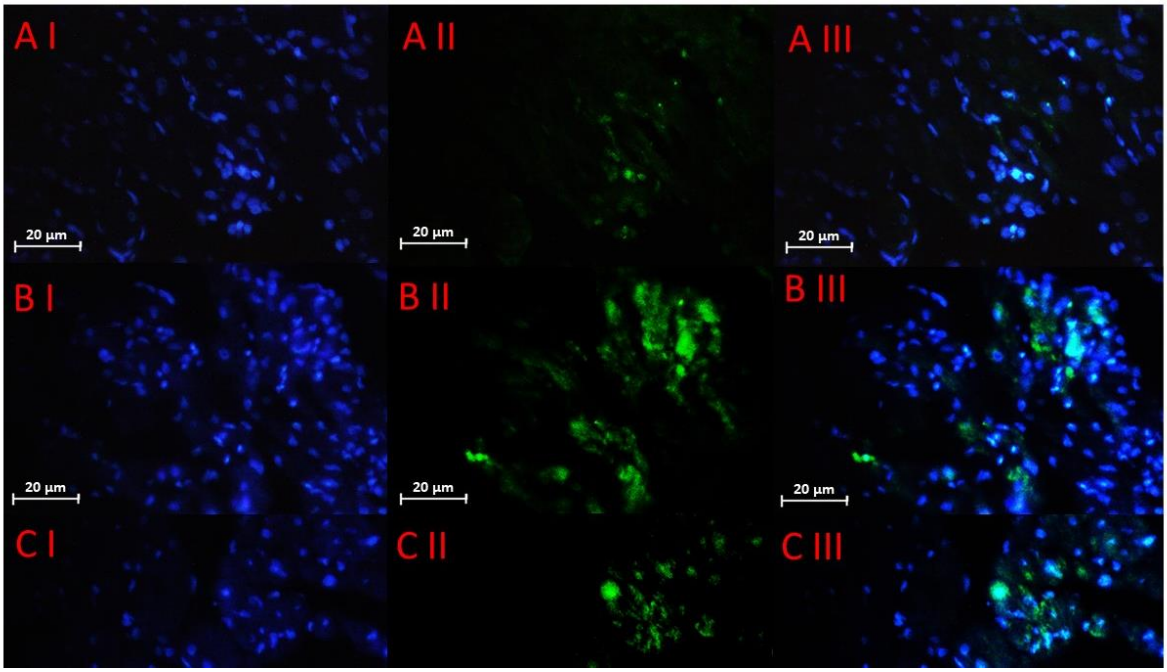

Fig.6. Representative immunostaining of TNF-α in atrial tissue arrays of saline and ouabain-injected vehicle and rosuvastatin-treated groups. A: vehicle+ saline, positive reaction 10%. B: vehicle+ ouabain, positive reaction 75%. C: rosuvastatin+ ouabain, positive reaction 40%. I: nuclei stained by DAPI; II: primary antibody to TNF-α; III: merge. Magnification 400X.

ouabain incubation) groups was not statistically alleviated the intensity of arrhythmias (Fig. 2B). The shape of arrhythmia in control group was typical bigeminy (twin spikes with strong force, repeatedly), which is the common manifestation of ouabain-induced arrhythmia (Fig. 2E). This arrhythmia lasted for several minutes and returned to the regular atrial rhythm in some samples. In rosuvastatin-treated group, irregularities between normal spikes were seen (Fig. 2F) and they occurred much later than control group.

Immunohistochemistry

Injection of ouabain significantly boosted the atrial expression levels of all studied inflammatory cytokines ($P \leq 0.0001$). On the other hand, pretreatment of rats with rosuvastatin could attenuate them, significantly ($P \leq 0.0001$, Fig. 3). Figure 4 shows the representative immunostaining of IL-1β in atrial tissue arrays of saline and ouabain-injected vehicle and rosuvastatin-treated groups. The positive reaction in saline-injected vehicle-treated group was 10%, while injection of ouabain elevated it to 75% and pretreatment of rats with rosuvastatin could significantly diminish it to 40%. Illustrating in Figure 5, the positive reaction for IL-6 in saline-injected vehicle-treated group was only 7%, while injection of ouabain boosted it to 75% and pretreatment of rats with rosuvastatin could significantly attenuate it to 40%. Similarly, the positive reaction for TNF-α in saline-injected vehicle-treated group was 10%, while injection of ouabain increased it to 75% and pretreatment of rats with rosuvastatin could significantly decrease it to 40% (Fig. 6).

Discussion

In the present experiment, we studied the involvement of inflammatory cytokines in anti-arrhythmic effects of rosuvastatin in both *in vitro* and *in vivo* examinations in rats. Results showed that administration of rosuvastatin delayed the onset of ouabain-induced arrhythmia. Furthermore, rosuvastatin attenuated the ouabain-induced over-expression of inflammatory cytokines.

Evidence show that ouabain boosts the production of pro-inflammatory cytokines (Chung et al., 2001, Vila et al., 2000, Vasan et al., 2003). For example, Matsumori et al. (2000) have reported that incubation of ouabain increases the levels of IL-1α, IL-1β, IL-6 and TNF-α in cultured human peripheral blood mononuclear cells. We also previously showed that ouabain could trigger the production of inflammatory

cytokines in rat atria (Tokazzabani Belasi et al., 2018, Najjari et al., 2018, Moradi et al., 2016). It is also reported that atrial fibrillation increases the production of IL-6 in patients (Ishida, 2006). Our data are in agreement with the aforementioned experiments. Besides, we also showed that rosuvastatin attenuates the over-production of inflammatory cytokines following ouabain injection, which corroborates our previous experiment on atorvastatin (Najjari et al., 2018).

The metabolic syndrome can be associated with increased risk of atrial fibrillation and other cardiac arrhythmias (Watanabe et al., 2008). Statins exert their anti-arrhythmic properties through reduction in corrected QT duration, inhibition of ventricular late potentials and improvement of heart rate variability (Pehlivanidis et al., 2001, Vrtovec et al., 2005, Kayikcioglu et al., 2003). Perioperative statin therapy is a therapeutic strategy for prevention of postoperative atrial fibrillation via inhibiting the inflammatory pathways (Sanchez-Quinones et al., 2008). Statins possess beneficial effects in acute myocardial infarction (Sicard et al., 2007, Baigent et al., 2008, Lenderink et al., 2006). The American heart association, American college of cardiology and European society of cardiology all suggest that statins should be prescribed following acute myocardial infarction (O'gara et al., 2013).

A recent clinical trial study in 2018 compared the effects of hydrophilic rosuvastatin and lipophilic atorvastatin on nonsustained ventricular tachycardia. They suggested that the effects of rosuvastatin on nonsustained ventricular tachycardia might be better than that of atorvastatin (Hu et al., 2018). Evidence show that adverse events of statins are dose-dependent (Jones et al., 2003). Given that a lower dose of rosuvastatin has a similar effect to higher doses of other statins, it is beneficial to use it (Guo et al., 2017). A high-dose of rosuvastatin can postpone ventricular remodeling, effectively suppress malignant remodeling of the heart, improve left ventricular systolic function, diminish the prevalence of adverse events and significantly improve the long-term prognosis (Guo et al., 2017).

Numerous experiments have reported the anti-inflammatory effects of statin (Blanco-Colio et al., 2003, S Antonopoulos et al., 2012, Mehrzadi et al., 2015). Inflammatory cytokines play a dramatic role in various pathological disorders. For example, London et al. (2003) have reported that TNF-α triggers the incidence of various atrial and ventricular arrhythmias. There are also other experiments indicating the involvement of TNF-α in pathophysiology of ventricular arrhythmia (Kowalewski et al., 2002, Duncan et al., 2010). Similarly, in the present experiment, our results are in agreement with the aforementioned reports indicating the anti-inflammatory properties of rosuvastatin. Moreover, various clinical trials report a marked correlation between atrial fibrillation and high levels of IL-6 (Ishida, 2006, Psychari et al., 2005, Wu et al., 2013). The ouabain-induced over-expression of IL-6 in our study also confirms the previous experiment.

Clinical practice guidelines suggest that cardiovascular benefits of statins generally outweigh non-cardiovascular adverse effects in patients suffering from cardiovascular disorders (Desai et al., 2014). The potentials of diverse therapeutic indications of statins could introduce the novel applications of this class of drugs in the future.

Conclusion

Our data suggest that rosuvastatin possesses anti-arrhythmic properties and diminution of atrial inflammatory cytokines expression might at least in part explain this response. Possible other mechanisms for anti-arrhythmic effects of rosuvastatin need for further experiments.

Acknowledgments

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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