ORIGINAL ARTICLE

The effect of statin administration on IL-6 and IL-1b expression in peripheral blood mononuclear cells of a hypertensive patient with SARS-CoV-2 spike protein stimulation

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ABSTRACT

Aim The infection of the SARS-CoV-2 virus potentially causes a cytokine storm with elevated IL-6 and IL-1β levels. Statin therapy was common among COVID-19 patients due to their cardiovascular comorbidities. However, the effect of statins on COVID-19 infection is unclear. The aim of this study was to evaluate the impact of statin administration on IL-6 and IL-1β level in peripheral blood mononuclear cells (PBMCs) after SARS-CoV-2 spike protein stimulation.

Methods The PBMCs were isolated from a hypertensive patient and stimulated by the SARS-CoV-2 subunit S1 spike protein. The PBMCs were then divided into four treatment groups and treated with simvastatin at various doses (10 μM, 25 μM, 50 μM, and control). IL-6 and IL-1β were measured from the supernatant using the ELISA method.

Results The stimulation of SARS-CoV-2 spike protein in PBMC cell culture statistically increased IL-6 and IL1β expression of 5.2 and 35.07 fold, respectively ($p<0.05$). The expressions of IL-6 and IL-1β were not statistically significant among three simvastatin doses and control.

Conclusion Statin administration did not have significant effect on IL-6 and IL-1β levels in PBMCs after SARS-CoV-2 spike protein stimulation in this study, a further study is needed.

Key words: cytokine release syndrome, interleukin-1 , interleukin-6, leukocytes, mononuclear, simvastatin

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INTRODUCTION

The COVID-19 pandemic caused by the SARS-CoV-2 virus has become a serious global problem and affected more than 260 million people, with more than 5 million deaths worldwide as of November 28, 2021 (1). SARS-CoV-2 is a pathogenic virus which primarily transmitted through respiratory droplets and close contact (2). Currently, there is no specific treatment for the SARS-CoV-2 virus. Thus, supportive treatment is the most recommended strategy to reduce the severity of COVID-19 and mortality (3).

Acute Respiratory Distress Syndrome (ARDS) is an immunopathological event and considered as the leading cause of death in COVID-19 patients. The mechanism of ARDS is initialized by the release of pro-inflammatory cytokines, which results in uncontrolled systemic inflammatory responses and cytokine storm (4). Previous evidence concluded that IL-6 and/or IL-1β are the main drivers of severe pathological conditions in COVID-19 patients (5). Therefore, anti-inflammatory drugs are expected to have beneficial effects in COVID-19 management.

Cardiovascular disease is the most common comorbidity in COVID-19 patients and associated with a higher mortality rate. A New York study involving 5.700 COVID-19 patients stated that hypertension was the most common comorbidity (56.6%), followed by obesity (47.1%), diabetes (33.8%), and coronary heart disease (11.1%) (6). A study in Wuhan included 138 patients and concluded that patients with more than one comorbidity had more severe presentation, up to 72% (7). This indicates that COVID-19 patients with the highest risk of mortality are those with cardiovascular comorbidities, particularly hypertension.

Statins are often used in cardiovascular disease management due to their numerous benefits among cardiovascular disease. Statins are known to have pleiotropic or anti-inflammatory effects (8). Among COVID-19 patients, the anti-inflammatory effects may promote clinical improvement for respiratory failure, elevated D-dimer, and IL-6 levels (9,10). Two observational studies reported 41% and 59%, respectively, reduction in 30-day all-cause mortality among hospitalized patients with influenza infection receiving statins (11,12). Recent molecular docking study suggested that statins may inhibit SARS-CoV-2 cell entry by binding directly to the main protease of the coronavirus (3).

Currently, an effect of statins on COVID-19 infection is still unclear, whether it is beneficial or detrimental. Scientific evidence is needed to answer whether statins are beneficial in COVID-19 management, especially regarding their anti-inflammatory effects.

The aim of this study was to evaluate the effect of statins on inflammatory cytokine IL-6 and IL-1β level in COVID-19 patients.

MATERIALS AND METHODS

Patients and study design

This study was a true experimental *in vitro* study with pre-test and post-test control group design. The period of investigation was from August until September 2021. Human peripheral blood mononuclear cells were a sample (PBMCs) taken and isolated from peripheral blood of patient with hypertension who was willing to participate in the study and had signed informed consent.

The inclusion criteria was a hypertension patient with the age >40 years old, who had a COVID-19 swab examination with negative results, and had no history of SARS-CoV-2 infection and had never received a Covid-19 vaccine, and had no other comorbidities besides hypertension. Exclusion criteria were patients with comorbidities other than hypertension, who had been vaccinated.

The isolated PBMCs cells obtained were then exposed to the SARS-CoV-2 spike protein and grouped randomly into 4 treatment groups: P0 - control group that was not given simvastatin, P1- treatment group with 10 μM simvastatin, P2 - treatment group with 20 μ M of simvastatin, P3 - treatment group with 50 μM simvastatin, and a negative control group (PBMC without spike protein stimulation). Three times replication was carried out in each treatment group (P0-P3) and the negative control group, resulting in total of 15 well plates. This process was repeated for each outcome measurement, namely IL-6 and IL-1 β expression.

This study follows the principles of the Declaration of Helsinki. The study protocol has been approved by the Institutional Ethics Committee of Faculty of Medicine Airlangga University

(approval number: 190/EC/KEPK/FKUA/2021). The patient gave his informed consent to be sampled for PMBCs and to participate in research activities. All information that could disclose the subject's identity was removed.

Methods

Peripheral blood mononuclear cells (PBMCs) isolation, culture and seeding. The 10 mL blood was collected in tube containing EDTA and processed in less than 2 hours after puncture. The PBMCs were isolated using density gradient centrifugation method. Collected blood was placed into a 50 mL centrifuge tube (Corning, Merck Chemicals, Indonesia), and diluted with phosphate buffer saline (PBS) with ratio of 1:1. Six millilitres of Ficoll solution (Ficoll Paque Plus, GE Healthcare, USA) were then added into the bottom of a 15 mL centrifuge tube (Corning); 6 mL of blood and PBS mixture were added to the Ficoll solution slowly to form two separate liquid phases. The tube was then centrifuged at 400xg at 20°C for 40 minutes. Plasma was collected and stored in a 1.5 mL tube (temperature -80°C). The buffy coat containing PBMC was collected using a Pasteur pipette and transferred to a new 15 mL centrifuge tube. Two wash procedures were carried out using PBS and centrifuged at 100xg at 20 °C for 10 minutes. The pellet was suspended in 1 mL of RPMI 1640 medium (Merck Chemicals, Indonesia) supplemented with 10% Fetal Bovine Serum (FBS) (Merck Chemicals, Indonesia), two mM 1-glutamine, 100 U/mL penicillin, and 100 ug/mL streptomycin (Merck Chemicals, Indonesia). PBMCs value was calculated using a hemacytometer and incubated in a 37°C incubator with 5% CO_2 for three days. Then, PBMCs were seeded into 24-well plates for each treatment and time of infection. Each observation was carried out in triples. Each well plate contains 100,000 cells.

SARS-CoV-2 spike protein. The SARS-CoV-2 spike protein subunit S1 (RayBiotech, Cat No. 230-01101) was used in this study. The concentration of the S1 subunit was at 28 nM, similar to previous studies (13). The SARS-CoV-2 spike protein was stored at -80°C and was stable at 4°C for two weeks.

Cell stimulation with SARS-CoV-2 spike protein. The SARS-CoV-2 subunit spike protein S1 was dissolved in Roswell Park Memorial Institute (RPMI) medium before being exposed to PBMC cell culture. The cell cultures were then exposed to S1 subunit protein at a dose of 28 nM and incubated for 30 minutes on ice and 30 minutes at 37.5°C. Then, 5 mL of RPMI 1640 was added, and the culture was stored at 37°C. After 24 hours, 0.5 mL of supernatant medium was collected and examined using the ELISA method to measure basal IL-6 and IL-1β levels.

Methods of treatment with statins. We used simvastatin (S6196) (Sigma-Aldrich) as statin treatment. The simvastatin was dissolved in 5 mM dimethyl sulfoxide (DMSO) and stored in the RPMI medium. After stimulated for 24 hours with spike protein SARS-CoV-2**,** the PBMCs were divided into 4 groups with the following statin concentration: 10 μM, 25 μM, 50 μM, and control (without statin). The control cultures were incubated with RPMI medium and DMSO at concentrations of 0.2% and 1% according to the concentrations added with the statin. Both control and treatment cultures were incubated with 10 ng/mL lipopolysaccharide for 24 h. At the end of the incubation period, in each statin level group, the cells were removed by centrifugation and supernatant medium was collected, stored at -70°C until further cytokine count testing.

ELISA assays to measure IL-6 and IL-1β expression. Measurements of IL-6 and IL-1β in stimulated PBMCs supernatants were carried out using specific ELISA tools, Human IL-6 ELISA kit (Sigmaaldrich, RAB0306, Germany) and Human IL-1 β ELISA kit (RAB0273). Nine standard controls were followed for which the manufacturing method had been provided from the kit. The control and PBMC samples supernatant were diluted in the diluent in a ratio of 1:10 for the measurement of IL-6 expression, and 1:2 for the expression of IL-1β. A total of 100 mL of wash the control and samples dilution were inserted into microplate (12x8 wells). The microplates were covered with a sealing cover and incubated at room temperature for 2.5 hours with gentle shaking. The microplates were then washed 4 times with 1x wash solution. Each well was washed and filled with wash buffer (300 μL) using a multi-channel pipette or autowasher. After washing, 100 μL of detection antibody was added to each well. The well was closed well and incubated for one hour at room temperature with gentle shaking. After each well was washed again, 100 μL of

streptavidin solution was added and incubated for 45 minutes at room temperature. After re-washing, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) one-step substrate reagent (Sigma-Aldrich, RAB0306, Germany) was added, incubated for 30 minutes at room temperature and dark cover. To each well 50 µL stop solution (Sigma-Aldrich, RAB0306, Germany) was then added. The intensity of the resulting colour was measured at a wavelength of 450 nm using a Microplate Photometer (Thermo Scientific, Indonesia). Cytokine levels were measured in all groups, PBMC cells without spike protein stimulation, PBMC cells with spike protein stimulation without simvastatin, and PBMC groups with spike protein stimulation and administration of simvastatin doses of 10 M, 25 M, and 50 M.

Statistical analysis

The data were not normally distributed, hence the Kruskal-Wallis comparison test was used to compare the differences between each group and compared to control group, and the Mann-Whitney post hoc test was used to compare the two groups. A $p<0.05$ was considered statistically significant.

RESULTS

The PBMC isolation process was carried out by the density gradient centrifugation method with FiColl solution. The centrifugation process produced a cloudy white ring-like between the plasma layer and Ficoll containing PBMC cells. The average number of viable PBMCs was 1.18 x 10^6 PBMC/mL.

The PBMCs cell culture was then incubated. After three days, PBMCs were evaluated under an inverted microscope with final density of 1.06 x 10^6 PBMC/mL (Figure 1).

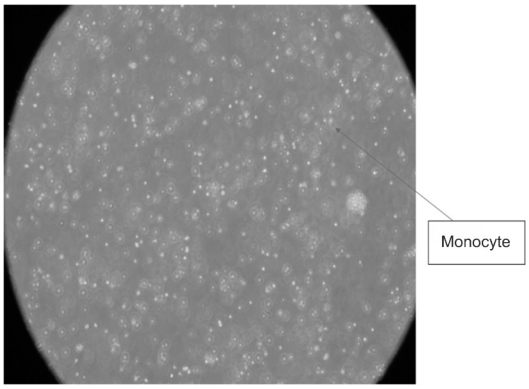

Figure 1. Peripheral blood mononuclear cells (PBMC) cells after incubation for three days (40x magnification)

Cytokine expression measurements were performed at 24 h of incubation after the SARS-CoV-2 spike protein stimulation. The expression of IL-6 was 5.2-fold higher compared to the negative control (p=0.037) (Figure 2).

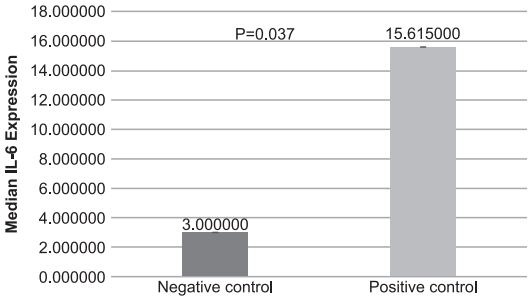

Figure 2. Differences in IL-6 expression in PBMCs without spike protein administration compared to SARS-CoV-2 spike protein stimulation

Similarly, the expression of IL-1β was 35.07-fold higher than negative control (p=0.037).

These findings indicated that the spike protein stimulation increased the expression of IL-6 and IL-1β in PBMCs (Figure 3).

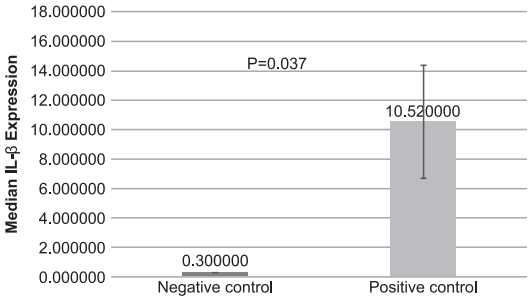

Figure 3. Differences in IL-1ß expression in PBMCs without spike protein administration compared to SARS-CoV-2 spike protein stimulation

The average IL-6 expression in PBMCs without simvastatin was 15.615 pg/mL. The administration of simvastatin decreased IL-6 expression in dose-dependent manner, but the difference was not statistically significant (Figure 4). The ave-

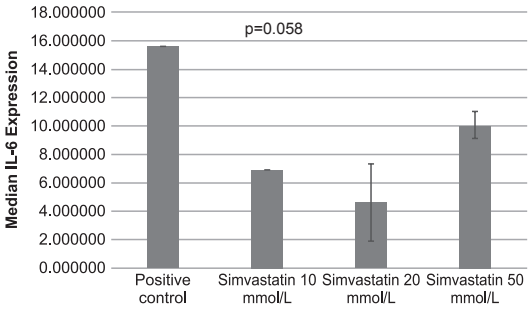

Figure 4. Differences in IL-6 expression in all doses of simvastatin and the control group

rage IL-6 expression in PBMCs treated with 10 μM, 25 μM, and 50 μM simvastatin was 15.040 pg/mL (3.7% less; p= 0.317), 14.708 pg/mL (5.8% less; p=0.121), and 14.461 pg/mL (7.4% less; p=0.121), respectively.

Similar to IL-6, the difference of IL-1β expression after simvastatin treatment was not significant (Figure 5). However, the expression was not in a dose-dependent manner. The highest IL-1β expression was in the control group with 8.44000 pg/mL, while the lowest was in the PBMCs receiving 25 μM simvastatin with 5.52667 pg/mL (34.6%less; p=0.513). The average IL-1β expression in simvastatin 10 μM and 50 μM was 6.94 pg/mL (17.8% less; p=0.513) and 8.823 pg/mL (4.5% less; p=0.827).

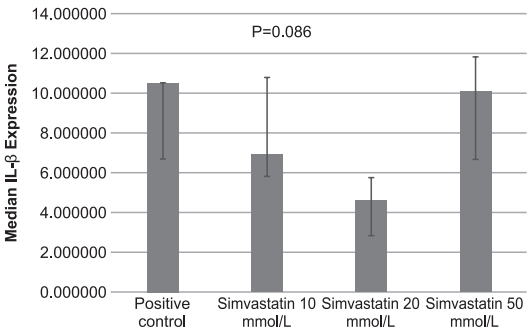

Figure 5. Differences in IL-1ßexpression in all doses of simvastatin and the control group

DISCUSSION

Our study result showed that the SARS-CoV-2 spike protein stimulation increased the expression of IL-6 and IL-1β in PBMCs. This result was similar to previous *in vitro* studies conducted on PBMC cells with SARS-CoV spike protein stimulation that showed a significant increase in IL-6 levels along with activation of the nuclear transcription factor p65/NF-kb. The study also proved that administration of TPCK, a NF-kb inhibitor, before spike protein transfection reduced the expression of IL-8 cytokines. Therefore, NF-kB was expected to take important roles in cytokine expression after spike protein transfection of PBMC cells (13).

In our study, PBMCs were derived from a patient with hypertension. Chronic inflammation of blood vessel walls is a major contributor to hypertension. However, the mechanisms responsible for the inflammatory response remain unclear. There was a suggestion that inflammasomes might play as important initiators of sterile inflammation inducing caspase-1 activation. Subsequently, the caspase-1 converts cytokines into their active form and triggers inflammation process (14).

Furthermore, based on our findings, simvastatin administration reduced expression of IL-6 and IL-1β in stimulated PBMCs, yet not statistically significant. Similarly, a Johns Hopkins observational study with 4.447 participants showed that the use of statins was not associated with reduced mortality (15). On the contrary, a meta-analysis of 147.824 COVID-19 patients concluded that statin use was associated with a significantly lower risk of death compared to non-statin users based on adjusted estimation. The subgroup analysis also showed that only chronic statin use was associated with a significant reduced risk of death (16). The duration of statin consumption is an important issue to highlight. The patients who received statin in long-term had a lower risk of death independently. This suggests that statins may be beneficial for COVID-19 patients after long-term consumption. While, in this study the exposure to statins was limited to merely 24 hours.

In contrast to the effect of simvastatin on IL-6 expression which was linear with increasing dose-dependent, IL-1β expression varied among three doses. One possibility is that, statins can trigger pro-inflammatory effects at certain dose to affect IL-1β expression response. This pro-inflammatory effect is related to statin effect in protein prenylation process by adding mevalonate or GGPP (an intermediate in the mevalonate pathway), which prevents the release of IL-1β (17). In vivo and in vitro studies have also identified novel pleiotropic properties of statins in modulating the adiponectin-AdipoR pathway in the human monocyte-macrophage lineage, wherein intensive statin therapy impairs the expression and function of adiponectin and its receptors, thereby increasing IL-1β release (18).

Although they have the same mechanism of action, several types of statins have different efficacy and effectiveness, especially their anti-inflammatory activity. Previously, a comparison of simvastatin to atorvastatin in patients with type 2 diabetes mellitus showed that atorvastatin had superior pleiotropic effect on oxidative stress and inflammation markers (19). Another study that compared the effects of simvastatin 20 mg/day with atorvastatin 10 mg/day in coronary heart di-

sease patients concluded that atorvastatin greatly reduced oxidative stress markers as compared with simvastatin (20). However, in vitro studies comparing the effectiveness of several statins demonstrated controversial results (21,22). This phenomenon is a major problem to researchers since there were mixing results on anti-inflammatory properties of statins. In this study, we used simvastatin since it is affordable and widely available in primary health care settings.

The limitation of this study is that we used only one type of statin (simvastatin), so the effect of other statins cannot be known, such as atorvastatin, rosuvastatin or pravastatin. Another limitation is that inflammatory parameters measured only IL-6 and IL-1beta, so statin effects on other parameters such as other cytokines, NF-kB, AP-1, or angiotensin II are unknown.

In conclusion, our study showed that statin administration did not have significant effect on IL-6 and IL-1β level in PBMCs after SARS-CoV-2 spike protein stimulation in 24 hours observation. A further study is needed to analyse statin effect in this population in a longer follow-up.

FUNDING

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TRANSPARENCY DECLARATION

Conflict of interest: None to declare.

REFERENCES

1. World Health Organization (WHO). Weekly Operational Update on COVID-19. Emerg Situational Updat 2021; 77:1–10.
2. Hu B, Huang S, Yin L. The cytokine storm and COVID-19. J Med Virol 2021; 93:250–6.
3. Zhang XJ, Qin JJ, Cheng X, Shen L, Zhao YC, Yuan Y, Lei F, Chen MM, Yang H, Bai L, Song X, Lin L, Xia M, Zhou F, Zhou J, She ZG, Zhu L, Ma X, Xu Q, Ye P, Chen G, Liu L, Mao W, Yan Y, Xiao B, Lu Z, Peng G, Liu M, Yang J, Yang L, Zhang C, Lu H, Xia X, Wang D, Liao X, Wei X, Zhang BH, Zhang X, Yang J, Zhao GN, Zhang P, Liu PP, Loomba R, Ji YX, Xia J, Wang Y, Cai J, Guo J, Li H. In-hospital use of statins is associated with a reduced risk of mortality among individuals with COVID-19. Cell Metab 2020; 32:176.
4. Dashti-Khavidaki S, Khalili H. Considerations for statin therapy in patients with COVID-19. Pharmacotherapy 2020; 40:484–6.
5. Maes B, Bosteels C, De Leeuw E, Declercq J, Van Damme K, Delporte A, Demeyere B, Vermeersch S, Vuylsteke M, Willaert J, Bolle L, Vanbiervliet Y Decuypere J, Libeer F, Vandecasteele S, Peene I, Lambrecht B. Treatment of severely ill COVID-19 patients with anti-interleukin drugs (COV-AID): a structured summary of a study protocol for a randomised controlled trial. Trials 2020; 21:556.
6. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, the Northwell COVID-19 Research Consortium, Barnaby DP, Becker LB, Chelico JD, Cohen SL, Cookingham J, Coppa K, Diefenbach MA, Dominello AJ, Duer-Hefele J, Falzon L, Gitlin J, Hajizadeh N, Harvin TG, Hirschwerk DA, Kim EJ, Kozel ZM, Marrast LM, Mogavero JN, Osorio GA, Qiu M, Zanos TP. Presenting characteristics, comorbidities, and cutcomes cmong 5700 catients cospitalized cith COVID-19 in the New York City area. JAMA 2020; 323:2052–9.
7. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 cospitalized patients with 2019 NovelCoronavirus–infected pneumonia in Wuhan, China. JAMA 2020; 323:1061.
8. Chansrichavala P, Chantharaksri U, Sritara P, Chaiyaroj SC. Atorvastatin attenuates TLR4-mediated NF-κB activation in a MyD88-dependent pathway. Asian Pasific J Allergy Immunol 2009; 27:49–57.
9. Kruger P, Bailey M, Bellomo R, Cooper DJ, Harward M, Higgins A, Howe B, Jones D, Joyce C, Kostner K, McNeil J, Nichol A, Roberts MS, Syres G, Venkatesh B. A multicenter randomized trial of atorvastatin therapy in intensive care patients with severe sepsis. Am J Respir Crit Care Med 2013; 187:743–50.
10. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. Intensive Care Med 2020; 46:846–8.
11. Vandermeer ML, Thomas AR, Kamimoto L, Reingold A, Gershman K, Meek J, Farley MM, Ryan P, Lynfield R, Baumbach J, Schaffner W, Bennett N, Zansky S. Association between use of statins and mortality among patients hospitalized with laboratory-confirmed influenza virus infections: a multistate study. J Infect Dis 2012; 205:13–9.
12. Laidler MR, Thomas A, Baumbach J, Kirley PD, Meek J, Aragon D, Morin C, Ryan PA, Schaffner W, Zansky SM, Chaves SS. Statin treatment and mortality: propensity score-matched analyses of 2007-2008 and 2009-2010 laboratory-confirmed influenza hospitalizations. Open forum Infect Dis 2015; 2: ofv028.
13. Dosch SF, Mahajan SD, Collins AR. SARS coronavirus spike protein-induced innate immune response occurs via activation of the NF-kappaB pathway in human monocyte macrophages *in vitro*. Virus Res. 2009; 142:19–27.

14. Krishnan SM, Sobey CG, Latz E, Mansell A, Drummond GR. IL-1β and IL-18: inflammatory markers or mediators of hypertension? Br J Pharmacol 2014; 171:5589–602.
15. Ayeh SK, Abbey EJ, Khalifa BAA, Nudotor RD, Osei AD, Chidambaram V, Osuji N, Khan S, Salia EL, Oduwole MO, Yusuf HE, Lasisi O, Nosakhare E, Karakousis PC. Statins use and COVID-19 outcomes in hospitalized patients. PLoS One 2021; 16:e0256899.
16. Diaz-Arocutipa C, Melgar-Talavera B, Alvarado-Yarasca Á, Saravia-Bartra MM, Cazorla P, Belzusarri I, Hernandez AV. Statins reduce mortality in patients with COVID-19: an updated meta-analysis of 147 824 patients. Int J Infect Dis 2021; 110:374.
17. Liberale L, Carbone F, Camici GG, Montecucco F. IL-1β and statin treatment in patients with myocardial infarction and diabetic cardiomyopathy. J Clin Med 2019; 8:1764.
18. Gasbarrino K, Hafiane A, Zheng H, Daskalopoulou SS. Intensive statin therapy compromises the adiponectin-adipoR pathway in the human monocyte-macrophage lineage. Stroke 2019; 50:3609–17.
19. Sathyapalan T, Shepherd J, Atkin SL, Kilpatrick ES. The effect of atorvastatin and simvastatin on vitamin D, oxidative stress and inflammatory marker concentrations in patients with type 2 diabetes: a crossover study. Diabetes Obes Metab 2013; 15:767–9.
20. Li QZ, Deng Q, Li JQ, Yi GH, Zhao SP. Valsartan reduces interleukin-1beta secretion by peripheral blood mononuclear cells in patients with essential hypertension. Clin Chim Acta 2005; 355:131–6.
21. Franzoni F, Quiñones-Galvan A, Regoli F, Ferrannini E, Galetta F. A comparative study of the in vitro antioxidant activity of statins. Int J Cardiol 2003; 90:317–21.
22. Greenwood J, Mason JC. Statins and the vascular endothelial inflammatory response. Trends Immunol 2007; 28:88–98.