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Effect of atorvastatin on pulmonary arterial hypertension in rats through PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of atorvastatin on pulmonary arterial hypertension (PAH) in rats and to observe its specific regulatory mechanism through the phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/AKT) signaling pathway.

MATERIALS AND METHODS: The model of PAH was successfully established in rats *via* hypoxia feeding. All rats were divided into three groups, including Control group (n=15), PAH model group (Model group, n=15) and atorvastatin treatment group (Ator group, n=15). Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and nitric oxide (NO) were detected by enzyme-linked immunosorbent assay (ELISA). Right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (RVHI) in each group were determined. Meanwhile, the pathological changes of lung tissues of rats were detected *via* hematoxylin-eosin (HE) staining. Furthermore, apoptosis in lung tissues in each group was detected *via* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. In addition, the expression levels of PI3K/AKT signaling pathway and apoptotic genes in lung tissues were detected *via* Quantitative Polymerase Chain Reaction (qPCR).

RESULTS: In Model group, the levels of TNF-α and IL-6 increased significantly, while the level of NO decreased. Both RVSP and RVHI in Model group were significantly higher than those of Control group and Ator group ($p<0.05$). The results of HE staining revealed that Model group showed significantly severe lung tissue injury (LTI). According to the results of TUNEL staining, the number of apoptotic cells in lung tissues in Model group was significantly smaller than that of Ator group ($p<0.05$). Meanwhile, the expression level of cysteinyl aspartate-specific proteinase-3 (Caspase-3) in Model group was markedly lower than that of Ator group ($p<0.05$). However, the expression level of B-cell lymphoma-2 (Bcl-2) in Model group was markedly higher than that of Ator group ($p<0.05$). In Ator group, the expression levels of PI3K and AKT in lung tissues were remarkably higher than those of Model group ($p<0.05$). All the above results indicated that atorvastatin could effectively up-regulate the expressions of PI3K and AKT ($p<0.05$).

CONCLUSIONS: Atorvastatin regulates the symptoms of PAH in rats through activating the PI3K/AKT signaling pathway.

Key Words:
Atorvastatin, Pulmonary arterial hypertension, PI3K/AKT signaling pathway, Rats.

Introduction

Pulmonary arterial hypertension (PAH) is a common cardiovascular disease, seriously threatening the health and affecting the life quality of patients[1]. However, the pathophysiological mechanism of PAH has not been fully elucidated yet. Currently, evidence has shown that after the occurrence of clinical symptoms, the vital activities of PAH patients are greatly restrained. Meanwhile, the physiological indexes are inhibited and the life cycle is shortened by about 2 years[2]. The major pathological features of PAH include abnormal increase of pulmonary vascular resistance, vascular cell proliferation, and enhanced resistance to apoptosis. This may eventually lead to a persistent proliferation of pulmonary arteries, declined cross-sectional area of pulmonary arteries, pulmonary artery sclerosis, decreased pulmonary blood flow, as well as changes in blood supply. As a result, pulmonary vascular remodeling occurs and pulmonary arterial pressure increases significantly. This causes a series of adverse reactions, ulti-

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mately inducing right heart failure[3,4]. Currently, targeted therapy can reduce severe PAH and delay the referral of patients receiving lung transplantation. However, transplantation is still an important choice for advanced PAH patients. However, such choice results in the treatment abandoning of many patients, seriously harming the development of social welfare[5]. Nowadays, the pathogenesis of PAH remains unclear. Multiple factors can lead to increased pulmonary circulation blood flow and resistance, thus inducing PAH. Therefore, the pathological state of PAH patients cannot be effectively improved by most treatment means, leading to its poor prognosis[6,7]. Furthermore, deeply analyzing the molecular mechanism of PAH pathogenesis and searching for new therapeutic targets are key issues in the effective treatment of PAH.

Phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/AKT) signaling pathway is involved in various biological functions throughout the whole life. The functions of the PI3K/AKT signaling pathway have been gradually studied. Scholars[8,9] have found that PI3K/AKT can mediate cell proliferation and a[illegible]sis. Moreover, PI3K/AKT exerts a sur[illegible]fect and inhibits abnormal death, which is [illegible]ly mediated by inhibiting various pro-apop[illegible] proteins such as Caspase-9[10]. The involvemen[illegible] PI3K/AKT pathway in the pathogenesis of PA[illegible] has been investigated as well[illegible] studies have indicated that the PI3K[illegible]T pa[illegible]ay may play a role in cell prolife[illegible]n and a[illegible]tosis in PAH. However, the pot[illegible]role [illegible] in the anti-apoptotic e[illegible]ct [illegible]atin is still unclear. Meanwhile[illegible] downs[illegible]elements of the PI3K/AKT si[illegible] remain to b[illegible]rmined. Currently, few [illegible]ve focused o[illegible]e regulatory effect [illegible]orva[illegible]n PI3K/AKT and its influence o[illegible] PAH.

In the [illegible]sent study, the [illegible] of PAH was succes[illegible]y established in rats *via* hypoxia feeding. [illegible] inflam[illegible]ory factors and morphological [illegible]ages [illegible]ung ti[illegible]s were detected *via* enzyme-[illegible] immu[illegible]rbent assay (ELISA) and hem[illegible]in-eo[illegible] (HE) staining, respectively. Right [illegible] systolic pressure (RVSP) and [illegible]t ventri[illegible] hypertrophy index (RVHI) were [illegible]ermined as well. The apoptosis level was de[illegible]minal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) [illegible]ing. Meanwhile, the changes in the. PI3K/A[illegible]athway in lung tissues were determined using [illegible]uantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). The effect of atorvastatin on PAH in rats was eval[illegible] thermore, the regulatory effect of a[illegible]statin the PI3K/AKT signaling pathwa[illegible]s explored. Our findings might provide an e[illegible]mental basis for subsequent researches and the [illegible]ment of new drugs.

Materials and Methods

Animal Groups and Modeling

Male Sprag[illegible]Dawley ([illegible]s w[illegible]ing 250-280 g were p[illegible]ased from the [illegible]ai Medical Laborator[illegible] Center. All [illegible]s were adaptively fe[illegible] the s[illegible] pathogen-free house for 1 week. After that, ra[illegible]e divided into Control gro[illegible]5), PAH mod[illegible]oup (Model group, n[illegible] and atorvastatin treatment group (Ator[illegible]up, n=15). The [illegible]odel of PAH was successfully established in[illegible] *via* hypoxia feeding. Rats in [illegible]trol group w[illegible]fed in a normal environment, w[illegible]hose in M[illegible]el group and Ator group were fed i[illegible]ncubator. This investigation was approved by the Laboratory Animal Ethics Com[illegible]ittee. All animal operations were performed [illegible]nce with the NIH Laboratory Animal [illegible]ide.

Establishment of the PAH model: rats were fed in a hypoxia incubator with mixed gas of 90% nitrogen and 10% oxygen for 8 h every day in a hypoxic environment. After 3 weeks, if the model has been successfully established in rats was evaluated (the duration of hypoxic treatment could be prolonged appropriately to ensure successful modeling).

Detection of Right Ventricular Pressure and Hypertrophy in Rats

Rats were fixed on an experiment table and anesthetized. Subsequently, RVSP in each group was detected and recorded. Right ventricular free wall (RV) and left ventricle + interventricular septum (LV + S) were weighed. Finally, RVHI was calculated.

Detection of Serum Cytokines in Rats

Serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and nitric oxide (NO) were detected according to the instructions of ELISA kit (R&D Systems, Minneapolis, MN, USA). Finally, the absorbance in each group was measured.

Pathological Changes in Lung Tissues of Rats

Lung tissues were first washed with running water for 24 h, dehydrated with gradient alcohol and routinely prepared into sections. After deparaffinization, the sections were hydrated with 95%, 90%, 80%, 75%, and 50% ethanol, respectively. Then, sections were stained with HE (Boster, Wuhan, China). Finally, pathological changes in lung tissues were observed under a light microscope.

TUNEL Apoptosis Assay

The sections were fixed with 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS) twice and infiltrated with 0.1% of Triton X-100. Then, apoptotic fragment DNAs were labeled with fluorescein isothiocyanate (FITC) according to the instructions of the TUNEL assay kit (Beyotime, Shanghai, China). Next, FITC-labeled TUNEL-positive cells were imaged at an excitation wavelength of 488 nm under a fluorescence microscope. Finally, the number of TUNEL-positive cells were counted in 10 fields of view.

Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-P

An appropriate number of frozen lung es were taken, added to liquid nitrogen and hon nized under low temperature at 2000 rpm for Total RNA was extracted from lung tissues usi TRIzol Reagent (Invitrogen, C A, USA). The RNA concentration was rmi sing an ultraviolet spectrophotome Hitachi vo, Japan). Subsequently, extra RNA transcribed into comp en ribose Nucleic Acid (cDNA) rding nstructions of PrimeScript™ MasterMix itrogen, Carlsbad, CA, expression l s of target genes wer tecte RT-PCR. Primers of target gene and glycera 3-phosphate dehydrogen (GAPDH) were ed according to Gen . The specific prime sequences used in th udy w shown in Table I. QRT-PCR rea cond s were follows: 94°C for 30 s, 55 , and 7 for 90 s, for a total of 40 cycles. elati xpression levels of genes in lung ti ch group we calculated by the $2^{-\Delta\Delta C}$ thod.

Table I. PCR primer sequences.

mRNA	Sequence
Caspase-3	F: 5'-CTACCGCACCCGG TAT-3' R: 5'-TTCCGGTTAACA GTGAG-3'
Bcl-2	F: 5'-GGTGCTCTTGAG GG-3' R: 5'-CCATCGAT TTCAG C-3'
PI3K	F: 5'-TGGTTCTT AAGTGA 3' R: 5'-CTGCTG GAAGTCCTG
AKT	F: 5'-TAGG CCTTC ACAGC-3 R: 5'-CGC GAGA GTGGA-3'
GAPDH	F: 5'-CAG GTCTC R: 5'- CAGTC 3'

Statistical Analysis

tistical Product and Service Solutions (SPSS) software (IBM, Armonk, NY, USA) was used all statistical ysis. The bar graph was plotusing Graph Prism 5.0. Experimental re- s re expr as mean ± standard deviation (χ±S ANOVA test was used to compare the differences among different groups, followed by st-hoc test (Least Significant Difference). $p<0.05$ ered statistically significant.

Results

General Data of Rats in Each Group

As shown in Table II, the body weight, activity, and food intake of rats were significantly reduced in Model group. Meanwhile, they were markedly improved in Ator group, but worse than those in Control group.

RVSP and RVHI in Each Group

After anesthesia, RVSP was determined *via* right cardiac catheterization. Meanwhile, the right ventricle was isolated to calculate RVHI. As shown in Figure 1, both RVSP and RVHI were significantly higher in Model group than those of Control group ($p<0.05$); however, they were significantly lower in Ator group than Model group ($p<0.05$).

Tabl General conditions of rats in each group.

	Case (n)	Manifestation
ntrol	15	Good mental state and normal food intake
	15	Decline in body weight loss, activity and food intake
A	15	Improvement in activity and food intake

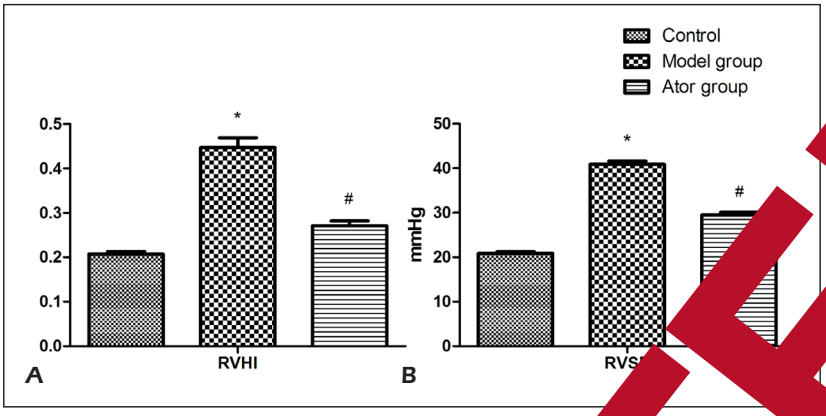

Figure 1. RVSP and RVHI of rats. Both RVSP and RVHI are remarkably highe[r] [i]n Model group than t[he] Control group. *p<0.05 *vs.* Control group

Table III. ELISA results in each group.

Group	IL-6 (mg/L)	TNF-α (fmol/m[L])	NO (mol/L)
Control	80.23±8.72	39.23±4.53	44[.]8.56
Model	190.35±15.05[a]	83.21±8.16[a]	22[.]6.57[a]
Ator	100.29±10.43[b]	46.58±6.23[b]	3[.]9.67[b]

[a]p<0.05 *vs.* Control group, [b]p<0.05 *vs.* Model group

ELISA Results in Each Group

As shown in Table III, the levels of IL-6 [a]nd TNF-α increased remarkably in Model grou[p] and Ator group (p<0.05). The[re] IL-6 and TNF-α in Ator group were [s]ignifi[c]antly lower than those of Model grou[p] (p<0.05). However, the level of serum NO [s]ignifi[c]antly in Ator group when [co]mpa[red] Model group (p<0.05).

Pathological Changes in Lung Tissues of Each Group

The pathological damage of lung tissues in each group was detected *via* HE staining. Results revealed that compared with Control group (A), pulmonary septal thickening, increased pulmonary shadow, unclear outline, and alveolar edema, fusion and hemorrhage were observed in Model group (B). In addition, lung tissue injury was significantly alleviated in Ator group (C) (Figure 2).

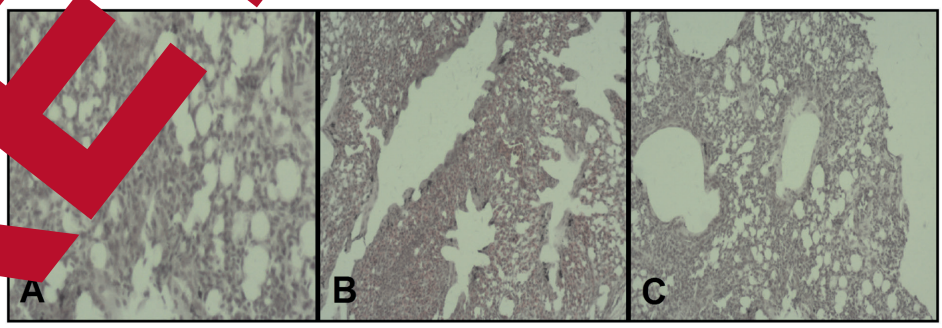

Figure 2. Pathological changes in lung tissues of each group detected via HE staining (magnification × 10). Lung tissue injury is significantly alleviated in Ator group.

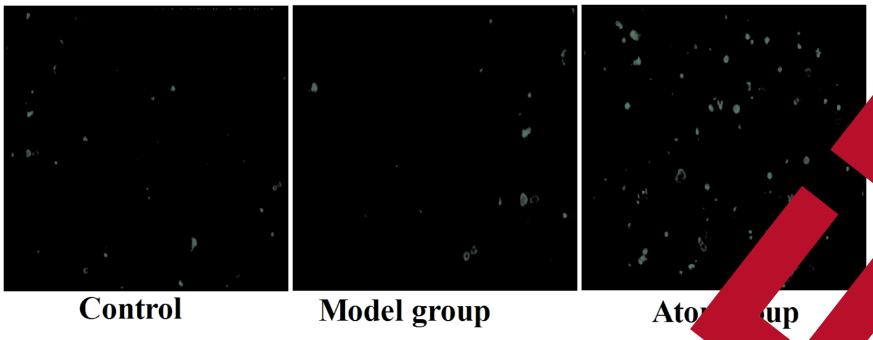

Figure 3. Apoptosis level of lung tissues in each group detected via TUNEL stainin aggi 200×). T mber of TUNEL-positive cells in lung tissues in Ator group is remarkably larger than that o el grou

Apoptosis Level of Lung Tissues in Each Group

No evident TUNEL-positive cells were observed in lung tissues of Control group and Model group. However, the number of TUNEL-positive cells in lung tissues of Ator group was remarkably larger than that of Model group ($p<0.05$) (Figure 3).

Expression Levels of Apoptosis Genes in Lung Tissues of Each Grou

The expression levels of apoptotic gen lung tissues of each group were detected *via* PCR. Results showed that the exp sion level Bcl-2 in lung tissues of Model s signifi- cantly higher than that of grou <0.05). However, the expression of C se-3 in Model group was signif lowe Ator group ($p<0.05$) (ure ove results indicated that apopt was in

Regulatory Effect orvastatin on AKT Signali athway-Related Genes

n Ator group he expression levels of PI3K AKT in lun ssues were remarkably high- an those of trol group and Model group (5) (Figu

Discussion

PA can be induced by a variety of factors, including chronic exposure to moderate hypoxia[12]. Meanwhile, PAH is a clinical syndrome characterized by pulmonary vascular occlusion lesions resulted from pulmonary vasoconstriction and structural changes in pulmonary arteries due to an increased pulmonary circulation pressure[13,14]. In most PAH patients, the apoptosis of pulmonary vascular endothelial cells decreases, while cell pro-

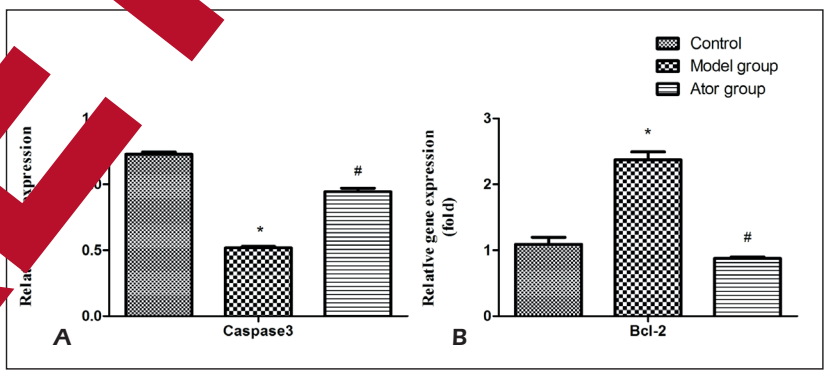

Fig Expression levels of apoptotic genes. Compared to those in Model group, the expression level of Caspase-3 in lung tissue creases significantly, while the expression level of Bcl-2 decreases significantly in Ator group. *$p<0.05$, #$p<0.05$

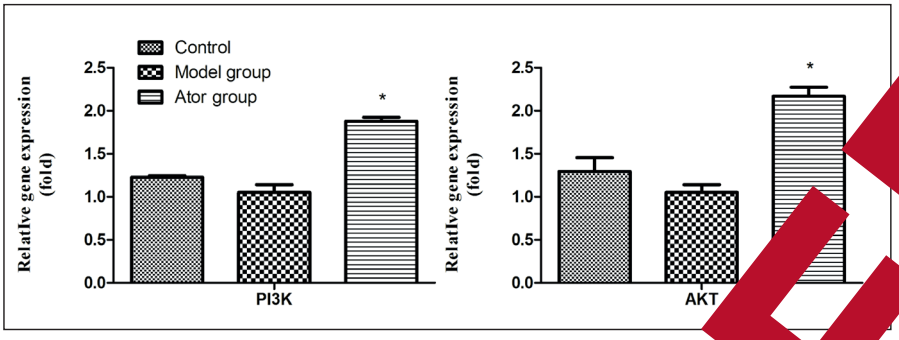

Figure 5. Expression levels of signaling pathway genes. The expression levels of PI and A ng tissu significantly up-regulated in Ator group. *$p<0.05$ *vs.* Control group and Model group.

liferation markedly increases. Meanwhile, the imbalance between apoptosis and proliferation leads to the remodeling of pulmonary arterial smooth muscle cells and pulmonary vascular occlusion. Eventually, this may result in right heart overload and even right heart failure[15,16]. Hypoxia feeding effectively simulates the pathogenesis of PAH in humans, which can be adopted to establish the rat model of PAH. Therefore, it is an important method to investigate the pathogenesis of PAH and to h for its therapeutic regimens[17]. In the prese SD rats were fed in a hypoxic environmen 21 days. Compared to normal rats, the rats in el group showed a significantly increased R and RVHI after modeling. This indicated that t model of PAH was successfull d in rats. At the same time, the HE st g of tissues showed that lung tissue inj n Mode up was severe. Sisniega et al[18] h nd th to secondary pulmona dys d lung tissue injury. Inflamm n plays dispensable role in the occur and develop of PAH as well, accom increased in matory cells[19]. In the ent the expression levels of IL-6 and TNF-α in M oup were significantly hi than those of group. However, the pressi n level of NO was significantly lowe n that ontrol group. After treatment wi vasta ne exp sion levels of IL-6 and TNF- n signi tly declined, while the expressio of N creased markedly. These sugge creased levels of IL-6 and TN and dec d level of NO might further pro the development of PAH. However, the ab was improved after treatment with vastatin, indicating that atorvastatin was effe ve for the PAH treatment. Our findings were c nt with the results of Pellicelli et al[20], Maki-P et al[21] and Gelosa et al[22]. To observe the changes ulmo terial pressure, RVSP and RVHI were further d d. Results manifested that nd RVHI we kedly declined, and l issue injury was alle iated in Ator group. above results rongly suggested that atorvas- n was involv n regulating PAH in rats and t affect the d lopment of PAH.

ty et a have found that atorvastatin can up-regulate the apoptosis level through mediating immune response and inflammatory response. In this work, the apoptosis g tissues of PAH rats was detected *via* UNE staining in this study. Results revealed that the apoptosis level in lung tissues of Model group increased significantly, which was consistent with the results in the previous studies. In addition, RT-PCR results demonstrated that the expression levels of apoptosis-related genes (Caspase-3 and Bcl-2) in lung tissues were significantly changed in Ator group. Omar et al[24] applied statins in the intervention treatment of PAH patients. They have found that statins can inhibit the proliferation of pulmonary arterial smooth muscle cells in a concentration-dependent manner, consistent with the data in this work.

AKT is a serine/threonine protein kinase, which is activated by various growth factors and cytokines in a PI3K-dependent manner[25]. The activation of the PI3K/AKT pathway exerts important influences on the differentiation, proliferation, and apoptosis of smooth muscle cells and vascular fibroblasts[26]. This is mainly mediated by the inhibition of various pro-apoptotic proteins such as Caspase-9. The involvement of the PI3K/AKT pathway in the pathogenesis of PAH has been widely studied. Therefore, the PI3K/AKT pathway may play an important role in cell proliferation and apoptosis in PAH. However, the potential role of PI3K/AKT in the anti-apoptotic effect of atorvas-

tatin is still unclear. Meanwhile, the downstream elements of PI3K/AKT signal remain to be determined[27-29]. In the current years, few authors have explored the regulatory effect of atorvastatin on PI3K/AKT and its influence on PAH. To further verify the effect of atorvastatin on the PI3K/AKT signaling pathway in PAH, the expression levels of pathway genes were detected in this study. It was found that the expression levels of PI3K and AKT in lung tissues decreased markedly in Model group, while remarkably increased in Ator group. Atorvastatin alleviated the symptoms of PAH in rats. Moreover, it significantly increased the expressions of PI3K and AKT in lung tissues. The above findings suggested that the activation of the PI3K/AKT signaling pathway might be an important reason for the decline in PAH. Although such an effect was observed in the present study, there were still some limitations. Cells should be cultured *in vitro*, and multiple cell lines could be introduced. Furthermore, more genes and proteins in the PI3K/AKT signaling pathway should be detected to further verify the effect.

Conclusions

We found that atorvastatin regulates the s toms of PAH in rats by activating the PI3K/A signaling pathway. Therefore, ato tatin can used as a therapeutic drug fo anwhile, the therapeutic effect and osis patients can be evaluated by the PI KT sig ag path-way. In subsequent rese more be introduced *in vitro* nd ble mecha-nisms of atorvastati n be f verified *via* flow cytometry a otein assay.

Conflict terests

The auth eclared that they have no conflict of interests.

Re ences

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