Effect of atorvastatin on the apoptosis of human umbilical vein endothelial cells and its drug mechanism

Yanmei Li[1], Hong Liu[2], Yanfang Wu[1] and Mingzhe Zhu[2]*
[1]Vascular surgery of Yantaishan Hospital, Yantai, China
[2]Department of Pediatrics, Yantai Affiliated Hospital of Binzhou Medical University, Yantai, China

Abstract: Recent studies have shown that statins can inhibit the apoptosis of vascular endothelial cells. Many pharmacological actions of statins have nothing to do with their cholesterol lowering effects. These effects are called non lipid lowering cardiovascular protective effects. In this study, by establishing a high glucose induced vascular endothelial cell apoptosis model, we explored the mechanism of the non lipid - related cardiovascular protective effect of atorvastatin. The results showed that atorvastatin could protect human umbilical vein endothelial cells from damage induced by new high glucose and inhibit apoptosis. High concentration atorvastatin can up regulate the expression of Bcl-2 and down regulate the expression of Bax protein ($P<0.05$). This suggests that statins may play a role in cardiovascular protection by inhibiting endothelial cell apoptosis. This result is confirmed by experiments, which can provide clues for clinical treatment, and combine drug therapy and lifestyle intervention to reduce blood sugar.

Keywords: Apoptosis, atorvastatin, high glucose induction, protein expression, glucose concentration.

INTRODUCTION

Diabetic angiopathy is a major cause of disability or death in diabetic patients. Dysfunction of vascular endothelial cells is closely related to diabetic vascular complications (Balmadrid *et al*., 2015). Numerous studies have shown that high glucose environment can induce apoptosis of endothelial cells, thereby promoting the development of atherosclerosis (Cahill *et al*., 2015). Statins are the most effective drugs for the treatment of hypercholesterolemia. Long-term use of statins can significantly reduce mortality and cardiovascular events in patients with hyperlipidemia and coronary heart disease, such as the incidence of myocardial infarction, sudden death, and unstable angina (Dai *et al*., 2010). Many pharmacological actions of statins have nothing to do with their cholesterol lowering effects (Hou *et al*., 2015). These effects are called non lipid lowering cardiovascular protective effects (Dindo *et al*., 2004). Recent studies have shown that statins can inhibit the apoptosis of vascular endothelial cells (Ghoneum *et al*., 2015). This provides a new research direction for statins in non lipid lowering related cardiovascular protection (Gunaldi *et al*., 2015). To this end, this study is based on the establishment of a high glucose induced apoptosis model of vascular endothelial cells, and the mechanism of the non lipid - related cardiovascular protective effect of atorvastatin.

Apoptosis refers to the autonomous and orderly death of cells controlled by genes in order to maintain stable internal environment (Jean *et al*., 2017). Apoptosis is different from cell necrosis. Apoptosis is not a passive process, but an active process (Inzucchi *et al*., 2015). It involves the activation, expression and regulation of a series of genes. It is not a phenomenon of autologous damage under pathological conditions, but a kind of death for better adaptation to the survival environment (Chen *et al*., 2009). Apoptosis is a natural mechanism regulating organisms within the environment stable indispensable (Larsen *et al*., 2013; Kargulewicz *et al*., 2016). Once the mechanism is regulated, it will lead to disease if it is out of control. Recent studies have shown that statins can inhibit the apoptosis of vascular endothelial cells (Chen *et al*., 2015). This provides a new research direction for statins in non lipid lowering related cardiovascular protection. To this end, the experiment is to establish the basis of high glucose induced vascular endothelial cell apoptosis model and to explore the mechanism of atorvastatin, a newly discovered non lipid-lowering cardiovascular protective effect.

MATERIALS AND METHODS

Flow cytometry (from American BD company), polyacrylamide gel electrophoresis vertical electrophoresis slot and transfer electrophoresis slot; Rabbit anti human Bcl-2 monoclonal antibody and Rabbit anti human Bax monoclonal antibody, Rabbit anti human beta -actin monoclonal antibody; DMEM cell culture medium; atorvastatin. This experiment was approved by ethics committee of Yantaishan Hospital, ethical approval number as 2015YTSHMD2.

Human umbilical vein endothelial cell line (HUVEC) is provided by our embryo teaching and research section. After adjusting the cell density to 1* 108 /L, the DMEM cell culture fluid containing 10% fetal bovine serum was inoculated in the culture bottle and incubating in 37°C 5%

**Corresponding author:* e-mail: 13156919099@126.com

CO_2 incubator. After the cell grew to the confluence, it was passed through the passage.

The experiment was divided into 5 groups as: control group (10% fetal bovine serum +glucose DMEM 5.6 mmol/L); medium sugar group (10% fetal bovine serum + glucose concentration of 17.6 mmol/L DMEM culture medium); high glucose group (10% fetal bovine serum + glucose concentration of 33.3 mmol/L DMEM culture medium); low concentration atorvastatin group (10% fetal bovine serum +33.3 mmol/L glucose and 0.2 mol/L atorvastatin DMEM medium); medium concentration atorvastatin group (10% fetal bovine serum +33.3 mmol/L glucose and 2 mol/L atorvastatin DMEM medium); and high concentration of atorvastatin group (10% fetal bovine serum +33.3 mmol/L glucose and 20 mol/L atorvastatin DMEM medium).

Observation of cell apoptosis by brominated pyrididine fluorescence staining

The disinfected cover glass was placed in the 6 Hole culture plate, and the cells were grouped into 1 x 108 /L cells with different concentration of glucose and atorvastatin DMEM culture. After 24 h culture in 37°C and 5% CO2 culture box, the cover glass was removed, and PBS liquid bleaching cells were 3 times, 3 min each time. After staining with acridine orange and bromopyridine, they were observed by fluorescence microscope.

Determination of the proliferation rate of human umbilical vein endothelial cells

$5*10^8$ /L cell suspension was made from the logarithmic growth period cells, inoculated in 96 hole culture plate, and inoculated into 96 hole culture plates with 100 micron holes per hole. Each group had three compound holes, zero holes and 100 μL culture liquid, and continued to cultivate as 12 h, 24 h, 48 h, before the end 4h was sucked to the supernatant, plus 0.5g/ L four methyl azazolazolium Blue 100 mu L. Remove all the supernatant, then add 200 μL two methyl submaple to each pore, shake it well, make the crystal fully dissolved, and measure the absorbance of each hole by enzyme-linked immunosorbent assay. The above experiments were repeated three times.

Determination of early apoptosis rate by flow cytometry

According to cell group, adjust the cell concentration by $5*10^5$ / inoculated in 6-well plates, each with three. After 24h culture, the cells were collected and PBS solution was rinsed for 3 times. The cells were suspended in a pre cooled 500μL buffer solution, 5μL AnnexinV-FITC and 5 μLPI were added to the cell. The number of early apoptotic cells was detected by flow cytometry in 1h, at room temperature, and the results were analyzed with CELL Quest software. The above experiments were repeated three times.

STATISTICAL ANALYSIS

The experimental data were all expressed by mean ± standard deviation (x±s). Using SPSS17.0 software processing and analysis, the comparison of the average number of multiple groups of data using the single factor analysis of variance (One-Way ANOVA) method, 22 data comparison between the LSD-t method analysis.

RESULTS

Morphological observation of apoptotic cells

Under fluorescence microscope, most of the chromatin in the control group was green and normal, and the color was lighter. The sugar group showed more nuclear chromatin bright green and showed early apoptotic cells pyknosis or bead like. In the high glucose group, there were lots of early apoptotic cells and late apoptotic cells with orange color. The apoptotic cells of the Atorvastatin group with different concentrations were significantly reduced. The late apoptotic cells in the low concentration atorvastatin group and the medium concentration atorvastatin group were significantly lower than those in the high glucose group, and only a few apoptotic cells were found in the high concentration atorvastatin group. As shown in fig. 1, A as control group, B as middle sugar group, C as high sugar group, D as low concentration atorvastatin group, E as medium concentration atorvastatin group, F as high concentration atorvastatin group.

Effect of atorvastatin on the proliferation of human umbilical vein endothelial cells induced by high glucose

When HUVEC was cultured for 12 h under different concentrations of glucose, there was no significant difference in the proliferation rate of the three groups, and there was no statistical difference between the medium sugar group and the control group after 24 h, while the proliferation rate of the high sugar group was lower than that of the control group ($P<0.05$). After 48 h culture, the middle glucose group was lower than that of the control group ($P<0.05$), while the cell proliferation rate of the high glucose group was significantly lower than that of the control group ($P<0.01$). Different concentrations of atorvastatin can significantly increase cell proliferation. The proliferation rate of HUVEC in the medium concentration atorvastatin group increased significantly after 48 h culture ($P<0.05$). The proliferation rate of HUVEC in 12h, 24h and 48h was significantly higher than that in the high glucose group and the low concentration atorvastatin group ($P<0.05$; table 1) after the treatment of atorvastatin with 20 mol/L.

Determination of apoptosis rate by flow cytometry

After 24 h treatment with different concentrations of glucose, the early apoptosis rate of endothelial cells increased with the increase of glucose concentration. The

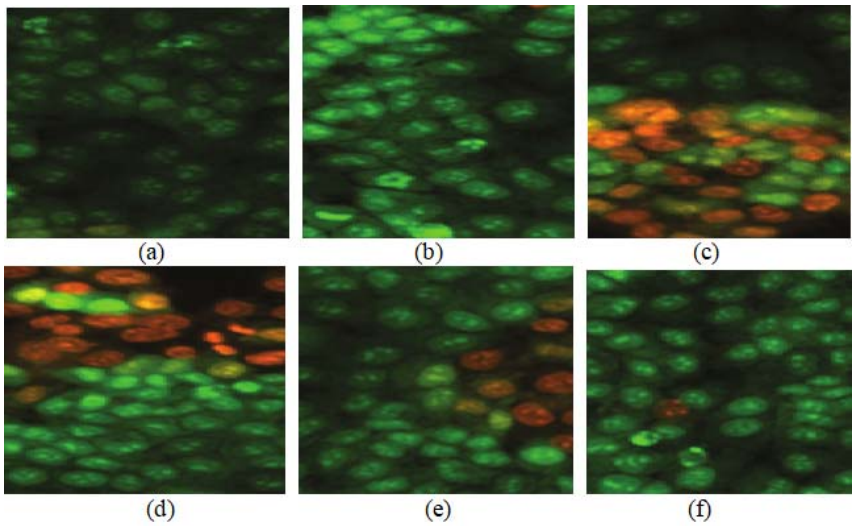

Fig. 1: Acridine orange / brominated fluorescence staining: A as control group, B as middle sugar group, C as high sugar group, D as low concentration atorvastatin group, E as medium concentration atorvastatin group, F as high concentration atorvastatin group.

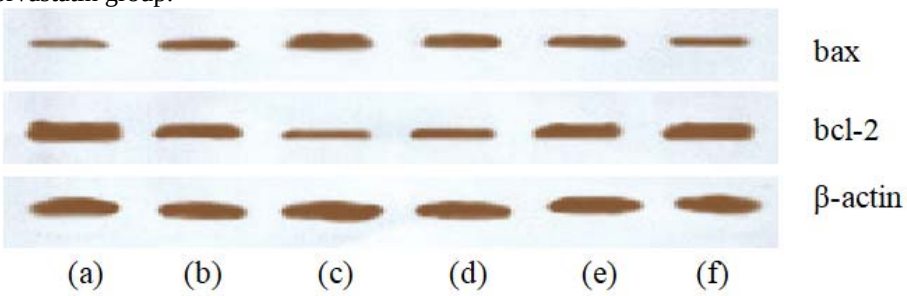

Fig. 2: Effect of atorvastatin and high glucose on the expression of Bcl-2 and Bax protein in endothelial cells.

apoptotic rate in the glucose group and the high glucose group were statistically different from those in the control group (P<0.01). Atorvastatin could significantly inhibit the apoptosis of endothelial cells induced by high glucose. The difference between the concentration of atorvastatin group and the high glucose group was statistically significant (P<0.05). The early apoptosis rate of the high concentration atorvastatin group was significantly lower than that in the high glucose group (P<0.01) (table 2).

Effect of atorvastatin on the expression of Bcl-2 /Bax protein in endothelial cells induced by high glucose

After the intervention of the endothelial cells with different concentrations of glucose, the expression of Bcl-2 protein decreased with the increase of glucose concentration, and there was a significant difference between the high glucose group and the control group (P<0.05). The expression of Bax protein increased with the increase of glucose concentration, and the difference between high glucose group and control group was statistically significant (P<0.05). Compared with the high glucose group, the high concentration of atorvastatin (20 mu mol/L) increased the expression of Bcl-2 protein (P<0.05) and decreased the expression of Bax protein (P<0.05) (table 3 and fig. 2).

DISCUSSION

Atherosclerosis is characterized by thickening of the wall of the arteries, reducing the elasticity of the vessels and reducing the lumen. The vascular involvement usually starts from the intima, and then there are a variety of complications (Liu *et al*., 2013). Then the middle layer of the artery is degenerated, and the arteriosclerosis thrombosis is seen in the lesion (Zhu *et al*., 2015). Now more scholars believe that chronic vascular inflammation is the process of generation and progression of AS disease (Muraki *et al.*, 1985; Lu, 2014). Oxidative stress is an important mechanism of its injury. At the same time, the typical manifestation of DM patients is increased blood glucose. The increased glucose environment can enhance oxidative stress and further aggravate endothelial cell injury. The effect of AS on coronary artery system can cause coronary atherosclerotic heart disease (Okuyama *et al.,* 2015). If the patient has high blood sugar and poor control, it will cause more vascular disease, increase the patient's difficulty and social medical burden. Therefore, it is of profound and important significance to understand the mechanism of endothelial cell injury, the mechanism of drug action, the target of the action and the promoting effect on the formation of atherosclerosis, and it is of

Table 1: Effect of atorvastatin on the proliferation of human umbilical vein endothelial cells induced by high glucose

Group	12h	24h	48h
Control Group	0.75±0.13	1.05±0.18	1.42±0.24
Middle Sugar Group	0.83±0.07	0.94±0.12	1.28±0.25
High Glucose Group	0.91±0.08	1.09±0.17	1.15±0.22
Low Concentration Atorvastatin Group	0.85±0.12	0.95±0.20	1.08±0.18
Medium Concentration Atorvastatin Group	0.92±0.13	1.06±0.18	1.20±0.15
High Concentration Atorvastatin Group	0.87±0.17	0.96±0.12	1.13±0.17

Table 2: Changes in the early apoptosis rate of endothelial cells after 24 h

Group	Apoptosis rate
Control Group	3.45±0.27
Middle Sugar Group	6.72±0.38
High Glucose Group	8.41±0.55
Low Concentration Atorvastatin Group	7.28±0.34
Medium Concentration Atorvastatin Group	6.52±0.27
High Concentration Atorvastatin Group	6.23±0.32

Table 3: Effect of atorvastatin on the expression of Bcl-2/Bax protein in endothelial cells induced by high glucose

Group	Bax/β -actin	Bcl-2 /β -actin
Control Group	0.423±0.071	0.946±0.132
Middle Sugar Group	0.485±0.056	0.917±0.125
High Glucose Group	0.531±0.051	0.724±0.105
Low Concentration Atorvastatin Group	0.608±0.064	0.851±0.062
Medium Concentration Atorvastatin Group	0.552±0.053	0.872±0.073
High Concentration Atorvastatin Group	0.519±0.046	0.961±0.095

great significance to the prevention and treatment of this kind of disease (Rosenthal *et al*., 2015). In this study, the flow cytometry was used to detect the human umbilical vein endothelial cells with different concentrations of glucose after 24 h. The early apoptosis rate of the cells in the control group, the middle sugar group and the high sugar group were 3.45±0.27%, 6.72±0.38% and 8.41± 0.55% respectively, and the difference between the two groups was statistically significant ($p<0.01$). Through the detection of apoptosis related protein Bcl-2 and Bax, it was found that the expression of Bcl-2 protein gradually weakened and the expression of Bax protein gradually increased with the increase of glucose concentration, and the expression of Bcl-2 protein and Bax protein in the high glucose group had a significant difference compared with the control group ($P<0.05$) after the glucose concentration increased (Souich *et al.,* 2013). These studies suggest that atherosclerosis may be associated with increased apoptosis of vascular endothelial cells in diabetic patients.

Statins are the most effective drugs for the treatment of hypercholesterolemia, but a large number of studies have shown that many pharmacological actions of statins have nothing to do with the cholesterol lowering effect (Szewczyk *et al*., 2015). These effects are also called non lipid lowering related cardiovascular protective effects, mainly including anti oxidation and improvement of vascular endothelial cell function. And anti-inflammatory effect, inhibiting neurohormonal system activity, improves the compliance of arteries (Shim *et al.,* 2010). The present study showed that atorvastatin could inhibit high glucose induced endothelial cell apoptosis ($P<0.05$), and 20 u mol/L atorvastatin could up regulate the expression of Bcl-2 protein and down regulate the expression of Bax protein. This suggests that statins may play a role in non lipid-lowering cardiovascular protection by inhibiting endothelial cell apoptosis (Yoshio *et al.*, 2013). The mechanism may be associated with statins that inhibit the induction of high glucose on the expression of Bcl-2 and Bax proteins.

The increase of vascular endothelial cell apoptosis will lead to the increase of vascular endothelial permeability and the imbalance of vascular regulation (Schneider *et al.*, 2011). The destruction of vascular endothelial integrity will undoubtedly contribute to the migration and deposition of lipid to the intima of blood vessels, and then the migration of monocytes and smooth muscle cells to the intima to phagocytic lipid, thus promoting the development of atherosclerosis (Tang *et al.,* 2014). Hyperglycemia may induce the apoptosis of vascular

endothelial cells by down regulation of the expression of Bcl-2 protein and up regulation of Bax protein expression, which provides a new way of thinking for the prevention and treatment of vascular complications in diabetes (Xuan, 2015). Atorvastatin may regulate the expression of Bcl-2 /Bax protein and inhibit the apoptosis of endothelial cells induced by high glucose, thereby playing a role in cardiovascular protection of non lipid lowering. This provides a new theoretical basis for clinical application of statins in the treatment of diabetic patients. However, in vitro experiments can not fully simulate the internal environment of patients with type 2 diabetes, hormone level, blood lipid level and individual factors will affect the inhibitory effect of statins on the apoptosis of endothelial cells induced by hyperglycemia. Therefore, further deep study on endothelial cell apoptosis will be of great clinical significance.

CONCLUSION

In conclusion, the experimental study showed that high glucose had obvious damage to endothelial cells and high glucose was more effective than persistent high glucose, and atorvastatin had a significant protective effect on the injury of human umbilical vein endothelial cells induced by new high glucose and inhibited apoptosis. Poor control of blood sugar, excessive frequent fluctuations in the corresponding target organs cause more serious damage, at the same time there will be more dangerous chronic complications, resulting in increased morbidity of diabetes fatal complications, the worse the prognosis. It is proved that this result can provide a clue for clinical treatment, and the combination of drug therapy and lifestyle intervention to reduce blood sugar and reduce sugar as far as possible, do not simply pursue the purpose of hypoglycemic and cause excessive blood glucose fluctuations.

REFERENCES

Balmadrid B and Hwang JH (2015). Endoscopic resection of gastric and esophageal cancer. *Gastroenterol. Rep.* (Oxf.), **3**(4): 330-338.

Chen X, Zhou T and Chen M (2015). Meta analysis of the association of cholesterol with pancreatic carcinoma risk. *J. Buon.*, **20**(1): 109-113.

Cahill G, Tran L, Huang YC and Ou CW (2015). Thoughts on the development of modern pharmaceutical logistics. *Busin. Rese.*, **1**(1): 27-38.

Chen J, Liu S, Pan J, Zheng X, Zhu K, Zhu J, Xiao J and Ying M (2009). Simulation model and optimization of medical delivery system. *Comp. Eng.*, **36**(3): 80-486.

Dai Y, Luk C, Siu H, Yiu H, Chan S and Lee S (2010). Mitochondrial dysfunction induced by statin contributes to endothelial dysfunction in patients with coronary artery disease. *Cardio. Toxicol.*, **10**(2): 130-138.

Dindo D, Demartines N and Clavien PA (2004). Marketing strategy and mode selection of pharmaceutical enterprises. *Disc. Mod. Eco.*, **240**(2): 205-213.

Ghoneum M, Felo N, Nwaogu OM, Fayanju IY, Jeffe JA and Margenthaler DB (2015). Logistics help pharmaceutical distribution competition. *Log. Tech. App.*, **1**(2): 73-82.

Gunaldi M, Kocoglu H, Okuturlar Y, Gedikbasi A, Karabulut M, Alis H and Hursitoglu M (2015). Heat shock protein 70 is a useful marker for predicting colorectal cancer. *J. Buon.*, **20**(6): 1464-1470.

Hou Y, Deng J and Zhang L (2015). Lower expression of PH domain leucine-rich repeat protein phosphatase 1 (PHLPP1) association with poor prognosis of gastric cancer. *Int. J. Clin. Exp. Med.*, **8**(11): 20481-20489.

Inzucchi S, Zinman B, Wanner B and Ferrari R (2015). SGLT-2 inhibitors and cardiovascular risk: Proposed pathways and review of ongoing outcome trials. *Diab. Vasc. Dis. Res*., **12**: 90-100.

Jean Y, John X, Stephen E and Claude G (2017). Canagliflozin in Conjunction With Sulfonylurea Maintains Glycemic Control and Weight Loss Over 52 Weeks: A Randomized, Controlled Trial in Patients With Type 2 Diabetes Mellitus. *Clini. Therap.*, **39**(11): 2230-2242.

Kargulewicz A, Szulinska M, Kujawska-Luczak E, Swora CK and Musialik M (2016). Improvement of serum adiponectin and leptin concentrations: Effects of a low-calorie or isocaloric diet combined with metformin or orlistat- a prospective randomized open-label trial. *Eur. Rev. Med. Pharmacol. Sci.*, **20**: 3868-3876.

Larsen S, Stride N, Hey-Mogensen M, Hansen C, Bang L, Bundgaard H and Nielsen L (2013). Simvastatin effects on skeletal muscle: Relation to decreased mitochondrial function and glucose intolerance. *J. Am. Coll. Cardiol.*, **61**(1): 44-53.

Liu G and Guo S (2013). Practice and experience of hospital drug supply chain optimization. *Chin. drug appl. Moni.*, **4**: 15-16.

Lu G (2014). Transformation of pharmaceutical logistics model. *J. phar. Prac.*, **3**: 79-84.

Muraki A, Miyashita K, Mitsuishi M, Tamaki M and Tanaka K (1985). Coenzyme Q10 reverses mitochondrial dysfunction in atorvastatin-treated mice and increases exercise endurance. *J. Appl. Physiol.* **113**(3): 479-486.

Okuyama H, Langsjoen T, Hamazaki Y, Ogushi R, Hama T and Kobayashi H (2015). Statins stimulate atherosclerosis and heart failure: Pharmacological mechanisms. *Expert. Rev. Clin. Pharmacol.*, **8**(2): 189-199.

Rosenthal N, Meininger K, Ways D and Polidori M (2015). Canagliflozin: A sodium glucose co-transporter 2 inhibitor for the treatment of type 2 diabetes mellitus. *Ann. N. Y. Acad. Sci.*, **1358**: 28-43.

Szewczyk A, Jarmuszkiewicz W, Koziel A, Sobieraj I,

Nobik W, Lukasiak A, Skup A and Bednarczyk P (2015). Mitochondrial mechanisms of endothelial dysfunction. *Pharmacol. Rep*., **67**(4): 704-710.

Souich P, Roederer G and Dufour R (2013). Myotoxicity of statins: mechanism of action. *Pharmacol. Ther.*, **175**: 1-16.

Shim YM, Kim HK and Kim K (2010). Chinese medicine circulation E-commerce. *Beijing: Peking University Medical Press*, **5**(5): 707-712.

Schneider M, Schmidt B, John S and Schmieder R (2011). Effects of statin treatment on endothelial function, oxidative stress and inflammation in patients with arterial hypertension and normal cholesterol levels. *J. Hypertens.*, **29**(9): 1757-1764.

Tang X, Luo Y, Chen H and Liu D (2014). Mitochondria, endothelial cell function and vascular diseases. *Front. Physiol*., 5:165-175.

Xuan Z (2015). Study of optimal control of drug stock in hospital. *Chi. Pha*., **1**: 59-66.

Yoshio T, Nishida T and Kawai N (2013). Gastric ESD under heparin replacement at high risk patients of thromboembolism is technically feasible but has a high risk of delayed bleeding: Osaka University ESD Study Group. *Gastroenterol. Res. Pract.*, **4**: 365-3700.

Zhu Y and Chen W (2015). Chinese herbal medicine logistics company access standards. *Med. Wor.*, **20**(6): 1585-1591.