Abstract. – OBJECTIVE: To study the protective effect of tacrolimus on vascular endothelium injured by oxidized low-density lipoprotein (ox-LDL) and its mechanism.

MATERIALS AND METHODS: Human umbilical vein endothelial cells were used as objects of study, and divided into control group, tacrolimus group and autophagy inhibition group. Control group received no ox-LDL, while tacrolimus group and autophagy inhibition group were treated with ox-LDL (100 μg/mL) for 3 h. Tacrolimus group was pre-treated with tacrolimus (100 nM) for 0.5 h, and the autophagy inhibition group was pre-treated with 3-methyladenine (3-MA) (10 mM) and tacrolimus (100 nM) for 0.5 h. The cell viability was detected via cell counting kit 8 (CCK8) assay, the cell apoptosis ratio was detected via flow cytometry and Hoechst staining, and the releases of superoxide dismutase (SOD), reactive oxygen species (ROS) and other cytokines were detected using the kit. Moreover, the autophagy level was detected via LC3 fluorescence staining, and the autophagy- and apoptosis-related molecules were detected via polymerase chain reaction (PCR) and Western blotting.

RESULTS: In the absence of ox-LDL, neither tacrolimus nor 3-MA had an effect on the cell viability. After the addition of ox-LDL, the cell viability was significantly decreased, whereas tacrolimus could alleviate such damage to cells. Flow cytometry and Hoechst staining proved that tacrolimus could reduce the proportion of apoptotic cells induced by ox-LDL, while PCR and Western blotting confirmed the decreased expression of apoptosis-related proteins in tacrolimus group. 3-MA could up-regulate the ratio of apoptosis and the expressions of apoptosis-related proteins. The detection of SOD and ROS showed that ox-LDL could induce the cell oxidative stress injury, whereas tacrolimus could inhibit such an effect. The addition of 3-MA inhibited the effect of tacrolimus. Besides, LC3 fluorescence staining, PCR and Western blotting revealed that ox-LDL could induce the autophagy, while tacrolimus could enhance the autophagy. After the addition of 3-MA, the intracellular autophagy level was significantly inhibited.

CONCLUSIONS: Tacrolimus protects vascular endothelial cells from ox-LDL damage through inducing the autophagy.

Key Words: Atherosclerosis, Ox-LDL, Vascular endothelial cells, Autophagy, FK506.

Introduction

The occurrence and development of atherosclerosis (AS) is a complex pathophysiological process involving many factors. Vascular endothelial cells cover the inner surface of vascular lumen playing important roles in maintaining the integrity of vascular structure and function. The injury and functional changes in vascular endothelial cells are initial links in the occurrence and development of AS, and they also play important roles in the progression of AS plaque. Vascular endothelial cells can induce endothelial cells to secrete a variety of cytokines, promote the local vascular inflammatory response, induce the proliferation of vascular smooth muscle cells, and aggravate the vascular injury. In addition, its toxic effect can lead to the proliferation of endothelial cells, promote the lipid infiltration, and accelerate the unstable progression of AS.

Dyslipidemia is one of the most common risk factors for AS, among which low-density lipoprotein (LDL) metabolic disorder is particularly important. A study has shown that defects often occur in LDL receptor genes of some patients with familial monogenic genetic disease, hypercholesterolemia, indicating that the single LDL meta-
bolic disorder is enough to cause AS. The pro-AS effect of LDL is closely related to its subendothelial-oxidized modification. After LDL enters arterial subendothelial tissues via the damaged endothelium, a series of chain reactions of oxygen free radicals will occur, forming oxidized-LDL (ox-LDL). Ox-LDL can directly damage the endothelial cells through its cytotoxic effect. In addition, ox-LDL can resist the degradation caused by lysosomal enzymes and cathepsin, and transform a large number of lipids accumulating in cells into foam cells. This feature is of great significance in promoting AS.

Tacrolimus (FK506) was originally developed and applied as a potent immunosuppressive agent in clinical practice. Researches have found that it can induce the autophagy and play a role in protecting cells. In investigations on tacrolimus in protecting endothelial cells, more attention has been paid to its effect of reducing inflammatory response in cells. However, how it down-regulates the inflammatory response is not studied deeply enough. Autophagy is a major cytoprotective pathway for eukaryotes to degrade and recycle cytoplasmic contents. Some researches have suggested that autophagy can protect cells via degradation of lipids and inflammatory factors in the occurrence and development of AS. This work, through the intervention in ox-LDL-induced endothelial cells, aimed to explicitly clarify that tacrolimus controls the endothelial cell inflammation mainly through inducing the autophagy.

**Materials and Methods**

**Experimental Materials**

Human umbilical vein endothelial cells (HUVECs) were provided by Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Tacrolimus (FK506) injection (1 mL each, containing 5 mg FK506) was purchased from Prograf, Fujisawa Ireland Ltd (Shenyang, China). 1640 medium was purchased from Gibco (Rockville, MD, USA), and LDH assay kit, superoxide dismutase (SOD) assay kit and reactive oxygen species (ROS) assay kit were purchased from Jiangsu Beyotime Biotechnology Institute (Nanjing, China).

**Cell Culture and Treatment**

HUVECs were cultured in 1640 medium containing 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO2. When cells were almost fused and covered more than 90% bottle wall, the experiment was performed.

The non-treatment group and ox-LDL group were further divided into blank group, tacrolimus group and autophagy inhibition group. Ox-LDL group was treated with 100 μM ox-LDL for 3 h, tacrolimus group was pre-treated with 100 nM tacrolimus for 0.5 h, and autophagy inhibition group was pre-treated with 10 nM 3-MA and 100 nM tacrolimus for 0.5 h.

**Cell Counting Kit 8 (CCK8)**

The cell viability was detected, and cells in the logarithmic growth phase were used for CCK8 assay (KeyGen BioTech, Nanjing, China). Then, cells were digested with 0.25% trypsin ethylene diamine tetraacetic acid (EDTA) and centrifuged, and the supernatant was discarded. After that, cells were resuspended in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), followed by counting and dilution into 1.0×10^5 cells/mL. Cells are inoculated into a 96-well plate. Cell viability assay: the cell suspension was inoculated into a 96-well plate (100 μL per well). After inoculation, cells were incubated at 37°C with 5% CO2 for 4 h until they were adherent to the wall, followed by intervention according to experimental grouping (4 wells per group). 10 μL CCK8 were added into each well of a 96-well plate and incubated with 5% CO2 for 4 h at 37°C. The optical density was measured at 450 nm using a microplate reader.

**Table I. CCK8 cell viability assay.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Blank</th>
<th>ox-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>FK506</td>
</tr>
<tr>
<td>Absorbance (by blank)</td>
<td>1.03±0.05</td>
<td>1.3±1.0</td>
</tr>
</tbody>
</table>

*p<0.05 vs. blank, **p<0.05 vs. ox-LDL, *p<0.05 vs. FK506 + ox-LDL.
Tacrolimus alleviates Ox-LDL damage through inducing vascular endothelial autophagy

Flow Cytometry
After grouping, cells were centrifuged at 1,000 rpm for 4 min at 4°C and collected, and the medium was discarded. Next, centrifuged cells were washed twice with cold phosphate-buffered saline (PBS), and suspended with 200 μL binding buffer at a concentration of approximately 1×10^6/mL. 10 μL Annexin V-fluorescein isothiocyanate (FITC) were added into the cell suspension and mixed gently, followed by incubation at room temperature for 15 min in a dark place. 5 μL propidium iodide (PI) were added and mixed gently, followed by detection via flow cytometry within 1 h. Results were acquired and analyzed using Cell Quest™ software (BD Biosciences, San Jose, CA, USA). The experiment was repeated for 3 times for each group.

SOD Detection
After grouping, the culture supernatant was collected. According to the instructions of SOD kit, the reagent was added and shaken, followed by water bath under constant temperature of 37°C. After that, 2 mL color developing agent were added, mixed evenly and placed for 10 min, followed by zero setting by distilled water, and detection of the optical density at 550 nm. SOD activity (U/mL) = (optical density of control tube – optical density of measured tube)/50% × dilution ratio of reaction system × dilution ratio before sample test.

ROS Detection
After grouping, the original medium was discarded. Cells were washed with pre-cooled D-Hanks solution at 4°C for 1-2 times, and diluted with serum-free 1640 medium at a ratio of 1:1000. The fluorescence probe DCFH-DA was added into cells, and the positive control group was added with Rosup. After incubation in an incubator for 30 min, cells were rinsed for 1-2 times, collected and resuspended using 200-μL serum-free medium. The fluorescence intensity of cells in each group was detected via flow cytometry. The average fluorescence intensity was analyzed using Cell Quest™ software (BD Biosciences, San Jose, CA, USA).

Autophagy Detection Using Autophagy Detection Kit Cyto-ID
Cyto-ID was used to detect the intracellular autophagy-associated LC3 protein (ENZO). Cells grew on the coverslip and when the density was 50-70%, the intervention was performed based on the experimental design. The negative control was set. The supernatant was removed, and cells were washed twice with 1 × assay buffer. After treatment with cationic liposomes for 24 h, the cells were stained by Cyto-ID Green reagent (dissolved in 5% FBS medium) for 30 min at 37°C. The reagent is a selective dye, which can stain autophagy bodies specifically. The autophagy flow is assessed by the autophagy corpuscle treated by different doses of cationic liposomes through the Cyto-ID mean fluorescence intensity reading value. 100 μL microscopy solution were used to cover the monolayer cells, followed by incubation in a dark place for 30 min at 37°C. 100 μL 1 × assay buffer were used to wash cells to remove excess buffer. The plate was sealed using the anti-quencher containing 4’,6-diamidino-2-phenylindole (DAPI) dye, and the cover glass was observed microscopically (FITC and DAPI, observed 60×).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)
After treatment, cells were collected and TRIzol was used to extract the total RNA. The concentration of each sample was measured, based on which the reverse transcription system was added for reverse transcription reaction. The first 40 cycles were used to synthesize complementary DNA (cDNA), and the conditions of reverse transcription reaction were set for PCR amplification. Fluorescence signals were collected in Real-time after each cycle, and amplification and solubility curves were recorded. The primers were designed by primer 3 software (http://primer3.ut.ee/). Bax forward 5’-TGCTTGAGTCTGGGAGTTCA-3’

<table>
<thead>
<tr>
<th>Table II. SOD and ROS levels.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>SOD (by blank)</td>
</tr>
<tr>
<td>ROS (by blank)</td>
</tr>
</tbody>
</table>

*p<0.05 vs. blank, **p<0.05 vs. ox-LDL, #p<0.05 vs. FK506 + ox-LDL.
reverse 5’-CTCACTGTGTTGCCCCAGG-3’; 
Bel-2 forward 5’-GGGTTCAAAATGGGTCGTA-3’ reverse 5’-TGCTTCTGTGTCGCTTGTA-3’.

**Western Blotting**

Cells in each group were collected. The cell protein was extracted using the protein extraction kit, and the protein concentration was determined using bicinchoninic acid (BCA) method. After protein quantification, 5 μL 5 × loading buffer solution were added and boiled at 99°C for 5 min, followed by separation via sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The concentration of spacer gel was 5%, while that of separation gel in the lower layer was 10%. The constant voltage separation was performed under 90 V for about 30 min for the upper-layer gel, and 150 V for about 50 min for the separation gel, until the bromophenol blue reached the bottom of gel. The gel, filter paper and polyvinylidene difluoride (PVDF) membrane were placed into the electrophoresis tank for membrane transfer. After that, the membrane was removed and labeled, then sealed in 5% skim milk powder for 1 h and incubated in a shaker at 37°C. After the membrane was washed with phosphate-buffered saline (PBS) for 3 times, the blocking solution was washed away and the primary antibody (diluted at 1:850, Invitrogen, Carlsbad, CA, USA) was added onto the membrane in a dark place at 4°C overnight. On the next day, the primary antibody was recycled, and the membrane was rinsed with Tris-buffered saline (TBST) for 3 times (5 min/time). Then, the secondary antibody horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG, diluted at 1:5000 (Invitrogen, Carlsbad, CA, USA) was added and incubated in a shaker at 37°C for 2 h. The secondary antibody was discarded, and the membrane was rinsed with TBS-T for 3 times (15 min/time). Finally, the color was developed in a developer, and the image grey scale was scanned using Image J (Rawak Software, Inc., Hamburg, Germany).

**Statistical Analysis**

Statistical Product and Service Solutions 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Measurement data were presented as mean ± standard deviation ( x±s); one-way analysis of variance was used for the comparisons among groups, and t-test was used for the pairwise comparisons. p<0.05 suggested that the difference was statistically significant.

---

**Results**

**Tacrolimus Protected the Vitality of Vascular Endothelial Cells**

The cell viability was detected by CCK8 assay in blank group, tacrolimus group, autophagy inhibition group, ox-LDL group, ox-LDL + tacrolimus group and ox-LDL + autophagy group. Results showed that tacrolimus and 3-MA had no significant cytotoxic effects on cells. After the addition of ox-LDL, the vitality of vascular endothelial cells in each group was significantly reduced, and there were statistically significant differences among groups. Compared with ox-LDL group, the addition of tacrolimus could protect the vascular endothelial viability (p<0.05). Compared with ox-LDL + tacrolimus group, the addition of 3-MA suppressed the protective effect of tacrolimus, and the viability of vascular endothelial cells was decreased (p<0.05) (Table I). Results proved that tacrolimus has a protective effect on vascular endothelial cytotoxicity induced by ox-LDL, while inhibiting the cell autophagy can inhibit such a protective effect.

**Tacrolimus Reduced The Number of Apoptotic Vascular Endothelial Cells**

The number of apoptotic cells was detected via flow cytometry and Hoechst staining. Results showed that compared with blank group, ox-LDL significantly increased the proportion of early apoptotic cells (33.2±5.3% vs. 1.7±1.2%, p<0.05) (Figure 1A-B), and Hoechst staining showed that the proportion of cells with karyopyknosis was significantly increased (p<0.05) (Figure 1C-D). After treatment with 100 nM tacrolimus, the proportion of early apoptotic cells was significantly decreased (12.6±2.4% vs. 33.2±5.3%, p<0.05) (Figure 1A-B), and Hoechst staining showed that the proportion of cells with karyopyknosis was decreased (p<0.05) (Figure 1C-D). After the addition of 3-MA, the protective effect of tacrolimus was inhibited, the proportion of early apoptotic cells was significantly decreased (12.6±2.4% vs. 33.2±5.3%, p<0.05) (Figure 1A-B), and the proportion of cells with karyopyknosis was also significantly increased (p<0.05) (Figure 1C-D). This suggests that ox-LDL significantly increases the proportion of early apoptotic cells, while tacrolimus can reduce the early apoptosis level, and inhibiting the cell autophagy, reduces the protective effect of tacrolimus.
Tacrolimus alleviates Ox-LDL damage through inducing vascular endothelial autophagy

Tacrolimus Reduced the Expressions of Vascular Endothelial Cell Apoptosis Factors

The apoptosis factor expressions were detected by PCR and Western blotting. The mRNA expressions of Bax and Bcl-2 were detected by PCR. Results showed that compared with blank group, ox-LDL significantly decreased the Bcl-2 mRNA expression but increased the Bax mRNA expression \((p<0.05)\). After the addition of tacrolimus, the expression of Bcl-2 was significantly increased, while the expression of Bax was significantly decreased \((p<0.05)\). After the addition of autophagy inhibitor, the effect of tacrolimus was reversed (Figure 2A). The expressions of Bcl-2, Bax, caspase3 and cleaved-caspase3 were detected by Western blotting. Results showed that the Bax/Bcl-2 and cleaved-caspase3/caspase3 ratios in ox-LDL group were significantly higher than those in blank group \((p<0.05)\). The Bax/Bcl-2 and cleaved-caspase3/caspase3 ratios in tacrolimus group were significantly decreased \((p<0.05)\), and they were further increased after autophagy inhibition \((p<0.05)\) (Figure 2B-C), proving that tacrolimus can reduce the expressions of apoptotic molecules, and inhibiting the autophagy, reduces the effect of tacrolimus.

Tacrolimus Reduced the Oxidative Stress of Vascular Endothelial Cells

The level of oxidative stress was observed by detecting the activity of SOD and the expression

![Figure 1. Apoptosis morphology. A, Detection of proportion of apoptosis in blank, ox-LDL, FK506 and inhibition groups via flow cytometry. B, Proportion of early apoptotic cells in flow cytometry. C, Detection of proportion of cells with karyopyknosis and karyorrhexis in blank, ox-LDL, FK506 and inhibition groups via Hoechst staining. D, Proportion of apoptotic cells in Hoechst staining. *\(p<0.05\) vs. blank, **\(p<0.05\) vs. ox-LDL, #\(p<0.05\) vs. FK506](image)
of ROS. Results showed that compared with blank group, ox-LDL significantly decreased the activity of SOD \((p<0.05)\) and increased the fluorescence intensity of ROS \((p<0.05)\). After treatment with 100 nM tacrolimus, the activity of SOD in cells was increased significantly \((p<0.05)\), while the expression of ROS was decreased significantly \((p<0.05)\). After the addition of 3-MA, the protective effect of tacrolimus was inhibited, and the activity of SOD was decreased, but the expression of ROS was increased (Table II). The above results suggest that ox-LDL significantly increases the cellular oxidative stress. Tacrolimus can reduce the cellular oxidative stress response, and the addition of 3-MA can inhibit the effect of tacrolimus on oxidative stress.

**Tacrolimus Induced the Autophagy of Vascular Endothelium**

The autophagy level of vascular endothelium was detected via cyto-ID and Western blotting. Results showed that ox-LDL could induce the accumulation of intracellular LC3 fluorescence, and increase the expressions of Atg5 and LC3II. After the addition of tacrolimus, the number of cells containing fluorescence was further increased, and there was a significant difference compared with that in ox-LDL group \((p<0.05)\). Both Atg5
Tacrolimus alleviates Ox-LDL damage through inducing vascular endothelial autophagy

and LC3II expressions were significantly higher than those in ox-LDL group. After the addition of autophagy inhibitor, the autophagic fluorescence was significantly inhibited (Figure 3A-B), and Atg5 and LC3II expressions were also inhibited ($p<0.05$) (Figure 3C-D). The above results suggest that ox-LDL can induce the autophagy of vascular endothelial cells, while tacrolimus can further increase this autophagic response; then, the autophagy is significantly inhibited after 3-MA is added.

**Discussion**

Ox-LDL, as an oxidative stress injury protein, plays an important role in the occurrence and progression of AS. It cannot be metabolized via the LDL receptor pathway, but it mainly recognizes and binds to ox-LDL receptors and endocytose, losing the normal cholesterol metabolism pathway$^{9,10}$. Ox-LDL uptake rate is 3-10 times that of natural LDL, which is not regulated by the intracellular cholesterol content$^{11}$. Ox-LDL can resist the degradation from lysosomal enzymes and cathepsin, and transform a large number of lipids accumulating in cells into foam cells. Ox-LDL into the cells can also induce a series of toxic reactions in cells, further increasing the endothelial cell uptake$^{12}$. Autophagy is a major cytoprotective pathway used by eukaryotes to degrade and recycle their cytoplasmic contents, during which some damaged proteins or organelles are encapsulated by autophagic vesicles with bilayer membrane structure, degraded and recycle in lysosome (animal) or vacuole (yeast and plant)$^{13,14}$. Moreover, it can promote the survival of cells by degrading harmful substances. FK506 was originally developed and applied as a potent immunosuppressive agent in clinical practice. In-vitro studies have shown that the potency of FK506 in inhibiting mixed lymphocyte reactions is several times that of cyclosporin A. FK506 binds to the corresponding FKBP receptor in T-lymphocytes$^{15}$. 

Figure 3. Detection of cell autophagy. A, Detection of LC3 fluorescence-positive cells in ox-LDL, FK506 and inhibition groups via cyto-ID. B, Statistical analysis of proportion of positive cells in each group. C, Detection of expressions of Atg5, LC3I and LC3II in each group via Western blotting. D, Statistical analysis of Atg5 expression and LC3II/LC3I ratio in each group. *$p<0.05$ vs. blank, **$p<0.05$ vs. ox-LDL, ***$p<0.05$ vs. FK506
The drug-receptor complex binds to calpain and inhibits its activity. Calpain is a kind of calcium/calmodulin-dependent protein phosphatase that is ubiquitous in all mammalian tissues, binding to drug-receptor complex to block the calcium-dependent signaling pathway in T-cells. Researches have shown that FK506 can up-regulate the autophagy via the mechanistic target of rapamycin (mTOR) pathway and protect hypoxic cells. We found that ox-LDL could promote the apoptosis of vascular endothelial cells, and induce the oxidative stress and other injuries, while FK506 could protect against ox-LDL-induced cell injury. Further detection of autophagy showed that ox-LDL could induce the increased autophagy of vascular endothelial cells, while FK506 further increased the cell autophagy. To demonstrate that the up-regulation of autophagy was related to the cytoprotective effect of FK506, 3-MA was used as an autophagy inhibitor for further study. Results showed that 3-MA significantly inhibited the level of autophagy, and the proportion of apoptosis and oxidative stress level were significantly increased. Therefore, the protective effect of FK506 on cells may be achieved via inducing the autophagy, increasing the clearance of intracellular ox-LDL and inhibiting its cytotoxic effect.

**Conclusions**

We showed that tacrolimus can protect vascular endothelial cells from ox-LDL damage through inducing the autophagy. Moreover, inhibiting the autophagy can aggravate the injury of vascular endothelial cells.

**Conflict of Interest**

The Authors declare that they have no conflict of interest.

**References**