



# Effect of Copper-Containing Stainless Steel on Apoptosis of Coronary Artery Smooth Muscle Cells

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## Abstract

**Background:** We aimed to investigate the effect of copper stainless steel on apoptosis of vascular smooth muscle cells in coronary artery.

**Methods:** The study was carried out in 2019 at Hubei University of Medicine, Xiangyang, China. The rat coronary artery smooth muscle cell was used for cell resuscitation and culture. MTT method was used to visualize cell growth curve and to detect the cell survival and growth. The incubated cells were randomly divided into copper-containing stainless-steel group, ordinary stainless-steel group, and control group. The cells were made into single cell suspension, which were intervened by experimental group and incubated in incubator with CO<sub>2</sub> for 48 hours. TUNEL method was used to detect the apoptosis. The number of apoptotic cells in five high power fields (×200) was counted. The expression of Fas protein in three groups of cells was detected by Western blot.

**Results:** The growth curves of rat coronary artery smooth muscle cells showed that the OD value of the cells reached the plateau 7 days after inoculation, indicating that the cells grew well. TUNEL staining showed the apoptosis in all three groups. The apoptotic index in copper-containing group was significantly higher than that in common stainless-steel group ( $P < 0.01$ ). The results of the Fas protein expression level through Western blot showed that the level in the copper-containing group was significantly higher than that in the common stainless-steel group ( $P < 0.01$ ).

**Conclusion:** Copper-containing stainless steel can promote apoptosis of coronary artery smooth muscle cells. The material could prevent stent restenosis.

**Keywords:** Copper-containing stainless steel; Coronary artery; Muscle cells; Apoptosis; Fas protein; Restenosis

## Introduction

With the change of people's food structure and living habits, more people begin to suffer from nutritional diseases, and the incidence of coronary atherosclerotic disease (CAD) as a metabolic disease is increasing year by year. Among patients with CAD, acute myocardial infarction (AMI) is

the first killer that endangers human life. How to reduce its incidence, reduce its complications and mortality, and improve the quality of life of patients has become one of the hot spots in medical research at present (1).



At present, the conventional treatment methods for CAD, especially AMI, mainly include basic medication, percutaneous transluminal coronary angioplasty (PTCA) and surgical treatment (1). Among them, percutaneous coronary intervention (PCI) is the most commonly used, convenient and effective method, which can restore the blood perfusion of infarcted area as quickly as possible, thus reducing the infarcted area. Although stents can open blood vessels quickly, there are still some unsolved problems after surgery. In-stent restenosis (ISR) is an urgent problem to be solved. The incidence of ISR is higher in complicated lesions, lesions after radiotherapy failure and lesions complicated with diabetes (1), which limits the clinical benefits of PCI. Therefore, how to avoid the occurrence of IRS has become a common clinical problem for cardiologists. In recent years, some research results showed that vascular smooth muscle cell (VSMC) have a close relationship with vascular related diseases, and the apoptosis acts in vascular remodeling, arteriosclerosis, IRS after PCI and other restenosis diseases (2-4).

Cell apoptosis is very common. VSMC apoptosis runs through the whole process of vascular formation and reconstruction after injury, and its apoptosis plays a decisive role in vascular structure and function, which is an important reason for the pathological changes of ISR after operation (5). Pathological manifestations of IRS are phenotypic change of neovascular endothelial cells, aggregation and fibrosis of extracellular matrix (ECM), proliferation and apoptosis of VSMC. Neointimal hyperplasia (NIH) is an important step in the pathological process of ISR, which can be caused by direct injury of vascular wall caused by balloon dilatation, injury of vascular wall caused by stent placement and long-term traction. One of the main causes of newborn NIH is the abnormal proliferation of VSMC and the accompanying insufficient apoptosis of VSMC (4).

At present, copper (Cu), as one of the essential trace elements of human body, seriously affects the cardiovascular system, especially in the formation of CAD. It has a significant inhibitory

effect. Copper-containing stainless steel can release trace copper locally and continuously under physiological conditions, and have good antiplatelet and anti-inflammatory reaction performance, which can inhibit the proliferation of VSMC, and have stable biocompatibility (6).

We used copper-containing stainless steel as cardiovascular stent material. An in vitro experiment was conducted on its influence on VSMC apoptosis, further investigated the role of copper-containing materials in ISR, and discussed the related mechanism. This study may provide scientific basis for developing a new generation of cardiovascular stent with the function of inhibiting restenosis, and add new content to the functional research of medical metal materials.

## Materials and Methods

This study was approved by the Ethics Committee of Xiangyang No.1 People's Hospital, Hubei University of Medicine, Xiangyang, China.

### *Cell line*

The Ac-21 cell line of rat coronary vascular smooth muscle cells was provided by Shanghai Institute of Cell Studies, Chinese Academy of Sciences.

### *Reagents and instruments applied in the experiment*

DMEM complete culture medium, MTT assay (Shanghai Jingke Chemistry Technology Co., Ltd); Fetal bovine serum, 0.25% trypsin digestive solution, 0.08% trypsin digestive solution (Gibco); Dimethyl sulfoxide (DMSO) cell freezing medium (Sigma); 0.1mmol/L phosphate buffer (PBS) (Central Laboratory of Xiangyang Hospital Affiliated to Hubei University of Medicine); Copper-containing stainless steel material leach liquor and ordinary stainless steel material leach liquor (Institute of Metal Research, Chinese Academy of Science); Rabbit anti-rat Fas/FasL polyclonal antibody, Anti-GAPDH antibody, standard protein marker (Abcom); Agarose and polyvinylidene fluoride (PVDF) membrane (Bei-

jing North TZ-Biotech Develop, Co. Ltd.); BAC protein concentration measuring kit and ECL kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd.); II antibody, Ethylene Diamine Tetraacetic Acid (EDTA), Sodium dodecyl sulfate (SDS), N'-Tetramethylethylenediamine (TEMED), Tris(hydroxymethyl)methyl aminomethane THAM (Tris) (Sigma); Various gel preparation solutions and buffers (Central Laboratory of Jinzhou Medical University).

TUNEL apoptosis in situ detection kit was provided by Boster Biological Technology Co. Ltd, Wuhan, China.

### Experimental grouping and detection methods

#### Experimental grouping

VSMCs with good growth were inoculated into culture bottle with  $5 \times 10^6$  cells / L, and cultured in DMEM complete medium. They were randomly divided into copper containing stainless steel group, ordinary stainless steel group and blank control group. Each group of cells was intervened according to experimental groups. A 5 ml of copper-containing stainless steel suspension was added to DMEM complete medium of copper-containing stainless steel group. Five ml of ordinary stainless steel extract was added to DMEM complete medium of ordinary stainless steel group, and PBS solution was added to DMEM complete medium of blank control group as control. Then, the three groups of cells were put into CO<sub>2</sub> incubator, cultured at 37 °C with 5% CO<sub>2</sub> and 95% humidity for 48 h, digested with 0.25% trypsin, centrifuged at 1000r / min, and then resuspended to make single cell suspension.

TUNEL cell apoptosis in situ detection kit was used to observe cell apoptosis, Western blot was used to detect the expression level of Fas protein, and the detection was performed in strict accordance with the operating instructions. Sodium dodecyl sulfonate-polyacrylamide (SDS-PAGE) gel denaturation electrophoresis detection includes four steps: SDS-PAGE electrophoresis, transfer membrane, immune response, ECL development and gel image analysis.

### Statistical analysis

SPSS 17.0 (Chicago, IL, USA) was applied for analysis. One-way ANOVA was applied for comparison among the three groups, SNK test and LSD-t test were used for comparison between the two groups, and the significance level was 0.05.

## Results

### VSMC cell growth curve

The OD value was measured by MTT method, and the growth curve of VSMC in rat coronary artery was visualized with time (d) as horizontal ordinate and OD value as vertical coordinate. It can be seen from the growth curve that in the first 2 days of cell culture, the curve was relatively flat and the cell proliferation was slow. From 3 days to 6 days, the curve started to get steep and the cell proliferation was rapid. After 6 days to 7 days, the OD value was stable and entered the platform stage, with the OD value between 0.2 and 1.2, indicating that the cell grows well (Fig. 1).

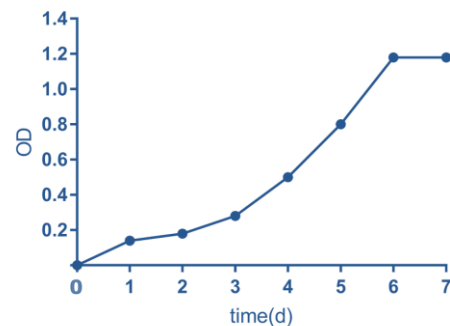


Fig. 1: Growth curve of rat coronary vascular smooth muscle cells

### Apoptosis in each group

TUNEL staining showed that the apoptosis index of copper-containing group was significantly higher than that of ordinary stainless steel group and control group ( $P < 0.01$ ), as shown in Table 1 and Fig. 2.

Table 1: Apoptosis of each group

Grouping	Number of cases	Apoptosis index	F value	P value
Copper - containing group	5	29.168±3.612	72.983	<0.001
Stainless steel group	5	9.034±1.868		
Control group	5	9.652±3.226		

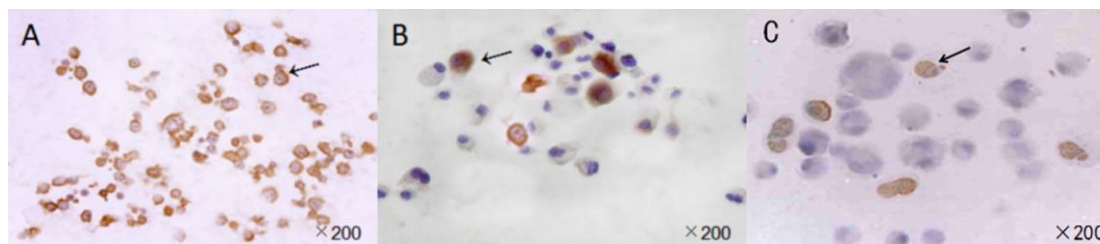


Fig. 2: Apoptosis. Note: A: copper group; B: stainless steel group; C: control group; arrow ←: apoptotic cells

**Fas protein expression level in each group**

Western blot showed that the expression level of Fas protein in smooth muscle cells of copper-

containing stainless steel group was significantly higher than that of ordinary stainless steel group ( $P < 0.01$ ), as shown in Fig. 3.

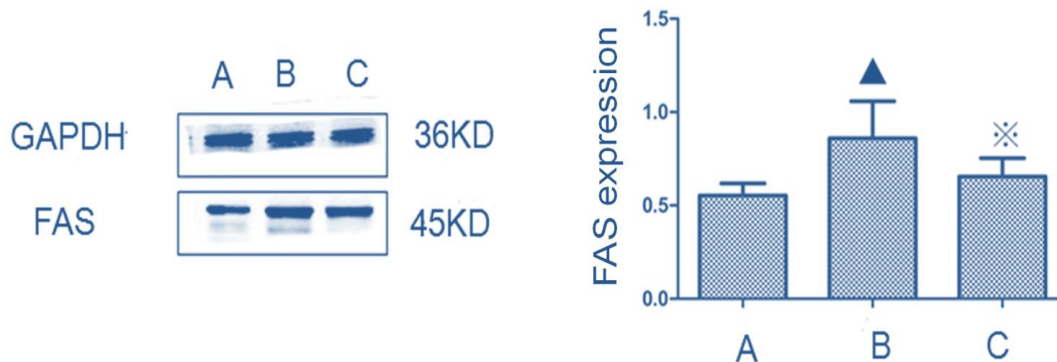


Fig. 3: Western blot was used to detect Fas protein expression in each group

Note: A: control group; B: copper-containing group; C: stainless steel group; ▲ copper-containing: stainless steel group:  $P < 0.01$ ; ✖ copper-containing: control group:  $P < 0.05$

**Discussion**

PTCA has the advantages of micro-trauma and high efficiency. Implanting stents into narrow heart vessels has become the main means of myocardial revascularization, and has rapidly become an effective method for treating CAD caused by vascular stenosis (7, 8). However, ISR

after interventional therapy is a common problem in the popularization and application of PTCA. The in-depth study of ISR mechanism by researchers (9, 10) showed that after balloon dilatation or stent implantation, the arterial vascular endothelium is destroyed, and the substances under the damaged intima are exposed to blood, which activates platelets. More platelets would be

activated by releasing thrombin, thus triggering the release of growth factors, promoting the change of intercellular components, mediating VSMC hyperproliferation to migrate from media to intima. At the same time, VSMC produces a phenotypic change of contractile differentiation to secretory type, and secretes EMC to thicken arterial intima, and form and develop atherosclerotic plaques in vascular lumen and stent (9, 10).

After VSMC apoptosis, it can resist the abnormal proliferation of cells, and then reduce the total number of cells, slow down the formation of neointima and the occurrence of intravascular restenosis. Therefore, promoting VSMC apoptosis is an important means to treat IRS after PCI. The abnormal hyperproliferation of VSMC can cause the stenosis of vascular lumen, while the increase of apoptosis can slow down the proliferation of cells and reduce the occurrence of vascular stenosis, which may solve vascular restenosis (RS) after injury (11). Animal models showed that when the blood vessels are stimulated by local external stress, for example, balloon dilation injury, the apoptotic signal expression and VSMC apoptosis are induced to increase, reducing and delaying vascular and stent RS after intervention (12). At the same time, the apoptosis of VSMC is mediated by various cell signal transduction pathways, among which Fas signal pathway of type I transmembrane protein is particularly important (13).

The pathological mechanism of CAD is atherosclerosis in arteries, which leads to stenosis or even closure of vascular lumen, resulting in myocardial ischemia, hypoxia or necrosis. Apoptosis is programmed. Under normal circumstances, VSMC can resist apoptosis induced by Fas and cytokines. VSMC plays an important role in maintaining the stability of atherosclerotic plaque, but this stability may be affected by proinflammatory mediators such as IFN- $\gamma$ , TNF- $\alpha$ , Il-1 $\beta$  and FasL. However, Fas is widely expressed in VSMC in arteriosclerosis, which is sensitive to some lipophilic statins and can strongly promote apoptosis (14). Tan NY et al (15) established the model of vascular injury by balloon dilatation of rat carotid artery, and then induced local Fas-L over-

expression of blood vessels after reaching the common carotid artery through adenovirus vector Fas gene. Furthermore, cascade reaction of apoptosis signaling pathway is activated to promote cell apoptosis, increase apoptosis of VSMC with excessive proliferation, resist proliferation, inhibit intimal thickening and effectively delay restenosis (15).

Copper is an important participant in the metabolic process of normal cells in human body. It plays an important role especially in heart, blood, internal environment of human body and cell function (16). Copper is an important factor to maintain cell structure and function and muscle contraction and relaxation. The lack of copper would lead to vascular elastic tissue degeneration, even rupture, and also lead to vascular smooth muscle proliferation, migration and degeneration. Finally, it may cause ventricular aneurysm or coronary artery aneurysm, which greatly increases the risk of sudden death (17), and becomes the main inducement of ISR after stenting. The new medical metal material has been improved in terms of scaffold material, which is biologically functionalized after adding copper. As a new type of implantable material, previous in-vivo and in-vitro studies showed that copper-containing stainless steel can release trace copper in situ and continuously, and copper can inhibit platelet aggregation and inflammatory reaction and improve the proliferation of vascular endothelium and smooth muscle cells (18-23), reducing the occurrence of restenosis in the blood vessels and stents to some extent.

This experiment showed that copper-containing stainless steel can promote VSMC apoptosis, and the apoptosis index of copper-containing stainless steel group was significantly higher than that of ordinary stainless steel group, indicating that copper-containing stainless steel can promote VSMC cell apoptosis. Combined with previous studies, it can be speculated that copper can promote apoptosis and inhibit anti-apoptosis pathway, so as to slow down the process of intimal formation, and further improve the restenosis of vessels and stents. At the same time, Fas protein level in copper-containing group was sig-



nificantly higher than that in ordinary stainless steel group, suggesting that its apoptosis-promoting effect may be realized through Fas signaling pathway.

## Conclusion

In this study, copper-containing stainless steel, as a new type of cardiovascular stent material, was creatively studied to promote the apoptosis of coronary artery smooth muscle cells in vitro. We preliminarily concluded that it might play a role in promoting apoptosis through the change of Fas protein level, and further explored its role in preventing restenosis in stent, providing a certain theoretical basis for the clinical application of new cardiovascular stent materials.

## Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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