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Effects of xiaoyu tablet on endothelin-1, nitric oxide, and apoptotic cells of atherosclerotic vessel wall in rabbits[1]

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KEY WORDS arteriosclerosis; endothelin-1; nitric oxide; apoptosis

ABSTRACT

AIM: To investigate the mechanism of xiaoyu tablet on reduction of smooth muscle cells (SMC) in atherosclerotic vessel wall. **METHODS**: The atherosclerotic model was performed in male New Zealand rabbits that were given high fat diet and abrasion of the abdominal aorta endothelial cells. The rabbits were then administered with xiaoyu tablet 0.16-0.32 $g \cdot kg^{-1} \cdot d^{-1}$ for 16 weeks. Changes in morphology, endothelin (ET)-1, nitric oxide (NO), and apoptotic cells of atherosclerotic vessel wall were determined by the microscopy, radioimmunoassay, colorimetric method, the techniques of DNA *in situ* end labeling, and image pattern analysis, respectively. **RESULTS**: After 16 weeks of xiaoyu tablet treatment, intimal thickness and SMC in atherosclerotic vessel wall were diminished, ET-1 was decreased by 8.2 % - 42.6 %, NO was increased by 7.5 % - 54.2 %, and labeled apoptotic nuclei were markedly decreased, the area and integral optical density of positive granule were (846 ± 308) μm^2 and 3425 ± 1374 in atherosclerotic group and (225 ± 60) μm^2 and 1445 ± 606 in xiaoyu tablet 0.32 g/kg group, respectively. **CONCLUSION**: Xiaoyu tablet not only inhibited proliferation of SMC through reducing ET-1 in atherosclerotic vessel wall, but also induced apoptosis of SMC by increasing NO in vessel wall.

INTRODUCTION

Xiaoyu tablet, a compound preparation of Chinese herbal medicines, consisted of Radix Salviae Miltiorrhizae and Fructus Crataegi extracts. The former is a drug of activating blood circulation to remove blood stasis, its extract contains 7.5 % danchensu and protocatechuic aldehyde. The latter can dissipate blood stasis, remove food retention, and promote digestion, its extract contains 20 % flavonoid components such as hyperoside, vitexin, quercetin, and rutine. Our previous studies have found that xiaoyu tablet possessed the inhibition of platelet aggregation[1], the regulation of blood lipid[2-4], and regression of atherosclerosis[5]. To investigate the possible mechanism of xiaoyu tablet on reduction of smooth muscle cells (SMC) in atherosclerotic vessel wall, effects of the drug on morphology, endothelin (ET)-1, nitric oxide (NO), and apoptotic cells of atherosclerotic vessel wall were observed.

MATERIALS AND METHODS

Animals Male New Zealand white rabbits (Grade Ⅰ, Certificate № 97018, weighing 2.0-2.5 kg) were obtained from Animal Breeding Center of Soochow University.

Drugs and reagents Xiaoyu tablet (batch number 960712) was supplied by Shekou Taiping Pharmaceutical Co Ltd (Shenzhen, China). Lipanthyl (batch number 442110B) was procured from Laboratoires Fournier SA (France). Cholesterol was produced by Nanjing Biochemical Pharmaceutical Factory. Lard was purchased from market. ^{125}I-ET-1 kit and NO detection kit were supplied by General Hospital of People's Liberation Army (Beijing, China). Protein k, nitro blue tetrazolium chloride (NBT)/ 5-bromo-4-chloro-3-indolyl phosphate (BCIP) stock solution, and *in situ* cell death detection kit were the products of Boehringer Mannheim (Mannheim, Germany). Other chemicals were of AR grade.

Preparation of atherosclerotic model Experimental atherosclerotic rabbits were induced by

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feeding high fat diet (cholesterol 0.5 g·kg^{-1}·d^{-1}, lard 0.5 mL·kg^{-1}·d^{-1}) and by performing abrasion of the abdominal aorta endothelial cells using 4F fogarty embolectomy catheter[6]. Eight weeks after injury, three rabbits were killed and abdominal aortae were taken for assessment of atherosclerotic development. After the model developed, the rabbits were randomly divided into four groups ($n = 6$): atherosclerotic model group, xiaoyu tablet 0.16 g/kg group, xiaoyu tablet 0.32 g/kg group, and lipanthyl 15 mg/kg group. A control group ($n = 6$) was added simultaneously. The rabbits were then fed on routine diet and routine diet supplemented with drug for 16 weeks, respectively. The rabbits were then killed, the abdominal artery was dissected, and a fraction was fixed in 10 % formalin and 4 % glutaraldehyde for *in situ* determination of apoptotic cells and microscopy, the rest was homogenized for measurement of ET-1 and NO.

***In situ* determination of apoptotic cells** Sections of paraffin embedded tissues were deparaffinated and rehydrated according to standard protocols, then treated with 3 % sodium citrate solution for 1 h and protein k 20 g/L for 20 min, and fixed with 4 % parafomaldehyde for 20 min at 22 ℃ ± 3 ℃. Sections were covered with tunel reaction mixture 50 μL for 1 h and converter-AP 50 μL for 30 min at 37 ℃. After the sections were washed in phosphate buffered solution (PBS 0.05 mol/L, pH 7.4) twice, the substrate solution (NBT/BCIP) was added, and the reaction was kept for 10 min and terminated by washing sections in PBS. Integral optical density (IOD) and area of labeled apoptotic nuclei were measured with KS-400 image pattern system.

Measurement of ET-1 and NO The abdominal artery was homogenized with acetic acid solution 1 mol/L for ET-1 detection and with Tris-HCl buffer 4.5 mmol/L for NO detection, respectively. The samples were then centrifuged, contents of ET-1 and NO in supernatant were determined by radioimmunoassay and colorimetric method according to the procedure provided, respectively. Protein in vessel wall was measured by modified Lowry's method[7].

Statistical analysis Data were expressed as $\bar{x} \pm s$, one-way ANOVA was used for the statistical evaluation of the results.

RESULTS

Effect on morphology of atherosclerotic abdominal aorta Light micrograph showed that intimal thickness, foam cells, SMC, and atheromatous necrotic substance in xiaoyu tablet treatment groups were markedly diminished (Fig 1), under electron microscopy, SMC arranged in order, macula densa and myofilament were richer, organelles were fewer, indicating a contractile phenotype (Fig 2).

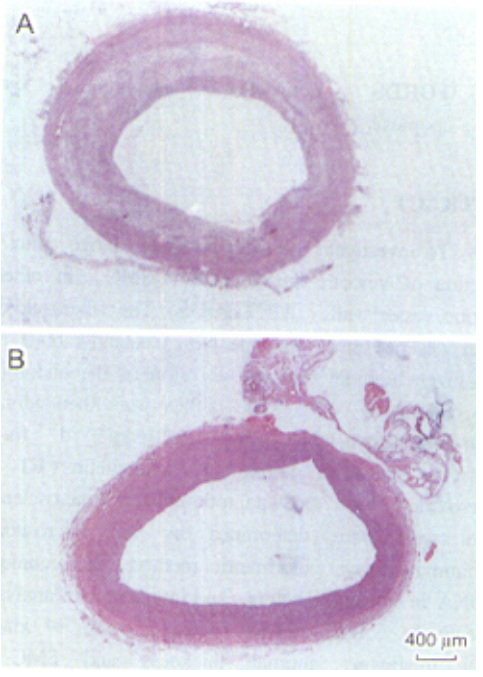

Fig 1. Light micrographs of rabbit abdominal aorta. A: atherosclerotic model group; B: xiaoyu tablet 0.32 g/kg treatment group. HE stain, × 24.

Effects on ET-1 and NO The ET-1 level of vessel wall tissue in atherosclerotic model group was significantly increased ($P < 0.01$), whereas the NO level was obviously decreased ($P < 0.05$) as compared with the control group. After treated with xiaoyu tablet for 16 weeks, ET-1 level was decreased and NO level of vessel wall was elevated, and the levels of ET-1 and NO in 0.32 g/kg group returned to baseline much more rapidly than those in 0.16 g/kg group ($P < 0.05$, Tab 1).

Effect on apoptotic cells The results showed that apoptotic nuclei in xiaoyu tablet groups were attenuated, in parallel, the IOD and area of labeled apoptotic nuclei were markedly decreased as compared with those in the atherosclerotic model group ($P < 0.05$ or $P < 0.01$, Tab 2).

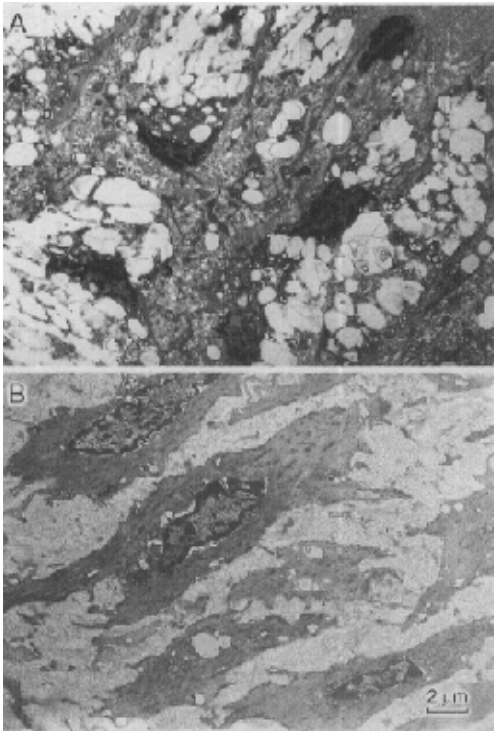

Fig 2. Electron micrographs of rabbit abdominal aorta. A: atherosclerotic model group (× 3700); B: xiaoyu tablet 0.32 g/kg treatment group (× 4000).

Tab 1. ET-1 and NO levels after treated with xiaoyu tablet for 16 weeks in atherosclerotic vessel wall of rabbits. $n=6$. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control. $^eP<0.05$, $^fP<0.01$ *vs* atherosclerotic model. $^hP<0.05$ *vs* xiaoyu tablet 0.16 g/kg group.

Group	ET-1/mg·g^{-1} protein	NO/μmol·g^{-1} protein
Control	2.9 ± 0.8	14 ± 3
Atherosclerotic model	6.1 ± 1.6^c	9.4 ± 2.2^b
Xiaoyu tablet 0.16 g/kg	5.6 ± 2.3^c	10.1 ± 1.9^b
Xiaoyu tablet 0.32 g/kg	3.4 ± 1.8^{eh}	14 ± 4^{fh}
Lipanthyl 15 mg/kg	4.5 ± 1.9	11 ± 4

DISCUSSION

The present results showed that xiaoyu tablet inhibited SMC proliferation and reduced intimal thickness of atherosclerotic abdominal aorta, and were in accordance with our previous observation[5], but its mechanism was not clear. It is known that ET-1 is a potent mitogen and stimulated proliferation of SMC[8,9].

Tab 2. Image pattern analysis of apoptotic cells after administration of xiaoyu tablet for 16 weeks in atherosclerotic vessel wall of rabbits. $n=6$. $\bar{x} \pm s$. $^cP<0.01$ *vs* control. $^eP<0.05$, $^fP<0.01$ *vs* atherosclerotic model.

Group	IOD	Area/μm^2
Control	235 ± 23	51 ± 8
Atherosclerotic model	3425 ± 1374^c	846 ± 308^c
Xiaoyu tablet 0.16 g/kg	1799 ± 590^{ce}	324 ± 141^{cf}
Xiaoyu tablet 0.32 g/kg	1445 ± 606^{ce}	225 ± 60^{ef}
Lipanthyl 15 mg/kg	2050 ± 573^{ce}	430 ± 98^{ce}

ET-1 in atherosclerotic vessel wall was significantly increased, after treated with xiaoyu tablet, the increase of ET-1 was obviously reduced. The inhibition of SMC proliferation by xiaoyu tablet might result from the suppressing synthesis and secretion of ET-1 in atherosclerotic vessel wall.

Apoptosis is another important mechanism of regulating the cell number. Recent studies have domonstrated that apoptosis was abundant in human atherosclerotic plaque[10,11], and apoptotic body could exacerbate the atherosclerosis when it could not be completely scavenged. Therefore, it is now well accepted that cell apoptosis plays a role in the occurrence and development of atherosclerosis[12,13]. In addition, NO, a mediator in the development of atherosclerosis, can block progression of atherosclerosis via inducing apoptosis in SMC[14]. Our experimental results showed that in xiaoyu tablet groups, apoptotic nuclei were decreased, while NO level in vessel wall was increased conversely. From the results, we speculated that the reduction of atherosclerotic degree by the drug might be associated with inducing apoptosis of proliferation SMC, and simultaneously enhancing the recognition and phagocytosis of phagocytes on apoptotic cells by increasing NO. However, the exact mechanism of the drug on reduction of apoptotic cells will be the subject of further research.

In sum, xiaoyu tablet could reduce SMC in atherosclerotic vessel wall, its mechanism included both inhibiting proliferation of SMC by decreasing ET-1 and inducing apoptosis of SMC by increasing NO in vessel wall.

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消瘀片对粥样硬化兔血管壁组织中内皮素-1、一氧化氮及细胞凋亡的影响[1]

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关键词 动脉硬化；内皮素-1；一氧化氮；细胞凋亡

目的：探讨消瘀片减少粥样硬化血管壁中平滑肌细胞数的作用机制. **方法**：雄性新西兰兔高脂饮食加腹主动脉剥脱术制成腹主动脉粥样硬化模型，通过显微镜检查、放免法、比色法、原位末端标记及图象分析技术分别测定连续给予消瘀片 0.16 - 0.32 $g \cdot kg^{-1} \cdot d^{-1}$治疗 16 周后血管壁组织的形态学、内皮素(ET)-1、一氧化氮(NO)含量及细胞凋亡的变化. **结果**：消瘀片治疗 16 周后，粥样硬化血管壁的内膜厚度和平滑肌细胞数明显减少，血管壁组织中的 ET-1 含量降低 8.2 % - 42.6 %，NO 含量增加 7.5 % - 54.2 %，凋亡细胞阳性反应颗粒明显减少，其所占的面积和积分光密度值在粥样硬化组是 (846 ± 308) μm^2 和 3425 ± 1374，消瘀片 0.32 g/kg 组是(225 ± 60) μm^2和 1445 ± 606. **结论**：消瘀片通过降低血管壁中的 ET-1 抑制平滑肌细胞的增生，通过增加血管壁中的 NO 诱导细胞凋亡.

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