Yiqi-Liangxue Recipe Improves Recovery of Injured Endothelia by Promoting the Proliferation and Migration of Vascular Endothelial Cells and Balancing Damage-associated in Flammatory Mediators

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ABSTRACT

Aim: Yiqi-Liangxue Recipe (YL) is a compound preparation of Chinese medicine used for preventing cardiovascular events after percutaneous coronary intervention (PCI) (Patent No. 200810240175.4). Maintaining the integrity of endothelia is one of the most effective approaches to prevent restenosis. Given its clinical protective effects on long-term prognosis, we investigated the mechanisms of YL in protecting vascular endothelial cells.

Methods: We prepared drugs serum and human umbilical vein endothelial cell (HUVEC) lines. The injured model was employed with Angiotensin II (Ang II). The YL group was employed with YL treatment. The ARB medicine group was employed with Losartan Potassium treatment. The combination of Chinese medicine and western medicine (YL + ARB) group was employed with both YL and ARB. The control group was unemployed with injury and medical treatment. For each group the cell migration rate (CM) and cell proliferation rate (CP) were measured. The concentration of Nitric Oxide (NO), Reactive Oxygen Species (ROS), Endothelin-1 (ET-1) were assessed. The gene expression level of ET-1 was observed.

Results: YL + ARB group significantly promoted the speed of the CM/CP of the injured HUVECs. Compared with model group, the concentration of NO increased after the drug intervention, and the concentration of ET-1 decreased. Compared with YL + ARB group, YL and ARB group each had the similar weaker effects, but did not have significant difference. The fluorescence of ROS in YL, ARB and YL + ARB group had no difference because the fluorescence was too strong. Compared with model group, the ΔΔCt values were decreased in the YL, ARB and YL + ARB group. The gene expression level of ET-1 was inhibited after the drug intervention, although with no significant difference.

Conclusion: Treatment with YL ameliorates the injury of vascular endothelial cells and YL + ARB has better curative effect. The mechanisms are associated with improving the speed of the CM/CP; increasing the release of NO and attenuating the concentration of damage-associated mediators like ROS, ET-1 and the gene expression level of ET-1. The results suggest that YL may be an option for preventing cardiovascular events after PCI.

Key words: Yiqi-Liangxue Recipe, PCI, Vascular endothelial cells, Cell migration, Damage-associated mediators

Introduction

Cardiovascular disease reported in China 2013 indicated that the prevalence of coronary heart disease (CHD) is increasing year by year in our country[1]. The mortality of PCI in emergency phase is around 4%~6% and many risk factors have the relationship with the cardiovascular events after PCI[2]. In many countries, clinical doctors are now focusing on side effects of PCI such as restenosis and cardiovascular events[3]. Our team in previous researches had found out Yiqi-Liangxue Recipe (YL) can reduce the prevalence of cardiovascular events after PCI and its clinical protective effects on long-term prognosis are significant[4].

The damage of vascular endothelial cell (EC) is the result of stent implantation in PCI and it can induce abnormality of cardiac function and cardiovascular events[5]. In the process of PCI, the continuity of EC in the coronary artery is scratched and the abnormal proliferation of vascular smooth muscle cell (VSMC) appeared. The lesions of tunica intima lead to the release of damage-associated mediators like NO, ROS, ET-1, AT, PEG, and so on. It’s well accepted that traumatic cytokines play an important role in the vascular inflammation and coronary atherosclerosis[6-7]. Protecting the integrity of endothelium as well as balancing the concentration of damage-associated mediators are considered as

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essential goals for the treatment of cardiovascular events after PCI.

YL is a compound Chinese medicine according to qi and blood theory, which currently used for preventing cardiovascular events after PCI. The main pharmacological components of it are Salvia, Cortex moutan, Astragalus and Honeysuckle. The clinical application of YL has been used for more than 10 years in dongfang hospital without any side effect. Its potential multiple curative effects make it a possible substitute for therapeutic measures after PCI.

Methods

1. Materials

Twenty-two-month-old male rabbits weighing (1.87±0.35) Kg were purchased from Beijing Vital River Animal Technique Limited corporation for the preparation of drugs serum (Certificate No: SCXK 2006-0008). All animals were prepared according to the guidelines of Beijing University of Chinese Medicine. The animals were bred in medical experimental center of China academy of traditional Chinese medicine at (22±2)°C and humidity of 35 ± 5% under a 12-hour light/dark cycle (Certificate No: SYXK (jing) 2010-0032).

We selected HUVECs provided by the medical experimental center, China academy of traditional Chinese medicine for experimental cells. All cells were handled according to the guidelines of medical experimental center and were bred in incubator (Thermo, Waltham, MA, USA) at 37°C, 5% CO₂ and Saturated humidity.

2. Grouping and Medicine

The rabbits were randomized into a YL group (n=4), an ARB medicine group (n=4), a combination group (YL+ARB, n=4) and a control group (n=4). YL group rabbits were given Yiqi-Liangxue Recipe (produced by Dongfang Hospital, Beijing, China) at a dose of 2.3 g·kg⁻¹·d⁻¹ treatment separately. Rabbits in ARB medicine group were given Losartan Potassium which were purchased by the medicine department of Dongfang Hospital (Merck & Co., Inc., Whitehouse Station, NJ, USA) at a dose of 115 mg·kg⁻¹·d⁻¹ treatment separately. Combination group rabbits were given both YL Recipe and Losartan Potassium (separated about half an hour). Rabbits in control group were given the same volume of 0.9% NaCl separately (purchased by medicine department of Dongfang Hospital). All drugs and 0.9% NaCl administration were performed via gastric twice a day until the end of the 7 days. Abdominal aorta blood sampling under aseptic condition, centrifugation of 1500 r (Bio-Rad, Hercules, CA, USA) for serum and inactivated 30 minutes at 56°C.

HUVECs were randomized into a model group, a YL group, an ARB medicine group, a combination group (YL+ARB) and a control group. Each group was added with separated drug serum.

3. Model Preparation and Drugs Serum Proportion

The model was induced by injecting Angiotensin II. MTT method (Ang II and MTT kits were purchased by Bainuwewi Pharmaceutical Co. Ltd; Sigma, St, Louis, MO, USA) was used for selecting the concentration of Ang II (1×10⁻⁸, 1×10⁻⁷, 1×10⁻⁶, 1×10⁻⁵, 1×10⁻⁴ mol/L) and drugs serum (5%, 10%, 15%, 20%, 25%). The results showed 1×10⁻⁶ mol/L Ang II and 15% drugs serum were the best concentration. The model and drugging preparation in five groups were as follows: model group (Ang II+control serum), YL group (Ang II YL serum), ARB medicine group (Ang II+ARB serum), combination group (Ang II+serum of YL+ARB) and control group (control serum and cell culture fluid instead).

4. CCK-8 Method for Cell Proliferation Rate (CP)

HUVECs of stable condition were manually purified by 0.25% trypsin plus 0.04% EDTA (1:1) (Bio-Rad, Hercules, CA, USA) and inoculated to culture plate of 96 holes as 6000/hole. CCK-8 kits were purchased by Dojindo (Co. Ltd, Japan) for cell proliferation assay. Drugs serum were added after 24 h of cell culture for the five groups (n=5 holes for each group) and CCK-8 solution were added as 10 μL after another 24 h for cell culture incubator. For 1~4 h the plates were tested in enzyme mark instrument as 450 nm for optical density (OD).

5. Live Cell Imaging System for Cell Migration Rate (CM)

HUVECs were inoculated to culture dishes as 1×10⁶/L in cell culture incubator for 24 h and then added drugs serum until overgrow (n=3 dishes for each group). The five groups were given to the cell scratch assay and rinsed 2~3 times in PBS. The HUVECs culture dishes were taken into live cell imaging system (Leica, Oskar-Barnack, Germany) for 12 h imaging and preservation. The cell dynamic migration conditions were recorded and CM of each group was tested. All the images were analyzed by Image Pro Plus 6.0 for analysis.

6. Nitrate Reductase Method for NO Concentration

HUVECs were inoculated to culture plates of 6 holes (n=5 holes for each group) and taken into cell culture incubator for 24 h and then added drugs serum until overgrow. The concentration of NO in cell supernatant of five groups was assayed by nitrate reductase method. The detection of each group was according to the steps of Nitric Oxide assay kits (Jiancheng Pharmaceutical Co. Ltd, Nanjing, China).

7. Fluorescence Probe Method for ROS

HUVECs were inoculated to culture dishes as 1×10⁶/L in cell culture incubator for 24 h and then added drugs serum for five groups (n=5 holes for each group). We added 10 μmol H2DCFH-DA (Sigma, St, Louis, MO, USA) with configuration of serum-free medium as probe. The HUVECs were bred in constant temperature water bath at 37°C and avoided light for 30 minutes. The concentration of ROS was tested by laser scanning confocal microscope (LSCM) after rinsing 2~3 times in serum-free medium.

8. ELISA for ET-1 Concentration

HUVECs were inoculated to culture plates of 6 holes (n=5 holes for each group) and taken into cell culture incubator for 24 h and
then added drugs serum until overgrow. The concentration of ET-1 in cell supernatant of five groups were assayed by ELISA. The procedures contain antibody-coated, sample adding, incubation, rinsing, adding substrate and termination, etc. All the steps were taken strictly according to the manufacture’s instruction of kits (Gusabio, Barksdale, Delaware, USA) and each group was tested in enzyme mark instrument as 450 nm for OD.

9. RT-PCR for Gene Expression Level of ET-1
The RNA of the five groups were extracted (n=5 for each group) and the concentration of RNA was detected as formula = [OD value (260 nm) × 40 × dilution ratio]/1000. The gene expression levels of ET-1 in five groups were observed by the method of RT-PCR. It contains the reverse transcription of RNA and the polymerase chain reaction. The experimental procedure was according to the manufacture’s instruction of PCR kit (Invitrogen, CA, USA). The RT-PCR primer sequences are as follows:

GAPDH-R (sequence 5′ to 3′): ACGACCAAATCCGTTGACTC.
GAPDH-f (sequence 5′ to 3′): CTCTGCTCCTCCTGTTCGAC
ET1-R (sequence 5′ to 3′): TCGGTTGTGGGTCACATAACG
ET1-f (sequence 5′ to 3′): ACATTATGGAGAAAGACTGG
Figure 3. The concentration of NO. #P<0.05 vs control group, * P<0.05 vs model group, ▲ P<0.05 vs YL and ARB.

Figure 4(a). The fluorescence of ROS. #P<0.05 vs control group.

Figure 4(b). The fluorescence images under LSCM in different groups.
Statistical Analysis

All data analyses were carried out by SPSS 17.0 software for Windows (SPSS, Chicago, IL) and parameters were expressed as means ± SD. Statistical analysis was performed using LSD and Bonferroni test of one-way ANOVA, followed by variance analysis for multiple comparisons. A probability of less than 0.05 was considered to be statistically significant.

Results

1. General Condition

Under microscopy, the HUVECs had typical morphology of EC and took stable condition on glass surface in RPM1640 medium with 10% FBS. The cells had round, spindle shape or irregular shape and had even cytoplasm.

2. YL Accelerating CP

Compared with the model group, YL group and ARB group both significantly enhanced the proliferation of injured endothelial cells and the YL+ARB group had better effects than the two (P<0.05). Each group was diluted into three concentrations as 10000, 5000 and 2500/hole and the cell proliferation curve showed YL+ARB group was statistically significant with others. (Figure 1(a) and Figure 1(b))

3. YL Accelerating CM

Compared with model group, ARB and YL+ARB group had significantly accelerated CM in 12 h. YL+ARB group was better than ARB group, but YL group showed no significant difference. (Figure 2(a) and Figure 2(b))

4. YL Increasing the Release of NO

Compared with model group, the content of NO increased after the drug intervention, and YL+ARB group had a statistically significant difference with YL and ARB group (P<0.05). (Figure 3)

5. YL Attenuating the Concentration of ROS, ET-1

The fluorescence of ROS in model group exhibited an obvious difference compared with the control group, but did not have significant difference in YL, ARB and YL+ARB group because the fluorescence was too strong. (Figure 4(a) and Figure 4(b))

The ELISA results showed significant decreased concentration of ET-1 in YL, ARB, YL+ARB group and YL+ARB group was better than the other two (P<0.05). (Figure 5)

6. YL Regulating Gene Expression Level of ET-1

The 2-ΔΔCt value of mRNA of ET-1 in model group was correlated with the cell injury. Compared with model group, the 2-ΔΔCt values were decreased in the YL, ARB and YL+ARB group. The result means the gene expression level of ET-1 was inhibited after the drug intervention, although with no significant difference. (Figure 6)

Discussion

PCI can rapidly alleviate the acute ischemia anoxic condition of coronary artery in CHD patients, but currently there are no effective prophylactic and therapeutic measures to reduce the incidence of long-time cardiovascular events. TCM therapies are on the basis of etiology and pathogenesis and have unique curative effects, which can relieve symptoms, improve ECG effects and reduce recurrence. PCI resulted in the injury of coronary artery endothelia and changes referred to morphological damage, inhibited propagation and abnormal damage-associated mediators, which lead to depress EC protection performance[8].

The good cellular growth state of EC is characterized by both normal cell proliferation and cell migration. Many studies are now focusing on the protective barrier functions of EC during the PCI procedure, and the role of the first defending line has been clearly demonstrated[9]. Many TCM herbs can inhibit the expression of cytokines in EC and result in a positive effect.
in anti-atherosclerosis effects for CHD patients\(^{[10]}\). A study showed that the Chinese herbs treatment groups improved the cell survival rate and enhanced the Cp\(^{[11]}\). The high speed of migration can reduce the structural damage of vascular endothelial cells in PCI, keep integrate state and prevent clinical complications\(^{[12]}\). In this study, the CM and CP were increased in YL+ARB group compared with YL and ARB group. The results showed that the degree of CM and CP were significantly accelerated when treated with YL.

Inhibiting EC injury, coronary artery lesions and clinical adverse cardiovascular events may occur through different mechanisms, and one of these mechanisms may be inhibiting the activation and the release of damage-associated mediators. NO is one of the vasorelaxation factors, which closely related to the cell lesions, and the expression of NO or eNOS can improve the vascular elasticity\(^{[13]}\). ET-1 plays an important part in lipid metabolism and vascular elasticity of EC, as well as pro-inflammatory signaling. ROS is as the second messenger for cell conduction and efficient activation, also results in metabolic disorders. The abnormal emission of damage-associated mediators may lead to disturbance of EC states and atherosclerosis. In this study, the concentration of NO increased after the intervention of drugs serum and YL+ARB group was significant compared with YL and ARB group. YL had also reduced the release of ET-1 and YL+ARB group had better effects of protection. However, the observation outcome of LSCM was not as we expected because the three drug groups did not have therapeutic effects as a successful model. The mechanisms of YL in preventing ROS lesions still need to be investigated furthermore. The present study about the balance of damage-associated mediators after injury demonstrated that YL had direct beneficial effects on the protection of EC and coronary artery.

CHD is as same as “Xiong bi” “Xin tong” in TCM theoretical system. Most chronic CHD patients have qi deficiency syndrome in clinical practice and most of the pathogenesis is about qi deficiency and blood stasis with the progress of the disease\(^{[14]}\). YL Recipe (drug composition is Salvia, Cortex moutan, Astragalus and Honeysuckle) is an empirical formula by professor Liao’s qi and blood theory and has already been used in clinical treatment for more than 10 years. In this recipe, Salvia and Cortex moutan cooling and activating blood are the sovereign medicinals of formula and Astragalus tonifying qi is the minister medicinal. Honeysuckle is assistant medicinal, which has heat-clearing and detoxifying effects, also can help removing heat-blood stasis toxicity and increasing the efficacy of other drugs. The compatibility of these four TCM herbs can cool the blood and support the vital energy.

In conclusion, YL has its own unique advantages to avoid cardiovascular events, side effects and improve long-term quality of life for PCI patients in more than 10 years clinical practice. The mechanisms for the function of YL are mainly on the protection of EC, promoting repair, accelerating CP and CM, increasing the release of vascular active substances NO, reducing the concentration and gene expression level of cell damage factor ET-1 and affecting the content of ROS.

**Limitation**

As a limitation of this study, it also should be explored from the expression of proteins, like western blot for the expression of some traumatic cytokines. More mechanisms about the protection of YL may be discovered. Furthermore, YL in preventing ROS lesions for EC is still needed to be investigated and other methods can be tried for the study.

**Conclusion**

According to the traditional Chinese medicine trauma recovery theory, the protective effects of YL on EC and coronary artery are meeting the needs of clinical popularization and application. The mechanisms may partly account for accelerating cell proliferation and cell migration, balancing the concentration of damage-associated mediators like NO, ET-1, etc. The therapy of the combination of Chinese medicine and western medicine has better effect on the damage repair of vascular endothelial cells. On the other hand, as a Chinese herbal compound, the specific mechanisms and therapeutic ingredients of YL still need further investigation.

**References**


