Basic Investigations

Effects of Chinese Herbal Medicine Serum on the Apoptosis of Sinoatrial Node Cells Induced by Simulated Ischemia-reperfusion

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Objective: To study the effect of Chinese herbal medicine Kangxin Fumai Granule (康心复脉颗粒 粒 颗粒 for heart diseases) serum on the primary cultured sinoatrial node (SAN) cell apoptosis induced by simulated ischemia-reperfusion (IR).

Methods: The SAN cells removed from SAN tissue of neonatal Wistar rats were cultured and purified with differential attachment and 5'-bromodeoxyuridine (BrdU) treatment. Simulated IR model was adopted. The obtained cells were morphologically observed with inverted microscopy. By using the method of serum pharmacology, the cell apoptosis was measured with TUNEL staining qualitatively and with flow cytometry quantitatively.

Results: Three kinds of cells were observed in the cultured SAN cells: spindle, triangle and irregular. The spindle cells comprised the greatest proportion. The SAN cells in the model group showed moderate positive brown staining in the nucleus, and the apoptosis rate increased significantly compared to that in the control group \( P < 0.01 \). While the SAN cells in the Kangxin Fumai Granule high-dos e group did not demonstrate positive staining in the nucleus, and the apoptosis rate decreased significantly compared to that in the model group \( P < 0.05 \).

Conclusion: Of the cells cultured from SAN, the spindle cells were pacemaker cells of SAN in rats. Blockade and/or inhibition of the SAN cell apoptosis might be one of the important mechanisms of Kangxin Fumai Granule in preventing and treating sinoatrial injury induced by simulated IR.

Keywords: Chinese herbal medicine serum pharmacology; ischemia-reperfusion; sinoatrial node; cell apoptosis

Apoptosis, or programmed cell death, is an initiative death of cells controlled by self-genes in physiological and pathological situations. Cell apoptosis has an important pathological significance during the injury of myocardial ischemia-reperfusion (IR), and great attention should be paid to the treatment of anti-apoptosis. Kangxin Fumai Granule (康心复脉颗粒 粒 颗粒 for heart diseases) is a new TCM preparation based on the formula of Liu Zhiming, a famous TCM veteran doctor, in treating sick sinus syndrome (SSS), with an excellent effect on SSS. Results from a previous experimental study showed that it increased the heart rate effectively. In the experiment, the effect of Kangxin Fumai Granule serum on the primary cultured sinoatrial node (SAN) cell apoptosis induced by simulated ischemia-reperfusion (IR) was observed.

MATERIALS AND METHODS

Animals

Wistar newborn rats (within 24 h), ether male or female, were purchased from the Institute of Experimental Animal of Chinese Academy of Medical Sciences.

Apparatuses and Reagents

The inverted microscope IX70 was purchased from Olympus, Japan; The flow cytometer FACS Calibur was from Becton Dickinson, USA; DMEM culture medium was from Gibco., USA; Fetal bovine serum from the Hyclong Co.; 5'-bromodeoxyuridine (BrdU), trypsin and collegenase II both from Sigma Co., USA. TUNEL apoptosis detection kit was purchased from Roche-B·M Co., Germany; Annexin V-FITC/PI Reagent Kit was purchased from Keygen Biotech. Co. LTD, Nanjing, China; Simulated ischemia solution (mmol/L): NaCl 98.5, KCl 10, NaH2PO4 0.9, NaHCO3 6.0, CaCl2 1.8, MgSO4 1.2, sodium lactate 40, HEPES 20, PH 6.8; simulated reperfusion solution (mmol/L): NaCl 129.5, KCl 5.0, NaH2PO4 0.9, NaHCO3 20, CaCl2 1.8, MgSO4 1.2, Glucose 55, HEPES 20, PH 7.4.

Drugs

Kangxin Fumai Granule, composed of 4 Chinese herbs including Fuzi (Radix Aconiti Praeparata), Danshen (Radix Salviae Miltiorrhiae), and Zhi Gancao (Radix Glycyrrhizae Praeparata), was supplied by the Department of Drug Preparation, Guang’anmen Hospital, China Academy of Chinese Medical Sciences, each gram

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containing 2.57 g of crude drug, batch number 20050321; Atropine, 0.3 mg/tablet, was supplied by Jiangsu Yancheng Pharmaceutical Co. LTD., batch number 20040801.

Preparation of Medicinal Serum
A total of 40 Wistar rats were randomized into 4 groups, the control group (distilled water, 4 mL/d), the atropine group (0.0006 mg/mL, 4 mL/d), the high-dose Kangxin Fumai Granule group (0.1725 g/mL, 4 mL/d), the low-dose Kangxin Fumai Granule group (0.0437 g/mL, 4 mL/d), 10 rats in each group. Administration of gastric infusion was executed twice a day, 2 mL each time, 7 days in total. Twelve hours fasting was done before the last administration. Two hours after administration, peripheral blood was sampled after decapitation. The serum was naturally separated, inactivated under 37 °C for 30 min, filtered to remove bacteria by 0.22 μm separate film, and preserved in -80 °C. The sera made above were the control serum, atroine serum, high-dose Kangxin Fumai Granule serum, and low-dose Kangxin Fumai Granule serum.

Separation and Culture of SAN Cells
Referring to Marvin’s method, in 20 Wistar newborn rats within 24 h, the thorax was opened to expose the heart. A 0.7 mm × 0.7 mm tissue was taken from the root of precaval vein lateral to the venous sinus in the middle of the terminal crest under anatomical microscope, and then put into carbon dioxide (CD) incubator at 37 °C for 5 min. After blowing for 1–2 min, the supernatant was discarded and the precipitate was added with 0.025% collagenase of 10 times of the tissue volume, and then put into CD incubator at 37 °C for 5 min. The solution was then blown for 1–2 min. After precipitation, the supernatant was filtered through a metal sieve with 400 meshes and then transferred to a centrifuge tube of 50 mL containing DMEM with 15% fetal bovine serum. The procedure above was repeated 4 or 5 times until the tissue was detached into single cells. The cell density was then adjusted to 1 × 10⁵ cells per mL. The cell suspension was centrifuged at 1200 rpm, washed once by DMEM, added with DMEM containing 15% drug serum before simulated ischemia solution, and then cultured for 30 min in aerobic environment with 5% CO₂ for 90 min. The purified SAN cells were attained by differential attachment technique. Trypan blue rejection test showed that the cell survival rate was over 95%. After the cell concentration was then adjusted to 1 × 10⁶ cells per mL, they were transferred to another 24-well culture plate for further culture. After 5 days, cells fused, and cells grew well were chosen for further experiment.

Modeling and Grouping
Referring to Koyama, simulated ischemia was made by oxygen-glucose deprivation, and simulated reperfusion was made by recovering the supply of oxygen and glucose. The old culture medium was replaced by the simulated ischemia solution saturated with nitrogen in advance, and the culture plate was put into a self-made closed box, which was continuously filled, with 10 L mixture gas about 10 L composed of 95% N₂ and 5% CO₂ for exhausting residual oxygen in the box, and was placed in the CO₂ incubator containing CO₂ for 30 min; then it was replaced by the simulated reperfusion solution for further culture for 3 h, by restoring supply of oxygen and glucose.

Cells were randomized into 5 groups: the control group, the atroine group, the high-dose Kangxin Fumai Granule group (HKFG), and the low-dose Kangxin Fumai Granule group (LKFG), six wells in each group, 10⁶ cells in each well. The cells were cultured 30 min under 37 °C. The cells in the atroine group, the HKFG group, and the LKFG group were cultured for 30 min in aerobic environment with DMEM containing 15% drug serum before simulated ischemia and reperfusion. The cells in the model group and the control group were also cultured for 30 min with DMEM containing 15% blank serum.

Morphological Identification of Cultured SAN Cells
The form, size and beating frequency of the cultured SAN cells were observed and the number was manually counted under an inverted microscope.

Qualitative Detection of Apoptosis of Cells
Based on the TUNEL staining, cell nuclear was observed to distinct staining degree. Cell nuclear with brown staining was the positive cell. Based on the staining degree, cells were divided into 5 grades: (-) negative; (+) light brown, weak positive; (++) brown, moderate positive; (+++) dark brown, strong positive.

Quantitative Measurement of Apoptosis of Cells
Apoptosis of cells was detected in quantity with flow cytometer. After digestion, cells were added with Annexin-V and PI labeled by fluorescein, and detected by flow cytometer, excitation wavelength: 488 nm, emission wavelength: 530 nm. Cells were filtered through a filter membrane with 400 meshes. The normal cells, the viable apoptotic cells, the advanced stage apoptotic cells and dead cells were distinguished by Annexin V-FITC/PI method, which made the apoptotic detection rate more specific.

Statistical Analysis
All results were expressed as mean ± SD. Student’s t test and Chi-square test were performed by using SPSS12.0 software.

RESULTS
Observation on Living Cells with Inverted Microscopy
On the 5th day of SAN cell culture, 3 types of cells with different forms were observed under the inverted microscope categorized as spindle, triangle and irregular. Spindle cells were the most abundant, accounting for
about 60%, with a beating frequency of 168±8 per minute, faster than other cells.

**TUNEL Staining**
The SAN cell nucleus in the control group did not have positive staining; the cell nucleus in the model group demonstrated moderate positive staining (++); the cell nucleus staining (++) in the atropine serum group were similar to that in the model group; while the cell nucleus in HKFG group did not showed positive staining and in LKFG group demonstrated weak positive staining (+). See Figure 1.

![TUNEL staining](image)

**Figure 1.** TUNEL staining in each group (TUNEL×100). A: the control group; B: the model group; C: the atropine serum group; D: the HKFG serum group; E: the LKFG serum group.

**Apoptotic Rate of SAN Cells Detected by Flow Cytometer**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Apoptosis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.96±0.03</td>
</tr>
<tr>
<td>Model</td>
<td>27.38±5.62**</td>
</tr>
<tr>
<td>Atropine</td>
<td>20.69±2.53*</td>
</tr>
<tr>
<td>HKFG</td>
<td>17.00±6.16*</td>
</tr>
<tr>
<td>LKFG</td>
<td>18.47±7.35</td>
</tr>
</tbody>
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Notes: Compared with the control group, *P<0.05, **P<0.01; Compared with the model group, *P<0.05.

The results showed that the apoptosis rate in the model group was 27.38±5.62%, increasing significantly compared to the apoptosis rate of 8.96±0.03% in the control group (P<0.01). The apoptosis rate in the atropine group was 20.69±2.53% with no significant change as compared with the model group (P>0.05). The apoptosis rate in the HKFG group was 17.00±6.16% and decreased significantly compared to that in the model group (P<0.05, Figure 2).

![Apoptotic rate detected by flow cytometer](image)

**Figure 2.** Apoptotic rate detected by flow cytometer (FCM). A: the control group; B: the model group; C: the atropine serum group; D: the HKFG serum group; E: the LKFG serum group.

**DISCUSSION**

**Sampling for Culture of Neonatal Rat SAN Cells**
Accurate location and sampling of SAN tissues from neonatal rats were one of key procedures of SAN cell culture because these two processes had direct influence on the proportion of cultured SAN cells. Based on Marvin’s method, the tissue from the root of precaval vein lateral to the venous sinus in the middle of the terminal crest was sampled under an anatomical microscope, and the SAN cells were then isolated and cultured, yielding 60% spindle cells in culture, which is consistent with other reports, indicating the reliability of this sampling method.

**Method for Culture of Neonatal Rat SAN Cells**
It is very important to remove impurity cells and increase the purity of SAN cells in SAN cell culture. In this experiment, differential attachment and BrdU treatment techniques in SAN cell culture were adopted to remove fibroblast and inhibit its growth, hence significant increase of the proportion of spindle cells.

**Apoptosis of Myocardial Cells Including Sinoatrial Node Cells**
In recent years, most of researches hold that there are two ways of cardiac myocyte death: necrosis and apoptosis. Apoptosis is an important way of early myocardial cell death. It has been confirmed that ischemia-reperfusion (IR) can cause myocardial cell apoptosis. Because sinoatrial node cells originate from primitive undifferentiated “working” myocardial cells and their structures and metabolism are similar to those of common myocardial cells, the mechanism of IR-induced apoptosis of SAN may be the same to that of
gradually. Time prolonged, the apoptosis rate of SAN cells increased. Apoptosis in cultured sinoatrial node cells, and with the time prolonged, the apoptosis rate of SAN cells increased gradually. A previous experimental study showed that Kangxin Fumai Granule atropine had no inhibitory effect on sinoatrial node cell apoptosis induced by simulated IR. This experiment studies the effect of Chinese herbal medicine Kangxin Fumai Granule serum on the primary culture from the newborn rat. The isolated sinoatrial node cell in primary culture from the newborn rat. Circ Res 1984; 55: 253-260.

Effects of Chinese Herb Kangxin Fumai Granule Serum
This experiment studies the effect of Chinese herbal medicine Kangxin Fumai Granule serum on the primary cultured SAN cell apoptosis induced by simulated IR, based on the former clinical experiments. Nowadays, in clinical practice, SSS is still treated symptomatically with atropine and other drugs used. Atropine can block muscarinic cholinergic receptor (MChR), relieve cardiac vagal tone, and increase the heart rate. It was shown in this experiment that atropine had no inhibitory effect on sinoatrial node cell apoptosis induced by simulated IR.

Kangxin Fumai Granule is a new TCM preparation based on the formula of Liu Zhiming, a famous TCM veteran doctor, in treating SSS, with an excellent effect on SSS, which is mainly composed of Fuzi (Radix Aconitum Lateralis Praeparata), Danshen (Radix Salviae Miltiorrhiae), and Zhi Gan Cao (Radix Glycyrrhizae Praeparata). It functions to warm yin and promote blood circulation. In this recipe, Fuzi (Radix Aconitum Lateralis Praeparata) warms and invigorates heart-yang, Danshen (Radix Salviae Miltiorrhiae) promotes blood circulation, and Zhi Gan Cao (Radix Glycyrrhizae Praeparata) invigorates qi and nourishes the heart. Combined use of these drugs can strengthen the body resistance to eliminate pathogenic factors. Modern pharmacological researches show that Fuzi (Radix Aconitum Lateralis Praeparata) has a tonic effect on the heart and effects of increasing blood flow of coronary artery, increasing the heart rate and lowering the oxygen consumption of myocardium; Danshen (Radix Salviae Miltiorrhiae) has been widely used in the treatment of cardio-cerebral vascular diseases. Salvianolic acid A (Sal-A), one of the components of Danshen (Radix Salviae Miltiorrhiae), has a strong antioxidation ation; Sodium glycyrrhetinic acid, the effective component of Zhi Gan Cao (Radix Glycyrrhizae Praeparata), reduces the concentration of cAMP and Ca2+ in myocardial cells and protects myocardium.

The previous experimental study showed that Kangxin Fumai Granule had the effect of inhibiting IR injury and this experiment showed that it had the effect of inhibiting sinoatrial node cell apoptosis induced by simulated IR, indicating that its mechanism in treating SSS is possibly related to inhibiting IR and relieving sinoatrial node cell apoptosis, thus the experiment provides the trustworthy experimental evidence for its preventing and treating IR injury of sinoatrial node, and provides scientific basis for its clinical treatment of SSS as well.

REFERENCES

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