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Research Paper

Effect of ecdysterone on heteroptopic heart transplantation in rats

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Abstract

Objective: To investigate the protective effects of ecdysterone(EDS) on the allograft heart transplant model of rats. **Methods:** Fifty healthy Sprayue-Dawley(SD) rats were divided into donors and recipients randomly. Hearts were harvested and placed heterotopically into allogenic recipients. All animals were divided into three groups: sham-operation group(only opening and closing the abdomen, n=10), EDS group(injected intraperitoneally with 20 mg/(kg • day) of EDS, n = 10), and control group (injected intraperitoneally with normal saline, n = 10). The pathological changes of myocardial tissue were analyzed by light microscopy and transmission electron microscopy and the levels of myocardial enzymes(GOT, LDH, CPK), SOD, ET-1, NO, MDA in serum were measured. Tissue samples underwent the detection of apoptotic cell death by in situ terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling for apoptotic index(AI) and also by immunohistochemistry method to study the expressions of Bcl-2 and Bax. **Results:** Pathological examination and transmission electron microscope observation showed greater myocardium damage in the control group. EDS group showed a decrease in the amount of myocardial enzymes, MDA, ET-1 and an increase in the levels of SOD, NO, compared to the control group. The AI of EDS group became lower than that of control group, meanwhile the EDS group increased the expression of Bcl-2 and decreased the expression of Bax. **Conclusion:** EDS has protective effects on heteroptopic heart transplantation, which provides a new idea for myocardial protection in heart transplantation. However, the mechanism of its protective effect needs further research.

Key words: ecdysterone; heart transplantation; myocardial protection; rats

INTRODUCTION

Ecdysteroids is a class of natural insect molting activity compounds, which can be isolated from plants. Ecdysteron(EDS) is one of the highest activities of its type. It has many functions such as oxidation resistance, promotion nucleic acid, protein synthesis, regulation of sugar and lipid metabolism, immunity regulation, reduction of vascular endothelial injury, improvement of microcycle, and promotion of wound recovery[1,2]. Based on the allograft heart transplant model of rats, we explored the protective effects of EDS on heart transplantation.

MATERIALS AND METHODS

Animal

Fifty SD rats of both genders(3-months-old, weight ranging 250~300 g) were divided into donors and recipients randomly. Donor heart grafts were transplanted onto the recipient abdominal aorta and inferior vena cava by the methods described by Ono[3]. There were three groups:sham-operated group(only opening and closing the abdomen, n = 10), control group(injected intraperitoneally with normal saline, n = 10), EDS group (injected intraperitoneally with 20 mg/(kg • day) of EDS, n = 10). Graft survival was monitored daily by palpation for the ventricular contraction. Cardiac graft function was expressed as the beating score, assessed by the Stanford cardiac surgery laboratory graft scoring system(0: no contraction; 1: contraction barely palpable; 2: obvious decrease in contraction strength,

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but still contracting in a coordinated manner, rhythm disturbance; 3:strong, coordinated beat but noticeable decrease in strength or rate, distention/stiffness; or 4: strong contraction of both ventricles, regular rate, no enlargement or stiffness)[4]. Operation was considered successfully by palpation scores≥3 at least for 7 days.

Reagents

Ecdysterone(purity 99.99%, No. 930619) was obtained from Kunming Institute of Botany, Chinese Academy of Sciences. Rat ET-1 kit was purchased from East Asian Immunity Technical Research Institute (Beijing, China). TUNEL Cell Apoptosis Detection Kit was purchased from Boster Biological Technology Co. Ltd(Wuhan, China). Terminal deoxynucleotidyl Transferase(TdT) was purchased from Promega Co. Ltd (USA). Rat Nitric Oxide Elisa Kit was obtained from Nanjing Jiancheng Bioengineering Institute(Nanjing, China).

Histopathology and Electron microscopy protocal

Grafts were removed for histological analysis on 7 days postoperative. Grafts harvested were fixed in buffered formalin for 24 hours. Thin hematoxylin and eosin (HE) stained sections of paraffin embedded samples were examined. After being dehydrated, embedded, sectioned and stained with uranyl acetate and lead citrate, the specimen was examined with a transmission electron microscope.

Biochemistry

The levels of serum myocardial enzymes which were glutamic oxaloacetic transaminase(GOT), lactate dehydrogenase(LDH), creatine phosphate kinase(CPK), were measured using biochemistry automatic analysis apparatus. Malondialdehyde(MDA) in serum, the intermediate product of lipid peroxide, was determined. superoxide dismutase(SOD) and endothelin-1(ET-1) were measured by radioimmunoassay. Nitric oxide(NO) concentration was measured by nitric acid reductase method.

Immunohistochemistry

Myocardial tissue was procured for sectioning and immunohistochemical analysis. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed according to the protocol of the TUNEL kit. Cells in each of 10 different isolated fields(×200) were observed under microscope. The number of positive cells/the total number of cells were calculated. The mean value was measured to express the apoptotic index. The protein expressions of Bax and Bcl-2 were detected by ABC immunochemical staining. The positive cells were identified under the light microscope.

Statistical analysis

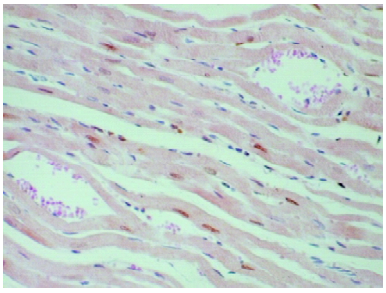
All analyses were performed with the SPSS. Data were expressed as the $\bar{x} \pm s$. F test was used to compare the significance of differences between the groups. $P < 0.05$ was demonstrated significant statistics difference, $P < 0.01$ was demonstrated highly significant statistics difference.

RESULTS

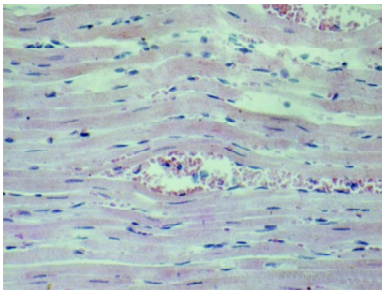
There was no case of death during and after operations, all groups of the transplantation were successful postoperatively after the 7th day.

Histopathology and transmission electron microscopy protocal

Myocardial degeneration, necrosis and infiltration of inflammatory cells were showed in the control group. There was some collagen proliferation presented around small vessel. The myocardial lesion in EDS group was slight, and the inflammatory reaction was diminished compared with that in control group(***Fig.1***), while the histopathological examination in the sham operated group showed no abnormal changes.



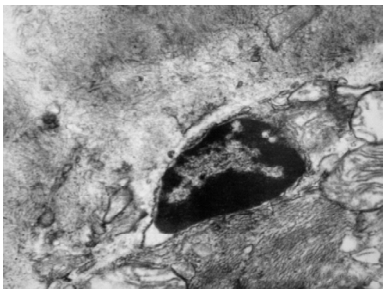
control group



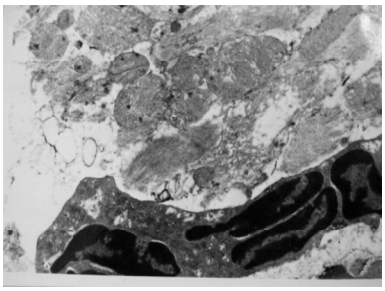
EDS group

Fig. 1 The myocardial lesion in the EDS group was slight, inflammatory reaction was diminished compared with that in the control group(HE,× 200)

Myocardial cells in the control group had abundant endoplasmic reticulum. Parts of the endoplasmic reticulum expanded into blebs and vesicles, undetectable nucleoli, chromatin condensed under the periphery of the nuclear envelope forming a dense apoptotic body, and even the ruptured envelopes under transmission electron microscope. Compared with the control group, cells displayed regular structures and showed little evidence of apoptosis in the EDS group(***Fig. 2***).



control group



EDS group

Fig. 2 Cells in the EDS group displayed regular structures and little evidence of apoptosis compared with those in the control group (transmission electron microscopy, × 25 000)

Biochemistry result

The levels of serum myocardial enzymes(GOT, LDH and CPK) were significantly decreased in the EDS group compared with those in the control group($P < 0.01$). Lipid peroxide(expressed as MDA) level was lower in the EDS group than that in the control group($P < 0.01$). The level of SOD was significantly higher in the EDS group than that in the control group($P < 0.01$). Compared with the control group, ET-1 increased in the EDS group($P < 0.05$). Meanwhile, the level of ET-1 in the EDS group was similar to that in the sham-operated group($P > 0.05$). The level of NO was elevated in the EDS group compared with that in the control group ($P < 0.05$), and was no difference between the EDS group and the sham-operated group(***Table 1***).

Table 1 **Levels of GOT, LDH, CPK, SOD, MDA, ET-1 and NO in serum**

GROUP	GOT(U/ml)	LDH(U/ml)	CPK(U/ml)	SOD(μg/ml)	MDA(nmol/ml)	ET-1(ng/L)	NO(μmol/L)
sham-operated	90.4 ± 13.3	70.7 ± 11.4	200.7 ± 28.2	47.4 ± 5.1	30.7 ± 4.9	113.73 ± 21.27	50.70 ± 11.25
control	218.6 ± 28.8*	130.3 ± 17.3*	441.8 ± 60.4*	22.5 ± 3.3*	84.9 ± 10.3*	159.47 ± 33.81	40.53 ± 9.47
EDS	174.4 ± 1.3*▲	109.4 ± 15.8*▲	374.5 ± 53.2*▲	38.5 ± 4.8*▲	67.2 ± 9.2*▲	124.29 ± 23.20■	53.50 ± 14.17■

Compared with the sham-operated group, *$P < 0.01$; Compared with the control group, ▲ $P < 0.01$; Compared with the control group, ■ $P < 0.05$.

Immunohistochemistry result

By using TUNEL method, the AI in the EDS group decreased significantly compared with that in the control group($P < 0.05$). However, there was no significant difference between the EDS group and the sham-operated group($P > 0.05$)(***Table 2***). Positive Bax and Bcl-2 staining showed a light-yellow color in the cytoplasm. Immunohistochemisty stain showed the positive expression of Bcl-2 increased(***Fig. 3***) and Bax decreased(***Fig. 4***) in the EDS group compared with those in the control group.

Table 2 Apoptotic index of each group

GROUP	AI
sham operated($n = 10$)	3.73 ± 2.27
control($n = 10$)	9.49 ± 3.81
EDS($n = 10$)	4.26 ± 2.20*

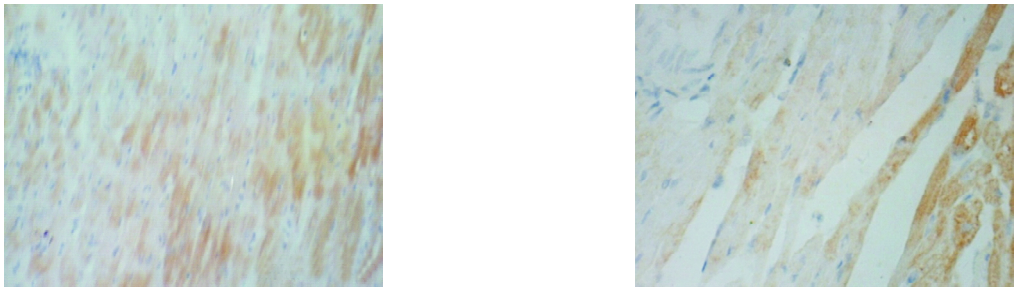
Compared with the control group,*$P < 0.01$.

DISCCUSION

In 1967, the first human to human heart transplantation was performed by Dr. Christian Barnard[5]. From then on, heart transplantation became one of treatments for terminal heart disease, and many surgeons co-opted the procedure world widely. With the development of immunosuppressant and myocardial protection, more transplant operations took place and increased patients' survival rates. Myocardial protection in basic and clinical research has been developing. Natural plant extracts have received the most attention, some being applied in basic research on heart transplantation. Ho and his colleagues[6] found that Tripterygium wilfordii Hook-F(TWHf) in T cells was mediated through both the downregulation of T cell receptor signaling pathway and induction of cell apoptosis, which was found to be defective in autoimmune diseases. The investigation of Zhang *et al.*[7] also reported that TWHf could prolong rat cardiac allograft

control group EDS group

Fig. 3 The expression of Bcl-2 increased in EDS group compared with that in the control group(IHE,× 200)



control group EDS group

Fig. 4 The expression of Bax decreased in EDS group compared with that in the control group(IHE,× 200)

survival. Astilbin which was the extract of smilax glabra, could induce apoptosis of activated T cell in heart transplantation, so that it decreased the rejection and prolonged the survival in mice heart transplantation[8].

EDS was found to exist in some chinese herbs such as:Twotooth Achyranthes Root, Radix Cyathuleae, Mulberryleaf, Ajuga decumbus and Rhizoma Alismatis. EDS can promote insects' growth and differentiation; it also performs many pharmacological activities in mammal[9]. Kuzmenko[10] demonstrated that EDS displayed antiradical properties by hydroxyl and amino groups in its molecule. Recent research showed that EDS could stimulate the expression of vascular endothelial growth factor(VEGF) in cardiac myocyte in hypoxic conditions, which played an important role in protective effect on myocardial cell apoptosis[11]. In the respect of donor protection in heart transplantation, whether EDS could display its functions of oxidation resistance and anti-apoptosis is a topic worth discussing.

In our study, the EDS group showed a decrease in the level of MDA and an increase in the level of SOD. In general, the level of MDA was conceded to reflect the lipin peroxidation damage, suggesting that the protective function of EDS was performed by inhibiting the free radicals which induced lipid peroxidation.

When the myocardial cells were damaged, myocardial enzymes such as LDH, GOT, especially CPK spilled from cadiocyte into serum. There was a positive correlation between the amount of myocardial enzymes and the degree of myocardial injury, so that an amount of myocrdial enzymes perhaps reflected indirectly the degree of myocardial damage after transplantation. The results showed that the myocardial enzymes in the EDS group decreased significantly. It is proved that EDS has a protective effect in allograft heart transplantations.

ET-1 may be the strongest vascular contracting substance. It caused the vasoconstriction, myocardial ischemia, metabolic disorders and cell proliferation, which are common pathogenic factors of vascular injury-related diseases. NO is a kind of vascular relaxing substances. It has many kinds of functions: expanding the coronary artery, improving the microcycle, resisting oxidation, preventing platelet aggregation and white blood cell adherence, plus suppressing the smooth muscle cell proliferation and so on. ET-1 and NO is a pair of antagonists with the effect of vasoactive substances, and the coordination role of their synthesis and release is the main factor of maintaining vessel tensity[12]. Our study showed that the level of ET-1 reduction and the level of NO elevation in the EDS group, which may contribute to the myocardial protection.

Pathological and transmission electron microscopic observation showed more serious myocardium damage in the control group compared with that in the EDS group, which proved that EDS could reduce the myocardial injury in heart transplantation.

Apoptosis also called programmed cell death and is

normal in multicelular organisms. Myocardial apoptosis plays an essential role after heart transplantation mediated by apoptosis specific genes, including Bax and Bcl-2. The TUNEL method can clearly display the apoptotic cells in situ and is reported as a sensitive and specific method for identification of apoptotic cells[13,14]. Bcl-2 was found to be the strongest apoptosis suppressor gene[15]. Bcl-2 can directly and indirectly inhibit Caspase which is the central element of the signal conduction in apoptosis, so that cell apoptosis was inhibited by blocking the last apoptosis gene pathway[16]. In general, immunohistochemical detection of Bcl-2 expression is negative in cardiac tissue. However, because of response to reperfusion injury, rejection and other positive endogenous protection effect after heart transplantation, expression of Bcl-2 can be measured(Bcl-2 excessively expressed; cell survived. Bax excessively expressed; cell died). Our study measured the apoptotic index, Bcl-2 and Bax expressions in each group, indicating that EDS has the effect of anti-apoptosis.

Our observations of the biochemistry, the pathology structure and the apoptosis suggested that EDS has some protective effects on heteroptopic heart transplantation in rats. This may provide new ideas for myocardial protection in heart transplantation. However, the mechanism of this protective effect still needs further research.

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