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Effects of metoprolol and small intestine RNA on marrow-derived endothelial progenitor cells applied for autograft transplantation in heart disease

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Abstract. – AIMS: The objective of this project was to improve the effect of EPC autograft transplantation and observe the tolerance of EPCs to I/R injury affected by metoprolol and small intestine RNA.

MATERIALS AND METHODS: We isolated bone marrow-derived EPCs and examined the effects of metoprolol and small intestine RNA on EPCs to ischemia at different time points after reperfusion. EPCs growth curve, secretion, apoptosis and mortality were also analyzed.

RESULTS: EPCs will be better protected if the blood can be recovered within 4 hours after ischemia for cardiac muscle cells and pretreatment of EPCs with metoprolol or small intestine RNA could protect and promote EPCs proliferation.

CONCLUSIONS: Our study demonstated that pretreatment of EPCs with metoprolol or small intestine RNA will increase the EPCs proliferation and may improve the EPCs autograft transplantation ability.

Key Words:
Endothelial progenitor cells, Metoprolol, small Intestine RNA, Autograft transplantation, Ischemia-reperfusion injury.

Abbreviations

EPC = endothelial progenitor cells; I/R = Ischemia/ Reperfusion; bFGF = basic Fibroblast Growth Factor; FBS DMEM = Fetal Bovine Serum Dulbecco Modified Eagle Medium; PBS = Phoshate Buffered Saline; NO = Nitric Oxide; NOS = Nitric Oxide Synthase; TNOS = Total NOS; cNOS = Constitutive NOS; iNOS = inducible NOS.

Introduction

Endothelial progenitor cells (EPCs) participate in postnatal vasculogenesis after ischemia injury of the heart[1-3]. Studies had shown that EPCs in bone marrow will enter the peripheral circulation system and then target to the region of blood-vessel to improve the angiogenesis when the tissues received ischemia injury[4-8]. Further studies showed that angiogenesis can be enhanced by transplanting mononuclear cells (MNCs) from bone marrow or from peripheral blood in many ischemia models[9,10]. The reason that MNCs can enhance the angiogenesis is that not only can MNCs secret vascular endothelial growth factors (VEGF), but the EPCs among them will participate in the angiogenesis directly. But the amount of EPCs among the MNCs is very low, which hinders the application of MNCs in the treatment of ischemia injury.

Recent research had shown that the microenvironment is more important than the stem cells themselves in determining their fates[11]. Changes in the microenvironment may play a pivotal role in directing the differentiation of the stem cells, but may also have an adverse effect on their proliferations and functions. Some other researchers pointed out that there are natural multifunctional stem cells in the heart which are probably engaged in the heart repair after injury[12-14]. These cells suffer the I/R injury and the subsequent changes in the microenvironment of the heart caused by I/R. Recent studies suggested that metoprolol may induce proliferation of EPCs[15]. In addition, RNA can also affect the microenvironment and influence the treatment of many diseases[16,17]. Small intestine RNA has been found having functions in the treatment of acute intestine injury and increasing the recovery rate after the irradiation[18].

In this study, we observed the effects of metoprolol and extrinsic RNA from small intestine on EPCs proliferations, secretion functions and biomarkers expression to try to obtain their possible

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useful applications. What's more, we provide some preliminary data to evaluate the autograft transplantation affected by EPCs in an acute myocardial infarction animal model.

Materials and Methods

Reagents and Animal Operations

VEGF and bFGF were purchased from Australia TBD science. Antibody CD133, CD31, and Flk-1 were from Santa Cruz Biotech. Antibody VIII factor and HRP labeled rabbit anti-mouse antibodies were from Zymed. TRITC labeled rabbit anti-goat antibodies and FITC labeled secondary antibodies were from Beijing Zhongshan Biotechnology Company, China. NO, NOS, LDH kits were from Nanjing Jiancheng Biotechnology Company. Metoprolol was provided by the Xijing Hospital, Fourth Military Medical University.

The animal treatment protocol was approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University.

Separation, Purification and Identification of Marrow-Derived EPCs

The posterior superior iliac spines of six healthy male Chinese mini pigs (weight 20.0±2.4 kg) were punctured with their bone marrows drawn after routine sterilization. The mononuclear cells were separated by Ficoll density-gradient technique and the live cells were cultured in 10% FBS DMEM medium. 24 h after adhesion screening, the non-adherent cells (set 2) were moved and cultured in another plate. Both of the cells (Set 1 is 24 h adherent cells) were cultured in the special DMEM medium (Recombinant porcine VEGF, 10 ng/ml, Australia TBD Science; Recombinant porcine bFGF, 10 ng/ml, Australia TBD Science) for further adhesion screening. The medium was changed every 3 days.

Experiment Grouping and Treatment

The cells were digested and cultured in good growth station at 1×106/ml in 6-well plate and the following groups were set up: (1) metoprolol pretreatment plus I/R injury group; (2) I/R injury plus metoprolol group; (3) sole metoprolol treatment group; (4) sole I/R injury group; (5) normal control group. The concentration of metoprolol in each group was 1×10^{-6} mol/l, which was verified by our previous experiments. Ischemia time was 3 h according to previous steps and reperfusion was 21 h. At this point, the cells were cultured at 1×10^{4}/ml in 24-well plate. The five groups were divided according to the previous steps and with four holes in each group. MTT observation was made 24 h after ischemia. The cell growth curve was drawn during one week observation.

Preparation of Small Intestine RNA

Small intestines from male rat were washed by PBS and sheared into small pieces. Small intestine tissues were then incubated in denature solution (194 g guanidine hydrochloride, 2.16 g sodium citrate, 1.44 g sodium sarcosine, 2.3 ml 2-mercaptoenthanol in 288 ml H_2O, pH 6.0) to get the homogeneous solution. Small intestine RNA was extracted by phenol-chloroform and precipitated by isopropanol.

I/R injury Model Construction and Observation of the Tolerance of EPCs to I/R Injury

The culture medium was replaced with the ischemic buffer after stabilizing the cells (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$.2 H_2O, 4 mM Hepes) and 10 mM deoxyglucose, 0.75 mM sodium dithionate, 12 mM KCl and 20 mM lactate were added to the buffer with pH 6.5. Reperfusion was performed by replacing the complete medium at 2, 3, 4, 5 and 6 h after the ischemia. The growth condition of cells was observed with optical microscope and MTT experiment at 24 h after the ischemia.

Measurement of NO, NOS and LDH in Culture Medium for Each Group

24 h after I/R injury, 400 μl culture solution was taken out from the 6-well plate of each of the five groups. Extracellular culture solution was examined with NO, NOS and LDH kits (Nanjing Jiancheng Biology-Engineering Institute).

Apoptosis Analysis

After being blocked with an Fc receptor blocker for 30 min, EPCs were labeled with phycoerythrin (PE)-conjugated CD133 (No. AC133, Santa Cruz Biotech, Inc, USA), PE-conjugated CD31 (No. 12-0311, eBioscience) and PE-conjugated c-Kit (No. 12-1171, eBioscience) antibodies. Flow cytometry was performed with 25,000 cells and data were analyzed using FCS Express (De Novo Software). Proper matched IgGs isotope were used as controls. Dead cells were excluded by 7-amino-actinomycin D counterstaining[19].

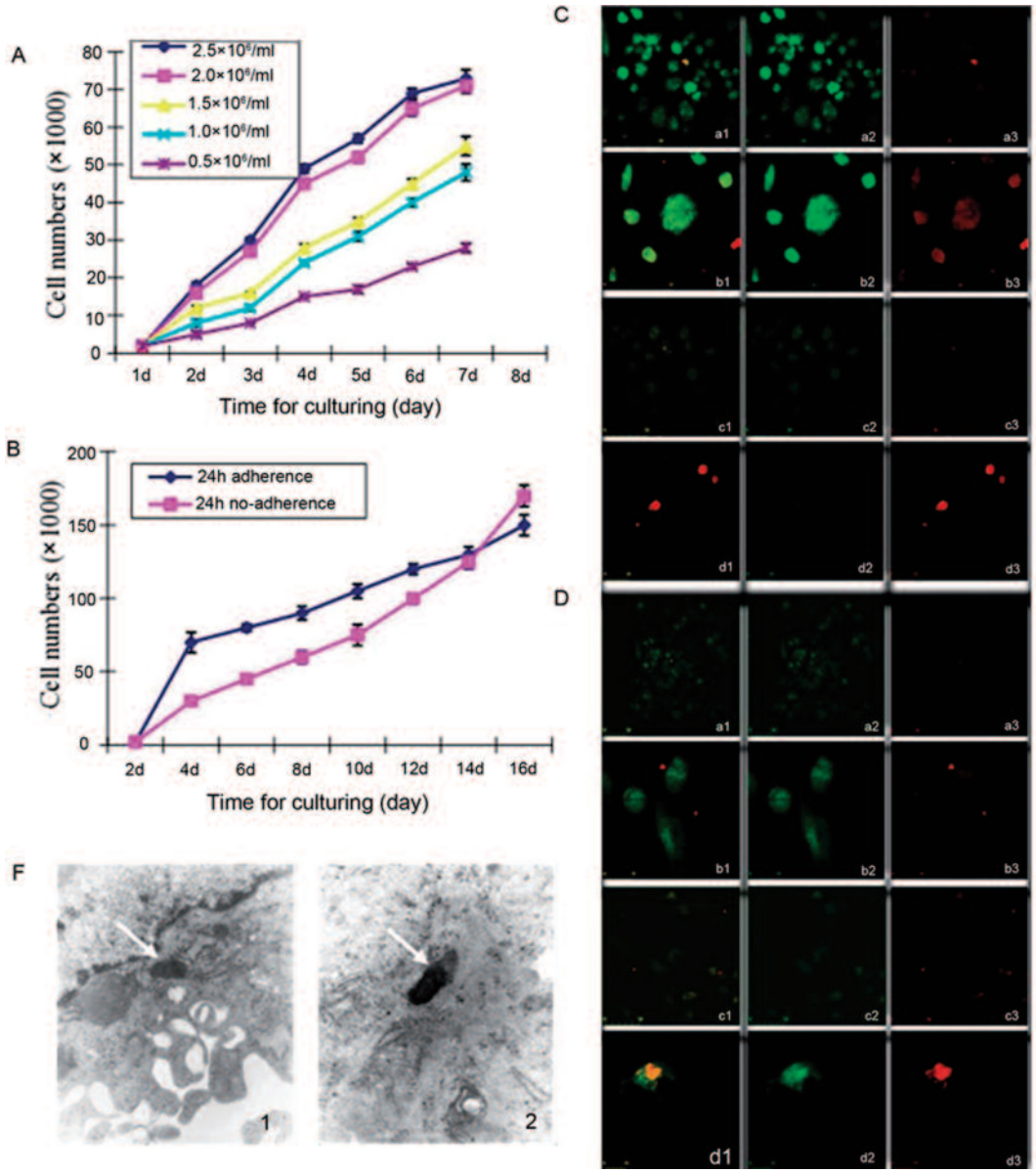

Figure 1. Validation of EPCs isolated from bone marrow of minipig. ***A***, growth curves for EPCs with different initial cell seeding concentrations. ***B***, growth curves for two different selection methods, 24 h non-adherent and 24 h adherent. ***C*** and ***D*** are immunofluorescence results (HE ×400) for fluorescence double labeling after culturing for 7 days for 24 h adherent and non-adherent sets, respectively. a1, CD133+ VIII double labeling; a2, VIII labeling; a3, CD133 labeling; b1, CD133+Flk-1 double labeling; b2, Flk-1 labeling; b3, CD133 labeling; c1, CD133+CD31 double labeling; c2, CD31 labeling; c3, CD133 labeling; d1, DiI-acLDL+CD31 double labeling; d2, CD31 labeling; d3, DiI-acLDL labeling. ***E***, electron microscope results for 24 h adherent (1) and 24 h non-adherent (2) EPCs sets. The arrow points to the Weibel-Palade.

Statistical Analysis

All experiments were performed at least three times. SPSS13.0 (SPSS Inc., Chicago, IL, USA) was used for all analyses. All data were expressed as mean ± standard deviation (SD, x ± s). The standard deviation was analyzed with one-factor analysis of variance. $p < 0.05$ was considered statistically significant.

Results

Validation of EPCs Isolated from Bone Marrow of Minipig

We first checked if our isolated EPCs have a healthy growth rate. There is a linear growth rate within 7 days after seeding the cells with different initial cell numbers (Figure 1A). Cells have a

Table I. Comparison of secret functions between two sets of cells (n = 6, mean SD).

Group	NO	TNOS	iNOS	cNOS
1 (24h adherent)	25.19 ± 1.06	4.19 ± 0.95	0.34 ± 0.18	3.86 ± 0.94
2 (24h non-adherent)	26.10 ± 1.09[a]	4.99 ± 1.07[a]	0.25 ± 0.11	4.74 ± 1.07[a]

[a]$p < 0.05$ 24h non-adherent set compared with 24h adherent set

faster growth rate when the initial cell number for seeding is increased. When comparing two sets of cells (set 1: 24 hours adherent cells; set 2: 24 hours non-adherent cells), we found that set 1 cells grow faster at the beginning, while proliferation rate is faster for set 2 cells after 10 days culture and the total cell number is higher for set 2 cells after 16 days (Figure 1B).

CD34+, Flk-1+, CD133+ are biological markers on the surface of EPCs[20-22]. We then detected if these markers are present in our isolated two sets of cells during the culture by immunofluorescence experiments (Figure 1C and D). The immunofluorescence results suggest that biological markers on the surface of EPCs can both induced in either set and the difference between these two sets is not significant. Weibel-Palade can be seen for both sets of cells detected by electron microscope (Figure 1E), but there are some cells in set 1 with some properties of cardiac muscle and fibrocyte.

Two sets of cells can both secret NO and NOS (Table I). This suggests that these two sets of cells possess the normal secret functions of EPCs. Table I also shows that set 2 cells have higher levels of NO, TNOS, and cNOS than that of set 1 cells.

By comparing these two sets of cells, we will use set 2 cells to further our studies since set 2 cells have a higher growth rate in a longer time with higher secretion levels of NO and NOS.

The Tolerance of EPCs to I/R Injury

No proliferation was detected in the cells for 4, 5 and 6 hours after ischemia according to our MTT assay and most of the cells were dead and floating after 4 hours. Partial cells were dead in the 2 and 3 h groups, but a certain number of cells were still alive. Though cells in the 3 h group were affected by I/R injury, their proliferation abilities were recovered after reperfusion. The results may suggest that I/R injury could lead to irreversible injury to the majority of EPCs ischemia for more than 4 h, which could cause dysfunction and even death of the cells.

The Growth Curve for Each Group and MTT Results After I/R Injury (IRI)

To get the optimized concentration of metoprolol and small intestine RNA in the treatment of EPCs, we measured the EPCs growth curve under different conditions (Figure 2A and B). Figure 2A showed that 1×10^6 mol/L metoprolol promote the cell proliferation most. And 20 μg/ml of small intestine RNA is the best condition for cell growth (Figure 2B). We will use the optimized concentrations of metoprolol and small intestine RNA obtained here to perform the following treatments.

For metoprolol treatment groups (Figure 2C and E), the proliferation ability of the metoprolol pretreatment group was stronger than those of the group with the I/R plus metoprolol and the sole I/R injury group. There was no significant difference compared the pretreatment group with that of the normal control group ($p > 0.05$), but it was significantly lower than that of the sole metoprolol treatment group. For the small intestine RNA treatment experiments (Figure 2D and F), it showed that the small intestine RNA pretreatment group has a better growth rate than that of I/R plus small intestine RNA group after I/R injury, but lower than that of normal control group ($p < 0.01$).

Result of NO, NOS and LDH Measurements

As can be seen from Table II and Table III, the LDH content of the metoprolol or small intestine RNA pretreatment group was lower than that of I/R injury group and I/R injury plus metoprolol or small intestine RNA treatment group ($p < 0.05$ and $p < 0.01$, respectively), but greater than that of the normal control and sole metoprolol or small intestine RNA treatment groups ($p < 0.01$).

The metoprolol pretreatment group had a lower level of NO than the sole I/R injury group ($p < 0.05$), but there was no statistical significance comparing it with I/R injury plus metoprolol treatment and the sole metoprolol treatment groups ($p > 0.05$). But the small intestine RNA

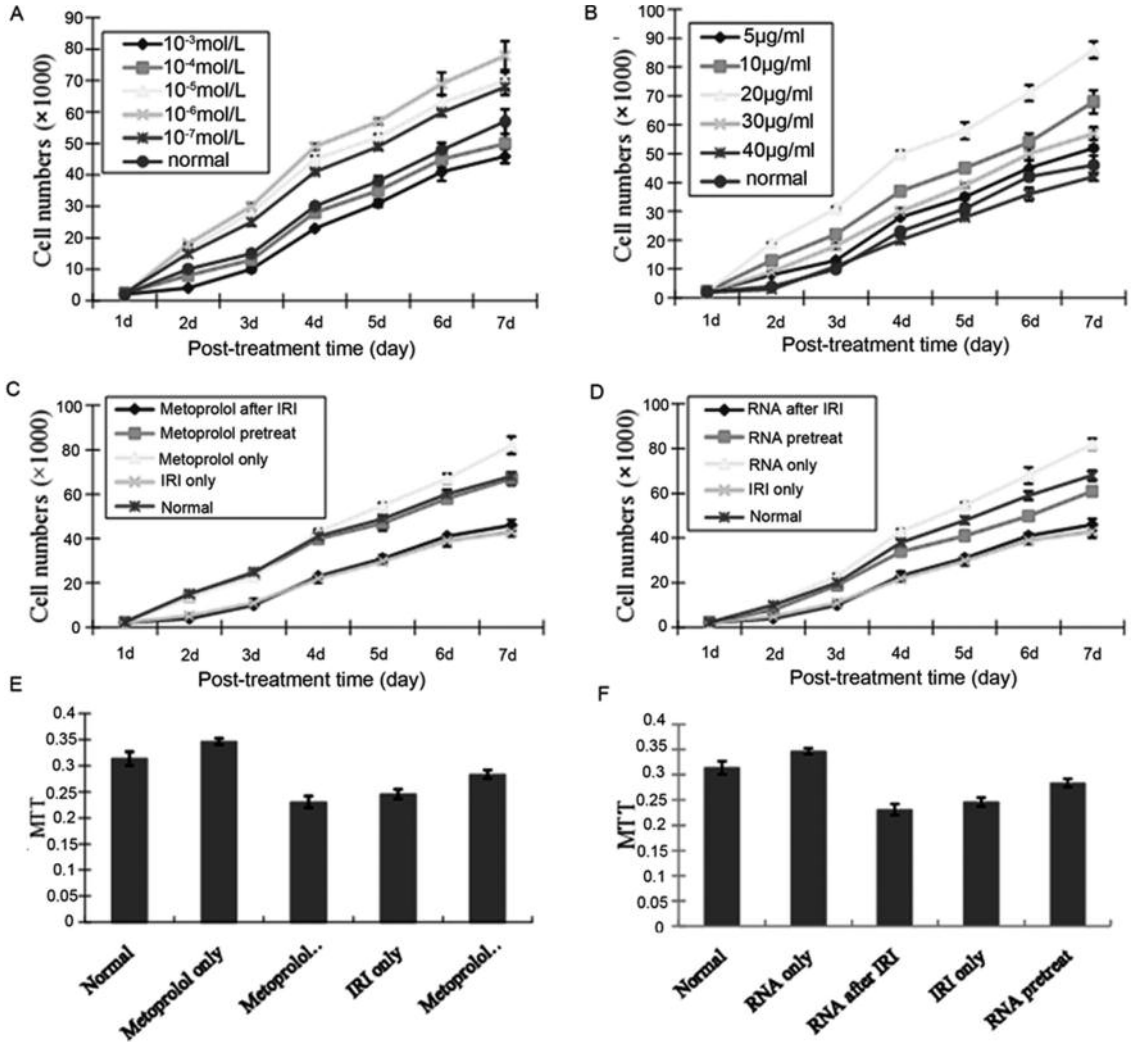

Figure 2. Growth curves for each group and MTT results after I/R injury. EPCs growth rates were affected by different concentration of metoprolol ***(A)*** and small intestine RNA ***(B)***. The numbers of cells were counted each day after I/R injury and the cell growth curves were plotted for each group for the treatment of metoprolol ***(C)*** and small intestine RNA ***(D)***. The effects of metoprolol ***(E)*** and small intestine RNA ***(F)*** after I/R injury were measured by MTT assay.

pretreatment group had a highest level of NO among all 5 groups ($p < 0.01$).

There was statistical significance among all the groups in the iNOS content ($p < 0.05$). Especially, the iNOS of the metoprolol pretreatment group was lowest among those of the sole I/R injury group and the I/R injury plus metoprolol treatment group. And the content of iNOS of the I/R injury plus metoprolol treatment group was the highest among all the other groups ($p < 0.01$).

Cell Apoptosis and Mortality

To further validate which kind of treatment is the best for EPCs proliferation after I/R injury, we examined the cell apoptosis and mortality in the next step. As shown in Figure 3A, the cell apoptosis rate in the sole metoprolol treatment group was lower than that of the normal control group and it was lower than that of the I/R injury related group. There was less cell apoptosis in the metoprolol pretreatment group than in the sole I/R injury group and a little lower than that of the group with addition of metoprolol after I/R injury. There was the highest cell mortality rate in the group with the addition of metoprolol after I/R injury. Small intestine RNA also has the same properties as showed in Figure 3B. Our data here suggested that pretreatment of EPCs with metoprolol or

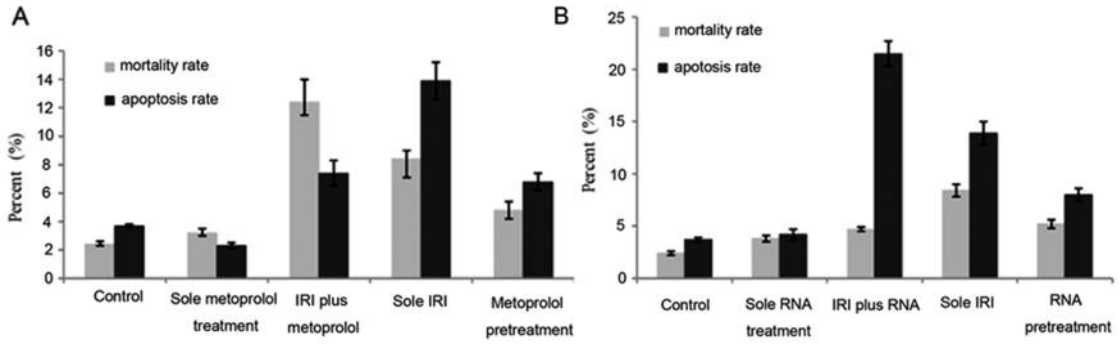

Figure 3. Cell apoptosis and mortality rate for every group. EPCs apoptosis and mortality rate were measured through FACS with annexin v-PI staining for different groups affected by metoprolol ***(A)*** and small intestine RNA ***(B)***.

small intestine RNA can prevent the cell apoptosis from I/R injury and reduce the mortality rate.

Discussion

EPCs play an important role in postnatal vasculogenesis and attract attentions of many researchers for cell transplantation. Recent researches had shown that only parts of cells come from marrow EPCs in vasculogenesis after injury and more and more researches showed that marrow-derived EPCs are more engaged in local injury recovery through their secretion functions rather than integrating into the target[23,24]. The recent researches indicated that the microenvironment is more important than the stem cells themselves in determining their fates. The changes in the microenvironment may play a pivotal role in directing differentiation of the stem cells, but may have a side effect on their proliferations and functions[25-27]. It is an urgent issue to deal with the "dual-side" effects in order to get the benefit in clinical usage of EPCs. In addition, EPCs transplantation is especially useful for the old patients or the patients with diabetes or hyperlipemia since these patients have lower responses to the cell/growth factors. Therefore, promotion of bioactivity of the EPCs with the suitable treatment *in vitro* is a key step in improving their therapeutic functions. In this study, we showed that EPCs could be cultured by two adherent methods (24 h adherent and 24 h non-adherent) and the 24 h non-adherent cells are better than those of 24 h adherent sets by comparing the biomarkers, the purity, maturity, and cell functions. Pretreatment of EPCs with metoprolol or small intestine RNA could reduce EPCs apoptosis rate and decrease the LDH and iNOS generation.

Metoprolol is now widely used in the treatment of ischemic heart diseases. Our study showed that the proliferation in the sole metoprolol group was similar to that of the normal group in a short time and became higher at about 1 week, which may be due to upregulation of VEGF secretion in EPCs by metoprolol. It is a new attempt to investigate its protective role in transplanted cells after I/R injury. The addition of

Table II. Effects of IRI (I/R injury) and metoprolol on EPCs secretions (n=6, mean SD).

Group	Normal control	Sole meloprolol	IRI plus metoprolol	Sole IRI	Metoprolol pretreatment
LDH (U/ml)	1997 ± 117[b]	1948 ± 81[b]	2893 ± 119[b]	2782 ± 127[b]	2339 ± 59
NO (μmol/L)	29.70 ± 3.68[b]	47.16 ± 3.30	55.82 ± 3.91	59.26 ± 3.80[a]	50.73 ± 4.41
TNOS (U/ml)	15.30 ± 1.23[b]	17.44 ± 0.64	18.74 ± 0.89	19.75 ± 1.35	19.16 ± 0.80
iNOS (U/ml)	1.72 ± 0.68[b]	3.95 ± 1.15[a]	16.04 ± 1.50[b]	13.09 ± 1.32[b]	6.88 ± 1.35
cNOS (U/ml)	13.58 ± 0.97	13.49 ± 1.51	2.71 ± 0.66[b]	6.66 ± 0.97[b]	12.28 ± 1.04
iNOS/TNOS%	11.12 ± 3.95[b]	22.77 ± 6.87[b]	85.41 ± 4.11[b]	66.27 ± 4.73[b]	35.79 ± 6.24

[a]$p < 0.05$, [b]$p < 0.01$ vs metoprolol pretreatment group.

Table III. Effects of IRI and small intestine RNA on EPCs secretions (n = 6, mean SD).

Group	Normal control	Sole RNA	IRI plus RNA	Sole IRI	RNA pretreatment
LDH (U/ml)	1997 ± 117[b]	2041 ± 83[b]	3133 ± 91[b]	2782 ± 127[b]	2456 ± 70
NO (μmol/L)	29.70 ± 3.68[b]	50.93 ± 3.06[b]	62.10 ± 3.85[b]	59.26 ± 3.80[b]	91.67 ± 3.40
TNOS (U/ml)	15.30 ± 1.23	13.41 ± 1.08	9.48 ± 1.21[b]	19.75 ± 1.35[b]	14.47 ± 0.79
iNOS (U/ml)	1.72 ± 0.68[b]	3.22 ± 0.89[b]	8.18 ± 1.08[a]	13.09 ± 1.32[b]	10.27 ± 0.98
cNOS (U/ml)	13.58 ± 0.97[b]	10.19 ± 1.54[b]	1.32 ± 0.51[b]	6.66 ± 0.97[a]	4.2 ± 0.91
iNOS/TNOS%	11.12 ± 3.95[b]	24.28 ± 7.74[b]	86.32 ± 5.25[b]	66.27 ± 4.73	70.99 ± 5.89

[a]$p < 0.05$, [b]$p < 0.01$ vs RNA pretreatment group

metoprolol after I/R injury facilitates little proliferation function within a short period of time, but causes much apoptosis and mortality. The levels of iNOS in cells for metoprolol pretreatment was the highest one among all the groups, which suggests that metoprolol pretreatment may benefit the patients, but the use of metoprolol right after I/R injury may be harmful. That is, pretreatment with metoprolol may promote cell proliferation and recover secretion function. Pretreatment also facilitates the reduction of both EPCs apoptosis and deleterious NO formation, thereby decreasing maleficent NO and LDH secretion so as to protect the EPCs. However, the mechanism still needs more study for clarity.

Conclusions

In our study, we found that EPCs have a similar proliferation rate treated with small intestine RNA in short period comparing with normal control group, but a higher growth rate appears after 1 week. This may be due to the increment of mRNA in EPCs induced by small intestine RNA. But pretreatment of EPCs with small intestine RNA after I/R injury will alleviate the effect of microenvironment changing and reduce the secretion of LDH and iNOS. It indicated that the mechanisms of effects on EPCs by metoprolol and small intestine RNA are different. In the future study, we will sequence and classify the purified small intestine RNA and continue to figure out the effective RNA with the function in EPCs for I/R injury. To further better understand and improve the EPCs transplantation after I/R injury, we will use pretreated EPCs with metoprolol or small intestine RNA and study the therapeutic functions of these EPCs in acute myocardial infarction animal model.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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