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Pioglitazone and low dose of cyclosporin A have synergistic effects in ameliorating rejection after heart transplantation in rats.

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Abstract

To investigate the effect of Pioglitazone (Pio) on the rejection reactions that occurs in heart transplantation. A total of 96 rats with heart transplantation were treated with cyclosporin A (CsA) alone, Pio alone, or the combination of both. The percentages of $CD25^+$ and $CD28^+$ T cell in the spleen of rats were measured using flow cytometry. Combined use of Pio and CsA prolonged the survival of transplanted hearts. In addition, pathologic lesions were relieved. Meanwhile, the contents of interleukin-2 in serum and the percentages of $CD25^+$ and $CD28^+$ lymphocytes in the receptor's spleens were significantly reduced by the combination of Pio and CsA. Of note, the effect of combined use of Pio and CsA was better than that of single use. The present study demonstrates that combined use of Pio and CsA reduces the dose of CsA, ameliorates rejection reactions, and improves the life quality of recipients with heart transplantation.

Keywords: Pioglitazone, Cyclosporin A, Rejection, Lymphokine.

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Introduction

Heart transplantation is surgical transplantation of hearts performed on patients with advanced congestive heart failure or severe coronary artery diseases [1]. It is widely used in clinical practice, as the most effective therapy for patients with end-stage heart diseases [1]. Due to advances in technology, the survival rate and duration of heart transplant recipients have been significantly increased [2,3]. As the main threat for successful heart transplantation, rejection reaction is the most difficult problem facing all heart transplant recipients [4-6]. Therefore, the development of drugs against rejection reaction attracts more and more attention from researchers.

Peroxisome Proliferator-Activated Receptor (PPAR) is expressed in macrophages, T cells, vascular smooth muscle cells and cardiomyocytes, participating in the regulation of various inflammatory responses [7,8], and the expression of tumor necrosis factor-α and inducible nitric oxide synthase [9]. The agonist of PPAR can improve heart function by inhibiting myocardial hypertrophy and fibrosis *via* lowering the activity of nuclear factor-kappa B [10]. Pioglitazone (Pio) is a highly selective agonist of PPAR that participates in immune regulation by inhibiting the proliferation of T cells *in vitro* [11]. Pio also plays important roles in inflammation, arteriosclerosis, and reperfusion injury [12], and promotes inflammatory cell apoptosis [13]. As an outstanding immunosuppressant, cyclosporin A (CsA) enhances the recent survival rate of solid organ transplantats, and becomes the first choice for immunosuppression and anti-rejection after organ transplantation [14-16]. In this study, we investigate the effect of Pio on the symptoms occurring in rat heart transplantation, the survival time of transplanted heart, and immunologic activities.

Materials and Methods

Animals

In the present study, Wistar rats (n=96) were used as donors, and Sprague-Dawley rats (n=96) were used as recipients to build the animal models for intraperitoneal heart transplantation. The recipient rats were randomly divided into 6 groups of 16 rats: Group I (normal control group) was not treated with CsA or Pio; Group II (normal amount CsA group) received intraperitoneal injection of 5 mg/kg CsA at 1 h before surgery and daily after surgery; Group III (normal amount CsA +Pio group) received intraperitoneal injection of 5 mg/kg CsA and intragastric administration of 10 mg/kg Pio at 1 h before surgery and daily after surgery; Group IV (half normal amount CsA group) received intraperitoneal injection of 2.5 mg/kg CsA at 1 h before surgery and daily after surgery; Group V (half normal amount CsA+Pio group) received intraperitoneal injection of 2.5 mg/kg CsA and intragastric administration of 10 mg/kg Pio at 1 h before surgery and daily after surgery; Group VI (Pio group) received intragastric administration of 10 mg/kg Pio at 1 h before surgery and daily after surgery. After surgery, heart beat conditions were determined by careful

touches by hand. All animal experiments were conducted according to the ethical guidelines of Qingdao University.

Tissues

On d 3, 7, 14 and 18, four rats in each group were sacrificed for laparotomy. Blood (2 ml) was collected from the inferior vena cava and centrifuged at 1500 rpm for 5 min. The separated serum was mixed with phenylmethanesulfonyl fluoride to prevent degradation, and frozen at -80°C for future ELISA. The excised heart tissues were fixed with 4% paraformaldehyde for pathological examinations. The spleen was made into cell suspension for flow cytometry.

Hematoxylin and eosin staining

After dewaxing by xylene, the sections were treated with high to low concentrations of ethanol, before rinsing with water, acid and ammonia. After washing with flowing water for 1 h, the sections were dehydrated using 70% and 90% ethanol for 10 min each. Then, the sections were stained with eosin for 3 min, before dehydration by pure ethanol and transparency by xylene. Stained slides were cover-slipped with Permount. Finally, the entire HE stained cells were examined under a bright light microscope using 100-400X magnification. The pathological examinations were performed according to the standard by International Society of Heart and Lung Transplantation (ISHLT).

Enzyme-linked immunosorbent assay (ELISA)

The level of interleukin (IL)-2 was measured using the ELISA kit (BD Biosciences, USA). The procedure was carried out according to the manufacturer's manual. Absorbance at 450 nm was measured using a microplate reader (DJ3022, Potenov Technology Co., Ltd., Beijing, China) within 15 min after stopping the reactions. The concentrations of IL-2 were calculated by plotting standard curves.

Flow cytometry

The spleen was collected from rats after transplantation for the examination of T-lymphocyte subsets. Single-cell suspensions (1×10^6 cells/ml) were stained in cell staining buffer at saturating concentrations according to standard procedures. Cells were stained for $CD25^+$ and $CD28^+$ monoclonal antibodies conjugated to Fluorescein Isothiocyanate (FITC) and Foxp3 monoclonal antibodies conjugated to Phycoerythrin (PE).

Statistical analysis

All statistical analyses were performed with SPSS 18.0 for Windows. Results were expressed as means ± standard deviation for test of normality. If the data conform to normal distribution and the variance is homogeneous, multigroup measurement data were analysed by ANOVA and Dunnett multiple comparison tests and two groups of data were compared by Student's t-test. If the data does not conform to normal distribution or variance is not uniform, multigroup data were checked by Kruskal-Wallis test and Tamhane's T2 or Dunnett's T3 methods, while two groups of data were examined by Mann-Whitney method. $P<0.05$ indicated statistically significant differences, while $P<0.01$ indicated differences that were even more significant.

Results

Pio alone prolongs the survival time of transplanted hearts

To investigate whether Pio affects the survival of transplanted hearts, we measured rat survival rate on different days. The data showed that rats in Group VI had significantly higher survival rates compared with those in Group I on the same day ($P<0.05$). The survival rate in Group VI reached 0 on d 12, while that in Group I reached 0 on day 9 (Figure 1). These data suggest that Pio alone prolongs the survival time of transplanted hearts.

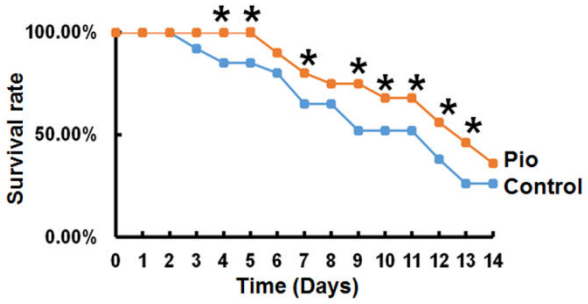

Figure 1. *Survival rates of rats in Group I and Group VI. Group I (normal control group) was not treated with CsA or Pio; Group VI (Pio group) received intragastric administration of 10 mg/kg Pio at 1 h before surgery and daily after surgery.* $^*P<0.05$.

Pio reduces the severity of rejection reactions of transplanted heart, having synergistic effects with CsA

To evaluate the pathology and rejection reactions of transplanted hearts, visual inspection and ISHLT classification were performed. On d's 3, 7, 14 and 28 after surgery, the size, shape, color and heartbeat power of transplanted hearts in Groups III and V appeared to be better than those in other groups. In addition, use of Pio in Groups III, V and VI ameliorated peritoneal adhesions (data not shown). On d 3 after surgery, the ISHLT grades of rejections in Groups II-V were significantly lower than that in Group I ($P<0.01$), rejection grade in Group VI was also significantly lower than that in Group I ($P<0.05$), and rejection grades in Groups III and V were significantly lower than that in Group VI ($P<0.05$) (Figure 2A). On d 7 after surgery, the ISHLT grades of rejections in Groups II-V were significantly lower than that in Group I ($P<0.05$), and rejection grades in Groups II-V were significantly lower than that in Group VI ($P<0.05$) (Figure 2B). On d 14 after surgery, rats in Groups I and VI were all grade 4, most rats in Group II were grade 3A, most rats in Group III were grade 2, most rats in Group IV were grade 3A, and most

rats in Group V were grade 1B. On d 28 after surgery, the grade of rejections in Group III was lower than Group II, while that in Group V was milder than Group IV. These results indicate that Pio reduces the severity of rejection reactions of transplanted heart, having synergistic effects with CsA.

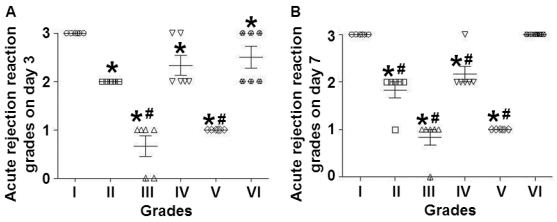

Figure 2. *Acute rejection reaction grades on (A) d 3 and (B) d 7 after surgery. *P<0.05 compared with Group I; #P<0.05 compared with Group VI.*

Combined use of half normal dose CsA and Pio has the best effect in reducing the levels of IL-2 after heart transplantation surgery

To determine the levels of serum IL-2, ELISA was performed. On d 3 after surgery, the levels of IL-2 in Groups II-V were significantly lower than that in Group I or Group VI (P<0.01), and that in Group VI was significantly lower than that in Group I (P<0.05). On d 7 after surgery, the levels of IL-2 in Groups II-V were significantly lower than that in Group I or Group VI (P<0.01), and that in Group VI was also significantly lower than that in Group I (P<0.01). In addition, the level of IL-2 in Group III was significantly lower than that in Group V (P<0.05). On d 14 after surgery, the levels of IL-2 in Groups III and V were significantly lower than that in Group I (P<0.01), and those in Groups II and IV were also significantly lower than that in Group I (P<0.05). In addition, the levels of IL-2 in Groups II, IV and VI were significantly higher than that in Group V (P<0.05) (Table 1). These results suggest that combined use of half normal dose CsA and Pio has the best effect in reducing the levels of IL-2 after heart transplantation surgery.

Pio alone, or its combination with CsA, delays the appearance of CD25+ and CD28+ T cells in spleen

To detect the spleen lymphocyte subtype markers in cells, flow cytometry was used. On d 3 after surgery, the percentages of $CD25^+$ cells in T lymphocytes in Groups II-VI were significantly lower than that in Group I (P<0.05). On d 7 after surgery, the percentages of $CD25^+$ cells in T lymphocytes in Groups II-VI were significantly lower than that in Group I (P<0.01), and those in Groups II-V were significantly lower than that in Group VI (P<0.05). On d 14 after surgery, no significant differences were observed between any two groups (P>0.05) (Table 2). On d 3 after surgery, the percentages of $CD28^+$ cells in T lymphocytes in Groups II-VI were significantly lower than that in Group I (P<0.01). On d 7 after surgery, the percentages of $CD28^+$ cells in T lymphocytes in Groups II-V were significantly lower than that in Group I or Group VI (P<0.01). On d 14 after surgery, no significant differences were observed between any two groups (P>0.05) (Table 3). These results indicate that Pio alone, or its combination with CsA, delays the appearance of $CD25^+$ and $CD28^+$ T cells in spleen.

Table 1. *Concentrations of interleucin-2 on d's 3, 7 and 14 after surgery (pg/ml) (means ± standard deviation).*

Groups	D 3	D 7	D 14
I	813.2 ± 28.4	935.2 ± 43.5	707.6 ± 35.5
II	603.5 ± 31.8$^{*\#}$	617.8 ± 35.4$^{*\#}$	612.4 ± 41.3$^{\Delta\$}$
III	562.4 ± 23.5$^{*\#}$	573.3 ± 29.6$^{*\#a}$	570.8 ± 32.2*
IV	623.8 ± 30.5$^{*\#}$	645.6 ± 28.4$^{*\#a}$	618.8 ± 34.5$^{\Delta\$}$
V	559.7 ± 21.3$^{*\#}$	568.4 ± 27.5$^{*\#}$	536.8 ± 27.8*
VI	729.5 ± 52.4$^{\Delta}$	737.2 ± 31.0*	685.5 ± 25.0$^{\$}$

Note: *P<0.01 compared with Group I; $^{\#}$P<0.01 compared with Group VI; $^{\Delta}$P<0.05 compared with Group I; ^{a}P<0.05 between each other; $^{\$}$P<0.05 compared with Group V.

Table 2. *The percentage of $CD25^+$ cells in T lymphocytes in spleen cell suspension on d's 3, 7 and 14 after surgery (%, means ± standard deviation).*

Groups	Day 3	Day 7	Day 14
I	15.77 ± 3.1	33.29 ± 5.2	10.79 ± 3.8
II	7.34 ± 2.4$^{\Delta}$	9.75 ± 3.6$^{*\#}$	8.62 ± 2.7
III	6.44 ± 2.8$^{\Delta}$	8.65 ± 3.1$^{*\#}$	11.35 ± 4.3
IV	7.29 ± 2.5$^{\Delta}$	12.12 ± 4.3$^{*\#}$	14.26 ± 2.4
V	6.22 ± 3.0$^{\Delta}$	7.48 ± 2.8$^{*\#}$	7.15 ± 2.3
VI	8.28 ± 3.5$^{\Delta}$	20.77 ± 5.6*	9.02 ± 3.7

Note: $^{\Delta}$P<0.05 compared with Group I; *P<0.01 compared with Group I; $^{\#}$P<0.05 compared with Group VI.

Table 3. *The percentage of $CD28^+$ cells in T lymphocytes in spleen cell suspension on d's 3, 7 and 14 after surgery (%, means ± standard deviation).*

Groups	Day 3	Day 7	Day 14
I	5.17 ± 1.32	7.24 ± 0.98	1.75 ± 0.24
II	1.82 ± 0.21*	1.93 ± 0.33$^{*\#}$	1.41 ± 0.32
III	1.39 ± 0.31*	1.65 ± 0.27$^{*\#}$	1.05 ± 0.25
IV	2.40 ± 0.42*	2.43 ± 1.02$^{*\#}$	2.24 ± 0.93
V	1.06 ± 0.29*	1.04 ± 0.41$^{*\#}$	1.03 ± 0.57
VI	2.46 ± 0.67*	7.82 ± 1.71	1.28 ± 0.43

Note: *P<0.01 compared with Group I; $^{\#}$P<0.01 compared with Group VI.

Discussion

The present study showed that rats treated with Pio had better recovery after surgery, longer survival time, and extenuated

rejection reactions. In addition, combined use of Pio and CsA showed longer survival time and milder rejection reactions compared with single use of CsA or Pio, demonstrating synergistic effects. IL-2 is a kind of cell factor with extensive biological activities. It enhances non-specific immune response and antigen-specific adaptive immune response by activating cytotoxic T cells and natural killer cells. After heart transplantation, serum levels of IL-2 are gradually elevated as the aggravation of rejection. After the peak time of rejection reactions, serum levels of IL-2 begin to reduce. In the present study, Pio delays the peak time of IL-2, and reduces the concentration of IL-2. Of note, combined use of Pio and CsA has better effect in lowering IL-2 levels compared with using CsA alone.

CD28 molecules can be expressed in all $CD4^+$ T cells or 50% $CD8^+$ T cells. In rejection reactions induced by organ transplantation, the activation of T cells must be triggered by the dual signals presented by antigen-presenting cells. CD28, as a receptor of B7, is mainly expressed on the surface of activated T lymphocytes. The binding of B7 with CD28 transfers activation signals, while the binding of B7 with CTLA4 (CD152) transfers inhibition signals [17]. The binding of B7 and CD28 increases the transcription of interleukin mRNA, enhancing the secretin of IL-2 and the proliferation and differentiation of T cells. In the deficiency of CD28, 90% of IL-2 mRNA can be degraded in 90 minutes, probably because the binding between B7 and CD28 enhances *IL-2* gene transcription and stabilizes IL-2 mRNA signals [18]. Rejection reactions in heart transplantation are caused by the direct killing effect by activated $CD8^+$ cytotoxic immune cells. Russell et al. reported that the survival rate of F344 rats with transplanted hearts in CTLA4-LgG treatment group was 64% on d 70, while that in CsA treatment group was 26% [19]. The frequency and severity of arteriosclerosis in CTLA4-LgG treatment group were significantly lower than those in CsA treatment group [19]. Dengler demonstrated that rats treated with CD28 monoclonal antibody had prolonged survival time after heart transplantation [20]. These reports show that CD28 is closely related to rejection reactions after heart transplantation. In the present study, the percentage of spleen $CD28^+$ cells in rats untreated with any drug was significantly increased on d 3 after surgery, and reached peak on d 7. After the acute phase of rejection, the percentage of $CD28^+$ T cells was quickly reduced. Pio delays the appearance of $CD28^+$ T cells in the spleen, while CsA alone or the combination of CsA and Pio significantly inhibited the appearance of $CD28^+$ T cells in the spleen.

Anti-CD25 monoclonal antibody is a novel drug used in organ transplantation. It effectively prolongs the survival time of transplanted organs, especially when it is used together with CsA [21]. CD25 participates in rejection reactions by increasing the expression of myosin heavy chain types I and II antigens, and by affecting the survival of transplanted organ *via* the regulation of cytokines [22]. Of note, CD25 can cause deviation of the expression of cytokines towards Th1 type, enhancing the expression of *IL-4, IL-5, IL-6* and *IL-10*, and prolonging the survival time of transplanted organs [23]. Immune deviation is demonstrated to be beneficial for the induction and formation of immunologic tolerance. High expression of *IL-2*, *IL-2R* and *interferon-γ* is detected in rejected organs. This phenomenon can be explained by the karyotype differentiation from Th1 to Th2 [24]. However, some other report argue that Th2 immune deviation promotes the occurrence of chronic rejection reactions in transplanted organs with relatively long survival time [25]. Therefore, the long survival of transplanted organs needs the absence of expression of Th1 and Th2 type cytokines. In the present study, higher levels of spleen $CD25^+$ correspond to more severe early rejection rejections. Pio alone, CsA alone or the combination of the two inhibits the early expression of $CD25^+$ in spleen. In conclusion, Pio ameliorates inflammatory responses after heart transplantation in rats, prolongs the survival time of transplanted hearts when used in combination with low dose of CsA, and exerts synergistic effects with CsA in inhibiting rejection reactions.

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Disclosures

All authors declare no financial competing interests. All authors declare no non-financial competing interests.

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