

# Changes in the action potential and transient outward potassium current in cardiomyocytes during acute cardiac rejection in rats

Wenqi Luo, Yixin Jia, Shuai Zheng, Yan Li, Jie Han, Xu Meng

Department of Cardiac Surgery, Beijing Anzhen Hospital, Capital Medical University, Beijing Institute of Heart Lung and Blood Vessel Disease, Beijing 100029, China

**Contributions:** (I) Conception and design: X Meng, Y Jia, W Luo; (II) Administrative support: X Meng, Y Li, J Han; (III) Provision of study materials or patients: X Meng, Y Jia; (IV) Collection and assembly of data: W Luo, S Zheng; (V) Data analysis and interpretation: Y Jia, W Luo, X Meng; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

**Correspondence to:** Xu Meng. The director of Department of Cardiac Surgery, Beijing Anzhen Hospital, Capital Medical University, Beijing Institute of Heart Lung and Blood Vessel Disease, No. 2 Anzhen Road, Beijing 100029, China. Email: mxheart09@sina.com.

**Background:** Acute cardiac rejection contributes to the changes in the electrophysiological properties of grafted hearts. However, the electrophysiological changes of cardiomyocytes during acute cardiac rejection are still unknown. An understanding of the electrophysiological mechanisms of cardiomyocytes could improve the diagnosis and treatment of acute cardiac rejection. So it is important to characterize the changes in the action potential (*AP*) and the transient outward potassium current (*I<sub>to</sub>*) in cardiomyocytes during acute cardiac rejection.

**Methods:** Heterotopic heart transplantation was performed in allogeneic [Brown Norway (BN)-to-Lewis] and isogenic (BN-to-BN) rats. Twenty models were established in each group. Ten recipients were sacrificed at the 2<sup>nd</sup> day and the other ten recipients were sacrificed at the 4<sup>th</sup> day after the operation in each group. Histopathological examinations of the grafted hearts were performed in half of the recipients in each group randomly. The other half of the grafted hearts were excised rapidly and enzymatically dissociated to obtain single cardiomyocytes. The *AP* and *I<sub>to</sub>* current were recorded using the whole cell patch-clamp technique.

**Results:** Forty grafted hearts were successfully harvested and used in experiments. Histologic examination showed mild rejection at the 2<sup>nd</sup> day and moderate rejection at the 4<sup>th</sup> day in the allogeneic group after cardiac transplantation, while no evidence of histologic lesions of rejection were observed in the isogenic group. Compared with the isogenic group, the action potential duration (*APD*) of cardiomyocytes in the allogeneic group was significantly prolonged (*APD<sub>90</sub>* was 49.28±5.621 mV in the isogenic group and 88.08±6.445 mV in the allogeneic group at the 2<sup>nd</sup> day, P=0.0016; *APD<sub>90</sub>* was 59.34±5.183 mV in the isogenic group and 104.0±9.523 mV in the allogeneic group at the 4<sup>th</sup> day, P=0.0064). The current density of *I<sub>to</sub>* was significantly decreased at the 4<sup>th</sup> day after cardiac transplantation.

**Conclusions:** The *APD* of cardiomyocytes was significantly prolonged during acute cardiac rejection in rats, which might be partly attributed to decreased current densities of *I<sub>to</sub>*.

**Keywords:** Acute cardiac rejection; action potential (*AP*); transient outward potassium current; patch-clamp technique

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## Introduction

Cardiac transplantation has become an established therapeutic option for eligible patients with end-stage cardiac diseases. However, acute cardiac rejection remains a frequent complication that can severely threaten the patient's survival in the early days after cardiac transplantation (1,2). Earlier research has revealed that the ventricular voltage is decreased (3,4), the intramyocardial impedance is increased (5), the resting potential (*RP*) and transmembrane action potential (*AP*) amplitude of the ventricular muscle is decreased (6), and the QRS amplitude of the autonomous intramyocardial electrograms (IMEG) and the maximum slope of the descending T wave of the ventricular evoked response is decreased during acute cardiac rejection in experimental models (7). All of these suggest that the electrophysiological characteristics of cardiomyocytes could be changed during acute cardiac rejection. However, the mechanism of electrophysiological changes in the cardiomyocytes is still unclear. The *AP* is a sign of cells in an excited state, and the modification of the shape and duration of the *AP* could lead to changes in the electrophysiological properties of cardiomyocytes. The transient outward potassium current ( $I_{to}$ ) is the main component of the *AP* repolarization phase 1 in myocardial cells. The modification of the current amplitude and kinetic characteristics can significantly change the *AP* and other ionic conductances (8,9). The characteristics of the *AP* and  $I_{to}$  in cardiomyocytes during acute cardiac rejection have not yet been established. To gain more insight into the electrophysiological changes and their possible ionic mechanism, our study was designed to investigate the changes in the *AP* and  $I_{to}$  in cardiomyocytes in an abdominal heart transplantation model in rats during acute cardiac rejection.

## Methods

### Animals

Eight-week-old male Brown Norway (BN) rats (n=60) and Lewis rats (n=20) with an average body weight of 230 g (200–250 g) were obtained from the experimental animal center of the Beijing Anzhen Hospital, Capital Medical University. Forty BN rats were used as donors, and 20 BN rats and 20 Lewis rats were used as recipients. Food and water were provided by placing food pellets and water bottles on a grid located on top of the tank. The water in the tank was changed daily until the experimental endpoints.

All surgical interventions and postoperative animal care were performed in accordance with the National Institutes of Health Guide-lines for the Care and Use of Laboratory Animals (National Research Council, 1996, USA) and were approved by the Chinese National Committee on the Use of Experimental Animals for Medical Purposes, Beijing Branch. All procedures were performed on animals in an unconscious state. All efforts were made to minimize the number of animals used and their suffering.

### Heterotopic heart transplantation

Heterotopic cardiac transplantation was performed as described by Ono and Lindsey (10), with some modifications as described elsewhere (11). The rats were anesthetized using 2% phenobarbital sodium (50 mg/Kg). The average cold ischemic time was less than 45 min. The graft function was assessed daily via abdominal palpation by a single investigator. The cessation of a palpable cardiac contraction was defined as graft loss, which was confirmed after laparotomy. When graft loss occurred before their experimental endpoints for various reasons, new matched rats were introduced to maintain a constant number of recipient animals within each group.

### Experimental design

The 20 allogeneic recipient rats (BN-to-Lewis) and 20 isogeneic recipient rats (BN-to-BN) were randomly separated into 4 groups respectively (n=5). Five grafted hearts were randomly harvested for pathological examination, and another five grafted hearts were harvested for electrophysiological examination at the 2<sup>nd</sup> day and the 4<sup>th</sup> day after the operation.

### Pathological examination

At the 2<sup>nd</sup> day and the 4<sup>th</sup> day after the operation, a laparotomy was performed under intraperitoneal anesthesia by 2% pentobarbital sodium and the grafted heart was harvested while still beating, fixed with 10% phosphate buffered formalin, and embedded in paraffin. The specimen was sliced into samples 3 or 4  $\mu$ m in thickness and then stained with hematoxylin and eosin (HE). One investigator, in a blind fashion, routinely evaluated the stained sections (HE) of grafted hearts with a semiquantitative scale for myocyte loss and the degree of inflammation [compatible with the ISHLT rejection grades of human allografts (12)],

in which 0 indicates no inflammation and myocyte loss (ISHLT grade 0); 1 indicates perivascular inflammation (ISHLT grade 1A); 2 indicates interstitial inflammation (ISHLT grade 1B); 3 indicates inflammation with focal myocyte loss (ISHLT grade 2); 4 indicates inflammation with multifocal myocyte loss (ISHLT grade 3A); 5 indicates inflammation with confluent foci of myocyte loss (ISHLT grade 3B); and 6 indicates inflammation with large areas of necrosis (>25% myocyte loss) and/or necrotizing vasculitis (ISHLT grade 4) (13,14).

### Electrophysiological measurements

#### Solution

A Tyrode's solution was prepared containing (in mM/L): NaCl 126, KCl 5.4, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 1.8, NaH<sub>2</sub>PO<sub>4</sub> 0.33, glucose 10 and 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, and the pH was adjusted to 7.4 with NaOH. A Ca<sup>2+</sup>-free Tyrode's solution was prepared by removing CaCl<sub>2</sub> from the aforementioned Tyrode's solution.

The enzyme solution used for the rat cardiomyocyte isolation contained 0.1 g/L collagenase (type II) and 0.5 g/L BSA in Tyrode's solution.

For recording the  $I_{to}$  current, the pipettes were filled with (in mM/L): K-aspartame acid 140, MgATP 4, MgCl<sub>2</sub> 1, ethylene glycol tetraacetic acid (EGTA) 10, guanosine triphosphate (GTP) 0.1, HEPES 10, and the pH was adjusted to 7.3 with KOH.

For recording  $I_{to}$  currents, the myocytes were superfused with a solution containing (in mM/L): NaCl 140, KCl 4, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 1, HEPES 10, glucose 5, with the pH adjusted to 7.4 with NaOH. Tetrodotoxin inhibits the Na<sup>+</sup> current; CdCl<sub>2</sub> inhibits the Ca<sup>2+</sup> current.

#### Cell preparations

A single cardiomyocyte was dissociated by an enzymatic dissociation as described previously. Grafted hearts were excised rapidly after the recipient rats were given an intraperitoneal injection of 2% pentobarbital sodium (50 mg/Kg). The hearts were perfused retrogradely on a Langendorff apparatus with a Ca<sup>2+</sup>-free Tyrode's solution after an incision was made in the right atrium. Five minutes later, the perfusate was switched to an enzymatic solution for fifteen minutes. The perfusates were bubbled with 95% O<sub>2</sub> + 5% CO<sub>2</sub> and maintained at 37 °C. The ventricles were cut into small chunks and gently agitated in Ca<sup>2+</sup>-free Tyrode's solution. The myocytes were filtered through a nylon mesh (pore size 200 μm) and stored in Ca<sup>2+</sup>-free

Tyrode's solution at 4 °C.

#### Patch clamp experiments

Quiescent, calcium-tolerant, rod-shaped cells with a clear cross-striation were used for the *AP* recordings at 35 °C. The transmembrane potentials and currents were recorded using the whole-cell patch-clamp technique with an EPC10 amplifier (HEKA Instruments, Germany). All signals were acquired at 5 kHz and analyzed by Patchmaster version 2.72 software (HEKA Instruments, Germany). The whole-cell currents and *APs*, obtained under voltage clamp, were filtered at 1–5 kHz and sampled at 5–50 kHz, and the series resistance was typically <5 mega ohms after approximately 70% compensation.

The *APs* were elicited using the current-clamp mode at a rate of 5.0 Hz of 30-train suprathreshold current pulses. The cardiomyocytes were electrically stimulated by intracellular current injection through patch electrodes using depolarizing pulses with duration of 5 ms and an amplitude of 1,500 pA. The action potential duration (*APD*) was measured at 50% and 90% of repolarization (*APD*<sub>50</sub> and *APD*<sub>90</sub>).  $I_{to}$  was recorded using the voltage-clamp mode. We used a pre-pulse to –40 mV for 50 ms to inactivate  $I_{Na}$ .  $I_{to}$  was recorded in voltage-clamp mode with 300-ms pulses from a holding potential of –90 mV, with different test potentials increasing from –40 mV to +60 mV with 10-mV steps.

#### Statistical analysis

All data are presented as the mean ± SD. The curves were fitted with Origin 6.0. The statistical significance was determined using a *t*-test to compare multiple groups. Statistical significance was defined by a P value <0.05.

## Results

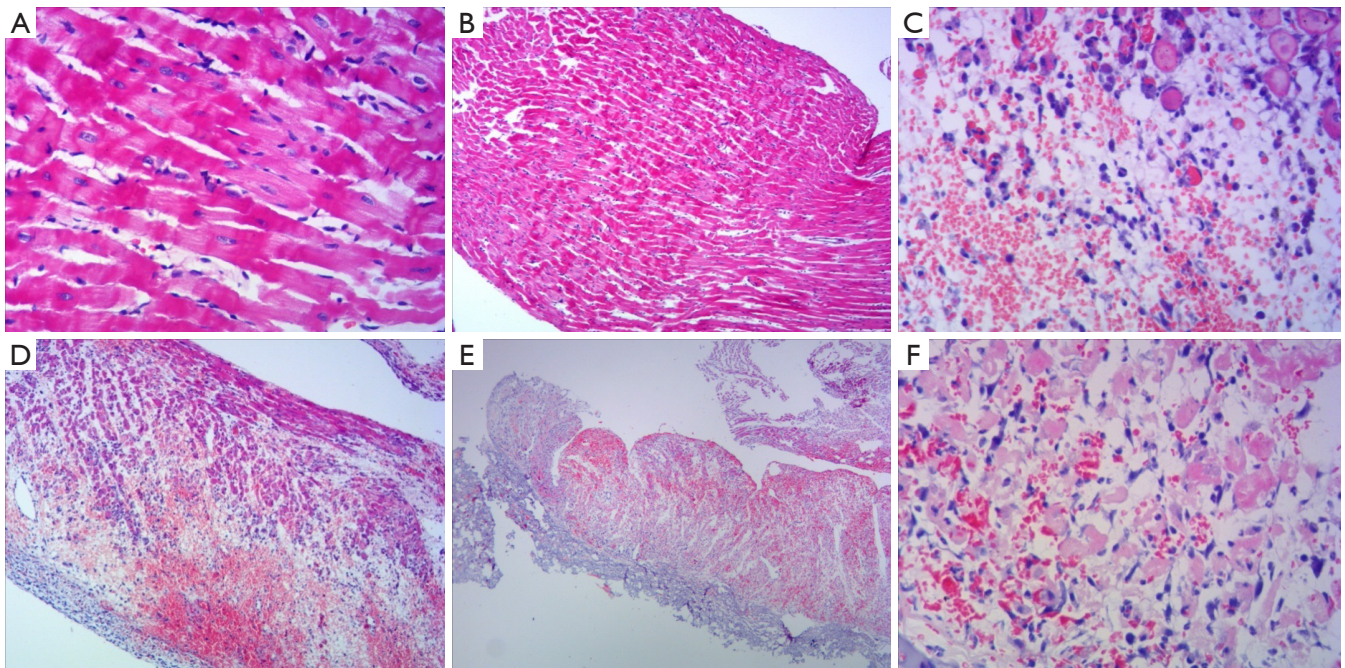
#### Heterotopic heart transplantation

All recipients survived excepted one recipient in allogeneic group died of anastomotic bleeding 6 hours after the operation. New matched rats (BN-to-Lewis) were introduced in the allogeneic group. And the grafted hearts were all beating before they were heaved.

#### Histology and rejection score of grafted heart

The histopathological findings showed an outer-to-inner lymphoplasmocytic infiltration; there was perivascular and





**Figure 1** Pathological examination of the grafted heart. (A,B) Showed that there is no evidence of acute cardiac rejection at the 2<sup>nd</sup> day and the 4<sup>th</sup> day in the isogeneic group (A, magnification,  $\times 100$ ; B, magnification,  $\times 40$ ). Inflammation with focal myocyte loss could be found in the allogeneic group at the 2<sup>nd</sup> day after cardiac transplantation (C,D) (C, magnification,  $\times 400$ ; D, magnification,  $\times 40$ ). Inflammation with confluent foci of myocyte loss could be found in the allogeneic group at the 4<sup>th</sup> day after cardiac transplantation (E,F) (E, magnification,  $\times 40$ ; F, magnification,  $\times 400$ ).

**Table 1** Semiquantitative rejection score at the 2<sup>nd</sup> and 4<sup>th</sup> day after cardiac transplantation

Time	Semiquantitative rejection score		P value
	Isogeneic group	Allogeneic group	
The 2 <sup>nd</sup> day	0, 0, 0, 0, 0	3, 2, 2, 1, 2	0.0002
The 4 <sup>th</sup> day	0, 0, 0, 0, 0	4, 4, 5, 4, 5	<0.0001

**Table 2** Changes of AP in the isogeneic group and allogeneic group at the 2<sup>nd</sup> day after cardiac transplantation

Parameter	Isogeneic group	Allogeneic group	P value
APD <sub>50</sub> (ms)	11.33 $\pm$ 1.857	21.46 $\pm$ 2.516	0.0136
APD <sub>90</sub> (ms)	49.28 $\pm$ 5.621	88.08 $\pm$ 6.445	0.0016
APA (mV)	128.4 $\pm$ 3.629	119.6 $\pm$ 3.391	0.1107
RP (mV)	-80.37 $\pm$ 1.626	-83.94 $\pm$ 1.839	0.1967

APD, action potential duration; APA, action potential amplitude; RP, resting potential.

**Table 3** Changes of AP in the isogeneic group and allogeneic group at the 4<sup>th</sup> day after cardiac transplantation

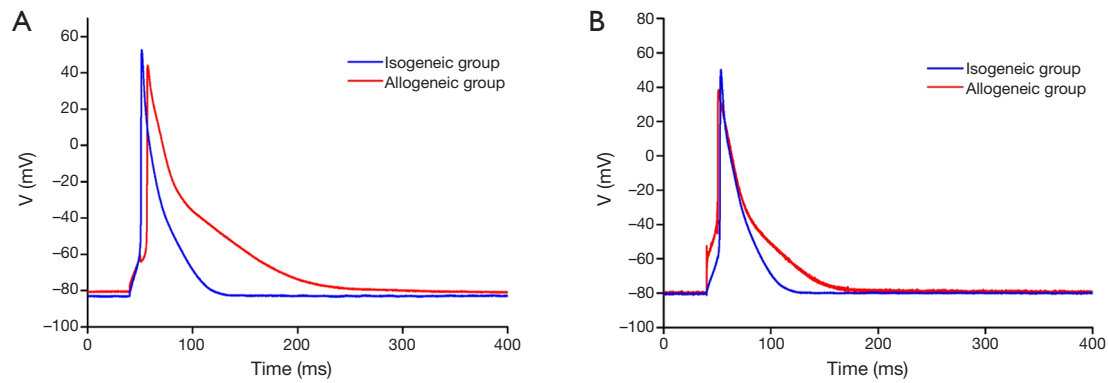
Parameter	Isogeneic group	Allogeneic group	P value
APD <sub>50</sub> (ms)	16.00 $\pm$ 2.687	19.17 $\pm$ 1.777	0.3272
APD <sub>90</sub> (ms)	59.34 $\pm$ 5.183	104.0 $\pm$ 9.523	0.0064
APA (mV)	117.0 $\pm$ 5.594	123.0 $\pm$ 2.824	0.3033
RP (mV)	-76.05 $\pm$ 0.8443	-79.89 $\pm$ 1.392	0.0781

APD, action potential duration; APA, action potential amplitude; RP, resting potential.

interstitial inflammation at the 2<sup>nd</sup> day after the operation. Inflammation with multifocal myocyte loss and confluent foci of myocyte loss could be found across the ventricular wall at the 4<sup>th</sup> day (Figure 1, Table 1).

### AP changes during acute cardiac rejection

The APD<sub>90</sub> of cardiomyocytes was significantly prolonged



**Figure 2** The morphology of AP of the cardiomyocytes. (A) The AP in rat cardiomyocytes of the isogenic group at the 2<sup>nd</sup> day after operation; (B) the AP in rat cardiomyocytes of the isogenic group at the 4<sup>th</sup> day after operation. AP, action potential.

in the allogeneic group at the 2<sup>nd</sup> day ( $P < 0.0016$ ) and 4<sup>th</sup> day ( $P < 0.0064$ ) after cardiac transplantation. There was no significant difference in action potential amplitude (APA) or RP (Tables 2,3 and Figures 2,3).

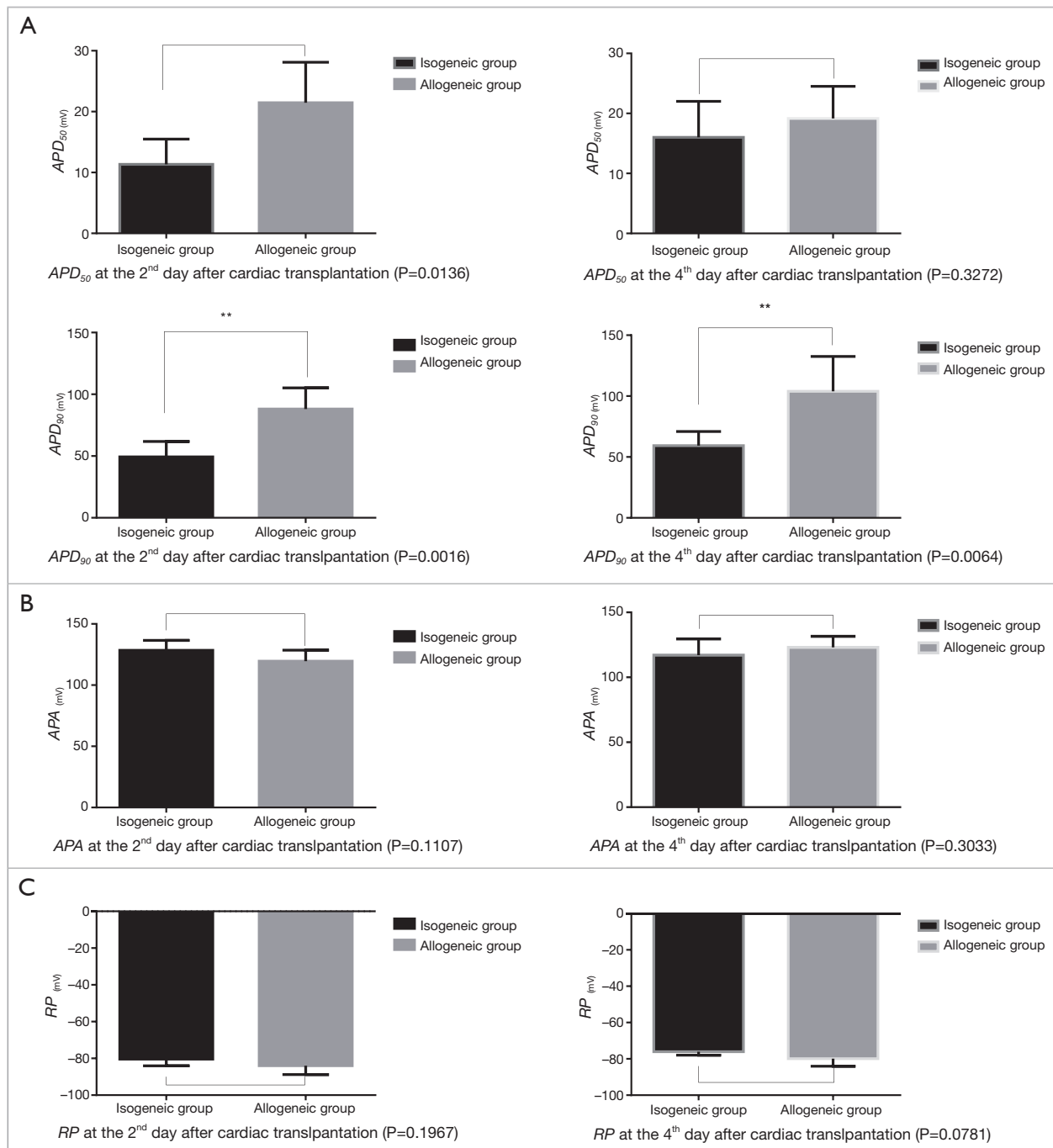
#### *I<sub>to</sub>* current changes during acute cardiac rejection

There was no significant difference in the current density of *I<sub>to</sub>* at the 2<sup>nd</sup> day after cardiac transplantation between the isogenic group and allogeneic group. At the 4<sup>th</sup> day, the *I<sub>to</sub>* density of the allogeneic group was significantly decreased compared to the isogenic group ( $P < 0.01$ ) (Figures 4,5).

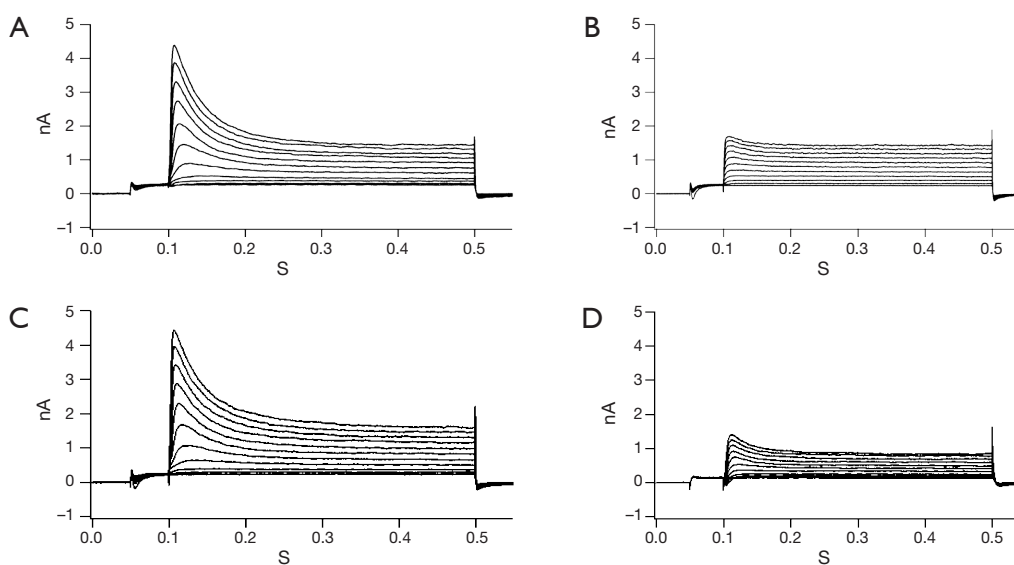
## Discussion

So far, the changes of the cardiomyocyte AP and the possible ionic mechanism during acute cardiac rejection in rats are not yet clear. In the present study, we first established a stable cardiac rejection model in allogeneic rats because the results obtained from cardiac transplantation studies in animal models must depend upon the model species involved, and particularly upon the stage of the rejection process studied (6). The isogenic model was also developed to eliminate the influence of the operation, anesthesia, cold ischemic preservation and withdrawal of autonomic nervous system control. On the 2<sup>nd</sup> day and the 4<sup>th</sup> day after the operation, the isogenic group showed no evidence of rejection, while the allogeneic group showed mild to moderate cardiac rejection without cardiomyocyte loss or necrosis. So the model of BN-to-Lewis heterotopic heart transplantation was suitable for electrophysiological studies in the early stage of acute cardiac rejection.

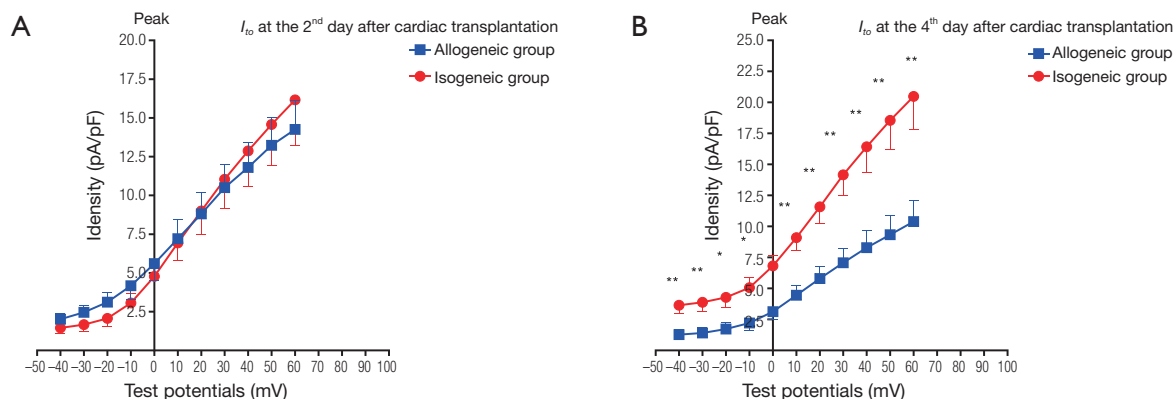
The cardiomyocyte AP is the most fundamental component of cardiac electrophysiology. The AP shape and APD are the most important features in AP. The present study showed that the *APD<sub>90</sub>* of cardiomyocytes was significantly prolonged during mild to moderate cardiac rejection, which suggested that the increased APD of cardiomyocytes may be a cellular mechanism underlying the electrophysiological changes. Binah *et al.* (15) found the APA, RP, and maximum rate of depolarization were clearly decreased in ventricular myocytes in the heterotopically transplanted rat heart using the standard microelectrode recording technique. However, Babuty *et al.* (4) observed that the APD and APA recorded from the right and left papillary muscles of the allogeneic heart significantly increased during early rejection in rats. Maybe the stage of the rejection process and the species involved in the rejection model led to the difference. However, there are no more detailed studies that could precisely characterize the electrophysiological changes in cellular preparations. In the present study, the grafted hearts in the allogeneic group and isogenic group all underwent the changes that take place in the abdominal cavity, such as fibrin layer formation, ascites, intestinal adhesion, and myocardial edema (16). We found that there was no significant difference in the APA and RP between the allogeneic group and isogenic group. So the prolongation of the APD may be the basic electrophysiological change in cardiomyocytes during the early stage of acute cardiac rejection. The changes would be expected to result in depressed conduction of the cardiac impulse through the ventricles, which would make contractile function less synchronous (17). Many studies have demonstrated that electrophysiological abnormalities



**Figure 3** Comparison of the *APD*, *APA* and *RP* of the cardiomyocytes between the two groups. (A) The *APD*<sub>90</sub> of cardiomyocytes was significantly prolonged in the allogeneic group at the 2<sup>nd</sup> day (P=0.0016) and 4<sup>th</sup> day (P=0.0064) after cardiac transplantation; (B,C) there was no significant difference in *APA* or *RPs* between the two groups. *APD*, action potential duration; *APA*, action potential amplitude; *RP*, resting potential.



**Figure 4**  $I_{to}$  of the cardiomyocytes. (A)  $I_{to}$  in rat cardiomyocytes of the isogenic group at the 2<sup>nd</sup> day after operation; (B)  $I_{to}$  in rat cardiomyocytes of the allogeneic group at the 2<sup>nd</sup> day after operation; (C)  $I_{to}$  in rat cardiomyocytes of the isogenic group at the 4<sup>th</sup> day after operation; (D)  $I_{to}$  in rat cardiomyocytes of the allogeneic group at the 4<sup>th</sup> day after operation.



**Figure 5** Comparison of the  $I_{to}$  of the cardiomyocytes between the two groups.  $I_{to}$  density in cardiomyocytes at the 2<sup>nd</sup> day (A) and the 4<sup>th</sup> day (B) after cardiac transplantation. The density of  $I_{to}$  in the allogeneic group was significantly decreased compared to the isogenic group at the 4<sup>th</sup> day (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

are closely connected with acute cardiac rejection, including rejection-related arrhythmias in the heart transplant patient (18), decreased ventricular voltage (3,4), increased intramyocardial impedance (5) and the characteristic changes in the IMEG (7,19).

The AP of a cardiomyocyte is generated by highly coordinated changes in different ionic conductances.  $I_{to}$  is a transient outward potassium current, which is rapidly activated and inactivated in response to the initial phase of the cardiomyocyte AP, resulting in repolarization.  $I_{to}$

is the key outward potassium current in the early stage of the AP, and the alterations of  $I_{to}$  would be expected to alter the morphology and duration of the AP (20). It has been confirmed that  $I_{to}$  is important in physiological and pathophysiological processes. In the present study, the current density of  $I_{to}$  was significantly decreased at the 4<sup>th</sup> day in the allogeneic group compared with the isogenic group after cardiac transplantation, which may explain the prolongation of the APD in cardiomyocytes during acute cardiac rejection. Additionally, more evidence has shown



that  $I_{to}$  has kinetic properties that make it especially effective in modulating the cardiac excitation-contraction coupling through its influence on  $I_{Ca,L}$  and possibly  $I_{Ca,NCX}$  (8,9,17,20). As a result, modification of  $I_{to}$  eventually leads to the impairment of cardiac systolic and diastolic function during acute cardiac rejection. The present study also observed that the cardiomyocyte *APD* in the allogeneic group was significantly prolonged at the 2<sup>nd</sup> day, while there was no significant difference in the current density of  $I_{to}$  between the two groups. The results indicate that some other factors may act on the  $I_{to}$ . Further studies are necessary to identify the mediators of the  $I_{to}$  in cardiomyocytes during acute cardiac rejection. An understanding of the electrophysiological mechanisms of cardiomyocytes could improve the diagnosis and treatment of acute cardiac rejection.

In conclusion, we suggest that the prolongation of the *APD* and the decrease of the  $I_{to}$  current density in cardiomyocytes might be basic electrophysiological mechanisms in the early stage of acute cardiac rejection in rats. The prolongation of the *APD* might be attributed to the decreased current densities of  $I_{to}$ .

### Limitations

We only focused on the changes of current density of  $I_{to}$  and did not investigate other ion channels such as those that give rise to  $Ca^{2+}$  currents, which are also involved in the duration of *AP*. Whether  $Ca^{2+}$  currents or other currents exert the same effect on *APD* during acute cardiac transplantation in rats requires further investigation.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The study was approved by the Chinese National Committee on the Use of Experimental Animals for Medical Purposes, Beijing Branch (No. 29971) and written informed consent was obtained from all patients.

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