Taxol stabilizes gap junctions and reduces ischemic ventricular arrhythmias in rats *in vivo*

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Abstract. The dynamic movements of connexin 43 (Cx43) are regulated by microtubules and their associated proteins. Dysfunction of Cx43 in the ischemic myocardium is correlated with ventricular arrhythmias (VAs). The present study aimed to determine the effects of microtubules on Cx43 expression and distribution in myocardial tissue, as well as to examine the susceptibility of the heart to VAs during acute myocardial ischemia and reperfusion. Rats were subject to left coronary artery occlusion for 20 min followed by 20 min reperfusion and received taxol at different concentrations (0.1, 0.3 and 0.9 μmol·kg^{-1} in 0.5 ml saline) intraperitoneally. Monophasic action potentials at the epicardium were recorded and analyzed using an electrocardiogram. Immunoblots and immunofluorescence staining were used to detect tubulin polymerization and Cx43 expression and distribution. Taxol pretreatment significantly ameliorated the depolymerization of microtubules, improved Cx43 expression and redistribution, reduced the occurrence of VAs, ameliorated shortening of 90% repolarization action potential durations $(APD)_{90}$ and improved APD dispersion during myocardial ischemia-reperfusion. The present study demonstrated that taxol reduced ischemic VAs and its mechanism may be correlated with the preservation of Cx43 by stabilizing microtubules.

Introduction

Ventricular arrhythmias (VAs) account for the majority of cardiovascular-associated mortalities in patients with ischemic heart disease. The mechanism of VAs is associated with gap junction (GJ) remodeling involving connexin 43 (Cx43) reduction at the intercalated disc (ID) and Cx43 lateralization (1). Previous studies have reported that the dynamic movements of Cx43 are associated with microtubules and their associated proteins (2,3). Considering that microtubular disruption is an early cellular reaction to hypoxia in cardiac myocytes (4,5), it is presumed that preservation of the microtubules may affect GJ functions and VAs during myocardial ischemia.

Taxol is a microtubular stabilizer that maintains polymerized microtubular structure and ameliorates hypoxia-induced myocyte injury (6). Taxol-proliferated microtubules are resistant to cold, Ca^{2+} and colchicines (7). Previous studies have revealed that microtubule stabilizers preserve cardiac function and prevent VAs in an isolated heart (8,9). However, the electrophysiological consequences of *in vivo* microtubule preservation during myocardial ischemia remains to be elucidated. In the present study, an attempt was made to determine the electrophysiological effects of taxol using an *in vivo* rat model with myocardial ischemia-reperfusion (IR).

Materials and methods

Animal model and experimental protocols. Experiments were approved by the Institutional Animal Care and Use Committee of Wannan Medical College (Wuhu, China). A total of 50 Sprague-Dawley rats (250 to 300 g) were randomly assigned to five groups: Control group, IR group and three taxol pretreatment groups. In the control and pretreatment groups, normal saline and taxol (Sigma-Aldrich, St. Louis, MO, USA; 0.1, 0.3 and 0.9 μM·kg^{-1} in 0.5 ml saline) were injected intraperitoneally (IP) 30 min prior to ischemia induction.

Each rat was anesthetized (pentobarbital sodium, 50 mg/kg IP; Sigma-Aldrich) and artificially ventilated. Once the pericardium was opened, a 6.0 silk atraumatic suture (Jinghua Co., Shanghai, China) was passed around the left anterior descending coronary artery at the level of the left atrial appendage. Ischemia was induced for 20 min by ligating the suture, following which it was confirmed by an elevated ST segment in the electrocardiogram (ECG) and changes in myocardial color from red to pale. Reperfusion was initiated by reopening the ligation for 20 min. The control group was established by following the same procedure, but without closing the suture.

ECG and monophasic action potentials (MAPs). ECG traces were recorded continuously, and signals <10 Hz and >100 Hz were filtered out. Epicardial MAPs were recorded at specific time points intermittently in normal sinus rhythm (10). MAP

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signals were amplified and recorded on a computer for subsequent analysis. A commercially available physiological signals recording and analysis system (RM6240; Chengdu Instrument Company, Chengdu, China) was used to digitalize, store and analyze MAP signals. The software was used to analyze action potential durations (APD_{90}) at 90% of repolarization. Dispersion of action potential duration (APDd) represented the absolute value of the time difference between the maximum and minimum APD.

Analysis of VAs. VAs were analyzed according to Lambeth conventions (11). The score system (9) used to quantify arrhythmias was as follows: 0, <10 ventricular premature contractions (VPCs); 1, ≥10 VPCs; 2, 1-5 episodes of ventricular tachycardia (VT); 3, >5 episodes of VT or 1 episode of ventricular fibrillation (VF); 4, 2-5 episodes of VF; 5, >5 episodes of VF; 6, VT or VF, or the two together with total combined duration ≤300 sec; 7, VT or VF, or the two together with total combined duration >300 sec.

Tubulin polymerization assay. A tubulin polymerization assay was performed using a previously described method (6). Myocardial tissue from the ischemic area (1 g) was minced and homogenized in different buffers. For the total tubulin fraction, the myocardium was homogenized in 1% SDS buffer containing 10 mM Tris·HCl (pH 7.4), 0.5 mM dithiothreitol and 1 mM Na_3VO_4. The mixture was boiled for 5 min and centrifuged at 16,000 x g at 4˚C for 10 min and then the supernatant was saved as the total protein fraction. For the free tubulin heterodimer and polymerized tubulin (microtubule) fractions, the myocardium was homogenized in a microtubule stabilization buffer containing 50% glycerol, 5% dimethyl sulfoxide, 10 mM Na_2HPO_4, 0.5 mM ethylene glycol tetraacetic acid and 0.5 mM $MgSO_4$ and then centrifuged at 100,000 x g at 25˚C for 20 min. The supernatant was saved as the tubulin heterodimer fraction, whereas the pellet was saved as the tubulin microtubule fraction. For immunoblotting, rabbit polyconal α-tubulin (1:2,000; ab125267; Abcam, Cambridge, MA, USA) antibody was used to detect the free tubulin heterodimer and polymerized tubulin.

Western blot analysis. Protein (100 μg) was denatured by heating at 95˚C for 5 min prior to resolution by SDS-PAGE and transferring to a polyvinylidene difluoride membrane (Shanghai Threebio Technology Co., Ltd, Shanghai, China). The membrane was blocked in phosphate-buffered saline (Shanghai Threebio Technology Co., Ltd) containing 0.2% Tween-20 (Shanghai Threebio Technology Co., Ltd) and 5% skimmed milk for 2 h at 37˚C and incubated overnight at 4˚C with primary polyclonal antibodies (rabbit anti-Cx43; 1:2,000; ab11370; Abcam). The housekeeping protein, glyceraldehyde-3-phosphate dehydrogenase (rabbit anti-GAPDH polyclonal antibody; ab9485; Abcam) was used as a loading control. Antibody binding was detected using horseradish peroxidase-conjugated secondary antibody (1:2,000; Sigma-Aldrich) and visualized using an enhanced chemiluminescence kit (Chemicon, Temecula, CA, USA).

Immunofluorescence staining. Frozen tissue sections fixed in cold acetone (-20˚C for 5 min) were used for immunofluorescence staining. Nonspecific antibody binding sites were blocked with 5% fetal bovine serum for 60 min. Tissue sections were labeled for 16 h at 4˚C with rabbit polyclonal anti-Cx43 (Cx43; 1:200, Abcam). Anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate was used for detection and images were observed using a Zeiss confocal microscope (LSM-510; Carl Zeiss, Gottingen, Germany).

Statistical analysis. Statistical analysis was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of the mean and were analyzed using analysis of variance followed by Newman-Keul's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of taxol on IR-induced VAs. In a preliminary experiment, it was observed that >2 μM taxol may lead to lethal ventricular bradycardias. Thus, the doses of taxol were reduced to 0.1, 0.3 and 0.9 μM. In the control group, 6/10 rats had ≥10 VPCs and only 1 had an episode of VT. In the IR group, all animals had >5 episodes of VT or several episodes of VF. The spontaneous VTs/VFs appeared most frequently (5 to 10 min) after ligation and then recovered gradually. Within 2 min after reperfusion, VPCs/VTs/VFs occurred transiently and automatically recovered rapidly. In three taxol treatment groups, fewer animals had VTs and VFs than in the IR group. However, no difference in VAs during reperfusion periods between the IR group and taxol-treated groups was observed (Fig. 1; Table I). The severity of VAs was significantly decreased in taxol-treated groups in a dose-dependent manner compared with the IR group based on the VA score system ($P<0.01$; Fig. 1).

Effects of taxol on IR-induced MAP changes. The MAP duration at APD_{90} of the ischemic epicardium in the IR group was significantly shorter than that of the control group (Fig. 2). When perfusion was restored, the APD_{90} rapidly recovered within 5 min and returned to baseline levels. The APDd was significantly increased in the IR group compared with that in the control group. Taxol treatment partially but significantly restored APD_{90} and reduced APDd during ischemia in the pretreatment groups. However, no significant difference in APD_{90} was observed during the reperfusion periods between the IR group and taxol-treated groups.

Effects of taxol on IR-induced microtubular depolymerization. The relative levels of free tubulin in the IR group increased significantly after the heart was exposed to IR compared with the control group (Fig. 3). In addition, polymerized tubulin in the IR group significantly decreased. Taxol treatment maintained tubulin polymerization significantly and reduced free tubulin in a dose-dependent manner ($P<0.01$).

Effects of taxol on IR-induced Cx43 distribution in the heart. The Cx43 staining signal in the non-ischemic heart was concentrated at the IDs (red arrows, Fig. 4A), whereas little Cx43 was located in the lateral cardiomyocyte surfaces (yellow arrows, Fig. 4A). In the hearts of the IR group, the

Table I. Effects of taxol on ischemia/reperfusion-induced ventricular arrhythmias.

	Ischemia			Reperfusion			
Group	VTs	VFs	VPCs	VTs	VFs	VPCs	VA Score
Control	1/10	0	7/10	-	-	-	0.8±0.63
IR	10/10[a]	6/10[a]	10/10	8/10	3/10	10/10	3.3±0.95[a]
IR + 0.1 μM taxol	10/10[a]	4/10[a]	10/10	7/10	3/10	10/10	2.7±0.48[ab]
IR + 0.3 μM taxol	10/10[a]	3/10[ab]	10/10	6/10	2/10	10/10	2.5±0.53[ab]
IR + 0.9 μM taxol	7/10[a]	0/10[ab]	10/10	6/10	2/10	10/10	1.7±0.45[ab]

[a]P<0.05 vs. control group and [b]P<0.05 vs. IR group. VT, ventricular tachycardia; VF, ventricular fibrillation; VPC, ventricular premature contractions; IR, ischemia-reperfusion.

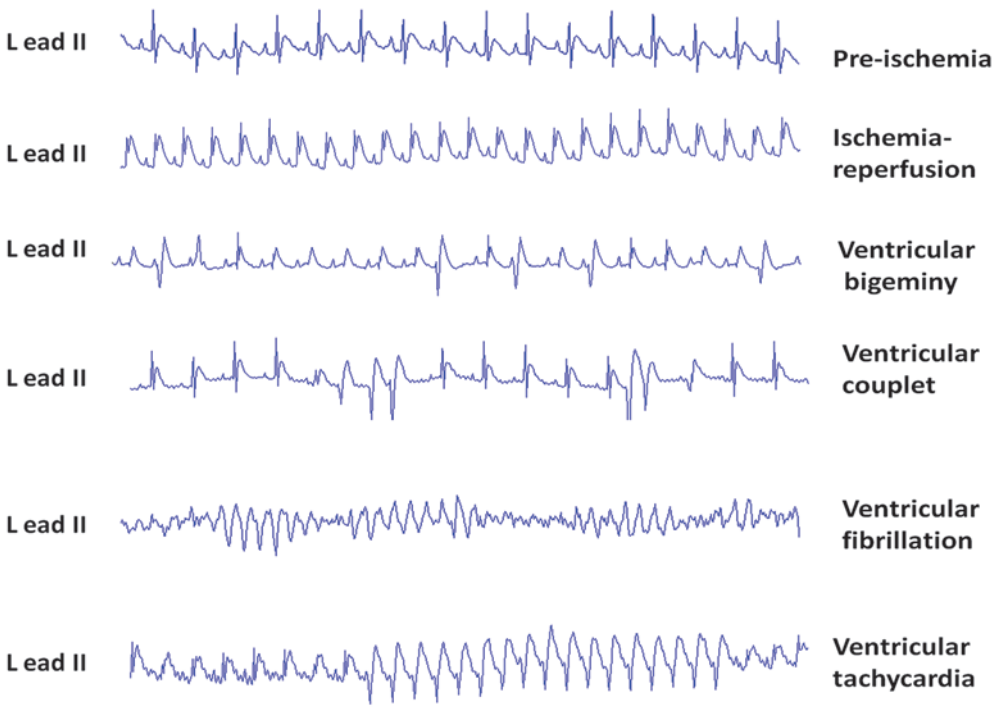

Figure 1. Ventricular arrhythmias induced by coronary occlusion and reperfusion. During left anterior descending coronary occlusion and reperfusion, animals developed ventricular arrhythmias, including ventricular bigeminy, ventricular couplet, ventricular tachycardia and ventricular fibrillation.

Cx43 signal was markedly reduced in ID and redistributed to the lateral cardiomyocyte surfaces (Fig. 4B). The hearts subjected to taxol pretreatment exhibited significantly improved Cx43 distribution compared with the IR groups (Fig. 4C, D and E).

Effects of taxol on IR-induced Cx43 expression. The relative levels of Cx43 protein expression in myocardial tissue were reduced significantly in the IR group compared with the control group (Fig. 5). Taxol treatment significantly improved Cx43 expression in a dose-dependent manner (P<0.01).

Discussion

Microtubules are important components of the cytoskeleton. They have important functions in protein synthesis, intracellular trafficking and intracellular signaling. During these processes, microtubules require a dynamic balance between free tubulin and polymerized dimers in the cytoplasm (12,13). Previous studies have demonstrated that microtubules are responsible for regulating Ca^{2+} channels, APD and membrane potential (14). In the present study, it was observed that susceptibility to VAs increased in an *in vivo* rat model with IR, however the susceptibility was also partly but significantly reduced by taxol pretreatment in a dose-dependent manner. The present results are in agreement with previous *ex vivo* experiments (8,9), indicating that stabilizing microtubules have significant anti-arrhythmic effects during myocardial IR *in vivo*.

Previous studies have revealed that during myocardial ischemia, either APD shortening or APDd enlargement generates pro-arrhythmic substrates (15,16). APD duration undergoes a

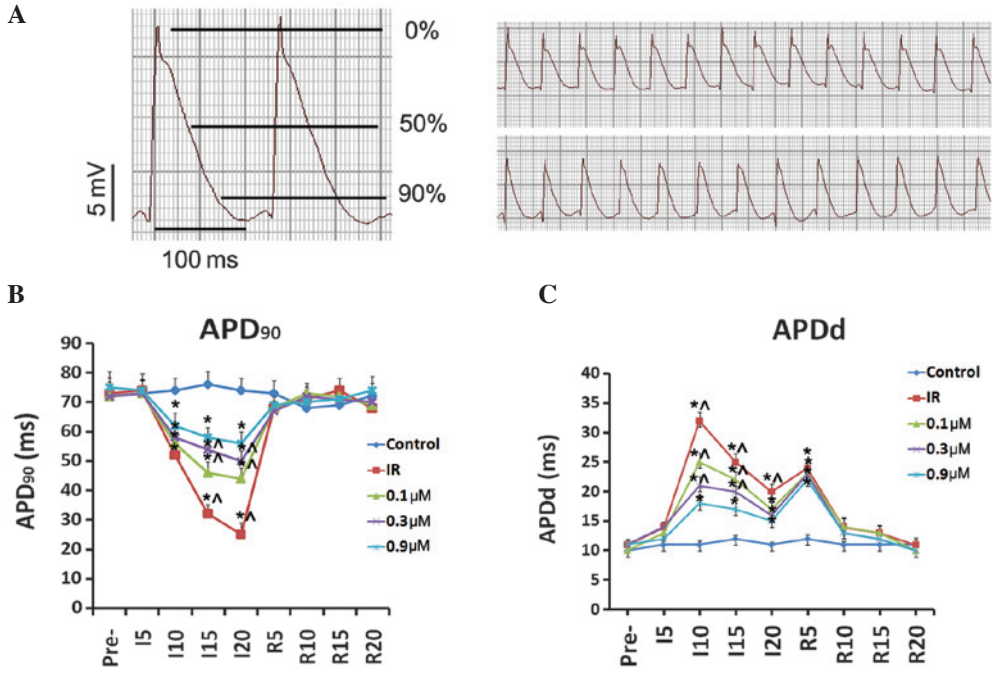

Figure 2. Epicardial monophasic APD and APDd is altered by IR. (A) MAP was recorded from the ischemia-reperfusion zone (apical). (B) APD_{90} is the 90% repolarization of MAP durations; (C) APDd is the absolute value of the time difference between the maximum and minimum of APD. *P<0.05, versus the control group; ^P<0.05, versus the IR group. IR, ischemia-reperfusion; APD, action potential duration; APDd, dispersion of action potential duration; APD_{90}, action potential duration 90% of repolarization; MAP, monophasic action potentials.

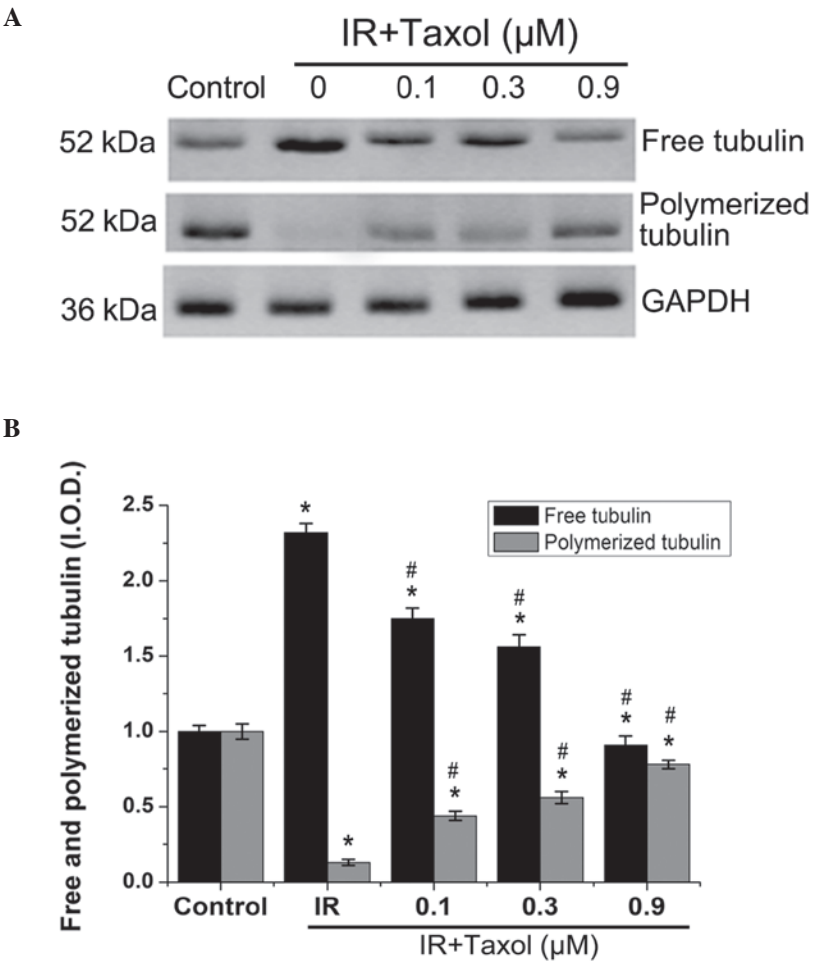

Figure 3. Taxol protects microtubules from depolymerization in the ischemic myocardium. Immunoblot examples from the apex of the left ventricle after myocardial ischemia-reperfusion. (A) Monoclonal anti-GAPDH antibody was used to load the control blot. (B) Relative quantitative levels of free and polymerized tubulin expression (IOD). *P<0.05, versus the control group; #P<0.01, versus the IR group. IR, ischemia-reperfusion; IOD, integrated optical density.

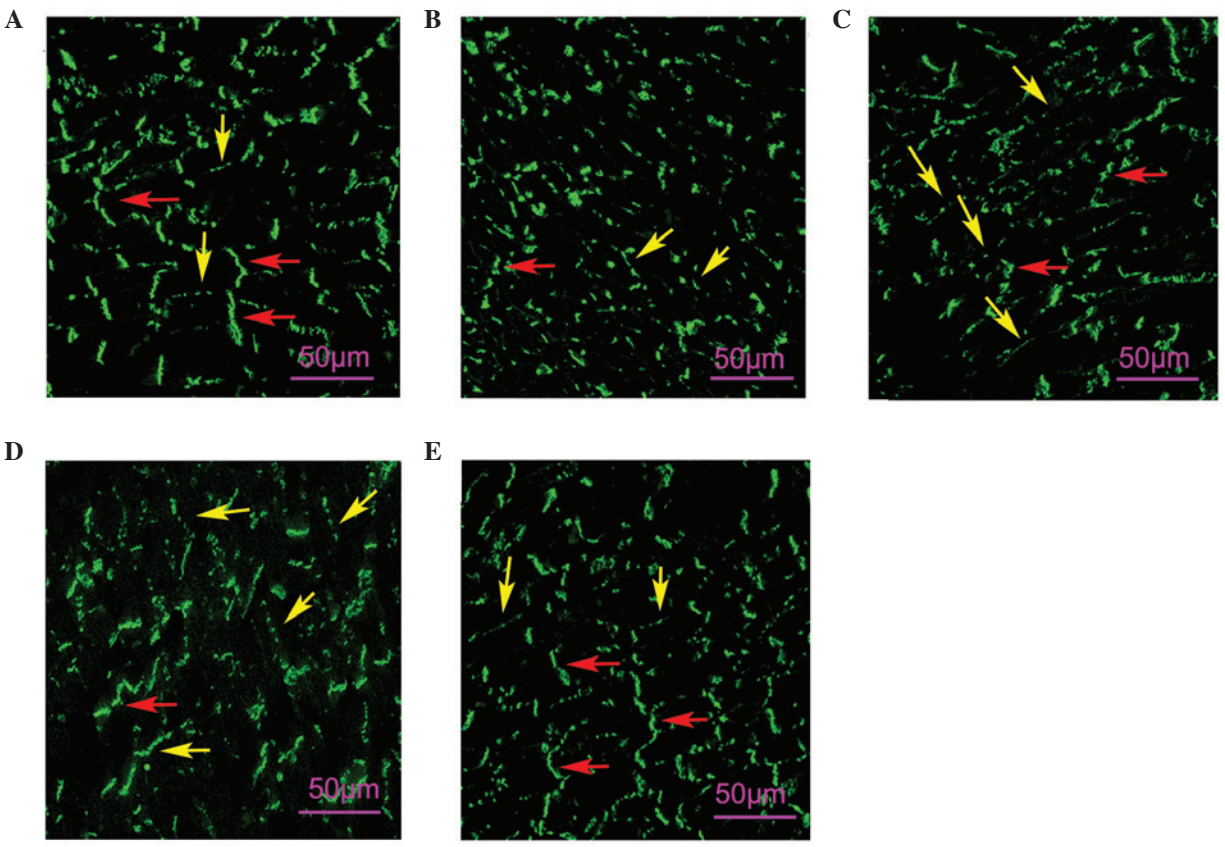

Figure 4. Taxol stabilizes Cx43 distribution in the ischemic myocardium. Representative immunofluorescence Cx43 images of sections from the apex of left ventricles from the (A) control group, (B) IR group and (C, D and E) 0.1, 0.3 and 0.9 μM taxol pre-treatment groups, respectively. Red arrows indicate Cx43 in intercalated disks, whereas yellow arrows indicate lateralized Cx43 in cardiac myocyte surfaces. IR, ischemia-reperfusion; Cx43, connexin 43.

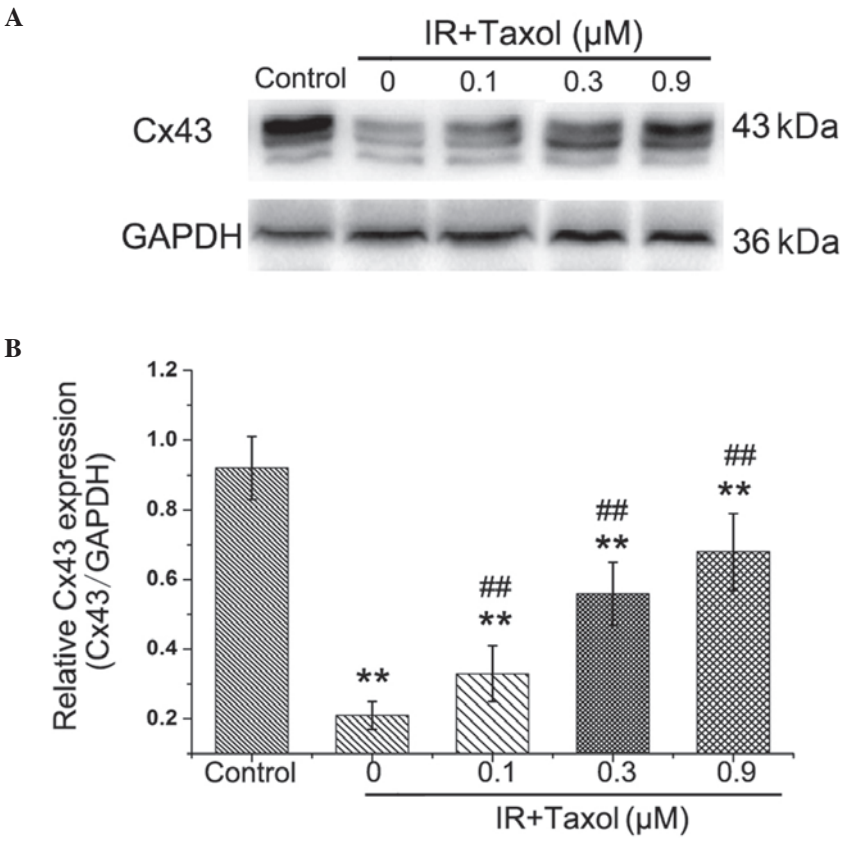

Figure 5. Taxol preserves Cx43 expression in the ischemic myocardium. Representative (A) western blots and (B) relative quantitative levels of Cx43 expression. **P<0.01, versus the control group; $^{\#\#}$P<0.01, versus the IR group. IR, ischemia-reperfusion; Cx43, connexin 43.

temporary prolongation and then gradually recovers following reperfusion (17). Considering that microtubules regulate APD and membrane potential (14), taxol may also affect APD duration and APDd during myocardial IR. In the present study, APD_{90} shortened rapidly following coronary occlusion, however was restored within 5 min after reperfusion. Taxol treatment significantly ameliorated ischemia-induced APD shortening and APDd, which can reduce ischemia-induced arrhythmias. Thus the beneficial effects of taxol on ischemia-induced arrhythmias may be due to the improvement in APD changes. In cultured myocytes, microtubular depolymerization is an early cellular reaction to hypoxia (4). Acute hypoxia leads to the collapse of microtubular networks and reversible cellular damage (18). The interruption of microtubules occurs prior to mitochondrial damage and myocyte injury (19,20). Taxol may stabilize polymerized microtubules and ameliorate hypoxia-induced myocyte injury (6). The present data demonstrated that taxol treatment significantly preserved polymerized tubulin and reduced free tubulin during IR.

GJ remodeling is associated with cardiac arrhythmias during myocardial ischemia. Previous studies have revealed that oxidative stress can affect microtubules and lead to subsequent perturbation of Cx43 delivery to the plasma membrane in the human and mouse myocardium (2,3). In the present study, it was observed that in the normal ventricular tissue, Cx43 was localized at IDs. It was also identified that taxol pretreatment significantly improved Cx43 distribution and expression in the ischemic myocardial tissue. The stabilization of Cx43 during ischemia may be associated with reduction of VAs through taxol pretreatment.

The present study has several limitations. The phosphorylation levels of Cx43 that may modulate GJ function during ischemia were not detected (21). In addition, the present study was not able to exclude the possibility that GJ preservation of taxol was secondary to the reduction of oxidative stress, which can reduce the levels of connexin passing to the plasma membrane (22). Taxol may also be able to modulate ion channels (14). Therefore, further studies should focus on identifying the ion channels preserved by taxol during ischemic injury.

The present study demonstrated that taxol pretreatment may stabilize microtubules, preserve GJs and reduce the severity of VAs during myocardial IR. These findings indicated that microtubule stabilization may be of benefit in modifying the VA susceptibility of ischemic hearts.

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