

Hemichannel-mediated volume regulation contributes to IPC-induced cardiomyocyte protection

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Abstract. Cx43 has been documented to be involved in ischemic preconditioning (IPC). However, the participation of Cx43-formed hemichannels in IPC and the potential underlying mechanisms remain unclear. The present study focused on cardiomyocytes' volume regulation during IPC to investigate the role of hemichannels in the IPC-induced cardioprotection. In the study, mice cardiomyocytes were respectively treated with a hemichannel blocker, octanol or 18a-Glycyrrhizic acid (18a-GA), and a Cx43-silenced lentivirus. They were subsequently cultured in hypotonic solution to simulate ischemic reperfusion (SIR) and systemic ischemic preconditioning (SIP). Cell morphology and volumetric (area) change were detected by inverted microscopy at 30 min following the addition of hypotonic solution. Cardiomyocyte mortality was assessed by trypan blue stain assay. The analyses revealed that regardless of the treatments, hypotonic solution aggravated cell edema: Compared with the initial condition (the moment before the solution addition, 0 min), the volumetric area increased significantly 30 min later (for hypotonic+DMSO, $5,050 \pm 1,511$ vs. $3,464 \pm 723 \mu\text{m}^2$; for hypotonic+scramble lentiviral vector, $5,517 \pm 1,128$ vs. $2,331 \pm 536 \mu\text{m}^2$; $P < 0.05$, respectively). Either treatment alleviated the edematous condition when a comparison was made between 30 min after the hypotonic addition and 0 min (for hypotonic+octanol, $2,990 \pm 765$ vs. $2,821 \pm 773 \mu\text{m}^2$; for hypotonic+18a-GA, $4,817 \pm 1,306$ vs. $4,762 \pm 1,271 \mu\text{m}^2$; for hypotonic+Cx43-silenced, $3,627 \pm 688$ vs. $3,419 \pm 814 \mu\text{m}^2$;

$P > 0.05$ for all). Notably, results indicated that the SIP group had lower mortality rates compared with its SIR counterpart; the hypotonic+octanol, hypotonic+18a-GA, and hypotonic+Cx43-silenced group showed markedly-declined mortality when compared with their respective control groups (respectively, 35.70 ± 1.02 , 30.76 ± 2.20 vs. $53.58 \pm 2.14\%$; 30.89 ± 2.37 vs. $54.12 \pm 2.55\%$; $P < 0.05$ for all). The results suggest that ischemic preconditioning may provide cardioprotection by blocking the opening of the hemichannels and further mediating the volume regulation of cardiomyocytes.

Introduction

Ischemic preconditioning (IPC) is a well-established adaptive response that briefly exposes cardiomyocytes to ischemia/reperfusion and dramatically improves myocardial tolerance of ischemic insult (1). During IPC, adenosine, kappa-opioid receptor, K^+ channels and Akt1 have been reported to be the responsible mechanisms (2-6), but none of them seem to elucidate IPC-induced cardioprotection in a convincing manner. On the other hand, connexin protein 43 (Cx43) has been documented to be implicated in preconditioning (7,8) and may serve as a potential end-effector of IPC-induced cardioprotection (9). However, it remains unclear which structure formed by Cx43 protein is involved in the preconditioning.

Cx43, a member of the connexin family (10), has been found extensively in connexon proteins of cardiac tissues (11,12). It consists of four transmembrane regions, two extracellular loops, and one intracellular loop. The amino- and carboxyl-termini are located on the cytoplasmic side of the membrane (13,14). The connexon of two adjacent cardiomyocytes constitutes a gap junction between the myocardial cells (15). However, for most of the time, the connexon of cardiomyocytes remains separate and do not form a gap junction. Thus, hemichannels are present in the non-junctional regions of individual cells. These structural domains mediate the intracellular and extracellular transport of small-molecule substances (16) and cell volume both in extracellular ischemia and physiological isosmotic situations (17). Cardiomyocytes regulate the hemichannel permeability by preventing water from entering the cells and subsequent cell death in a hypotonic extracellular environment, which is called volume regulation.

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During ischemia, metabolic by-products of anaerobic glycolysis accumulate in the cytoplasmic solute, which creates an osmotic load in cardiomyocytes (15). During the cell death process, hemichannels have been documented to serve as potential toxic pores (18). We suspect that IPC may provide myocardial protection by blocking the hemichannels, which in turn mediates the cell volume of cardiomyocytes. Cx43 is present not only in the inner and outer membranes of mitochondria, but also in the inner and outer membranes of ventricular myocytes (19,20). Many studies have investigated the mechanism of IPC with Cx43 in mitochondria (21) and gap junction on cell membrane (22,23). However, none of the research completely explains the IPC-induced cardiomyocyte protection, suggesting that there may be other mediators or terminal effectors in the IPC. So this paper focuses on whether Cx43-formed hemichannels on the cell membrane are involved in IPC and its mechanism.

The current study employed a lentivirus with Cx43-silencing shRNA and a hemichannel blocker, octanol or 18a-Glycyrrhizic acid (18a-GA) to block these channels so as to investigate the mechanism involved in IPC-induced protection. The study found that both treatments reduced the mortality of cardiomyocytes.

Materials and methods

Experimental protocol. To investigate the effects of hemichannels on volume regulation of cardiomyocytes, mouse cardiomyocytes (HL-1; Saiqi Biological Engineering Company, Shanghai, China), cultured *in vitro* under a hypotonic condition (at an osmotic pressure of 150 mOsm/l), were divided into 6 groups: Control, Hypotonic+dimethylsulphoxide (DMSO) (10 μ l; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); Hypotonic+Octanol or 18a-GA groups, respectively cultured with hemichannel blockers (octanol and 18a-GA, dissolved in DMSO, both at 100 μ mol/l, 10 μ l; Sigma-Aldrich; Merck KGaA); Lentiviral vector, and Cx43-silenced groups, respectively transfected with a scramble lentiviral vector (MOI=100) and a lentivirus transfected with Cx43-silencing shRNA (MOI=100). A normal control group was cultured with an isotonic solution (at an osmotic pressure of 308 mOsm/l). After the treatments, cardiomyocytes were collected to assess cell morphological feature and volumetric (replace with area) changes at 0 min (the moment before the addition of hypotonic solution) and 30 min after the intervention.

Further, we evaluated the effects of hemichannel-induced volume regulation on cardioprotection conferred by ischemic preconditioning *in vitro*. Simulated ischemia-reperfusion (SIR) was induced by a 7-h anoxia and a subsequent 6-h reoxygenation and the cardiomyocytes were divided into 6 groups: Control, SIR+DMSO, SIR+octanol, SIR+18a-GA, SIR+scramble lentiviral vector, and SIR+Cx43-silenced groups; Simulated ischemic preconditioning (SIP) was achieved by exposing the cells to a 1-h anoxia and a 30-min reoxygenation before a subsequent 7-h anoxia and 6-h reoxygenation. The cardiomyocytes received the same treatments as SIR groups and were divided into: Control, SIP+DMSO, SIP+octanol, SIP+18a-GA, SIP+scramble lentiviral vector, and SIP+Cx43-silenced groups. A normal control group was

cultured in an isotonic solution (at an osmotic pressure of 308 mOsm/l). The mortality of each group was averaged from 3 samplings by trypan blue stain assay after the experiment.

Cell culture. Mouse cardiomyocytes were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% penicillin and streptomycin at 37°C and in a moist atmosphere with 5% CO₂. Cardiomyocytes in logarithmic growth phase were seeded in 6-well plates (1x10⁵ cells/well) and cultured in DMEM for 10-12 h. Three fragments of Cx43-silencing shRNA lentiviral vectors [LV-Gja1-RNAi (51660-1), LV-Gja1-RNAi (51661-2) and LV-Gja1-RNAi (51662-1)] and scramble lentiviral vector (CON077; Gene Company Ltd., Shanghai, China) were then introduced (MOI=100, for both). The transfection efficiency of the lentiviral vectors was observed by inverted fluorescence microscopy (Olympus Corporation, Tokyo, Japan) after puromycin (Sigma-Aldrich; Merck KGaA) was added to filter successfully-transfected cells after 72 h. Cardiomyocytes with the highest transfection rate in the three fragments were prepared for subsequent experiments after sampling checking by western blotting and qPCR.

Cell volumetric regulation. To verify the role of hemichannels in volume regulation, we simulated the extracellular hypotonic environment after ischemia-reperfusion. Mouse cardiomyocytes in logarithmic growth phase were seeded in 12-well plates (5x10⁴ cells/well), including two wells of cardiomyocytes transfected with scramble lentiviral vectors and two wells with Cx43-silenced lentivirus (see cell culture, above). Hypotonic solution and Octanol or 18a-GA were added to corresponding groups simultaneously under normal ambient conditions. Cell morphological feature and volumetric (replaced with area) change were observed and photographed by inverted microscopy within 30 min after the addition of the solution. The 20 best adherent cells in each group were chosen to calculate and compare the changes of the areas (at 0 and 30 min after the solution adding) using Image-Pro Plus 6.0.

Mortality of cardiomyocytes. To evaluate the role of hemichannels in IPC, we simulated the ischemic reperfusion (IR) and IPC conditions with the cardiomyocytes. Briefly, the cells in the 12-well plates as previously described were divided into 6 SIR and 6 SIP groups. They were cultured in hypotonic solution (at an osmotic pressure of 150 mOsm/l), in which the anoxic condition was conducted in a hypoxic device (containing 95% CO₂ and 5% N₂, at 37°C) and the reoxygenation condition in a normal cellular incubator (containing 5% CO₂, at 37°C). Octanol or 18a-GA was administered into the matching groups just before the experiment began. A normal control group was cultured in an isotonic solution (at an osmotic pressure of 308 mOsm/l). The mortality of each group was averaged from 3 samplings by trypan blue stain assay after the experimental intervention.

Western blot. To assess the transfection efficiency of the Cx43-silenced lentivirus, we determined the total protein and activated (phosphorylated) protein of Cx43 by western

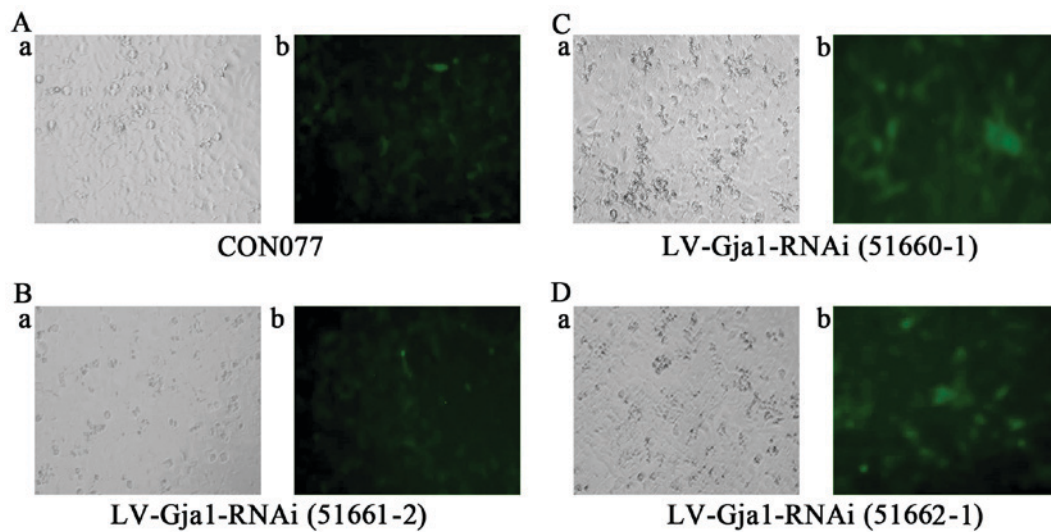


Figure 1. Fluorescence outcomes of four lentiviral vectors at 72 h after the transfection (in pairs). (Aa and Ab) Scramble lentiviral vector (CON077), (Ba and Bb) lentiviral vector LV-Gja1-RNAi (51661-2), (Ca and Cb) LV-Gja1-RNAi (51660-1) and (Da and Db) LV-Gja1-RNAi (51662-1). (a) observed by inverted microscopy (magnification, x200) and (b) stimulated by blue light under the fluorescence microscope (magnification, x200). Cells with green fluorescence indicate successful transfection.

blotting. Normal and transfected cardiomyocytes were lysed in RIPA buffer containing 1% protease inhibitor and phosphatase inhibitor (Sigma-Aldrich; Merck KGaA). Protein concentrations of each sample were determined by Quantitative nucleic acid-protein Analyzer (Biomate 5; Thermo Fisher Scientific, Inc.). Equal load of proteins was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred electrophoretically to nitrocellulose membranes (Whatman International Ltd., Maidstone, UK), and then rocked in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% milk at room temperature for 2 h. Membranes were incubated at 4°C overnight with specific primary antibodies: rabbit anti-Cx43 (1:1,000), rabbit anti-phospho-Cx43 (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-GADPH antibody (1:2,000; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The membranes were further incubated in the horseradish peroxidase-conjugated secondary antibodies (1:5,000) (Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 1 h at room temperature after four rinses in TBST. The blots were visualized with a chemiluminescent detection kit. The bands were quantified using Image J software.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR analysis was essential to further confirm the expression of Cx43. Total ribonucleic acids (RNAs) of normal and transfected cardiomyocytes were isolated with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and their concentration and purity were quantified by spectrophotometry with the software of Quantitative nucleic acid-protein Analyzer. Reverse transcription and amplification were performed respectively with the Eastep RT Master Mix and Eastep qPCR Master Mix (Promega Corporation, Madison, WI, USA) by qPCR in the ABI PRISM 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Amplification process was a three-step procedure of 40 cycles, including denaturation at 95°C for

2 min, annealing at 95°C for 15 sec, and extension at 60°C for 50 sec followed by a melting-curve procedure of 1 min at 60°C. β -actin and the target samples were performed in triplicate. $2^{-\Delta\Delta C_q}$ method was used for analysis. The primers were listed as follows: Cx43 forward, 5'-TTCATGCTGGTGGTGTCC-3'; reverse, 5'-TTGGCATTCTGGTTGTC-3'; β -actin forward, 5'-AGCGAGCATCCCCAAAGTT-3'; reverse, 5'-GGGCACGAAGGCTCATCATT-3'.

Statistical analysis. Results were presented as mean $\pm$ standard deviation (SD) and analyzed by Paired Student's t-test or One-way analysis of variance (ANOVA) followed by a least significant difference (LSD) for multiple comparisons. A value of $P < 0.05$ indicated the statistic significance. Data were processed by SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Transfection efficiency of Cx43-silenced lentivirus. After the Cx43-silenced lentivirus was transfected into the cardiomyocytes, green fluorescence was observed under an inverted fluorescence microscope at 72 h after the transfection. A rough estimate of the photoluminescent ratios of the four lentiviral vectors was well over 80% (Fig. 1).

Western blot analysis was repeated 3 times. The analysis revealed that abundant total protein and phosphorylated protein of Cx43 were present in normal cardiomyocytes but both decreased in the cells transfected with Cx43-silenced lentivirus, especially in the LV-Gja1-RNAi (51662-1) group (Fig. 2). The transfection efficiency was validated again through RT-qPCR. The RNA expression level coincided with the previous results (Fig. 3). Compared with that of the normal cardiomyocytes, the Cx43 expression in cells transfected with Cx43-silenced lentivirus declined in the following sequence: Lentiviral vectors LV-Gja1-RNAi (51661-2), LV-Gja1-RNAi

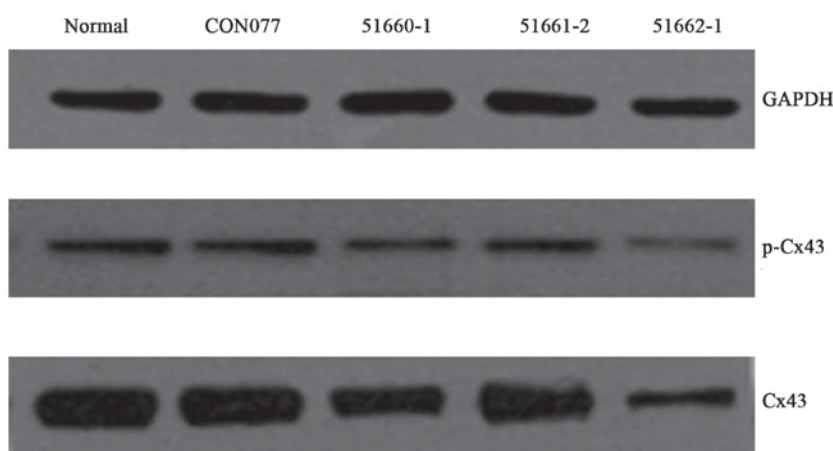


Figure 2. Protein expression of Cx43 and phosphorylated Cx43 by western blotting. Representative western blot images manifested these protein expression levels in the five groups: The normal group, Scramble lentiviral vector (CON077) group, lentiviral vectors LV-Gja1-RNAi (51661-2) group, LV-Gja1-RNAi (51660-1) and LV-Gja1-RNAi (51662-1) group. Expression of proteins (both total and activated protein of Cx43) was markedly alleviated in the LV-Gja1-RNAi (51662-1) group when compared with that of the scramble lentiviral vector group.

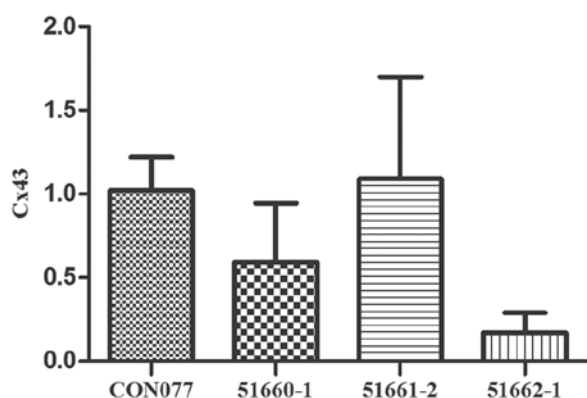


Figure 3. Gene expression of Cx43 in the four lentiviral vectors in comparison with normal cells by RT-qPCR. $n=3$ in each group. All results were presented as means $\pm$ SD. Gene expression in LV-Gja1-RNAi (51662-1) was lower compared with that of scramble lentiviral vector ($P<0.05$). Scramble lentiviral vector (CON077), lentiviral vector LV-Gja1-RNAi (51660-1), LV-Gja1-RNAi (51661-2) and LV-Gja1-RNAi (51662-1) were indicated.

(51660-1) and LV-Gja1-RNAi (51662-1) ($P<0.05$). Therefore, the LV-Gja1-RNAi (51662-1) was used for subsequent experiments.

Hemichannels-mediated volume regulation. The morphological and volumetric changes of the cells were determined at 30 min after the addition of hypotonic solution. Within the 30 min after the solution addition, all cells remained adherent and showed good viability. When a comparison was made between the two time points (30 min after the solution addition vs. 0 min), the cellular area in the hypotonic, hypotonic+DMSO, hypotonic+scramble lentivirus group augmented markedly ($3,973\pm1,720$ vs. $2,363\pm619 \mu\text{m}^2$, $5,050\pm1,511$ vs. $3,464\pm723 \mu\text{m}^2$, $5,517\pm1,128$ vs. $2,331\pm536 \mu\text{m}^2$, $P<0.05$, respectively; on the contrary, the area of the remaining four groups (Isotonic, hypotonic+octanol, hypotonic+18a-GA, hypotonic+Cx43-silenced lentivirus) showed no conspicuous changes ($3,804\pm737$ vs. $3,313\pm543 \mu\text{m}^2$, $2,990\pm765$ vs. $2,821\pm773 \mu\text{m}^2$, $4,817\pm1,306$ vs. $4,762\pm1,271 \mu\text{m}^2$, $3,627\pm688$ vs. $3,419\pm814 \mu\text{m}^2$, $P>0.05$, respectively; Fig. 4).

These volumetric changes indicate that cell edema can be substantially avoided by obliterating the Cx43-formed hemichannels, demonstrating their function of accommodating cell volume.

IPC-induced protection by blocking hemichannels. In both SIR and SIP conditions, the normal control group reported excellent cell viability and zero cell death but the SIR and SIP groups displayed varied mortality rates: the SIR group reported a mortality of up to $71.50\pm3.12\%$; both SIR+octanol and SIR+18a-GA group showed a much lower death rate when compared with the SIR+DMSO group (50.19 ± 0.97 , 46.20 ± 1.93 vs. $68.83\pm1.93\%$, $P<0.05$, respectively); similarly, the mortality of the SIR+Cx43-silenced group decreased noticeably when compared with that of SIR+scramble lentivirus group (47.17 ± 1.41 vs. $74.78\pm1.88\%$, $P<0.05$; Fig. 5). Likewise, the mortality of the SIP+octanol and SIP+18a-GA group declined considerably when compared with that of the SIP+DMSO group (35.70 ± 1.02 , 30.76 ± 2.20 vs. $53.58\pm2.14\%$, $P<0.05$, respectively); that of SIP+Cx43-silenced group also decreased distinctly when compared with the SIP+scramble lentivirus group (30.89 ± 2.37 vs. $54.12\pm2.55\%$, $P<0.05$; Fig. 6).

Of note, the SIP group had a much lower mortality rate when in comparison with the SIR group, suggesting the successful modeling and the protective effect of IPC on cardiomyocytes (52.44 ± 1.53 vs. $71.50\pm3.12\%$, $P<0.05$). Taken together, these findings demonstrate that blocking the hemichannels can further consolidate the IPC-induced cardiomyocyte protection.

Discussion

The principal aim of the present study was to investigate the role of the hemichannels of cardiomyocytes in the protective effect of ischemic preconditioning on the ischemic/reperfusion injury as a result of capacity regulation. In this research, we demonstrated that cellular edema was deterred by blocking hemichannels with blockers or by silencing Cx43 gene, which apparently enhanced the role of IPC protection. Therefore,

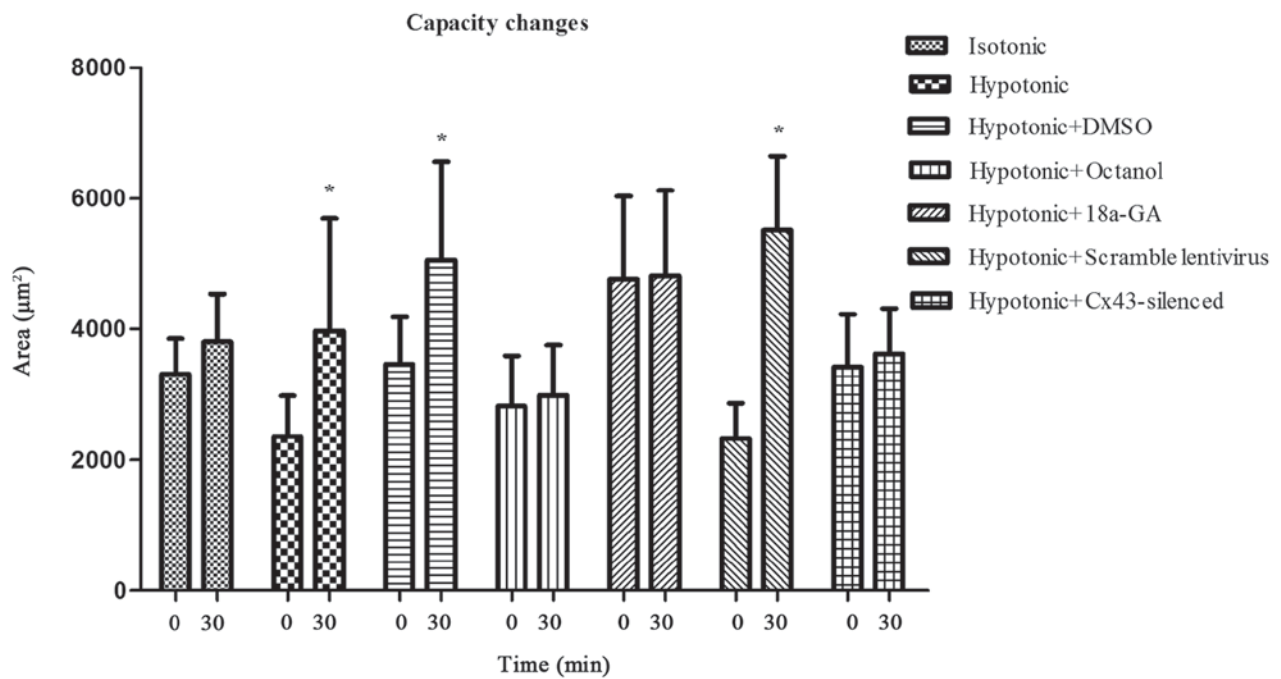


Figure 4. Volume changes of cardiomyocytes in the hypotonic solution. Areas are presented as means $\pm$ SD. The cell areas of the groups before the addition of hypotonic solution (baseline) and 30 min after the addition were shown in the first and second column, respectively. * $P<0.05$ vs. their corresponding baseline groups (0 min). 18a-GA, 18a-Glycyrrhizic acid; DMSO, dimethylsulphoxide.

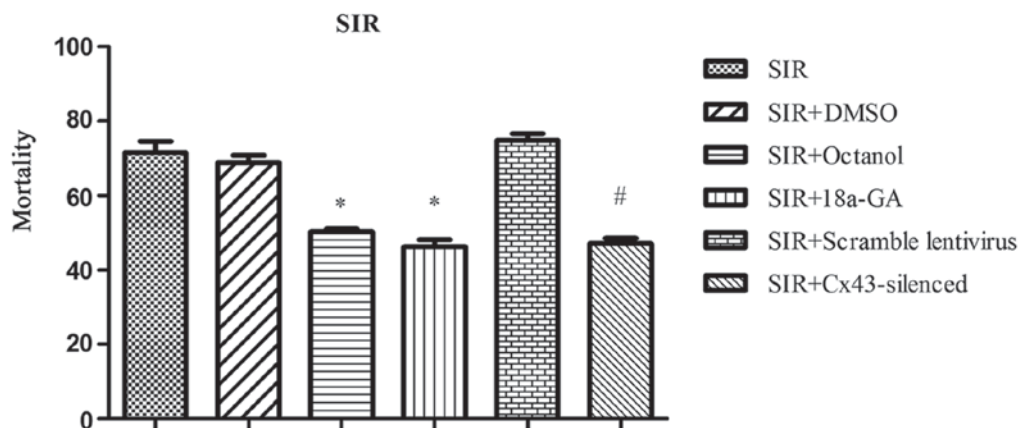


Figure 5. Mortality of cardiomyocytes in SIR groups. The mortality rates were expressed by means $\pm$ SD. * $P<0.05$ as compared with the SIR+DMSO group. # $P<0.05$ as compared with the SIR+scramble lentivirus group. SIR, simulate ischemic reperfusion; 18a-GA, 18a-Glycyrrhizic acid; DMSO, dimethylsulphoxide.

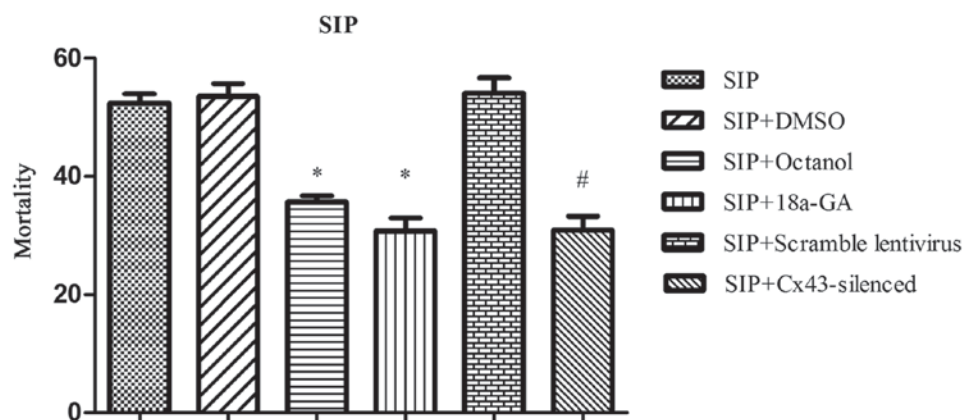


Figure 6. Mortality of cardiomyocytes in SIP groups. The mortality rates were expressed by means $\pm$ SD. * $P<0.05$ as compared with the SIP+DMSO group. # $P<0.05$ as compared with the SIP+scramble lentivirus group. SIP, systemic ischemic preconditioning; 18a-GA, 18a-Glycyrrhizic acid; DMSO, dimethylsulphoxide.

these results suggest that the IPC-induced cardiomyocyte protection may be mediated by hemichannels.

IPC has been reported as a myocardial protection, in which after a brief, repeated, nonlethal ischemia/reperfusion, the myocardial infarction area caused by the subsequent prolonged ischemia decreased by 75% (1). This IPC-induced protection is not complicated at all but a decisive elucidation of its underlying mechanism remains such a challenge that our understanding up to date is still modest in the available literature. In the course of ischemia, intracellular osmotic pressure increases when metabolites accumulate in cardiomyocytes (24). This increased pressure creates an osmotic gradient between the intracellular and extracellular environment and, in turn, leads to cell distension (25). In cardiomyocytes, Cx43-formed hemichannels control the intra- and extra-cellular transfer of water (17). Thus, Cx43 protein is definitely involved in the protective effect against ischemia (preconditioning) (22). Our results of SIR and SIP groups showed that the mortalities of hemichannel-blocked groups decreased noticeably compared with those of control groups, indicating that the hemichannels participate in the death caused by ischemia, and that blocking the hemichannel can reduce the cell mortality. Therefore, the current study confirms that the involvement of Cx43 in the IPC-induced protection is achieved by blocking the hemichannels.

Diaz *et al* speculated that the main myocardial protective mechanism of IPC is cell volume regulation (26). Their study focused on cell volume regulation from the direction of chloride channels, which is still controversial (27) and awaits further verification. Naitoh *et al* supposed that IPC has distinct effects on interaction of gap junction Cx43 with PKCepsilon, p38MAPKalpha, and Src during ischemia (28). Miura *et al* presumed that PKC-mediated Cx43 phosphorylation contributes to IPC-induced protection (29). Such studies ignore the fact that a hemichannel state is prevalent for most of the cardiomyocytes. The present study probed into the hemichannel-mediated volume regulation of cardiomyocytes in hypotonic solution and demonstrated that the cell volume is regulated by hemichannels, which echoes the findings of our previous research (30). On the other hand, Azzam *et al* claimed that an alleged death factor after ischemia may spread from cell to cell through gap junctions formed by Cx43 (31). However, the hemichannel blocker, heptanol, functioning as gap junction uncoupling (32), can restrain the propagation of this factor-the spatial process of cell death (33-35). Conversely, some research asserted that cardioprotection of IPC was lost in heterozygous Cx43-deficient mice (36), indicating that IPC protection is independent of Cx43-formed gap junctions or cell-to-cell communication. Unlike those previous studies (36), which were conducted *in vivo* and did not completely knock out the Cx43 gene, the present study was implemented *in vitro*, with the Cx43 gene utterly silenced.

Moreover, the current study found that the mortality of groups with hemichannels blocked by chemicals and gene deletion was distinctly lower than that of their corresponding counterparts in SIR/SIP experiments, which directly addresses the role of volume regulation in IPC. These findings correspondingly imply that cell swelling and the loss of cell volume regulation play important roles in ischemic injury in the myocardium, which is consistent with the findings of

other previous studies (37,38). Similar research of gap junction ascertained that heptanol can interfere with gap junction opening (32) and in turn reduces the final myocardial infarct size during reoxygenation or reperfusion (7), which lends substantial support to our conclusion. On the basis of the present findings, we attempt to propose for the first time that hemichannel-mediated cell volume regulation is most likely involved in the IPC-induced cardiomyocyte protection.

However, the inhibition of hemichannel transmission is not the only mechanism of IPC-induced protection. The mortality of cardiomyocytes was only partly decreased in SIP groups with hemichannels blocked by chemicals or by silencing Cx43 gene. Hence, other mechanisms, other than hemichannel-mediated capacity regulation, may be involved in the IPC-induced protective effect and have not been elucidated. An experiment conducted in isolated cardiomyocytes verified that mitochondrial ROS was engaged in the IPC protection (39). Another research showed that during ischemia, IPC confers the cardioprotection by the gap junction protein Cx43-mediated signaling pathway of PKCepsilon, p38MAPK, and Src (28). Further studies are still required to understand other contributory mechanisms involved in the IPC-induced protection.

There are few methods, other than microscopy, to accurately determine the capacity of individual cells (40,41). In this research, the thickness of cardiomyocytes, which were closely adherent to the plate, was so thin that it was negligible. Thus, the volume change was replaced with the area when cells were still active. In the current study, before the addition of hypotonic solution, the area of cardiomyocytes was calculated as the baseline. At 30 min after the solution addition, obvious volumetric change was evident while the cell viability and plate adherence were maintained. Another technical difficulty in the current study was to measure the volumetric transformation right at the incidence of cell death. Nevertheless, the disparity in mortality rates is sufficient to demonstrate the mechanism of IPC-induced cardiomyoprotection.

Together, the present study comes up with an explicit interpretation of how IPC protects myocardium from ischemia injury. This finding provides a new perspective into ischemic protection and has a great significance for the protection of clinical patients with myocardial infarction.

In summary, our results demonstrate that Cx43-formed hemichannels participate in the volume regulation of cardiomyocytes and that swelling of cardiomyocytes can be alleviated by blocking these hemichannels in a hypotonic environment. This hemichannel-mediated volume regulation contributes to the IPC-induced cardiomyoprotection.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JF, YL and WW designed the study. DZ and HL performed the cell culture. JH, HC and TY participated in the cell area data analysis. WW analyzed the data and drafted the manuscript. All authors reviewed the manuscript and approved the final version.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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