Protective Effect of Grape Seed Proanthocyanidins Extracts on Reperfusion Arrhythmia in Rabbits

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Summary Reperfusion arrhythmia (RA) is one of the main complications which are also an important cause of sudden cardiac death. The aim of this study was to clarify whether grape seed proanthocyanidins extracts (GSPE) were therapeutic agents against RA. The models of cardiac ischemic reperfusion injury were established in rabbits. GSPE (100 mg/kg, and 250 mg/kg body weight/d, respectively) were administered for 3 wk. The incidence rates of arrhythmias before and after reperfusion of each group were recorded, cardiac infarction area and microstructures of cardiac cells of each rabbit were observed, and the expression of connexin 43 (Cx43) was detected by immunohistochemistry. Data were analyzed using the Leica Qwin V3 image analysis system. Reperfusion induced arrhythmia. Ventricular fibrillation (VF) occurred during the early phase of reperfusion after ischemia. Our results showed that GSPE treatment significantly reduced the incidence of VF and the infarction size compared with the model control group. Moreover, the intercalated disks in the model control group showed collapse, displacement and even the formation of cisterns. After being treated by GSPE, the intercalated disks were improved and there were less collapse and displacement. The expression of Cx43 was improved by GSPE treatment, and high dose of GSPE resulted in significant improvement. The study suggests that GSPE has a protective effect on myocardial ischemic reperfusion arrhythmias, which may be mediated by inhibiting the degradation of Cx43 and enhancing gap junctional conductance.

Key Words grape seed proanthocyanidin extracts, myocardial ischemic reperfusion injury, reperfusion arrhythmias, gap junction, connexin 43

Since reperfusion treatment has become a prime strategy in the treatment of ischemic cardiac diseases, more attention has been paid to myocardial reperfusion injury. Reperfusion arrhythmia (RA) is one of the main complications and is also an important cause of sudden cardiac death. The mechanisms of reperfusion arrhythmia may include heterogeneous recovery of conduction and a refractory period of incomplete reperfusion, reentry, abnormal automaticities, and activities triggered by Ca2+ overload and free radicals (1–4). However, the details of the mechanisms remain unclear and RA has not received satisfactory treatment. It is reported that gap junctional uncoupling plays a trigger role in the antiarrhythmic effect of ischemic preconditioning (5). Gap junctions (GJ) are clusters of intercellular channels localized to intercalated disks that mediate electrical and chemical signaling throughout the cardiovascular system (6, 7). The predominant composition of GJ in the ventricular cells of mammals is connexin 43 (Cx43), which plays a key role in maintaining normal gap junction, cardiac electric activity and coordination of cardiac mechanical activities (8). Up to now, there have been few reports on the change of gap junctional conductance and the expression of Cx43 in acute myocardial ischemic reperfusion injuries.

Grape seed proanthocyanidin extracts (GSPE), derived from grape seeds, are polyphenols and have a strong antioxidant effect, polymerized by monomers of catechin or epicatechin. They have been reported to possess a variety of potent properties, including protective effects against cardiac disorders (9) such as coronary artery sclerosis, and against myocardial reperfusion injury, which may be mediated through their antioxidative and anti-apoptosis effect (10). GSPE could provide significant protection against myocardial ischemia-reperfusion injury and doxorubicin-induced cardiotoxicity, while the mechanisms are different (11–13). In this study, we tried to determine whether GSPE have a protective effect against RA and to detect the role of GSPE in maintaining the integrity of intercalated disks, so as to provide a new strategy for the treatment of myocardial ischemic reperfusion arrhythmias.

MATERIALS AND METHODS

Materials. GSPE (56% dimeric proanthocyanidins, 12% trimeric proanthocyanidins, 6.6% tetrameric proanthocyanidins, and small amounts of monomeric and high-molecular-weight oligomeric proanthocyanidins and flavanols; Lot No G050412) were provided by

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Jianfeng, Inc. (Tianjin, China). Nitroblue tetrazolium (NBT) was provided by Shanghai Forward Huaxueshijian; primary anti-Cx43 polyclonal rabbit antibody was provided by Boter Bioengineering Corp., Wuhan, China; secondary antibody horseradish-marked IgG of rabbit from sheep was provided by Boter Bioengineering Corp., Wuhan, China; formaldehyde, paraffin and all the other chemicals used were of the highest grade available commercially.

Experimental groups. Forty-eight male New Zealand rabbits were purchased from the Laboratory Animal Center of Shandong University (Shandong, China). The animals were housed in cages and received a normal standard laboratory diet 100–150 g/d in a constant environment (room temperature 22 ± 1.5°C, room humidity 55 ± 5%) with a 12 h light,12 h dark cycle. The animals were kept under observation for 1 wk before the experiment. All procedures were approved by the animal ethics committee of Shandong University. The animals were housed in cages and received a normal standard laboratory diet 100–150 g/d in a constant environment (room temperature 22 ± 1.5°C, room humidity 55 ± 5%) with a 12 h light,12 h dark cycle. The animals were kept under observation for 1 wk before the experiment. All procedures were approved by the animal ethics committee of Shandong University. The rabbits with a body mass of 1.5 to 2.0 kg were randomized into 4 groups after 1 wk: rabbits in the control group with a sham operation (group A, n = 12), the model control group (group B, n = 12) receiving saline intragastrically, the model groups with low (group C, n = 12) or high dose (group D, n = 12) GSPE treatment. On the basis of our previous study the last received GSPE 100 mg/kg and 200 mg/kg intragastrically every day for 21 d; the lavage volume was 2 mL/kg.

Twenty-four hours after the last administration, animals were anesthetized with 0.6% pentobarbital sodium 20 mg/kg and underwent thoracotomy. All animals except those in group A underwent advanced ischemic surgery as previously described (14). Once the pericardium was opened and separated, a loose silk suture was placed around the left anterior descending coronary artery approximately 1/3 below its origin. A small silicon ring (diameter: 2 mm) was inserted in the silk thread below the knot to facilitate the subsequent removal of the suture. Ischemia was induced by tightening the coronary suture and was maintained for 30 min. Reperfusion was obtained by cutting the ligature around the coronary artery and the duration of reperfusion was predetermined as 120 min. Animals in group A receiving the sham operation had the breast removed and had no further surgery.

Sample preparations. At the end of reperfusion, the hearts were quickly removed, the left ventricle was excised along the coronary sulcus and the extension of the left ventricle undergoing damage caused by ischemic reperfusion was determined by the nitro blue tetrazolium dye exclusion method. The anterior left ventricular wall, 3 mm distal to the ligature, was excised for morphological studies in the infarct region.

Arrhythmia study. ECG was continuously recorded throughout the experiment. The ECG data were retrospectively analyzed, in a blind manner, for the incidence, time to onset, and duration of ventricular fibrillation (VF) during reperfusion. All analyses were carried out in accordance with the Walker report (15). VF was defined as a signal in which individual QRS deflections could no longer be distinguished from one another and the heart rate could not be determined. Data were collected before ligation, 30 min after ligation, 60 min, and 120 min after reperfusion.

Calculation of myocardium infarction area. The heart was removed and washed with PBS. The left ventricle was weighed and excised into 4 approximately equal parts from apex to basement along the atrioventricular groove. The myocardium was treated by nitroblue tetrazolium (NBT) dye reduction test and the area of the damaged myocardium was determined by planimeter. The NBT dye reduction method was performed as described previously for cell suspensions (using triplicate samples for each data point) in 250 μL buffer containing 0.2% NBT. The assay was scored under light microscopy after 30-min incubation by counting the percentage of cells that had aggregates of reduced NBT. The infarction area was calculated according to weighting method (infarction area=weight of damaged myocardium/weight of left ventricle×100%).

Morphology observation. The harvested myocardium was routinely fixed in 10% formaldehyde, embedded in paraffin, cut into 4-μm-think sections for light microscopy, and then stained with hematoxylin-eosin at a magnification of ×200 with a JEM-1200EX light microscope. Furthermore, part of the LV myocardium was fixed in 3% glutaraldehyde. Ultrathin sections cut from the embedded blocks were stained with uranylacetate and lead citrate and were examined with a HITACHI H-800 electron microscope.

Immunohistochemistry. The myocardium was prepared in wax sections and cardiac cells undergoing reperfusion were detected by Cx43 polyclonal antibody for rabbit. Gradation of 10 randomly chosen campus visuals (×400) of different sections in different groups were analyzed by Leica Qwin V3 image analysis software. The expression of Cx43 was negatively correlated with its gradation.

Western blot analysis. Samples of myocardium were obtained from the 4 groups of rats. Equal amounts of proteins were separated by electrophoresis in a 12% SDS-polyacrylamide gel. After the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), the blot was blocked with 5% (w/v) non-fat milk in TBST (Tris-buffered saline and 0.05% Tween-20) for 1 h at room temperature and then probed with anti-Cx43 (1:1,000) polyclonal antibody and anti-GAPDH (1:500), followed by incubation with the secondary antibody, horseradish peroxidase conjugated affinity anti-mouse IgG (1:7,500) for 1 h. Signal detection was performed via exposing the blots to enhanced DAB color reagent for 5 min. Quantification of the luminosity of each identified protein band was performed using a densitometric analysis (Digital Protein DNA Imagineware, Huntington Station, NY, USA).

Statistic analysis. Results are shown as means ± SD on the basis of at least 3 separate experiments. Statistic analysis was performed using SPSS 11.5 software. One-way ANOVA was used to determine the significance of...
Table 1. Effect of GSPE on reperfusion arrhythmias.

| Group | n   | 60 min after reperfusion | 120 min after reperfusion |
|-------|-----|--------------------------|--------------------------|
|       | VT  | VF | Morbidity (%) | Mortality (%) | VT  | VF | Morbidity (%) | Mortality (%) |
| A     | 12  | 0  | 0             | 0              | 0   | 0  | 0             | 0              |
| B     | 12  | 12 | 4             | 100**           | 0   | 10 | 2             | 8.3#           |
| C     | 12  | 6  | 1             | 50             | 0   | 2  | 0             | 16.7           |
| D     | 12  | 2  | 0             | 16.7*          | 0   | 0  | 0             | 0              |

**p<0.01, #p<0.05, group B vs A; *p<0.05, group D vs B. A: control; B: ischemic-reperfusion group; C: low dose GSPE-treated group; D: high dose GSPE-treated group. VT: ventricular tachycardia; VF: ventricular fibrillation.

Fig. 1. The morphology of rabbit myocardium (HE ×200). A: control; B: ischemic-reperfusion group; C: low dose GSPE-treated group; D: high dose GSPE-treated group.

Fig. 2. Electron microscopic examinations of rabbit myocardium nucleus, sarcomere and mitochondria. A: control; B: ischemic-reperfusion group; C: low dose GSPE-treated group; D: high dose GSPE-treated group.
differences among treatments. \( p < 0.05 \) was considered statistically significant.

**RESULTS**

Effect of GSPE on the occurrence of reperfusion arrhythmias

Reperfusion-induced arrhythmia VF occurred during the early phase of reperfusion after ischemia and no arrhythmia occurred in the non-operative group. The incidence of VF was 100% 60 min after reperfusion in group B, while that after 120 min was 83.3%, and it decreased with treatment with GSPE (as shown in Table 1). There was no significant difference in the occurrence of VF between group D and group C, but the occurrence of VF in group D was significantly decreased by treatment with high doses of GSPE.

Effect of GSPE on the infarction area

Following NBT dyeing, the normal myocardium showed an intense blue staining reaction, whereas infarction regions remained unstained. As shown in Fig. 1, myocardium samples from group B showed a larger infarction area compared to group A (13.8±2.7 vs 2.7±2.0, \( p < 0.01 \)), indicating that animals in group B had undergone serious ischemia. Moreover, GSPE treatment in both low and high dose could apparently protect ischemic myocardium from infarction compared with group B (9.1±4.2 vs 13.8±2.7, \( p < 0.05 \); 6.8±3.7 vs 13.8±2.7, \( p < 0.01 \)).

Effect of GSPE on the change of myocardium structure

The myocardium structure showed obvious changes in different experimental groups as shown in Fig. 2. In the stained integrated cell membrane, degeneration, and edema of group A, we observed derangements, cloudy swelling, pyknosis and cytolysis of cardiac cells. We also observed intercellular exudation and numerous neutrophils migrated into the perivascular connective tissue in the ischemic-reperfusion hearts in group B, which were obvious expressions of infarction compared with group A, while in the GSPE-treated groups, the morphology of cardiac tissue was much better, especially that of group D which was treated with a high dose of GSPE.

Effect of GSPE on the change of myocardium ultrastructure

As shown in Fig. 3, specimens from group A showed normal features: the cardiac cells were rich in mitochondria arranged between myofibrils in rows with myofibrils arranged evenly and normal intercalated disks. The sarcomere was of the same length. Nuclear membrane was integrated and chromatin was normally distributed. In group B, disarrangement, breakage and local absence of myofibrils were observed. Numerous disarranged mitochondria substituted for myofibrils. Mitochondria vacuolization and crista loss were observed. Images of cell necrosis were also observed. Images of abnormal atrophy such as myofibril disarrangement, different lengths of sarcomere, and abnormality of mitochondria were still observed in group C; however, compared with group B, there was no myofibril breakage, and the structure abnormalities of nuclear and mitochondria were apparently reduced.

High dose of GSPE showed much more apparent differences. The most protective effect was acquired by high doses of GSPE in that there were only moderate mitochondrial and nucleolus abnormalities in cardiac myocytes shown in group D.

Effect of GSPE on gap junction

We further observed the effect of GSPE on intercalated disks, which comprised gap junctions, by using a transmission electron microscope, and we found that GSPE played a protective effect on the gap junctions. As shown in Fig. 3, pictures of intercalated disks in group B showed collapse, displacement and even formation of cisterns, yet in group C, the intercalated disks were...
improved and there were little collapse or displacement. Strikingly, the intercalated disks of animals with high doses of GSPE were normal just as in the normal group; there was no evident injury.

**Effect of GSPE on the expression of Cx43 in experimental groups**

Cx43 stained buffy appeared in strip distribution located on intercalated disks. As shown in Fig. 4, sections from group A were evenly stained with uniform positive coloring points. On the other hand, staining of sections from group B were much lighter showing disarranged positive coloring points with different sizes and colors, resulting in higher gradation compared with group A (p<0.01). It is interesting that images from group C and group D showed apparent improvement of Cx43 expression, and a high dose of GSPE resulted in significant improvement (shown in Fig. 4), indicating that GSPE treatment could improve Cx43 distribution during ischemic-reperfusion injury compared with those without treatment (p<0.05). By Western blot, it was found that the protein expression of Cx43 in group B decreased compared to that of control rats (p<0.01); after treatment with GSPE, the expression of Cx43 in group C and group D increased compared to group B (Fig. 5).

**DISCUSSION**

Although reperfusion treatment has been proved to be the most effective way in dealing with heart ischemia, ischemic-reperfusion injury would further exacerbate tissue damage. It has been reported that 25–50% of the infarction area was caused by reperfusion itself (16). Moreover, reperfusion could lead to arrhythmias, myocardial stunning, and fatal reperfusion injury and therefore result in heart failure and even sudden death (17). So, it would be helpful to provide new effec-
tive drugs and explain the related actions.

GSPE has been reported to have protective effects against various forms of cardiac disorders (18, 19), such as arteriosclerosis and diabetic cardiomyopathy. It has also been reported to have a protective effect against myocardial reperfusion injury which might be mediated through its antioxidative and anti-apoptosis effect (20). However, the mechanism is still unclear, so we designed this study in order to elucidate its protective mechanism.

Abnormal excitability of cardiac cells was once thought to be the main reason for arrhythmias, but recent studies indicated that intercellular electrical coupling dysfunction play a much more important role in the occurrence of arrhythmias (21). The functional connecting region of the end-to-end connections between cardiac cells are called intercalated disks, the intercellular gap junction of which is the basic structure of intercellular electrical coupling. Each gap junction formed by thousands of special different gap junction channels is characterized by hydrophilia, low selectivity and electric resistance. Gap junctions mediate normal electric and chemical communication between individual cardiac cells to preserve normal cardiac functions and so they are called communication junctions. Dysfunction of gap junctions plays a more important role in the occurrence of arrhythmias than dysfunction of ionic channels. Each gap junction is created by stable, tight junctions of two hemichannels known as connexon, which are basic units of the gap junction. Each connexon is composed of six connexin proteins (Cx) forming a 1.5 nm central channel permitting small molecules (<1 kDa) and ions to pass through (22). In the mammalian heart, gap junctions are mainly composed of three different Cx: Cx43, Cx40 and Cx45. Cx43 is the main constituent of cardiac gap junction expressed in all atrial and ventricular cells, except the sinusatrial node, atroventricular node and parts of the conducting system. Cx43 is the only connexin expressed in adult ventricular cells, and they are distributed mainly on the intercalated disks. Abnormal expression and distribution of Cx43 indicates functional and structural injury of cell membranes and intercellular junctions and results in dysfunction of electrical coupling, which has been implicated in cause changing of conductive velocity and direction leading to the occurrence of conductive block and re-entry (23).

Degradation and disturbance of the distribution of Cx43 has been proved to be closely related with arrhythmias in chronic cardiac ischemia and old myocardial infarctions (24). The ultrastructure of intercalated disks from chronic ischemic cardiac cells of canine origin showed that the number and area of gap junctions per unit length were obviously reduced (25). Previous studies showed that the disturbance of distribution of Cx43 was much more serious in regions bordering ischemic myocardium, resulting in the curling of the wave front of the cardiac cells and reentry was induced. In the center of infarction regions, complete absence of Cx43 formed an anatomical block of electrical conduction contributing to a reentrant helix wave. It has also been reported that Cx43 depredated during acute ischemic-reperfusion infarction; the damage of gap junction gradually migrated from the center of infarction to the non-ischemic regions and caused heterogeneity of conductive velocity. All of these data suggested that the changed distribution and expression of Cx43 played a key role in the occurrence of malignant arrhythmias. Researchers reported that they detected the area of gap junction in ischemic and reperfusion myocardium by patch clamp, immunofluorescence and laser copolymerized microscope and found that the area of gap junction was reduced and gap junction channel conductive velocity slowed down, respectively (26, 27). Decoupling between cardiac cells was more serious 30 min after reperfusion than when early ischemia caused heterogeneity of conduction and electrical activity of cardiac cells; therefore malignant arrhythmias occur frequently at this moment.

Our study showed that in the ischemic-reperfusion group the area of infarction was larger and the occurrence of ventricular arrhythmias was higher compared to the normal control. Ischemic-reperfusion caused serious damage to the structure of cardiac cells, especially intercalated disks displayed as disturbance of distribution and degradation of Cx43 resulting in reperfusion arrhythmias. On the other hand, GSPE treatment both in low and high dose could significantly reduce the
infarction area after reperfusion, protect the integrity of cardiocytes, and improve the Cx43 expression and distribution, especially in the high dose GSPE-treated group. Both the occurrence of arrhythmias and Cx43 expression levels in GSPE-treated groups were significantly better than in the untreated ischemic group.

Based on this study, we propose that the anti-reperfusion arrhythmia effect of GSPE may be mediated by its protection of intercalated disks, due to the inhibition of Cx43 degradation and abnormal distribution, so as to improve intercellular communication and therefore reduce heterogeneity of conduction which is the anatomical basis of reentry. The protective effect of GSPE appeared in a dose-dependent manner. We inferred that the protective mechanism of GSPE on cardiac gap junctions may be attributed to its protection of the integrity of intercalated disks and inhibiting the degradation of Cx43. Our study first confirmed the protective effect of GSPE on cardiac gap junctions, and provided a new strategy for preventing cardiac reperfusion injury. However, the limitation of this study was not to explore the molecular mechanism of GSPE, so its anti-ischemic effects should be studied further before a firm conclusion is drawn.

Moreover, this cytoprotective action may be derived from some other proteins induced by GSPE. Nuclear factor E2-related factor 2 (Nrf2) is a critical regulator of cytoprotective gene expression, including those involved in Phase II detoxification and antioxidant gene expressions. Nrf2-dependent gene expressions may be attributed to its protection of the integrity of intercalated disks and inhibiting the degradation of Cx43. Attenuation of renal ischemia-reperfusion injury by Procyanidins from Vitis vinifera seeds display cardioprotection in an experimental model of ischemia-reperfusion damage. Drugs Exp Clin Res 29: 207–216.

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