Cardiovascular Topics

Salidroside protects the cardiac function of exhausted rats by inducing Nrf2 expression

Peng Xu, Yang Wang, Weiwei Sun, Yawei Sun, Wei Lu, Yumei Chang, Zheng Ping, Yang Li, Xuebin Cao

Abstract

Objective: To investigate whether salidroside (Sal) protected the rat heart from exhaustive exercise-induced injury by inducing nuclear factor erythroid 2-related factor 2 (Nrf2) expression.

Methods: Forty-eight male Sprague-Dawley rats were divided into four groups (n = 12 rats per group): the control, the exhaustive swimming (ES) group, the low-dose Sal plus acute exhaustive swimming (SLE) group, and the high-dose Sal plus acute exhaustive swimming (SHE) group. In the SLE and SHE groups, 15 and 30 mg/kg Sal were administered, respectively, once a day. The rats in the control and ES groups were administered the same amount of physiological saline, respectively, once a day. On the 14th day, the rats in the ES, SLE and SHE groups underwent exhaustive swimming training once. Then cardiac function parameters and electrocardiograms were recorded. Biomarkers of myocardial injury in the serum and oxidative stress factors in the myocardial tissue were evaluated using ELISA tests. The levels of Nrf2, nuclear Nrf2 and Kelch-like ECH-associated protein 1 (Keap1) messenger RNA and proteins were assessed in the myocardium using q-PCR and Western blotting, respectively.

Results: Compared to the control group, the ES group showed remarkable increases in serum brain natriuretic peptide (BNP), cardiac troponin I (cTnI) and reactive oxygen species levels, but significant decreases in catalase and glutathione levels (p < 0.05). Compared to the ES group, the Sal treatment decreased serum BNP and cTnI levels and alleviated the changes in levels of oxidative stress-related factors. After treatment with Sal, nuclear and intracellular levels of Nrf2 protein were increased in the myocardial cells, while the level of Keap1 protein was decreased (p < 0.05).

Conclusion: Sal protected the heart from exhaustive exercise-induced injury, and it may improve cardiac function and cardiac bioelectricity in exhausted rats by inducing Nrf2 expression.

Keywords: exhaustive exercise, heart function, nuclear factor erythroid 2-related factor 2, oxidative stress, rats

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High-intensity exercise, such as exhaustive exercise (exercise intensity or duration exceeding the body’s limit), not only influences performance in competition but also impairs the physical and mental health of athletes and military personnel. Therefore it is important to explore performance and the pathogenesis of exercise-induced heart injury to improve its treatment.

Exhaustive exercise can cause destruction of the myocardial ultrastructure, abnormal energy metabolism and a reduction in cardiac function and electrocardio-electric changes. Exhaustive exercise also increases oxidative stress levels, potentially causing heart damage.

Nuclear factor erythroid 2-related factor 2 (Nrf2) plays a leading role in activating or encoding anti-oxidant enzymes and an important role in regulating the oxidation–anti-oxidation states of cardiac and vascular endothelial cells. High-intensity exercise affects Nrf2 levels, but the effect remains controversial. Acute intense exercise increased the messenger RNA (mRNA) transcription of Nrf2 target genes in skeletal muscle and most antioxidant enzyme genes in cardiac myocytes in mice. Levels of Nrf2 protein exhibited little change in the skeletal muscle of rats exposed to exhaustive treadmill exercise, and the expression and enzyme activity of its target proteins also exhibited little or decreased change.

Kelch-like ECH-associated protein 1 (Keap1) is a negative regulator of Nrf2. In response to oxidative stress, a conformational
change in Keap1 causes Nrf2 to dissociate from Keap1, which is the most common method for activating Nrf2. Upon activation, Nrf2 is transported to the nucleus, then antioxidant enzyme-encoding genes are expressed. The expression of the antioxidant enzymes superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) is mainly induced by activated Nrf2.

Exhaustive exercise also increases oxidative stress levels. Reactive oxygen species (ROS) activate the stress-response kinases in the MAPK family; many protein kinases, such as the extracellular signal-regulating kinase (ERK) and p38MAPK are located upstream of Nrf2. These kinases phosphorylate Nrf2 to regulate Nrf2 transcriptional activity and alter the cellular distribution of Nrf2, while exhaustive exercise alters the levels of p38MAPK and ERK. Therefore we hypothesised that exhaustive exercise may impact on the heart by modulating Nrf2 expression.

Salidroside (Sal) is an effective extract obtained from *Rhodiola rosea* that induces Nrf2 expression. The intervention of Sal improved left ventricular function, decreased myocardial ischaemia–reperfusion injury, and improved the myocardial ultrastructure and energy metabolism in exhausted rats. Sal altered myocardial levels of proteins in the MAPK pathway, MAPK activation improved cardiac arrhythmia and other cardiac diseases. Therefore we hypothesised that the protective effect of Sal on the exhausted heart was related to Nrf2 expression. In these experiments, we aimed to explore the effects of exhaustive exercise on myocardial levels of Nrf2 and Keap1.

Methods

Forty-eight male Sprague-Dawley rats (385 ± 34 g) were provided by the Academy of Military Medical Sciences (Beijing). National standard rodent dry feed was provided *ad libitum*, the indoor temperature was maintained at 18 to 22°C, and the relative humidity was maintained at 40 to 55%.

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals and reviewed and approved by the Ethics Committee for the Use of Experimental Animals at No. 252 Hospital of the Chinese People's Liberation Army.

The main reagents used in this study are listed below. The 98% rhodionine powder was purchased from Nanjing Zelang Pharmaceutical Technology Co., Ltd. Solutions containing specific concentrations of Sal were generated by dissolving the Sal powder in sterile normal saline. The cardiac troponin I (cTnI), brain natriuretic peptide (BNP), CAT and GSH enzyme-linked immunoassay kits were obtained from BD Biosciences (New York, USA). The ROS enzyme-linked immunoassay kit was purchased from R&D Systems (USA) and the anti-Nrf2 and anti-Keap1 antibodies were purchased from Abcam (UK). The TRizol total RNA extraction reagent was purchased from Tiangen Biotech Co., Ltd. The PrimeScript™ RT reagent kit with genome DNA Eraser, SYBR® Premix Ex Taq™ II, DL2 and DNA marker were purchased from TaKaRa Co., Ltd.

The following main instruments were used in this study: a PowerLab signal acquisition and analysis system, MultiscanGO enzyme standard instrument (Thermo, USA), Sigma 3k15 high-speed refrigerated centrifuge (Sigma, Germany), pressure–volume catheter (SPR-838, Millar Company, USA), fluorescence quantitative PCR platform (ABI 7500, Applied Biosystems), vertical electrophoresis system (BIO-TEK, USA), transfer electrophoresis system (BIO-TEK, USA), gel imaging system (BioSpectrum), image analysis system (Image-Pro Plus 4.1), PowerLab data acquisition and analysis system (AD Instruments, Australia), bioelectric amplifier (AD Instruments, Australia) and a needle electrode (AD Instruments, Australia).

Sprague-Dawley rats were randomly divided into four groups (*n* =12 rats per group): the control, an acute exhaustive swimming group (ES), a low-dose Sal plus acute exhaustive swimming group (SLE), and a high-dose Sal plus acute exhaustive swimming group (SHE). Six of the 12 animals in each group were used for the pressure–volume catheter detection of cardiac function, which was an invasive experiment. These animals were euthanised after the experiment.

Serum, electrocardiogram and myocardial specimens were collected from the remaining animals (*n* = 6 rats per group). Each group was administered the Sal solution (15 or 30 mg/kg/d) or the same amount of normal saline for 14 days. The adaptive swimming exercise was performed three times (20 min/time) during the irrigation period. The control group did not exercise.

The rats in the ES, SLE and SHE groups were submitted to one exhaustive swimming training session after the 14-day treatment. Because eating would increase the time an animal would be required to swim to reach exhaustion, rats were fasted for 12 hours before training. The water temperature was maintained at 32°C, and the temperature fluctuated by no more than 1°C. Each rat in the exhaustion groups carried a tin wire (3% body weight) on the tail. The exhaustive swimming exercise was performed until exhaustion was achieved.

The experimental animal model of exhaustive exercise-induced damage was established according to the standards described by Thomas: animals were unable to return to the surface of the water for 10 seconds and when placed upside down, they were unable to complete a righting reflex. Their fur was dried with a heater immediately after exhaustion was reached.

Rats were subjected to abdominal anaesthesia with pentobarbital sodium (40 mg/kg), the chest was opened, and blood was collected from the inferior thoracic vena cava. The blood was centrifuged at 3 000 rpm for 20 minutes and the supernatant was collected and stored in a −80°C freezer until detection of the serum indicators.

The hearts were quickly removed and washed with cold saline. Tissues were stored individually at −80°C until q-PCR and Western blot analysis was done.

**Determination of cardiac function parameters with a pressure–volume catheter**

Rats were anaesthetised with pentobarbital sodium (40 mg/kg, intraperitoneal), and the closed-chest approach was chosen for catheter insertion. The animal was fixed in the supine position on the operating table. The skin of the neck was disinfected prior to a midline neck incision, and the trachea was separated and intubated. The right carotid artery was separated from the common carotid artery. Two 4-0 silk threads were sewn through the common carotid artery, and one of the silk threads was used to ligate the proximal end of the carotid artery. A cut was made at the end of the heart to complete the knot.

The pressure–volume catheter was inserted through the incision into the left chamber along the inverse blood flow of the
carotid artery and calibrated with MPVS control software. The left ventricular pressure-volume waveform of the anaesthetised rats was recorded with Chart® software in real-time. Vessels and catheters were fixed with another silk thread. Baseline data were recorded for 15 minutes.

The abdominal skin was disinfected, a median incision was made, the inferior vena cava was occluded, and changes in the waveform were recorded. A 20-μl solution of 30% NaCl was rapidly injected into the anterior jugular vein and pressure-volume waveform changes were recorded. The first four holes of a calibration cuvette with known diameters (provided by the manufacturer) were quickly filled and the catheter tip was submerged in warm, fresh heparinised blood. The conductance changes in the volume channel were recorded and the volume was then calculated.

The heart rate (HR), end-systolic pressure (Pes), end-diastolic pressure (Ped), end-systolic volume (Ves), end-diastolic volume (Ved), stroke volume (SV), ejection fraction (EF), peak rate of the increase in pressure (dP/dt max), peak rate of the decrease in pressure (−dP/dt min), slope of the end-systolic pressure-volume relationship (ESPVR), relaxation time constant (Tau), and slope of the end-diastolic pressure-volume relationship (EDPVR) were detected. The pressure–volume loop (PV loop) was drawn, with pressure on the y-axis and volume on the x-axis.

**Electrocardiography**

Adaptive electrocardiography (ECG) training was performed in all experimental rats. ECGs were recorded from rats in the control group in a quiet state for five minutes after 14 days of intraperitoneal injections of normal saline. In the EE, SLE and SHE groups, ECGs were recorded for five minutes immediately after exhaustive swimming. Wide-awake rats were placed in the rat cage, and both sets of limbs and the right forearm were routinely disinfected. Subcutaneous punctures in the extremities were created to insert the electrodes (the left hind leg was used as the positive electrode, the right foreleg as the negative electrode, and both sets of limbs and the right forearm were intermittently homogenised with an electric homogenate machine for one minute, incubated on ice for 30 minutes, and submerged in warm, fresh heparinised blood. The conductance changes in the volume channel were recorded and the volume was then calculated.

**Enzyme-linked immunoassays for ROS, CAT and GSH levels in the myocardium**

The heart was removed from a −80°C freezer and left ventricular myocardial tissue was sheared, weighed and diluted to produce a 10% homogenate in phosphate-buffered saline (PBS) (0.01 mol/l, pH 7.2). All procedures were performed on ice. The mixture was centrifuged at 5 000 rpm for 10 minutes at a low temperature, pH 7.2. (All procedures were performed on ice. The mixture was centrifuged at 5 000 rpm for 10 minutes at a low temperature, pH 7.2.) All procedures were performed on ice. The mixture was centrifuged at 5 000 rpm for 10 minutes at a low temperature, drained and placed in a new EP tube for storage.

Enzyme-linked immunosorbent assays were performed according to the instructions included in the kits. The optical density (OD) of each sample was measured at 450 nm. The OD value for the standard was measured, and a standard curve was constructed with the OD value on the y-axis and concentration on the x-axis. The concentration of the indicated marker in each sample was obtained from the standard curve.

**q-PCR of mRNA levels in rat myocardial tissues**

All gene sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov) and primers were synthesised by Invitrogen, Beijing. The upstream primer for Keap1 was GTGGAGACAGACAGACCTGGACCTTC, its downstream primer was TGTCAGCCGGCTCCTCTCCT, and the product size was 178 bp. The upstream primer for Nrf2 was AAGAGGCTTGTACTTGGAGACTGT, its downstream primer was GAAAAATAGCTCTCTCACCCTAAT, and the product size was 223 bp. The upstream primer for actin was CCTAAGGCAACCGTGAAAA, its downstream primer was GACCAGAGGCATAACAGGGACA, and the product size was 106 bp.

The TRIzol total RNA extraction reagent was used to extract RNA from the samples, and real-time polymerase chain reaction (PCR) was performed according to the instructions for cDNA reverse transcription and PCR. In the reaction system, the fluorescent dye SYBR Green I was added for real-time monitoring, and the relative expression level of the target gene was analysed using the 2−ΔΔCT method. The reaction system was: 2X mix, 10 μl; upstream primer (10 μM), 0.5 μl; downstream primer (10 μM), 0.5 μl; template 2, 10 μl; and sterilised distilled water to a 20-μl total volume. The following amplification procedure was used: 95°C for 30 seconds, followed by 45 cycles of 95°C for five seconds and 60°C for 40 seconds.

**Western blot analysis of Keap1 and total and nuclear Nrf2 levels in the left ventricular myocardium**

The heart was removed from a −80°C freezer, left ventricular myocardial tissue was sheared on ice, minced with fine scissors, and 50 mg was removed and mixed with lysis buffer containing protease inhibitors and a phosphatase inhibitor. The solution was intermittently homogenised with an electric homogenate machine for one minute, incubated on ice for 30 minutes, and centrifuged at 12 000 rpm for 20 minutes at 4°C. The supernatant was then placed in a 0.5-ml centrifuge tube.

The nucleoproteins were extracted according to the manufacturer’s instructions. Protein concentrations were determined using the bicinchoninic acid (BCA) method with bovine serum albumin as the standard. Then the protein samples were diluted to the same volume and heated at 100°C for five minutes after the addition of an equal volume of loading buffer. The denatured protein samples were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for two hours and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with blocking buffer containing 5% skim milk at room temperature for one hour and then incubated with primary antibodies overnight at 4°C.

After the membranes were washed three times with Tris-buffered saline (TBS) containing Tween, they were incubated with secondary goat anti-mouse IgG antibodies conjugated to horseradish peroxidase for one hour at room temperature, and then exposed to ECL for one to two minutes to detect the bands. A gel imaging system was used to capture images and for the quantitative analysis, and grayscale values were determined.

**Statistical analysis**

The data are presented as means ± SD. SPSS 17.0 statistical
software was used to analyse all experimental data. A single-factor analysis of variance was used for comparisons of multiple means after a one-way ANOVA and homogeneity test were first performed. Comparisons of mean values between two groups were performed using the LSD test if the variance was equal or Dunnett’s T3 method if the variance was unequal. A correlation analysis was performed by calculating Pearson’s correlation coefficients. A single-factor regression analysis was performed; \( p < 0.05 \) was considered to indicate a significant difference.

**Results**

The highest serum BNP (298.15 ± 36.98 ng/l) and cTnI (180.32 ± 19.69 pg/ml) levels were detected in the ES group. Significantly lower levels were detected in the Sal intervention groups.

Compared with the control group, significantly higher myocardial ROS levels were observed in the ES, SLE, SHE groups than in the control group (\( p < 0.05, n = 6 \)). Significantly lower ROS levels were detected in the SLE (6.25 ± 0.36 ng/mg) and SHE (4.91 ± 0.74 ng/mg) groups than in the ES group (7.66 ± 0.81 ng/mg, \( p < 0.01 \)). Significantly lower ROS levels were observed in the SHE than the SLE group. Myocardial oxidative stress levels were significantly increased by the acute exhaustion produced by swimming, and myocardial ROS levels were reduced by Sal.

Myocardial CAT levels were significantly lower in the SLE, SHE and ES groups than in the control group (0.60 ± 0.04 ng/mg). Compared with the ES group, significantly higher myocardial CAT levels were observed in the SLE and SHE groups (\( p < 0.01, n = 6 \)).

Significantly lower myocardial GSH levels were detected in the ES, SLE and SHE groups than in the control group (3.30 ± 0.45 U/mg). A significantly higher GSH level was observed in the SHE than in the ES group (\( p < 0.05, n = 6 \)) (Fig. 1).

Compared with the control group, the HR, Pes, dP/dt\(_{max}\) and –dP/dt\(_{min}\) were lower in the ES group, and the Ved, Ves, Ped, EDPVR and Tau were all higher. The differences in SV, Ved, Ped and Tau were significant between the Sal intervention groups and the control group. The Pes, dP/dt\(_{max}\)–dP/dt\(_{min}\), Tau and EDPVR were substantially higher in the SLE and SHE groups than in the ES group. Compared with the control group, a lower HR was recorded in the SLE and ES groups.

Non-significant differences in EF and ESPVR were observed between the groups (\( p > 0.05, n = 6 \)). Non-significant differences in EF and ESPVR were also observed between the SLE and SHE groups (Table 1).

HR was increased in the ES group (\( p < 0.05, n = 6 \)). The PR and QTc intervals were significantly longer in the ES, SLE and SHE groups than in the control group. A larger R amplitude was observed in the ES group (\( p < 0.01, n = 6 \)), but a smaller amplitude was observed in the SLE and SHE groups than in the ES group (\( p < 0.01 \)). However the QT interval and P amplitude were significantly higher in the ES and SLE groups. The ST-segment of the ES group was changed (Table 2).

Compared with the control group (0.71 ± 0.17), the change

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**Fig. 1.** The effect of Sal on serum BNP, cTnI and ROS levels, CAT and GSH in the myocardium after exhaustive exercise. The data are presented as means ± SD, \( n = 6 \) animals per group. BNP: brain natriuretic peptide; cTnI: cardiac troponin I; ROS: reactive oxygen species; CAT: catalase; GSH: glutathione; Con: control group; ES: acute exhaustive swimming group; SLE: low-dose salidroside plus exhaustive swimming group; SHE: high-dose salidroside plus exhaustive swimming group. \(* p < 0.05 \) and \(* * p < 0.01 \) compared with the control group; \(* p < 0.05 \) and \(* * p < 0.01 \) compared with the ES group; \( \# p < 0.05 \) and \( \# \# p < 0.01 \) compared with the SLE group.
in expression of Nrf2 mRNA was not statistically significant. Compared with the ES group (0.57 ± 0.13), a non-significant change in expression of Nrf2 mRNA was observed in the SLE group (0.59 ± 0.18) and SHE groups (0.54 ± 0.06) after the application of Sal. A non-significant change in expression of Keap1 mRNA was observed (Fig. 2).

Compared with the control group (0.39 ± 0.07), the level of Nrf2 protein in the ES group (0.43 ± 0.06) did not change significantly. However, significantly higher levels of Nrf2 protein were detected in the myocardium and nuclei of the SLE and SHE groups (p < 0.01, n = 6). Compared with the SLE group (0.48 ± 0.11), nuclear Nrf2 levels were significantly increased in the SHE group (0.61 ± 0.08) (p < 0.01, n = 6).

Compared with the control group (0.96 ± 0.03), Keap1 protein was expressed at lower levels in the other groups (p < 0.01, n = 6).

Fig. 2. The effect of Sal on expression of Nrf2 and Keap1 mRNA after exhaustive exercise. The data are presented as means ± SD, n = 6 animals per group. A: Relative levels of Nrf2 mRNA in rat myocardium. B: Relative levels of Keap1 mRNA in rat myocardial tissue. Con: control group; ES: acute exhaustive swimming group; SLE: low-dose salidroside plus exhaustive swimming group; SHE: high-dose salidroside plus exhaustive swimming group.

The level of Keap1 protein was significantly reduced in the SLE group (0.66 ± 0.06) and SHE groups (0.52 ± 0.03) compared to the ES group (0.72 ± 0.04), and it was expressed at significantly lower levels in the SHE than in the SLE group (p < 0.01, n = 6) (Fig. 3).

In the control group, Pearson’s correlation coefficient between the T amplitude and level of Nrf2 protein was −0.944 (p < 0.05), indicating a negative correlation. In the ES group, Pearson’s correlation coefficient for dP/dt max with nuclear Nrf2 was 0.836 (p < 0.05). Nuclear Nrf2 levels positively correlated with the P amplitude, and Pearson’s correlation coefficient was 0.921 (p < 0.01). Keap1 level was positively correlated with the QT interval (r = 0.934, p < 0.05). In the SLE group, nuclear Nrf2 levels were positively correlated with the P amplitude, with a correlation coefficient of r = 0.875 (p < 0.05). In the SHE group, the P amplitude was negatively correlated with levels of Nrf2 protein (r = −0.817, p < 0.05) (Table 3).

Expression levels of target proteins were divided by the expression level observed in the control group to calculate the relative mRNA and protein levels.

### Table 1. The effect of Sal on cardiac function parameters in exhausted rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ES</th>
<th>SLE</th>
<th>SHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV (μl)</td>
<td>122.10 ± 0.77</td>
<td>166.70 ± 16.71</td>
<td>176.14 ± 22.69</td>
<td>183.74 ± 19.34</td>
</tr>
<tr>
<td>VSS (μl)</td>
<td>71.88 ± 0.38</td>
<td>96.21 ± 9.36</td>
<td>83.22 ± 14.99</td>
<td>85.34 ± 14.80</td>
</tr>
<tr>
<td>VED (μl)</td>
<td>183.60 ± 29.82</td>
<td>249.09 ± 10.46</td>
<td>237.48 ± 11.43</td>
<td>262.40 ± 19.12</td>
</tr>
<tr>
<td>Pes (mmHg)</td>
<td>93.05 ± 11.68</td>
<td>71.45 ± 9.93</td>
<td>92.11 ± 6.04</td>
<td>99.25 ± 5.57</td>
</tr>
<tr>
<td>Ped (mmHg)</td>
<td>5.01 ± 3.30</td>
<td>7.88 ± 2.04</td>
<td>6.01 ± 0.86</td>
<td>6.60 ± 1.2</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>735 ± 31</td>
<td>290 ± 41</td>
<td>309 ± 24</td>
<td>345 ± 12</td>
</tr>
</tbody>
</table>

### Table 2. The effect of Sal on changes in ECG parameters in exhausted rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ES</th>
<th>SLE</th>
<th>SHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR interval (ms)</td>
<td>15.70 ± 0.97</td>
<td>14.04 ± 1.27</td>
<td>15.49 ± 2.02</td>
<td>15.56 ± 1.14</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>392 ± 21</td>
<td>444 ± 37</td>
<td>412 ± 33</td>
<td>405 ± 30</td>
</tr>
<tr>
<td>PR interval (ms)</td>
<td>4.18 ± 0.28</td>
<td>4.64 ± 0.43</td>
<td>4.77 ± 0.39</td>
<td>4.61 ± 0.48</td>
</tr>
<tr>
<td>P duration (ms)</td>
<td>1.64 ± 0.24</td>
<td>1.48 ± 0.24</td>
<td>1.54 ± 0.27</td>
<td>1.65 ± 0.23</td>
</tr>
<tr>
<td>QRS interval (ms)</td>
<td>1.96 ± 0.19</td>
<td>1.96 ± 0.18</td>
<td>1.98 ± 0.33</td>
<td>1.84 ± 0.38</td>
</tr>
<tr>
<td>QT interval (ms)</td>
<td>5.96 ± 0.55</td>
<td>6.96 ± 0.95</td>
<td>7.02 ± 0.97</td>
<td>6.83 ± 0.98</td>
</tr>
<tr>
<td>QTc interval (ms)</td>
<td>14.62 ± 1.73</td>
<td>19.70 ± 1.87</td>
<td>18.07 ± 3.14</td>
<td>18.42 ± 2.44</td>
</tr>
<tr>
<td>P amplitude (mV)</td>
<td>0.069 ± 0.022</td>
<td>0.120 ± 0.036</td>
<td>0.112 ± 0.028</td>
<td>0.096 ± 0.017</td>
</tr>
<tr>
<td>R amplitude (mV)</td>
<td>0.514 ± 0.073</td>
<td>0.722 ± 0.107</td>
<td>0.578 ± 0.088</td>
<td>0.560 ± 0.084</td>
</tr>
<tr>
<td>ST height (mV)</td>
<td>0.033 ± 0.072</td>
<td>0.015 ± 0.050</td>
<td>0.064 ± 0.067</td>
<td>0.045 ± 0.019</td>
</tr>
<tr>
<td>T amplitude (mV)</td>
<td>0.140 ± 0.070</td>
<td>0.174 ± 0.059</td>
<td>0.156 ± 0.033</td>
<td>0.143 ± 0.041</td>
</tr>
</tbody>
</table>

The data are presented as means ± SD, n = 6 per group.

ES: acute exhaustive swimming group; SLE: low-dose salidroside plus exhaustive swimming group; SHE: high-dose salidroside plus exhaustive swimming group.

*p < 0.05 and **p < 0.01 compared with the control group; "#p < 0.01 compared with the ES group.
0.05). Moreover, nuclear translocation of Nrf2 also increased with increasing Sal concentrations \((y = 0.1195x + 1.557)\), while the expression of Keap1 decreased \((y = -0.0068x + 0.7592)\); however, the differences were not significant \((p > 0.05)\) (Fig. 4).

**Discussion**

In this study, Sal improved cardiac function and electrocardiography in exhausted rats. Regarding the mechanism, for the first time, we revealed that Sal induced Nrf2 expression and increased nuclear translocation of Nrf2. Sal intervention did not affect levels of Nrf2 or Keap1 mRNA in the myocardium of exhausted rats. However, it decreased the Keap1 level in the myocardium. Therefore more Nrf2 was transported to the nucleus to induce the expression of antioxidant enzymes in the heart, reduce oxidative stress reactions in the exhausted myocardium, and protect the heart from exhaustion.

Under the high circulatory conditions observed during exhaustive exercise, the strength of cardiac stroke volume must increase to meet the needs of the increased metabolism of organs throughout the body. Ved and Ves increased, ventricular diastolic function decreased. Stroke volume is always adapted to the Ved phase, and the EF therefore did not show a significant difference. Notably, the \(-dP/dt_{\mathrm{min}}\) was reduced, while Tau was noticeably longer in the exhaustive exercise groups, suggesting that left ventricular diastolic function was decreased.

The results of this experiment are similar to the findings described in the study by Alexiou.\(^23\) After applying Sal, Ved decreased, Pes increased, \(dP/dt_{\mathrm{ms}}\) and \(-dP/dt_{\mathrm{ms}}\) recovered, and Tau was reduced. Based on these results, Sal improved systolic and diastolic function in exhausted hearts.

On ECG, HR showed a compensatory increase, the RR interval became shorter, and an increase in the ST-segment height caused by myocardial ischaemia was observed during the process of exhaustion. Myocardial ischaemia altered the activity of the heart conduction system, prolonged the PR and QT intervals and increased the risk of arrhythmia. The R amplitude change in inhibition or activation of the target protein was obtained. Sal: salidroside; *\(p < 0.05\) compared with the control group; **\(p < 0.01\) compared with the ES group; ***\(p < 0.01\) compared with the SLE group.

**Table 3. Pearson’s correlation analysis for some parameters (\(r\))**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ES</th>
<th>SLE</th>
<th>SHE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T amplitude (mV)</td>
<td>R amplitude (mV)</td>
<td>QT interval (ms)</td>
<td>dP/dt&lt;sub&gt;ms&lt;/sub&gt; (mmHg/s)</td>
</tr>
<tr>
<td>Nrf2</td>
<td>-0.944**</td>
<td>0.041</td>
<td>0.333</td>
<td>-0.362</td>
</tr>
<tr>
<td>Nuclear Nrf2</td>
<td>0.157</td>
<td>0.921**</td>
<td>0.699</td>
<td>0.836*</td>
</tr>
<tr>
<td>Keap1</td>
<td>0.445</td>
<td>0.453</td>
<td>0.934*</td>
<td>0.153</td>
</tr>
</tbody>
</table>

The data show Pearson’s correlation coefficients \((r)\), \(n = 6\) animals per group. \(*p < 0.05\) and \(**p < 0.01\). ES: acute exhaustive swimming group; SLE: low-dose salidroside plus exhaustive swimming group; SHE: high-dose salidroside plus exhaustive swimming group; dP/dt<sub>ms</sub>: peak rate of the increase in pressure.

**Fig. 3.** The effect of Sal on levels of total and nuclear Nrf2 and Keap1 proteins in rat myocardium after exhaustive exercise \((x \pm s, n = 6)\). A: Ratio of Nrf2/β-actin levels in rat myocardium. B: Ratio of nuclear Nrf2/β-actin levels in rat myocardium. C: Ratio of Keap1/β-actin levels in rat myocardium. Con: control group; ES: acute exhaustive swimming group; SLE: low-dose salidroside plus exhaustive swimming group; SHE: high-dose salidroside plus exhaustive swimming group.

**Fig. 4.** The effects of different concentrations of Sal on the inhibition of Keap1 and the activation and nuclear translocation of Nrf2 in the myocardium were analysed using a single-factor regression analysis \((n = 6)\). The level of target protein was divided by the level observed in the control group, and the multiple of change in inhibition or activation of the target protein was obtained. Sal: salidroside; *\(p < 0.05\).
was clearly reduced in the Sal-treated groups. Sal reduced the instability in cardiac electrical activity and conduction dysfunction caused by myocardial ischaemia, and prevented the occurrence of arrhythmias.

As shown in the study by Zhao investigating ECG data, the levels of ST–T changes and arrhythmia differed after exhaustive exercise.24 In our experiments, the anaesthetised and awake states exerted opposite effects on HR that are potentially related to the inhibitory effect of anesthetic drugs on the exhausted heart.

Exhaustive exercise increased ROS levels, and the accumulation of ROS in myocardial cells leads to structural and functional damage in the heart.7 The accumulation of ROS activated Nrf2 during exhaustive exercise, but did not produce a sufficient level of activation to prevent oxidative damage, and drugs were required to further activate Nrf2. Nrf2 was expressed at significantly higher levels after the Sal treatment. However, the expression of Nrf2 mRNA was not affected by Sal, indicating that Sal did not regulate Nrf2 expression at the transcriptional or translational level, but instead at the post-translational level.

According to Numazawa, inducers rarely promote the biosynthesis of Nrf2.25 The epigenetic regulation of Nrf2 activity might involve a long-term or basic regulatory mechanism. Sal may either inhibit Keap1 expression or promote its degradation. The abundance of Nrf2 was inhibited and its degradation was reduced, increasing the level of Nrf2 protein. The level of Nrf2 in the nucleus increased significantly as the concentration of Sal increased. Sal induced nuclear translocation of Nrf2.

Devling et al. used an siRNA to antagonise the Keap1 mRNA and its expression decreased significantly. The authors observed a significant increase in the level of Nrf2 transported to the nucleus. Moreover, the levels of antioxidant enzymes were also increased significantly.26,27

The antioxidant enzyme system downstream of Nrf2 consists of SOD, CAT and GSH and plays a role in preventing cardiac remodelling and cardiac function disorders.28,29 In the control group, a positive correlation was observed between EDPVR and GSH, while dP/dt max and GSH were positively correlated in the ES group. Therefore GSH exerted a protective effect on the heart and improved cardiac function parameters.

The observed decrease in levels of GSH and other enzymes after exhaustive exercise was due to their function in reversing ROS levels. The levels of SOD, CAT and GSH all increased with increasing concentrations of Sal. Sal induced Nrf2 expression and increased levels of downstream antioxidant enzymes by increasing the amount of Nrf2 protein and activating its nuclear translocation.

Conclusion
Sal protected the heart from exhaustive exercise-induced injury, and it may improve cardiac function and cardiac bioelectricity in exhausted rats. Sal improved the antioxidant capacity by activating Nrf2.

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