Medicine

Transplantation of exogenous mitochondria mitigates myocardial dysfunction after cardiac arrest

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eLife Assessment

In this **valuable** report, the authors investigated the effect of mitochondrial transplantation on post-cardiac arrest myocardial dysfunction (PAMD), which is associated with mitochondrial dysfunction. They **convincingly** demonstrated that mitochondrial transplantation enhanced cardiac function and increased survival rates after the return of spontaneous circulation (ROSC). They have also shown that myocardial tissues with transplanted mitochondria exhibited increased mitochondrial complex activity, higher ATP levels, reduced cardiomyocyte apoptosis, and lower myocardial oxidative stress post-ROSC.

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Abstract

The incidence of post-cardiac arrest myocardial dysfunction (PAMD) is high, and there is currently no effective treatment available. This study aims to investigate the protective effects of exogenous mitochondrial transplantation. Exogenous mitochondrial transplantation can enhance myocardial function and improve the survival rate. Mechanistic studies suggest that mitochondrial transplantation can limit impairment in mitochondrial morphology, augment the activity of mitochondrial complexes II and IV, and raise ATP level. As well, mitochondrial therapy ameliorated oxidative stress imbalance, reduced myocardial injury, and thus improved PAMD after cardiopulmonary resuscitation (CPR).

Introduction

Cardiac arrest (CA) is a life-threatening event and that is followed by post-cardiac arrest myocardial dysfunction (PAMD) after the return of spontaneous circulation (ROSC). PAMD exhibits degrees of systolic and diastolic dysfunction. The heart requires up to 30 kg of ATP daily to maintain systolic function (Hall, et al.,2014). Due to the high energy requirements of the

myocardium, mitochondria make up approximately one-third of the volume of cardiomyocytes (Cao, et al.,2023). The occurrence of PAMD is associated with mitochondrial damage with increased mitochondrial volume, accumulation of calcium ions, a diminution in complex activity, and a decrease in phosphate synthesis (Huang, et al.,2015; McCully, et al.,2023; Su, et al.,2021; Tsai, et al.,2021). Myocardium, as a high energy-consuming tissue, is very susceptible to energy depletion due to mitochondrial dysfunction. After mitochondrial injury, cytoplasmic and mitochondrial calcium overload prevents full relaxation of cardiomyocytes and can release cytochrome C to activate mitochondrial apoptotic pathways. This process contributes to the development of PAMD (Gazmuri and Radhakrishnan,2012; Su, et al.,2021). Therefore, maintaining cardiomyocyte mitochondrial function after cardiopulmonary resuscitation (CPR) could be useful for cell survival and tissue homeostasis.

Transplantation of mitochondria isolated from non-ischemic or allogeneic tissues promoted myocardial recovery after regional ischemia (McCully, et al.,2009). Although the safety and efficacy of this approach have been verified (Ali, et al.,2021; Jia, et al.,2022), effectiveness of mitochondrial transplantation in treating myocardial injury following CA remains to be demonstrated. Herein, we performed mitochondrial transplantation in rats following CPR to observe its effects on cardiac function and prognosis and explored its potential mechanism of action.

Results

CA-CPR model

No significant differences in weight or temperature were found between any groups before modeling. There was no significant difference in CA induction or CPR duration among the normal saline (NS), respiration buffer (Vehicle), and mitochondrial suspension (Mito) groups. Compared with the Sham group, the other three groups tended to receive less anesthetic (**Table 1**).

Mitochondrial transplantation improves cardiac function and does not alter hemodynamics in rats

Echocardiography revealed no differences in cardiac function among the groups prior to CA-CPR. Compared with the baseline, the left ventricular ejection fraction of rats subjected to CA-CPR significantly decreased at 1 hour after ROSC, and gradually improved over time ($P<0.05$; **Fig. 2A, 2B**). Of note, the level of cardiac-function impairment in the Mito group was significantly reduced 4 hours after ROSC compared with the NS and Vehicle groups ($P<0.05$).

There was no statistically significant difference between MAP and HR at any observational timepoints ($P>0.05$, **Fig. 2C**). In the NS, Vehicle and Mito groups, the mean arterial pressure (MAP) and heart rate (HR) decreased at 1, 2, 3, and 4 hours after ROSC, reaching their nadir at 1 hour. Subsequently, MAP and HR increased gradually but did not show any statistically significant differences compared with the Sham group.

Mitochondrial transplantation improves the 72-hour survival rate after CPR in rats

In animals not treated with mitochondria, Kaplan-Meier analysis found a rapid drop in the survival rate of rats within 24 hours after ROSC. In contrast, the survival rate of the Mito group was significantly higher than that of the NS and Vehicle groups ($P<0.05$, **Fig. 2D**).

Table 1

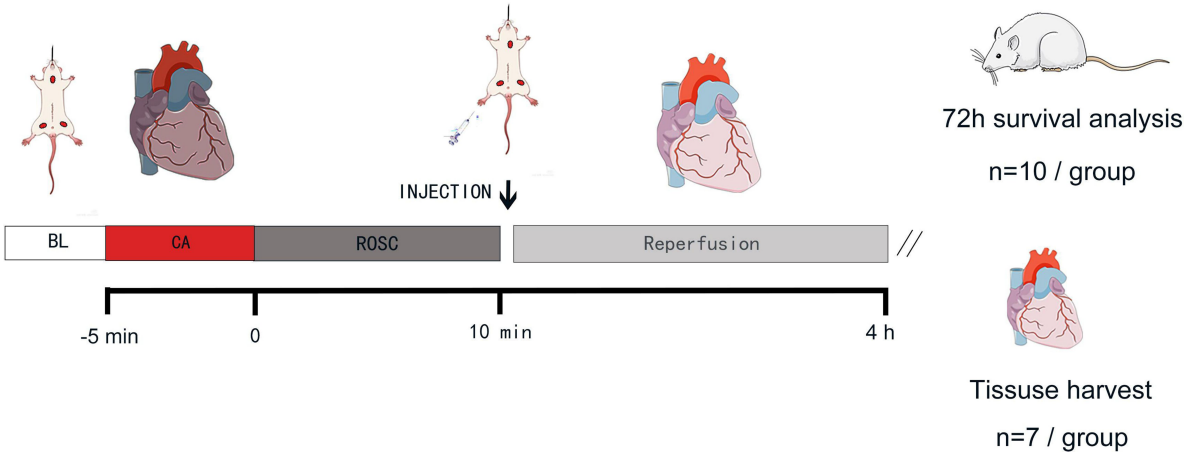
Baseline characteristics of rats and resuscitation characteristics ($\bar{x} \pm s$)

Variable	n	Weight (g)	Temp (°C)	CA-induced time (sec)	CPR time (sec)	Adrenaline (mcg)	Pentobarbital sodium (mg)
Sham	17	321.89±14.09	37.20±0.36	-	-	-	22.82±2.01
NS	17	318.65±10.00	37.45±0.43	234.29±22.89	128.41±12.50	12.75±0.40	21.63±1.35
Vehicle	17	326.80±11.64	37.33±0.37	247.06±23.61	138.82±14.64	13.07±0.47	21.79±0.98
Mito	17	320.64±14.32	37.45±0.39	241.29±23.38	131.88±14.51	12.83±0.57	21.92±1.05
***P*-Value**		0.29	0.21	0.57	0.10	0.13	0.07

n = 17 animals per group. CA, cardiac arrest; CPR, cardiopulmonary resuscitation. All data are expressed as Mean ± SD.

Figure 1.

Experimental design of the *in-vivo* study, Rats in all groups except the Sham group underwent cardiac arrest for 5 minutes and then received the corresponding intervention 10 minutes after ROSC. Four hours after ROSC, the 4-hour group was used to collect myocardial tissue and blood samples for detection, while the 72-hour group was used for survival detection. BL, baseline; CA, cardiac arrest; ROSC, return of spontaneous circulation.



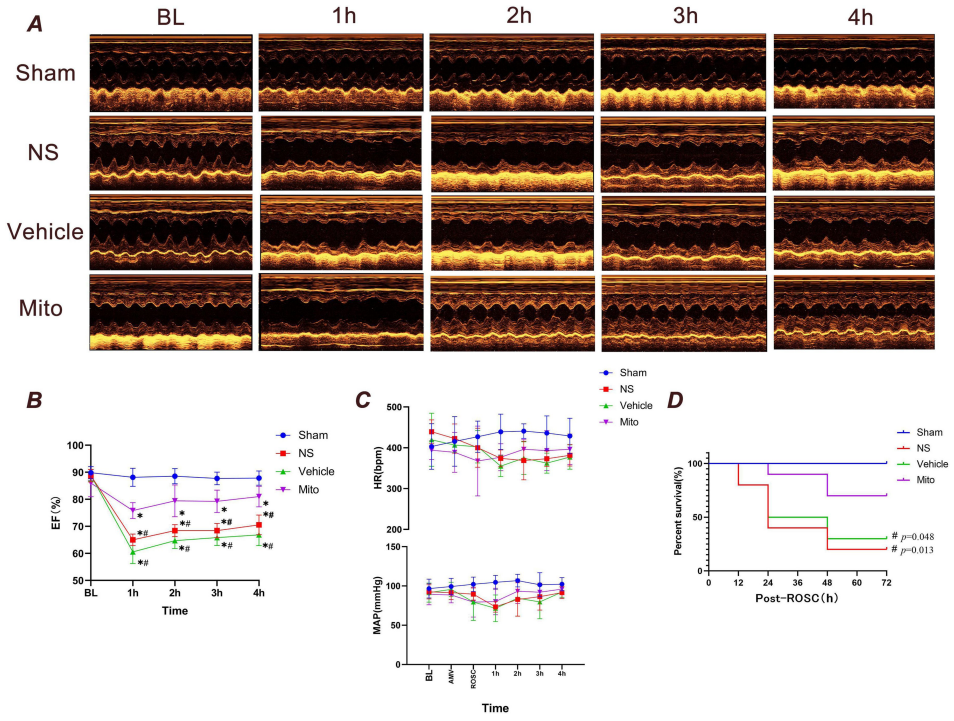


Figure 2.

Observational results at 4 and 72 hours after cardiopulmonary resuscitation in rats. (A) Echocardiograms of rats in each group from baseline to 4 hours following ROSC (n=7). (B) EF of rats in each group from baseline to 4 hours following ROSC (n=7). (C) HR and MAP changes during post -ROSC in 4 hours (n=7). (D) Survival rate during the first 72 hours following ROSC (n=10). Myocardial function between groups was compared by time-based measurements in each group using repeated-measures ANOVA. The survival rate between groups was compared by the Kaplan-Meier survival analysis test. * $P<0.05$ vs. the Sham group and # $P<0.05$ vs. the Mito group. BL, baseline; EF, ejection fraction; HR, heart rate; MAP, mean arterial pressure; bpm, beats per minute; mmHg, millimeters of mercury; AMV, after mechanical ventilation; ROSC, return of spontaneous circulation.

Viability and of isolated mitochondria

To evaluate the mitochondrial membrane potential (ΔΨm), JC-1 dye was used JC-1 dye aggregates in healthy mitochondria and fluoresces red. Upon the decrease of ΔΨ m, JC-1 can only exists as monomers and fluoresces green. A more pronounced red fluorescence indicates a higher number of normal mitochondria. This finding suggests that the majority of mitochondria extracted from the gastrocnemius muscle can maintain normal mitochondrial function (**Fig. 3A**). Mitochondria purity was confirmed by immunoblotting for TOM20 (a mitochondrial-specific protein) and COX-IV in both skeletal samples and isolated mitochondrial proteins. GAPDH was only detected in skeletal muscle proteins (**Fig. 3B**).

Effects of mitochondrial transplantation on cardiomyocyte mitochondria

Transplanted Mitochondria are internalized by cardiomyocytes

To verify the transfer of mitochondria to cardiomyocytes, we labeled the mitochondria with Mito-Tracker Red and transplanted them into the myocardium via the femoral vein. We primarily seek evidence of mitochondrial internalization within the endocardium, as initial injury occurs in this region during myocardial ischemia (Kuwada and Takenaka,2000). Four hours after injection, analysis of tissue sections found labelled mitochondria in endocardium (**Fig. 4**). The uptake of exogenous mitochondria was also observed in the myocardium and epicardium (supplement Fig. 1).

Mitochondrial transplantation does not alter the mass of myocardial mitochondria

The TOM20 protein is constitutively expressed in the outer membrane of mitochondria, and its abundance correlates with the amount of mitochondrial mass (Abrigo, et al., 2023). To investigate the potential increase of mitochondrial mass in myocardial tissue through mitochondrial transplantation, the expression of TOM20 was assessed in each group using tissue immunofluorescence staining. After 4 hours of ROSC, there was no significant difference in TOM20 level (as reflected by fluorescence intensity) among all groups, suggesting that mitochondrial transplantation did not increase the mass of mitochondria in heart. (**Fig. 5A, 5B**).

Mitochondrial transplantation improves cardiac mitochondrial structure

Transmission electron microscopy (TEM) micrographs of heart tissues were presented in **Fig. 5C**. By using the Flameng method, the extent of mitochondrial damage in heart tissues across the four groups was assessed and the result was presented in **Fig. 5D**. A higher score indicates more severe injury. The scores in the NS and Vehicle groups were significantly higher than those in the Mito group ($P<0.05$), indicating that mitochondrial damage in the Mito group was mitigated by mitochondrial transplantation. In the NS and Vehicle groups, the myocardial myofibrils exhibited a disordered arrangement on TEM, with variation in sarcomeres length and loss of Z-lines. Translucent and swollen mitochondria were visible, exhibiting a larger gap between membranes, distorted cristae, and accumulated calcium. In contrast, the mitochondria sarcomeres of the Mito group were more distinct, and the myocardial myofibrils were arranged in a more orderly fashion. The M-lines and Z-lines were clearly visible, and the number of mitochondrial cristae had increased, with their structure appearing to be complete.

Figure 3.

Assessment of the viability and purity of isolated mitochondria. (A) JC-1 staining of mitochondria after isolation from muscle. The staining of isolated mitochondria by JC-1 is visible either as red for J-aggregates or green for J-monomers. The intensity of the red color indicates that the isolated mitochondria had a high membrane potential, confirming their quality for transplantation. Scale bar = 100 μm. (B) SDS/PAGE analysis of fractions obtained during the purification of muscle mitochondria. GAPDH is only expressed in muscle, confirming its purity for transplantation.

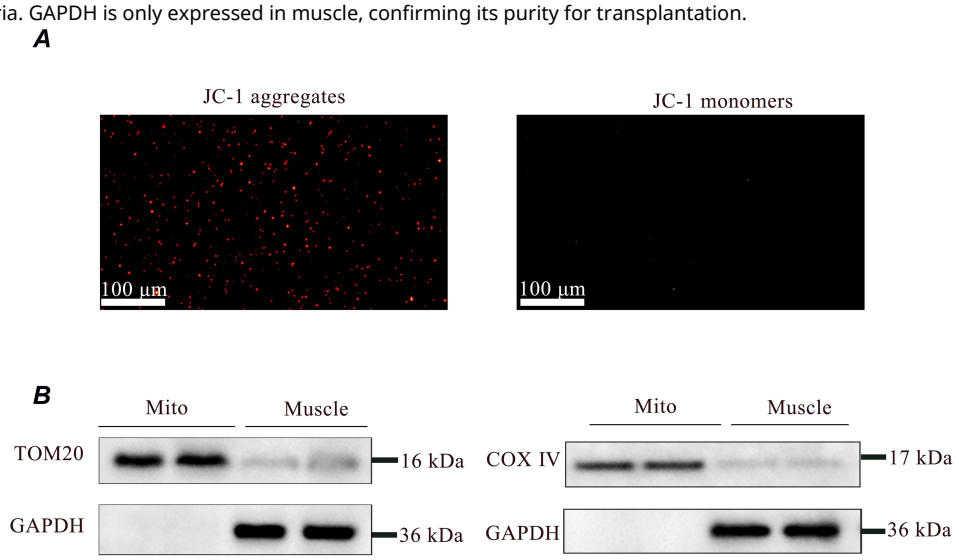
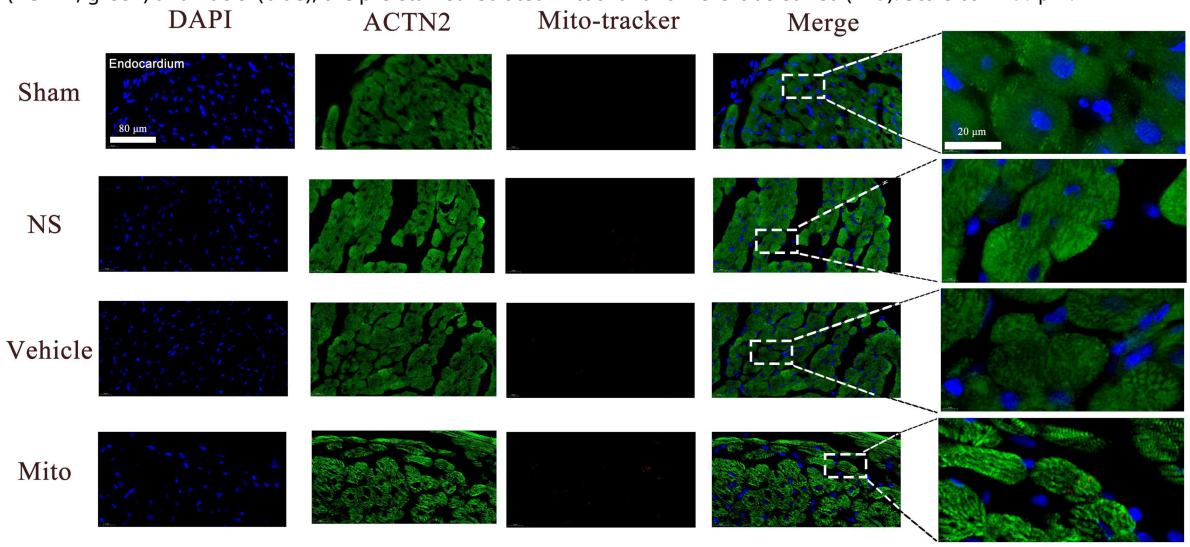


Figure 4.

Localization and uptake of transplanted mitochondria in endocardium, myocardial tissue was stained for anti-α-actinin 2 (ACTN2; green) and nuclei (blue); the pre-stained isolated mitochondria were labeled red (n=3). Scale bar = 80 μm.



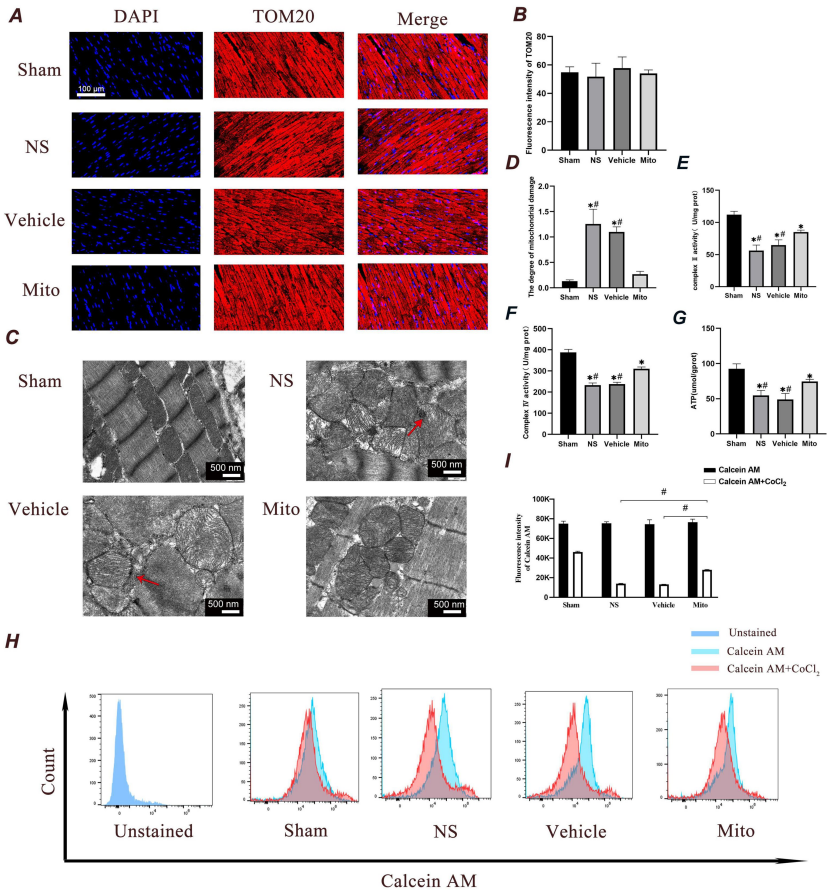


Figure 5.

Administration of mitochondria ameliorates ischemia reperfusion-mediated mitochondrial alterations in cardiomyocytes four hours after ROSC. (A and B) A and B represent the detection of fluorescence intensity of TOM20. A represents the image, while B represents the quantitative data. Scale bar = 100 μm (n = 3). (C and D) C shows representative photographs of mitochondrial morphology obtained through TEM examination, with arrows indicate calcium accumulation. D illustrates the degree of mitochondrial damage (n=3). Scale bars = 500 nm. (E and F) Changes in myocardial mitochondrial complex II and IV enzyme activities in hearts (n=7). (G) The ATP content in myocardial tissue was measured by colorimetry (n=7). (H and I) mPTP opening was detected by Calcein staining. I represent the quantitative analysis of the mean fluorescent intensity acquired in H (n = 3). Analyses were performed using ANOVA with Tukey's post hoc test. mPTP opening detection between groups was compared by time-based measurements in each group using repeated-measures ANOVA. The data were expressed as the mean ± standard deviation (SD). * P<0.05 vs. the Sham group and # P<0.05 vs. the Mito group.

Mitochondrial transplantation improves mitochondrial metabolism of cardiac tissue after CA-CPR

The mitochondrion supplies nearly all of the cell's energy through oxidative phosphorylation, which occurs via the mitochondrial respiratory chain. In instances of global ischemia, there is a deficiency of oxygen available to the mitochondria, which inhibits oxidative phosphorylation (Rousou, et al., 2004). The activity of mitochondrial complexes II and IV, along with ATP content in cardiac tissue, were assessed in our research to reflect mitochondrial metabolism. Compared with the Sham group, complex II and IV activity and ATP content were significantly attenuated in the other three groups (P<0.05). In contrast, the activity of complexes II and IV and ATP content were significantly elevated in the Mito group compared with the NS and Vehicle groups, suggesting that transfer of exogenous mitochondria enhanced function and energy metabolism of resident mitochondria (P<0.05, **Fig. 5E, 5F, 5G**).

Mitochondrial transplantation limits mitochondrial permeability transition pore (mPTP) opening

The opening of the mPTP induces mitochondrial permeability transition, resulting in the uncoupling of oxidative phosphorylation, an increase in mitochondrial volume (swelling), and the dissipation of the mitochondrial membrane potential. Following calcein acetoxymethyl ester (Calcein AM) staining and cobalt chloride (CoCl2) treatment, the opening level of mPTP was assessed using flow cytometry. In this assay, stronger fluorescence intensity indicates lower mPTP opening (**Fig. 5H, 5I**). The rats in the Mito group demonstrated lower fluorescence intensity, which means a higher mPTP opening level compared with the Sham group. The rats of NS and Vehicle groups showed further increased mPTP opening level compared with the Mito group. Mitochondrial transplantation caused a significant reduction in mitochondrial swelling.

Effects of mitochondrial transplantation on cardiomyocytes

Mitochondrial transplantation alleviates myocardial oxidative stress injury after CA-CPR

Increased level of reactive oxygen species (ROS) during reperfusion are a critical factor in the development of reperfusion injury. Consequently, oxidative stress in the ischemic myocardium of rats undergoing CA-CPR was evaluated. First, we evaluated the level of ROS in myocardial mitochondria. We used probe DCFH-DA, which releases green fluorescence in response to esterase oxidation, to measure ROS level based on fluorescence intensity. As shown in **Fig. 6A**, the ROS content in the myocardial mitochondria increased after 4 hours of reperfusion, and decreased with the transplantation of isolated mitochondria. Second, the production of malondialdehyde (MDA), which is widely used as 'footprints' of ROS generation, was also monitored. MDA level in the myocardial tissue of rats 4 hours after ROSC was decreased in animals given exogenous mitochondria (**Fig. 6B**, P<0.05). Third, our results indicated that the activity of superoxide dismutase (SOD) was significantly elevated in the Mito group compared with both NS and Vehicle groups (**Fig. 6C**, P<0.05).

Mitochondrial transplantation reduces cardiomyocyte apoptosis

Apoptotic indices and the apoptotic rate, as assessed by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and flow cytometry, increased 4 hours after ROSC. Compared with the NS and Vehicle groups, the apoptotic indices and apoptotic rate in the Mito group were lower (P<0.05), which linked exogenous mitochondrial transplantation and cardiomyocyte apoptosis reduction after CPR (**Fig. 6D, 6E, 6F**). The expression of cleaved caspase-3 protein in the Mito group was lower than that in the NS and Vehicle groups. (**Fig. 6G, 6H**)

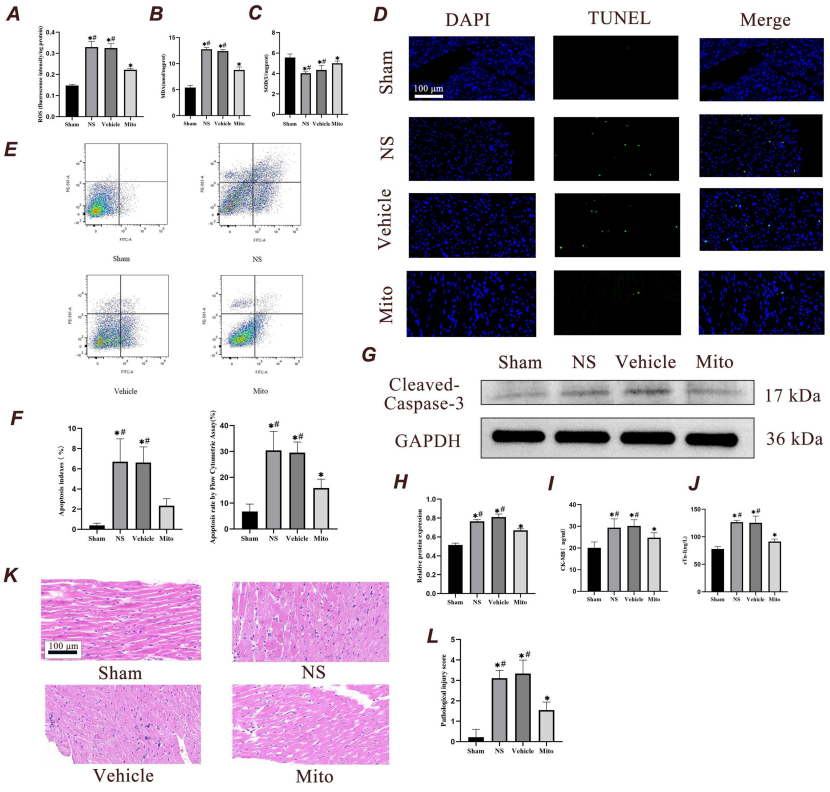


Figure 6.

Mitochondrial transplantation reduces myocardial damage 4 hours after ROSC. (A) Detection of mitochondrial ROS in various groups (n=3). (B and C) Changes in malondialdehyde and superoxide dismutase activity level in cardiac tissue (n=7). (D) Myocardial apoptosis level was examined using TUNEL (n=3). Scale bar = 100 μm. (E) The percentage of myocardial apoptosis was examined using flow cytometry (n=3). (F) Quantitative analysis of myocardial TUNEL apoptosis index and flow apoptosis rate (n=3). (G and H) Immunoblotting and quantitative analysis of the expression level of cleaved caspase-3 in the myocardium 4 hours after ROSC (n=3). (I and J) The changes in CK-MB and cTn-I level in the serum of rats were examined using ELISA (n=7). (K and L) Representative histological sections of the myocardium stained with hematoxylin and eosin. Myocardium from each experimental group were subjected to histological evaluation (n=3), Scale bar = 100 μm. Analyses were performed using ANOVA with Tukey's post hoc test. The data were expressed as mean ± standard deviation (SD). * P < 0.05 vs. Sham group and # P < 0.05 vs. Mito group. CK-MB, creatine kinase-MB fraction; cTn-I: cardiac troponin-I.

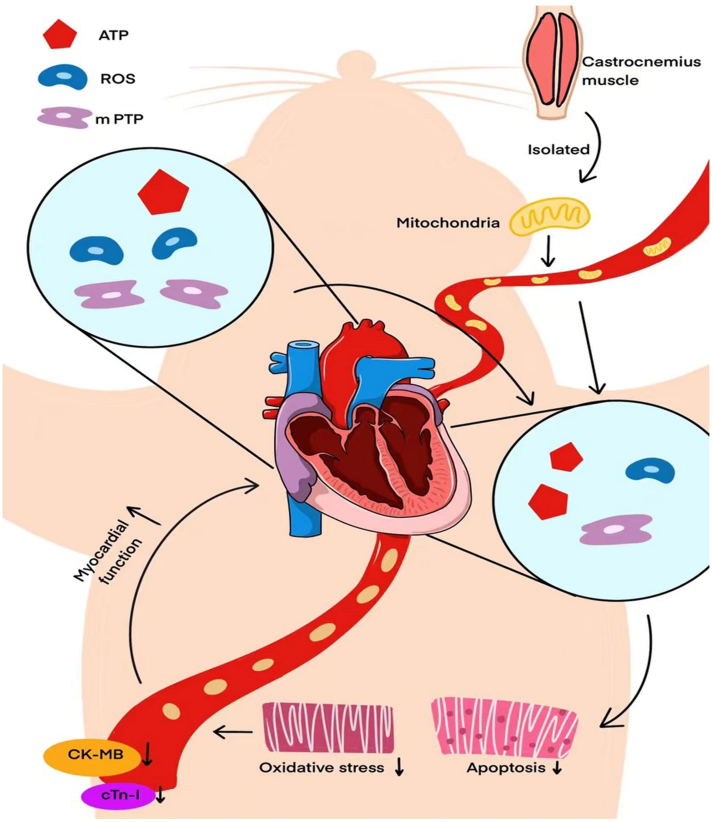


Figure 7.

Exogenous mitochondrial transplantation improved cardiac function after CPR. The specific mechanism involved may be related to the improvement in mitochondrial function, thus reducing the oxidative-stress response and apoptosis of myocardial cells. These dates suggest possible advantage in mitochondrial transplantation following CPR.

Mitochondrial transplantation reduces the markers of myocardial injury

ELISA was used to detect the expression of myocardial injury markers, Creatine Kinase MB isoenzyme (CK-MB) and Cardiac Troponin I (cTn-I), at 4 hours after ROSC. Compared with the Sham group, the level of CK-MB and cTn-I in the serum of the other three groups showed a significant rise ($P<0.05$). However, both CK-MB and cTn-I level in the serum of the Mito group were lower than those in the NS and Vehicle groups, suggesting that myocardial injury was reduced ($P<0.05$, **Fig. 6I, 6J**).

Mitochondrial transplantation attenuates myocardial histopathologic changes

Four hours after ROSC, myocardium from each experimental groups were subjected to histological evaluation (**Fig. 6K, 6L**). Tissue samples from the Sham group exhibited a well-organized structure of myocardial fibers, with no apparent inflammatory cell infiltration into the stroma. In the NS and Vehicle groups, the myocardial fibers were disordered, with some myocardial cells exhibiting thickening, rupture, and vacuolization. Also, there was inflammatory cell invasion in the stroma. The myocardial arrangement in the Mito group was more neatly arranged than that in the NS and Vehicle groups although thickening of myocardial cells was noted.

Discussion

PAMD is a common complication following resuscitated. It is characterized by systolic and diastolic insufficiency, arrhythmias, and recurrent CA. Circulatory dysfunction occurs in approximately two-thirds of CA subjects, which is associated with a poor outcome (Han and Lee, 2022). Unlike myocardial depression caused by regional ischemia characterized by localized ventricular wall-motion abnormalities, myocardial depression caused by PAMD after CA manifest as global left ventricular systolic dysfunction accompanied by reduced ejection fraction, left ventricular diastolic dysfunction, and right ventricular dysfunction (Lazzarin, et al., 2022; Xu, et al., 2008). Of these, global left ventricular systolic dysfunction is the most significant manifestation.

In the present study, we tested the hypothesis that the administration of exogenous mitochondria is cardioprotective effects after CPR. The rationale was to enhance the energy-dependent contractility of the myocardium. We isolated mitochondria from allogeneic gastrocnemius muscle tissue of healthy rats and maintained optimal mitochondrial activity and therapeutic effects. CA is an occasional event, making it impossible to predict when it will occur. Consequently, it is consistent with clinical practice to perform mitochondrial transplantation interventions only after spontaneous circulation has recovered and stabilized. The dosage of mitochondria is determined based on previous studies (Blitzer, et al., 2020; Guariento, et al., 2020). In previous research, isolated mitochondria (1×10^9) were delivered to the left coronary ostium in pigs, and can be a viable treatment in cardiac ischemia-reperfusion injury (Blitzer, et al., 2020; Guariento, et al., 2020). Additionally, the dose of 1×10^9 mitochondria achieves the maximal hyperemic effect when administered via intracoronary injection (Shin, et al., 2019). Considering that Sprague-Dawley (SD) rats are smaller than pigs and that there is a loss of mitochondria during pulmonary circulation, we adopted a mitochondrial transplantation dose of 5×10^8. Subsequently, uptake of the delivered mitochondria occurred in the heart and myocytes, perhaps via micropinocytosis (Kami and Gojo, 2020). AS well, mitochondrial internalization occurred through actin-dependent endocytosis (Pacak, et al., 2015). Internalized mitochondria promote degradation of resident via lysosomes to maintain an energy mitochondrial balance (Jia, et al., 2022; Liu, et al., 2022). Notably, Cowan et al. (Cowan, et al., 2017) indicated that isolated mitochondria are transported to endosomes and lysosomes; however, most of these mitochondria escape from these

compartments and subsequently fuse with the endogenous mitochondrial network. Internalized mitochondria facilitated respiratory restoration in recipient cells with depleted mtDNA, and improved cellular function by increasing ATP level and oxygen-consumption rates (Pacak, et al., 2015).

In our results, increased numbers of mitochondria were not found in the hearts of treated animals. Possibly because CPR caused ischemia/reperfusion injury to organs throughout the body, with non-specific distribution and uptake of the delivered mitochondria. Moreover, mitochondria possess the ability to adjust their mass and function to maintain cellular homeostasis. This includes mitochondrial dynamics such as fusion and fission, as well as biogenesis and degradation through mitophagy, which are crucial for regulating the morphology, mass, and function of mitochondria (Quiles and Gustafsson, 2020). These mechanisms may regulate the continuous remodeling of mitochondrial numbers to maintain mitochondrial mass homeostasis following mitochondrial transplantation (Jia, et al., 2022). After mitochondrial transplantation, the energy supply of myocardial mitochondria was restored, and autophagy was enhanced, resulting in an increased clearance of damaged mitochondria (Xu, et al., 2024). This also clarifies why the overall mass of mitochondria did not increase.

Masuzawa et al. (Masuzawa, et al., 2013) proposed that the internalization of mitochondria is not critical, as cardioprotection occurs rapidly within 10 minutes of reperfusion, the presence of viable mitochondria in cardiac tissue is all that is required. The nonviable mitochondria, mitochondrial fractions, mitochondrial deoxyribonucleic acid, ribonucleic acid, and exogenous adenosine diphosphate and ATP are incapable of providing protection to the ischemic heart (Masuzawa, et al., 2013). Shin et al. (Shin, et al., 2019) also suggested that the number of mitochondria required for cardioprotection is not dependent on the absolute number of transplanted mitochondria. Therefore, an increase in mitochondrial mass may not be the mechanism underlying the cardioprotective effects of transplanted mitochondria. The cardioprotective mechanisms of isolated mitochondria involve increased ATP production. The results of our study were consistent with other date that the energy metabolism of myocardial mitochondria improved after mitochondrial transplantation along with increased complex activity and ATP content (Jia, et al., 2022; Pacak, et al., 2015). Mitochondrial transplantation is being pursued as a therapeutic strategy, as it does not result in coronary occlusion, autoimmunity and arrhythmia attacks (Masuzawa, et al., 2013; Shin, et al., 2019). The clinical application of mitochondrial transplantation can enhance the prognosis of pediatric patients requiring extracorporeal membrane oxygenation following cardiac surgery, the improvement of cardiac function in patients helps them successfully wean off ECMO support (Emani and McCully, 2018). It is worth noting that the method of mitochondrial isolation may better meet the needs of clinical settings where an interventional time below 60 minutes is desired (Ali, et al., 2021). After the isolation, mitochondria can be immediately used for transfer and internalization (Ali, et al., 2021). Still concerns regarding mitochondrial hemocompatibility and stability in serum have been raised. In this line, serum calcium did not adversely impact isolated mitochondria (Maleki, et al., 2023).

ROS is generated with ischemia and then targets the normal physiologic functions of cells by damaging the structures of proteins, lipids, and even DNA (Zhu, et al., 2024). As well, oxidative stress promotes cardiac ischemia-reperfusion injury and was implicated in pro-inflammatory responses and apoptosis, both of which worsen myocardial cell injury (Patel and Karch, 2020). In a regional ischemia-reperfusion model, mitochondrial transplantation reduced oxidative stress in cells following reperfusion (Zhang, et al., 2019). Similarly, in the present study, mitochondrial transplantation reduced the production of ROS, decreased the level of myocardial oxidative markers, and increased the activity of antioxidant enzymes after 4 hours of ROSC.

Ischemia/reperfusion injury of myocardial tissue releases CK-MB and cTn-I, which were commonly used indicators of early myocardial injury in clinical settings (Zhang, et al., 2021). The concentrations of CK-MB and cTn-I rose significantly 4 hours after resuscitation in the CA-CPR model, while mitochondrial transplantation significantly reduced this effect. During ischemia, the loss of mitochondrial membrane potential leads to mitochondrial swelling and release of cytochrome C, followed by caspase-mediated cell death during reperfusion (Lu, et al., 2023; Milliken, et al., 2022). Apoptosis is the process by which cardiomyocytes die (Kunapuli, et al., 2006). Increase apoptosis was found in rats after CA-CPR along with increased caspase-3 expression after resuscitation (Wu, et al., 2021). Similar results were reported in pigs that underwent CA-CPR (Wang, et al., 2023). In our results, the level of apoptosis and the opening of mPTP in cardiomyocytes increased significantly after CPR, whereas administration of healthy mitochondria significantly reduced both apoptosis and mPTP opening. Moreover, exogenous mitochondrial transplantation significantly reduced the disordering of myocardial fiber arrangement and inflammatory cell infiltration.

In conclusion, exogenous mitochondrial transplantation improved cardiac function after CPR. The specific mechanism involved may be related to the improvement in mitochondrial function, thus reducing the oxidative-stress response and apoptosis of myocardial cells. These dates suggest possible advantage in mitochondrial transplantation following CPR.

Limitations

Hemodynamic observation was carried out only for 4 hours after ROSC, Thus, the impact of mitochondrial transplantation on long-term cardiac function following CPR could not be assessed. In this study, the animal model consisted of healthy rats. However, in clinical practice, CA is primarily caused by cardiac issues. Healthy rats tend to exhibit a higher recovery ability after a CA attack, which may lead to an overestimation of the therapeutic effects of mitochondrial transplantation. In our next phase, we plan to utilize various CA models to conduct comprehensive studies to validate the effectiveness of mitochondrial transplantation in different pathological models.

Methods

Animals

The experimental protocol (**Fig.1**) was approved by the Animal Experiment Committee of the General Hospital of Central Theater Command (No.2023017) and conformed to the Guide for the Care and Use of Experimental Animals published by the National Institutes of Health, USA (NIH Publication No. 5377-3, 1996). Adult male SD (7 to 8 weeks old) rats weighing 250 to 350g were purchased from Hunan Silaikejingda Experimental Animal Co., Ltd. (No. 430727231102711675, Changsha, China). Male rats were selected to avoid estrous cycle interference. Animals were housed in standard cages with a 12-hour light-dark cycle at a room temperature 22°C.

Animals were randomly assigned to four groups using the random number table method: a Sham group, NS group, Vehicle group, and Mito group (n=17). Each group was further divided into two survival-analysis subgroups: a four-hour group (n=7) and a 72-hour group (n=10). Rats in the NS, Vehicle and Mito groups underwent CA induced by asphyxia and CPR, while rats in the Sham group underwent tracheal intubation and arteriovenous puncture only, without CA and CPR.

Experimental protocol

CA-CPR model

Establishment of the CA-CPR model was as previously published (Huang, et al., 2020). Rats were anesthetized with pentobarbital sodium (45 mg/kg) via intraperitoneal injection. A 16-G intravenous catheter was used for endotracheal intubation. The left femoral artery and right femoral vein were subsequently inserted using a 24-G venous indwelling needle for dynamic blood pressure detection and venous access establishment. Blood pressure, HR, and rectal temperature were monitored using the ALC-MPA monitoring system (Alcott Biotech, Shanghai, China). After 10 minutes of mechanical ventilation, asphyxia-mediated CA was induced by blocking the tracheal tube. CA was defined as a decrease in systolic blood pressure to 25 mmHg. Five minutes after CA, external chest compression (200 bpm) and mechanical ventilation (tidal volume, 0.60 mL/100 g, respiratory rate, 80 bpm) were initiated, and epinephrine (40 mcg/kg) was administered intravenously. ROSC was defined as the spontaneous restoration of sinus rhythm with a systolic blood pressure of >60 mmHg that was maintained for 10 minutes. Subsequently, corresponding treatments were administered. Animals that did not regain autonomic circulation within 5 minutes were excluded from the study. After ROSC, hemodynamics and echocardiograms were recorded for 4 hours. Basal body temperature was maintained at 37°C throughout the intervention. Pentobarbital sodium (10 mg/kg) was given if animals urinated or showed increased limb-muscle tension. Twenty-eight animals of 4-hour group completed the full protocol and were humanely euthanized with excess pentobarbital sodium 4 hours after ROSC. Myocardial tissue and blood samples were collected for detection. The remaining 40 animals of 72-hour group were monitored for 72-hour to track survival.

Intervention measures

Ten minutes after ROSC, 0.5 mL of normal saline, 0.5 mL of respiration buffer, and 0.5 mL of 1×10^9/mL mitochondrial suspension were injected through the femoral vein in the NS, Vehicle, and Mito groups, The Sham group received 0.5 mL of NS at 30 minutes postoperatively.

Echocardiography

To assess myocardial function, an ultra-high resolution, small-animal ultrasound imaging system (SigmaVET, Esaote, Genoa, Italy) was used to capture M-mode echocardiographic images at the parasternal long axis and/or parasternal short axis near the papillary muscle at 1-hour, 2-hour, 3-hour, and 4-hour post-ROSC.

Survival analysis

In the 72-hour survival study cohort, the catheters were extracted 4 hours post-ROSC, and the surgical incisions were closed. Animals were placed back in their cages and administered subcutaneous injections of 0.5mL of 1% lidocaine for analgesia. Animals were monitored every 2 hours during the initial 24 hours following ROSC, and then at 48 and 72 hours. Animals that exhibited signs such as wheezing or a respiratory rate below 5 breaths per minute were deemed moribund and humanely euthanized (Su, et al., 2021). At the end of the time rats were euthanized.

Isolation and identification of mitochondria

Mitochondrial isolation and labeling

Mitochondria were isolated as published (Preble, et al., 2014). In brief, 180 mg of gastrocnemius muscle from healthy male SD rats aged 7 to 8 weeks were placed into a centrifuge tube containing 5 mL of pre-chilled homogenizing buffer (300 mM sucrose, 10 mM K-HEPES, and 1 mM K-EGTA), homogenized, and incubated on ice with *Bacillus subtilis,* a protease (P5380, Sigma Aldrich,

Darmstadt, Germany), for 10 minutes. The mixture was filtered through a 40-μm cell filter, and then bovine serum albumin (ST023-50 g, Beyotime, Shanghai, China) was added and incubated for 5 minutes. The homogenate was then filtered through a 40-μm screen and then through a 10-μm screen, and centrifuged at 9000 g for 10 minutes at 4L. The precipitate was reconstituted in 1 mL of pre-chilled respiration buffer (250 mM sucrose, 2 mM potassium dihydrogen phosphate, 10 mM magnesium chloride, 20 mM K-HEPES, and 0.5 mM K-EGTA). Mitochondrial amounts were determined as previously published and were quantified using a bacterial counter (0650010, Marienfeld, Lauda-Königshofen, Germany) (McCully, et al., 2009). The isolated mitochondria were incubated with Mito-Tracker Red CMXRos (C1049B-50μg, Beyotime, Shanghai, China) at 37L for 15 minutes and then washed three times with the mitochondrial respiration buffer.

Detection of mitochondrial membrane potential

A mitochondrial membrane potential detection kit (JC-1) (C2003S; Beyotime, Shanghai, China) was used to characterize function. Purified mitochondria were incubated with JC-1 staining solution at 37°C for 20 minutes, followed by imaging with a fluorescence microscope (BX53, OLYMPUS, Tokyo, Japan) equipped with a WHN 10/22 eyepiece. The image analysis software used was OLYMPUS cellSens Standard version 3.2.

Western blotting

Tissue samples and isolated mitochondria were lysed in RIPA buffer (P0013B; Beyotime, Shanghai, China) containing 1% PMSF (ST505; Beyotime, Shanghai, China) on ice to extract proteins. Protein concentration was determined by the BCA method. Protein was boiled in a water bath for 10 minutes. Electrophoresis was performed using a 12% SDS-PAGE gel (P0012A, Beyotime, Shanghai, China), followed by transfer to a PVDF membrane (FFP70, Beyotime, Shanghai, China). Non-specific binding was blocked with 5% skim milk powder at room temperature for 2 hours. Membranes were exposed to primary antibodies overnight at 4L: anti-Caspase-3 (1:1000, 14220T, Cell Signaling Technology, Beverly, MA, USA), anti-TOM20 (1:1000, AF5206; Affinity, Changzhou, China), anti-COX-IV (1:1000; AC610, Beyotime, Shanghai, China) and anti-GAPDH (1:3000; LF206, Epizyme, Shanghai, China) on a shaker at 4L for 12 hours. After washing, membranes were incubated with horseradish peroxidase-labeled secondary antibody (1:2000, A0208, Beyotime, Shanghai, China) for 2 hours and developed with the ECL chemiluminescence method. Results from immunoblots were scanned using the chemiluminescence imaging system (SH-523, Shenhua, Hangzhou, China; JY-Clear ECL, JUNYI, Beijing, China;). The relative band intensities were quantified using ImageJ software (version 1.54f; National Institutes of Health, USA).

Mitochondrial distribution and uptake

Mitochondrial co-localization was determined as published (Masuzawa, et al., 2013). Tissue sections were incubated with 0.25% Triton-X-100 for 10 minutes at room temperature, blocked with normal bovine serum albumin, and incubated with primary antibody generated against alpha-actinin 2 (1:500; GTX103219; GeneTex, Irvine, USA) at 4L overnight. After washing with PBST, sections were incubated with an Alexa Fluor 488-labeled secondary antibody (1:1000, HZ0176; Huzhen, Shanghai, China) for 1 hour. DAPI (P0131-5 ml; Beyotime, Shanghai, China) was applied to stain nuclei. Images were acquired using the OLYMPUS fluorescence microscope.

Analysis of cardiac mitochondria

Complex activity

Mitochondria were extracted from 0.1 g myocardial tissue using differential centrifugation. The mitochondrial suspension was disrupted by ultrasonic waves in an ice bath releasing the mitochondrial respiratory chain complex. The working solution from mitochondrial complex activity test kit (AKOP008M, AKOP006M; Boxbio, Beijing, China) was mixed with the samples and

the absorbance at 605 nm and 550 nm measured values at various timepoints. The variance between the two timepoints was calculated and recorded as ΔA1 and ΔA2. Protein concentration (C) of each sample was measured. Mitochondrial complex II activity was calculated as 476.19 × ΔA1/C, and mitochondrial complex IV activity was calculated as 1099.48 × ΔA2/C.

ATP determination

ATP level was determined using an ATP-content test kit (A095-1-1; Nanjing Jiancheng, Nanjing, China).

TOM20 labeling

Tissue sections were labeled with TOM20 antibody (1:100, AF5206; Affinity, Changzhou, China), incubated with a horseradish peroxidase-coupled secondary antibody (1:400, 5220-0336; SeraCare, Massachusetts, USA), subjected to tyramide signal amplification (TSA), stained with DAPI and images acquired using an OLYMPUS fluorescence microscope.

Transmission electron microscope

Fixed hearts were rinsed with phosphate-buffer ed saline (PBS) for 45 minutes and exposed to 1% osmium tetroxide for 2 hours. After dehydration in graded ethanol concentrations, tissue samples were immersed in epoxy resin, embedded, and cut into ultrathin sections (60-80 nm). The sections were stained with uranium and lead, and examined and imaged using a TEM (HT7800; Hitachi, Tokyo, Japan). The degree of mitochondrial damage in the 4 groups was assessed using the Flameng classification method. The evaluation is conducted by trained experimenters who are unaware of the group assignments. Mitochondrial damage was evaluated on a scale from 0 to 4, where 0 indicates a normal structure, 1 indicates normal with slight swelling, 2 indicates mitochondrial swelling, 3 indicates serious swelling and cristae disorder, 4 indicates mitochondria membrane breach and vacuolization. Based on this scale, the degree of mitochondrial damage in rats across the four groups was scored, and the average scores were calculated from three random fields of view per rat. A higher score indicates more severe injury (Zhang, et al., 2017).

The determination of ROS

The extraction of mitochondria from myocardial tissue was performed using a mitochondrial isolation kit (S3606; Beyotime, Shanghai, China). Rat myocardial tissues were homogenized with mitochondrial isolation reagent A and then centrifuged at 600 g for 5 minutes at 4°C. The supernatant was subsequently centrifuged again at 11,000 g for 5 minutes at 4°C. ROS was determined by a fluorescent probe DCFH-DA using the ROS assay kit (S0033S; Beyotime, Shanghai, China) based on the manufacturer's directions. After removing the supernatant, the pelleted materials were suspended in incubation buffer (20 mM MOPS, 0.1 M KCl, 10 mM ATP, 10 mM $MgCl_2$, 10 mM sodium succinate, and 1 mM EGTA); then DCFH-DA (1: 1000) was added. Next, the contents were mixed and incubated at 37°C in the dark for 20 minutes. Finally, the fluorescence intensity was measured on a flow cytometry with excitation and emission wavelengths of 485 nm and 525 nm, respectively. The results were presented as the fluorescent intensity per nanogram of protein.

Quantification of MDA and SOD detection

The MDA and SOD activity level of myocardial samples were determined with an MDA test kit (A003-1; Nanjing Jiancheng, Nanjing, China) and SOD test kit (A001-3; Nanjing Jiancheng, Nanjing, China).

Mitochondrial swelling assay

Myocardial tissue was initially isolated. The tissue was digested using 0.1% collagenase and 0.125% trypsin, followed by hydrolysis at 37℃ for 30 minutes. The mixture was filtered through a 200-mesh screen at 1,500 rpm and then centrifuged for 15 minutes to collect the sediment. mPTP Assay Kit (C2009S; Beyotime, Shanghai, China) was used to determine the opening of the mPTP in cardiomyocytes. This kit provides a more straightforward approach for assessing mPTP opening. In brief, cardiomyocytes were treated with Calcein AM for 30 minutes at 37 ℃, which easily entered the cells and accumulated in various cytosolic compartments, including the mitochondria. Once inside, intracellular esterases broke down the acetoxymethyl esters, releasing the highly polar fluorescent dye calcein. The fluorescence from cytosolic calcein was diminished when CoCl2 was added, while the fluorescence from mitochondrial calcein remained unaffected. However, when the mPTP opened, mitochondrial calcein was released into the cytosol, where it was quenched by CoCl2, leading to a notable decrease in calcein fluorescence. A decrease in absorbance reflects mitochondrial swelling and the opening of the mitochondrial mPTP. Subsequently, flow cytometry was employed to detect the opening of the mPTP in 1×10^6 cardiomyocytes.

Detection of myocardial injury

TUNEL assay

Paraffin-embedded tissue sections were processed with an *in-situ* cell-death detection kit (11684795910; Roche, Basel, Switzerland) for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling. The ratio of TUNEL-positive cells to total cells was determined.

Flow cytometry

To prepare a single-cell suspension, the separation of cardiomyocytes is described as previously mentioned. Cells were washed with PBS, suspended in the binding buffer (from Annexin V-FITC/PI apoptosis detection kit below-mentioned) and stained with AnnexinV-FITC and PI (Annexin V-FITC/PI apoptosis detection kit; A211-02; Vazyme, Nanjing, China). Apoptosis was assessed using flow cytometry.

Markers of ischemic injury

Blood samples were collected from the femoral artery and centrifuged for 15 minutes at 3000 rpm to obtain serum. The level of myocardial-injury markers was measured using the CK-MB kit (MM-0625R1; Meimian, Yancheng, China) and the cTn-I kit (MM-0426R2; Meimian, Yancheng, China).

Histology

Hearts was fixed in 4% paraformaldehyde for 24 hours, dehydrated, rendered transparent, embedded in paraffin and cut into thin sections. After dewaxing and rehydration, the sections were stained with hematoxylin and eosin, and images acquired using a light microscope. A histological score was utilized to evaluate the extent of myocardial injury, based on the criteria established by Rona et al. (RONA, et al., 1959). (Supplemental Table1).

Statistics and data analysis

IBM SPSS Statistics software (V26.0, IBM Corp, New York, USA) was used for data analysis. All data are expressed as mean ± standard deviation (Mean ± SD). Sample size for the 4-hour group was calculated as published (Dell, et al., 2002). The ejection fraction of the first three surviving rats among the groups was measured. The population standard deviation of the variable is 11.34. With

a magnitude of difference set at 15, an error rate of 0.1, and a power of 80%, the smallest acceptable sample size was determined to be five animals. Considering an animal loss of approximately 20% after 4 hours of CPR, seven animals were selected for each group. The sample size for the 72-hour group was calculated using StatBox (StatBox-Open 0.1.0; Cloud Powered Clinical Trial, Hebei, China). Set the survival rate at 20% for survival rate 1 and 80% for survival rate 2 (with power = 0.8 and α = 0.05). Accordingly, 10 was the smallest acceptable animal sample size. The comparison of time-based measurements was evaluated using repeated-measures tow-way ANOVA, with groups as the between-subjects factor and time as the within-subjects factor. One-way ANOVA and Tukey's multiple-comparison test were employed to compare multiple groups. Survival was characterized using the Kaplan-Meier survival analysis test. Statistical significance was defined at $P<0.05$.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Additional information

Funding

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Author contributions

X.Z. and G.S.G. supervised the study and designed the experiments. Z.W. and J.Z performed the experiments. Z.W and J.Z. collected, analyzed and interpreted the data. M.D.X. and J.Y.Y. supervised the experiments. X.Y.M. and M.Z.S. accessed and verified the data. Z.W. and J.Z. wrote the manuscript. X.Z. and G.S.G. revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics

The animal experiments were approved by the Animal Experiment Committee of the General Hospital of Central Theater Command (No.2023017).

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Additional files

Supplementary Figure 1. *Localization and uptake of transplanted mitochondria in myocardium and epicardium, myocardial tissue was stained for anti-α-actinin 2 (ACTN2; green) and nuclei (blue); the pre-stained isolated mitochondria were labeled red (n=3). Scale bar = 20 μm.*

Supplementary table S1. *Details of antibodies used in the methodology.*

Supplementary table S2. *Scoring standard of myocardial pathological injury.*

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Reviewer #1 (Public review):

Summary:

In this study, the authors investigate the effect of mitochondrial transplantation on post-cardiac arrest myocardial dysfunction (PAMD), which is associated with mitochondrial dysfunction. The authors demonstrate that mitochondrial transplantation enhances cardiac function and increases survival rates after the return of spontaneous circulation (ROSC). Mechanistically, they found that myocardial tissues with transplanted mitochondria exhibit increased mitochondrial complex activity, higher ATP levels, reduced cardiomyocyte apoptosis, and lower myocardial oxidative stress post-ROSC.

Strengths:

Previous studies have reported that mitochondrial transplantation can improve myocardial recovery after regional ischemia, but its potential for treating myocardial injury following cardiac arrest has not been tested yet. Therefore, the findings are somewhat novel. Remarkably, the increased survival in mitochondria treated group post ROSC is very promising and highlights its translational potential.

Comments on revisions:

My concerns are adequately addressed.

https://doi.org/10.7554/eLife.98554.2.sa2

Reviewer #3 (Public review):

In this manuscript titled "Transplantation of exogenous mitochondria mitigates myocardial dysfunction after cardiac arrest", Zhen Wang et al. report that exogenous mitochondrial transplantation can enhance myocardial function and survival rates. It limits mitochondrial morphology impairment, boosts complexes II and IV activity, and increases ATP levels. Additionally, mitochondrial therapy reduces oxidative stress, lessens myocardial injury, and improves PAMD after cardiopulmonary resuscitation. The results of this manuscript clearly demonstrate that mitochondrial transplantation can effectively improve PAMD after cardiopulmonary resuscitation, highlighting its significant scientific and clinical value. The findings shown in this manuscript are interesting to the readers. However, further experiments are needed to confirm this conclusion. In addition, the results should be rewritten to describe and discuss the relevant data in detail.

Major comments from the original round of review:

(1) Can isolated mitochondria be transported to cultured cardiomyocytes, such as H9C2 cells, in vitro?

(2) The description of results in the manuscript is too simple. It lacks detail on the rationale behind the experiments and the significance of the data.

(3) The authors demonstrate that mitochondrial transplantation reduces cardiomyocyte apoptosis. Therefore, Western blot analysis of apoptosis-related caspases could be provided for further confirmation.

(4) Do donor mitochondria fuse with recipient mitochondria? Relevant experiments and data should be provided to address this question.

(5) In Figure 5A, the histograms are not labeled with the specific experimental groups.

Comments on revisions:

The revised manuscript quality has been improved, and most of my concerns were addressed and resolved.

https://doi.org/10.7554/eLife.98554.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer 3 (Public review):***
>
> *Major comments:*
>
> *(1) Can isolated mitochondria be transported to cultured cardiomyocytes, such as H9C2 cells, in vitro?*

Thank you for this insightful question. Mitochondria are highly dynamic organelles that play a crucial role in cellular energy metabolism. When cells encounter various stressors and increased energy demands, they can benefit from the incorporation of exogenous mitochondria. In 2013, Masuzawa et al. (Masuzawa, et al.,2013) were the first to demonstrate that transplanted mitochondria are internalized by cardiomyocytes 2 to 8 hours after transplantation, significantly contributing to the preservation of myocardial energetics. Ali et al. (Ali, et al.,2020) discovered that exogenous mitochondria could be internalized by H9C2

cardiomyocytes as quickly as 5 minutes after co-incubation, resulting in an acute enhancement of normal cellular bioenergetics following mitochondrial transplantation. Pacak et al. (Pacak, et al.,2015) established that the internalization of mitochondria into cardiomyocytes is time-dependent and occurs through actin-dependent endocytosis.

Collectively, these evidences illustrate that exogenous mitochondria can be effectively internalized by H9C2 cells and other cardiomyocytes, our experiments further confirmed that mitochondrial transplantation can be incorporated by the myocardium in *vivo*.

> *(2) The description of results in the manuscript is too simple. It lacks detail on the rationale behind the experiments and the significance of the data.*

Thank you for this suggestion. We have realized that the results in the submitted manuscript have not been adequately interpreted. We have added necessary details on the rationale behind the experiments and the significance of the data to the results section (Lines 57~59, 69~73, 81~88, 91~98, 100~102, 103~104, 10^9~115, 124~129, 135~146, 149~157, 159~161, 168~169, 178~179). We would like to express our gratitude to the reviewers once again and hope that our modifications will meet their requirements.

> *(3) The authors demonstrate that mitochondrial transplantation reduces cardiomyocyte apoptosis. Therefore, Western blot analysis of apoptosis-related caspases could be provided for further confirmation.*

Thank you for this constructive comment. We fully agree with the reviewer's perspective on the detection of apoptosis-related caspases and have conducted a Western blot assay to investigate the impact of mitochondria on myocardial tissue. Our new evidence indicates that rats receiving mitochondrial transplantation exhibited reduced expression of cleaved caspase-3 compared with those in the NS and Vehicle groups (Fig. 6G, 6H, Lines 168~169), suggesting that mitochondrial transplantation decreased the level of apoptosis in the myocardium.

> *(4) Do donor mitochondria fuse with recipient mitochondria? Relevant experiments and data should be provided to address this question.*

This is a very helpful comment. Investigating the fate of transplanted mitochondria in myocardial cells after CA is of great significance. The internalization of exogenous mitochondria has been observed across various cell types (Liu, et al.,2021; Shanmughapriya, et al.,2020). Notably, a recent study indicated that after being incorporated into host cells, isolated mitochondria are transported to endosomes and lysosomes. Subsequently, most of these mitochondria escape from these compartments and fuse with the endogenous mitochondrial network (Cowan, et al.,2017). We have discussed this in the manuscript. (Lines 217~220)

Oxidative stress, a pathophysiological phenomenon common to cells suffering from ischemia/reperfusion insults after CA/CPR, was implicated to promote internalization and survival of exogenous mitochondria (Aharoni-Simon, et al.,2022). In our study, we confirmed that mitochondrial transplantation can enhance the metabolism of cardiomyocytes, increase ATP level, and reduce reactive oxygen species (ROS). Our results indirectly confirm that isolated mitochondria can successfully fuse with myocardial mitochondria.

> *(5) In Figure 5A, the histograms are not labeled with the specific experimental groups.*

We apologize for this oversight. We have labeled the specific experimental groups in the histograms presented in Figure 6B and 6C (originally Figure 5A).

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) The age, gender, and strain of the donor rats should be specified in the Methods section. Additionally, it is not obvious what doses of mitochondria were injected into the rats and how the dosage was initially determined.*

Thanks for your suggestion. We have included relevant information about the donor rats in the Methods section（Lines 361~362）.

In Mito group, each animal received 0.5 mL of 1×10^9/mL mitochondrial suspension. (Lines 342~345). Considerable amounts of data have demonstrated the efficacy of mitochondrial transplantation in cellular, animal, and human research (Alemany, et al.,2024; Kaza, et al.,2017; Liu, et al.,2023). However, there is currently no evidence to determine the optimal dosage for transplantation. In previous research, isolated mitochondria (1×10^9) were delivered to the left coronary ostium in pigs, and can be a viable treatment modality in cardiac ischemia-reperfusion injury (Blitzer, et al.,2020; Guariento, et al.,2020). Additionally, the dose of 1×10^9 mitochondria achieve the maximal hyperemic effect when administered via intracoronary injection (Shin, et al.,2019). Considering that Sprague-Dawley (SD) rats are smaller than pigs and that there is a loss of mitochondria during pulmonary circulation, we adopted a mitochondrial transplantation dose of 5×10^8. We will explore the optimal dosage in our future research.

> *(2) In Figure 4a, the number of transplanted mitochondria appears to be very low. Considering the high number of mitochondria present in cardiomyocytes, it is unclear whether this small amount of transplanted mitochondria can significantly impact complex II activity and ATP levels in myocardial tissues, as shown in Figures 4b-d, or improve survival post-ROSC, as shown in Figure 2d. Could the observed benefits of mitochondrial transplantation be due to the indirect effects of the injected mitochondria, such as the release of mitochondrial contents, rather than the mitochondria themselves, as discussed by Bertero et al. (2021, Circ. Research)? This issue should be addressed in the manuscript.*

Thanks for this wonderful comment. As presented in Fig. 4 (originally Figure 4A), our results indicated the internalization of mitochondria by myocardium, shown by colocalization of Mito-tracker and myocardium marker. We would like to make our points here regrading to Fig. 4:

(1) Significant left ventricular systolic and diastolic dysfunction that occurs in the myocardium shortly after the return of ROSC is referred to post-cardiac arrest myocardial dysfunction (PAMD) (Laurent, et al.,2002). It has demonstrated the efficacy of mitochondrial transplantation for the heart following ischemia-reperfusion injury in cellular, animal, and human studies, despite inadequate mitochondrial internalization (Liu, et al.,2023). A low number of transplanted mitochondria may improve cardiac function.

(2) Only biologically active mitochondria can be specifically labeled with Mito-tracker. Therefore, cardiomyocytes uptake mitochondria that possess complete functionality. Previous results have demonstrated that mitochondrial contents, such as nonviable mitochondria, mitochondrial fractions, mitochondrial deoxyribonucleic acid, ribonucleic acid, exogenous adenosine diphosphate and ATP, do not provide protection to the ischemic heart (McCully, et al.,2017; McCully, et al.,2009).

(3) The specific mechanism for mitochondrial internalization has yet to be fully elucidated. We totally agree with reviewer's opinion pertaining the presence of other mechanisms of mitochondria transplantation that play a role in cardiac protection. Multiple mechanism may involve in the cardiac protection effect of mitochondria transplantation, and we are actively

seeking reasonable approach to verify these hypotheses in an underway study (Lines 236~246).

> *(3) In Figure 4g, the claims regarding sarcomere length, mitochondrial structure, the number of cristae, accumulated calcium etc. seem to rely on the visual interpretation of representative images. To ensure a reliable interpretation of the data, a blinded quantification of each image in each group should be conducted. The same applies to the claims made in Figure 5E.*

Thanks for this suggestion. We have quantitatively evaluated the electron microscope images and HE images of the myocardium to ensure reliable interpretation. Corresponding supplements have been added to the methods (Lines 433~441, 494~496), results sections (Lines 10^9~115, 178~179), and Figures 5C, 5D, 6K and 6H (originally Figures 4G and 5E).

> *(4) In line 69, it is unclear why the authors claim that MAP and HR decrease at 1, 2, 3, and 4 hours after ROSC in all groups compared to the Sham group, despite stating in line 72 that "MAP and HR did not differ at any observational time points (P>0.05, Figure 2C)."*

We apologize for our inaccurate phrasing. In the presented study, there was no statistically significant difference between MAP and HR at any observational timepoints (P>0.05, Figure 2C). In the NS, Vehicle and Mito groups, the MAP and HR decreased at 1, 2, 3, and 4 hours after ROSC, reaching their nadir at 1 hour. Subsequently, MAP and HR increased gradually but did not show any statistically significant differences compared with the Sham group. (Lines 69~73).

> *(5) The absence of increased mitochondrial content in the mito-groups should be discussed further in the manuscript.*

Thank you for your suggestion. We discussed the reasons why the mass of isolated mitochondria did not increase in Lines 224~235.

> *(6) The N in Figure 5d should be provided.*

Thanks for your suggestion. We have revised the figure legend to include N of Figure 6F (originally Figures 5D).

> *(7) Figure 6 demonstrates content beyond the findings in this manuscript. This reviewer recommends limiting the graphical abstract to the findings specifically in this paper.*

Thanks for your great advice. We have revised Figure 7 (originally Figure 6) and restricted the graphical abstract to the findings presented in this paper.

> *Minor issues:*
>
> *(8) The order of data in Figure 4 should be consistent with the text in the manuscript. Figures 4E-F-G are described before Figures 4B-C-D in the text. Similarly, Figure 5F was described before Figure 5E in the text.*

Thanks for your great advice. We have rearranged the order of the pictures to align with the text. Thank you for your proposal.

> *(9) In Figure 4A, the locations of the epicardium, muscle, and endocardium should be indicated for clarity. Also, it is not obvious where the close-up box refers to in the actual image.*

Thank you for your suggestion. We primarily seek evidence of mitochondrial internalization within the endocardium, as injury occurs first during myocardial ischemia (Kuwada and Takenaka,2000). The close-up box in Fig. 4 refers to the endocardium.

> *(10) In Figure 5A, the group annotations are missing from the MDA and SOD graphs. The standard deviation bars for the SOD vehicle and SOD mito groups (3rd and 4th columns) appear to overlap. Can the authors provide the actual p-values?*

We apologize for the mission of group annotations in the MDA and SOD graphs. The p-value between the Vehicle group and the Mito group was 0.004. The SOD activity level of myocardial samples in the groups are presented in Table 1.

Author response table 1.

The SOD activity levels of myocardial samples in groups (U/mgprot)

	Sham	NS	Vehicle	Mito
n	7	7	7	7
Mean±SD	5.55±0.35	4.02±0.15	4.34±0.45	5.00±0.22

> *(11) In line 58, NS abbreviation is used without defining what NS is.*

We apologize for not including the full name of NS. NS is the abbreviation of normal. It has now been marked in the manuscript. (Line 58)

> *(12) In line 118, what MDA stands for is not described until line 348. MDA should be defined in the text for the general audience.*

We apologize for this. We have defined it in the manuscript. (Lines 156~157)

> *(13) In line 192, the authors state that "mitochondrial transplantation... increased the expression of antioxidant enzymes after four hours of ROSC," while only SOD activity levels were assessed in the manuscript. Increased activity levels do not necessarily imply an increase in expression levels. This discrepancy should be addressed in the Discussion section.*

Sorry for confusing the ‘activity’ with ‘expression’. Although mitochondrial transplantation has been shown to be involved in the restoration of manganese superoxide dismutase levels after ischemic insults, the changes in antioxidant enzyme expression level were not evaluated at the protein level in this paper (Tashiro, et al.,2022). To avoid misunderstandings, we have replaced the term ‘expression’ with ‘activity’ as appropriate. (Lines 268~271)

> *(14) Mitochondria from non-ischemic gastrocnemius muscle of health donor animals were isolated and a manner that maximized their healing potential. This sentence is not clear.*

We apologize for the confusing sentence in the original manuscript. To improve clarity, we have revised that sentence. We isolated mitochondria from allogeneic gastrocnemius muscle tissue of healthy rats and maintained optimal mitochondrial activity and therapeutic effects. (Lines 199~201)

> *Minor grammar issues:*
>
> *In line 153, mitochondrial should be mitochondria.*
>
> *Figure 2D: Percent servival should be percent survival.*
>
> *There should be a blank in complex IIactivity Figure 4B, and complex IV activity in Figure 4C.*
>
> *In line 134, Four hours of ROSC, Tissue samples from. Tissue is capital.*
>
> *In line 190, Similaerly should be similarly.*

Thank you for your valuable comments. We apologize for the grammatical issues caused by our oversight. We have made the necessary corrections in the manuscript and figures. (Lines 198, 179, and 268), Figure 2D, Figure 5E (originally Figure 4B); Figure 5F (originally Figure 4C).

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Some details are lacking clarity, such as the rationale behind choosing certain doses or time points for interventions.*

Thank you for this valuable suggestion. We have explained the rationale behind the selection of the dosage and the timing of the intervention. (Lines 201~212)

> *I would suggest verifying mitochondrial function using the seahorse experiment oxygen consumption, and to check mitochondrial oxidative stress. I would also suggest checking the mitochondrial permeability transition pore opening, using for example calcein cobalt quenching or simply a kit to examine this further.*

Thank you for your valuable advice. In our manuscript, we added results regarding mitochondrial reactive oxygen species (ROS) and the mitochondrial permeability transition pore (mPTP) opening. As anticipated, mitochondrial transplantation reduced the increase in mitochondrial ROS and the mPTP opening in ischemic myocardium. (Lines 135~146, 149~157, 442~455, 460~476, Figure 5H, 5I, 6A)

We agree that seahorse experiment oxygen consumption would be beneficial for understanding the intricacies of their interactions and enhancements. Additionally, Ali et al. (Ali, et al.,2020) have demonstrated that introducing non-autologous mitochondria from healthy skeletal muscle cells into normal cardiomyocytes results in a short-term improvement in bioenergetics, as measured using a Seahorse Extracellular Flux Analyzer. In our results, we have not yet conducted cellular experiments, The process of isolating cells from the myocardial tissue of adult SD rats for Seahorse analysis can lead to secondary damage to the myocardial cells (Jacobson, et al.,1985). In this experiment, we measured ATP content and the activity of mitochondrial complexes to evaluate energy changes after mitochondrial transplantation. We will conduct cell experiments and utilize Seahorse measurements to further clarify the alterations in myocardial energy in future.

> *For Figure 3B, it would be beneficial to include the relative quantification of the mitochondrial marker COX-IV. Additionally, if feasible, I suggest verifying the representation of the mitochondria outer membrane TOM20 or VDAC.*

Thank you for your great suggestion. As suggested, we added TOM20 to assess the purity of the isolated mitochondria and reached the same conclusion: the isolated mitochondria

exhibited high purity (Figure 3B). TOM20 was expressed in both muscle lysates and isolated mitochondria, whereas GAPDH was exclusively found in the muscle lysate. (We re-validated the purity of the mitochondria by using relative quantification of TOM20 and COX VI.)

> *In Figure 2C, the clarity of the graphs depicting both arterial pressure (MAP) and heart rate (HR) is lacking and could potentially confuse the reader. I recommend incorporating color coding instead of relying solely on symbols, or by presenting the data in a more comprehensible format and that aligns with graph B as well.*

Thank you for your constructive comments. We have color-coded the diagrams in Figure 2B and 2C.

> *In Figure 4A, please include high-magnification of the mitochondria to provide a more detailed examination.*

Thank you for this insightful comment. We have provided a high-magnification image of the mitochondria in Figure 4.

> *Regarding lines 81-82, I recommend specifying the sentence more precisely for better clarity and understanding.*

Thank you for your comments. We have revised the sentences in lines 83~86 to enhance their clarity for readers.

In the Materials and Methods section, it is crucial to provide precise details. For instance, when staining the exogenous mitochondria with MitoTracker Red, it is important to specify the duration of staining, such as the standard 20 minutes for example. Additionally, it is advisable to mention the number of times these mitochondria were washed with the respiratory solution to ensure thorough removal of excess MitoTracker, thus preventing unintended staining of endogenous mitochondria with MitoTracker red upon injection of pre-labeled mitochondria.

> *Thank you for your suggestion. We have added the necessary details regarding Mito-Tracker Red dyeing. (Lines 373~376) In addition, we also added other details in necessary (Lines 373~376, 379~382, 395~396, 397~400, 487~488). We appreciate your suggestion once again.*

The sensitivity of JC-1 dye to temperature and pH fluctuations underscores the necessity for meticulous experimental conditions. It is crucial for the authors to elucidate why they chose to maintain the samples at 4 {degree sign} C for 60 minutes, especially considering the dye's optimal operating temperature of 25 {degree sign} C. Providing a rationale behind this deviation from standard protocol would enhance the scientific rigor and reproducibility of the study. Please add more information on the objectives used in the fluorescence microscope (BX53, OLYMPUS, Tokyo, Japan) and the software used.

We sincerely apologize for the mistake in this sentence. The purified mitochondria, which are stained with JC-1, should be stored at 4°C and examined using a fluorescence microscope within 60 minutes. Purified mitochondria were incubated with JC-1 staining solution at 37°C for 20 minutes. The fluorescence microscope used in our experiment is equipped with a WHN 10/22 eyepiece, and the software version is OLYMPUS cellSens Standard 3.2. (Lines 379~382)

> *Moreover, in the context of immunoblotting, it is imperative for the authors to furnish detailed information regarding the preparation of muscle tissue homogenates. Specifically, clarification is needed regarding the solution utilized for tissue grinding. Did the authors employ ice-cold RIPA lysis buffer or an alternative lysis buffer, supplemented*

> *with a protease inhibitor cocktail? Such details are pivotal for methodological transparency.*

Thanks for this wonderful comment. In the methods section, we added detailed information about protein extraction. (Lines 383~385)

> *Furthermore, it would be beneficial for the authors to specify the instrument employed for scanning the immunoblots, as well as the software utilized for subsequent analysis of the immunoblot images. Providing this information would not only enhance the reproducibility of the findings but also facilitate the evaluation of the experimental results.*

Thank you for your suggestion. We have included the instrument used for scanning the Western blot, as well as the software used for image analysis in the manuscript. (Lines 397~400)

> *Authors must exercise caution against copy-pasting. In line 282, there's a query regarding how the mitochondria were isolated. It is recommended to cite a specific reference and offer more comprehensive details. Despite the authors referencing a number within the text, the absence of numbered references makes it challenging to cross-reference.*

Thank you for pointing this out; we have updated the citation accordingly (Line 361).

> *Figure 5C please double check some misspelling label errors (e.g: Vehicle and not Vehucle).*

We apologize for the misspelling in Figure 6E (originally Figure 5C) and have corrected it. Additionally, we have thoroughly reviewed the text for spelling errors and sincerely apologize once again for the previous mistakes. (Lines 249~252, 322)

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