

# Cardioprotective effects of triiodothyronine supplementation against ischemia reperfusion injury by preserving calcium cycling proteins in isolated rat hearts

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**Abstract.** Hypothyroidism is associated with profound left ventricular dysfunction. Triiodothyronine (T<sub>3</sub>) supplementation may improve cardiac function after ischemic reperfusion (I/R) injury. In the present study, the effect of T<sub>3</sub> on major calcium cycling proteins and high-energy phosphate content during I/R was evaluated. Isolated perfused rat hearts were divided into 5 groups: Sham Control (Sham, n=10), Control (n=8), T<sub>3</sub> 10 nM (T<sub>3</sub>-10, n=10), T<sub>3</sub> 25 nM (T<sub>3</sub>-25, n=10) and T<sub>3</sub> 50 nM (T<sub>3</sub>-50, n=10). T<sub>3</sub> was administrated for 60 min before 30 min of ischemia and 120 min of reperfusion. The protein contents of Ca<sup>2+</sup>-release channels (RyR2), Ca<sup>2+</sup>-adenosine triphosphatase (SERCA2a), phospholamban (PLB), sarcolemmal Ca<sup>2+</sup>-adenosine triphosphatase (PMCA) and sodium-calcium exchanger (NCX), as well as the high-energy phosphate content

in heart tissues were measured by western blot analysis. The results revealed that T<sub>3</sub> improved the contractile recovery (left ventricular developed pressure; +dP/dt, -dP/dt) after I/R. Western blotting assays demonstrated that I/R depressed the contents of RYR2, SERCA2a and phosphorylated RYR2 and PLB; there were no effects on the contents of PLB, PMCA and NCX. T<sub>3</sub> reversed I/R-induced degradation of RyR2 and SERCA2a, restored the phosphorylation of RyR2 and PLB, and preserved the high-energy phosphate contents of ATP and creatine phosphate. T<sub>3</sub> supplementation protected the heart against I/R injury via the preservation of Ca<sup>2+</sup>-cycling proteins and high-energy phosphate content.

## Introduction

Thyroid hormones have profound effects on the heart and cardiovascular system (1,2). Cardiopulmonary bypass (CPB) leads to a 'euthyroid-sick' state (3). This low triiodothyronine (T<sub>3</sub>) state manifests as low circulating levels of T<sub>3</sub> with hemodynamic abnormalities (4). There is a growing body of experimental data that suggests that T<sub>3</sub> supplementation improves hemodynamic parameters after ischemic injury in animal models of CPB (5,6) and in isolated heart studies (7,8). Previously, studies using experimental models of ischemia reperfusion (I/R) revealed that T<sub>3</sub> administration improved the recovery of post-ischemic cardiac performance and reduced the pro-fibrotic process that leads to adverse cardiac remodeling (2,9-11).

Cytosolic calcium (Ca<sup>2+</sup>) overload plays a major role in the development of myocardial injury during I/R. The sarcoplasmic reticulum (SR) is critical in Ca<sup>2+</sup> uptake via Ca<sup>2+</sup>-ATPase (SERCA2a) and Ca<sup>2+</sup> release via the Ca<sup>2+</sup> release channels (ryanodine receptors; RyRs). Human cardiomyocyte calcium handling and phenotype are strongly influenced by T<sub>3</sub>. 'Low T<sub>3</sub> syndrome' is observed in the progression of I/R injury (12). It is possible that myocardial protection induced by T<sub>3</sub> supplementation involves the protection of SR function. The effects of T<sub>3</sub> supplementation on myocardial contractility may be attributed to their effects on intracellular Ca<sup>2+</sup> homeostasis. The

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**Abbreviations:** T<sub>3</sub>, triiodothyronine; I/R, ischemic reperfusion; RyR2, Ca<sup>2+</sup>-release channels; SERCA2a, Ca<sup>2+</sup>-adenosine triphosphatase; PLB, phospholamban; PMCA, sarcolemmal Ca<sup>2+</sup>-adenosine triphosphatase; NCX, sodium-calcium exchanger; CPB, cardiopulmonary bypass; Ca<sup>2+</sup>, calcium; SR, sarcoplasmic reticulum; RyRs, ryanodine receptors; LVP, left ventricular pressure; CF, coronary flow; MVO<sub>2</sub>, myocardial O<sub>2</sub> consumption; ATP, adenosine triphosphate; CP, creatine phosphate; LVDP, Left ventricular developed pressure; CaMKII, calmodulin-dependent kinase II; PKA, protein kinase A

**Key words:** triiodothyronine, cardiac function, ischemic reperfusion injury, calcium cycling protein, high-energy phosphate

authors have previously reported a marked  $\text{Ca}^{2+}$  overload in the mitochondrial compartment during I/R (13). The ability of  $\text{T}_3$  supplementation to improve cardiac function, induce the activation of sarcolemma and SERCA2a, and decrease cytosolic ( $\text{Ca}^{2+}$ ) accumulation have been reported previously (13). However, the mechanisms that underlie these cardioprotective effects of thyroid hormone remain largely unknown. In the present study, it was hypothesized that the cardiac protection of  $\text{T}_3$  supplementation against I/R injury is associated with the preservation of  $\text{Ca}^{2+}$ -cycling proteins and high-energy phosphate contents.

## Materials and methods

**Animals.** Adult male Sprague-Dawley rats (n=50; weight, 350–425 g) were obtained from the Animal Center of Soochow University (Suzhou, China). Rats were fed a standard diet and were housed at 25°C with 60% humidity under a 12 h light-dark cycle. All rats were allowed access to food and water *ad libitum* for one week before experiments. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing University.

**Langendorff isolated heart preparation and measurements.** The methods employed have been previously described (13). Briefly, rats were anesthetized and decapitated when unresponsive to noxious stimulation. The hearts were excised and perfused in the Langendorff mode at a perfusion pressure equivalent to 80 mmHg. Perfusate and bath temperatures were maintained at  $37.2 \pm 0.1^\circ\text{C}$  using a thermostatically controlled water circulator (Lauda E100; Lauda Dr R Wobser GmbH & Co., KG). Left ventricular pressure (LVP) and coronary flow (CF) were measured at a constant temperature and perfusion pressure (100 mmHg). Coronary inflow and coronary venous  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and pH were measured offline with an intermittently self-calibrating analyzer system (Radiometer Copenhagen ABL 505; Radiometer, Ltd.). Coronary outflow (coronary sinus)  $\text{O}_2$  tension was also measured online continuously with a Clark-type  $\text{O}_2$  electrode (203B; Instech Laboratories, Inc.). Myocardial  $\text{O}_2$  consumption ( $\text{MVO}_2$ ) was calculated as [(coronary flow/g heart weight)  $\times$  (arterial  $\text{pO}_2$ -venous  $\text{pO}_2$ )]  $\times$  24  $\mu\text{l O}_2/\text{ml}$  at 760 mmHg; and cardiac work efficiency was calculated as [systolic-diastolic LVP  $\times$  HR]/ $\text{MVO}_2$ . At the end of the experiments, the hearts were freeze-clamped and stored at  $-80^\circ\text{C}$  until subsequent use in western blot assays.

**Experimental group establishment.** Animals were randomly divided into 5 groups. The untreated sham (non-ischemic) group (Sham, n=10) was perfused for 225 min and after 15 min the equilibration/stabilization of functional parameters were employed; hearts were not subjected to ischemia. Ischemia groups underwent 15 min of stabilization and 60 min perfusion with or without  $\text{T}_3$  administration at three different doses (10, 25 and 50 nM), followed by 30 min ischemia and 120 min reperfusion [n=10 each for the control (CTL),  $\text{T}_3$ -10,  $\text{T}_3$ -25 and  $\text{T}_3$ -50 groups]. The range of doses was selected according to a previous study (14). A three-way stopcock, located immediately above the aortic cannula, allowed the induction of global, no-flow ischemia.

**Western blot assays for SR and sarcolemmal  $\text{Ca}^{2+}$ -cycling and -regulating proteins.** Western blot assays were conducted as previously described (15). Briefly, aliquots of homogenate samples (20  $\mu\text{g}$  protein/lane) were solubilized in Laemmli sample buffer (cat. no. S3401; Sigma-Aldrich; Merck KGaA) and fractionated by SDS-PAGE using a 4–20% gel. After transfer to nitrocellulose membranes, membranes were blocked with 5% nonfat milk for 1.5 h at room temperature in phosphate-buffered saline and probed with primary antibodies against RyR2 (cat. no. MA3-916; 1:2,000), SERCA2a (cat. no. 2A7-A1; 1:2,000), Phospholamban (cat. no. 2D12; PLB; 1:1,000), and sarcolemmal  $\text{Ca}^{2+}$ -adenosine triphosphatase (PMCA; cat. no. 5F10; 1:1,000). The above antibodies were purchased from Affinity Bioreagents, Inc. The sodium-calcium exchanger (cat. no. ab3516P; NCX; 1:1,000) was obtained from Sigma-Aldrich; Merck KGaA. RyR-ser2809 and PLB-Thr17 were purchased from Badrilla., Ltd., (1:1,000). Secondary antibodies (m-IgGk BP-HRP, cat. no. sc-516102, Santa Cruz Biotechnology; 1:20,000) were conjugated to horseradish peroxidase. Immunoreactive bands were visualized by enhanced chemiluminescence (SuperSignal™ west pico PLUS chemiluminescent substrate, cat. no. 34577; Pierce; Thermo Fisher Scientific, Inc.). The amount of protein was determined by densitometry using Kodak 1D software (version 3.4.5; Sigma-Aldrich; Thermo Fisher Scientific, Inc.) and normalized to protein load. Positive (purified proteins) and negative (blocking peptide or blot without primary antibodies) controls were used to establish the specificity of the protein signals.

**Biochemical analysis.** At the end of reperfusion, the hearts were freeze-clamped with aluminum tongs pre-cooled with liquid nitrogen as described previously (16), to measure myocardial ATP and creatine phosphate (CP) levels. Briefly, frozen ventricles were pulverized and mixed with 0.3 M  $\text{HClO}_4$  and 0.25 mM EDTA under liquid nitrogen cooling. The extract was centrifuged at 8,000  $\times$  g for 15 min at 4°C and the resulting supernatant was sampled to measure myocardial ATP and CP using the high pressure liquid chromatography method as previously described (16). Myocardial CP was converted to ATP via the creatine kinase enzymatic reaction.

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard deviation. Statistical analysis used SPSS 20.0 (IBM Corp.). A two-way analysis of variance was used to assess the overall difference between groups. Student Newman Keuls' post hoc test was used for multiple comparisons between the different groups. All experiments were repeated three times.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Cardiac performance.** Table I summarizes the changes in the indices for coronary flow, heart rate,  $\text{MVO}_2$  and cardiac efficiency in the Sham, CTL,  $\text{T}_3$ -10,  $\text{T}_3$ -25 and  $\text{T}_3$ -50 groups before 30 min ischemia, and at 120 min of reperfusion. The heart rates of all the groups were similar prior to ischemia, but decreased after I/R. There was no significant difference among the different groups. Coronary flow was much lower

Table I. Cardiac effects in Sham, CTL, T<sub>3</sub> 10 nM, T<sub>3</sub> 25 nM and T<sub>3</sub> 50 nM before and after ischemia reperfusion.

Group	Sham	CTL	T <sub>3</sub> 10 nM	T <sub>3</sub> 25 nM	T <sub>3</sub> 50 nM
HR (beat/min)					
Baseline	266±14	269±15	262±10	265±16	271±18
Reperfusion 120'	261±16	241±24	240±21	245±14	248±19
CF (ml/min)					
Baseline	12.2±0.8	11.9±0.8	12±0.7	12.2±0.9	11.9±0.7
Reperfusion 120'	11.8±1.0	5.6±0.6 <sup>a,b</sup>	6.6±0.5 <sup>a,b</sup>	7.9±0.6 <sup>a-c</sup>	8.0±0.7 <sup>a-c</sup>
MVO <sub>2</sub> (mmHg)					
Baseline	115±8	112±11	111±10	112±12	115±10
Reperfusion 120'	112±11	58±7 <sup>a-c</sup>	69±7 <sup>a,b</sup>	73±8 <sup>a-c</sup>	75±7 <sup>a-c</sup>
Cardiac efficiency (mmHg·beat·0.1 μl O <sub>2</sub> ·g <sup>-1</sup> )					
Baseline	16.5±1.3	16.6±1.5	16.2±1.6	16.3±1.1	16.5±1.4
Reperfusion 120'	15.6±1.2	6.3±0.7 <sup>a,b</sup>	7.2±0.8 <sup>a,b</sup>	8.6±0.8 <sup>a-c</sup>	8.7±0.8 <sup>a-c</sup>

The HR, CF, MVO<sub>2</sub> and Cardiac efficiency before ischemia and after 30 min ischemia and 120 min reperfusion. Values are the mean ± standard deviation. <sup>a</sup>P<0.05 vs. the baseline; <sup>b</sup>P<0.05 vs. the Sham group. <sup>c</sup>P<0.05 vs. the CTL group. Sham (non-ischemia; n=10); CTL (Ischemia; n=10); T<sub>3</sub> 10 nM (n=10); T<sub>3</sub> 25 nM (n=10); T<sub>3</sub> 50 nM (n=10). CF, coronary flow; HR, heart rate, MVO<sub>2</sub>, myocardial O<sub>2</sub> consumption; Cardiac efficiency, cardiac work efficiency; T<sub>3</sub>, Triiodothyronine.

than baseline throughout reperfusion in each of the ischemia groups, but it was increased in all of the T<sub>3</sub> treated groups (returned to 55% in T<sub>3</sub>-10, 64% in T<sub>3</sub>-25 and 67% in T<sub>3</sub>-50) compared with the CTL group (47%) throughout reperfusion. After 120 min of reperfusion, MVO<sub>2</sub> and cardiac efficiency were increased in the T<sub>3</sub> administrated groups compared with in the CTL group, but remained lower than the baseline in all of the ischemic groups. Both parameters of MVO<sub>2</sub> and cardiac efficiency in the T<sub>3</sub>-25 and T<sub>3</sub>-50 groups were significantly increased compared with those in the CTL group (P<0.05; Table I).

Left ventricular developed pressure (LVDP) was decreased after I/R compared with the baseline in each group. LVDP was increased in the T<sub>3</sub> treated groups compared with in the CTL group upon reperfusion. The improvement in LVDP was significantly different in the T<sub>3</sub>-25 and T<sub>3</sub>-50 groups compared with the CTL group (P<0.05). There was no significant difference in the T<sub>3</sub>-10 group (Fig. 1A). Hearts subjected to global ischemia for 30 min showed a marked increase in LVDP. However, a significant reduction in LVEDP was observed with T<sub>3</sub> 25 or 50 nM treatment (P<0.05; Fig. 1B). Cardiac contractility +dP/dt and relaxation -dP/dt (Fig. 1C and D) were reduced during ischemia in all groups. Upon reperfusion, contractility increased but still remained lower than that recorded before ischemia throughout reperfusion in each group. T<sub>3</sub> administration at doses of 25 and 50 nM significantly improved contractile recovery in I/R injury hearts (P<0.05). This was evidenced by a greater recovery in LVDP and +dP/dt and -dP/dt, respectively, when compared with the pre-ischemic values.

*Effect of T<sub>3</sub> supplementation on the SR Ca<sup>2+</sup> regulatory proteins.* There was a significant reduction in the density of RyR2 and the phosphorylation status of RyR2 at S2809 in the CTL group (P<0.05). Treatment with T<sub>3</sub> in I/R hearts

significantly prevented the attenuation of RyR2 density and Ser-2809 (P<0.05 Fig. 2). Fig. 3 presents the protein density of SERCA2a in the control and I/R hearts that were treated with or without T<sub>3</sub> supplementation. Compared with the non-ischemic values, SERCA2a expression decreased significantly after I/R injury (P<0.05).

*Effect of T<sub>3</sub> supplementation on sarcomemmal Ca<sup>2+</sup>-regulatory protein.* The density of PLB was lower in all the ischemia groups; however, there was no significant difference when compared with the Sham group (Fig. 4). The phosphorylation of PLB at Thr-17 significantly decreased after 30 min of ischemia compared with the non-ischemia group (Sham group; P<0.05). T<sub>3</sub> treatment at all three doses before ischemia attenuated this decrease and a significant difference was detected in all T<sub>3</sub> dose groups compared with the ISC group (P<0.05). The immunoblots of the protein expression of sarcomemmal PMCA and NCX revealed that there were no changes in PMCA and NCX after I/R in the CTL and T<sub>3</sub> supplementation groups.

*ATP and CP content.* The ATP and CP content of transmural sections taken from the left ventricular free wall at the end of reperfusion were determined. In the CTL and T<sub>3</sub> groups, the ATP contents were 6.7±0.9, 9.3±1.1, 11.3±1.6 and 12.2±1.9 μmol/g, respectively; the CP contents were 8.6±1.2, 11.5±2.3, 13.7±1.8 and 14.5±1.7 μmol/g at the end of reperfusion, respectively. The contents of ATP and CP were better preserved in the T<sub>3</sub> (10, 25 and 50 nM) treated groups compared with in the CTL group (Fig. 5).

## Discussion

A number of studies have confirmed that a cardiopulmonary bypass affects thyroid hormone metabolism to reduce circulating free T<sub>3</sub>, which leads to a 'euthyroid-sick' state. It is not

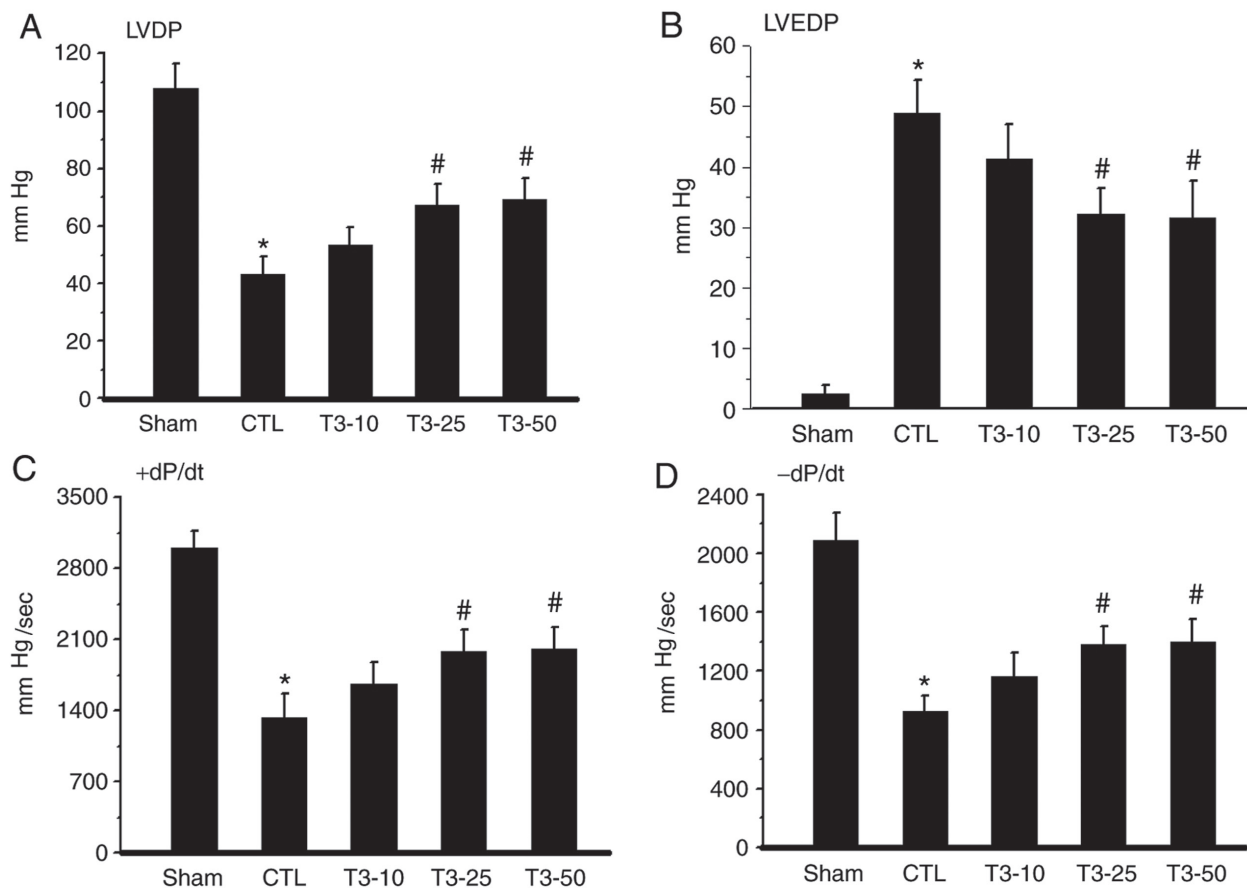


Figure 1. LVDP, LVEDP, +dP/dt and -dP/dt after 30 min ischemia and 120 min reperfusion. (A) LVDP values in the Sham, CTL, T<sub>3</sub>-10, T<sub>3</sub>-25 and T<sub>3</sub>-50 groups. (B) LVEDP values in Sham, CTL, T<sub>3</sub>-10, T<sub>3</sub>-25 and T<sub>3</sub>-50 groups. Cardiac contractility (C) +dP/dt and relaxation (D) -dP/dt values in Sham, CTL, T<sub>3</sub>-10, T<sub>3</sub>-25 and T<sub>3</sub>-50 groups. Values are presented as the mean  $\pm$  standard deviation. \*P<0.05 vs. the Sham group; #P<0.05 vs. the CTL group. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt, rate of ventricular pressure development; CTL, ischemia control group; Sham group, non-ischemic group; T<sub>3</sub>-10, T<sub>3</sub> 10 nM; T<sub>3</sub>-25, T<sub>3</sub> 25 nM; T<sub>3</sub>-50, T<sub>3</sub> 50 nM; T<sub>3</sub>, Triiodothyronine.

clear how acute stress-induced hypothyroidism affects the myocardium pathophysiological change; however, previous studies (4,17) have suggested that T<sub>3</sub> is a positive inotrope and acts by improving the 'aerobic capacity' of the myocardium after ischemia. This then leads to an enhanced availability of myocardial high-energy phosphates for contractile work (4). The results of the present study demonstrated that the recovery of myocardial function was improved after ischemia by treatment with T<sub>3</sub> (at 10, 25 and 50 nM). The supplementation of thyroid hormone improved the recovery of cardiac function and persisted throughout the reperfusion interval. This is consistent with data reported by Klemperer *et al* (18), in which acute administration of T<sub>3</sub> to post-ischemic hearts improved the preload recruitable stroke work area, but had no effect on the preload stroke work area of the non-ischemic hearts. In the present study, T<sub>3</sub> at 10 nM, ten times the physiological dose, produced a measurable effect. T<sub>3</sub> at 25 nM had a greater effect on post-ischemic recovery, but there was no additional recovery at 50 nM. T<sub>3</sub> had a similar salutary effect on the recovery of the diastolic chamber, with a direct relationship between the dose of T<sub>3</sub> required and the severity of the injury. The observation that T<sub>3</sub> (50 nM) did not make additional improvements is important. It suggests the need to further define the effective therapeutic range for T<sub>3</sub> supplementation.

T<sub>3</sub> supplementation could improve cardiac functions and limit infarct size against I/R injury (10). Previous studies have clearly shown that in addition to binding to nuclear-localized receptors, T<sub>3</sub> can also activate signaling processes at the plasma membrane, in mitochondria or within the cytosol by targeting T<sub>3</sub> receptors or other proteins (2,19). Although the heart may be capable of synthesizing 1 mg of protein per g of heart tissue per h (20), the rapid post-ischemic recovery of cardiac function evident in the present study suggests that the site of action targeted by T<sub>3</sub> is more likely to be in the plasma membrane or cytosol. There is also strong evidence that the accumulation of cytosolic Ca<sup>2+</sup> during I/R is highly correlated with the severity of injury (13). The present study using isolated hearts revealed a marked improvement in cardiac function by T<sub>3</sub> supplementation before I/R, which is consistent with previous studies (2,8,21). As the inhibition of calpain prevented the I/R-induced degradation of key SR Ca<sup>2+</sup> cycling proteins (such as RyR2 and SERCA2a) and improved contractile function (22), the present study measured the protein contents of the Ca<sup>2+</sup> release channel, Ca<sup>2+</sup> uptake and other Ca<sup>2+</sup>-regulating proteins. The results demonstrated that an I/R-induced depression in cardiac performance was associated with a downregulation of the major SR Ca<sup>2+</sup>-cycling proteins. This downregulation could be attenuated by T<sub>3</sub> supplementation before ischemia.

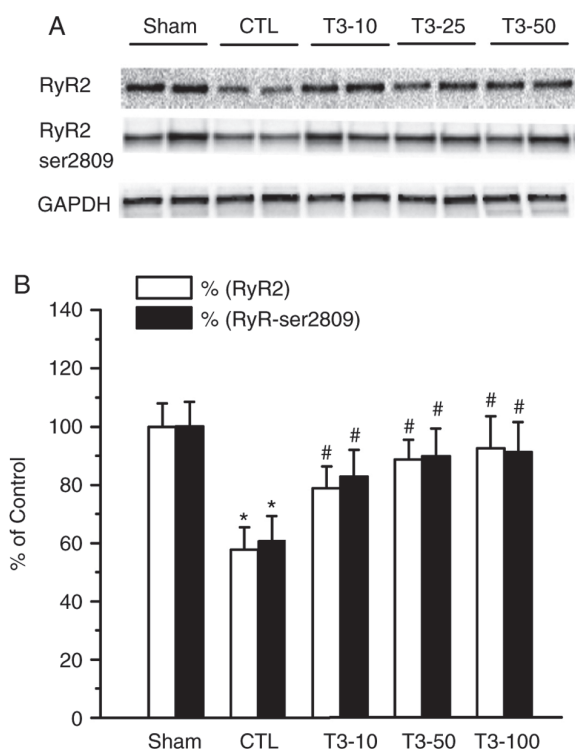


Figure 2. Western blot analysis of RyR2 and the phosphorylation of RyR2 at Ser-2809 in the homogenate of myocardial tissue from rat hearts. The data are presented as the percentage of controls. (A) Western blots presenting the levels of RyR2 and RyR2-Ser-2809 protein. Bands are quantitative immunoblots of representative samples from the hearts of the different groups run on the same gel. (B) Group results. Values (mean  $\pm$  standard deviation) in the  $T_3$  treatment groups are expressed as the percentage of the Sham group. \* $P < 0.05$  vs. time control group (Sham); # $P < 0.05$  vs. the CTL group. RyR2, ryanodine receptor 2; CTL, ischemia control group;  $T_3$ , Triiodothyronine.

RyR2 is a protein found primarily in cardiac muscle. The RyR2 protein functions as the major component of a calcium channel located in the SR that supplies ions to the cardiac muscle during systole to initiate contraction (23). In the present study,  $T_3$  supplementation improved cardiac function recovery in I/R hearts and was accompanied with the preservation of RyR2 protein content. To date, it has been demonstrated that Ser-2809 is one of the main sites of phosphorylation on RyR2. Phosphorylation of RyR2 by CaMKII, or protein kinase A (PKA), at Ser-2809 induced channel function changes *in vitro*. These changes include an increase in the probability of being open (24). In the present study a low level of Ser-2809 phosphorylation was observed in the CTL group, but marked Ser-2809 phosphorylation was expressed following supplementation with  $T_3$ . The results suggest that  $T_3$  may facilitate the phosphorylation of RyR2 by CaMKII or PKA.

SERCA2 in cardiac muscle plays an important role in the muscle's overall contractility status. Improved cardiac SR function by  $T_3$  supplementation against I/R injury may partly contribute to a higher level of the protein content of major SR  $Ca^{2+}$  uptake proteins including SERCA2a and PLB, a 52 amino acid phosphoprotein. Unphosphorylated PLB inhibits SERCA2a, but the phosphorylation of PLB prevents the inhibition of SERCA2a at either Ser-16 by PKA or Thr-17 by CaMKII, thereby increasing SERCA2a activity and the rate of  $Ca^{2+}$  uptake by the SR (25). The present results showed

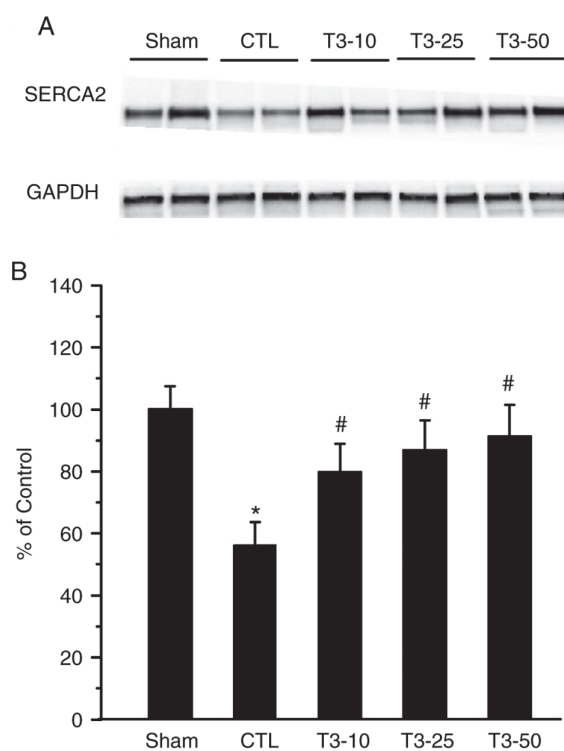


Figure 3. Western blot analysis of SERCA2 and the phosphorylation of RyR2 at Ser-2809 in the homogenate of myocardial tissue from rat hearts. The data are presented as the percentage of the Sham group. (A) Western blots presenting the levels of RyR2 and RyR2-Ser-2809 protein. Bands are quantitative immunoblots of representative samples from the hearts of different groups run on the same gel. (B) Group results. Values (mean  $\pm$  standard deviation) in the  $T_3$  treatment groups are expressed as percentage of the Sham group. \* $P < 0.05$  vs. time control group (Sham); # $P < 0.05$  vs. the CTL group. SERCA2,  $Ca^{2+}$ -adenosine triphosphatase; RyR2, ryanodine receptor 2; CTL, ischemia control group;  $T_3$ , Triiodothyronine.

that the protein level of PLB was decreased in I/R hearts and SERCA2a levels were reduced even more. Therefore it is in agreement with a previous study in which the PLB/SERCA2a interaction controlled the calcium content of the SR and ultimately controlled cardiac contractility (25). A decrease in the phosphorylation of PLB, along with an increase in the PLB/SERCA2a ratio was observed (data not shown) in the CTL group, suggesting the strong inhibitory effect of PLB on SERCA2a in I/R hearts. Phosphorylation of the PKA substrate PLB is a critical determinant of  $Ca^{2+}$  re-entry into the SR and is coordinated by CaMKII and PKA. In the present study, PLB phosphorylation on Thr-17 was decreased during I/R. PLB phosphorylation was downregulated in I/R hearts, suggesting that the rate of  $Ca^{2+}$  uptake by the SR decreased due to the inhibition of SERCA2a. The present results demonstrated that  $T_3$  supplementation improved PLB phosphorylation at Thr-17 in I/R hearts.  $T_3$  supplementation increased the PLB/SERCA2a ratio by valid preservation of SERCA2a. The phosphorylation of PLB by  $T_3$  reduced the inhibition of SERCA2a and therefore enhanced SR  $Ca^{2+}$  uptake in I/R hearts.  $T_3$  supplementation inhibited ATP-dependent  $Ca^{2+}$  uptake in isolated cardiac SR vesicles (26). There is evidence that alterations in SR  $Ca^{2+}$  cycling function are components of the impaired SR  $Ca^{2+}$  uptake,  $Ca^{2+}$  release and the content of  $Ca^{2+}$ -cycling proteins (23,27); the beneficial effect of  $T_3$  supplementation on

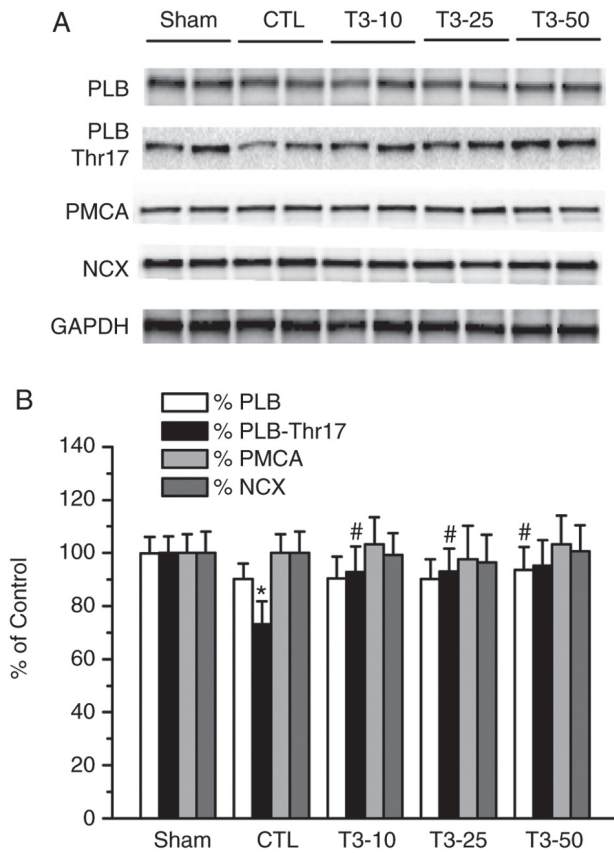


Figure 4. Western blot analysis of PLB and its phosphorylation at Thr-17 (PLB-Thr-17), PMCA and NCX in the homogenate of myocardial tissue from rat hearts. The data are presented as the percentage of controls. (A) Western blots presenting the levels of PLB, PLB-Thr-17, PMCA and NCX protein. Bands are quantitative immunoblots of representative samples from the hearts of the different groups run on the same gel. (B) Group results. Values (mean  $\pm$  standard deviation) in the treatment groups are expressed as the percentage of the control group. \* $P < 0.05$  vs. time control group (Sham); # $P < 0.05$  vs. the CTL group. PLB, phospholamban; PMCA, plasma membrane  $\text{Ca}^{2+}$ -adenosine triphosphatase; NCX, sodium-calcium exchanger; CTL, ischemia control;  $T_3$ , Triiodothyronine.

SR  $\text{Ca}^{2+}$  cycling function may contribute to the attenuation of I/R-induced changes in SR function and protein content.

Cytosolic  $\text{Ca}^{2+}$  regulates several cellular processes and its concentration is, in turn, finely regulated by various channels, pumps and exchangers. The NCX and the PMCA pump are two concurrent mechanisms for  $\text{Ca}^{2+}$  extrusion from the cell. There is a very large transmembrane electrochemical  $\text{Ca}^{2+}$  gradient driving the entry of the ion into cells, yet it is very important that they maintain low concentrations of  $\text{Ca}^{2+}$  for appropriate cell signaling. Thus, it is necessary for cells on pumps to remove  $\text{Ca}^{2+}$ . PMCA and NCX together are the main regulators of intracellular  $\text{Ca}^{2+}$  concentrations (25). In this manner, intracellular  $\text{Ca}^{2+}$  and SR  $\text{Ca}^{2+}$  content are regulated and maintained. The present results revealed no alterations in PMCA and NCX contents after I/R. Furthermore,  $T_3$  supplementation did not affect the content of PMCA or NCX.

The results obtained from measuring myocardial ATP and CP in the present study suggested that  $T_3$  induces inotropic effects via the rapid replacement and maintenance of high energy phosphate stores within the myocardium. The administration of  $T_3$  led to the return of normal mitochondrial function, reactivation of the tricarboxylic acid cycle and full aerobic metabolism;

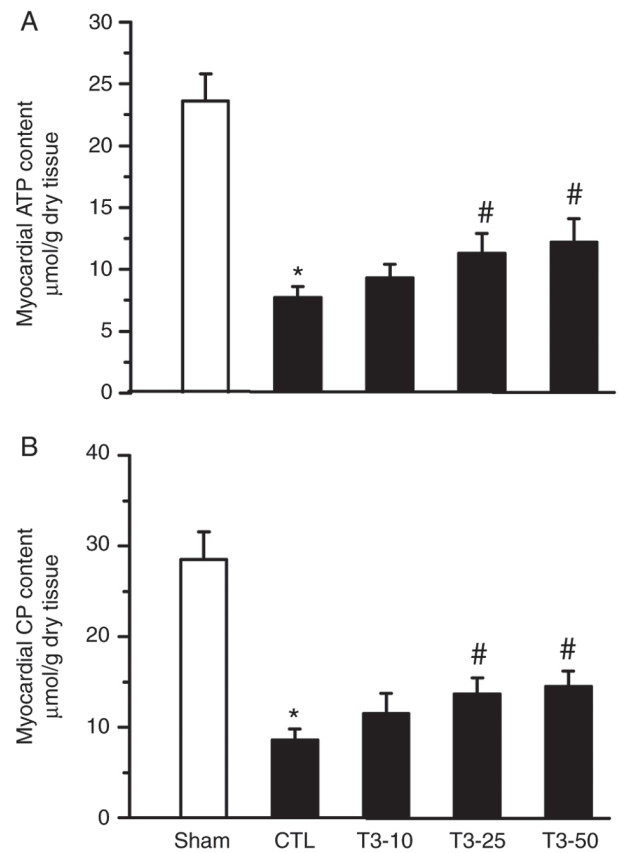


Figure 5. ATP and CP content of transmural sections taken from the left ventricular free wall of rat hearts. (A) Myocardial ATP and (B) CP contents. Values (mean  $\pm$  standard deviation) in all groups are expressed as  $\mu\text{mol/g}$  dry tissue. \* $P < 0.05$  vs. time control group (Sham); # $P < 0.05$  vs. the CTL group. ATP, adenosine triphosphate; CP, creatine phosphate; CTL, ischemia control;  $T_3$ , Triiodothyronine.

tissue lactate levels were reduced and high energy phosphate stores were more rapidly replaced (4).  $T_3$  may lead to both the increased synthesis of high energy stores and increased utilization of such stores, which results in improved myocardial function. This hypothesis is also supported by previous studies (2,4). Sterling *et al* (28) demonstrated the rapidly increased synthesis of ATP production secondary to mitochondrial stimulation in both euthyroid and hypothyroid rats treated with  $T_3$ .

In conclusion, the results of the present study demonstrated that  $T_3$  supplementation improves left ventricular function after I/R in isolated rat hearts by preserving major  $\text{Ca}^{2+}$  cycling proteins. Rapid cardiac functional improvements by  $T_3$  supplementation suggested that these effects may be mediated through  $T_3$  binding at the plasma membrane or SR rather than at the nuclear level. Increased synthesis of myocardial high energy phosphate ATP and CP are attributed to the improvement of myocardial function.  $T_3$ -induced preservation of calcium cycling proteins potentially involves the direct inhibition of protease activities. However, the mechanisms underlying these effects require further elucidation.

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## Availability of data and material

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

## Authors' contributions

Conceived and designed the experiments: LF, LL and JA. Performed the experiments: LF, ZX, JL, LH and SQ. Analyzed the data: LF, LL and JA. Contributed reagents/materials/analysis tools: LF, ZX, JL, LH and SQ. Wrote the paper: LF, LL and JA. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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