

# Cardioprotective effects of phosphocreatine on myocardial cell ultrastructure and calcium-sensing receptor expression in the acute period following high level spinal cord injury

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**Abstract.** Phosphocreatine (PCr) mobilizes high-energy phosphates in cardiac muscles, which is potentially useful as a cardioprotective agent in patients with spinal cord injury (SCI). The cardioprotective effects of PCr on myocardial cell ultrastructure and calcium-sensing receptor (CaSR) expression following high-level spinal cord injury (SCI) were investigated. Healthy adult male Sprague-Dawley (SD) rats (n=54) weighing 250-300 g were subjected to C7 SCI injury by Allen's method with or without treatment by abdominal injection of PCr (200 mg/kg) at 6, 12, 24 or 48 h (SCI + treatment and SCI-only groups, respectively; 6 rats/group/time point). Right apical tissues were sampled 2 h following each time interval. Surgeries without SCI were performed in 6 control rats (sham operation group). Cardiac troponin I (cTnI), serum creatine kinase (CK) and creatine kinase (CK-MB) levels were assessed automatically. Myocardial morphology was examined by transmission electron microscopy (TEM). Quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis were used to determine myocardial tissue calcium-sensing receptor (CaSR) mRNA and protein expression, respectively. Normal myocardial ultrastructure was observed in the sham operation group, while SCI-only groups exhibited progressive and extensive damage to myofibrils, sarcomere structure, mitochondrial membranes and vacuole structures, occasionally accompanied by punctured cell membranes, nuclear chromatin condensation and cavitation. SCI + treatment groups, however, exhibited significantly relieved ultrastructural abnormalities and reduced the levels of CaSR, cTnI, CK and CK-MB mRNA and protein expression at all time intervals ( $P < 0.05$ ). In the SCI rat model, PCr exhibited cardioprotection by relieving myocardial ultrastructural

abnormalities and preserving the normal metabolic energy balance, including calcium regulation.

## Introduction

Following high-level spinal cord injury (SCI), defined broadly as injury above the plane of the chest, complications of the cardiovascular, respiratory and urological systems with highly varying severities have been reported in acute and long-term SCI patient care (1,2). Immediately following SCI, the majority of patients exhibit post-traumatic hypotension coupled with a parallel decline in cardiac output (CO), which may be associated with direct myocardial injury (3). However, the association between myocardial injury and SCI remains controversial, with different studies reporting varying severities of myocardial ultrastructural change following SCI (4,5). Thus, therapeutics that limit the degree and severity of myocardial ultrastructural change in SCI patients are highly desirable in order to reduce cardiovascular symptoms in the acute phase of SCI.

The current gold standard method for the detection of myocardial injury is measuring serum levels of cardiac troponin T (cTnT) and troponin I (cTnI), the cardiac regulatory proteins responsible for the control of actin and myosin interactions by calcium mediation (6). Although troponins are expressed by the cardiac and skeletal muscles of healthy individuals, the levels of cardiac troponins remain undetectably low under normal circumstances. However, extremely small degrees of cardiac necrosis may be detected using monoclonal antibodies to epitopes of cTnI and cTnT with little cross-reactivity for skeletal troponins, thereby indicating myocardial injury within 4-12 h of injury (7). Furthermore, troponin levels may peak several days following injury (7). Combined, testing of cTnI and the mitochondrial enzymes serum creatine kinase (CK) and creatine kinase (CK-MB) may allow for cost-effective and accurate myocardial injury assessment, however, these tools may not fully indicate myocardial infarction risk and ultrastructural change leading to coronary plaque rupture or occlusion (7). Thus, clinical management and peri- and postoperative strategies for cardiac protection are required for patients with any indication of myocardial injury.

Phosphocreatine (PCr), also referred to as creatine phosphate (CP), is an important resource for rapid mobilization of reserves of high-energy phosphates in skeletal muscles, including muscles of the heart and has recently been employed

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for perioperative cardioprotection (8,9). Furthermore, recent studies have indicated that CaSR, a G protein-coupled receptor superfamily C family member, may be used to indicate the degree of myocardial cell damage leading to infarction (10,11). Wang *et al* (10) suggested that CaSR mRNA and protein expression were significantly upregulated with the severity of myocardial damage, suggesting that CaSR may be a useful biomarker for myocardial ischemia and reperfusion injury. Similarly, Chen *et al* (11) confirmed the upregulation of CaSR expression in a rat model of SCI. Thus, PCr may be a useful agent for myocardial protection following injuries, including SCI.

The present study assesses the cardioprotective effects of PCr following SCI by examining changes in CaSR mRNA and protein expression as well as ultrastructural changes in myocardial tissues in rats. This study provides preliminary evidence for the use of PCr in developing clinical cardioprotective agents.

## Materials and methods

**Study design.** Healthy adult male SD rats (n=54) weighing 250-300 g were purchased from the Animal Laboratory of the Fuzhou General Hospital of the Nanjing Military Area Command (Fuzhou, Fujian, China) and housed in the Animal Breeding Room of the Institute of Hypertension Research, The First Affiliated Hospital of Fujian Medical University (Fuzhou, Fujian, China) under 12/12 h light/dark conditions at 25-30°C with *ad libitum* access to food and water. A group of 6 rats was subjected to sham operation without SCI (sham operation group). The remaining rats were divided into equal groups and subjected to C7 SCI injury by Allen's method with or without treatment by abdominal injection of PCr (200 mg/kg) at 6, 12, 24 or 48 h (SCI + treatment and SCI-only groups, respectively; 6 rats/group/time point). For treated (SCI + treatment groups) and untreated (SCI-only groups) right apical tissues were sampled 2 h following treatment at each treatment time interval. All animal experiments were conducted in compliance with all national and state guidelines and were approved by the Animal Use Committee of Fujian Medical University.

**SCI model establishment.** Rats had no access to food or water for 12 h prior to the procedure. Each rat was fixed in place on an arched table and anesthetized by intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg). The injury site was shaved and disinfected with 10% povidone-iodine. A midline incision was made to expose the C7 spinous process (5 cm) and a longitudinal incision in the skin and subcutaneous tissue was made to expose the approximate region of the C6 to T1 spinous process and lamina. In the sham operation group, a similar operation was performed to expose the C7 region, however, SCI was not performed.

In the SCI + treatment and SCI-only groups, SCI was established according to the Allen's method (12). Briefly, the C7 dural surface was padded with a plastic gasket along the curvature of the spinal cord. A 10 g weight was delivered by vertical free-fall along a 5 cm hollow glass tube, impacting the C7 region of the spinal cord. Successful models exhibited immediate darkening of the spinal cord, fluttering of the lower extremities, body retraction and spasticity in the tail

muscles. Immediately following SCI, bleeding at the surface of the spinal cord was stopped using a gelatin sponge and skin and muscle were sutured. Following each surgery, rats were treated with intraperitoneal injection of 5 ml of normal saline (40 ml/kg) and intramuscular injection of penicillin (200,000 U) twice/day for 2 days. Successful models were housed as previously described and provided daily artificial urination and defecation care.

**Animal treatment.** Rats in the SCI + treatment groups were treated at 6, 12, 24 or 48 h following SCI with intraperitoneal injection of PCr (200 mg/kg). Rats in the sham operation group and SCI groups were treated with intraperitoneal injection of the equivalent volumes of normal saline. A midline and longitudinal incision in the skin and subcutaneous tissue was made 2 h following treatment administration, to expose the chest organs and the pericardium was cut to expose the heart. Myocardial tissues from the right apical structures were collected using ophthalmic scissors. Samples were placed in sterile tubes and stored by freezing at -80°C. Simultaneously, the abdominal aorta was exposed and arterial blood (~2 ml) was sampled by scalp needle aspiration and then stored in refrigerated tubes.

**Assessment of serum cTnI, CK and CK-MB levels.** The level of cTnI was determined using an AU2700 automated analyzer provided by Olympus (Tokyo, Japan). The levels of CK and CK-MB were measured using an ADVIA Centaur automated analyzer provided by Olympus (Tokyo, Japan).

**Ultrastructural examination by TEM.** A portion of each apical sample was cut into 1x1x1 mm cubes with a surgical-grade blade and fixed immediately in 0.1 M PBS (pH 7.2) with 3% glutaraldehyde and 1.5% paraformaldehyde provided by the Laboratory of Electron Microscopy of Fujian Medical University (Fuzhou, Fujian, China) at 4°C for at least 2 h. Samples were then rinsed with 0.1 M PBS (3 times), fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanate at 4°C for 2 h and rinsed again 3 times in 0.1 M PBS. Then, samples were subjected to graded acetone dehydration by 50% alcohol for 10 min, 70% alcohol saturated with uranyl acetate dye at 4°C overnight, 90% alcohol for 10 min, 90% alcohol-acetone for 10 min, 90% acetone for 10 min and 3 rounds of 100% anhydrous acetone for 10 min. Samples were then saturated with anhydrous acetone and epoxy 618 embedding medium (1:1) for 1.5 h and embedded in pure 618 embedding medium at 35°C for 3 h, with polymerization conditions of 35°C for 12 h, 45°C for 12 h and 60°C for 3 days. Resultant samples were then sectioned into ultra-thin sections with an ultra-thin slicer (70-80 nm), stained with uranyl acetate and lead citrate for 5 min and washed thoroughly with distilled water. Samples were observed using an EM208 YEM microscope (Philips, Amsterdam, Netherlands).

**qRT-PCR detection of CaSR mRNA expression.** Samples of ~0.1 g were ground at low temperatures and total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA purity was determined at 260 and 280 nm (A260/280). cDNA was synthesized using a Revert Aid First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). Specific mRNA quantification was performed by real-time PCR using SYBR Premix Ex TaqII (Takara Bio, Inc.,

Dalian, Liaoning, China) in a real-time PCR system (Thermal Cycler Dice, USA), according to the manufacturer's instructions. qRT-PCR detection was conducted with primers provided by Dalian Biological Engineering Co., Ltd. (Shanghai, China): CaSR forward primer, 5'-TTCGGCATCAGCT TTGTG-3', reverse primer 5'-TGAAGATGATTTCTGCTTCC-3' and amplification fragment length was 38 bp; GAPDH forward primer 5'-CTCAACTACATG GTCTACATG-3', reverse primer 5'-TGGCATGGACTGTGGTCATGAG-3' and amplification fragment length was 420 bp.  $2^{-\Delta\Delta Ct}$  was calculated to represent the relative mRNA expression of target genes, as previously described (13).

**Western blot analysis of CaSR protein expression.** Samples of ~0.1 g were ground and subjected to treatment with protein RIPA lysis buffer (BioTeke Biotechnology Co., Ltd., Beijing, China). The supernatant was collected following centrifugation at 14,167 x g and 4°C for 5 min. Protein quantification was conducted using a BCA Protein Assay kit (BioTeke Biotechnology Co., Ltd.). For western blot analyses, equal amounts of protein were separated by 10% SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Invitrogen, Grand Island, NY, USA). Non-specific sites on each blot were blocked with 5% milk powder diluted in TBS with 0.05% Tween (TBST). Following overnight incubation with rabbit anti-CaSR polyclonal antibody and rabbit anti-β-actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), each blot was washed three times with TBST buffer. Blots were then incubated with HRP-labeled goat anti-rabbit secondary antibody (Beijing Zhongshan Golden Bridge Corporation, China) for 1 h. Proteins were detected using Ultra ECL reagent (BioTeke Biotechnology Co., Ltd.). Band intensity was quantified using Quantity One 4.6.2 software (Bio-Rad, Hercules, CA, USA). CaSR expression was normalized to β-actin.

**Statistical analysis.** All data were reported as the means ± SD. All data were analyzed with SPSS v.11.5 for Windows (SPSS, Inc., Chicago, IL, USA). Statistical significance was evaluated by one-way analysis of variance (ANOVA) and with the Dunnett t-test for post hoc analysis. P<0.05 were considered to indicate a statistically significant difference.

**Results**

**Serum cTnI, CK and CK-MB levels are reduced by PCr treatment.** The serum cTnI, CK and CK-MB levels in treated and untreated SCI rats were significantly higher than those of the sham operation group rats at all time points (P<0.05). In the SCI-only group, levels of cTnI decreased from 6 to 48 h, with 48 h values significantly greater than those of the sham operation group (P<0.05). Serum CK content in the SCI-only and the SCI + treatment group were highest at 24 h and levels at 48 h remained significantly greater than those of the sham operation group (P<0.05). CK-MB levels in the SCI-only and SCI+treatment group were highest at 12 h and levels at 48 h remained significantly greater than those of the sham operation group (P<0.05). At all time points, rats in the SCI + treatment group exhibited significantly lower cTnI, CK and CK-MB levels than the SCI-only group (P<0.05; Table I).

Table I. Serum cTnI, CK and CK-MB levels.

	Sham operation group (n=6)			SCI-only groups			SCI + treatment groups				
	6 h (n=6)	12 h (n=6)	24 h (n=6)	6 h (n=6)	12 h (n=6)	24 h (n=6)	48 h (n=6)	6 h (n=6)	12 h (n=6)	24 h (n=6)	48 h (n=6)
cTnI	0.004±0.002 <sup>*\$</sup>	0.056±0.001 <sup>*&amp;#</sup>	0.031±0.002 <sup>*&amp;#</sup>	0.026±0.001 <sup>*&amp;#</sup>	0.011±0.003 <sup>*&amp;#</sup>	0.047±0.003 <sup>*&amp;#</sup>	0.023±0.002 <sup>*&amp;#</sup>	0.018±0.006 <sup>*&amp;#</sup>	0.009±0.001 <sup>*&amp;#</sup>	0.009±0.001 <sup>*&amp;#</sup>	0.009±0.001 <sup>*&amp;#</sup>
CK	520.521±122.135 <sup>*\$</sup>	3005.021±632.173 <sup>*&amp;#</sup>	4089.032±348.006 <sup>*&amp;#</sup>	5307.124±256.781 <sup>*&amp;#</sup>	2344.124±901.346 <sup>*&amp;#</sup>	2781.324±456.093 <sup>*&amp;#</sup>	3012.115±783.289 <sup>*&amp;#</sup>	5012.112±478.387 <sup>*&amp;#</sup>	1988.09±456.326 <sup>*&amp;#</sup>	5012.112±478.387 <sup>*&amp;#</sup>	1988.09±456.326 <sup>*&amp;#</sup>
CK-MB	552.833±98.053 <sup>*\$</sup>	1150.092±309.325 <sup>*&amp;#</sup>	2713.121±349.489 <sup>*&amp;#</sup>	1345.032±387.587 <sup>*&amp;#</sup>	564.083±793.328 <sup>*&amp;#</sup>	1023.013±893.389 <sup>*&amp;#</sup>	2012.398±674.398 <sup>*&amp;#</sup>	1012.144±498.236 <sup>*&amp;#</sup>	1249.932±345.908 <sup>*&amp;#</sup>	1012.144±498.236 <sup>*&amp;#</sup>	1249.932±345.908 <sup>*&amp;#</sup>

Data are presented as the means ± SD. <sup>\*</sup>P<0.05, SCI + treatment vs. sham operation; <sup>#</sup>P<0.05, 6 h-SCI-only group, 12 h-SCI-only group, 24 h-SCI-only group vs. 48 h-SCI-only; <sup>\$</sup>P<0.05, SCI only vs. sham operation; <sup>@</sup>P<0.05, 6 h-SCI + treatment, 12 h-SCI + treatment, 24 h-SCI + treatment vs. 48 h-SCI + treatment; <sup>&</sup>P<0.05, SCI-only vs. SCI + treatment groups at each time interval. SCI, spinal cord injury; cTnI, cardiac troponin I; CK, creatine kinase; CK-MB, creatine kinase isoenzyme MB.

### Ultrastructure abnormalities and necrosis in myocardial cells induced by SCI are relieved by PCr treatment

**Sham operation group.** In the sham operation group, myocardial cells exhibited normal nucleus and nucleolus regions, cardiac myofibrils were arranged in neat rows, sarcomeres were clear and the mitochondrial membrane was intact (Fig. 1).

**SCI-only groups.** From 6 to 48 h, progressive necrotic change was observed in myocardial cells of the SCI-only groups. In the 6 h-SCI-only group, the visible portion of the cardiac sarcoplasm exhibited edema, myofibril dissolution, fracture, swelling of the membrane and slight membrane breakage. Additionally, these cells exhibited significant displacement of the mitochondria, cellular contents and other damage indicative of necrosis, however, overall cellular structure remained intact without significant swelling or vacuole degeneration. The basic ridge structure was clear and rich with glycogen granules in the mitochondria (Fig. 2A). By 12 h (12 h-SCI-only group), the structure of visible myocardial filaments had dissolved, mitochondrial swelling was apparent and cristae dissolution in vacuoles indicated necrotic processes. Furthermore, nuclear chromatin at 12 h was marginalized, with highly coiled chromatin in the nucleus and cavitation in vacuolar structures with apoptotic morphological changes in the myocardium (Fig. 2B). By 24 h (24 h-SCI-only group), changes in myocardial apoptotic morphology were readily apparent, including highly coiled chromatin in the nucleus (marginalization), high degrees of nuclear chromatin condensation and apparent cavitation in numerous vacuolar structures. Furthermore, there were a large number of broken myocardial filaments, indicating significant myocardial cell damage (Fig. 2C). By 48 h (48 h-SCI-only group), necrotic changes in myocardial cell structure were commonplace, including complete, or near complete, myofilament dissolution and sarcomere fault, significant mitochondrial swelling, dissolution of mitochondrial cristae and fusion and deposition of electron dense particles in cavitation. Furthermore, the cell membrane was visibly swollen with slight ruptures in multiple regions and interconnected myocardial cells of the intercalated disc were apparent throughout the intercellular space (Fig. 2D).

**SCI + treatment groups.** From 6 to 48 h, progressive necrotic change was relieved in the SCI + treatment groups compared with the SCI-only groups. In the 6 h-SCI + treatment group, the majority of the cardiac muscle fibers were neatly arranged and myocardial filaments were clear. Furthermore, the sarcomere structure was clear, only slight swelling of the cell membrane was apparent, mitochondria were displaced and occasionally condensed, however generally intact, no swelling and vacuolar degeneration was present and the basic ridge structure was clear and rich with glycogen granules in the mitochondria (Fig. 3A). By 12 h (12 h SCI + treatment group), the structure of the nucleus, mitochondria, myocardial fibers and sarcomere were basically intact, although some dissolution of filaments was apparent (Fig. 3B). By 24 h (24 h SCI + treatment group), the structure of the majority of the myocardial cells and filament sarcomeres remained intact and mitochondrial structure remained normal, although slight edema was observed in the sarcoplasmic region of myocardial cells. Notably, dissolution of myofibrils was apparent by 24 h, with fracture, collapse, cell swelling and slight breakage along with displaced and

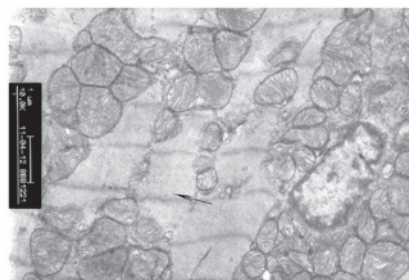


Figure 1. Rat myocardial cells in the sham operation group (magnification, x10,000). Rats in the sham operation group were treated with intraperitoneal injection of normal saline. Myocardial cells exhibit normal nucleus and nucleolus regions, neat cardiac myofibrils, clear sarcomeres and intact mitochondrial membranes. The arrow depicts the myoneme by TEM. TEM, transmission electron microscopy.

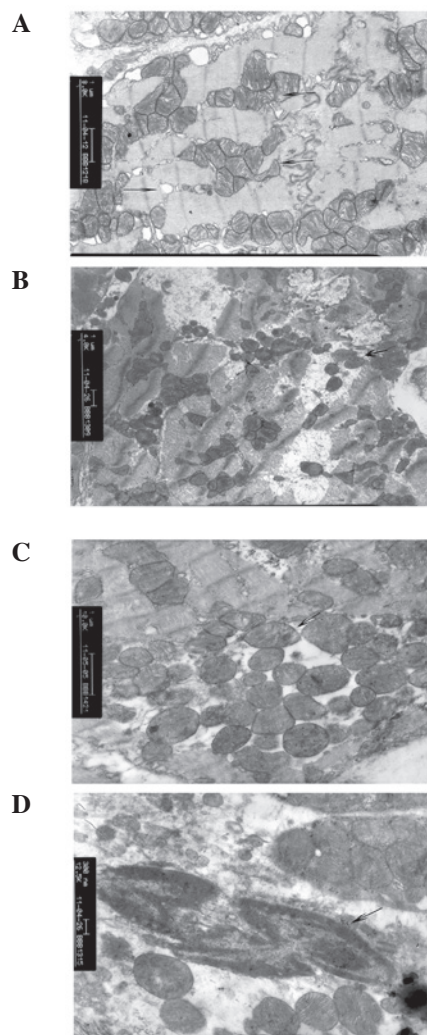


Figure 2. Rat myocardial cells in the SCI-only groups (magnification, x10,000). Rats in the SCI-only groups were treated at 6, 12, 24 or 48 h following SCI with intraperitoneal injection of normal saline. Myocardial cells were shown after (A) 6 h showing mitochondria; (B) 12 h showing mitochondria; (C) 24 h showing mitochondria and (D) 48 h showing nucleus by TEM. SCI, spinal cord injury; TEM, transmission electron microscopy.

condensed mitochondria, however, these features were less prominent than in the 24 h-SCI-only group. Additionally, the ridge structure was defined and intact, with only slight

cavitation (Fig. 3C). By 48 h (48 h-SCI + treatment group), the majority of the myocardial cell nuclei and sarcomere structures remained notably more complete than in the 48 h SCI-only group. Furthermore, myofilaments were predominantly intact, mitochondria structure was generally normal and no swelling or vacuolar degeneration was observed. Only slight dissolution of the mitochondrial crest, indicated by fusion or electron dense particle deposition was apparent, revealing minimal cavitation. Connections between myocardial cell structures of the intercalated disc remained intact, with only a small fraction of myocardial cells exhibiting sarcoplasmic edema, myofibril dissolution, membrane swelling and slight bursting. While certain mitochondrial positions were shifted, none appeared to have punctured the plasma membrane and minimal necrosis was observed (Fig. 3D).

*CaSR mRNA and protein levels are reduced following PCr treatment.* All SCI-only and SCI + treatment groups' CaSR mRNA expression levels at all time intervals were significantly higher than values observed in the sham operation group (all  $P < 0.05$ ), with SCI + treatment groups exhibiting significantly lower CaSR mRNA expression levels than that in the SCI-only groups at all time intervals (all  $P < 0.05$ ). In the SCI-only group, CaSR mRNA expression increased from 6 to 24 h then decreased to 48 h, however, the SCI + treatment group exhibited increases up to 12 h and decreases thereafter (all  $P < 0.05$ ). Relative CaSR mRNA expression among the sham operation group, SCI-only groups and SCI + treatment groups are displayed in Fig. 4. A similar trend in relative CaSR protein expression determined by western blotting is displayed in Fig. 5. Relative CaSR protein expression among the sham operation group, SCI-only groups at 6, 12, 24 or 48 h and SCI+treatment groups at 6, 12, 24 or 48 h following SCI were  $0.331 \pm 0.021$ ,  $0.470 \pm 0.004$ ,  $0.627 \pm 0.008$ ,  $0.808 \pm 0.012$ ,  $0.392 \pm 0.005$ ,  $0.439 \pm 0.011$ ,  $0.505 \pm 0.007$ ,  $0.524 \pm 0.005$  and  $0.358 \pm 0.023$ , respectively.

## Discussion

The present study demonstrated that treatment with intraperitoneal injection of 200 mg/kg PCr following SCI in rats preserved myocardial ultrastructure, reduced CaSR mRNA and protein expression levels, and produced consistently lower cTnI, CK and CK-MB levels when administered within the first 48 h following injury. Furthermore, myocardial necrosis, capsular swelling and abnormal mitochondrial characteristics were notably reduced in all rats treated with PCr, which may preserve function of these tissues and have beneficial cardioprotective effects. Thus, PCr merits further study as a potentially useful clinical agent for cardioprotection in the acute stages of SCI.

Varying degrees of myocardial injury and necrosis have been reported following SCI and these mechanisms are generally controversial and poorly characterized (5). Since cardiovascular failure and dysfunction remain leading causes of mortality and disability in patients with SCI, numerous potentially cardioprotective compounds have been recently investigated in animal models, including FPTIII, MG132 or Ang-(1-7) (14). Unfortunately, the majority of successful treatments require immediate administration, generally

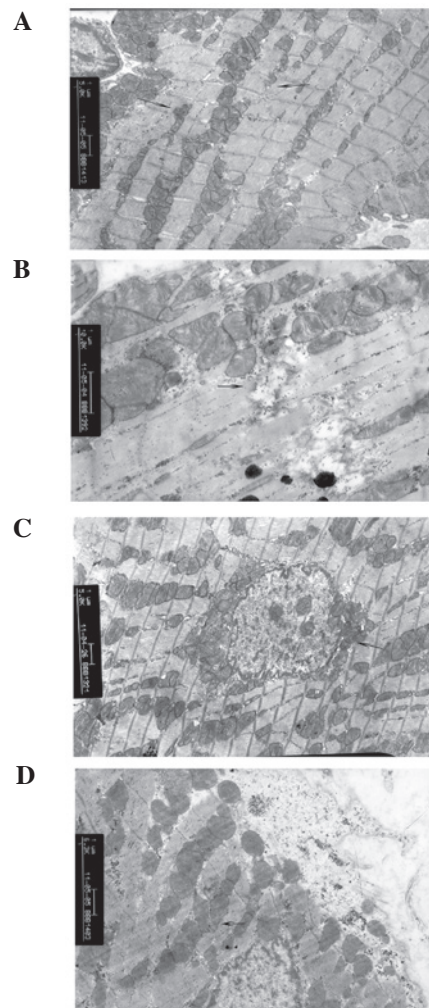


Figure 3. Rat myocardial cells in the SCI + treatment groups (magnification,  $\times 10,000$ ). Rats in the SCI + treatment groups were treated at 6, 12, 24 or 48 h following SCI with intraperitoneal injection of PCr (200 mg/kg). Myocardial cells were shown after (A) 6 h showing sarcomere structure, (B) 12 h showing nucleus, (C) 24 h showing sarcomere structure and (D) 48 h showing mitochondria by TEM. SCI, spinal cord injury; TEM, transmission electron microscopy; PCr, phosphocreatine.

directly following the initial SCI assault or within the first 6 h. Preliminary studies in the last two decades have indicated that post-SCI administration of PCr, however, is able to effectively improve functional heart contractile recovery, lower diastolic pressure and mediate abnormal myocardial enzyme release relatively late in the acute SCI period (15). Similarly, it has been reported that phosphorous-containing compounds, including PCr, are capable of reducing demyelination in nerve fibers following SCI, resulting in overall improvement in systemic nervous and cardiovascular function (16). Furthermore, a benefit to cerebral tissues, including reduced morphological change and apoptosis, has been demonstrated using PCr during cerebral ischemic reperfusion injury (17), which may be analogous to cardiological damage during SCI. The current study, which examines rats within the first 48 h following SCI, provides additional supporting evidence that PCr may have beneficial cardioprotective effects by limiting abnormal changes in cell ultrastructure.

It is well established that administration of PCr is safe, reaching its peak activity within 20 to 40 min following

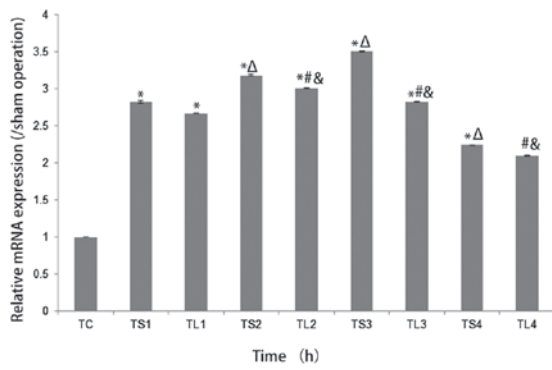


Figure 4. Effect of PCr treatment on CaSR mRNA expression following high level SCI in rats. Total RNA of right apical tissues was extracted and assayed for CaSR mRNA by qRT-PCR analysis. GAPDH was used as an internal control. The data are represented as the means  $\pm$  SD of three independent experiments. \* $P$ <0.05 vs. sham operation group; # $P$ <0.05, SCI + treatment groups vs. SCI groups at different time intervals;  $\Delta P$ <0.05 vs. 6 h-SCI only group within SCI groups and  $\delta P$ <0.05 vs. 6 h-SCI + treatment group within SCI + treatment groups. SCI, spinal cord injury; PCr, phosphocreatine; CaSR, calcium-sensing receptor; qRT-PCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation.

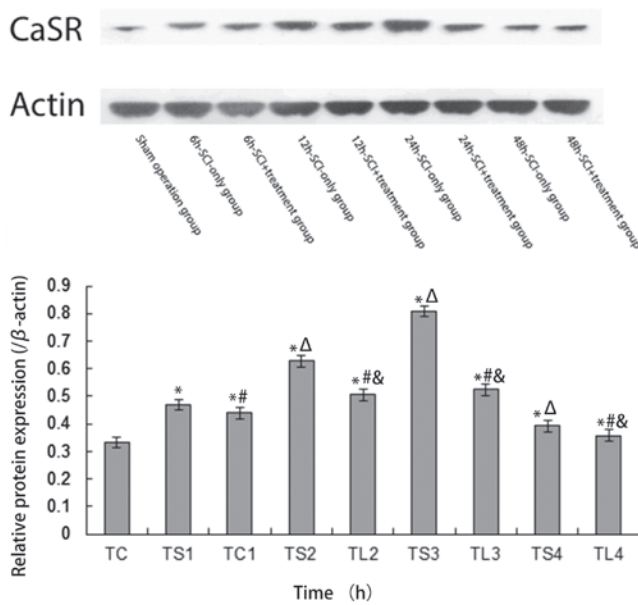


Figure 5. Effect of PCr treatment on CaSR protein expression following high level SCI in rats. Total protein of right apical tissues was extracted and assayed for CaSR protein by western blot analysis.  $\beta$ -actin was used as an internal control. The data are represented as the means  $\pm$  SD of three independent experiments. \* $P$ <0.05 vs. sham operation group; # $P$ <0.05, SCI + treatment groups vs. SCI groups at different time intervals;  $\Delta P$ <0.05 vs. 6 h-SCI only group within SCI groups and  $\delta P$ <0.05 vs. 6 h-SCI + treatment group within SCI + treatment groups. SCI, spinal cord injury; PCr, phosphocreatine; CaSR, calcium-sensing receptor; SD, standard deviation.

intramuscular administration or 30 to 120 min following intravenous administration in humans (8). Furthermore, PCr stays active in the human body for up to 250 min following administration (8). It has been suggested that PCr has a similar activity in rats (17), which was used as a basis for the 2 h post-treatment sampling time selected for this study. In addition to providing energy during this period, Liu *et al* (9) demonstrated that PCr administration during the acute phase following injury

has a beneficial protective effect on mitochondrial membranes, consistent with the results observed in the current study. Furthermore, upregulation of CaSR, raising intracellular  $\text{Ca}^{2+}$  levels, has been demonstrated to cause calcium overload and endoplasmic reticulum imbalance (18). As a result, elevation of CaSR associated with SCI may indirectly increase myocardial cell apoptosis by increasing stress on the membranes of the endoplasmic reticulum, which further increases G protein-phospholipids enzyme C-inositol triphosphate signaling and intracellular  $\text{Ca}^{2+}$  ion release (18). Thus, the beneficial effects of PCr treatment in reducing CaSR levels in the present study may be central to the mechanism by which PCr reduces overall morphological change and apoptosis in myocardial tissues following SCI and other traumatic injuries.

Mechanistically, administration of PCr has been previously demonstrated to act through several different intracellular mechanisms involving inhibition of lysophosphoglyceride accumulation in the ischemic myocardium and cardiac cell sarcolemma preservation by PCr-related zwitterionic interactions as well as ATP regulation and inhibition of adenine nucleotide degradation (15). The current study provided novel ultrastructural support for these mechanisms, suggesting that the myocardium and sarcolemma are, indeed, preserved structurally and functionally by PCr administration throughout the acute period, even later (up to 48 h) following the initial SCI incident. Furthermore, PCr may also act to maintain the extracellular ATP levels and increase cell penetrance of key compounds required to sustain cellular electrolyte balance (15), consistent with findings in the serum that support this sustained extracellular action. These mechanisms, however, require further verification in larger and more extensive biochemical studies prior to their confirmation.

Notably, the current study is limited by the relatively small group and use of a rat animal model, which may have PCr activity peaks that slightly vary from those of human patients. However, the cardioprotective benefits of preoperative PCr have been demonstrated in preliminary studies of human patients undergoing open heart surgery (19), providing strong evidence that the currently demonstrated cardioprotective PCr benefit in SCI rats may similarly be applicable in humans that have experienced SCI. Furthermore, it has been demonstrated that cellular energy metabolism, impacted by PCr levels, following SCI is non-linear and highly variable (20). Thus, further study is required to determine the optimal dosages and treatment times for administration of PCr in human patients, necessitating further clinical study prior to implementation.

The current study demonstrates that PCr has numerous cardioprotective benefits for increasing energy, reducing morphological changes and preserving the normal ultrastructural features in myocardial cells in the acute period ( $\leq 48$  h) following SCI. Furthermore, PCr-treated SCI rats exhibited lower CaSR levels at all studied intervals than rats that underwent SCI without treatment, suggesting improvements in calcium balance that may contribute to reduced apoptosis by preserving intracellular membrane structures in myocardial cells. Thus, early treatment with PCr may potentially contribute to better functional recovery and fewer cardiovascular complications, thereby meriting further study and evaluation for clinical implementation. The full impact of

CaSR regulation and its mechanism following PCr treatment, however, requires further study.

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