Phosphocreatine Restores High-Energy Phosphates in Ischemic Myocardium: Implication for Off-Pump Cardiac Revascularization

Ganga Prabhakar, MD, Linda Vona-Davis, PhD, David Murray, PhD, Paresh Lakhani, BS, Gordon Murray, MD, FACS

BACKGROUND: High-energy phosphate metabolism is altered in the ischemic myocardium. We investigated the effects of in vivo administration of phosphocreatine (PCr) in a transient ischemic rat model to emulate off-pump myocardial revascularization.

STUDY DESIGN: Rats received either PCr (100 mg/kg) or saline intravenously 1 hour before surgery. Regional ischemia was maintained for 12 minutes by ligation of the left anterior descending artery and compared with sham-operated animals. Cardiac tissue was studied for ATP, PCr, and inorganic phosphate (P*i*) using ^{31}P-cryo-NMR. Results were compared by ANOVA.

RESULTS: Levels of ATP were significantly ($p < 0.01$) lower in the ischemic hearts compared with controls; P*i* and PCr remained unchanged. The PCr/P*i* ratio was altered in ischemic hearts, reflecting an increased energy demand. PCr administration significantly ($p < 0.01$) elevated the content of PCr and ATP in both normal and ischemic hearts.

CONCLUSIONS: PCr restores high-energy phosphates and attenuates metabolic stress during periods of myocardial ischemia in the rat. Preconditioning with PCr may serve as a useful adjunct to off-pump coronary revascularization. (J Am Coll Surg 2003;197:786–791. © 2003 by the American College of Surgeons)

Recently there has been a great deal of interest in the application of phosphocreatine (PCr) for medicine and muscle physiology. PCr is intimately involved in the energy metabolism of all muscle cells, including the heart.[1] Together with adenosine triphosphate (ATP), these energy-rich molecules are shuttled from mitochondria to contractile proteins in the muscle. ATP is essential for muscle work and PCr is capable of acting as an energy buffer, protecting the ATP concentration.[2] In addition to its role in cellular metabolism, PCr also stabilizes the membrane phospholipid bilayer by binding to the polar heads and decreasing membrane fluidity. Stabilizing the membrane attenuates some of the damage caused by transient ischemia and hypoxia and helps prevent cytoplasmic leakage.[1,3]

No competing interests declared.

Presented at the American College of Surgeons 88th Annual Congress, San Francisco, CA, October 2002.

Received January 8, 2003; Revised April 25, 2003; Accepted May 24, 2003.
From West Virginia University, Department of Surgery and National Institute of Occupational Safety and Health (NIOSH), Morgantown, WV.
Correspondence address: Ganga Prabhakar, MD, West Virginia University, Department of Surgery, PO Box 9238, Morgantown, WV 26506-9238.

Current myocardial protective strategies to alleviate ischemia-reperfusion injury during cardiac surgery include the use of compounds added as preconditioning agents, substrates added during cardioplegic arrest, or during reperfusion. Adenosine-supplemented blood cardioplegia attenuates postischemic dysfunction after severe regional ischemia,[4] but it does not attenuate coronary artery endothelial dysfunction unless administered during reperfusion. Clinical trials have shown that using glucose-insulin-potassium during and after cardiac surgery improves ischemic cardiac dysfunction and reduces injury.[5] But increasing glycolytic substrate with high glucose and insulin appears to be beneficial in low-flow ischemia and in patients with acute myocardial infarction.[5,6] These results were verified by Cave and colleagues,[7] who showed improved ATP synthesis during low-flow ischemia with addition of a glycolytic substrate. But in their model of ischemia, the addition of glucose and insulin failed to use more of the available phosphocreatine to slow the rate of ATP depletion. More benefit may be obtained by adding a source of high-energy phosphate, such as phosphocreatine, which can more directly affect the efficiency of energy transfor-

ISSN 1072-7515/03/$21.00
doi:10.1016/j.jamcollsurg.2003.05.001

Abbreviations and Acronyms
NMR = nuclear magnetic resonance
PCr = phosphocreatine
P*i* = inorganic phosphate

mation. In order to prevent losses in cardiac muscle energetics, PCr has been given to patients by intravenous injection as part of the treatment for ischemia and myocardial infarction.[3] It preserves myocardial energy and decreases electrical anomalies that sometimes lead to lethal arrhythmias. PCr has been used as a cardioplegic cardioprotective agent during cardiopulmonary bypass and ischemic arrest.[1,8] This suggests that PCr, as an energy buffer during cardiac ischemia, is important to the energy metabolism of the myocardium. Though off-pump coronary artery bypass grafting has had a recent resurgence, its wider application has been limited partly because of associated myocardial dysfunction, which can be significant.[9] Methods to alleviate this dysfunction are needed if the benefits of off-pump coronary artery bypass grafting are to be available to a wider group of patients.

Because PCr has potent beneficial effects on muscle energy, particularly in buffering ATP concentration, the present study tested the hypothesis that PCr will increase high-energy phosphates in the ischemic cardiac muscle. Specifically, this study investigates whether chemical preconditioning with PCr, before transient regional ischemia, will alter high-energy phosphates in the cardiac muscle. This could have therapeutic implications in transient ischemic conditions created by off-pump myocardial revascularization.

METHODS

Animal preparation

Seventeen pathogen-free male Sprague-Dawley rats (275 to 350 g) were housed and maintained in the animal facility at West Virginia University as approved by the Animal Care and Use Committee, under guidelines of the National Institutes of Health for the treatment of experimental animals. Food and water were provided ad libitum, and a 12-hour light/dark cycle was used. Rats were anesthetized with Telazol (30 mg/kg, intraperitoneally). Additional Telazol was administered as needed. Rats were placed on an electric heating pad to maintain body temperature. Rats received either PCr (100 mg/kg) or saline intravenously 1 hour before surgery. Control animals were sham-injected with an equal volume of 0.9% normal saline. A catheter was placed in the trachea to allow artificial ventilation. The chest was opened by left thoracotomy at the fourth intercostal space and the fourth and fifth ribs were sectioned approximately 2 mm from the left margin of the sternum. Immediately after opening the chest, the animals were ventilated with room air using a stroke volume of approximately 2 mL/100 g and a rate of 54 strokes/minute. Regional ischemia was maintained for 12 minutes by ligation of the left anterior descending artery and compared with sham-operated animals.[10] After the ligation period, samples of cardiac tissue were taken for measurement of high-energy phosphates with animals still under general anesthesia.

^{31}P nuclear magnetic resonance

Cardiac tissue was immediately freeze-clamped with precooled aluminum tongs at liquid nitrogen temperature for cryo-nuclear magnetic resonance (NMR) analysis as previously described.[11] Briefly, a weighed amount of frozen tissue was mixed gently with 1 mL of 2-mM methylenediphosphonic acid (MDP) in 60% (v/v) ethylene glycol, ground in a liquid nitrogen bath with a mortar and pestle, transferred to a 10-mm NMR tube and equilibrated with 1 mL of 40 mM edetic acid (EDTA) (pH 7.4) for 1 hour at 243 K. NMR measurements were obtained with a Bruker Avance DMX300 spectrometer using a 10-mm QNP probe maintained at 243 K. Single-pulse ^{31}P-NMR spectra (600 scans, 1 second delay, ^{1}H decoupling) were acquired at 121.4936 MHz over a 20-kHz spectral width. ^{31}P chemical shift was referenced externally to 85% D_3PO_4. Metabolite concentrations were determined for ATP, phosphocreatine (PCr), inorganic phosphate (P*i*), monophosphate esters, and other phosphate metabolites by comparing integrated resonance areas to those of an external 10-mM PCr solution. Methylene-diphosphonic acid was used as an internal standard for chemical shift determinations and area calculations. Relative peak areas were compensated for T_1 saturation effects (25% to 45% loss). The pH_i was calculated from the relative chemical shift of P_i with respect to PCr.

Statistical analysis

Statistical analyses were performed with analysis of variance (ANOVA) incorporating repeated measures. If the ANOVA showed significant differences, individual

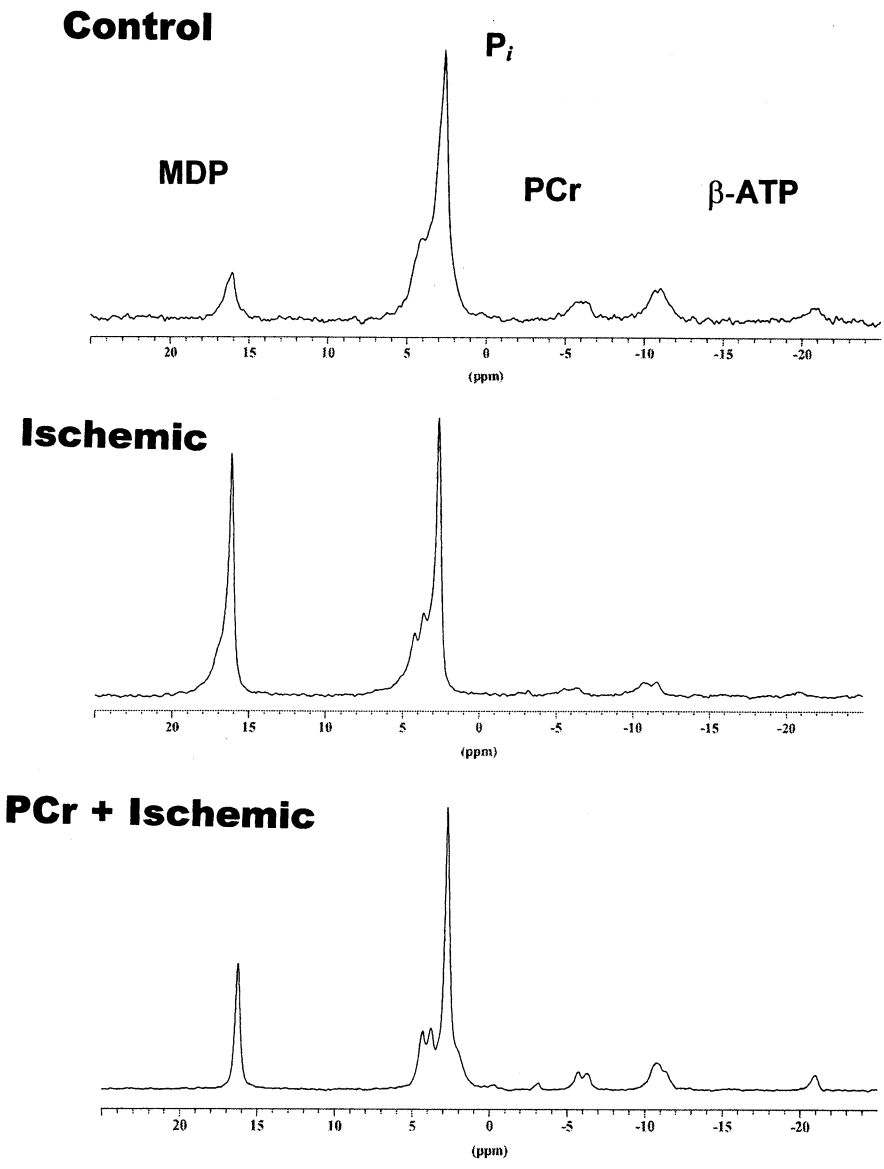

Figure 1. ^{31}P-NMR scan of the cardiac muscle. Results of phosphorus 31-labeled magnetic resonance spectroscopy of cardiac tissue from rats after regional ischemia and pretreated for 1 hour with either PCr or saline. The peaks include: P_i, inorganic phosphate; PCr, phosphocreatine; β-ATP, adenosine triphosphate; note the peak MDP, methylene-diphosphonic acid, which represents a known marker.

group means were compared with the control using orthogonal contrasts. All data in the text and figures are presented as mean ± SEM. A p value of 0.05 or less was considered statistically significant.

RESULTS

Cardiac tissue homogenates were prepared from rats subjected to either phosphocreatine (100 mg/kg, iv) or sham injection with normal saline 1 hour before they underwent 12 minutes of transient ischemia. ^{31}P-NMR analysis was performed within 1 hour of tissue removal. Representative spectra (Fig. 1) were collected for each treatment group in order to display changes in high-energy phosphate content for the individual ATP, PCr, and P*i* metabolites. The ^{31}P spectrum of ATP and phosphocreatine shows that each of the three phosphorous nuclei of ATP is distinguishable from one another and visible in the spectra from control, ischemia, and PCr-

Table 1. High-Energy Phosphate Ratios and Intracellular pH in Cardiac Tissue from Saline and PCr-Treated Rats after Regional Ischemia

	P*i* (mM)	PCr (mM)	ATP (mM)	ADP (mM)	PCr/P*i* ratio	pH
Control rats						
−PCr (n = 4)	6.94 ± 1.21	8.05 ± 2.06	14.62 ± 3.01	0.46 ± 0.06	1.32 ± 0.42	7.59 ± 0.04
+PCr (n = 3)	3.26 ± 0.67	18.35 ± 5.74*	44.10 ± 16.55*	0.25 ± 0.02	5.80 ± 1.35*	7.58 ± 0.05
Ischemic rats						
−PCr (n = 5)	4.35 ± 1.81	4.06 ± 1.66	6.02 ± 2.25†	0.11 ± 0.03†	0.93 ± 1.03	7.57 ± 0.04
+PCr (n = 5)	8.25 ± 2.69	14.66 ± 2.29*	16.25 ± 2.27*†	0.45 ± 0.12*	1.77 ± 0.69	7.58 ± 0.04

Data are presented as mean ± SEM of 3–5 animals in each group.
*$p < 0.01$ with PCr.
†$p < 0.05$ versus control.
ADP, adenosine diphosphate; ATP, adenosine triphosphate; PCr, phosphocreatine; P_i, inorganic phosphate.

ischemia groups. Concentrations of the various phosphate metabolites are provided in Table 1.

Levels of ATP were significantly ($p < 0.01$) lower in the ischemic hearts compared with controls. ATP concentrations were reduced by nearly 60%, from 14.62 ± 3.01 mM to 6.02 ± 2.25 mM. Tissue PCr was also reduced by 50% from 8.0 ± 2.06 mM to 4.06 ± 1.66 mM, although this change was not statistically different. P*i* and ADP concentrations were both reduced in the ischemic hearts. Only ADP levels were significantly ($p < 0.01$) reduced by 75% in the ischemic hearts compared with the controls. Hearts subjected to ischemia showed a 30% reduction in the PCr/P*i* ratio, an index of oxidative metabolism, reflecting an increased energy demand.

Pretreatment with PCr before ischemia significantly ($p < 0.01$) elevated the content of tissue PCr and ATP in both normal and ischemic hearts. In the ischemic hearts, tissue PCr (4.06 ± 1.66 mM to 14.66 ± 2.29 mM) and ATP (6.02 ± 2.25 mM to 16.25 ± 2.27 mM) were increased with pretreatment by 3.6- and 2.7-fold, respectively. There was a fourfold ($p < 0.01$) increase in the content of ADP (0.114 ± 0.026 mM to 0.449 ± 0.119 mM) in the ischemic heart with pretreatment. There was a twofold increase in the P*i* in the cardiac tissue with in vivo administration of PCr, but this increase was not statistically significant. Measurements of intracellular pH were not significantly different between control and experimental animals.

DISCUSSION

In this study, cardiac ischemia was imposed for 12 minutes by ligation of the left anterior descending artery in the rat. Samples of the cardiac muscle were taken in the area of the occlusion and measured for phosphate metabolites (ATP, PCr, ADP, and P*i*) and intracellular pH using ^{31}P-NMR spectroscopy. ^{31}P-NMR spectroscopy and imaging techniques have proved reliable and sensitive indicators of deficiencies of myocardial energy metabolism in intact cells, organs, and animals.[12,13] In addition, NMR technology compares favorably with chemical indices when assessing the distribution of phosphate-containing metabolites in muscle tissue and in intact beating hearts during ischemia and reperfusion studies.[7,14]

Ischemic conditions can create a state of energy deficiency in the heart. Alterations in myocardial energetics vary according to the type, duration, and degree of ischemia imposed, but it generally compromises heart function. The model of ischemia imposed in this study reflects creation of a relatively severe ischemia (zero-flow) in a region of the heart. By comparison, we would expect the metabolic pathways for glycolysis and oxidative phosphorylation to be more inhibited than in less severe ischemia models. In this transient ischemic model, cardiac tissue PCr was reduced by 50% and ATP by 60% with 12 minutes of ligation; P*i* remained unchanged or did not accumulate. This agrees with total ischemia models, where PCr falls within minutes to very low levels in order to slow the rate of ATP depletion.[7] Although we observed a decline in PCr, tissue concentrations remained at 50% of preischemic values, even when ATP had declined by 60%. Experiments with labeled phosphate have shown that newly synthesized PCr is being formed only from mitochondrial ATP, explaining why ATP can still be found during ischemia after all contractile activity has ceased.[15] Interestingly enough, with transient ischemia, we did not observe changes in intracellular pH of the tissue even after 12 minutes of ligation. Although we observed a decline in ATP and PCr levels,

there was not an accumulation of free protons, and pH fell by less than 0.1 pH unit. This would indicate that hypoxia had not yet begun to compromise the cardiac tissue with ischemia as substrate availability was declining.

A major result of this study is that pretreatment with PCr increased ATP concentrations in the cardiac tissue with ischemia. In addition, pretreatment with exogenous phosphocreatine maintained phosphocreatine content in the cardiac tissue near control levels at 14.66 ± 2.29 mM after ischemia. The same was true for ATP as concentration was maintained at 16.25 ± 2.27 mM with PCr administration. This is in agreement with Sharov and coworkers,[16] who showed protection of ischemic myocardium by exogenous phosphocreatine and complete restoration of ATP and PCr after 35 minutes of total ischemia. Exogenous phosphocreatine (200 mg/kg) given to cats 5 minutes before coronary ligation and for 3 to 6 hours after ligation showed a decrease in the size and severity of ischemia.[17] Although the mechanism of metabolic regulation was not defined in this study, administration of PCr stimulated oxidative phosphorylation by increasing both ADP and P*i* concentrations in the cardiac tissue. With this in mind, it seems reasonable to conclude that PCr administration was able to stabilize ATP concentrations sufficiently through oxidative phosphorylation reactions in order to sustain mitochondrial respiration.

Ischemic preconditioning as a method to improve myocardial protection is not currently popular.[9] To achieve ischemic preconditioning brief periods of occlusion and reperfusion is followed by a longer period of occlusion to perform the coronary artery bypass grafting to improve myocardial protection in the area supplied by the coronary artery. Because most patients require multiple anastomoses this technique has limitations in the clinical setting. The chemical methods to attenuate myocardial dysfunction are attractive because they can be administered before surgery and they do not increase the operative time if successful.

The cardioprotective effects of phosphocreatine are well documented.[18] One of the key mechanisms of PCr action is its interaction with the sarcolemmal membrane and its ability to stabilize membrane phospholipids into a more structured gel-like state. In addition, PCr is an important high-energy phosphate compound for cardiac contractile function, as demonstrated by chronic phosphocreatine deficiency studies.[19] In clinical studies where PCr was given in the blood cardioplegia to valve replacement patients with acquired heart disease, a more rapid recovery of hemodynamics after release of the aortic cross-clamp was achieved. There was also decreased frequency of fibrillation and frequent restoration of sinus rhythm.[20]

The molecular and cellular mechanisms of creatine phosphate for cardioprotection have been studied for more than 50 years.[21] Results of these studies on administered PCr have identified that the efficiency of exogenous PCr for cardiac protection is related to the transient movement from the extracellular space into the heart rather slowly. PCr is administered by intramuscular or intravenous route because it is broken down in the gastrointestinal tract. Its pharmacodynamic effects last for 2 to 5 hours. The pharmacokinetics of PCr after a single-dose injection shows a biphasic distribution of PCr in the blood with clearance half-times of 7 and 50 minutes.[16] Although the clearance rate is rapid initially, PCr is specifically absorbed by the heart and remains bound to extracellular binding sites because of its high affinity. In addition, PCr has membrane-stabilizing effects that oral creatine does not have, and oral creatine has to be administered for many days to obtain full benefit.[22]

A limitation of this study was that it examined normal rat hearts, which are different from ischemic human hearts that have been preconditioned with chronic ischemic heart disease. The reperfusion injury was not evaluated in this study. Attenuation of reperfusion injury and stunning, if shown, would provide a strong role for PCr in the clinical field.[9] Whether improved intramyocardial phosphate levels translate into improved energy and functional capability remains unproved.

In summary, PCr administration restores high-energy phosphates and attenuates metabolic stress during periods of myocardial ischemia in the rat. Because its peak pharmacodynamic activity is 2 to 5 hours after intravenous administration, it is well suited for perioperative use. Because the safety of PCr is well documented, preconditioning with PCr might serve as a useful adjunct to off-pump revascularization.

Author Contributions

Study conception and design: Prabhakar
Acquisition of data: Vona-Davis, D Murray, Lakhani
Analysis and interpretation of data: Vona-Davis
Critical revision: G Murray
Statistical expertise: Vona-Davis
Supervision: Prabhakar

REFERENCES

1. Saks VA, Kuznetsov AV, Kupriyanov VV, et al. Creatine kinase of rat heart mitochondria. The demonstration of functional coupling to oxidative phosphorylation in an inner membrane-matrix preparation. J Biol Chem 1985;260:7757–7764.
2. Wallimann T, Wyss M, Brdiczka D, et al. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem J 1992;281:21–40.
3. Sharov VG, Saks VA, Kupriyanov VV, et al. Protection of ischemic myocardium by exogenous phosphocreatine. I. Morphologic and phosphorus 31-nuclear magnetic resonance studies. J Thorac Cardiovasc Surg 1987;94:749–761.
4. Thourani VH, Ronson RS, Van Wylen DG, et al. Adenosine-supplemented blood cardioplegia attenuates postischemic dysfunction after severe regional ischemia. Circulation 1999;100: II376–II383.
5. Apstein CS. Glucose-insulin-potassium for acute myocardial infarction: remarkable results from a new prospective, randomized trial. Circulation 1998;98:2223–2226.
6. Saupe KW, Eberli FR, Ingwall JS, Apstein CS. Metabolic support as an adjunct to inotropic support in the hypoperfused heart. J Mol Cell Cardiol 2001;33:261–269.
7. Cave AC, Ingwall JS, Friedrich J, et al. ATP synthesis during low-flow ischemia: influence of increased glycolytic substrate. Circulation 2000;101:2090–2096.
8. Robinson LA, Braimbridge MV, Hearse DJ. Creatine phosphate: an additive myocardial protective and antiarrhythmic agent in cardioplegia. J Thorac Cardiovasc Surg 1984;87:190–200.
9. Puskas JD, Vinten-Johansen J, Muraki S, Guyton RA. Myocardial protection for off-pump coronary artery bypass surgery. Semin Thorac Cardiovasc Surg 2001;13:82–88.
10. Selye H, Bajusz E, Grassos S, Mendell P. Simple techniques for the surgical occlusion of coronary vessels in the rat. Angiology 1960;11:398–407.
11. Ikai I, Okuda M, Doliba N, Chance B. Visibility of ATP and ADP in freeze-trapped tissue from perfused rat liver during normoxia and ischemia using 31P-cryo-NMR. Biochim Biophys Acta 1991;1074:289–293.
12. Ingwall JS, Atkinson DE, Clarke K, Fetters JK. Energetic correlates of cardiac failure: changes in the creatine kinase system in the failing myocardium. Eur Heart J 1990;11[Suppl B]:108–115.
13. Hassinen I, Ito K, Nioka S, Chance B. Mechanism of fatty acid effect on myocardial oxygen consumption. A phosphorus NMR study. Biochim Biophys Acta 1990;1019:73–80.
14. Loster H, Keller T, Grommisch J, Grunder W. Effects of L-carnitine and its acetyl and propionyl esters on ATP and PCr levels of isolated rat hearts perfused without fatty acids and investigated by means of ^{31}P-NMR spectroscopy. Mol Cell Biochem 1999;200:93–102.
15. Yang WC, Geiger PJ, Besman SP. Formation of creatine phosphate from creatine and 32P-labelled ATP by isolated rabbit heart mitochondria. Biochem Biophys Res Commun 1977;76: 882–887.
16. Sharov VG, Afonskaya NI, Ruda MY, et al. Protection of ischemic myocardium by exogenous phosphocreatine (neoton): pharmacokinetics of phosphocreatine, reduction of infarct size, stabilization of sarcolemma of ischemic cardiomyocytes, and antithrombotic action. Biochem Med Metab Biol 1986;35: 101–114.
17. Kryzhanovskii SA, Kandelaki IN, Sharov VG, et al. Effect of exogenous phosphocreatine on the size of experimental myocardial infarction. Kardiologiia 1988;28:88–91.
18. Saks VA, Dzhaliashvili IV, Konorev EA, Strumia E. Molecular and cellular aspects of the cardioprotective mechanism of phosphocreatine. Biokhimiia 1992;57:1763–1784.
19. Kapelko VI, Kupriyanov VV, Novikova NA, et al. The cardiac contractile failure induced by chronic creatine and phosphocreatine deficiency. J Mol Cell Cardiol 1988;20:465–479.
20. Semenovsky ML, Shumakov VI, Sharov VG, et al. Protection of ischemic myocardium by exogenous phosphocreatine. II. Clinical, ultrastructural, and biochemical evaluations. J Thorac Cardiovasc Surg 1987;94:762–769.
21. Conway MA, Clark JF. Creatine and creatine phosphate: Scientific and clinical perspectives. San Diego, CA: Academic Press, Harcourt Brace & Company; 1996.
22. Clark JF. Creatine and phosphocreatine: A review of their use in exercise and sport. J Athletic Training 1997;32:45–51.