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REVIEW

Cyclocreatine protects against ischemic injury and enhances cardiac recovery during early reperfusion

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ABSTRACT

Introduction: A critical mechanism of how hypoxia/ischemia causes irreversible myocardial injury is through the exhaustion of adenosine triphosphate (ATP). Cyclocreatine (CCr) and its water-soluble salt Cyclocreatine-Phosphate (CCrP) are potent bioenergetic agents that preserve high levels of ATP during ischemia.

Areas covered: CCr and CCrP treatment prior to the onset of ischemia, preserved high levels of ATP in ischemic myocardium, reduced myocardial cell injury, exerted anti-inflammatory and anti-apoptotic activities, and restored contractile function during reperfusion in animal models of acute myocardial infarction (AMI), global cardiac arrest, cardiopulmonary bypass, and heart transplantation. Medline and Embase (1970 – Feb 2019), the WIPO databank (up to Feb 2019); no language restriction.

Expert opinion: This review provides the basis for a number of clinical applications of CCrP and CCr to minimize ischemic injury and necrosis. One strategy is to administer CCrP to AMI patients in the pre-hospital phase, as well as during, or after Percutaneous Coronary Intervention (PCI) procedure to potentially achieve protection of the myocardium, reduce infarcted-size, and, thus, limit the progression to heart failure. Another clinical applications are in predictable myocardial ischemia where pretreatment with CCrP would likely improve outcome and quality of life of patients who will undergo cardiopulmonary bypass for coronary revascularization and end-stage heart failure patients scheduled for heart transplantation.

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Cardioprotection; reversible ischemia; acute myocardial infarction; heart transplantation; cyclocreatine; cyclocreatine phosphate; ischemia-reperfusion injury; adenosine triphosphate; cardiac nourin; orphan drug designation

1. Introduction

1.1. Myocardial tissue injury

The myocardium is among the most highly sensitive tissues to ischemia and hypoperfusion. These conditions particularly affect the heart that is undergoing transplantation and this creates a substantial risk of morbidity and mortality in the postoperative period. An often overlooked but critical mechanism of how hypoxia/ischemia causes irreversible myocardial injury is through the exhaustion of the high-energy source ATP. To date, there have been no clinical or experimental proposals aimed at improving and/or preserving myocardial ATP levels in transplanted hearts. Thus, the development of a pharmacologic agent that has the ability to maintain and restore myocardial energetics would address a very important unmet need in cardiac transplantation.

In general, current therapies are focused on timely restoration of blood flow to the ischemic myocardium to enable the effective synthesis of ATP and to prevent a reduction in contractile function and irreversible injury [1–5]. Although, multiple proposed therapies to attenuate ischemia/reperfusion injury and improve contractile function have shown promise in early preclinical trials, none have proved to be beneficial in clinical settings.

Myocardial apoptosis and inflammation are the hallmarks of the tissue response to ischemia/reperfusion injury. Apoptosis is characterized by the initiation of a cascade of Caspase activation leading to intracellular proteolysis. Reduced ATP levels have adverse effects beyond just the immediate demand of the heart for energy in order for it to maintain its proper contractile function. Depletion of ATP during ischemia is one of the major factors that accelerates the apoptotic process of healthy myocardial tissue. Reduction of ATP and elevated levels of the inflammatory mediator tumor necrosis factor-α (TNF-α) are major contributors of myocardial apoptosis [6–8].

As described here in this review, ATP depletion of ischemic hearts resulted not only in tissue apoptosis, but also in the release of our newly identified cardiac-derived early inflammatory mediator, Nourin. In earlier publications, we referred to Nourin as cardiac-derived neutrophil chemotactic factor which is released by ischemic myocardium within 5 min of the onset of ischemia while heart muscles are still *alive* (i.e. sick tissue,

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Article highlights box

- Ischemia causes irreversible myocardial injury through the exhaustion of the high-energy source ATP.
- Reduced ATP levels during ischemia is associated with the release of the cardiac-derived inflammatory mediator, named Nourin, tissue inflammation, apoptosis, and reduction of contractile function.
- The administration of the bioenergetic agents, CCr and CCrP preserved high levels of myocardial ATP and reduced myocardial cell injury, circulating Nourin, tissue inflammation and apoptosis, as well as restored immediate contractile function.
- CCr and CCrP showed their cardioprotective properties in animal models of AMI, global cardiac arrest, cardiopulmonary bypass, and heart transplantation.
- CCrP can be administered to acute myocardial infarction patients in the *pre-hospital phase*, as well as *during*, or some hours *after* PCI procedure to potentially achieve protection of a greater amount of myocardium, reduce the infarcted scar size, and, thus, limit the deleterious remodeling that leads to heart failure.
- CCrP can be administered to *predictable* myocardial ischemia where pretreatment of surgical patients with CCrP would likely improve outcome and quality of life of patients who will undergo cardiopulmonary bypass for coronary revascularization and end-stage heart failure patients scheduled for heart transplantation procedure.
- The U.S. Food & Drug Administration (FDA) has awarded CCrP the Orphan Drug Status with the designation of: '*Prevention of Ischemic Injury to Enhance Cardiac Graft Recovery and Survival in Heart Transplantation*'.
- CCr can be administered *prophylactically* to likely protect against ischemia-induced heart damage in ischemic heart disease (IHD) patients and patients with high risk for cardiovascular diseases.

but not dead) and that treatment by the cardioprotective CCr and CCrP inhibited its formation and release [9–35].

The rapid release of Nourin by reversible ischemia is associated with marked cardiac inflammation during post-ischemic early reperfusion. As a potent inflammatory mediator, Nourin stimulates leukocyte chemotaxis (migration) and activates human monocytes, neutrophils and vascular endothelial cells to release of a number of cytokine and chemokine mediators, adhesion molecules, digestive enzymes and free radicals [12–14,16,18–30]. Specifically, Nourin activates human monocytes to release high levels of TNF-α, a key stimulant of apoptosis (20,24,27).

As reported in this review, CCr and CCrP treatment preserved high levels of myocardial ATP in ischemic myocardium, reduced circulating Nourin level, myocardial inflammation, apoptosis, acidosis, edema and tissue injury, as well as restored immediately contractile function at reperfusion [9–35]. Dog and rat models of regional and global myocardial ischemia were used to demonstrate the cardioprotective benefits of CCr and CCrP in models of: a) AMI, b) warm global cardiac arrest, c) cardiopulmonary bypass surgery for coronary revascularization, and d) heart transplantation models (in vivo rat syngeneic heterotopic cardiac transplant model, non-heart beating ex vivo heart preservation, and prolonged ex vivo heart preservation). We, therefore, believe that an effective therapeutic approach targeting the preservation of ATP in ischemic myocardium is likely to mitigate the impact of inflammation and apoptosis and help restore post-ischemic contractile function.

Figure 1 describes our proposed mechanism of action of the cardioprotective benefits of CCr and CCrP through the

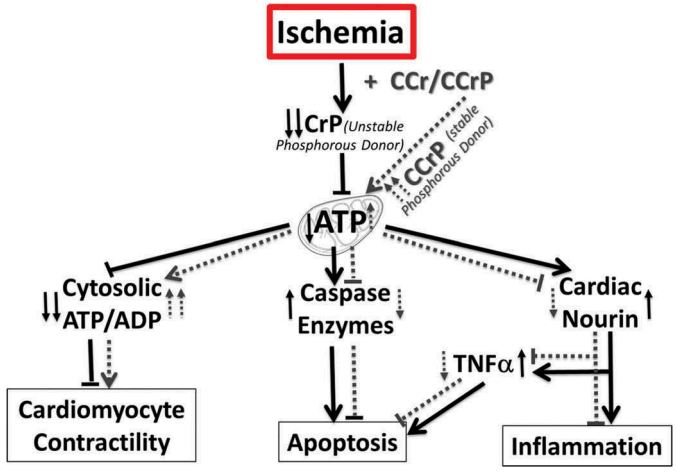

Figure 1. Proposed mechanism of action of cyclocreatine (CCr) and cyclocreatine phosphate (CCrP) [no need for 'Copyright Permission'].

preservation of high levels of myocardial ATP during *ischemia* and reduction of tissue injury, circulating Nourin, and acidosis. During *reperfusion*, CCr and CCrP will also reduce tissue inflammation, apoptosis, and edema resulting in immediate restoration of post-ischemic contractile function, without arrhythmias.

1.2. Contractile elements

The heart has the largest metabolic demands of ATP of any organ in the body. Although ATP is utilized primarily for contraction it also, to a lesser degree, is utilized for ionic homeostasis [4]. Because of its central role in cardiac metabolism and performance, abnormalities in ATP have been characterized in many animal models of hypertrophy and heart failure [3,36]. Therefore, an understanding of the vital role that ATP plays in the heart is critical for the development of new therapeutic options that help cardiomyocytes meet their ATP need to maintain contractile function and viability [37].

Under ischemic conditions, the heart alternatively shifts to anaerobic glycolysis for its requisite energy production, which unfortunately is quite inefficient. During ischemia, ATP levels decrease by 65% at 15 min and by 90% at 40 min [2]. Research has shown that contractile performance in vivo decreases precipitously and ceases when only *20% of ATP* and 75% of CrP are depleted [38].

A number of studies have established that the decline in ATP associated with ischemia could have many adverse consequences, including loss of ionic gradients resulting in a calcium overload and activation of endogenous phospholipases or proteases. Catabolites of lipid degradation may act as a *detergent* and damage cell membranes, leading to edema. Adenosine nucleotides and bases accumulate and might be a major source of free radicals via the xanthine oxidase reaction [39].

In summary, there is a need for a new therapy focused on reducing reperfusion injury to preserve myocardial cell viability for contractility since necrotic myocardium due to ischemic and reperfusion injury is replaced by fibrous scar tissue, which does not contribute to myocardial contractile function and that the degree of the impaired contractile function is

determined by the infarction scar size; large scars result in progressive chronic heart failure.

1.3. Cyclocreatine

Creatine (Cr) is the naturally occurring compound necessary for myocardial contractility. CCr is a synthetic analogue of Cr and it acts as a potent *bioenergetic* protective agent by preserving high levels of ATP in ischemic myocardium. In the heart, Cr and CCr are converted to CrP and CCrP, respectively, by the mitochondrial Creatine Kinase enzyme [40]. When CCr is administered to animals before ischemia, it gets stored in myocardial tissue as CCrP, while when CCrP is administered intravenously, it loses its phosphorous group in circulation and becomes CCr. In the heart, CCr is converted to CCrP and stored in the myocardium until an ischemic event, in which it will generate ATP by phosphorylating adenosine diphosphate (ADP).

During ischemia, the generation of ATP is through the CrP system (i.e. mitochondrial Creatine Kinase enzyme) as well as, glycolysis where the heart alternatively shifts to anaerobic glycolysis for its requisite energy production. Unfortunately, glycolysis is quite inefficient because ischemic heart catabolizes glucose and produces lactic acid. The generated tissue acidity results in a quick reduction of CrP function. On the other hand, CCrP is much more stable and is a superior long-acting phosphagen than CrP as it sustains ATP synthesis *longer* during ischemia by continuing phosphorylating ADP at low acidity.

We demonstrated that the administration of CCr and CCrP 30 to 60 min before the induction of myocardial ischemia resulted in the continuation of ATP synthesis during ischemia with over 85% preservation of myocardial ATP with and a loss of only 15% [9,10]. In a comparison study between CCrP and CrP using rat hearts exposed to hypothermic cardioplegic arrest, CCrP exerted significant cardioprotection while CrP failed [34]. CCrP also has a long intracellular half-life compared to CrP, because it does not follow the typical CrP metabolism pathway.

Independent studies by Walker JB et al. also reported that CCr is effective when administered prior to the induction of ischemia. In the hearts, massive amounts of CCrP are accumulated indicating phosphorylation and intracellular storage of CCr as CCrP [39,41–43]. Because CCrP is a long-acting phosphagen, it helps to sustain ATP levels longer during ischemia compared to controls containing CrP as the sole phosphagen. Walker JB et al. demonstrated that the long-term feeding of rats and chickens (up to 3 weeks) with 1% CCr significantly delayed the reduction of myocardial ATP and onset of rigor tension during cardiac ischemia. Upon reperfusion, the number of hearts recovering mechanical function was significantly higher in CCr-treated rats compared to controls. They showed that CCrP possesses a substantially less negative Gibbs standard free energy of hydrolysis than CrP and, therefore, it continues to thermodynamically buffer the adenylate system at lower pH values and lower cytosolic phosphorylation potentials that occur during the later stages of ischemia, conditions in which CrP is no longer effective.

Similarly, feeding of CCr to rats for 10 days delayed ATP depletion and the onset of ischemic contracture (rigor) in ischemic isolated hearts [39,41–43]. The reduction of rigor was associated with delayed development of acidosis and that CCr-treated hearts spontaneously defibrillated sooner during reperfusion compared to control hearts. It is interesting to note that similar to studies Walker JB et al. regarding spontaneously defibrillated hearts, we have reported that hearts of CCr-treated dogs in the coronary bypass model resumed spontaneous sinus rhythm shortly after release of the aortic cross-clamp (i.e. 1 to 2 min), while all control hearts required defibrillation within the first 10 min [34].

Osbakken M, et al. reported that the half-lives of ATP were 19 min for control and Cr-treated rats, while 37.5 min for CCr group [44]. One factor in the mechanism of protection by CCr may be the prolonged maintenance of phosphagen due to the higher equilibrium concentration of CCrP which in turn provides a substrate for continued synthesis of ATP during and after ischemia, thus defining CCr as a *bioenergetic* protective agent.

A major limitation of CCr, however, is that it is water insoluble and thus, all above reported studies were conducted by feeding CCr for an extended period of time *before* the induction of myocardial ischemia. Another limitation is that the commercially available CCrP was very unstable and expensive. To overcome these limitations, we used our recently patented procedure to prepare a water-soluble and stable CCrP [45]. In this new soluble preparation, CCr represents only 40% of CCrP and thus, the required effective dose was reduced by 40%, which will minimize any potential side effects. Furthermore, new clinical applications are now possible by administering soluble CCrP shortly *after* the onset of ischemia in AMI patients to save heart muscle, reduce infarction scare size and, thus, minimize the progression of patients to heart failure. We are currently testing this application in an animal of ischemia-induced heart failure.

In summary, to date there are no proven clinical options that directly address the preservation of ATP during tissue hypoxia and ischemia. Thus, the development of a pharmacologic agent that has the ability to maintain and restore myocardial energetics in the setting of ischemia would address a very important unmet need in the clinical care of patients with cardiac disorders. An effective therapeutic approach targeting the preservation of ATP in ischemic myocardium is likely to mitigate the impact of inflammation and apoptosis and to improve post-ischemic contractility function. Furthermore, using a blood test like the Nourin assay will be of a great benefit for early diagnosis of cardiac inflammation associated with ischemic injury.

This review describes the cardioprotective benefits of our compound CCr and CCrP and their significant improvement of post-ischemic contractile function by maintaining high levels of myocardial ATP during ischemia.

1.4. Current therapies for reducing ischemic injury

After an ischemic event, approximately 15% to 20% of the hypoperfused myocardial zone, previously perfused by the culprit coronary artery, undergoes necrosis within minutes, but it takes 3 to 4 h for the remaining 80% to 85% to progress from ischemic damage to permanent necrosis [46]. Timely reperfusion produces a greater amount of salvaged myocardium; but it is also a major component of reperfusion injury [46]. The greater clinical

emphasis on rapid reperfusion of ischemic myocardium opens a window of opportunity for new cardioprotective therapies to address the associated pathophysiology. Although many well-controlled experimental studies were reported, to date, there are no available pharmacologic therapies that effectively reduce reperfusion injury [47–51].

Inflammation is an important contributor to the pathogenesis of early and late myocardial reperfusion injury, and it also plays a key role in the healing process essential for cardiac repair and scar formation. Therefore, it is critical to achieve the right balance between limiting the early '*harmful*' inflammation in the first few minutes to hours after reperfusion and allowing the '*beneficial*' inflammation required for tissue repair.

The beneficial healing is largely mediated by neutrophils, and it starts 12 to 24 h after reperfusion and occurs again 3 days later. Neutrophil-derived oxygen free radicals are a major mechanism of reperfusion injury. Influx of macrophages starts 5 to 7 days after reperfusion, and they are responsible for removing the necrotic tissue and replacing it with fibroblasts.

Results of animal studies indicated a significant reduction of myocardial infarction size with therapeutic strategies designed to inhibit the early inflammatory process at the time of myocardial reperfusion using antibodies against cell-adhesion molecules (ICM-1) and the inhibition of complement activation; C5a [52,53]. However, corresponding clinical studies using this therapeutic approach have been largely negative [54–56]. Investigators also tested the gold standard anti-inflammatory agents, corticosteroids for their role in treating acute myocardial infarction. Although some studies have shown an increase in patients' survival in the first 3 days of hospitalization, concern was raised in other studies regarding the potential for corticosteroids to impair and retard healing after 3 days resulting in wall thinning and rupture [57,58].

Several experimental studies have reported that administering anti-apoptotic and anti-inflammatory agents are cardioprotective, however, these findings have been only partially confirmed in humans [59–67].

Similarly, although reperfusion strategies to reduce ischemic injury and increase survival have been successful experimentally, pharmacologic therapies to reduce reperfusion injury have failed to show clinical benefit [1,68–76].

A major limitation is the fact that the 'onset' of myocardial infarction is usually unclear in clinical settings, in extreme contrast to an animal model, with stuttering chest pain occasionally confusing the exact timing. Also, preceding episodes of ischemia may play a role in 'pre-conditioning' of the affected myocardial territory, allowing for better tolerance of the acute infarction [66,77]. Other factors that may limit the application of these new technologies to the real world include the time to reperfusion as relates to the onset of infarction. This is usually variable depending on the geography and the availability of resources (catheterization laboratory and interventional capability), despite clear guidelines for the management of acute myocardial infarctions. If new therapies are administered during or after reperfusion, then all these logistic limitations would apply to them as well [78–81].

Having available therapies like CCrP that targets ischemic injury, and can be administered expeditiously, even in the pre-hospital phase, can be extremely beneficial in limiting infarct size, and may be easily applicable to a large population of patients.

We are currently studying the use of CCrP as an anti-apoptotic and anti-inflammatory agent along with Cyclosporin H (CsH-Nourin specific competitive antagonist) as a potent anti-inflammatory to reduce *early* inflammation and control reperfusion injury without affecting crucial tissue repair and thus, reducing infarct size and minimizing deleterious remodeling [31,32].

1.5. Nourin, a cardiac-derived early inflammatory mediator

Nourin is a small peptide rapidly released within 5 min by myocardial tissue in response to *reversible ischemic injury before cardiac cell death*, as well as by necrotic cells when ischemia persists [12,13,17,19,20]. We demonstrated that the release of Nourin by well-preserved ischemic dog hearts during 1-h cardioplegic arrest, is associated with a neutrophil influx in the right and left ventricles after 2 h of reperfusion [12,13]. Nourin was purified from cardioplegic solutions collected during cardiac arrest from over 80 patients who underwent cardiopulmonary bypass surgery for coronary revascularization [12,21].

Chemically, Nourin is a 3 KDa formyl peptide confirmed by mass spectrometry analysis [19,20]. Since formylated peptides and formyl peptide receptors (FPR) are important potential therapeutic targets to control post-ischemic inflammation, we tested a variety of pharmacological antagonists for their capability to inhibit Nourin chemotactic activity both in vitro and in vivo [20,82]. We, therefore, developed six anti-inflammatory compounds to specifically inhibit the inflammatory response induced by the cardiac-derived Nourin.

Since the tissue-derived Nourin is an N-formyl peptide that works on leukocyte FPR similar to the bacterial product formyl Met-Leu-Phe (f-MLP), we have used several of the low molecular weight peptides which are known to block the effects of f-MLP. As listed below, blockers of the bacterial-derived f-MLP at the receptor level on leukocytes have also inhibited the inflammatory actions of Nourin in vitro and in vivo.

As indicated in Table 1, we demonstrated that the six *specific* inhibitors/antagonists of Nourin work through three different approaches including; local myocardial tissue, competitive antagonists for FPR on leukocytes and monoclonal antibodies in blood circulation [19–30,83–90]. Interestingly, we also identified a potent *endogenous inhibitor* of Nourin released by gastric tissues subjected to alcohol treatment [21].

As a potent inflammatory mediator, Table 2 describes the high levels of interleukin-8 (IL-8) (12,000 ng/ml), interleukin-1β (IL-1β) (400 pg/ml) and TNF-α (400 pg/ml) released by human monocytes in response to Nourin incubation for only 4 h. Stimulating high levels of TNF-α is detrimental since it is known to play a key role in cardiac apoptosis. It has been reported that when patients' serum TNF-α concentrations is in excess of 1 ng/ml, it is frequently predictive of a lethal outcome. Nourin stimulated the release of 0.4 ng/ml TNF-α by human monocytes when incubated for only 4 h (Table 2).

Table 1. Specific nourin antagonist and primary treatment approaches [no need for 'Copyright Permission'].

Treatment Approach	Local Anti-Inflammatory Activity to Myocardial Tissue	Competitive Antagonism of FPR on Leukocytes				Systemic Monoclonal Antibodies
Nourin Antagonists	**Cyclocreatine** A synthetic small molecule preserves high levels of myocardial ATP during ischemia and thus inhibits the intracellular formation of Nourin by ischemic heart tissue. As described in Figure 2, the administration of Cyclocreatine reduced circulating Nourin level resulting in inhibition of post-ischemic cardiac inflammation.	**Cyclosporine H** A "synthetic" small molecule works as a competitive antagonist for FPR on leukocytes and it is a member of a family of the "Generic" compound Cyclosporin A with well characterized biodistribution and safety. Unlike Cyclosporin A, which is an immunosuppressant, Cyclosporin H is a potent anti-inflammatory agent.	**FPR-17 aa loop peptide** A "natural" small molecule and it is Nourin's FPR soluble receptor fragment	**Spinorphine** An endogenous small molecule works as a competitive antagonist for FPR on leukocytes.	**t-Boc-Phe-D. Leu-Phe-D. Leu-Phe** A "synthetic" small molecule works as a competitive antagonist for FPR on leukocytes	**Anti-NOURIN I mAb** A murine monoclonal IgG antibody directed against Nourin blocked its leukocyte chemotactic activity and the release of IL-8 by leukocytes
References	18, 22,28	76-83	26-28			36,37,28

Table 2. Nourin stimulates the release of cytokines and chemokines (IL-8, IL-1β and TNF-α) by human monocytes after 4-hour incubation [19,20 our patents – no need for 'Copyright Permission'].

	Treatment	
Inflammatory Mediator	Nourin	Control Media
Interleukin-8 (ng/mL)	12,000	2,000
Interleukin-1β (pg/mL)	400	10
TNF-α (pg/mL)	400	<10

We have also shown that after 24-h incubation, Nourin stimulates: a) neutrophils to release IL-8 (5,500 ng/ml) and the adhesion molecule LECAM-1 (20 ng/ml) as well as Collagenase type IV, N-acetyl-B-glucosaminidase, Gelatinases and superoxide anion; and b) human aortic vascular endothelial cells (HAVEC) to release IL-8 (11,000 ng/ml) and the adhesion molecules ICAM-1 (2 ng/ml) and ELAM-1 (2.8 ng/ml) [19–30]. These results further indicate the important role that Nourin would play as a potent inflammatory mediator.

Activated neutrophils exert potent cytotoxic effects through the release of proteolytic enzymes, free radicles (oxidative stress), and adhesion molecules such as Intercellular Adhesion Molecule-1 (ICAM-1) [91–93]. The chronically sustained presence of inflammation/cytokines lead to myocyte phenotype transition and activation of matrix metalloproteinases, which in turn alters the local collagen composition and the integrins that constitute the interface between myocytes and the matrix. Thus, modulation of inflammation/cytokines through future therapies could promote improved healing and cardiac remodeling post-myocardial infarction.

Additionally, it has been reported that reperfusion of the infarcted areas is associated with the release of a number of inflammatory mediators including, TNF-α, IL-8, IL-1β, IL-6, and C5a activation. These mediators have a crucial role in recruiting and activating a large number of neutrophils into the ischemic and reperfused myocardium, resulting in the early '*no-reflow*' phenomenon following PCI [91–93]. Thus, individuals with an overactive and prolonged post-infarction inflammatory response might exhibit left ventricular dilatation and systolic dysfunction and, these patients might benefit from targeted anti-IL-1 or anti-TNF-α therapies [94–96].

In summary, since Nourin is rapidly released by local myocardial tissue following ischemia and contributes to the initiation and amplification of myocardial inflammation, it can be characterized as an *Alarmin* that promotes the innate immune response by stimulating the *initiation* and *amplification* of post-reperfusion myocardial inflammation. As such, Nourin can be an important therapeutic target as we demonstrated here using CCr and CCrP to control early and late post-reperfusion inflammation and injury. Furthermore, since leukocytes and inflammatory mediators play a role in reperfusion injury, a major therapeutic goal is to design anti-inflammatory strategies like Nourin inhibitors/antagonists aimed at *minimizing* post-reperfusion injury without interfering with crucial cardiac *repair* following myocardial infarction.

2. Cyclocreatine clinically relevant benefits

This section briefly describes various cardioprotective activities of CCr and CCrP.

2.1. A potent energy source

We demonstrated that when CCr was administered to dogs 60 min before the induction of myocardial ischemia (Left Anterior Descending (LAD) occlusion for 1 h), ATP synthesis continued during ischemia and its depletion was delayed resulting in over 85% preservation of ATP with a loss of only 15%; a crucial preservation since ATP depletion of more than 20% ceases contractility [38]. Control saline-treated hearts maintained only 66% of the ATP with a loss of 34%.

As described below, the observed preservation of ATP by CCr and CCrP treatment resulted in: (a) inhibition of intracellular formation of Nourin and the level of post-ischemic myocardial inflammation (Figure 2); (b) reduction in the level of Caspase enzyme and apoptosis (Figure 3); (c) *immediate full restoration* of strong contractile function during the 2-h reperfusion following 1 h of LAD occlusion in treated dogs (Figure 4) and reduction in myocardial cell injury (Figure 5), while contractile function in control saline-treated hearts were ceased completely and never recovered during reperfusion; (d) reduction in heart weight after 6 h of cold storage for heart transplantation (Figure 6); and (e) significant

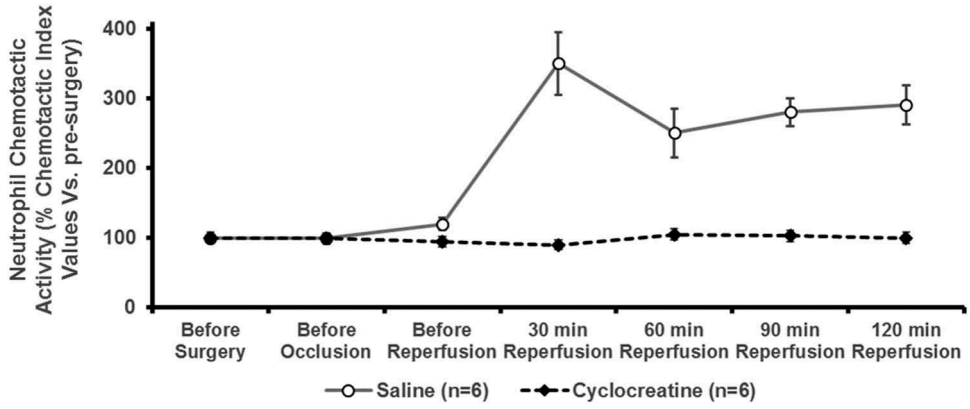

Figure 2. Cyclocreatine inhibits nourin levels in plasmas in the Intact AMI dog model (LAD occlusion for 1 h followed by reperfusion for 2 h) [11 'Copyright Permission' has been obtained].

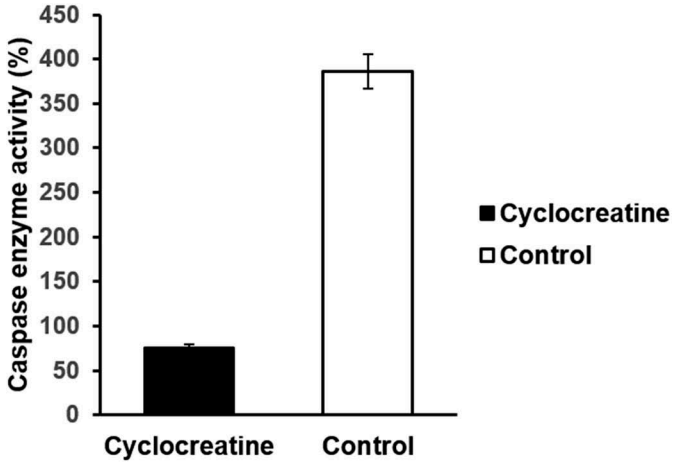

Figure 3. Cyclocreatine reduces apoptotic enzyme activity in the non-heart beating dog model of heart transplantation. Dog hearts underwent 1 h of global warm ischemic arrest then hearts were explanted and perfused ex vivo for an additional 4 h with a cold lactated ringers solution containing cyclocreatine while control hearts received cold-lactated ringers solution alone [21 our patent – no need for 'Copyright Permission'].

protection of donor hearts against prolonged ischemic injury during harvesting and 22 h of cold storage resulting in fast recovery and an increase in graft survival (Table 3).

2.2. Anti-inflammatory activity

The release of the potent inflammatory mediator Nourin by the myocardium in response to ischemic injury suggests a role of *local tissue* in the pathogenesis of post-ischemic cardiac inflammation.

To determine whether the release of Nourin is ischemia-dependent, we identified the CCr analogue, CCr as a compound that can modify the extent of ischemic injury by maintaining a high level of the energy source ATP. We tested the hypothesis that if ischemia is important for the formation and release of Nourin by myocardial tissue, then blocking (or delaying) ischemic changes by CCr should inhibit the release of Nourin. Using rabbit and dog models of ischemia/reperfusion, we demonstrated that the pre-administration of CCr before the induction of ischemia resulted in the preservation of high levels of myocardial ATP and significantly reduced the level of circulating Nourin and cardiac inflammation. As described in Figure 2, the pre-administration of CCr intravenously 60 min before ligating the LAD coronary artery for 1 h followed by reperfusion for 2 h, resulted in a significant reduction of the levels of Nourin detected in dog plasma collected 30 min after reperfusion and for an additional 2 h. Marked reduction of neutrophil infiltration into ischemic myocardium was evident after the 2-h reperfusion [10,11,21]. Similarly, in a separate study using rabbits, the pre-administration of CCr intravenously 60 min before the removal of hearts, significantly reduced the level of Nourin released in the perfusate of isolated ischemic rabbit hearts compared to control hearts treated with saline.

2.3. Anti-apoptotic activity

2.3.1. Non-beating donor heart – preservation of hearts during 4-h storage for transplantation

Most hearts for transplantation are procured from brain-dead, cadaveric donors. In these standard circumstances, hearts are quickly arrested and the period of warm myocardial ischemia is kept to a minimum. A non-beating donor heart is a novel, potential new source of hearts for transplantation where the patient's ventilator support is discontinued and the heart develops asystole as a result of hypoxia. For this strategy to be clinically feasible for clinical heart transplantation, the warm ischemic injury that inevitably occurs to the myocardium while waiting for the heart-beat to stop would need to be reversed or mitigated. Improvements in the current cardioprotective agents are clearly needed for this approach to have an impact on clinical heart transplantation.

We tested the effectiveness of CCr pretreatment at improving the quality of hearts from a non-heart beating donor in a standard dog model. CCr was injected intravenously into a dog 1 h before the induction of global warm ischemia induced by exsanguination. Control dogs (n = 3) received saline prior to exsanguination. After the blood pressure was documented at <10 mm Hg, dogs underwent 1 h of the global warm ischemic arrest. After global ischemia, hearts were explanted, and then perfused ex vivo for an additional 4 h with a cold lactated ringers

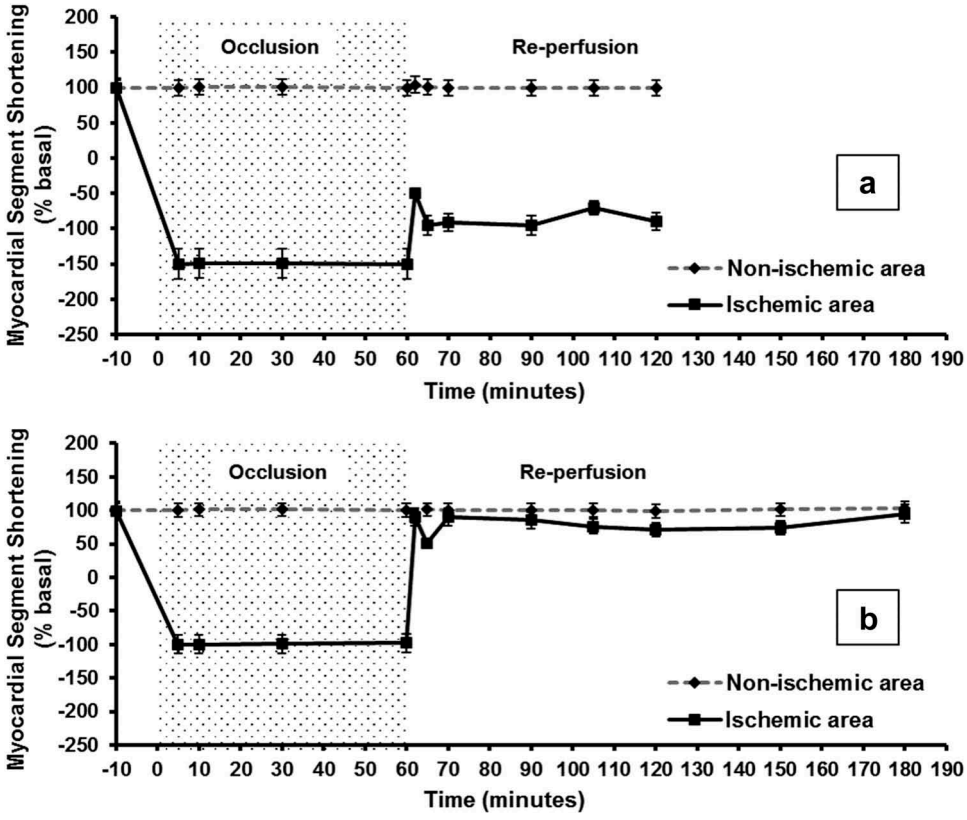

Figure 4. Cyclocreatine (B) restores heart contractile function compared to control saline (A) in the intact AMI dog model (LAD occlusion for 1 h followed by reperfusion for 2 h) [9 our patent – no need for 'Copyright Permission'].

solution containing 1% CCr (20 gm in 2 l) while control hearts received cold-lactated ringers alone.

To simulate transplantation, hearts were placed on the Langendorff working-heart model and perfused with oxygenated blood so that contractile function could be measured. Regional myocardial tissue pH was measured continuously using a pH probe implanted into the anterior and posterior myocardium. Serial biopsies were also taken from these same regions for a variety of biochemical analyzes. Myocardial MRI was obtained on the ex vivo perfused heart [21].

After exsanguination to induce global warm ischemia, the heart of the CCr-treated dog took 9 min to stop beating and develop asystole. In contrast, control hearts completely stopped beating after an average of only 2 min. Similarly, the myocardium of the CCr dog maintained a tissue pH of 7.04 ± 0.1 during the warm ischemia period of 1 h and throughout the ex vivo perfusion interval, which was close to its baseline level of 7.11. On the other hand, tissue pH in control hearts fell to a nadir of 6.00 ± 0.25 during the induction of warm ischemia and never returned back to baseline levels during the ex vivo preservation period [21].

Additional biochemical and functional analyses were obtained to assess the capability of CCr solution to preserve the heart during the prolonged 4-h cold perfusion. When compared to controls, CCr treatment demonstrated the following results:

(1) Three-fold increase of myocardial ATP content compared to controls,
(2) Reduced intracellular edema compared to control as measured by diffusion-weighted imaging on MRI,
(3) Reduced myocardial tissue lactic acidosis compared to control as measured by spectroscopic imaging on MRI,
(4) Reduced level of the cell injury marker Malondialdehyde compared to controls,
(5) Significant reduction in apoptosis in CCr heart compared to controls as measured by Caspase enzyme activity. Figure 3 describes the reduction of the Caspase enzyme activities in the CCr group (25% reduction of baseline) compared to the significant stimulation observed in control dogs (3.86-fold increase over baseline). Interestingly, the significant reduction of Caspase activities in the CCr group indicates that the enzymes are present more in the 'inactive proenzyme' forms [21].

2.4. Restoration of post-ischemic contractile function by CCr and CCrP

We used five different animal models of ischemia-reperfusion injury to demonstrate the *consistent* and *immediate* restoration of post-ischemic contractile function by the treatment of CCr and CCrP compared to buffer controls. The administration of CCr and CCrP to dogs and rats minutes before the induction of regional and global myocardial ischemia, significantly pro-

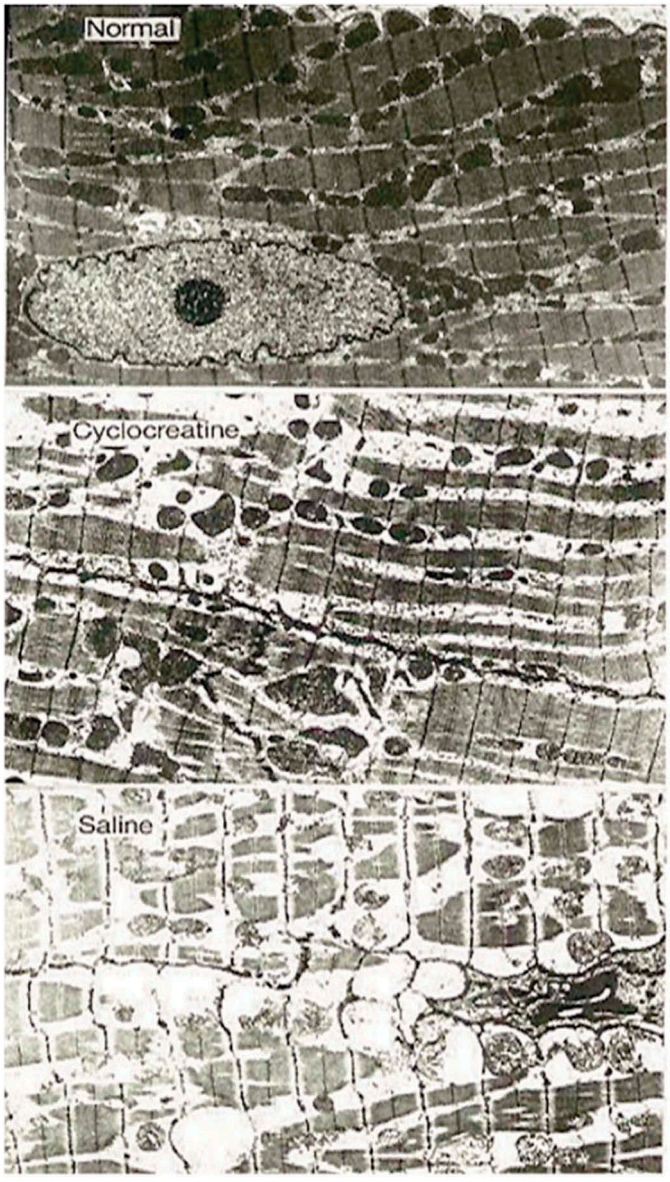

Figure 5. Cyclocreatine significantly reduces myocardial cell injury in the intact AMI dog model (LAD occlusion for 1 h followed by reperfusion for 2 h) [9 our patent – no need for 'Copyright Permission'].

tected the hearts against ischemic injury and restored immediate contractile function during early reperfusion.

2.4.1. Regional warm ischemia of AMI – intact dog model

Using a canine model of AMI, we demonstrated that intravenous injection of CCr (500 mg/kg) 60 min before occluding the LAD for 1 h preserved 85% of pre-ischemic ATP level (loss of only 15%) and 97% of the CrP (loss of 3%) during ischemia, as well as *immediately* restored over 80% of contractile function during the 2 h of reperfusion (Figure 4) [10,21]. On the contrary, control saline-treated hearts showed 66% of pre-ischemic ATP level (loss of 34%) and 18% of the CrP (loss of 83%) and their contractile function were ceased completely after LAD occlusion and never recovered during reperfusion. Histologically, the CCr-treated hearts showed markedly less myocardial cell injury when compared to the control (saline) group (Figure 5) [10,21]. Eosinophilic changes and patches of contraction bands associated with ischemia were evident in control hearts. CCr-treated hearts, on the other hand, showed only occasional small foci of contraction bands and no significant eosinophilic changes [10,21]. These studies indicate that the short-term administration of CCr is safe for hearts in the AMI intact dog model. Similar safety results were obtained using the intact dog model of cardiopulmonary bypass surgery.

2.4.2. Global warm cardiac arrest – isolated rat heart model

We tested whether CCrP administered before the induction of global warm cardiac arrest is an important cardioprotective agent. For this study, rats were injected intravenously with 1 ml saline or CCrP at 500 mg/kg (containing only 300 mg CCr) 1 hour before the induction of global warm ischemia by cross-clamping the aorta for 7, 9, and 10 min. Results indicate that when CCr dose was reduced to half in the soluble CCrP preparation (from 500 mg/kg to 300 mg/kg), CCrP treatment continues to significantly improve the contractile functional recovery after prolonged global-warm cardiac arrest (40% to 78%

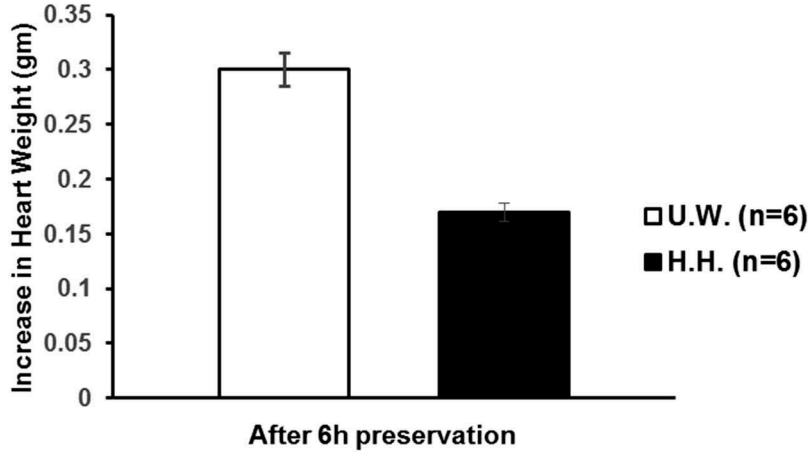

Figure 6. Cyclocreatine phosphate (CCrP) reduces heart weight after 6 h of cold storage in HH solution (UW + CCrP) compared to control (UW) [35 'Copyright Permission' has been obtained].

Table 3. Cyclocreatine phosphate protects rat grafted hearts against ischemic injury during harvesting and prolonged cold storage for 22 h and 24 h (saline = 5 and CCrP = 6) [no need for 'Copyright Permission'].

				ECHO Analyzes		
Treatment	Rat #	Incubation Time (hours)	Dose (g/kg)	Wall Thickness and Left Ventricular Mass	Heart Beating Score	Potential Graft Survival
Saline	1	22	–	Loss	1+	Poor
	2	24	–	Partial loss	1+ – 2+	Poor
	3	24	–	Loss	1+ – 2+	Poor
	4	22	–	Loss	1+	Very Poor
	5	22	–	Loss	1+	Poor
CCrP	6	22	1.5	Preservation	3+	Excellent
	7	22	1.2	Preservation	4+	Excellent
	8	24	0.5	Partial Preservation	2+	Average
	9	24	0.5	Preservation	3+	Very Good
	10	22	0.8	Preservation	4+	Excellent
	11	22	0.8	Partial Preservation	2+	Very Good

baseline) compared to controls (13% to 50% baseline) [21,33].

2.4.3. Dog cardioplegic arrest for cardiopulmonary bypass surgery – intact dog model

In an intact canine model of cold cardioplegic arrest, adult mongrel dogs underwent aortic cross-clamping for 1 h and 3 h followed by reperfusion on bypass for 45 min and then off bypass for 4 h. Dogs were injected intravenously with saline or CCr (500 mg/kg) for 1 h before initiating the experiment. Post-bypass cardiac output was significantly better in CCr-treated hearts compared to that of controls [21,33,34]. For the 1-h cardiac arrest, CCr-treated hearts achieved over 90% of the baseline function throughout the 4 h of reperfusion while control hearts achieved only 60% of the baseline function [21,33,34]. For the 3-h cardiac arrest, CCr-treated hearts achieved 91% of the baseline function during the 3-h reperfusion, while control hearts showed weak contractility and stopped beating after 2 h [21,33,34].

2.4.4. Rat cardioplegic arrest for cardiopulmonary bypass surgery – rat heart model

We tested the cardioprotective effect of CCrP using rat models of hypothermic (2.5 h at 22°C) and normothermic (40 min at 37°C) cardioplegic arrest [34].

2.4.4.1. Hypothermic cardioplegic arrest for 2.5 h at 22°c.

Results indicate that the recovery of contractile function measured as Aortic Flow, in the Langendorff working-heart mode for 30 min was significantly better in the CCrP-treated group (68% to 80% baseline) compared to that of control hearts (30% to 40% baseline). We also tested the cardioprotective effect of CCr at three doses, 600 mg/kg, 300 mg/kg and 150 mg/kg. Similar cardioprotection was observed when CCr was used at 600 and 300 mg/kg. No cardioprotection was observed at the low dose of 150 mg/kg. Similar results were obtained if CCr was injected 30 min or 60 min before the induction of ischemia [34].

2.4.4.2. Normothermic cardioplegic arrest for 40 min at 37°c.

Similar findings were observed in the normothermic cardioplegic arrest [34].

2.4.5. Heart transplantation

We tested the cardioprotective effect of CCr and CCrP on the following in vivo and ex vivo models of heart transplantation and preservation:

2.4.5.1. In vivo rat syngeneic abdominal heterotopic heart transplantation.

Currently, approximately 7 hearts from every 10 organ donors go unutilized, in part because of lack of current protocols to protect the heart from ischemic injury during harvesting and to adequately preserve the organ during the prolonged cold storage [91–93]. To date, there have been no clinical or experimental proposals that successfully improved and/or preserved myocardial ATP levels in transplanted hearts. Thus, the development of a pharmacologic agent that has the ability to maintain and restore myocardial energetics would address a very important unmet need in cardiac transplantation. Furthermore, frequent heart biopsy is currently used to screen for rejection. Recent efforts are made to develop non-invasive blood tests using angiogenesis factors as biomarkers of allograft vasculopathy in cardiac transplant recipients [94]. We believe that the Nourin blood test can monitor the levels of Nourin in heart transplant patients as a predictor of cardiac inflammation. Early detection of cardiac inflammation will permit crucial clinical intervention to avoid losing the graft.

We demonstrated the in vivo cardioprotective benefits of CCrP in the standard rat model of abdominal heterotopic heart transplantation by increasing the survival of grafted hearts compared to control hearts when transplanted into recipient rats for 3 days. Donor rats were injected IV with CCrP (4 doses) immediately before removing the hearts. CCrP-treated donor hearts were flushed with University of Wisconsin (UW) solution containing CCrP and then hearts were incubated in the same CCrP/UW solution for 22 h and 24 h at cold temperature. Prior to their surgical transplantation in recipient rats, donor hearts were flushed with CCrP/saline solution to provide additional heart protection during the surgical procedure. Control rats were injected IV with saline before removing and incubating the hearts in UW solution alone for 22 hours and 24 h. Before transplantation, donor hearts were flushed with saline then surgical transplanted in recipient rats. CCrP and control heart grafts were allowed to survive for 3 days. The IV injection of CCrP to donor rats right before the removal of the hearts resulted in the protection of

donor hearts from ischemic injury during the harvesting as well as during the prolonged cold storage for 22 h and 24 h in UW solution containing CCrP. The CCrP treatment also increased the survival of the grafted hearts in recipient rats for 3 days.

Lewis rats were used for both the donors and recipients to avoid immunologic rejection. This approach allowed us to focus at determining the in-vivo cardioprotective benefits of CCrP treatment and to evaluate whether CCrP would restore cardiac contractility after prolonged cold storage and increase graft survival.

A total of 13 grafted rat hearts were conducted as follows: a) Saline-treated group (n = 7) and b) CCrP-treated group (n = 6). Lewis rats were used for donors and recipients to avoid immunologic rejection. Donor rats were injected IV with CCrP or saline while recipient rats did not receive any treatments. One important change that we made to facilitate future CCrP's use in clinics is that we eliminated the waiting time of 1 to 2 h after the IV injection of CCrP before removing the hearts. In the present protocol, we removed donor hearts immediately after CCrP injection and the hearts were stored at UW solution containing CCrP for 22 h and 24 h before transplantation.

For the control group (n = 7), donor rats were injected intravenously with 1 ml saline.

For the CCrP group (n = 6), rats received 1 ml CCrP solution prepared in saline at doses of .5 gm/kg, .8 gm/kg, 1.2 gm/kg and 1.5 gm/kg. Saline donor hearts were incubated in 40 ml of cold UW solution for 22 h and 24 h. For the CCrP-treated hearts, 800 mg CCrP were added to the 40 ml UW solution. After the prolonged cold storage, donor hearts (saline & CCrP) were transplanted into Lewis recipient rats and allowed to survive for 3 days.

Grafted rat hearts were examined using photos and videos taken within the first 2 min after transplantation at day 0 and then after 3 days before sacrifice. Echocardiography (ECHO) analyzes were also conducted 2 h after transplantation at day 0 and at day 3 before sacrifice. All 13 rat-grafted hearts (photos, videos, and ECHO) were evaluated *BLINDLY* by two Echocardiographer Cardiology specialists without the knowledge of the rat treatment. The study ECHO's evaluation and conclusions were confirmed by an independent cardiologist expert in rat ECHO.

As summarized in Table 3, the IV injection of CCrP at doses .5 gm/kg, .8 gm/kg, 1.2 gm/kg and 1.5 gm/kg prior to the removal of donor hearts, protected the hearts from ischemic injury during harvesting and the prolonged 22 h and 24 h of cold storage. CCrP treatment showed significant cardioprotection against early reperfusion injury after transplantation as illustrated by the *absence of delayed heart function in the first 1 min* and the restoration of strong contractile function in all CCrP-treated hearts minutes after transplantation. In the contrary, saline-treated control donor grafts showed a slow start of a heart beating with weaker contractile function.

CCrP-treated hearts at doses of .8 gm/kg, 1.2 gm/kg and 1.5 gm/kg showed strong beating scores of 4+ and 3+, respectively, at both day 0 and day 3 (score of +4 is the highest). However, the low dose of .5 gm/kg, showed strong heart beating scores of 4+ and 3+ right after transplantation at day 0 but partial myocardial protection by day 3 with beating scores ranged from 2+ to 3 + . Saline-treated control donor rats were evidently dilated with an increase in the sizes of both ventricles and atria. Additionally, the color was mildly cyanotic and the contractility was poor and irregular in rhythm. In most control grafted hearts, their heart beating score ranged from 1+ to 2+ at day 0 and day 3.

In general, CCrP grafted hearts after 22 h and 24 h of incubation had *good preservation of myocardial color and perfusion as well* contractile function as indicated by *preservation of the myocardial wall thickness and mass* compared to control saline grafted hearts.

We, therefore, demonstrated the ability of CCrP to exert in vivo cardioprotective benefits and to extend the survival of grafted hearts compared to control grafts after prolonged 22 to 24 h of cold storage. This is a significant benefit of this compound.

As also indicated in Table 3, this protection was shown in most of CCrP grafted hearts at day 3 where the myocardial color and the consistency of the degree of contractility were almost the same as day zero. Additionally, the day 3 ECHO showed the continued *preservation of the myocardial wall thickness and mass* which are the main criteria that determine the degree of myocardial ischemia over a period of time. Most the control grafted hearts, on the other hand, continued to show evidence of ischemia as well as *loss of the wall thickness and the cardiac mass* by day 0 and day 3.

The general overall survival of the cardiac tissue of 'CCrP-grafted hearts' was very good to excellent, while the general overall survival of the cardiac tissue of 'control-grafted hearts' was poor. Based on these preclinical efficacy studies, the U.S. FDA has awarded the Orphan Drug Designation (ODD) status for CCrP for the: '*Prevention of Ischemic Injury to Enhance Cardiac Graft Recovery and Survival in Heart Transplantation*'. As requested by the FDA, we are currently preparing to conduct animal safety studies using a Good Laboratory Practice (GLP) preparation of CCrP. Results of CCrP safety studies will be submitted as an Investigational New Drug (IND) application to FDA to initiate Phase I study on end-stage heart failure patients. The second clinical application is to administer CCrP shortly *after* the onset of ischemia in AMI patients to save heart muscle and reduce the progression to heart failure.

2.4.5.2. Ex vivo rat heart preservation during prolonged 6-h storage for transplantation.

In the rat heart preservation model, CCrP was given 2 h before removal of the heart. Hearts were removed and stored at standard UW solution alone (control) and in UW solution with added CCrP (HH). Both CCrP (HH) hearts and control (UW) hearts were stored at 4°C for 6 h, then were tested for the recovery of contractile function. Results indicated that the recovery of contractile function was significantly better in the CCrP treated-group (HH) compared to saline controls [35]. Furthermore, there was a higher weight gain in control hearts (UW) after 6 h of cold storage compared to the CCrP-treated hearts (HH) [35]. As indicated in Figure 6, CCrP-treated hearts (HH) weighted only 0.25 gm while control hearts (UW) weighed 0.31 gm. The observed reduction of heart edema in the CCrP hearts (HH) is crucial for the restoration of contractile function during reperfusion at the end of 6-h storage.

2.4.5.3. Ex vivo dog non-heartbeating donor – heart preservation for transplantation. The dog model is described above under the anti-apoptotic activity of CCr. For this study, CCr-treated heart continued to show strong contractile function throughout the one-hour period of analyzes, while the control hearts showed contractile function only during the first 15–20 min then the contractility declined gradually and ceased [21].

2.5. A cardioprotective comparison between CCr/CCrP and Cr/CrP

We conducted a number of studies to compare the beneficial effects of CCr and CCrP to the natural product Cr and its salt CrP on preserving ischemic hearts. In general, the administration of CCr and CCrP showed constant superiority over Cr and CrP in protecting hearts against ischemic injury as follows:

2.5.1. Release of cardiac nourin

Creatine treatment 30 min before removing rabbit hearts showed a marked increase (double) in the level of Nourin in the perfusate compared to rabbits treated with saline, suggesting a toxic effect of excessive Creatine on myocardial tissues [15]. On the contrary, CCr treatment completely inhibited the release of Nourin by ischemic hearts perfused or incubated in the buffer for 2 h at room temperature [15].

2.5.2. Myocardial levels of ATP & CrP

CCr treatment 30 min before removing rabbit hearts preserved high levels of ATP (39%) and CRP (30%) by the end of the 2-h perfusion at room temperature. On the other hand, hearts isolated from CCr-treated and control buffer-treated rabbits, showed loss of over 95% of both ATP and CrP with a preservation of only 5% [15].

2.5.3. Restoration of contractile function

Extensive work on myocardial preservation by CrP was published between 1980 and 1995, primarily by Saks VA in Russia and Hearse DJ in the United Kingdom [95–99]. We have demonstrated that intravenous injection of rats with CCrP and CrP before removing the hearts and subjecting them in vitro to normothermic arrest for 40 min at 37°C significantly improved the recovery of contractile function compared to control saline-treated hearts [100]. However, only CCrP treatment to rat hearts continued to exert restoration of contractile function when hearts were exposed to hypothermic arrest for 2.5 h at 22°C. CrP failed to exert any cardioprotection under hypothermic conditions (2.5 h at 22°C) [34]. Results of this study demonstrate the superiority of CCrP over CrP in protecting hearts against ischemic injury.

3. Conclusions

Cardiovascular events are the leading causes of death in the world and AMI is one of the most lethal diseases society faces. Therefore, developing strategies aimed at minimizing myocardial necrosis, and optimizing cardiac repair following myocardial infarction, is one of the major therapeutic goals of modern cardiology. We believe that the administration of CCrP in *predictable* myocardial ischemia will likely improve outcome and quality of life of patients who will undergo surgical procedures including, cardiopulmonary bypass for coronary revascularization and end-stage heart failure patients scheduled for heart transplantation. Similarly, the administration of CCrP in the *pre-hospital phase*, as well as *during*, or shortly *after* PCI procedure will potentially provide a crucial therapeutic window to allow a cardioprotective intervention that can be beneficial in limiting infarct size. Additionally, CCr can be administered *prophylactically* to likely protect IHD patients and high-risk patients against ischemic damage.

Current pharmacologic and mechanical revascularization have been successful to ensure early reperfusion to reduce ischemic injury and to increase survival. However, the first few minutes of reperfusion constitute a critical phase of reperfusion injury, and pharmacologic therapies to reduce this injury so far have not been so successful. Also critical is energy depletion of ATP, which occurs during myocardial infarction and in the next 3- to 4-h period after reperfusion leading to apoptosis and progression to necrosis and heart failure.

The current available options for addressing myocardial ischemia injury are all directed at restoring tissue perfusion in the myocardium. However, mechanism of myocardial sensitivity to hypoxia/ischemia is through the exhaustion of the high-energy source ATP that leads to tissue inflammation, apoptosis, contractile dysfunction, and irreversible injury. To address this very important unmet need in the clinical care, we developed the pharmacologic agent CCr and CCrP as the potent preserver of myocardial ATP, and thus, maintained and restored myocardial energetics in the setting of ischemia and reperfusion.

Myocardial reperfusion injury may account for up to 50% of the final myocardial infarct size, and the lack of effective pharmacologic therapies remains a challenge. Large infarct size, and ventricular remodeling also contributes to reduced ventricular function and development of heart failure. This represents a significant socioeconomic burden on health-care systems. Therefore, a new therapy aimed at minimizing reperfusion injury to preserve contractile function is crucial. The ideal therapy would promote effective tissue repair and reduce the deleterious remodeling that leads to heart failure.

Using animal models of ischemia/reperfusion in dogs, rats and rabbits, our data demonstrated that the treatment by CCr and CCrP exerted cardioprotection against ischemic-reperfusion injury. Specifically, we demonstrated that initiating treatments 30 to 60 min before the induction of ischemia preserved high levels of ATP in ischemic myocardium and reduced tissue injury, acidosis, edema, inflammation, and apoptosis. This cardioprotection resulted in immediate restoration of strong post-ischemic contractile function during perfusion. The models of ischemia reperfusion studied include AMI, global warm cardiac arrest, cardiopulmonary bypass for coronary revascularization and heart transplantation models (prolonged heart preservation and non-heartbeating donor hearts). Furthermore, in an intact canine model of cardiopulmonary bypass, CCr-treated hearts resumed spontaneous contractility without defibrillation during early reperfusion compared to control-hearts, which required defibrillation within the first 10 min.

Microvascular dysfunction occurs in a substantial proportion of AMI patients (more than 30%) despite aggressive therapy with thrombolytic agents and/or revascularization techniques. Patients with impaired microvascular perfusion after immediate reperfusion therapy have a poor prognosis. Leukocyte influx and edema during early reperfusion have been proposed as major contributors for microvascular obstruction ('no-reflow' phenomenon). Currently, there is no effective therapy to reduce microvascular obstruction in patients who have undergone PCI procedure.

As reported here, CCr treatment preserved high levels of ATP and reduced both tissue injury and the formation/release of Nourin by ischemic myocardium. The reduction of circulating Nourin was associated with a reduction of early myocardial inflammation and edema. Therefore, it is likely that the observed reduction in cardiac inflammation resulted in: (a) less leukocyte influx and plugging of the capillaries and (b) less edema and compression, leading to less microcirculation obstruction as evident by the immediate restoration of contractile function during reperfusion. Thus, CCr as an anti-inflammatory/anti-apoptotic agent warrants further development as a new pharmacologic therapy aimed at protecting the microvascular network from obstruction and reducing the deleterious remodeling which results in the progression of AMI patients to heart failure.

Current challenges for end-stage heart failure patients scheduled for transplantation surgery are the low utilization of donor hearts (7 out of 10 donor hearts) because of the 4-h limit of transportation time from donor site to recipient; lack of protection from ischemic injury during donor heart harvesting; and lack of adequate preservation during transportation beyond 4 h. Some of the clinical impacts of using the cardioprotective CCrP are: (a) expand the transportation time beyond 6 h and, thus, increase utilization of donor hearts; (b) faster and better heart recovery after transplantation surgery; (c) improve survival time of heart grafts and patients; (d) reduce length of hospital stay and save money; and (e) improve patients' outcomes and quality of life.

Improvement of heart protection and recovery has been a target of research since the first patient that received a heart transplant in 1967. Poor cardiac output leading to shock after cardiac transplantation (i.e. primary graft failure) has been the leading cause of postoperative death and major morbidity within the first 30 days, even for donor hearts classified as 'optimal'. The most common reason for this problem is poor preservation of the donor heart during the ex vivo transport period; the 2–6 h. period the heart is outside the body and stored on ice.

With the long track record of failure in this line of investigation, it is important to emphasize the potentially novel advantages of CCrP as a potent supplier of myocardial energy during ischemia (harvesting and transportation). The depletion of ATP during cold storage is the final common pathway that triggers myocardial injury, acidosis, edema, inflammation, apoptosis or any other cause of ischemia-reperfusion injury post-transplantation.

Without ATP depletion in donor heart tissues, there would be few pathogenic mechanisms able to cause poor cardiac recovery. Our preclinical data show that a single dose of CCr to donor dog has been very effective at restoring donor heart high-energy phosphates in a challenging transplant model. Similarly, we demonstrated that a single dose of CCrP is a potent cardioprotective drug which showed strong recovery of contractile function immediately after transplantation which was persistent for an additional 3 days compared to the weak control hearts stored at current standard UW solution. Based on this preclinical efficacy study, the U.S. FDA has awarded the ODD status for CCrP with the unique designation of: '*Prevention of Ischemic Injury to Enhance Cardiac Graft Recovery and Survival in Heart Transplantation*'. Currently, we are preparing an IND application to be submitted to the FDA to initiate Phase I study on end-stage heart failure patients.

Furthermore, since frequent heart biopsy is currently used to screen for myocardial inflammation and rejection in heart transplantation patients, recent efforts are made to develop non-invasive blood tests using angiogenesis factors as biomarkers of allograft vasculopathy in cardiac transplant recipients. We believe that the non-invasive Nourin blood test can monitor the levels of Nourin in heart transplant patients as a predictor of cardiac inflammation. Early detection of cardiac inflammation will permit crucial clinical intervention to avoid losing the graft.

Thus, an effective therapeutic approach targeting preservation of ATP in ischemic myocardium is likely to mitigate the impact of inflammation and apoptosis and to improve post-ischemic contractile function and that the use of the early biomarker, Nourin to monitor the health of transplanted hearts, will be of a great benefit to predict cardiac recovery and therapy response.

4. Expert opinion

We demonstrated that preservation of ischemic myocardial ATP by CCr and CCrP treatment resulted in a reduction of tissue injury, acidosis, edema, inflammation, and apoptosis leading to immediate recovery of strong contractile function during post-ischemic perfusion. Cardioprotection by the administration of CCr and CCrP were shown in animal models of AMI, global cardiac arrest, cardiopulmonary bypass, and heart transplantation. Therefore, there are three possible clinical scenarios for CCr and CCrP. *first*, where myocardial ischemia is *predictable* and pretreatment of patients with CCrP would be possible, such as patients undergoing cardiopulmonary bypass surgery for coronary revascularization and end-stage heart failure patients scheduled for heart transplantation. The U.S. FDA has recently awarded the ODD status for CCrP with the unique designation of: '*Prevention of Ischemic Injury to Enhance Cardiac Graft Recovery and Survival in Heart Transplantation*'.

Second, another clinical scenario for CCrP treatment is for AMI patients in the *pre-hospital phase*, as well as *during*, or some hours *after* PCI procedure which will potentially provide a crucial therapeutic window to allow a cardioprotective intervention that can be beneficial in limiting myocardial damage. Early treatment with CCrP during the transfer of suspect to the hospital then administrating CCrP intracoronary during reperfusion will likely protect the adequacy of microcirculations and achieve protection of a greater amount of myocardium, reduce infarct size, and, thus, limit the progression to permanent damage. Myocardial protection by CCrP within the first crucial 3- to 4-h period is particularly critical for patients with long transport times to the hospital and for patients who cannot get timely PCI. Protecting AMI patients against

ischemic injury by CCrP will likely reduce the incidence of chronic heart failure and improve patients' outcome and quality of life. *Third*, CCr can be administered *prophylactically* to patients with IHD and patients at high risk for cardiovascular diseases to potentially protect against ischemia-induced heart damage.

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Declaration of interest

SA Elgebaly is the Founder and CEO of Nour Heart, Inc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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