

ORIGINAL PRE-CLINICAL SCIENCE

Creatine supplementation attenuates pulmonary and systemic effects of lung ischemia and reperfusion injury



Francine Maria Almeida, MS,^a Manoel Carneiro Oliveira-Junior, BSc,^b
Renato Aparecido Souza, PhD,^{b,c} Ricardo Costa Petroni, PhD,^d
Sonia Fatima Soto, MS,^a Francisco Garcia Soriano, MD PhD,^d
Paulo Tarso Camillo de Carvalho, PhD,^b Regiane Albertini, PhD,^b
Nilsa Regina Damaceno-Rodrigues, PhD,^e
Fernanda Degobbi Tenorio Quirino Santos Lopes, PhD,^a
Hugo Caire Castro-Faria-Neto, MD, PhD,^f Milton Arruda Martins, MD, PhD,^a
Marisa Dolhnikoff, MD, PhD,^g Rogerio Pazetti, PhD,^h
and Rodolfo Paula Vieira, PhD^b

From the ^aDepartment of Clinical Medicine (LIM 20), School of Medicine, University of São Paulo, São Paulo, São Paulo, Brazil; ^bLaboratory of Pulmonary and Exercise Immunology (LABPEI), Nove de Julho University (UNINOVE), São Paulo, São Paulo, Brazil; ^cGrupo de Estudos e Pesquisa em Ciência da Saúde (GEP-CS), Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas Gerais (IFSULDEMINAS), Campus Muzambinho, Muzambinho, Minas Gerais, Brazil; ^dDepartment of Clinical Medicine (LIM 51), School of Medicine University of São Paulo, São Paulo, São Paulo, Brazil; ^eDepartment of Pathology (LIM 59), School of Medicine University of São Paulo, São Paulo, São Paulo, Brazil; ^fLaboratory of Immunopharmacology, Oswaldo Cruz Foundation, Oswaldo Cruz Institute, Rio de Janeiro, Rio de Janeiro, Brazil; ^gDepartment of Pathology (LIM 05), School of Medicine, University of São Paulo, São Paulo, São Paulo, Brazil; and the ^hDepartment of Cardiopneumology (LIM 61), School of Medicine, University of São Paulo, São Paulo, São Paulo, Brazil.

KEYWORDS:

creatine supplementation;
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BACKGROUND: Creatine (Cr) is a dietary supplement that presents beneficial effects in experimental models of heart and brain ischemia and reperfusion (I/R) injury. It can improve adenosine 5'-triphosphate generation and reduce cell damage. This study evaluated the effects of Cr supplementation in a model of lung I/R.

METHODS: Forty male Wistar rats were divided into 4 groups: sham operated, Cr+sham, I/R, and Cr+I/R. We investigated the effects of 5 days of Cr supplementation (0.5 g/kg/day by gavage) before left pulmonary artery ischemia (90 minutes) and reperfusion (120 minutes) on pulmonary and systemic response.

RESULTS: Cr inhibited the I/R-induced increase in exhaled nitric oxide ($p < 0.05$), total cells ($p < 0.01$), and neutrophils ($p < 0.001$) in bronchoalveolar lavage fluid and in the systemic circulation ($p < 0.001$). The levels of interleukin-1 β ($p < 0.05$), tissue damping, and tissue elastance ($p < 0.05$) were also minimized. Cr also inhibited pulmonary edema formation (total proteins in bronchoalveolar lavage fluid,

Reprint requests: Rodolfo Paula Vieira, PhD, Universidade Nove de Julho (UNINOVE), Laboratório de Imunologia Pulmonar e do Exercício, Rua Vergueiro 239/245, 01504-001-São Paulo, SP, Brazil. Telephone/Fax: +55-11-3385-9001.

E-mail address: rodrelena@yahoo.com.br

$p < 0.001$; histologic edema index, $p < 0.001$) and neutrophils accumulation in lung tissue ($p < 0.001$). As possible mechanisms underlying Cr effects, we observed a reduced expression of caspase 3 ($p < 0.05$), reduced expression of Toll-like receptor (TLR) 4, and increased expression of TLR7 in lung tissue ($p < 0.001$).

CONCLUSIONS: Cr supplementation presents pulmonary and systemic protective effects in acute lung injury induced by I/R in rats. These beneficial effects seem to be related to the anti-inflammatory and anti-oxidant properties of Cr and modulation of TLRs.

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Ischemia and reperfusion (I/R) is a common procedure in organ transplantation and organ resection.¹ During the period of ischemia, a severe impairment of oxidative phosphorylation occurs, reducing adenosine 5'-triphosphate storage, contributing to cellular death.^{2–4} After the ischemia, the reperfusion period even impairs the cellular metabolism and integrity, and exacerbates free radicals pool⁴ and cytokines production^{1,5} while reducing the amount of growth factors.⁶ In all of these, the Toll-like receptors (TLRs) play a central role.^{7–9}

One of the possible repercussions of I/R injury is the development of acute respiratory distress syndrome (ARDS).⁹ ARDS is a clinical syndrome characterized by pulmonary hypoxemic failure, bilateral inflammation and edema, and compliance failure, without cardiac pressures abnormalities.¹⁰ The inflammatory response in ARDS presents intense systemic and pulmonary neutrophilic inflammation and increases the production of pro-inflammatory cytokines and nitric oxide (NO).¹¹

Creatine (Cr) is an amine synthesized by the kidneys and liver using the amino acids arginine, glycine, and methionine. It is essential for cellular metabolism and is a substrate for adenosine 5'-triphosphate generation.¹² Synthetic Cr is the most common dietary supplement and is used to increase strength and muscle mass.¹² Cr supplementation also presents prophylactic and therapeutic effects for some muscular, neuromuscular, cardiovascular, and neurologic disorders.¹² Previous studies have demonstrated that Cr supplementation increases the expression of insulin-like growth factor 1 (IGF-1) in skeletal muscle^{13,14} and in lungs^{15,16} and modulates the expression of TLRs.¹⁷ Cr supplementation reduces I/R injury in the brain and heart.^{18,19} These effects are likely mediated by the anti-oxidant and anti-inflammatory effects of Cr.^{2,20,21} In the present study, we hypothesized that Cr supplementation could inhibit the lung injury induced by I/R and investigated the effects of Cr supplementation on the pulmonary and systemic response in a lung model of I/R.

Methods

All experimental procedures were approved by the University of São Paulo Ethical Committee (protocol 179/10) and were conducted in accordance with National Institutes of Health guidelines for the ethical treatment of animals.

Animals and experimental groups

Forty male Wistar rats (250–300 g) were obtained from the central animal facility of the University of São Paulo and maintained at

standard conditions. The animals were randomized into 4 groups: sham operated (Control), Cr supplemented + sham operation (Cr), I/R, and Cr supplemented+I/R (Cr+I/R).

Cr supplementation protocol

Cr monohydrate (Sigma-Aldrich, Steinheim, Germany), 0.5 g/kg/day diluted in water, was administered by gavage for 5 days before the surgical procedure. The Control group received only water.^{15,16,22}

I/R procedures and lung mechanics evaluation

Twenty-four hours after the last Cr administration and under anesthesia (pentobarbital, 50 mg/kg), the rats underwent tracheotomy and mechanical ventilation (FlexiVent; Scireq, Montreal, QC, Canada).²³

Measurements of pulmonary mechanics (airway resistance, tissue elastance, and tissue damping) were performed using a model of forced oscillation.²⁴ These measurements were collected for 3 other different periods during the procedures: immediately after arterial clamping (ischemia), after the ischemia period (90 minutes), and after the reperfusion period (120 minutes).

After the first measurement of pulmonary mechanics, the rats underwent thoracotomy, and a microvascular clamp was used to occlude the left pulmonary artery, vein, and bronchi for 90 minutes. This was followed by 120 minutes of reperfusion.²³

Exhaled NO

After reperfusion and the last lung mechanical measurement, the exhaled air was collected for 5 minutes in a Mylar bag connected to the expiratory port of the ventilator. The exhaled NO (NO_{ex}) concentrations were measured by chemiluminescence using a fast-responding analyser (NOA 280; Sievers Instruments, Boulder, CO).^{25–27}

Blood collection and systemic inflammation analysis

Blood samples (~5 mL) were collected from the inferior vena cava. A total and differential leukocytes count was performed, and the remaining blood samples were centrifuged at 4°C for 5 minutes, with the plasma stored at –80°C for interleukin (IL)-1β measurement via enzyme-linked immunosorbent assay (ELISA).

Lung inflammation in bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) was collected using 10 mL of phosphate buffered saline. The recovered volume was used to determine the number of total cells with a hemacytometer (Neubauer chamber; Carl

Roth, Karlsruhe, Germany). Cytospins slides were prepared and stained with the Diff Quick kit (Medion Diagnostics, Dündingen, Switzerland) for differential cells analysis.^{27,28}

Plasma and BAL fluid measurements of IL-1 β

The levels of IL-1 β in plasma and in BAL fluid were measured by ELISA (R&D Systems, Minneapolis, MN).

Pulmonary vascular permeability through total proteins in BAL

The total proteins level in BAL is a marker of alveolar vascular permeability²⁹ and was performed using bicinchoninic acid (BCA) method (BCA Protein Assay kit; Thermo Scientific, Rockford, IL).

Lung histology and Histomorphometric Study

The lungs were excised in bloc and routinely sectioned and stained with hematoxylin and eosin for analysis of the density of polymorphonuclear cells in the lung parenchyma.^{25,30,31} The edema index was determined with the histomorphometric technique.²⁷

Quantitative analysis by image analysis of Immunohistochemistry Study

Histologic sections were incubated with anti-IGF-1 (diluted 1:800; sc-9013, Santa Cruz Biotechnology, Santa Cruz, CA) and with anti-caspase-3 (diluted 1:500; sc-7148, Santa Cruz Biotechnology). The ABC Vectastain Kit (Vector Elite; Vector Laboratories, Burlingame, CA) was used as a secondary antibody.

The quantitative analysis of IGF-1 and caspase-3 was performed by image analysis using Image Pro-Plus software (Media Cybernetics, Silver Spring, MD), as previously described.^{16,32} The measurements were performed in 5 entire pulmonary artery walls and in 5 entire bronchial walls of each rat at original magnification $\times 400$ in a blinded fashion. The results were expressed as percentage of positive-stained area in relation to the total area of the artery wall and of the bronchial epithelium.^{16,32}

Western blotting

The left lungs were homogenized in radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology). We used the BCA method for protein quantification (BCA Protein Assay kit). Here, 50 μg of protein (a pool of the 4 groups) was loaded in NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane. Primary antibodies anti-TLR4 (1:500, rabbit polyclonal immunoglobulin G, sc-30002; Santa Cruz Biotechnology) and anti-TLR7 (1:500, rabbit polyclonal immunoglobulin G, sc-30004; Santa Cruz Biotechnology) were visualized using horseradish-conjugated secondary antibody (1:500, Santa Cruz Biotechnology) and enhanced by chemiluminescence (Pierce/Thermo Scientific). An additional probe, using actin as a control for the amount of proteins, was performed using monoclonal anti-actin antibody clone C4 (MP Biomedicals, Solon, OH). The densitometric analysis of the bands was performed using ImageJ software (National Institutes of Health).

Statistical analysis

Data are expressed as mean \pm standard deviation. Statistical analysis used 1-way analysis of variance, followed by appropriate post hoc tests (Student-Newman-Keuls for parametric data or Dunn's for non-parametric data). Values of $p < 0.05$ were considered significant.

Results

Cr supplementation prevents the impairment of lung mechanics after I/R-induced ARDS

Figure 1 shows the results for tissue damping (GTIS) and tissue elastance (HTIS). Cr supplementation significantly inhibited increases in GTIS and HTIS both at post-ischemia (Figure 1A and B, respectively) and post-reperfusion (Figure 1C and D, respectively), compared with non-treated I/R animals ($p < 0.05$).

Cr supplementation reduces I/R-induced increase in NOex

I/R significantly increased the levels of NOex compared with the sham group ($p < 0.05$; Table 1). Cr supplementation also significantly reduced the levels of NOex compared with I/R group ($p < 0.05$).

Cr supplementation decreases I/R-induced pulmonary inflammation and edema

Figure 2 presents the number of total cells and neutrophils in BAL (Figure 2A), the number of neutrophils in lung tissue (Figure 2B), the levels of total proteins in BAL (Figure 2C), the edema index in lung tissue (Figure 2D), and illustrative photomicrographs of the control (Figure 2E), Cr (Figure 2F), I/R (Figure 2G), and Cr+I/R (Figure 2H) groups. Cr supplementation significantly reduced I/R-induced increases in the number of total cells ($p < 0.01$) and neutrophils ($p < 0.001$) in BAL, the number of neutrophils in lung tissue ($p < 0.001$), the levels of total proteins in BAL ($p < 0.001$), and the edema index in lung tissue ($p < 0.001$).

Cr supplementation inhibits I/R-induced systemic inflammation

Figure 3 shows the number of total leukocytes and neutrophils in peripheral blood and the plasma levels of IL-1 β . The results demonstrate that I/R injury significantly increased the number of total leukocytes ($p < 0.05$; Figure 3A) and neutrophils ($p < 0.01$; Figure 3B) in peripheral blood as well as plasma levels of IL-1 β ($p < 0.05$; Figure 3C) vs the other groups. Cr supplementation also significantly inhibited the I/R-induced increases in total leukocytes ($p < 0.05$; Figure 3A) and neutrophils ($p < 0.001$; Figure 3B) in peripheral blood as well as the increased plasma levels of IL-1 β ($p < 0.05$; Figure 3C) compared with the I/R group.

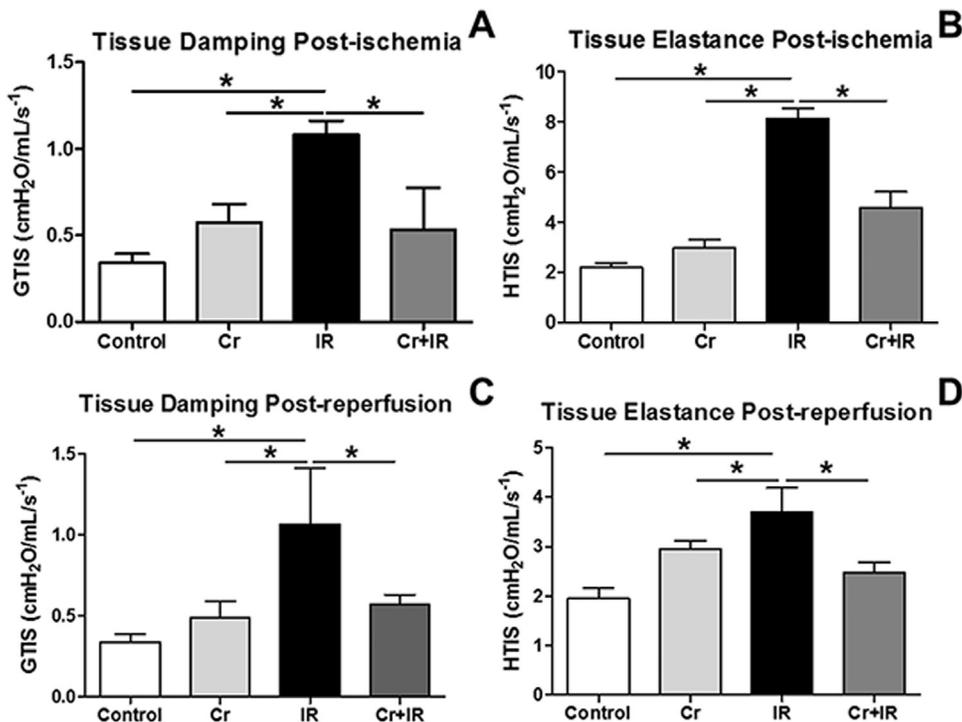


Figure 1 Results are shown for (A) tissue damping (GTIS) and (B) tissue elastance (HTIS) of the Control, creatine (Cr), ischemia/reperfusion (IR), and Cr+IR groups post-ischemia and (C) GTIS and (D) HTIS post-reperfusion. The bars show the standard deviation. * $p < 0.05$.

Cr supplementation inhibits apoptosis (caspase-3) and increases IGF-1 expression

Figure 4 shows the expression of caspase-3 in lung tissue. The results demonstrate that I/R injury significantly increased the apoptosis of bronchial epithelial cells ($p < 0.001$; Figure 4A) and vascular endothelial cells ($p < 0.001$; Figure 4B) vs the control group. The results also demonstrated that Cr supplementation significantly inhibited the apoptosis of bronchial epithelial cells ($p < 0.001$; Figure 4A) and vascular endothelial cells ($p < 0.001$; Figure 4B) compared with the I/R group. Figures 4C to F show representative photomicrographs of caspase-3 immunostained slides of the Control, Cr, I/R, and Cr+I/R groups, respectively.

Cr supplementation increased the IGF-1 expression in bronchial epithelial cell ($p < 0.001$; Figure 5A) and in vascular endothelial cells ($p < 0.001$; Figure 5B) vs the I/R group. Figures 5C to 5F show representative photomicrographs of IGF-1 immunostained slides of the Control, Cr, I/R, and Cr+I/R groups, respectively.

Cr supplementation modulates the expression of toll-like receptors

I/R injury significantly increased the expression of Toll-like receptor (TLR) 4 in lung tissue ($p < 0.001$; Figure 6A) vs all other groups. The results also demonstrated that I/R injury reduced the expression of TLR7 ($p < 0.001$; Figure 6B) and that Cr supplementation reestablished the expression of TLR7 only partially ($p < 0.05$; Figure 6B). Figures 6C and D

show the representative Western blots for TLR4 and TLR7 in the Control, Cr, I/R, and Cr+I/R groups, respectively.

Discussion

The present study shows that Cr supplementation results in protective effects in acute lung injury induced by pulmonary artery I/R. Cr supplementation became a popular ergogenic aid to increase muscle performance,¹² with more than 2,500 metric tons used in 2000.³³ Although Cr displays several beneficial therapeutic effects,^{2,12–16,20,21,34} other studies have also found that Cr supplementation does not have a beneficial effect on the respiratory system during respiratory diseases.^{15,35–37} This study, however, indicates that Cr supplementation presents beneficial effects after lung I/R injury, including improvements in lung mechanics and inflammation. These contradictory findings in the context of the respiratory diseases are probably related to the pathophysiologic differences between the respiratory diseases under study (i.e., chronic obstructive pulmonary diseases vs acute lung injury).

Table 1 Levels of Exhaled Nitric Oxide

Group	Exhaled nitric oxide (Mean \pm SD ppm)
Control	6.43 \pm 1.72
Creatine	7.01 \pm 4.65
Ischemia/reperfusion	12.61 \pm 6.06 ^a
Creatine + ischemia/reperfusion	6.6 \pm 2.51

SD, standard deviation.

^a $p < 0.05$ compared with the other groups.

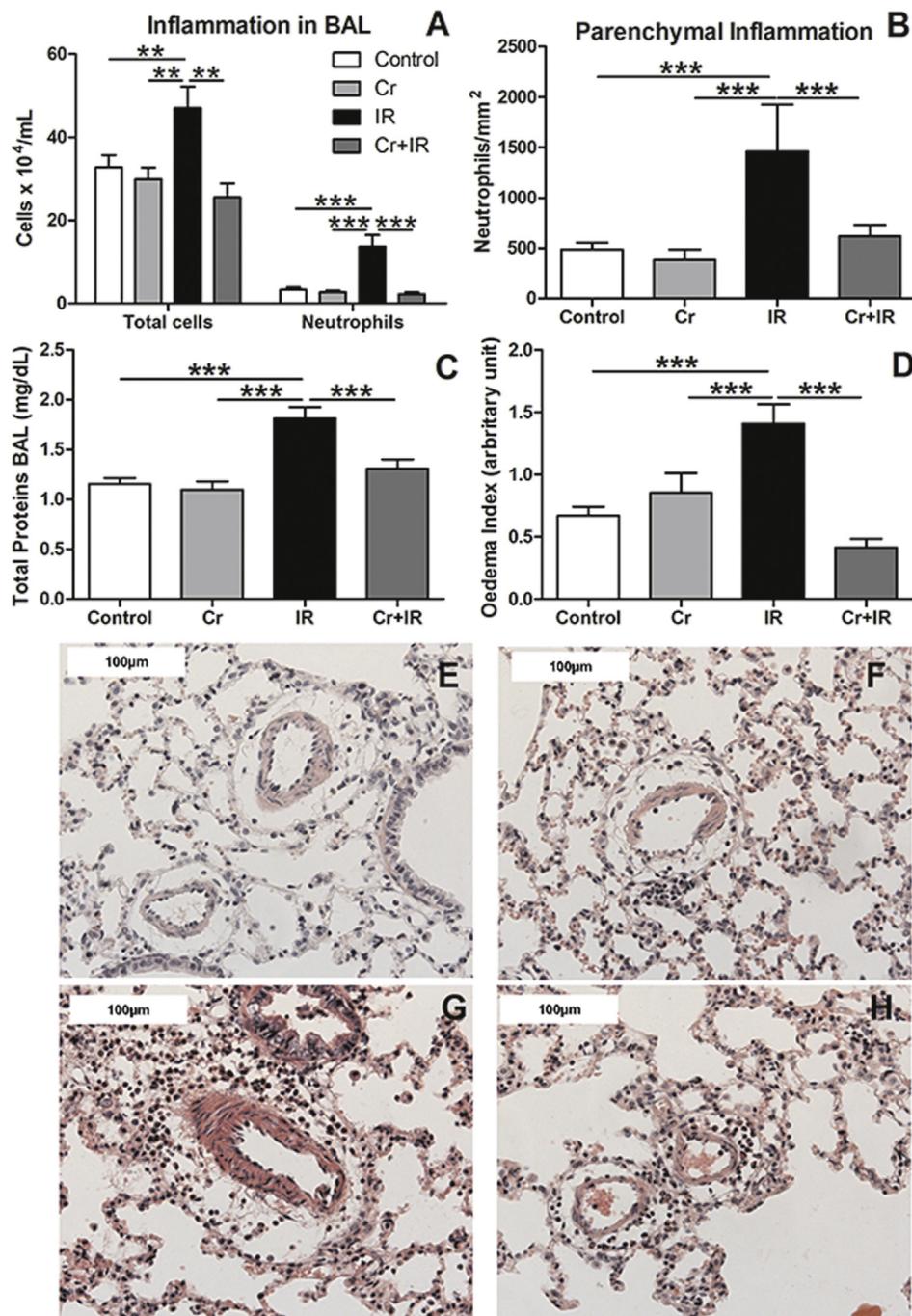


Figure 2 Results are shown for (A) the total number of leukocytes in bronchoalveolar lavage (BAL), (B) the number of neutrophils in the lung parenchyma, (C) the levels of total proteins in BAL, and (D) the edema index. The bars show the standard deviation. Cr, creatine; IR, ischemia/reperfusion. ** $p < 0.01$ and *** $p < 0.001$. (E–H) Representative photomicrographs are shown of lung inflammation and edema (hematoxylin and eosin stain).

In recent years, Cr was shown to have neuroprotective effects in a mouse model of stroke¹⁸ and to increase myocardial stability after I/R injury.¹⁹ The exact mechanisms of these potential effects are unknown and need further investigation. However, Cr protection during I/R injury is probably associated with improved phosphocreatine and glycogen storage, which effectively reduces ischemic injury.¹⁹

Lung transplantation can be a life-saving procedure for those with end-stage lung diseases.³⁸ The most common indications for transplantation are chronic obstructive

pulmonary disease, idiopathic pulmonary fibrosis, cystic fibrosis, α -1 antitrypsin deficiency, and idiopathic pulmonary arterial hypertension.³⁸

A barrier to transplant success is severe I/R injury, also known as primary graft dysfunction. This dysfunction is associated with high morbidity and mortality in the first 30 days after transplantation.³⁹ Unfortunately, long-term graft and patient survival are limited by acute and chronic allograft rejection, with a median survival of just over 6 years.⁴⁰

Increasing the I/R during organ transplantation leads to early primary graft failure.⁹ Severe I/R-induced lung injury

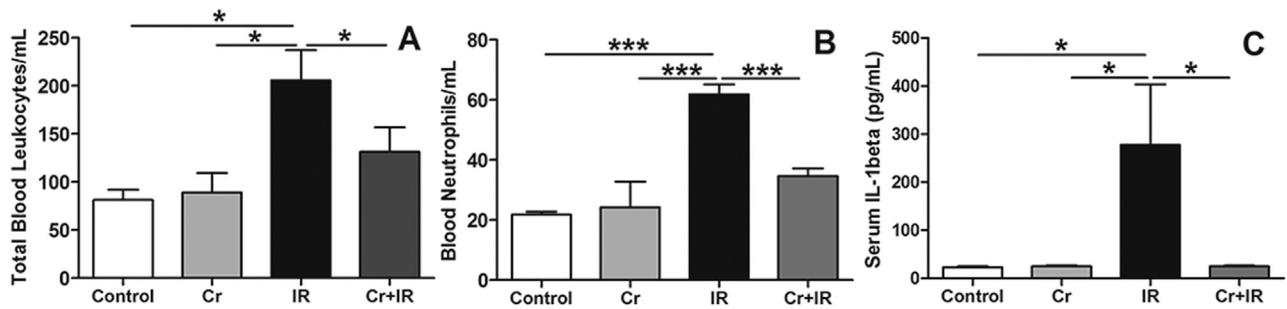


Figure 3 Results are shown for the total number of (A) leukocytes and (B) neutrophils in the blood, and the levels of (C) interleukin-1 β (IL-1 β). The bars show the standard deviation. Cr, creatine; IR, ischemia/reperfusion. * $p < 0.05$; *** $p < 0.001$.

occurs in approximately 25% of all lung transplants and is clinically characterized by poor oxygenation, tissue damage, and pulmonary edema.⁴¹ I/R injury models can reproduce several pulmonary changes observed in patients undergoing lung transplantation, such as increased vascular permeability and pulmonary edema, immune system activation with

lymphocyte and neutrophil infiltration, alveolar damage, and hypercoagulation state.^{1,23,42,43} In I/R injury models, ischemia is usually induced for only 60 to 90 minutes. Thus, it may not completely reproduce the actual conditions experienced during lung transplantation. Nevertheless, these models are useful in studying the mechanisms underlying

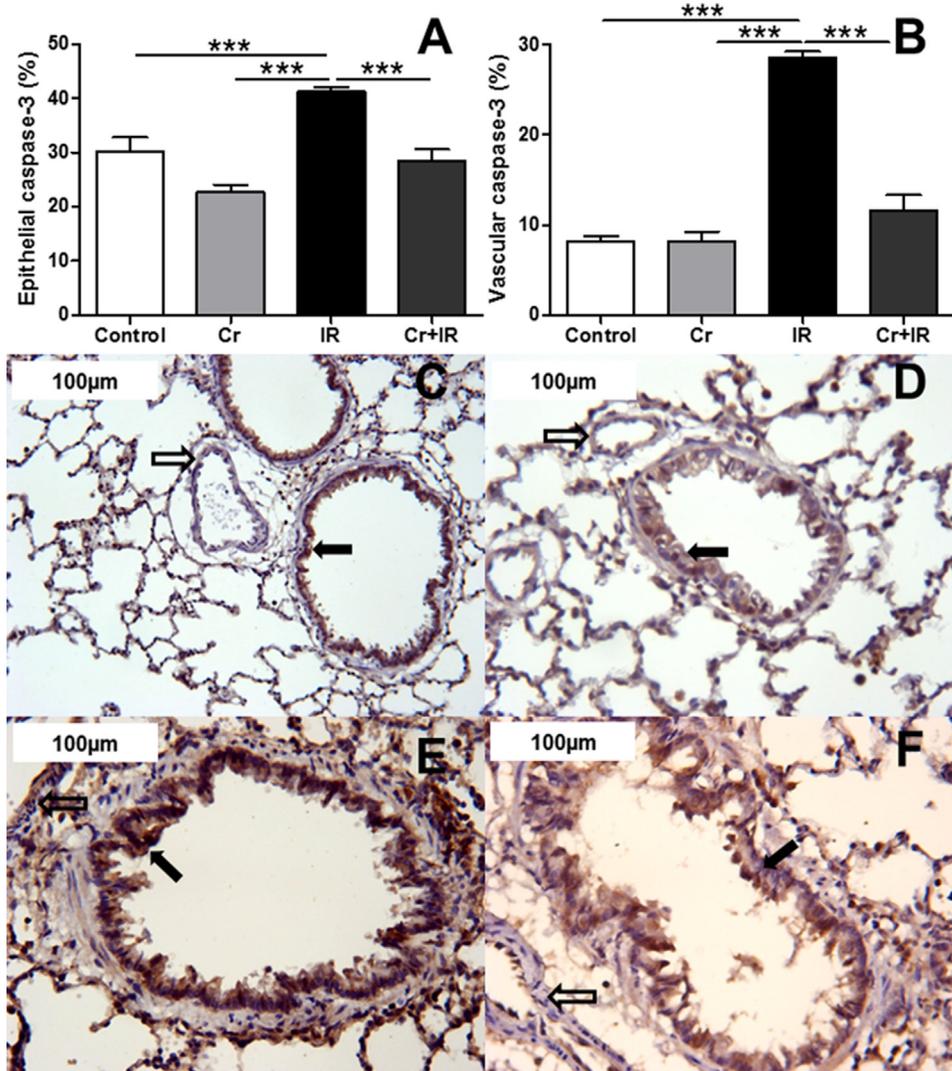


Figure 4 The expression of caspase-3 is shown in the (A) airway epithelial cells and in the (B), vascular wall. Cr, creatine; IR, ischemia/reperfusion. The bars show the standard deviation. *** $p < 0.001$. Representative photomicrographs are shown of the (C) Control, (D) Cr, (E) I/R, and (F) Cr+I/R groups. The black arrows show the epithelial positivity for caspase-3 in airway epithelial cells and the open arrows show the positivity for caspase-3 in the vascular wall (hematoxylin and eosin stain).

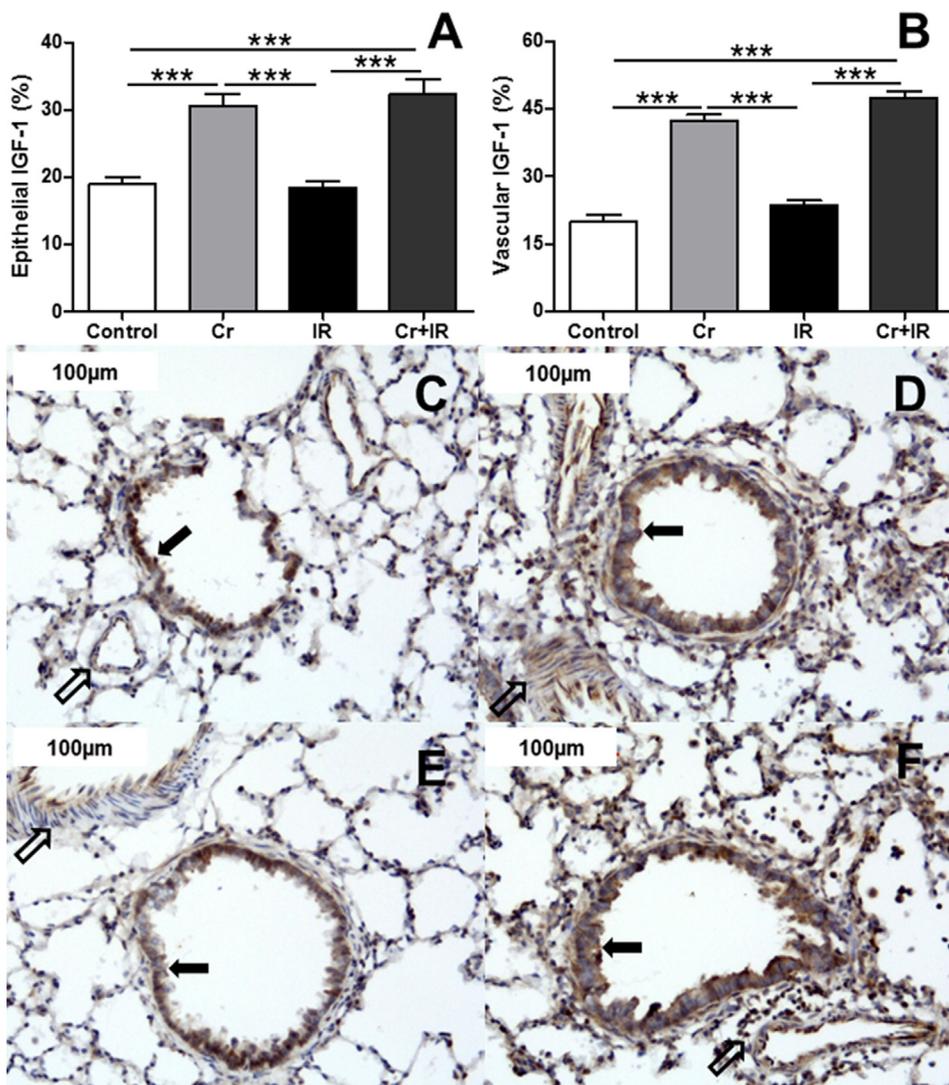


Figure 5 Expression of insulin-like growth factor 1 (IGF-1) is shown in the (A) airway epithelial cells and (B) vascular wall (***) $p < 0.001$; bars show the standard deviation) and in representative photomicrographs of the (C) Control (D) creatinine (Cr), (E) ischemia/reperfusion (IR) and (F) Cr+IR groups. The black arrows show the epithelial positivity for IGF-1 in airway epithelial cells, and open arrows show the positivity for IGF-1 in vascular wall (hematoxylin and eosin stain).

pulmonary I/R and the conditions of transplant patients. Our model of lung I/R induced acute lung injury that was characterized by pulmonary inflammation and edema, alveolar protein leakage, increases in NOex, and changes in pulmonary mechanics.

Finding an effective drug to prevent pulmonary I/R injury is essential because it is the major cause of primary graft dysfunction that can lead to early death after lung transplantation.⁴¹ Although many potential drugs that target specific pathways or specific cell types have been tested in animal models, there are still no clinically effective drugs. The process of pulmonary I/R is very complicated and includes inflammatory cell infiltration and activation, release of inflammatory mediators, and lung cell death as major contributing factors.^{10,42,44} The present study showed that Cr supplementation attenuated all of these principal I/R outcomes.

Intense neutrophilic lung inflammation, lung edema, and the release of pro-inflammatory cytokines impair lung mechanics in ARDS patients.¹¹ This study showed that Cr supplementation inhibited I/R-induced impairments in lung

mechanics, especially in HTIS and GTIS. This was seen after both ischemia and reperfusion and indicates an important functional effect of Cr supplementation. Studies have suggested that Cr possesses anti-inflammatory properties, stabilizes mitochondrial membranes, and has antioxidant effects.^{2,20,21,45} Our results show that Cr supplementation decreased I/R-induced pulmonary inflammation and edema, suggesting that the improvements in lung mechanics in the Cr supplemented group may be related to its anti-inflammatory properties.

Reactive oxygen and nitrogen species mediate inflammation in ARDS.⁴⁶ NO is a highly reactive free radical gas that reacts with a wide range of biomolecules to produce reactive nitrogen species.⁴⁶ In this study, we showed that the experimental model of lung I/R injury had increased the NOex levels, but that Cr supplementation had anti-oxidant effects and reduced the levels of NOex, as suggested in previous studies.^{2,20,21}

Besides reactive oxygen and nitrogen species, massive production of pro-inflammatory cytokines^{1,4,5} and reduced

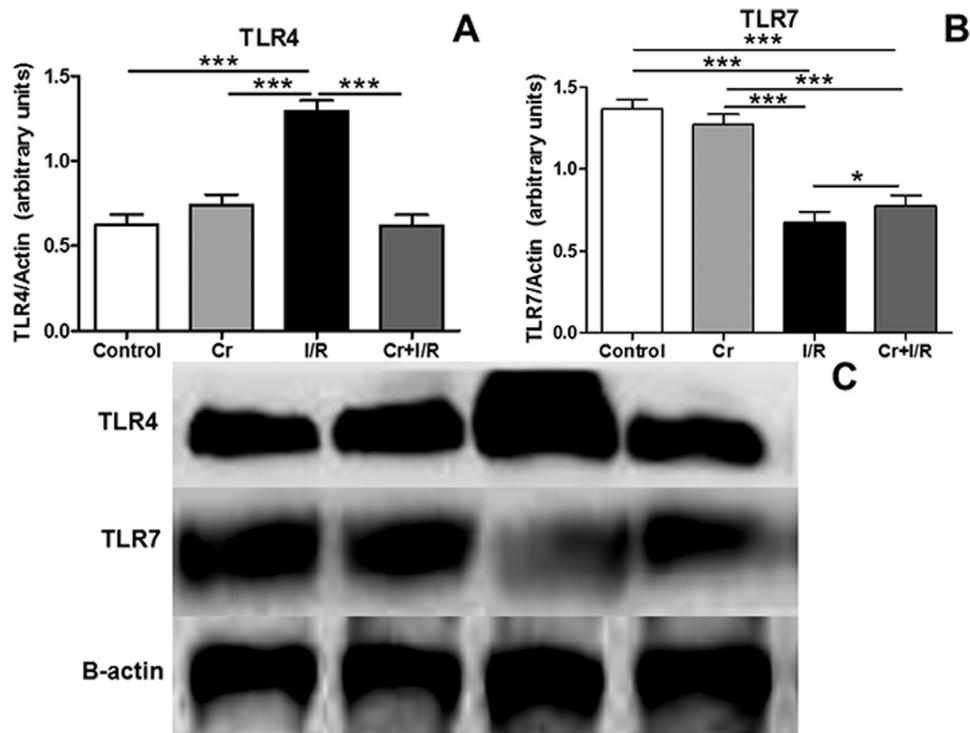


Figure 6 Expression of (A) Toll-like receptor (TLR) 4 and (B) TLR7 in lung tissue. The bars show the standard deviation. * $p < 0.05$, *** $p < 0.001$. (C) Representative Western blots are shown for TLR4 (top lane), TLR7 (middle lane), and β -actin (bottom lane).

expression of growth factors⁶ plays a central role in mediating inflammation in ARDS.^{1,5} For instance, IL-1 β , a potent pro-inflammatory cytokine, is centrally involved in the mechanism of lung injury and regulates microvascular endothelial permeability.⁴⁷ Here, we observed that Cr supplementation reduced I/R-induced high plasma levels of IL-1 β , which was followed by reduced pulmonary perivascular edema.

Growth factors are also involved in the early and late response in ARDS.⁶ Reduced expression of IGF-1 contributes to apoptosis of pulmonary fibroblasts⁶ and endothelial cells.⁴⁸ Although we found that Cr supplementation increased the IGF-1 expression in bronchial epithelial cells and in pulmonary vascular cells, IGF-1 did not seem to play an important role in our I/R model. Furthermore, our results indicate that Cr may inhibit apoptosis of bronchial epithelial and endothelial cells, a phenomenon that involved in the formation of pulmonary edema.

The modulation of TLRs is essential for development of injury mediated by I/R.⁷⁻⁹ On one hand, TLR4 activation leads to nuclear factor- κ B activation, which mediates lung inflammation and injury.⁷ On the other hand, TLR7 preconditioning mediates protection against ischemic injury in the brain of mice.⁸ Of note, a recent study showed that Cr and Cr derivatives modulate the expression of TLRs in a mouse macrophage cell line.¹⁷ This suggested that Cr may affect the ability of immune cells to sense a wide array of viral and bacterial pathogens.¹⁷ In our study, we showed that Cr supplementation resulted in decreased expression of TLR4 and increased TLR7 expression in lung tissue. Overall, these data support the hypothesis that Cr possesses anti-

inflammatory properties in the lungs by affecting the sensing arm of the innate immune system and by modulation of TLR4 and TLR7 expression.

In our experimental protocol, the control I/R group received only water. Although the observed changes in Cr+I/R can be attributed to the supplemented Cr, we cannot exclude the possibility that other dietary supplements may induce similar effects.

In conclusion, Cr supplementation offers pulmonary and systemic protective effects in a model of acute lung injury induced by lung I/R in rats.

Disclosure statement

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None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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