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Gadolinium decreases inflammation related to myocardial ischemia and reperfusion injury

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Abstract

Background: The lanthanide cation, gadolinium ($GdCl_3$) protects the myocardium against infarction following ischemia and reperfusion. Neutrophils and macrophages are the main leukocytes responsible for infarct expansion after reperfusion. $GdCl_3$ interferes with macrophage and neutrophil function in the liver by decreasing macrophage secretion of inflammatory cytokines and neutrophil infiltration. We hypothesized that $GdCl_3$ protects against ischemia and reperfusion injury by decreasing inflammation. We determined the impact of $GdCl_3$ treatment for reperfusion injury on 1) circulating monocyte and neutrophil counts, 2) secretion of inflammatory cytokines, and 3) influx of monocytes and neutrophils into the myocardium.

Methods: Rats ($n = 3-6/gp$) were treated with saline or $GdCl_3$ ($20 \mu mol/kg$) 15 min prior to a 30 min period of regional ischemia and 120 min reperfusion. Sham rats were not subject to ischemia. Blood was collected either after 30 min ischemia or 120 min reperfusion and hearts were harvested at 120 min reperfusion for tissue analysis. Blood was analyzed for leukocytes counts and cytokines. Tissue was analyzed for cytokines and markers of neutrophil and monocyte infiltration by measuring myeloperoxidase (MPO) and α -naphthyl acetate esterase (ANAE).

Results: $GdCl_3$ did not affect the number of circulating neutrophils prior to ischemia. Two hours reperfusion resulted in a 2- and 3- fold increase in circulating monocytes and neutrophils, respectively. $GdCl_3$ decreased the number of circulating monocytes and neutrophils during reperfusion to levels below those present prior to ischemia. Furthermore, after 120 min of reperfusion, $GdCl_3$ decreased ANAE and MPO activity in the myocardium by 1.9-fold and 6.5-fold respectively. $GdCl_3$ decreased MPO activity to levels below those measured in the Sham group. Serum levels of the major neutrophil chemoattractant cytokine, IL-8 were increased from pre-ischemic levels during ischemia and reperfusion in both control and $GdCl_3$ treated rats. Likewise, IL-8 levels increased throughout the 3 hour time period in the Sham group. There was no difference in IL-8 detected in the myocardium after 120 min reperfusion between groups. In contrast, after 120 min reperfusion $GdCl_3$ decreased the myocardial tissue levels of macrophage secreted cytokines, GM-CSF and IL-1.

Conclusion: GdCl₃ treatment prior to ischemia and reperfusion injury decreased circulating monocytes and neutrophils, macrophage secreted cytokines, and leukocyte infiltration into injured myocardium. These results suggest GdCl₃ decreased monocyte and neutrophil migration and activation and may be a novel treatment for inflammation during ischemia and reperfusion.

Background

The lanthanide cation, gadolinium (GdCl₃) protects the myocardium against infarction following ischemia and reperfusion (IR) *in vivo* [1], although this preconditioning is not observed in a buffer perfused, isolated heart model of acute reperfusion injury (unpublished observation). This discrepancy suggests that GdCl₃-induced cardioprotection is dependent upon factors found only *in vivo*, such as blood cells, proteins or hormones among others.

Inflammatory cells are important in the pathophysiological response to injury associated with IR. While crucial to healing, the influx of inflammatory cells, specifically macrophages and neutrophils, results in tissue injury beyond that caused by ischemia alone. Many studies have focused on the acute myocardial inflammatory reaction as a mediator of ischemia-reperfusion injury [2]. Monocytes and other leukocytes infiltrate the area at risk soon after the onset of ischemia. Activated macrophages secrete cytokines that promote tissue damage and recruit neutrophils [3]. Accordingly, the influx of neutrophils into ischemic tissue increases tissue necrosis by releasing proteolytic enzymes and reactive oxygen species and expands the zone of infarction [4].

Strategies aimed at reducing the levels of inflammatory cytokines [5] or the infiltration of leukocytes [6] attenuate myocardial damage associated with reperfusion. Evidence suggests that GdCl₃ interferes with macrophage and neutrophil function in the liver by decreasing macrophage secretion of inflammatory cytokines and toxic oxygen radicals [7] and by inhibiting neutrophil infiltration [8]. The role GdCl₃ plays in monocyte and neutrophil infiltration during myocardial ischemia and reperfusion is unknown. Accordingly, this study tests the hypothesis that GdCl₃ modulates leukocyte function either directly by interfering with migration or indirectly by decreasing the generation of inflammatory cytokines and chemokines, thereby decreasing the signal that triggers leukocytes to infiltrate into the injured tissue.

Methods

Male Sprague Dawley rats at 8 weeks of age (250-300 g) were used in this study and received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). This project was granted approval by the local IACUC review board.

Instrumentation, ischemia-reperfusion protocol and GdCl₃ treatment

Rats were anesthetized with 20-40 mg/kg intraperitoneal sodium pentobarbital. The right jugular vein was cannulated for delivery of saline solution. A catheter was inserted in the left femoral artery to measure both blood pressure and heart rate and to withdraw blood. Pressure and rate measurements were monitored using a Gould PE50 or PE23 pressure transducer connected to a Grass model 7 polygraph. The rats were intubated with a 14-gauge catheter and ventilated at 38-45 breaths/min (Harvard Apparatus, model 683; South Natick, MA) with supplemental oxygen. Atelectasis was prevented by maintaining a positive end-expiratory pressure of 5-10 mm H₂O. Arterial pH, pCO₂ and pO₂ were monitored with an AVL 995 pH/blood gas analyzer, and normal values were maintained by adjusting respiratory rate, tidal volume and/or oxygen flow. Body temperature was maintained between 35 and 37°C using a heating pad.

A left thoracotomy was performed, the pericardium was opened and the left coronary artery was identified. A ligature (6-0 Prolene) was passed around the proximal segment of the left coronary artery, and the ends of the suture were threaded through a propylene tube to form a snare. Regional left ventricular ischemia was induced by tightening the snare for 30 min. Coronary artery occlusion was confirmed by epicardial cyanosis and a decrease in blood pressure. Reperfusion was achieved by releasing the snare and was confirmed by a marked hyperemic response of the myocardium. The heart was reperfused for 120 min then excised and assessed for extent of tissue injury.

Gadolinium chloride hexahydrate (20 μmol/kg dissolved in 0.9% NaCl solution; Sigma, Milwaukee, WI) was given as an intravenous (i.v.) bolus 15 min before inducing myocardial ischemia [1]. Experimental groups are shown in Figure 1A and included Sham (no treatment, no ischemia), Sham + GdCl₃ treated (treated, no ischemia), Control (no treatment but subject to ischemia and reperfusion) and GdCl₃ (treated and subject to ischemia and reperfusion) groups. Infarct size was also measured using this protocol and serves as a positive control for this study (Figure 1B).

Myocardial Tissue Myeloperoxidase Activity Assay

Myeloperoxidase (MPO) activity was assayed as a measure of neutrophil activity in hearts using a modified protocol [9]. The heart was homogenized in 50 mM potassium

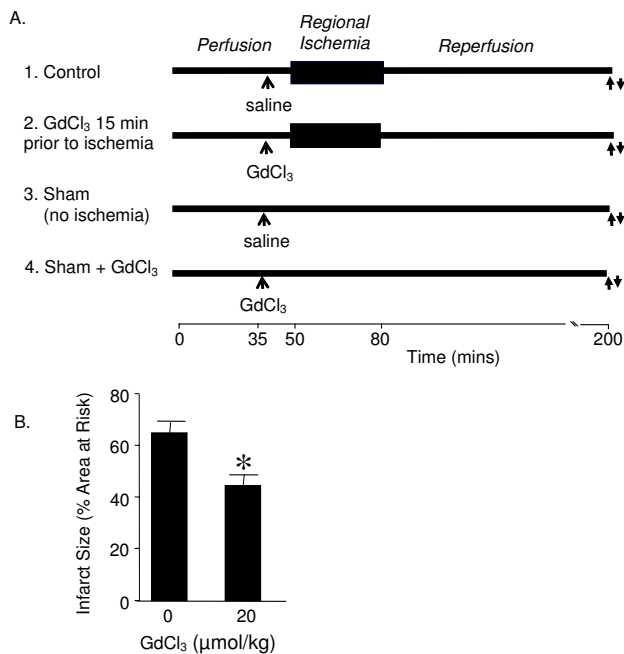


Figure 1
In vivo rat model of ischemia and reperfusion injury.
A) Rats were treated with either saline or GdCl₃ (20 μmol/kg) 15 minutes prior to a 30 minute period of regional ischemia and 2 hours reperfusion. ↑ Blood collection. ↓ Harvest the free wall of the left ventricle. **B)** Measurement of infarct size as a percentage area at risk using this protocol.

phosphate buffer (pH 6.0) and centrifuged. The pellet was washed twice in 5 mM potassium phosphate buffer. After washing, the pellets were resuspended in extraction buffer (50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammoniumbromide), followed by three rounds of freeze-thawing. The suspension was incubated at 4°C for 20 minutes and then centrifuged at 13,000 rpm at 4°C for 15 minutes. The supernatant (100 μL) was mixed with 100 μL of reaction buffer (50 mM potassium phosphate buffer (pH 6.0) containing 0.6 mg/mL O-dianisidine dihydrochloride and 0.03% hydrogen peroxide). Absorbance was measured at 450 nm after 5 minutes of incubation. After normalization for protein concentration, the MPO content was expressed as units of MPO activity per milligram of protein.

Myocardial Tissue α -Naphthyl Acetate Esterase Assay

The activity of α -naphthyl acetate esterase (ANAE), a marker enzyme of monocytes and macrophages was detected using a previous published protocol [10]. In brief, frozen specimens from Sham, Sham + GdCl₃, Control and GdCl₃ groups were separately homogenized in ice-cold 0.25 mol/L sucrose (1:5; weight to volume) for 2 × 5 seconds. The samples were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was further sonicated for

90 seconds in ice, centrifuged at 105,000 g for 90 minutes at 4°C, and assayed for protein. An equivalent of 25 mg of supernatant protein for each sample and 5 mL of 200 mmol/L α -naphthyl acetate (Sigma-Aldrich, St. Louis, MO), dissolved in 95% ethanol to give a final concentration of 0.5 mmol/L, was added to a final volume of 2 mL of saline solution. Blanks received no substrate. After 10 minutes of incubation at 37°C, the reaction was stopped by adding 116 mL of 12.5% w/v sodium dodecyl sulfate solution. Subsequently, 5 mL of 200 mmol/L α -naphthyl acetate was added to the blanks. Finally, 30 mL of fast red solution (10 mg/mL distilled water; Fast Red B, Sigma-Aldrich, St. Louis, MO) was added to the sample, followed by an incubation period of 15 minutes at room temperature. Optical density absorption at 490 nm was used to estimate the metabolism of α -naphthyl acetate to α -naphthol. Alpha-naphthyl acetate esterase activity was estimated as absorption at 490 nm per 25 mg of protein.

Cytokine Assays

Blood was collected either 30 min after ischemia or 120 min after reperfusion. Plasma was separated by centrifuging the sample at 14,000 rpm × 10 min at 4°C and frozen at -80°C until analysis. Left ventricular free wall tissue homogenates were processed and quantitated using methods described previously [11]. Interleukin (IL)-8 levels were determined using an IL-8 (Rat cytokine-induced neutrophil chemoattractant (CINC)-1) enzyme-linked immunoassay kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. CINC-2, CINC-3, granulocyte monocyte colony stimulating factor (GM-CSF), Interferon (INF)- γ , IL-1 α , IL-1 β , IL-4, IL-6, IL-10, Monocyte chemoattractant protein (MCP)-1, Macrophage inflammatory protein (MIP)-3A, and Tumor necrosis factor (TNF)- α tissue levels were determined using RayBio® Rat Cytokine Antibody Array 1 (Norcross, GA) according to the manufacturer's instructions. Each dot on the immunoblot representing a cytokine was quantitated using ImageJ 1.37v software.

Statistical Analysis

Data are reported as mean ± SEM. Statistical analyses were performed by the Student's t test for paired values and a one-way Analysis of Variance (ANOVA) for differences between treatment groups. If significant, a Newman-Keuls multiple comparison test was used to perform pair wise comparisons. Data were considered significant at a p < 0.05. Statistics were performed using WINKS SDA Software (Texassoft, Cedar Hill, TX).

Results

GdCl₃ Attenuates IR-induced Increases in Circulating Monocytes and Neutrophils

We first determined whether GdCl₃ decreases circulating monocytes and neutrophils following ischemia and reperfusion. We have previously shown that the optimal cardi-

oprotective dose of $GdCl_3$ was 20 $\mu\text{mol/kg}$ (Figure 1B) [1]. Therefore, we used this dose for the experiments in this study. Rats were treated with either saline or $GdCl_3$ 15 minutes prior to a 30 minute period of regional ischemia followed by 120 min reperfusion. A complete blood count and differential was performed after 120 min reperfusion. $GdCl_3$ did not affect the number of circulating monocytes or neutrophils prior to ischemia. Two hours reperfusion resulted in a 2-fold increase in circulating monocytes and a 3-fold increase in circulating neutrophils. $GdCl_3$ decreased the number of circulating monocytes (Figure 2A) and neutrophils (Figure 2B) during reperfusion to levels below those present prior to ischemia. In addition, $GdCl_3$ given to the Sham group also decreased the number of circulating monocytes, but not

neutrophils when compared to the untreated Sham group.

Macrophage Infiltration in Myocardium

Using α -naphthyl acetate esterase (ANAE) content as an indicator of myocardial tissue monocyte/macrophage infiltration, ischemia and reperfusion increased ANAE activity by 3.6-fold in the IR control group when compared the Sham control group (Figure 3). $GdCl_3$ partially reversed increase in ANAE activity after ischemia and reperfusion injury by 1.9-fold but this was still above Sham values. $GdCl_3$ did not change ANAE activity in the Sham- $GdCl_3$ group when compared to the Sham control group.

Neutrophil Infiltration in Myocardium

Using myeloperoxidase (MPO) content as an indicator of myocardial tissue neutrophil infiltration, ischemia and reperfusion increased MPO activity by 3.8-fold in control hearts when compared to a Sham procedure (Figure 4). $GdCl_3$ not only reversed this increase in MPO activity after ischemia and reperfusion injury by 6.5-fold when compared to IR control but also decreased MPO activity below Sham control values by 1.7-fold. In the Sham groups, $GdCl_3$ decreased MPO activity 2.4-fold.

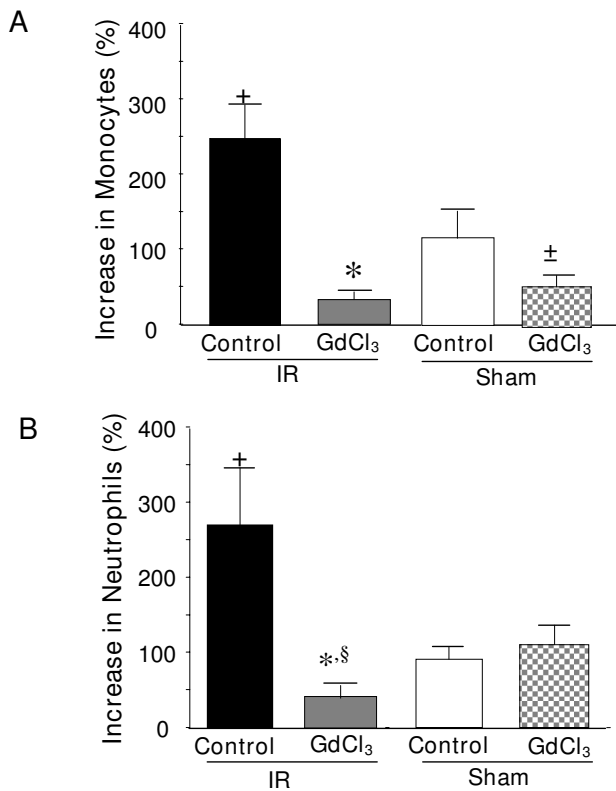


Figure 2
Gadolinium chloride decreases circulating monocytes and neutrophils following ischemia and reperfusion. Rats were treated with either saline or $GdCl_3$ (20 $\mu\text{mol/kg}$) 15 minutes prior to a Sham procedure or a 30 minute period of regional ischemia and 2 hours reperfusion. **A)** Increase in monocytes. **B)** Increase in neutrophils. Data mean \pm SD, $n = 3-6/\text{gp}$, $+ = p < 0.05$, Sham control vs. Ischemia-Reperfusion (IR) control, $* = p < 0.05$, IR Control vs. IR $GdCl_3$, $\pm = p < 0.05$, Sham Control vs. Sham $GdCl_3$, $\S = p < 0.05$, IR $GdCl_3$ vs. Sham $GdCl_3$

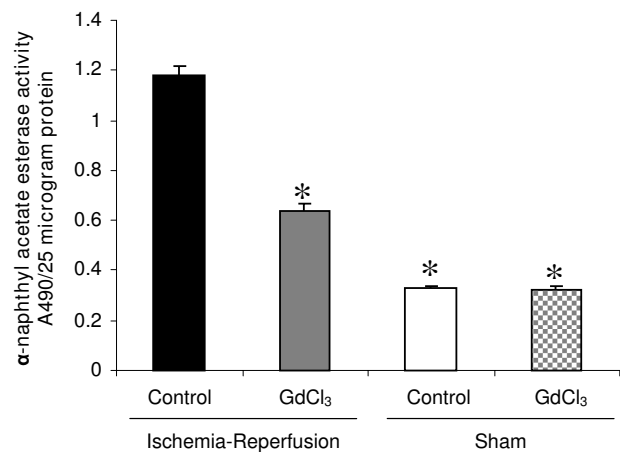


Figure 3
Gadolinium chloride decreases Alpha naphthyl acetate esterase activity in the reperfused myocardium. Rats were either treated with vehicle or $GdCl_3$ either before 30 min ischemia and 120 min reperfusion or a Sham procedure. Alpha naphthyl acetate esterase activity was measured after 120 min reperfusion in IR Control and IR + $GdCl_3$ groups and at a comparable time point in Sham Control and Sham + $GdCl_3$ groups. Data is mean \pm SD, $n = 6/\text{group}$, $* = p < 0.05$ vs. IR control.

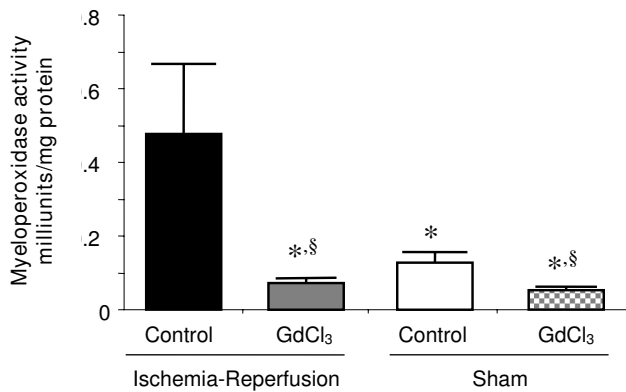


Figure 4
Gadolinium chloride decreases myocardial tissue myeloperoxidase activity. Rats were either treated with vehicle or GdCl₃ either before 30 min ischemia and 120 min reperfusion or a Sham procedure. Myeloperoxidase activity was measured after 120 min reperfusion in IR Control and IR + GdCl₃ groups and at a comparable time point in Sham and Sham + GdCl₃ groups. Data is mean ± SD, n = 3/group, * = p < 0.05 vs. IR control; § = p < 0.05 vs. Sham control.

GdCl₃ Modulation of Cytokine Levels

Inflammatory cytokines were then measured from either serum or myocardial tissue at the end of ischemia or reperfusion periods. There was no difference in IL-8/CINC-1 levels in the serum or the tissue when measured after 30 min ischemia (serum) or 120 min reperfusion (serum and tissue) (Figure 5A and 5B). Interestingly, IL-8/CINC-1 serum levels increased throughout the study in all groups when compared to the respective baseline levels.

A macroarray was performed using homogenized tissue after 120 min reperfusion to look for the tissue levels of CINC-2, CINC-3, GM-CSF, INF-γ, IL-1α, IL-1β, IL-4, IL-6, IL-10, MCP-1, MIP-3A, and TNF-α. GdCl₃ treatment did not reduce the major monocyte chemoattractant, MCP-1. However, GdCl₃-treatment did reduce the macrophage secreted cytokines such as GM-CSF and IL-1 after 120 min reperfusion (Figure 6). In addition, an increase in TNF-α was observed in the GdCl₃ groups after 120 min reperfusion.

Discussion

The important finding in this study is that a single treatment of GdCl₃ prior to ischemia decreased the numbers of circulating monocytes and neutrophils following reperfusion and reduced infiltration of these leukocytes into the injured myocardium. In addition, GdCl₃ decreased production of cytokines that are typically secreted by monocytes. These findings are associated with GdCl₃-mediated reduction in infarct size after ischemia and reperfusion [1].

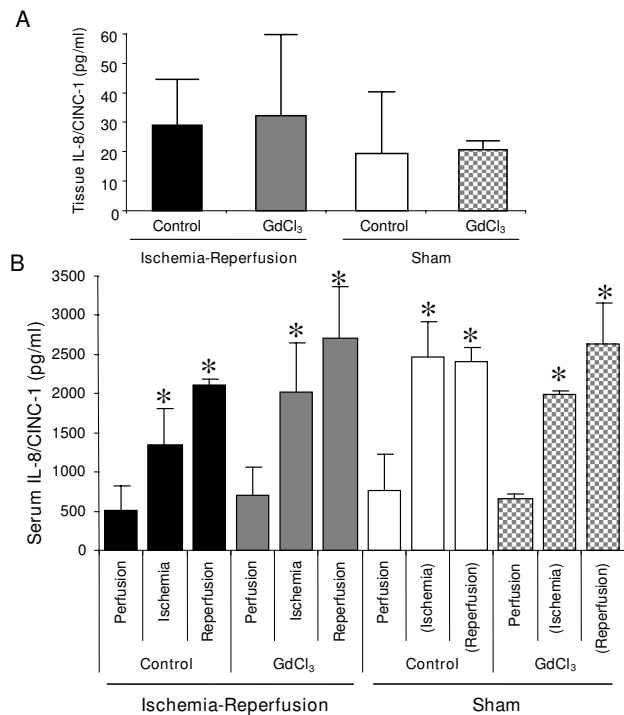


Figure 5
Gadolinium chloride does not decrease IL-8/CINC-1 production in the myocardium or serum during ischemia and reperfusion. Rats were either treated with vehicle or GdCl₃ either before 30 min ischemia and 120 min reperfusion or a Sham procedure. (Ischemia) and (Reperfusion) in the Sham groups is equal to the time period that correlates with Ischemia and Reperfusion in the Control and GdCl₃ groups. IL-8/CINC-1 was measured from the serum at 30 min ischemia and 120 min reperfusion and from the tissue at 120 min reperfusion. **A)** Quantitation of tissue IL-8/CINC-1 **B)** Quantitation of serum IL-8/CINC-1. Data mean ± SD, n = 3-4/gp, * = p < 0.05 vs. perfusion. No significant difference between IR vs. Sham groups or Control vs. GdCl₃ groups.

The early phase of myocardial infarction is associated with tissue infiltration of circulating leukocytes in response to chemotactic factors [12]. As the leukocytes infiltrate the tissue and become activated, they release more cytokines thereby recruiting further leukocytes to the injured area. Activated leukocytes within the tissue cause further tissue injury by releasing reactive oxygen species and proteases and cause capillary plugging leading further tissue hypoxia. Inhibiting neutrophil infiltration using anti-neutrophil antibodies [13], neutrophil depleting antimetabolites [14] or neutrophil filters [15] have been successful in limiting myocardial infarct size. We have shown that GdCl₃ is also a potent monocyte and neutrophil depleting compound as it prevents these leukocytes from circulating and infiltrating into the myocardium.

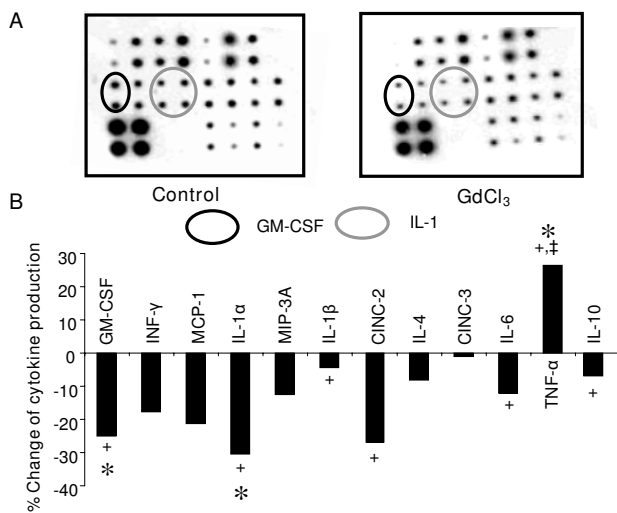


Figure 6
Gadolinium chloride decreases macrophage secreted cytokines after ischemia and reperfusion in rats. (A) Representative blots from cytokine arrays from control hearts vs. GdCl₃-treated hearts. The GM-CSF and IL-1 dots are indicated. Dots were quantitated and normalized to the positive control dots (lower left 4 dots) and results displayed above **(B)**. Data mean ± SD, n = 3-4/gp, * = p < 0.05, GdCl₃ vs. control, + = macrophage secreted cytokines, † = cardiomyocyte secreted cytokines.

Gadolinium is known to modulate inflammatory responses by liver macrophages (Kupfer cells) [7,16]. Gadolinium causes a 70% reduction in the phagocytosis of radiolabeled bacteria [17], and GdCl₃ has been shown to prevent the release of both inflammatory cytokines and toxic oxygen radicals such as superoxide anion produced by activated Kupffer cells [18,19]. Gadolinium also upregulates secretion of TNF-α from Kupfer cells in response to endotoxemia.

In the present study, GdCl₃ treatment pre-ischemia was associated with attenuation of the IR-induced increases in circulating monocytes and neutrophils, and it decreased circulating monocytes in animals not exposed to IR. The selective action of GdCl₃ on monocytes is based on the fact that this compound is readily dissolved in normal saline; however, when it is injected into the bloodstream, it rapidly aggregates into relatively large colloidal particles at neutral pH. The particles of GdCl₃ are taken up exclusively by circulating phagocytic mononuclear cells of a monocyte lineage (CD11b+, CD13+, and CD14+) [20,21]. Once inside the cell and after exceeding the threshold concentration, GdCl₃ causes cell apoptosis [22]. The GdCl₃ aggregates are not expected to cross the endothelial barrier and thus would not be taken up by resident tissue phagocytic cells.

This decrease in circulating leukocytes was not associated with any changes in the levels of the major monocyte chemoattractants, MCP-1 and the neutrophil chemoattractants IL-8/CINC-1, CINC-2, and CINC-3 suggesting that GdCl₃ modulates leukocyte trafficking to the heart via some other, as yet undefined mechanism following myocardial IR. Interleukin-8/CINC-1 serum levels increased over baseline levels during ischemia and reperfusion. Interestingly, there was a similar increase in serum levels at the corresponding time points in the Sham group. Although the Sham animals do not undergo LAD occlusion, they still receive a tracheostomy and a thoracotomy. These two invasive surgical procedures may be enough to induce an inflammatory response in these rats.

Gadolinium triggered an increase of TNF-α levels in the myocardium after 120 min reperfusion (Figure 6B). TNF-α is a potent cytokine which is elevated in a variety of inflammatory conditions. Endogenous TNF-α has been correlated with the deterioration of myocardial performance, while blockade of TNF-α with a neutralizing antibody preserves myocardial function after IR [23]. Somewhat paradoxically, exogenously added TNF-α is protective during ischemia and reperfusion through mechanisms which involve the JAK/STAT3 pathway, K_{ATP} channels and the mitochondrial permeability transition pore [24,25]. This protective effect of TNF-α may be related to our previous data, which showed that GdCl₃-induced cardioprotection is dependent upon the activation of JAK-STAT3 and K_{ATP} channels [1].

We have previously shown that GdCl₃ given 24-72 hours prior to the index ischemic event is also able to attenuate reperfusion injury as determined by infarct size [1]. Interesting, in that study even though GdCl₃ was intravenously administered to the rats *in vivo*, infarct size was determined in isolated buffer perfused hearts subject to IR injury. Since the isolated heart model of IR does not have circulating blood components, the infarcts are not likely solely caused by the inflammatory response. However, it still could be possible that GdCl₃-mediated up-regulation of TNF-α in the myocardium could be part of the mechanism of GdCl₃-mediated delayed cardioprotection. TNF-α was not measured in the delayed cardioprotection studies but this hypothesis forms the basis of further studies.

Gadolinium also attenuated IR-induced expression of the macrophage-secreted cytokines GM-CSF and IL-1 in this study, suggesting that attracted monocytes may not be differentiating into activated macrophages. Treatment with GdCl₃ also did not significantly alter levels of INF-γ and MIP-3 which are activators of macrophage function. In support, we observed a decrease in ANAE activity in the myocardium which suggests decreased infiltration and activation of monocytes into the myocardium. Similarly,

we observed decreased MPO activity in the myocardial tissue which suggests a decrease of neutrophil infiltration into the myocardium although the levels of circulating and tissue IL-8/CINC-1 are not modified by GdCl₃ treatment. This suggests that GdCl₃ may be inhibiting leukocyte chemotaxis and in essence "paralyzing" the monocytes and neutrophils at their storage locations. Swirski et al. have recently identified the splenic subcapsular red pulp as the reservoir for monocytes [26]. From their splenic reservoirs, the monocytes are deployed to inflammatory sites such as the infarcted myocardium [26]. The reservoir of neutrophils is yet to be verified but the neutrophils are thought to reside in the post-capillary venule sites in the periphery such as the bone marrow [27]. Although previous studies have shown that GdCl₃ does not affect the migration of neutrophils [28], inhibition of monocyte migration has not been reported.

One limitation of this study is that we did not study the *in vitro* migration of isolated monocytes or neutrophils treated with GdCl₃. However, the decrease in monocytes and neutrophils into the circulation after GdCl₃ treatment indicates that the leukocytes are either not migrating from their reservoirs or are being scavenged prior to the 120 min reperfusion time point.

In summary, a single dose of GdCl₃ when given prior to ischemia and reperfusion decreased circulating monocytes and neutrophils as well as decreased the infiltration of these leukocytes into the myocardium. Furthermore, this was not associated with a change in tissue levels of the major monocyte chemoattractant MCP-1 or neutrophil chemoattractant IL-8/CINC-1 but was associated with an up-regulation of TNF- α .

List of abbreviations

(GdCl₃): Gadolinium; (IR): Ischemia and Reperfusion; (IL): Interleukin; (CINC): cytokine-induced neutrophil chemoattractant; (GM-CSF): monocyte colony stimulating factor; (INF): Interferon; (MCP): monocyte chemotactic protein; (MIP): macrophage inflammatory protein; (TNF): tumor necrosis factor; (ANOVA): Analysis of Variance.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JLS conceived the study, designed the experiments, performed the cytokine assays, data analysis, and prepared the manuscript. KVR performed the MPO and ANAE assays. AH performed the rat studies needed. ACN contributed to the development of the study and revising the manuscript critically for important intellectual content. JEB supervised the experiments and oversaw manuscript

construction with JLS. All authors read and approved the final manuscript.

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