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# Humanized cobra venom factor decreases myocardial ischemia reperfusion injury

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## Abstract

Cobra venom factor (CVF) is a complement activating protein in cobra venom, which functionally resembles C3b, and has been used for decades for decomplementation of serum to investigate the role of complement in many model systems of disease. The use of CVF for clinical practice is considered impractical because of immunogenicity issues. Humanization of CVF was recently demonstrated to yield a potent CVF-like molecule. In the present study, we demonstrate that mice treated with recombinant humanized CVF (HC3-1496) are protected from myocardial ischemia-reperfusion (MI/R) injuries with resultant preservation of cardiac function. Also, C3 deposition in the myocardium following MI/R was not observed following treatment with HC3-1496. HC3-1496 led to complement activation and depletion of C3, but preserved C5 titers. These data suggest, unlike CVF, HC3-1496 does not form a C5 convertase in the mouse, similar to recent studies in human sera/plasma. These results suggest that humanized CVF (HC3-1496) protects the ischemic myocardium from reperfusion injuries induced by complement activation and represents a novel anti-complement therapy for potential clinical use.



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**Disclosures**

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### Keywords

complement; inflammation; infarction

## Introduction

Myocardial ischemia and reperfusion (MI/R) injury occurs after restoration of blood flow following ischemia in the heart (Walport, 2001b; Walport, 2001a). Complement was shown to be an important contributor to myocardial inflammation and tissue injury following MI/R for 30 years (Hill and Ward, 1971). Protease inhibitors, specific anti-complement biologics, and the use of genetically modified animals demonstrated that complement inhibition decreases inflammation and tissue injury following MI/R (Hill and Ward, 1971; Buerke *et al.*, 1995; Walsh *et al.*, 2005; Weisman *et al.*, 1990; Pinckard *et al.*, 1980). A recent clinical trial also suggests an important role of complement in humans (Armstrong *et al.*, 2006; Granger *et al.*, 2003). Thus, the development of biologics to inhibit complement activation following MI/R may represent a novel cardioprotective mechanism for the clinic.

Cobra venom factor (CVF) is a structural analog of the complement component C3 and is functionally similar to C3b. CVF is used to deplete complement to investigate the role of complement activation in many disease models (Vogel and Fritzinger, 2007; Vogel, 1991). Several studies demonstrated the therapeutic potential of CVF in the setting of MI/R (Hill and Ward, 1971; Pinckard *et al.*, 1980; MacLean *et al.*, 1978; Maroko *et al.*, 1978), but the immunogenicity of this protein, as well as its ability to cleave C5, which generates the potent proinflammatory C5a anaphylatoxin, has not allowed its continuation into clinical development (Gowda *et al*., 1994; Gowda *et al*., 2001). Recently, we generated a human C3 derivative with CVF-like functions by replacing a short stretch of amino acids from the C-terminus of the C3 α-chain with the homologous sequences from CVF. The resulting hybrid proteins, termed "humanized CVF," form a stable C3 convertase similar to CVF, which activates human complement component C3 but not C5 (Vogel and Fritzinger, 2007). Humanized CVF also exhibits partial resistance to the complement regulatory proteins factors H and I, and has been shown to deplete serum complement activity in vitro as well as in rodents and primates (Fritzinger *et al*., 2008a; Fritzinger *et al*., 2009; Fritzinger *et al*., 2008b). In the present study, we tested a humanized CVF protein (HC3-1496) for its ability to protect the mouse myocardium from complement activation and tissue injury following MI/R.

## Material and Methods

All C57BL/6 mice used were 8–12 weeks old, weighed between 20–30g, and came from the Charles River Laboratories. The Institute's Animal Care and Use Committee (IACUC) reviewed all procedures. We performed all experiments in accordance with the IACUC and the standards and principles set forth in the Guide for the Care and Use of Laboratory Animals (revised 1996).

### Proteins for Decomplementation

CVF was prepared from lyophilized venom from *N. kaouthia* as previously described (Vogel and Muller-Eberhard, 1984). HC3-1496 is a human C3/CVF hybrid protein containing a 168 amino acid residue substitution of CVF sequence at the C-terminus of the α-chain of C3 (humanized CVF). The plasmid preparation, protein expression, and purification were performed essentially as previously described (Fritzinger *et al.*, 2009; Vogel and Fritzinger, 2007).

### Experimental myocardial ischemia-reperfusion (MI/R) model

C57BL/6 mice were treated with either 100μl of intra-peritoneal (IP) PBS, 250μg/kg of the recombinant humanized CVF (HC3-1496) or 250μg/kg purified CVF in 100μl of PBS two hours prior to induction of anesthesia. Experimental MI/R was performed as previously published (Walsh *et al.*, 2005; Busche *et al.*, 2008). Briefly: mice were intubated, ventilated, and anesthesia maintained with isoflurane. The chest was opened and a suture was placed around the left anterior descending coronary artery (LAD) and tightened. After 30 minutes of ischemia, the ligation was loosened and the myocardium reperfused for 4 hours. An electrocardiogram (modified lead III) was evaluated before, during, and after ischemia and was used to verify ischemia and reperfusion. Left ventricular discoloration/dyskinesis and its reversal were also visualized for additional documentation of ischemia and reperfusion, respectively.

### Transthoracic Echocardiography (TTE)

Echocardiography (Philips Sonos 5500; Philips Medical Systems, Bothell, WA, USA) was performed (7–12 MHz animal transducer; Agilent Technologies, Santa Clara, CA, USA) following MI/R to assess cardiac function. We previously demonstrated myocardial injury via histological infarct analysis is directly correlated to loss of cardiac function as measured by echocardiography (Busche *et al.*, 2008; Walsh *et al.*, 2005). Ejection fraction (EF) was calculated via left ventricular M-mode measurements as well as by 2D imaging via long and short axis area measurements of the left ventricle (LV) (Sahn *et al.*, 1978; Kenchaiah *et al.*, 2004). Only M-mode data for EF are present, as both methods produced identical results, which we previously published (Busche *et al.*, 2008).

### Measurement of infarct size and area at risk (AAR)

Following reperfusion, a median sternotomy was performed and the LAD ligation suture re-tightened. A right oblique laparotomy was performed, the inferior vena cava isolated, 200μg of heparin administered and the vena cava transected for exsanguination. Then, vessels of the aortic arch were ligated, the descending aorta partially transected, polyethylene 10 tubing inserted, and 100–200μl of 1% Evans Blue injected for antegrade perfusion and negative staining of the AAR as we described (Busche *et al.*, 2008; Walsh *et al.*, 2005). Hearts were excised and cross-sectioned into 1-mm slices using a coronal acrylic matrix (Roboz). Sections were placed into 6-well plates (Costar) and incubated in 1% triphenyltetrazolium chloride (TTC) at 37°C for 15 minutes as we previously published (Walsh *et al.*, 2005). Following TTC staining, sections were fixed in formalin, imaged, and analyzed using a Nikon SMZ800 stereoscopic zoom microscope and SPOT imaging software (Diagnostic Instruments). The myocardial infarct size was determined by calculating the total areas of the LV, as well as the nonischemic and ischemic area within the AAR. Infarct was expressed as a percentage of the total LV area or the AAR.

### Immunochemistry

Following MI/R, some hearts were mounted in Tissue-Tek optimum cutting temperature (O.C.T.) compound, cut into 5μm sections and stained for C3 deposition. Primary and secondary antibodies consisted of goat anti-mouse C3 antibody (MP Biomedicals) and donkey anti-goat IRDye800 (Rockland), respectively. Sections were scanned and quantified by Odyssey (LiCor), as we published and validated previously (Busche *et al.*, 2008; Walsh *et al.*, 2005).

### Functional mouse C5 analysis following CVF or HC3-1496 treatment

CVF is known to lead to the depletion of C3, as well as C5, resulting in production of C5a anaphylatoxin (Vogel, 1991). On the other hand, HC3-1496 does not form a C5 convertase in

human sera (Vogel and Fritzinger, 2007). In order to establish whether C5 was present in mouse sera following PBS, CVF or HC3-146 treatment, sera were collected following the MI/R studies. Human serum (20%, 90 μl) depleted of C5 (Comptech, Tyler, TX) was supplemented with 10 μl of untreated mouse serum or serum from CVF, PBS or HC3-1496 treated mice following MI/R and immediately incubated with sensitized chicken RBCs (30 μl, Colorado Serum, Denver, CO) as described (Vakeva *et al.*, 1998). Cells were separated from the sera after 30 min at 37°C and hemolytic activity was evaluated from serially diluted samples done in triplicate as described (Vakeva *et al.*, 1998) .

### Statistics

All statistical analysis was performed using Sigma Stat software version 3.0 (SPSS). All data were evaluated using one-way ANOVA and post hoc analysis using the Student-Newman-Keuls method. Values are expressed as the mean±SE.

## Results

### Analysis of cardiac function

Following 30 minutes of ischemia and 4 hours of reperfusion, we observed a significant decrease in LV function in WT mice treated with PBS (i.e., vehicle) compared to HC3-1496 or CVF treatment (Figure 1). Utilizing M-mode echocardiography, fractional shortening in the long axis was significantly reduced following MI/R in the mice treated with PBS compared to CVF or HC3-1496 treatment. Similarly, when we evaluated LV ejection fraction by TTE, we obtained similar results (Figure 2). These data demonstrate that complement depletion with HC3-1496 or CVF significantly protected LV function following MI/R compared to PBS treatment.

### Infarct analysis

To investigate if the decrease in LV function was a result of tissue injury, we quantified infarct size. The area at risk (AAR) of ischemia compared to the entire LV was not statistically different between the groups (56±14%, 55±8%, and 48±14% for PBS, CVF, and HC3-1496, respectively). Infarct size, normalized to the LV (Figure 3A) or to the AAR (Figure 3B), was significantly larger in vehicle-treated mice compared to HC3-1496 or CVF treatment. Infarct size in HC3-1496 or CVF treated mice was not significantly different (Figure 3). Thus, infarct size was reduced by complement depletion with HC3-1496 to the same extent as CVF.

### Myocardial C3 deposition analysis

We evaluated complement C3 deposition in mice following MI/R. As shown in Figure 4A, control mice treated with PBS had more myocardial C3 deposition compared to HC3-1496 or CVF treated mice. Figure 4B summarizes quantitative evaluation of C3 deposition. Complement depletion with either HC3-1496 or CVF resulted in significantly less myocardial C3 deposition compared to PBS-treated mice. Thus, complement depletion with CVF or HC3-1496 results in decreased myocardial C3 deposition following MI/R.

### HC3-1496 does not decrease mouse C5 levels

CVF is known to deplete complement through the formation of a stable C3/C5 convertase, which decreases C3 and C5 titers (Vogel, 1991). We recently demonstrated that the humanized CVF does not decrease C5 levels in human or primate sera (Vogel and Fritzinger, 2007; Fritzinger *et al.*, 2008a; Fritzinger *et al.*, 2009). To determine if HC3-1496 has similar properties in mouse serum, serum from PBS, HC3-1496 or CVF-treated mice were used as a source for C5 and added to C5-depleted human serum, which was then subjected to a CH50 assay. As shown in Figure 5, C5-depleted human serum does not result in significant hemolysis

of sensitized chicken RBCs. Addition of 10 μl of normal mouse sera (NMS) or sera from mice treated with PBS or HC3-1496 results in significant hemolytic activity. In contrast, addition of 10 μl serum from CVF-treated mice had virtually no hemolytic activity. These data demonstrate that unlike mice treated with CVF, the amount of C5 in mice treated with HC3-1496 (250μg/kg) does not differ from normal mouse sera. Thus, HC3-1496 does not measurably activate C5 in mouse serum.

## Discussion

Complement depletion by CVF has long been the “gold standard” for the evaluation of complement playing a role in disease. Complement depletion with CVF allows the determination of the role of complement in disease models by generating a C3/C5 convertase that is both physiochemically stable and resistant to the complement inhibitors/proteases present within biological systems, leading to the depletion of these components and a temporary inactivation of the complement system. While CVF has been effective in demonstrating the role of complement in many settings, the immunogenicity of CVF has limited its ability to progress as a clinically viable biologic (Gowda *et al.*, 1994).

CVF was shown to be effective in reducing the infarct size in a number of animal models of MI/R (Hill and Ward, 1971; Maroko *et al.*, 1978; Crawford *et al.*, 1988; Pinckard *et al.*, 1980). In this study, we demonstrate that decomplementation with the humanized CVF protein, HC3-1496, is similarly effective in reducing infarct size in a mouse model of MI/R injury. Previous studies by our laboratory demonstrated that MBL initiates complement activation following I/R, which is then amplified by the alternative pathway leading to tissue inflammation and injury (Jordan *et al.*, 2001; Stahl *et al.*, 2003; Walsh *et al.*, 2005; Hart *et al.*, 2005). Interruption of the complement activation initiation by blocking MBL or inhibition of the amplification by the alternative pathway leads to similar protective actions in animal I/R models (Jordan *et al.*, 2001; Stahl *et al.*, 2003; Walsh *et al.*, 2005; Hart *et al.*, 2005). In the present study, we show that complement depletion via the formation of a stable C3 convertase containing the humanized CVF protein, HC3-1496, leads to similar cardioprotective action that we observed by inhibition of the alternative or MBL-dependent lectin complement pathways (Jordan *et al.*, 2001; Stahl *et al.*, 2003; Walsh *et al.*, 2005; Hart *et al.*, 2005).

While CVF is known to activate complement components C3 and C5, HC3-1496 does not activate human C5 *in vitro* (Vogel and Fritzinger, 2007; Fritzinger *et al.*, 2008a; Fritzinger *et al.*, 2009). In the present study, we developed an *in vitro* assay to test the hypothesis that HC3-1496, unlike CVF, does not activate murine C5. Using C5-depleted human serum, we used the C5 present in normal mouse serum to demonstrate that murine C5 can replace human C5 to lyse sensitized chicken RBCs. The hemolytic activity of C5-depleted human serum could be restored by normal mouse serum, or serum from mice that were treated with HC3-1496 or PBS. In contrast, serum from mice treated with CVF did not restore the hemolytic activity, indicating that little or no C5 was present. Thus, while both HC3-1496 and CVF can attenuate MI/R injury, HC3-1496 does this by only depleting C3 without formation of the potent anaphylatoxin, C5a.

In summary, we demonstrate that a humanized chimeric form of CVF provides similar cardioprotective actions *in vivo* following MI/R in mice. Unlike, CVF, HC3-1496 does not activate C5 and may represent a novel therapeutic biologic for the treatment of complement mediated diseases including myocardial infarction.

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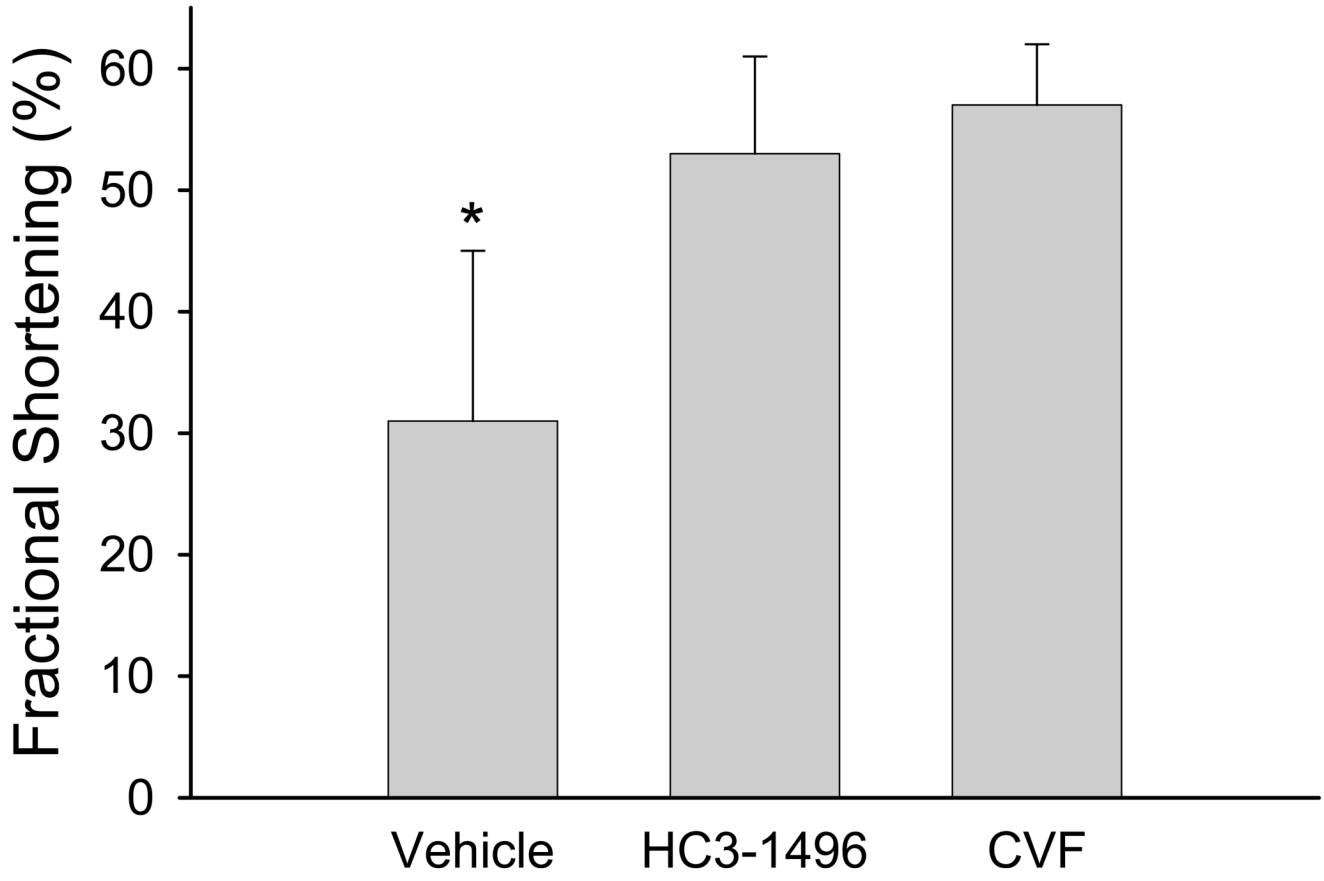


**Figure 1. Left ventricular function following MI/R**

Summary of fractional shortening measured by echocardiography for PBS (Vehicle; n=5), HC3-1496 (n=6), or CVF (n=5) treatment following MI/R. * $p<0.05$ compared to HC3-1496 or CVF. Bars and brackets represent mean±SE.

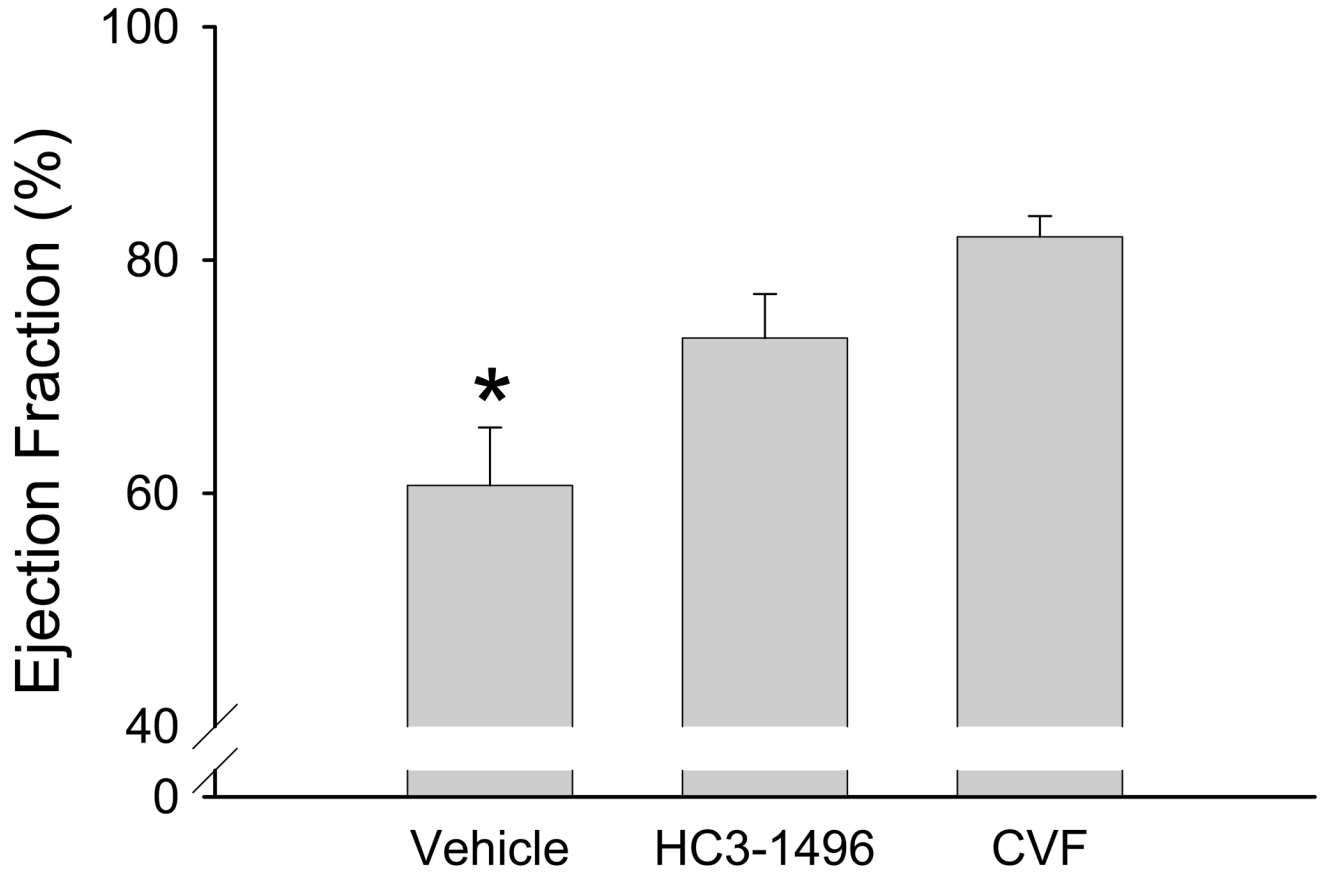


**Figure 2. Left ventricular ejection fraction following MI/R**

Ejection fraction (EF) measured by TTE following MI/R in PBS (Vehicle; n=5), HC3-1496 (n=6), or CVF (n=5) treated mice. *p<0.05 compared to HC3-1496 or CVF. Bars and brackets represent mean±SE.

Figure 3A

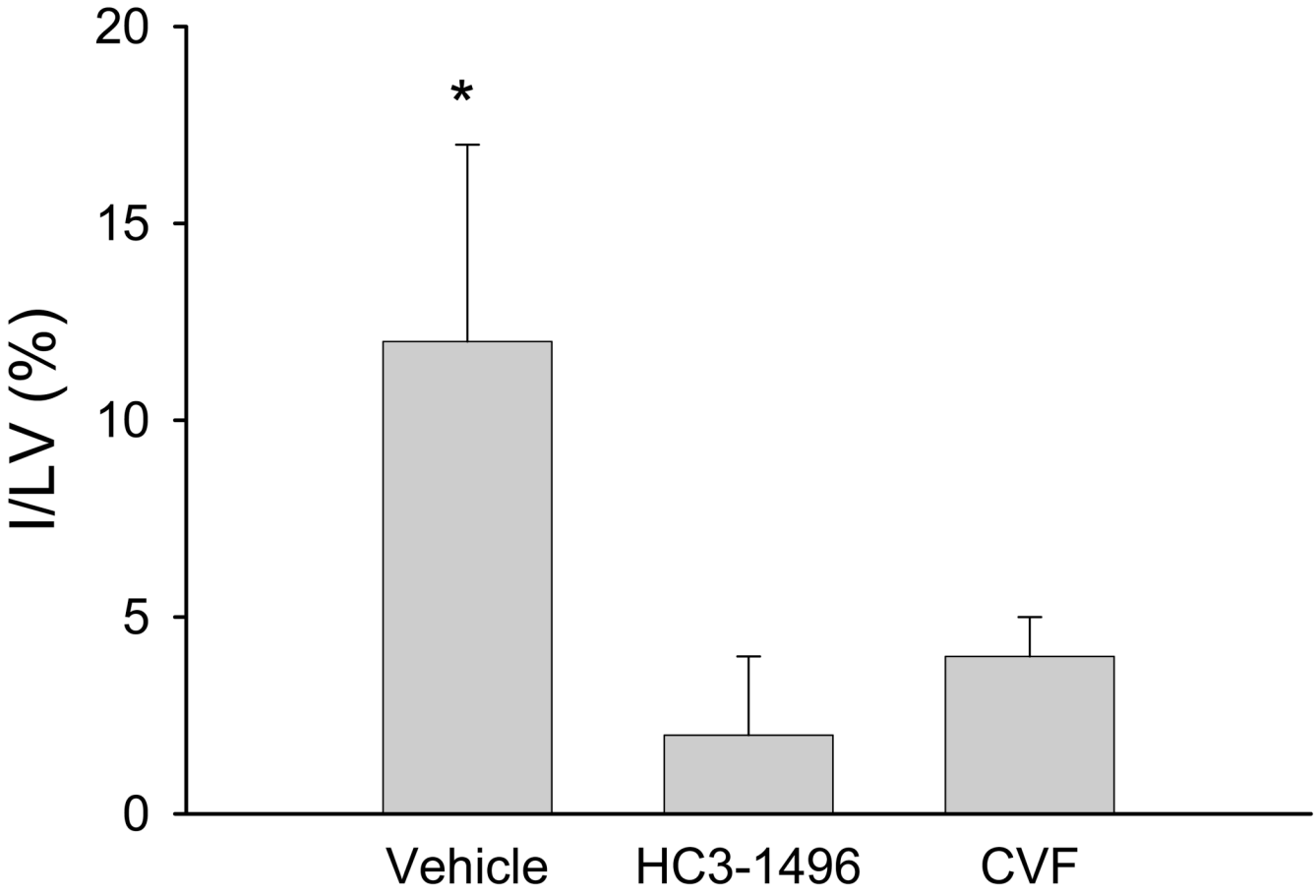

Figure 3B

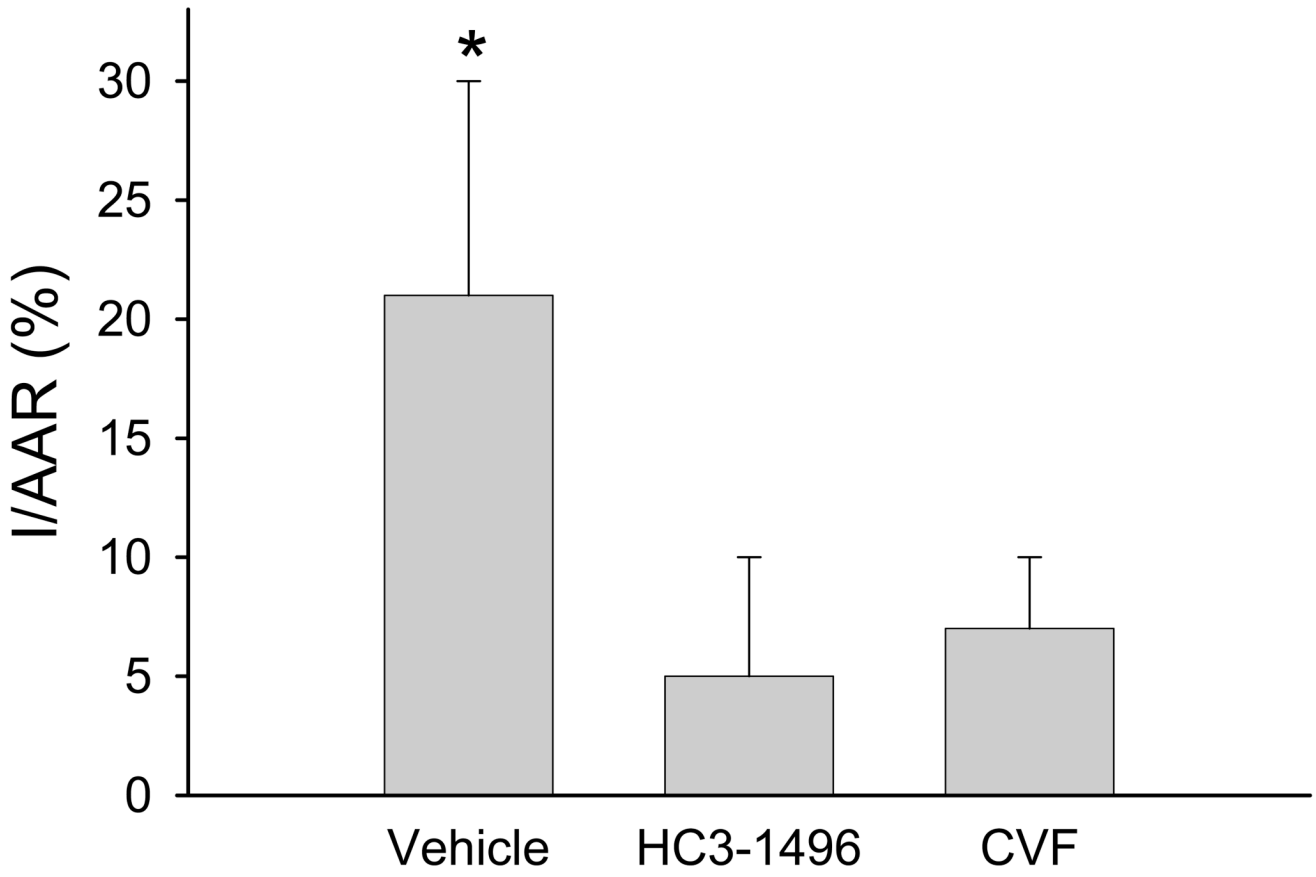


**Figure 3. Myocardial infarct analysis**

Myocardial infarct assessment following MI/R in mice treated with PBS (Vehicle; n=5), CVF (n=5), and HC3-1496 (n=5).

Figure 3A. Infarct size to total left ventricle area. *p<0.001 compared to CVF or HC3-1496.

Figure 3B. Infarct size to area at risk. *p<0.002 compared to CVF or HC3-1496. Bars and brackets represent mean±SE.

Figure 4A

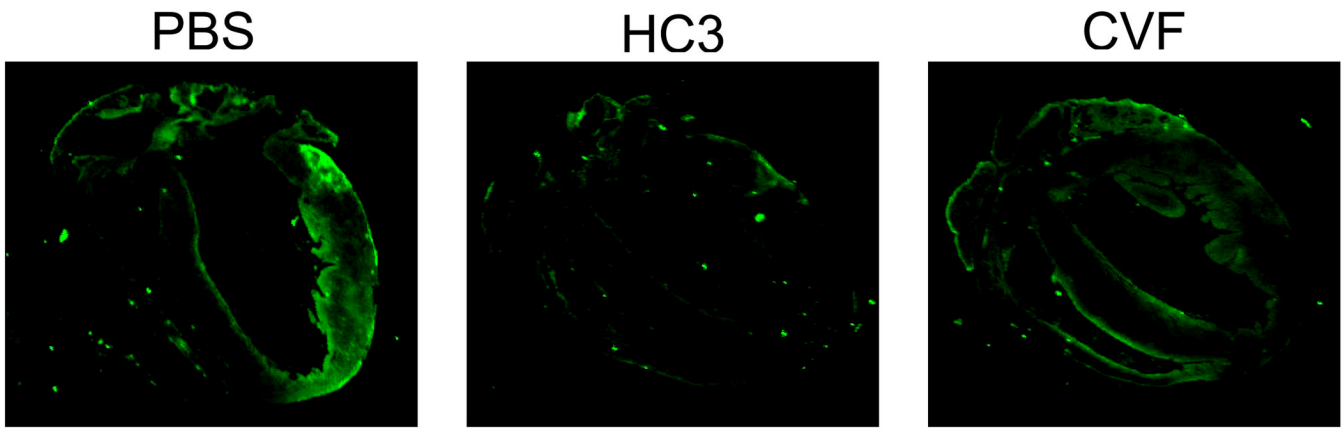

Figure 4B

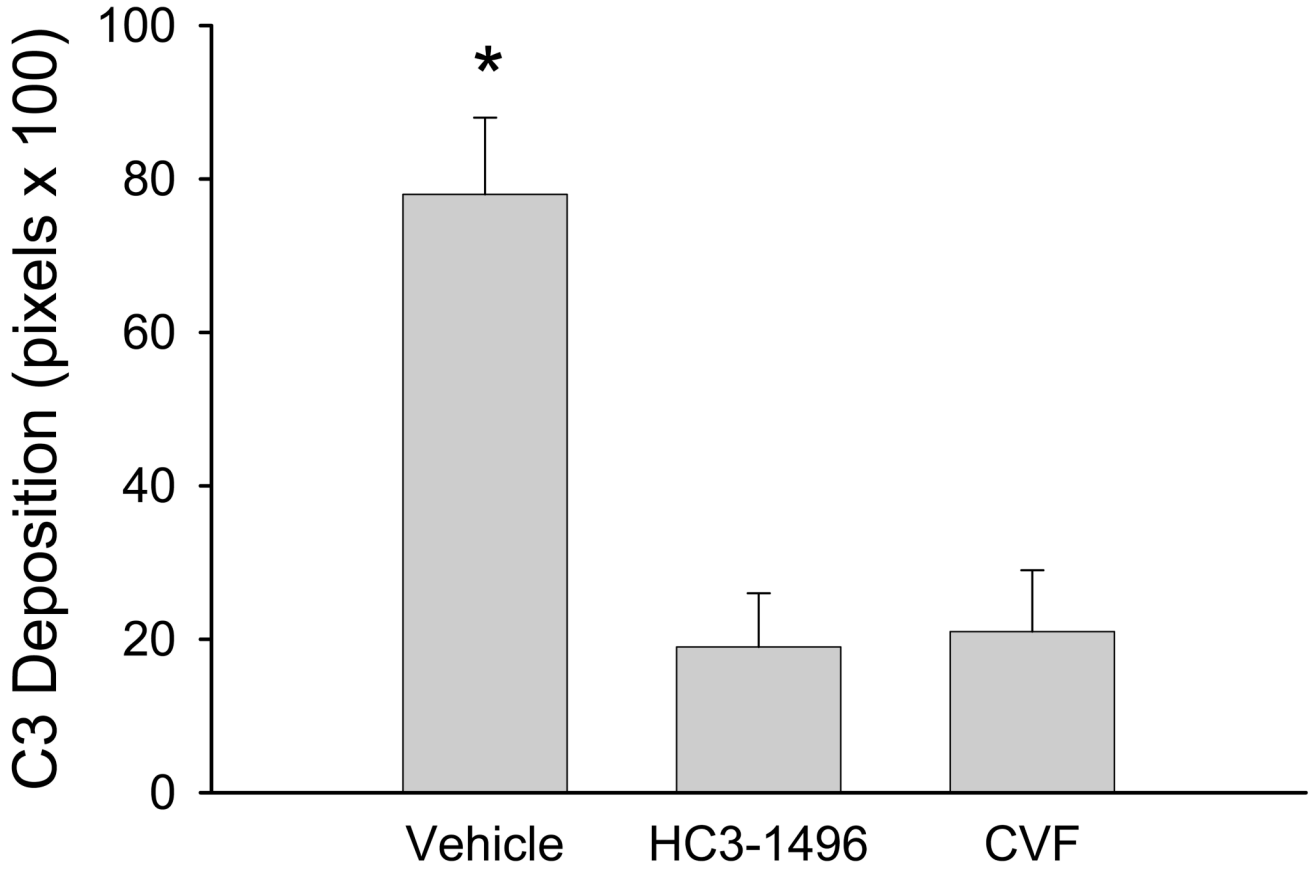


**Figure 4. Myocardial C3 deposition following MI/R**

Figure 4A. Representative myocardial C3 deposition following MI/R in PBS (Vehicle; n=3), HC3-1496 (n=3), or CVF (n=3) treated mice.

Figure 4B. Quantitative summary of myocardial C3 deposition. *p<0.001 compared to CVF or HC3-1496. Bars and brackets represent mean±SE.

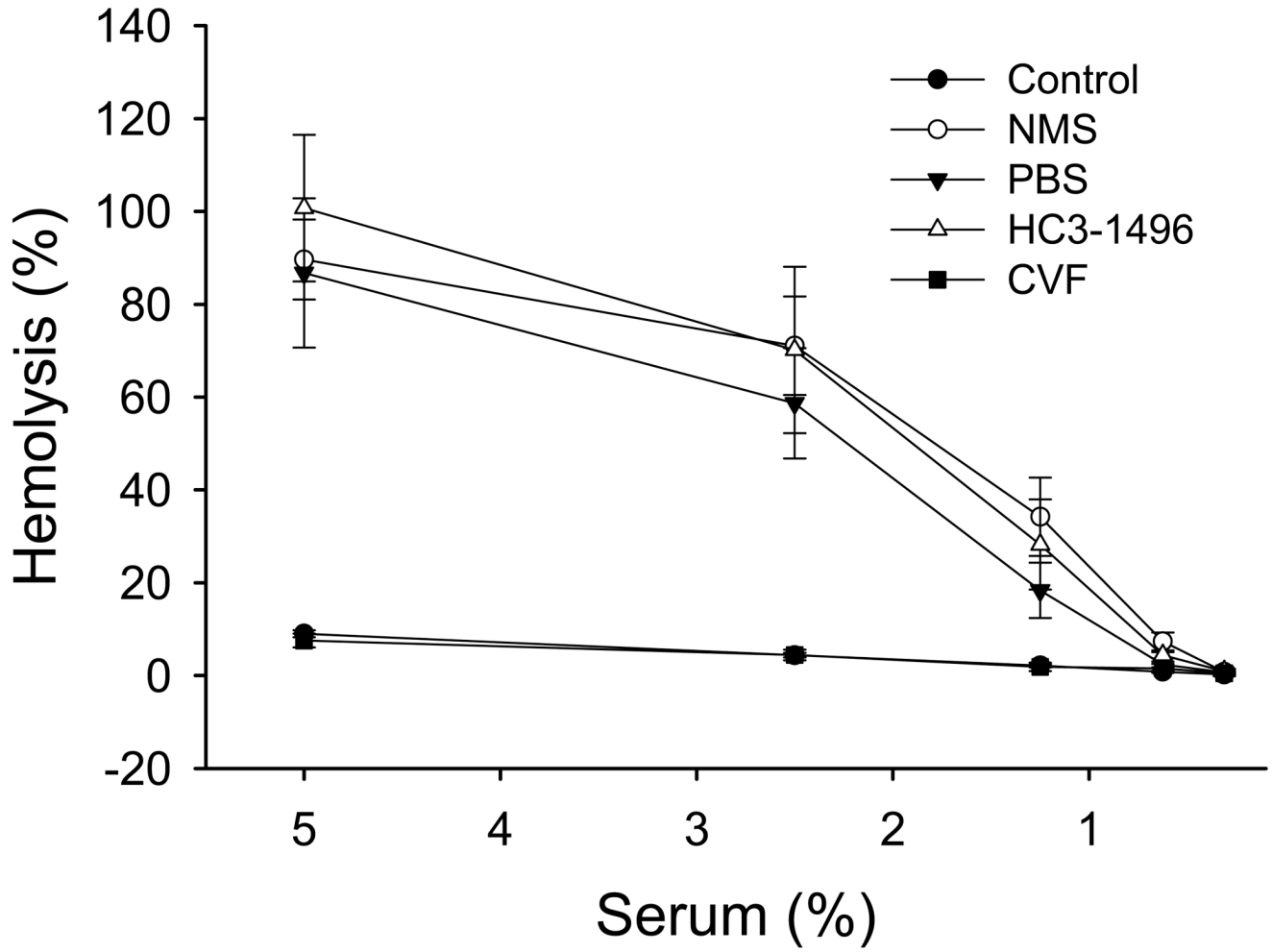


**Figure 5. Preservation of murine C5 following HC3-1496 treatment in vivo**

Mouse sera were collected from mice treated with PBS, HC3-1496 (250μg/kg), or CVF (250μg/kg) following MI/R. Human serum depleted of C5 (Control) was used to lyse sensitized chicken RBCs following addition of 10μl of PBS (Control), normal mouse serum (NMS) or serum from mice treated with PBS, CVF or HC3-1496. Symbols and brackets represent mean ±SE from 3 separate experiments per group.