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Protective Mechanisms of a Metalloporphyrinic Peroxynitrite Decomposition Catalyst, WW85, in Rat Cardiac Transplants

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Running title: Peroxynitrite Decomposition and Allotransplantation

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Abbreviations: inducible nitric oxide synthase (iNOS); International Society for Heart and Lung Transplantation (ISHLT); mitochondrial permeability transition (MPT); poly(ADP-ribose) polymerase (PARP); posttransplant day (POD); reverse transcriptase polymerase chain reaction (RT-PCR); tumour necrosis factor α (TNFα)

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Abstract

NO derived from iNOS has been implicated in cardiac rejection. However, little is known about the role of the reactive nitrogen species, peroxynitrite. We examined the protective actions of a peroxynitrite decomposition catalyst WW85 in an experimental model of acute cardiac rejection. Heterotopic, abdominal transplantation of rat donor hearts was performed. Groups included: isografts, allografts or allografts treated with either WW85, cyclosporine or cyclosporine + WW85. We determined graft survival, histological rejection and graft function (by *in situ* sonomicrometry). Intragraft biochemical analysis of cytokines, pro-apoptotic and anti-apoptotic gene expression using RT-PCR were determined. Treatment with WW85 or cyclosporine alone prolonged graft survival, improved graft function and decreased histological rejection. Graft survival was further significantly ($P<0.001$) enhanced by combination treatment. A decrease was also shown in nitrotyrosine, PARP activation and lipid peroxide formation by WW85 that was potentiated when given in combination with cyclosporine. Benefits could not be ascribed to changes in intragraft myeloperoxidase activity. Only combination therapy produced significant decreases in inflammatory cytokine gene expression suggesting that WW85 acted primarily downstream of these stimuli. In general, WW85 had no direct action on expression of the pro-apoptotic gene, Fas ligand; however, WW85 given alone or with cyclosporine enhanced expression of anti-apoptotic genes, Bcl-2 and Bcl-xL. Collectively, these findings suggest a protective action of the peroxynitrite decomposition catalyst, WW85, on graft rejection that is independent of any action on leukocyte sequestration and cytokine gene expression. Rather, effects appear to be downstream on decreased protein nitration, decreased lipid peroxidation and decreased PARP activation.

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Introduction

Nitric oxide (NO) formed by the inducible NO synthase (iNOS) has been implicated in cardiac rejection. However, the nature of this action at the molecular level is not fully understood. Previous attempts to understand the role of iNOS in cardiac rejection have utilized enzyme inhibitors for iNOS or gene deletion strategies. Another strategy to protect the myocardium in cardiac rejection is to influence downstream actions of NO derived from iNOS rather than to alter iNOS expression or NO bioactivity. Among these potential downstream pathways is that derived via NO-derived peroxynitrite formation. Peroxynitrite is formed by the reaction of NO and superoxide anion radical. Potential biological down-stream actions of peroxynitrite include: cardiac depression, lipid peroxidation, nitration of tyrosine residues on proteins, activation of poly(ADP-ribose) polymerase (PARP) and apoptosis.

Peroxynitrite formation has been suggested based upon increased nitrotyrosine staining in experimental cardiac rejection in rats (Sakurai et al., 1999). Apoptosis of cardiac myocytes in acute cardiac rejection was associated with iNOS and nitrotyrosine suggesting that reactive nitrogen species (i.e. NO or peroxynitrite) may contribute to apoptosis and graft failure (Szabolcs et al., 1996). Furthermore in human cardiac transplantation, evidence of increased apoptosis was more likely to be associated with increased cardiac dysfunction (Birks et al., 2000). Clinically, the importance of peroxynitrite formation was also suggested from previous studies showing increased immunostaining for nitrotyrosine in biopsies of human cardiac grafts with Grade III rejection scores but not in biopsies with Grade 0 histological rejection scores (Szabolcs et al., 1998). In a recent study, we have shown that neutralizing NO directly limits the

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extent of protein nitration in rat cardiac transplants and prolongs graft survival (Pieper et al., 2004). Despite strategies to limit NO bioactivity to protect cardiac transplants, there has not been to date a direct strategy to evaluate protection of grafts following transplantation by limiting the actions of peroxynitrite.

Recently, it has been recognized that water-soluble iron (III) porphyrin derivatives are highly-reactive towards peroxynitrite with rate constants as high as 5.0 x $10^7 M^{-1}s^{-1}$ (Shimanovich and Groves, 2001). These agents catalyze the isomerization of peroxynitrite resulting in its decomposition to the less reactive anion, nitrate, thereby, decreasing the levels of the potent oxidizing and nitrating species, peroxynitrite. In previous studies, iron (III) metalloporphyrin-based peroxynitrite decomposition catalysts including 5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III] or FeTPPS and FeCl tetrakis-2-(triethylene glycol monomethyl ether) pyridyl porphyrin or FP15 have been shown to have protection in models of myocardial infarction (Bianchi et al., 2002), cytokine-induced (Ferdinandy et al., 2000) or doxorubicin-induced (Pacher et al., 2003) or endotoxin-induced (Lancel et al., 2004) cardiac dysfunction.

In the present study, we examined the anti-rejection, anti-inflammatory and anti-apoptotic mechanisms of action of the novel metalloporphyrinic peroxynitrite decomposition catalyst, WW85, in experimental cardiac transplantation.

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Methods

Animal model and treatments. Lewis (Lew: $RT1^{l}$) and Wistar-Furth (WF:$RT1^{u}$) rats were chosen to represent genetic disparity at both the major and minor histocompatibility loci for donor-to-recipient combinations of Lew → Lew (isografts) or WF → Lew (allografts). Heterotopic transplantation of donor hearts to the abdominal aorta and vena cava of recipient rats was performed by established microsurgical techniques as described (Ono and Lindsay, 1969). Allograft recipients were either untreated or received 0.3 or 1.0 mg/kg WW85 (i.p. three times daily) or 2.5 mg/kg daily of cyclosporine (i.p.) or a combination of WW85 + cyclosporine beginning the day of surgery until the day of harvesting. Graft survival was monitored twice daily for presence or absence of palpable activity and was confirmed upon direct inspection following laparotomy. In another set of experiments, the protocol was terminated on posttransplant day 6 (POD6). At this time, cardiac graft function quantitated *in situ* using sonomicrometry from ultrasonic crystals placed on the external surface of the heart at the mid-level to determine short-axis dimension/graft function (Sonometrics Corp., London, Ontario, Canada). Grafts were then harvested for histological rejections scoring, biochemical analysis and gene expression analysis. This study conformed with the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health.

Histological Rejection Scoring. Tissue was harvested from grafts at the indicated postoperative days upon arrest and flushing with cold, University of Wisconsin solution and used for either biochemical analysis or histological examination. For histology, tissue was fixed initially in 4% phosphate-buffered formalin. Paraffin-embedded sections were stained with hematoxylin and eosin. Histological rejection was scored blinded using

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criteria established by the International Society for Heart and Lung Transplantation (ISHLT) as modifed to a linear score system to allow statistical analysis and as described previously (Pieper et al., 2002; Szabolcs et al. 2002).

Immunostaining for nitrotyrosine and poly (ADP-ribose). Tissue sections were deparaffinized and rehydrated by passing through xylene and a graded series of ethanol. Antigen retrieval was performed for 20 min in sodium citrate buffer (pH 6.4) in a microwave oven. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% H_2O_2 for 15 min. After 30 min of incubation in normal goat serum, tissue sections were incubated with chicken polyclonal antibody against poly (ADP-ribose) (1:250; Tulip BiolLabs, Inc.) or rabbit polyclonal antibody against nitrotyrosine (2.5 μg/ml; Upstate) overnight and then incubated with biotinylated secondary antibody and avid-biotin-peroxidase complex kit (Vector Laboratories). Color was developed using Ni-diaminobenzidine. The section was counterstained with nuclear fast red (Xiao et al., 2004).

Malondialdehyde Assay. To quantify lipid peroxidation of cardiac grafts, we performed assays of thiobarbituric acid-reactive material. Tissues were homogenized as processed as described (Szabo et al., 2002).

Myeloperoxidase Assay. To quantify neutrophil accumulation within cardiac grafts, myeloperoxidase activity was assayed in cardiac homogenates. Tissues were homogenized (50 mg/ml) in 0.5% hexadecyltrimethylammonium bromide in 10 mM 3-(*N*-morpholino)propanesulfonic acid and the homogenates were centrifuged at 15,000 x g for 40 min. An aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM H_2O_2. Activity was measured spectrophotometrically as

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the change in absorbance at 650 nm in a Spectramax microplate reader (Spectra MAX 250, Molecular Devices). Results are expressed as mU activity/mg protein (Szabo et al., 2002).

Gene Expression. Grafts were harvested by arresting and flushing with cold University of Wisconsin solution, minced and frozen in liquid nitrogen. Tissues were stored at -80°C for RT-PCR. Total RNA was purified from approximately 60 mg of frozen tissue using the Promega SV total RNA isolation kit (Promega, Madison, WI). Total RNA was purified from approximately 60 mg of frozen tissue using the Promega SV total RNA isolation kit (Promega, Madison, WI). One μg RNA was reverse transcribed using the Invitrogen Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA) with oligo (dT) primers. Briefly, 1 μL of cDNA was mixed with 25 pmol of each primer and Invitrogen supermix (Invitrogen, Carlsbad, CA) to a volume of 25 μL and incubated in an Applied Biosystems Gene Amp® PCR System 9700 (Foster City, CA) under the following conditions: for iNOS; 94°C (60s), 60°C (60s), 72°C (60s) for 30 cycles; for interferon-γ; 95°C (30 s), 60°C (30 s) and 72°C (60 s) for 35 cycling times; for interleukin-6 and interleukin-10 and TNF-α; 95°C (30s), 60°C (30 s) and 72°C (60 s) for 35 cycling times, for Bcl-2 and Bcl-xL, 95°C for 5 min, 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute for 30 cycles, and 37 cycle, respectively, and for Fas ligand, 95°C for 5 min, 95°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute for 31 cycles. The PCR products were resolved on a 1% agarose gel and visualized with ethidium bromide staining followed by densitometry and normalized to a β-actin housekeeping gene antibody. Immunoreactive protein was visualized using enhanced chemiluminescence.

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Data. Results were expressed as the mean ± S.E.M. Statistical analysis included ANOVA with Student Newman Keuls test for multiple group means or *t*-test for differences between two group means. Significance was set at $P<0.05$.

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Results

Cardiac allograft survival was significantly (P<0.001) enhanced by treatment with both 0.3 mg/kg or 1.0 mg/kg WW85 doses comparable to that seen with low-dose cyclosporine (Figure 1). As there was no difference in the prolongation of graft survival between these two doses, all subsequent studies were performed in recipients treated with the lower dose of WW85. When this dose was given concurrently with cyclosporine, there was a significant (P<0.001) further enhancement in graft survival greater than that achieved by low dose cyclosporine or WW85 given singly (Figure 1).

In other studies, untreated and treated recipients were examined at postoperative day 6 (POD6) for *in situ* graft function using sonomicrometry and then grafts were harvested for histological rejection scoring. Heart rate was decreased in allografts compared to isograft controls (Figure 2). In addition, both end diastolic and end systolic transverse segment lengths were enhanced with decreased % fractional segment shortening in untreated allografts. Diastolic and systolic dimensions were normalized by WW85 or cyclosporine alone and by combination treatment with WW85 plus cyclosporine. % fractional segment shortening and heart rate were increased by either cyclosporine alone or in combination with WW85.

Using a modifed, linearized ISHLT rejection scoring system, we found a significant (P<0.01) increase in histological rejection scores in untreated allografts vs. isograft controls (Figure 3). Both cyclosporine or WW85 significantly (P<0.05) decreased histological rejection compared to untreated allografts.

Both nitrotyrosine and PAR staining were enhanced in cardiac allografts and nitrotyrosine staining was less frequent in recipients treated with either cyclosporine or

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WW85 alone and essentially eliminated in recipients receiving combination treatment (Figures 4 and 5). To show efficacy of WW85 on downstream actions of peroxynitrite on lipid peroxidation in graft tissue, we determined lipid peroxidation by malondialdehyde levels in the various groups. Malondialdehyde levels were significantly ($P<0.001$) enhanced in untreated cardiac allografts vs. isografts (Figure 6). Treatment with WW85 produced a significant ($P<0.001$) inhibition of malondialdehyde while the increase in malondialdehyde were completely blocked by either cyclosporine alone or in combination with WW85. Myeloperoxidase activity was enhanced in allografts vs. isografts. Myeloperoxidase activity in recipients treated with either cyclosporine or cyclosporine plus WW85 was not significantly different from the untreated group (Figure 6).

We next examined iNOS and inflammatory cytokine gene expression normalized to β-actin in untreated and treated recipient groups. Expression for iNOS was increased in allografts but was not altered by treatment with the peroxynitrite decomposition catalyst, WW85 (Figure 7). In contrast, cyclosporine or cyclosporine plus WW85 partially attenuated iNOS gene expression. Likewise, interferon-γ, interleukin-6 and interleukin-10 gene expression was not altered by treatment with WW85 alone while decreases were seen only with combination treatment with cyclosporine plus WW85 (Figures 7).

To examine pro-apototic gene expression, we examined Fas ligand and TNFα gene expression. The increase in Fas ligand gene expression in allografts was not altered by treatment with WW85 or cyclosporine alone or cyclosporine plus WW85 (Figure 8). To examine anti-apoptotic gene expression, we examined Bcl-xL and Bcl-2. Expression

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for Bcl-xL was not altered in allografts vs. isograft controls (Figure 8). However, both WW85 or cyclosporine alone increased expression of Bcl-xL but this action was lost in combination treatment. In contrast, expression of the anti-apoptotic gene, Bcl-2 was decreased in cardiac allografts compared to isograft controls (Figure 8). The decrease in Bcl-2 gene expression was unaltered by WW85 or cyclosporine alone while the decrease of Bcl-2 expression was reversed by combination treatment with cyclosporine plus WW85.

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Discussion

The present study showed that treatment with the peroxynitrite decomposition catalyst WW85 decreased lipid peroxidation, increased anti-apoptotic gene expression, decreased PARP activation, decreased nitrotyrosine, and improved allograft survival and function. These findings collectively suggest that peroxynitrite plays a role in the pathology of acute cardiac transplant rejection. Previous studies have focused on the role of NO derived from iNOS in cardiac transplant rejection but have not examined the potential role of other reactive nitrogen species such as peroxynitrite. Our studies suggest that downstream actions of iNOS-dependent peroxynitrite might explain some of the effects previously attributed to by NO.

Peroxynitrite is a potent nitrating species and initiator of lipid peroxidation and apoptosis. Traditionally, immunodetection of nitrotyrosine has been used as evidence of peroxynitrite formation in biological tissue. More recently, it has been shown that protein nitration may, under some conditions, arise independently of peroxynitrite via the action of MPO and nitrite (Sampson et al., 1998; Eiserich et al., 1998; Gaut et al., 2002). While the precise pathways contributing to nitration in acute cardiac rejection have not yet been determined with certainty, a role of iNOS in nitrotyrosine formation has been indicated.

Protein Nitration and Peroxynitrite in Acute Cardiac Transplant Rejection. Studies using a selective iNOS dimerization inhibitor showed decreased nitrotyrosine levels and apoptosis (Szabolcs et al., 2002). Since this intervention also decreased iNOS expression, it is possible that decreased nitration resulted secondarily from decreased production of NO, a substrate for peroxynitrite formation. Other studies from the same group using iNOS knockout mice showed decreased apoptosis and lack of nitrotyrosine

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formation when $iNOS^{-/-}$ donor and recipients were used compared to presence of nitrotyrosine when $iNOS^{+/+}$ allografts were used (Szabolcs et al., 2001). These findings are complemented by our studies showing that limitation of NO by two mechanisms a) by inhibiting iNOS activity but not expression and b) decreasing iNOS expression by immunosuppressant therapy both decreased protein nitration (Pieper et al., 2004). Together, these findings support a role of iNOS in nitrotyrosine formation in acute cardiac allograft rejection.

On the other hand, one cannot exclude the possible nitration of protein via iNOS-dependent but peroxynitrite-independent pathways. Indeed, myeloperoxidase in the presence of nitrite and H_2O_2 can cause nitration of proteins (Sampson et al., 1998). Based upon our current knowledge, it cannot be excluded that nitrite derived from increased iNOS activity could contribute to peroxynitrite-independent protein nitration in our model of cardiac allograft rejection.

We found that treatment with WW85 alone limited nitrotyrosine formation but not myeloperoxidase activity. This contrasts with previous findings using iron-based metalloporphyrinic agents as peroxynitrite decomposition catalysts which resulted in decreased myocardial myeloperoxidase activity in septic rats (Lancel et al., 2004) and decreased myeloperoxidase activity in lung reperfusion injury (Naidu et al., 2003). These discrepancies are possibly related to differences in the type of catalyst used or differences in experimental models. Nevertheless, our findings using a peroxynitrite decomposition catalyst are significant in providing the first known evidence suggesting that myeloperoxidase-derived nitration is probably not a major source of nitrotyrosine

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formation in this transplant model. Rather, it is more likely that nitrotyrosine derives predominately from a peroxynitrite-dependent pathway.

PARP Activation. Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme that is activated by single strand DNA breaks. This enzyme is increased in tissue injury and catalyzes the transfer of ADP-ribose subunits to proteins associated with DNA damage. Peroxynitrite is a potent activator of DNA strand breaks and PARP activation (Virág and Szabó, 2002; Szabó 2003). Detection of poly (ADP-ribose) is used to indicate PARP activation.

PARP activation has been shown after reperfusion injury in rat cardiac transplants (Szabo et al. 2002) or following alloimmune activation and rejection in rat tracheal (Farivar et al., 2004) and cardiac allografts (Liu et al., 2004). The observation that 5-aminoisoquinoline, an inhibitor of PARP, attenuated rejection scores and improved graft survival in a rodent model of cardiac allograft rejection suggests the importance of PARP activation (Liu et al., 2004). In our study, we found that WW85 given alone decreased poly (ADP-ribose) suggesting that it acted, in part, by decreasing PARP activation. To our knowledge this is the first suggestion that PARP activation in acute cardiac rejection may be related, at least in part, to peroxynitrite formation.

FasL Expression. Expression of CD95 antigen ligand or Fas ligand is chemotactic for neutrophils (Ottonello et al., 1999) and is one potential pathway for apoptosis during alloimmune activation in transplanted organs. Upregulation of Fas ligand has been shown in a variety of studies of cardiac allograft rejection. The precise molecular events in upregulation of Fas ligand are incompletely understood.

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The specific regulatory role of NO or NO-derived species on Fas ligand expression has not been developed in any detail. Studies in iNOS knockout mice suggest that NO does not appear to alter Fas ligand expression in cardiac transplant rejection (Köglin et al., 1999). Whether or not peroxynitrite regulates Fas ligand expression is unknown. We found that Fas ligand expression was dramatically increased in allografts vs. isograft controls. Furthermore, we found no action of WW85 used alone or in combination with cyclosporine on Fas ligand expression. Since, FasL is a chemoattractant for circulating leukocytes, the finding of unchanged Fas ligand expression after treatment with WW85 is consistent with our other finding that WW85 did not limit myeloperoxidase activity, an index of intragraft neutrophil sequestration. Taken together, these data suggest that WW85 provides benefits distally to these stimulatory pathways.

Inflammatory Cytokine Gene Expression. Alloimmune activation was associated with upregulation of interferon-γ, interleukin-6 and interleukin-10. Previously, we showed that agents that inhibited inflammatory cytokine gene expression promote increased graft survival and/or improved graft function (Pieper et al., 2002; Pieper et al., 2005). In the present study, we found that cytokine gene expression was unaltered by treatment with WW85 suggesting that benefits cannot be ascribed to modulation of inflammatory cytokine gene expression. In contrast, gene expression was decreased or prevented (depending on the specific gene) when WW85 was given with cyclosporine. In general, this decrease was greater than for cyclosporine alone. This anti-inflammatory activity could explain its beneficial actions on graft function and survival used in combination therapy.

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Pro- and Anti-Apototic Gene Expression. We found that cytokine gene expression and FasL (stimulants for apoptosis) were unchanged by treatment with WW85 alone. Apoptosis is also regulated by anti-apoptotic factors. In this context, increases in Bcl-2 and Bcl-xL have been argued to counteract apoptosis in cardiac transplant models. While oxidants such as H_2O_2 can induce Bcl-xL expression in cardiac myocytes (Valks et al., 2003), our current understanding of the signaling molecules responsible for regulating expression of these anti-apoptotic genes in cardiac rejection are incomplete.

Likewise, the effect of reactive nitrogen species on anti-apoptotic genes is not well understood. Deletion of iNOS gene in mouse cardiac transplants increased expression of both Bcl-2 and Bcl-xL (Köglin et al., 1999). These findings suggest that NO may limit anti-apoptotic gene expression. However, it is unclear whether this is a direct consequence of limiting the actions of NO or secondarily to downstream peroxynitrite formation. Our study may shed some new understanding on this possibility.

Specifically, we found that the peroxynitrite decomposition catalyst WW85 had a discriminatory effect on anti-apoptotic gene expression depending on the specific gene and/or condition. Alloimmune activation decreased Bcl-2 expression while treatment with WW85 alone did not alter this decreased expression. In contrast, alloimmune activation did not alter expression of Bcl-xL but treatment with WW85 caused a marked increase in expression of this anti-apoptotic gene. The increase in Bcl-xL expression by WW85 was lost in combination therapy with cyclosporine; however this action was countered by findings that combination therapy prevented the decrease in Bcl-2 expression in allografts. Collectively, these studies indicate that peroxynitrite

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decomposition catalysts may prove beneficial, in general, by stimulating anti-apoptotic gene expression.

High Ca^{2+} induces mitochondrial permeability transition (MPT) pore opening that is enhanced in myocytes isolated from allografts vs isografts (Raisky et al., 2004). While cyclosporine *ex vivo* can inhibit MPT pore opening, these authors showed that a non-immunosuppressive analog of cyclosporine inhibited the MPT pore opening but did not alter rejection. They concluded that acute rejection occurs upstream of cardiomyocyte apoptosis and that inhibiting MPT opening may provide a mechanism to prevent actual graft failure. In addition to high Ca^{2+}, reactive oxygen and peroxynitrite trigger MPT pore opening in liver mitochondria *ex vivo* (Brookes and Darley-Usmar, 2004). Based upon these findings, we cannot exclude the possibility that WW85 might provide cardiac protection by a mechanism involving inhibition of peroxynitrite-mediated MPT pore opening leading to diminished apoptosis.

Overall, our findings demonstrate that neutralization of peroxynitrite, when WW85 is applied together with low-dose cyclosporine therapy, prolongs the life of cardiac allografts and inhibits the activation of many cytotoxic pathways of injury (including tyrosine nitration and PARP activation). This is likely to produce a down-regulation of the subsequent immunological and inflammatory response which may be responsible for the benefits of this approach. By allowing a reduction in the amount of cyclosporine used for transplant therapy, co-application of a peroxynitrite catalyst at the early stages of transplant rejection may be a useful approach to limit the cyclosporine-induced side-effects.

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Footnotes:

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Figure legends

Figure 1. Increase in cardiac allograft survival by treatment with either WW85 or low-dose cyclosporine (CsA) alone and with CsA + WW85 (n=10-12 each group). ¶P<0.001 vs. untreated; (¶)P<0.001 vs groups B and C

Figure 2. Improvement by treatment with either WW85 or low-dose CsA alone and with a combination (Comb) of WW85 + CsA *in situ* graft function at POD6 (n =5 each group) determined by sonomicrometry. *P<0.05 vs. isograft (iso)

Figure 3. Effect of drug treatments on histological rejections scores in grafts (n =5 each) harvested at POD6. ‡P<0.01 vs. isograft (iso); (*)P<0.05 vs. untreated allografts (allo).

Figure 4. Decrease in PARP activation determined by poly(ADP)-ribose staining in recipients treated with either WW85 or CsA alone or WW85 + CsA in combination (Comb). Examples shown are representative of n=3-4 grafts each.

Figure 5. Decrease in nitrotyrosine staining in recipients treated with either WW85 or CsA alone or in combination (Comb). Examples shown are representative of n=3-4 each.

Figure 6. Effect of treatment (n = 5 each group) with WW85, low-dose CsA or WW85 + CsA on intragraft malondialdehyde (MDA) levels (upper panel) or myeloperoxidase (MPO) activity (lower panel). *P<0.05, ¶ and ‡P<0.001 and P<0.01, respectively, vs. isografts (iso); (¶)P<0.001 vs. untreated allografts (allo)

Figure 7. Effect of drug treatment (n = 3 each group) with WW85, low-dose CsA or WW85 + CsA on intragraft gene expression of iNOS and inflammatory cytokines*P<0.05, †P<0.025, ‡P<0.001 and ¶P<0.001 vs. isografts (iso); (¶)P<0.001 and (‡)P<0.01 vs untreated allografts (allo)

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Figure 8. Effect of drug treatment (n =3 each group) with WW85, low-dose CsA or WW85 + CsA on intragraft expression of pro-apoptotic (FasL and TNFα) and anti-apoptotic (Bcl-2 and Bcl-xL) genes. $P<0.05$ and ‡$P<0.01$ vs. isograft controls (iso); (*)$P<0.05$ vs. untreated allografts (allo)

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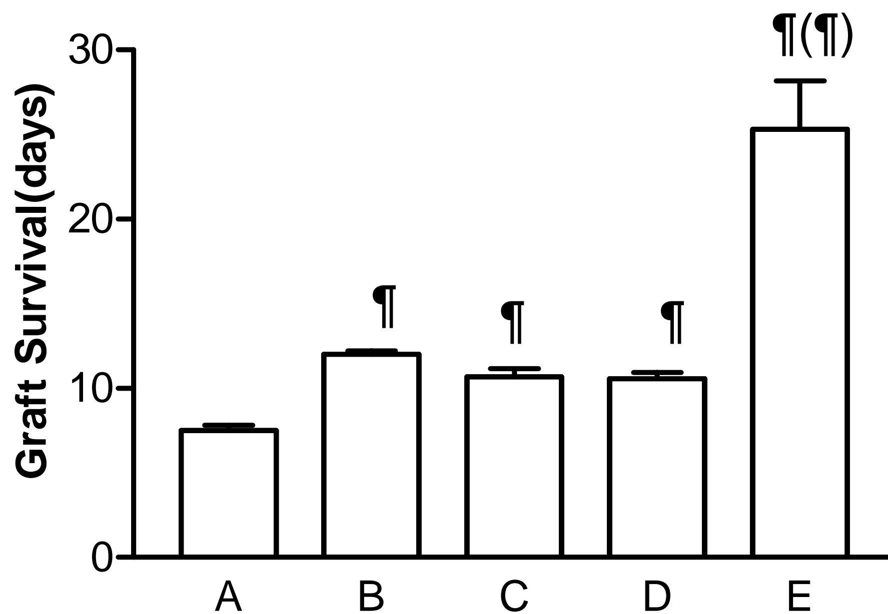
Figure 1



A: untreated

B: CsA @ 2.5 mg/kg

C: WW85 @ 0.3 mg/kg tid

D: WW85 @ 1.0 mg/kg tid

E: CsA + WW85 @ 0.3 mg/kg tid

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Figure 2

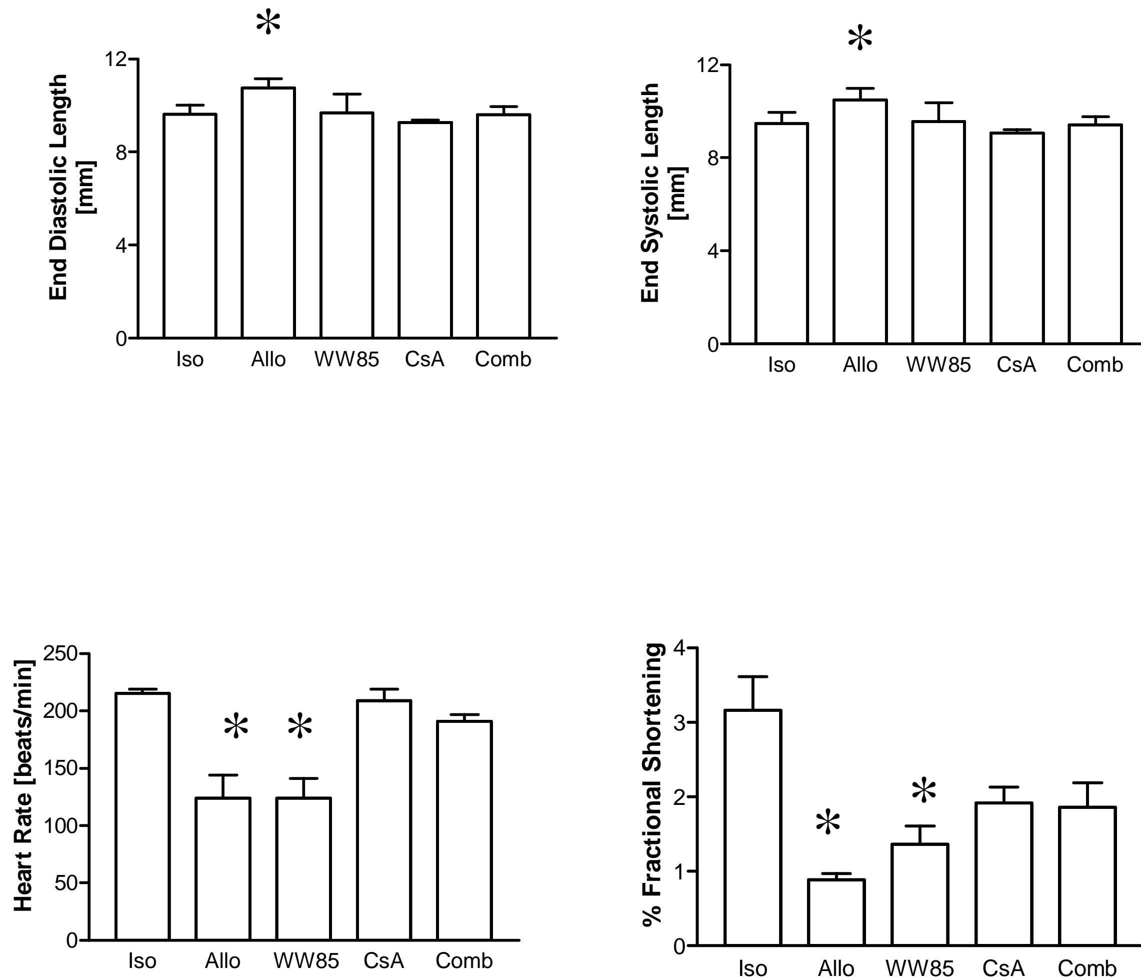

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Figure 3

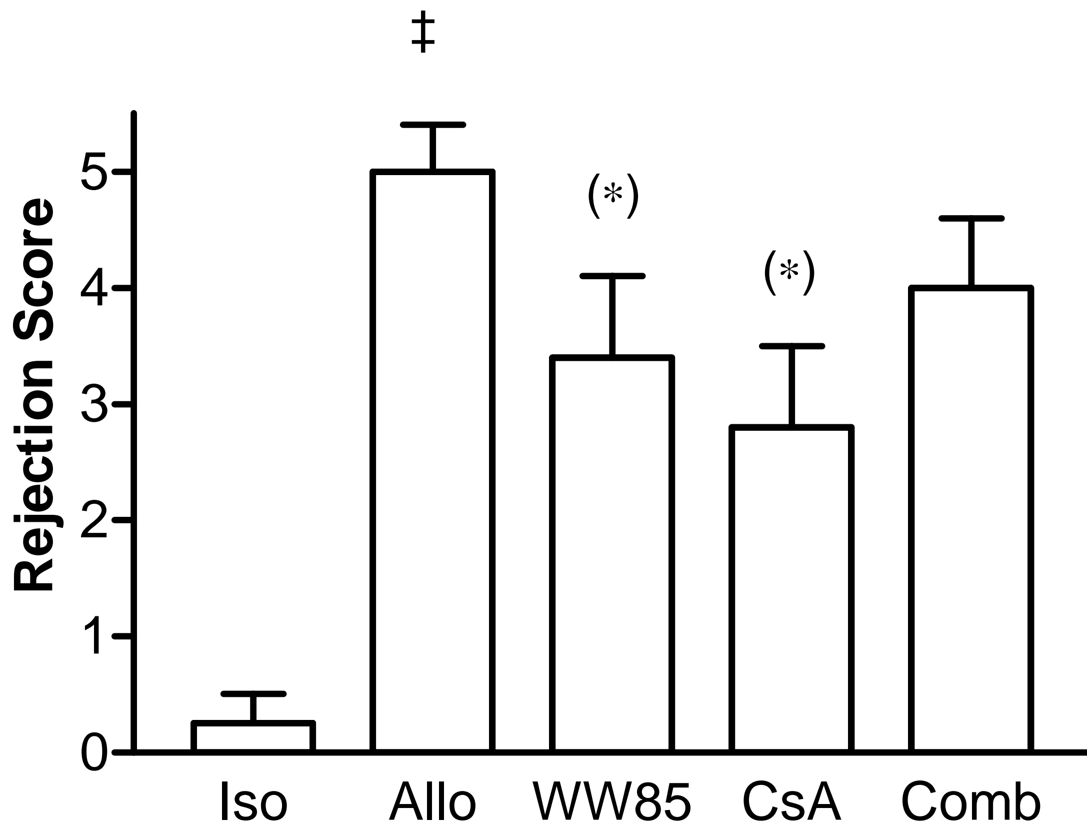

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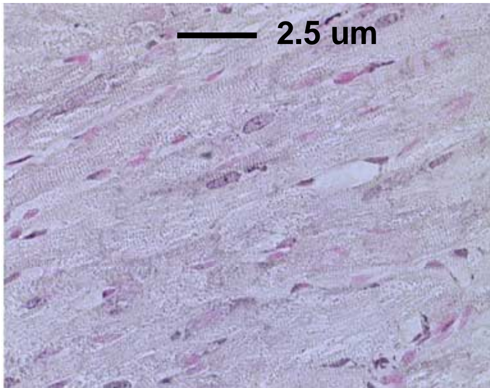
Figure 4

Intragraft Immunostaining for PAR

Iso



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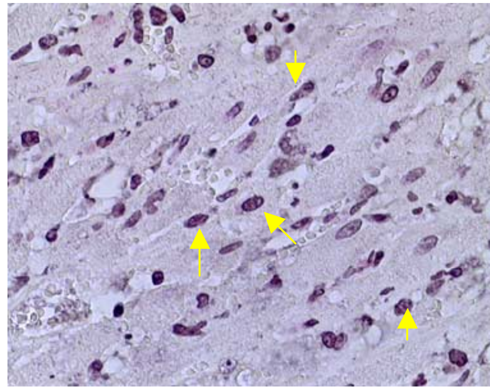
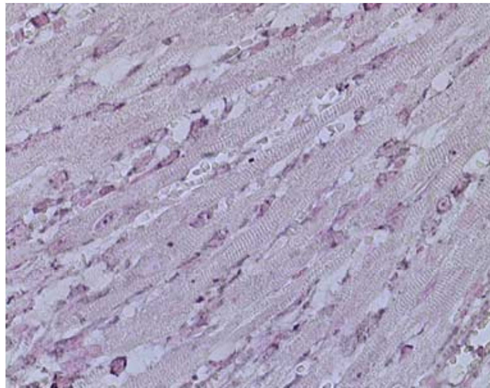
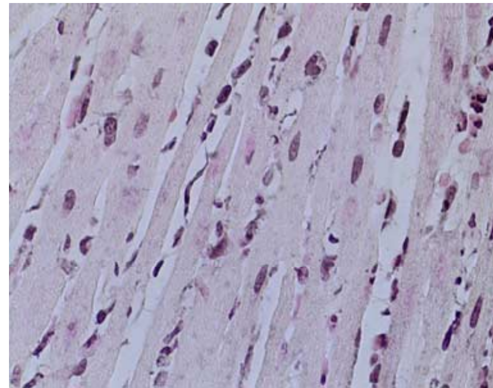


WW



CsA



Comb

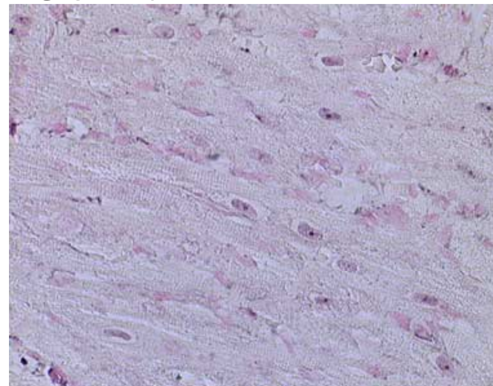


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Figure 5

Nitrotyrosine Immunostaining

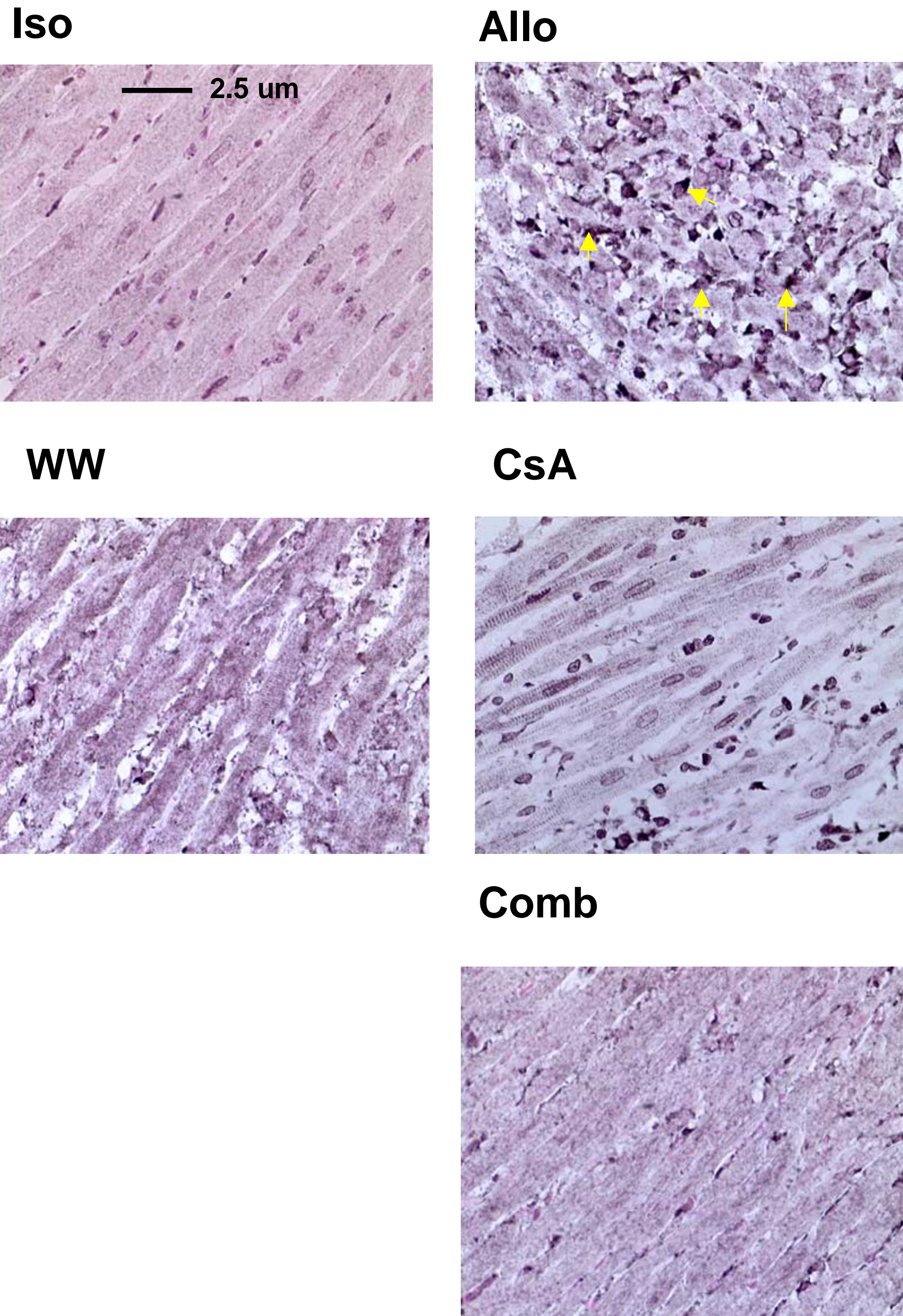

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Figure 6

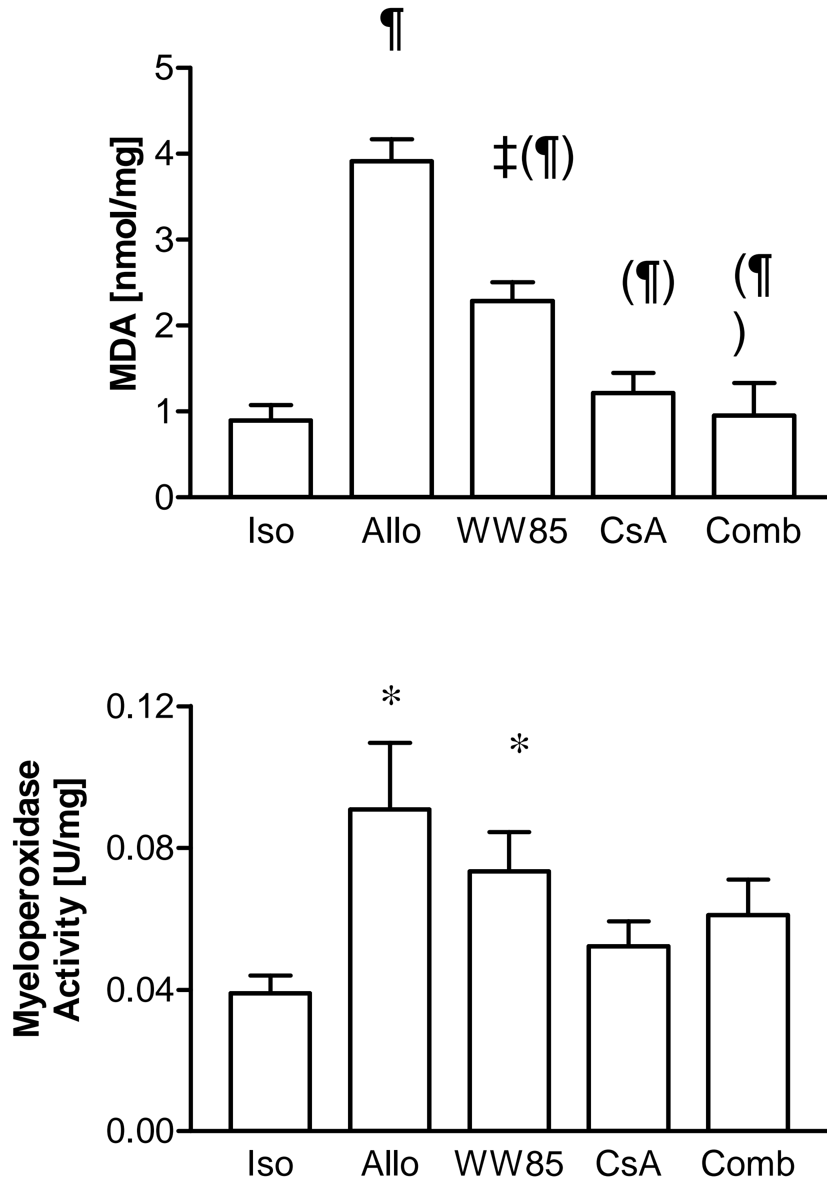

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Figure 7

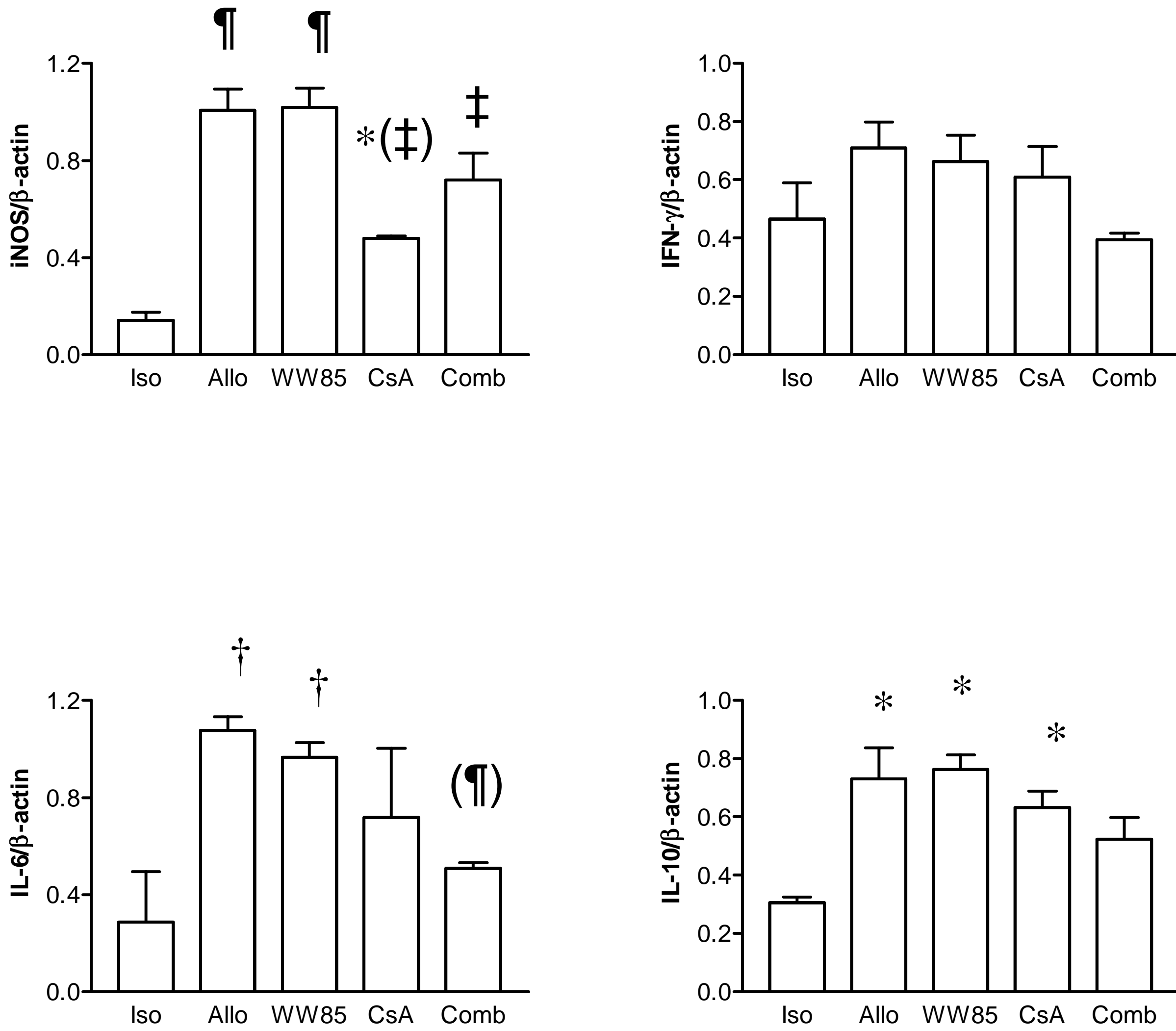

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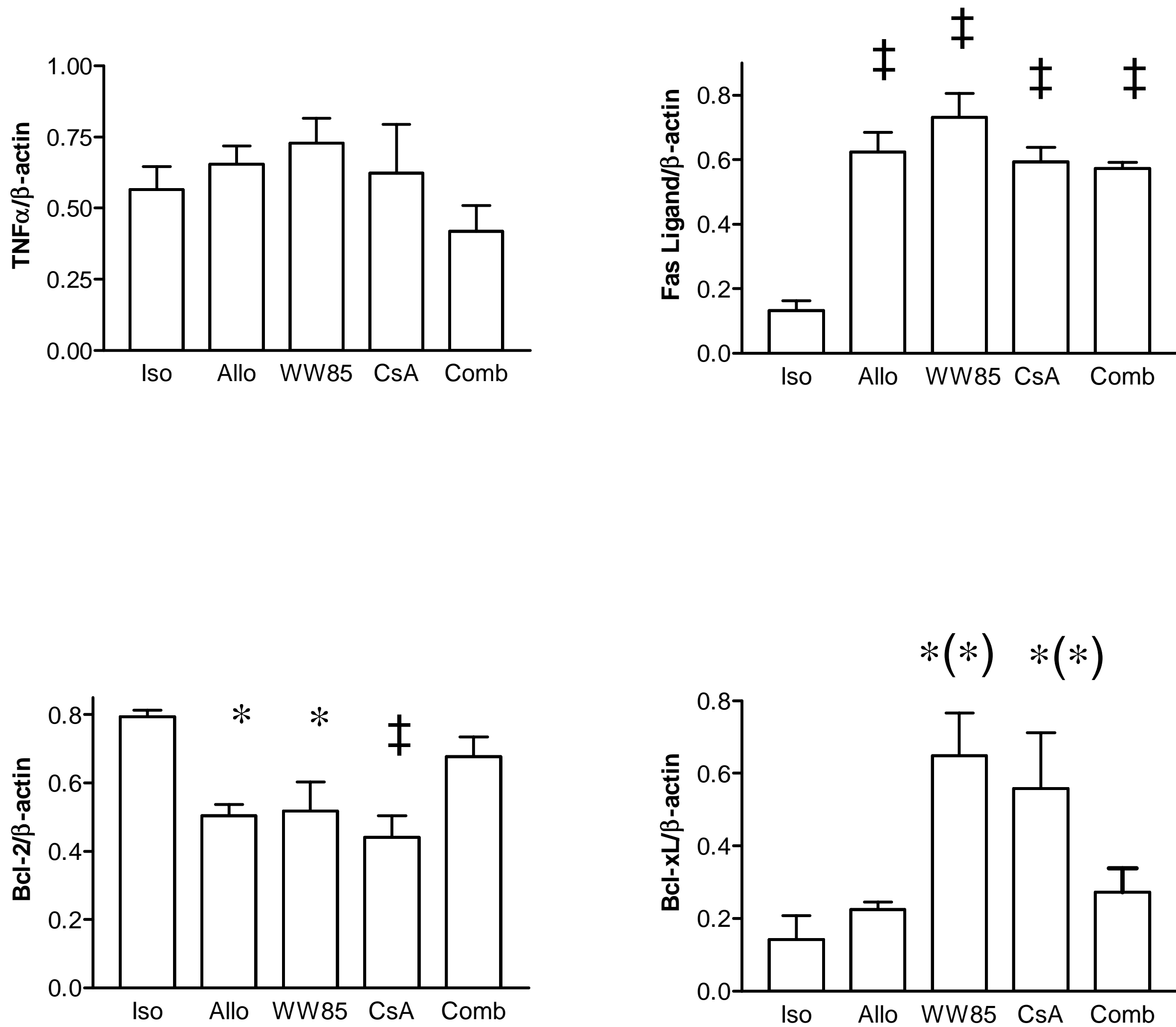
Figure 8



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