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Effects of Direct Mechanical Ventricular Actuation on the Apoptotic Signaling of a Failing Heart

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Effects of Direct Mechanical Ventricular Actuation on the Apoptotic Signaling of a Failing Heart

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

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B.S., University of Dayton, 2005

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ABSTRACT

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Effects of Direct Mechanical Ventricular Actuation on the Apoptotic Signaling of a Failing Heart.

Cardiovascular disease accounts for more than 40% of all deaths in the United States (AHA-2004 report). A non blood contacting ventricular assist device (VAD) can be used to treat heart failure without the complications that arise from blood contacting VADs. This study used cellular markers of heart failure as indicators of heart function in an attempt to assess if direct mechanical ventricular actuation (DMVA) support lessened the impact of heart failure in rabbits. Cell signaling proteins were monitored using enzyme activity measurements and quantitative immunoblotting with antibodies against intrinsic and extrinsic apoptotic pathways during heart failure with and without DMVA support.

New Zealand White rabbits were treated as sham or heart failure, with or without DMVA assistance. Animals had heart failure induced by esmolol, with or without DMVA support for 30, 60, or 120 minutes. At all time points, animals were recovered for 30 minutes after which the hearts were excised. Tissue extracts were prepared and measurements were made using the following groups; acute model with sham and 30
minute groups or an extended support/failure model with 60 minute and 120 minute groups. Cell extracts from left and right ventricles were used for immunoblot analysis to determine protein levels for the following heart failure markers; the extrinsic apoptotic pathway comprised of tumor necrosis factor receptor (TNFR) and caspase-8, the intrinsic apoptotic pathway comprised of caspase-9, cytochrome-C, or the stress related marker of heat shock protein-70 (Hsp70). Enzymatic activity was used to monitor the extrinsic pathway with caspase-8, or markers of stress with superoxide dismutase (SOD), and matrix metalloproteinase-9 (MMP-9) in all heart extracts.

In the left ventricle acute model there was a rise in both 30 minute groups compared to the sham group for all the markers, except in the caspase-8 enzymatic assay where both groups showed less activity. The extended support/failure model showed that the use of DMVA during heart failure significantly attenuated the increase of protein content for TNFR, Hsp70, and caspase-8 at the 120 minute time point. The caspase-8 enzymatic assay showed a significant decrease in 120 minute DMVA assisted group compared to the heart failure group. For every marker in the extended support/failure model there was an increase in all the markers as the duration of the experiment increased.

The right ventricle acute model exhibited an increase in both 30 minute groups for TNFR content. A significant rise in SOD levels for the 30 minute heart failure compared to both the sham and 30 DMVA groups. MMP-9 levels for both 30 minute groups rose
significantly. In the extended support/failure model, the right ventricle showed a significant increase of both groups at 120 minutes when compared to the 60 minute heart failure group in TNFR and Hsp70 protein content. In the caspase-8 enzyme assay the 120 minute DMVA group had a significant rise when compared to the 60 minute heart failure group.

DMVA provided evidence of the ability to attenuate the increase of pro-apoptotic signaling during heart failure. Evidence of this was mostly observed in the left ventricle samples, often significantly different. Both ventricles exhibited trends of attenuation by DMVA in many markers that did not differ significantly. It should also be noted that at no time point did DMVA appear to have a negative effect on the heart during failure. The validity of the heart failure model being used was also confirmed by the continuing increase in activation of known markers over time.
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List of Abbreviations

Ac-IETD-AMC - N-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methyl coumarin

AMC - 7-amino-4-methyl coumarin

CD95L - CD95 Ligand

DISC - Death-Inducing Signaling Complex

DMSO - Dimethyl sulfoxide

DMVA - Direct Mechanical Ventricular Actuation

DTT - Dithiothreitol

ECM - Extracellular Matrix

EDTA - Ethylenediaminetetraacetic acid

FADD - Fas-Associated Death Domain

FasR - Fas Receptor

GAPDH - Glyceraldehydes 3 Phosphate Dehydrogenase

H₂O₂ - Hydrogen Peroxide

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hsp70 - Heat Shock Protein 70

LVAD – Left Ventricular Assist Device

LV – Left Ventricle

MMP - Matrix Metalloproteinase

MMP-9 - Matrix Metalloproteinase 9
$O_2$ - Oxygen

Omi/HtrA2 - High Temperature Requirement Protein A2

PMSF - Phenylmethylsulphonyl fluoride

PVDF - Polyvinylidene fluoride

RV – Right Ventricle

RVAD - Right Ventricular Assist Devices

SMAC/DIABLO - Second Mitochondria-Derived Activator of Caspase/Direct IAP Binding Protein with low pI

SOD - Superoxide Dismutase

TBSTB - Non-fat dry milk in Tris buffered saline with 5% Tween-20

TNFR - Tumor Necrosis Factor Receptor

TRADD - TNFR-Associated Death Domain

TRAIL - TNF-Related Apoptosis-Inducing Ligand

VAD - Ventricular Assist Device
I. Introduction

Overview of Ventricular Assist Devices

Cardiovascular disease accounted for 36.3% of all deaths in the United States in 2004, more than any other major cause of death. (Rosamond, 2007). The necessity for a method of supporting the failing heart has been and still is an ever-growing concern. One such method is the use of left ventricular assist-devices (LVADs) to provide circulatory support during heart failure by unloading the heart (Miller, 2007). Current methods for supporting a failing heart, which include LVADs and right ventricular assist devices (RVADs), are broken into two groups based on their purpose. One group is for long term support of over one month. These devices are large and expensive units, but are very reliable for improving systemic blood-flow. Short term devices are used in cases where patients are expected to recover or require suscitating support to evaluate prognosis or consider further therapies (Catena, 2007). The fundamental configuration of an LVAD includes an inflow cannula which diverts blood entering the LV into a prosthetic ventricle that pumps the blood back into systemic circulation by means of an outflow cannula positioned in the aorta. In general RVADs normally pump blood received by the right
ventricle into pulmonary circulation via the pulmonary artery (Rose, 2001). The benefits of this type of device relate to its ability to restore hemodynamics toward normal. This can result in improved survival compared to relatively futile efforts of medical management. An investigation of nontransplant-eligible patients was done in 2007 comparing optimal medical therapy to using LVAD support. The results showed that using LVAD support significantly improved the survival rates after 6 months (46% vs. 22%; p = 0.03) and 12 months (27% vs. 11%; p = 0.02). Another positive aspect seen in this study was that 85% of the patients that received LVAD support also showed minimal to no heart failure symptoms. Despite these benefits, adverse effects associated with VAD support remain problematic. These often stem from embolic events due to thrombus formation on blood contacting surfaces despite the use of anticoagulants. Anticoagulants used to prevent thrombo-embolic events are associated with an increased risk for bleeding complications which require re-operation in up to 50% of patients. Often these side effects that increase the mortality rates of LVAD supported patients (Rogers, 2007). Other studies have shown that infection can become a problematic side effect from the LVAD which can lead to fatal sepsis was common. However, it is felt that these adverse effects were outweighed by the LVADs ability to improve mortality rates compared to medical therapy at one year (52% vs. 25%) and at two years (23% vs. 8%). In this older study, the cause of death in LVAD supported patients was mainly due to
sepsis and device failure, both of which are continually improved on aspects of the
device, as seen in the Rogers study described earlier (Rose, 2001).

**Direct Mechanical Ventricular Actuation**

A direct mechanical ventricular actuation device (DMVA) differs significantly
from the typical VAD. DMVA is non-blood contacting and provides biventricular
support. It is placed directly over the heart and pumps the ventricular chambers. Unlike
conventional LVADs that *unload* the heart, DMVA augments ventricular pump function
of the *loaded* heart. The DMVA (Fig. 1) is a cup shaped device that encompasses the
ventricle portion of the heart. This design allows it to be applied to both ventricles
without incisions in the heart or contact with the circulating blood. From the cup there
are two lines that connect it to a drive system. One line is at the apex of the heart and is a
vacuum that assures the device stays in place on the heart. The other line is connected to
a pulse pressure system that inflates and deflates a flexible membrane to simulate systolic
and diastolic forces. The device actively engages the ventricles and augments ventricular
function, somewhat like the use of open heart massage (Anstadt, 2002). Unlike heart
massage, DMVA is further unique in its ability to provide diastolic support. DMVA does
not require blood contact, and blood contact often leads to complications due to
coagulation of the blood. Therefore, DMVA does not require anticoagulation drugs,
which reduces the risk of bleeding complication. Because VADs pump blood through
Figure 2

Diagram of the direct mechanical ventricular actuation cup (DMVA). The DMVA is a non-blood contacting ventricular assist device.
prosthetic ventricles, anticoagulants are required to prevent the blood from coagulating. Blood contacting devices are associated with an increase for the risk of thrombo-embolic events, hemorrhage, and infection (Lowe, 1991). Other problems with conventional VADs relate to cannulation techniques and the blood’s flow to and from the VAD system. For example, tissue can collapse over the inlet of the inflow cannula. This can occur in a variety of places such as the interventricular septum, the left ventricular free wall, or the mitral valve leaflets leading to inadequate VAD filling and low flow states (Reesink, 2007). The DMVA system does not draw or push the circulating blood by means of a vacuum, thus those complications do not appear to arise.

**Esmolol Induced Acute Heart Failure**

In this study heart failure was induced by using a short acting beta-blocker, esmolol, to reduce contractility of the heart and lower cardiac output. Esmolol was an appropriate candidate for this due to its moderate selectivity to beta-1 receptors, which are predominately found in the heart. The other positive characteristic of esmolol is its short half-life when compared to other beta-blockers. Esmolol has a half-life of 9 minutes, compared to 6-9 hours with Atenolol and 2.5-4.5 hours with Metoprolol, both also have moderate selectivity to beta-1 receptors. The effects of esmolol are also relatively quick, in 2 minutes effects can be seen and peak at around 5 minutes. Esmolol is a drug commonly used for treating arrhythmias and hypertension (Frakes, 2001).
Esmolol has had limited use as an acute heart failure model, but a 1997 study did show its potential to induce arrest for a period of 120 minutes while still allowing for satisfactory recovery within 35 minutes after infusion was stopped. Esmolol induces heart failure by inhibition of catecholamine binding to beta-adrenergic receptors in the cell membrane, which results in the decrease in heart rate and contractility. Heart failure can thereby be modeled without direct injury to the myocardium. The metabolites from esmolol are extremely weak acting and essentially do not exert any further effects on other functions in the body. Toxic levels of esmolol infusion have been reported to cause severe bradycardia and hypotension (Ede, 1997). Another study had shown esmolol to be useful in inducing minimal myocardial contraction. This study did however conclude that in cases longer than 2 hours the metabolites from esmolol could accumulate and cause less favorable fluid filtration out of the myocardial capillaries. Esmolol was chosen due to its ability to avoid the induction of ischemia, myocardial edema formation, and post bypass cardiac function (Warters, 1998).

**Ventricular Remodeling**

As a result of heart failure, a process known as ventricular remodeling can occur in the heart. Ventricular remodeling is related to local and systemic compensatory responses that initially allow surviving muscle to compensate and maintain better hemodynamics. Eventually the muscle initially unaffected becomes dysfunctional
because of continued compensatory responses, termed maladaptive cell signaling (Friehs, 2006) (Drakos, 2007). These affects also can be seen at a cellular level, known as myocardial remodeling. At this level the cardiomyocytes are undergoing alterations in the contractile apparatus, cell size (hypertrophy), cell shape, and cell survival (Fedak, 2005). Although once thought to be an irreversible process, it has been shown that with proper recovery of cardiac function with the use of devices such as LVADs, the process of remodeling can be reversed by a process aptly named reverse remodeling. Reverse remodeling, the reversal of cardiac remodeling to a point where patients no longer require mechanical support, has been shown to be possible when a device or method is able to decrease LV end diastolic volume, increase LV wall thickness and ejection fraction, and improve hemodynamic function (Wohlschlaeger, 2005), (Drakos, 2007). The main focus this study will examine how DMVA affects maladaptive cell signaling in the acutely failing heart.

**Overview of Apoptosis**

Apoptosis is programmed cell death which is in response to cell injury and is defined by the removal of the damaged cell while trying to preserve surrounding cells. The affected cells in apoptosis are phagocytosed, preventing the release of intracellular material and its accompanying inflammatory response (Scarabelli, 2006). Apoptosis is viewed as the principal form of cellular death associated with heart failure (Cook, 1999).
The Intrinsic Pathway of Apoptosis. Initiated by intracellular signals, the intrinsic pathway is a result from the interaction between Bcl-2 proteins and the mitochondria. The mitochondria then release different factors, such as cytochrome c, SMAC/DIABLO, and Omi/HtrA2 to form the apoptosome. The apoptosome then activates caspase-9 starting the caspase cascade and apoptosis.

Adapted from Jin, 2005.
For the cellular detection of apoptosis it is necessary to first focus on the initial markers of the apoptotic pathway. There are two main groups known as the intrinsic and the extrinsic pathways. The intrinsic pathway (Fig. 2) comes from an intracellular signals; oxidative stress, DNA damage, and protein misfolding. Those signals cause a family of Bcl-2 proteins to interact with the mitochondria. The interactions create the opening of the mitochondrial permeability transition pore or can rupture the outer mitochondrial membrane. Once the membrane is ruptured the mitochondria then release different factors, such as cytochrome c, second mitochondria-derived activator of caspase/direct IAP-binding protein with low pi (SMAC/DIABLO), and high temperature requirement protein A2 (Omi/HtrA2) (Crow, 2004). Those factors initiate the formation of an apoptosome, which in turn activates caspase-9 and finally caspase-3 resulting in cell death (Riedl, 2007).

**The Extrinsic Pathway of Apoptosis**

The extrinsic pathway (Fig. 3) is initiated by extracellular signals and stress on the cell. The main signaling for apoptosis in the heart occurs by two death receptors, the tumor necrosis factor receptor 1 (TNFR1) and the Fas receptor (FasR). There are corresponding ligands; the CD95 ligand (CD95L), TNFα, and TNF-related apoptosis-inducing ligand (TRAIL), which bind to the receptors (Scarabelli, 2006). Once the appropriate ligands bind to a receptor death domain proteins are recruited; TNFR-
Figure 3

The Extrinsic Pathway of Apoptosis. Also known as the death receptor pathway, it is characterized by the presence of death receptors. Ligands bind to the corresponding receptor, which recruit death domains and eventually form a
associated death domain (TRADD) and the Fas-associated death domain (FADD). It is at this time point that the caspase pathway is initiated. Those complexes recruit pro-caspase-8 to form the death-inducing signaling complex (DISC). The formation of the DISC allows for the activation of pro-caspase-8 to caspase-8 by self-cleaving. The caspases recruited early in apoptosis are called initiators; the initiators will act on other downstream caspases known as effectors. In cardiac apoptosis there are two main initiators (caspase-8 and caspase-9) along with one effector (caspase-3) (Jin, 2005).

**Heat Shock Protein 70 and Its Relationship to Heart Failure**

Another response found in a failing heart is the increase in heat shock protein 70 (Hsp70) activation. The purpose of Hsp70 is to reactivate denatured enzymes and prevent protein denaturation when in the presence of stress (Fig.4). This increase has been seen in previous rabbit models of cardiac ischemia (Knowlton, 1991). There are multiple factors that are observed in a failing heart that have been linked with increases of Hsp70 like TNFα, stretch, and decreased shortening in the ventricles. Hsp70 has been show to interact in the caspase pathway by preventing Apaf-1 from recruiting caspase-9 in the intrinsic pathway. There is also a caspase independent manner in which Hsp70 has been shown to inhibit apoptosis. That way involves Hsp70’s ability to inhibit c-Jun N-terminal kinase, which is used in apoptosis. However, the caspase independent manner that Hsp70 can work by has yet to be studied in cardiac tissue (Latchman, 2001).
Figure 4

The Role of Hsp70. Heat shock protein 70 recognizes unfolded and misfolded proteins. Heat shock protein 70 then reacts with them and by using ATP can restore them back to their native state.

Adapted from Wilhelmus, 2007.
an elongated period of time the recruitment of Hsp70 is lessened due to this constant stimulation and it then becomes less and less effective over time (Knowlton 1998). The body’s natural reaction to curb the use of Hsp70 can be over-ridden and when Hsp70 is over expressed it is able to protect against ischemia from occurring (Li, 1991).

Changes in the Extracellular Matrix

The extracellular matrix (ECM) is a collection of fibrillar collagen, elastin, microfibrillar proteins, proteoglycans, and adhesive proteins used to support the three dimensional structure of most cells. The ECM is a structure that under different conditions can alter its own composition to adapt to those stimuli. In the myocardium of the heart the ECM is mostly composed of fibrillar collagen. The restructuring of the components in fibrillar collagen is thought to play a part in ventricular remodeling. A typical response to injury such as heart failure is to increase the amount of collagen and reinforce the area. The accumulation of myocardial collagen can lead to myocardial fibrosis. The left ventricular dilation that causes cellular hypertrophy is also associated with myocardial fibrosis. As heart failure progresses, the activation of matrix metalloproteinases (MMP) becomes an important part of myocardial matrix remodeling. These zinc-dependant enzymes degrade the ECM during remodeling and triggers left ventricular dilation, wall thinning, and cardiac dysfunction. Of the different MMP species, MMP-9 is one that has been commonly associated with heart failure. MMP-9 is
an enzyme that degrades collagen types I, II, and III. A chronic heart failure study in mice showed that the deletion of the gene encoding MMP-9 attenuated left ventricular dilation and the accumulation of collagen. This shows the effects that activation of MMP-9 can have on hastening the progression of heart failure (Ducharme, 2000). The reasoning behind how MMP-9 is involved in the progression of heart failure lies in the fact that it breaks down the collagen in the ECM. In response to this breakdown more collagen is synthesized and eventually this exceeds the rate of degradation leading to an accumulation of oxidized ECM. As the ECM accumulates, the distance between myocytes increases and that negatively affects the contractility of the heart and its overall function (Ovechkin, 2005).

**Oxidative Stress Results of Heart Failure**

During heart failure there is degradation in the proper contractility and overall function of the ventricles. This ventricular dysfunction leads to improper oxygen supply to the myocardial tissue as well as a loss of contractile myocytes. During the progression of heart failure these factors lead to exaggerated levels of oxidative stress produced by various cytokines and growth factors. The results of oxidative stress are the creation of harmful reactive oxygen species. Superoxide dismutase (SOD) is an early defense mechanism to scavenge superoxide anions (O$_2^-$), which occur under normal conditions in the body and increase under environmental factors that cause stress. Since reactive
oxygen species, such as O$_2^-$, occur naturally there is also a normal level of SOD that is always present, but in the heart this level is normally low. As there is an increase in oxidative stress from heart failure from ventricular dilation there is an increase in the amount of SOD that is needed to scavenge the free O$_2^-$. It is important for this to occur because O$_2^-$ is able to cause cellular death by binding to DNA, protein, and lipids (Casserot and Doul). The result of SOD is the creation of oxygen (O$_2$) and hydrogen peroxide (H$_2$O$_2$), which are the body’s primary cellular defense against the formation of O$_2^-$ (Fig. 5). Once this reaction occurs there is a need for further reactions since the newly created H$_2$O$_2$ is also a reactive oxygen species, although not as effective as O$_2^-$ is in harming cells. As these reactions continue to occur during heart failure the antioxidant reserve is slowly being compromised. As this reserve is compromised, the myocytes and the extra cellular matrix of the heart are predisposed to more damage (Haibo, 2007).

Oxidative stress has also been linked to another route of apoptosis involving the production of TNF. TNF is a proinflammatory cytokine that is associated with cardiac dysfunction. A previous study linked the relationship between H$_2$O$_2$ and oxidant-sensitive proteins that could initiate the production of TNF. The study showed that H$_2$O$_2$ was able to induce TNF production even without ischemia and reperfusion (Meldrum, 1998). One possibility for this link is that physical forces such as ventricular dilation during heart failure release cytokines and growth factors that may induce the production of reactive oxygen species. One study saw an increase in superoxide production under
Figure 5

**Superoxide Dismutase Reaction.** Superoxide dismutase reacts with harmful superoxide anions to create oxygen and hydrogen peroxide. This process is increased in response to oxidative stress that is brought on by heart failure.
$2 \text{O}_2 \cdot + 2 \text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2$
conditions of high tension in the heart. The study also saw an increase in the expression of the Fas receptor protein, a member of the TNF family, in the myocytes. The conclusion was that there was a link between the generation of reactive oxygen species and the activation of genes that lead to cell death by apoptosis (Cheng, 1995). Other studies have come out more recently confirming that an increase in reactive oxygen species is an important determinant in activating the apoptotic cascade. Initially a small increase in the amount reactive oxygen species present assisted in causing hypertrophy, and then as the amount increases the occurrence of apoptosis becomes more prevalent. This study also showed that stretch induced in ventricular myocytes caused an increase in superoxide anion production (Pimentel, 2001). Oxidative stress is a good marker to use because it can be shown as an early marker in two main causes of heart failure; stress related and pro-apoptotic signaling.

Specific Aims

This thesis will test the following hypothesis: The non-blood contacting direct mechanical ventricular actuation device (DMVA) can improve ventricular pump function of the failing rabbit heart while attenuating maladaptive cell signaling that characterizes heart failure.

This thesis will be accomplished by using the following specific aims:
1. The use of a DMVA device is able to reduce the myocardial stress that occurs during heart failure.
   
a) The expression of Hsp70 will be evaluated by immunoblotting. This will evaluate the amount of stress and stretch that is being exerted on the ventricles at each stage.

b) The activity of superoxide dismutase will be determined by an activity assay. Increases in superoxide dismutase are an early signal that there is an increase in oxidative stress.

2. The DMVA device will impede pro-apoptotic cellular signaling.
   
a) The extrinsic pathway of apoptosis will be analyzed through the expression of TNFR and caspase-8 will be evaluated by immunoblotting and fluorimetric activation. Both of these components are upstream activators of the extrinsic caspase cascade involved in apoptosis. Due to the short duration of the experiment these upstream activators are the most reliable to see early changes in protein expression.

b) The intrinsic pathway of apoptosis will be analyzed through the expression of caspase-9 and will be evaluated by immunoblotting. Caspase-9 represents the furthest upstream caspase in the intrinsic pathway of apoptosis.

3. The use of DMVA will attenuate the stress placed on the extracellular matrix.
   
a) The activation of MMP-9 will be evaluated by immunoblotting and an activity assay. The activation of MMP-9 evaluates the breakdown of the ECM of the ventricles due to the dysfunction of loading and unloading during heart failure.
II. Materials and Methods

**Rabbit Surgery**

New Zealand White Rabbits (4-5 kg) were to be randomly chosen to undergo one of three procedures; a control group receiving no surgery or induction of heart failure, have heart failure induced for a set amount of time with no assistance, or have heart failure induced for a set amount of time with the assistance of direct mechanical ventricular actuation (DMVA). In the control group six rabbits were euthanized and the hearts were extirpated immediately and quick frozen to be stored at -80°C until needed. The remaining animals, ranging from 5 to 7 animals per sample group, underwent the same initial procedures to induce heart failure. Anesthesia was induced by using 1-2.5% isoflurane in oxygen, given by way of a mask and maintained throughout the procedure. Once the animal was anesthetized incisions were made on the hind legs and femoral intravenous lines were put in place. These lines were used to maintain proper doses of phenylephrine and esmolol, which were used to maintain blood pressure and to simulate heart failure by decreasing cardiac output. A Millar catheter (Millar Instruments, Houston, TX) was inserted into the internal carotid artery for measuring arterial pressure. A median sternotomy was performed and an ultrasonic flow probe (Transonic Systems, Ithaca, NY) was positioned on the ascending aorta for measuring cardiac output. Electrocardiogram, hemodynamics, pulse oximetry, end-tidal CO2, and rectal temperature were recorded with a LifeWindow 6000 system (Digicare, Boynton Beach, FL).
Once all instrumentation was in place a baseline cardiac output was determined from the flow probes. From that reading a value 60% of the baseline was considered “heart failure” for that animal. Phenylephrine was administered intravenously to maintain a mean arterial pressure between 40mmHg and 50 mmHg. To induce heart failure esmolol, a short acting beta blocker, was also administered intravenously both with a running dose and by using boluses to maintain the heart failure state. At this point the treated animals were either kept in the heart failure state for 30 minutes, 60 minutes, or 120 minutes, or could receive DMVA assistance by means of the Myovad cup used for this study. The total duration of the cup treatment was also for 30 minutes, 60 minutes, or 120 minutes, each a doubling of the previous time frame. During the DMVA studies, the cup was to be removed every 15 minutes for observation of the heart to ensure that the equipment was working properly and that the animal remained in the heart failure state, if the rabbit was no longer in the “heart failure” state the appropriate adjustments were made to the infusion of esmolol and phenylephrine to again reach that point and the trial would continue. The action of removing the cup may have caused some additional trauma by removing and reattaching, but this was necessary to maintain the integrity of the heart failure state. Once a study had reached the necessary time span the animals were then observed for 30 minutes in a recovery state. During this recovery state the esmolol was no longer administered, but general anesthesia continued to be administered and those parameters continued to be measured. At all points during the surgery echocardiography was used to monitor each phase of the experiment. Once the recovery period was over the animals were euthanized under deep anesthesia, 5% isoflurane, by extirpating the hearts. The hearts were sliced into six sections laterally and quick frozen
in isopentane cooled in liquid nitrogen for fixation and future study.

In this study two distinct groups were analyzed, the acute group and the extended support/failure group. The acute group was made up of the sham group and the 30 minute groups of heart failure with or without DMVA assistance. The extended support/failure model was comprised of the 60 minute and the 120 minute groups of heart failure with or without DMVA assistance. It was necessary to separate the acute model from the extended support/failure model to eliminate the possible differences in cellular response to apoptosis due to those time differences.

**Protein Extraction**

Tissue was thawed and then homogenized with 300 µl T-PER Tissue Protein Extraction Reagent (Pierce), 10 µl Halt Protease Inhibitor Cocktail Kit (Pierce), 3 µl EDTA (Pierce), and 3 µl PMSF (Pierce), then centrifuged at 13,000 RPM for 5 minutes. The supernatant was recovered from the homogenate and the protein concentrations of the supernatant was determined by using the Bradford method (BioRad Laboratories, Hercules, CA) (138).

**Immunoblotting**

Each sample was loaded on SDS-Page 12% Tris-HCl Ready gel, 8.6 x 6.8 cm (BioRad Laboratories, Hercules, CA), and run on electrophoresis at 80 volts for 20 minutes and then at 180 volts until the dye front nears the bottom of the gel. Each gel was loaded according to time duration to better directly compare the difference of heart failure versus direct mechanical ventricular actuation at each time point. Proteins were
transferred from the gel to a PVDF Immobilon-P transfer membrane (Millipore) using semi-dry transfer with a Trans-Blot SD (Biorad) at 20 volts for one hour. The PVDF membranes were blocked with 5% Non-fat dry milk in tris buffered saline with 5% Tween-20 (TBSTB). Then they were incubated with the primary antibody, either tumor necrosis factor receptor I, heat shock protein 70, caspase-8, caspase-9, or glyceraldehyde 3 phosphate dehydrogenase, diluted to the optimal amount in TBSTB for two hours. Membranes were then washed three more times with TBST and then probed with the secondary horseradish peroxidase linked antibody (antibody specific to marker) diluted in TBSTB. Then there will be three more washes of the membrane with TBST. Samples were visualized via enhanced chemiluminescence substrate (Pierce) and captured by the Fuji LAS3000 imager. The relative amount of protein of interest was determined by normalizing that protein to glyceraldehyde 3 phosphate dehydrogenase. Glyceraldehyde 3 phosphate Dehydrogenase is considered a housekeeping gene that is expressed in most tissues and cells, making it appropriate as a control. Actin was not used due to its relation to the contractile apparatus in muscle cells, which may be affected during heart failure.

**Protein Transfer Analysis**

To evaluate the ability of the total protein to effectively transfer from the Tris-HCl gel to the membrane, tests were run with the gel and the membrane. First the semi-dry transfer method was evaluated by running a time course transfer. For this a gel was loaded with three different amounts of total protein; 15µg, 30µg, and 45µg. Lanes were set up so that 15µg was in lane four, 30µg was in lane five, and 45µg was in lane six.
Lanes 8-10 and 12-14 mirrored the set up from lanes 4-6. Gel was run as described previously in western blotting. Before the gel was run in the semi-dry, transfer lanes 1-7 were removed and stained in Coomasie Blue to visualize the amount of protein that ran through the gel. The remaining gel was run in the semi-dry transfer method for one hour. At that point lanes 8-11 were removed and placed in Coomasie Blue stain. The remaining gel continued to run for one additional hour and was also stained in the Coomasie Blue.

The other test run to evaluate transfer efficiency was to stain the membrane after the semi–dry transfer to visualize the amount of protein retained on the membrane. To do this a gel was run with different concentrations and transferred for one hour. The resulting membrane was incubated in Ponceau S stain (Pierce) for 10 minutes. This was followed by three washes of five minutes with 20% acetic acid to remove any excess stain. The resulting protein was analyzed visually.

**Caspase-8 Activity Assays**

Tissues were thawed and then homogenized with 200 µl of lysis buffer provided by Sigma with the Caspase-8 assay kit containing 25mM HEPES, pH 7.4, 5mM CHAPS, and 5mM DTT. The resulting homogenate was then centrifuged at 13,000 RPM for 5 minutes. The supernatant was recovered from the homogenate and the protein concentrations of the supernatant were determined by using the Bradford method (BioRad Laboratories, Hercules, CA) (138).

The reaction was carried out in a 96-well plate. Each well was designated as a
blank, positive control, positive control plus the inhibitor, or sample. The blank well contained 90ul of the assay buffer; 20mM HEPES, pH 7.4, 0.1% CHAPS, 5mM DTT, 2mM EDTA, and 5% sucrose. The positive control contained 5ul of 10ug/ml caspase-8 along with 85ul assay buffer. The positive control plus the inhibitor contained 5ul of caspase-8 and 83ul of assay buffer along with 2ul of caspase-8 inhibitor, 25uM Ac-IEDT-CHO. The samples were loaded according to the protein content of the sample so that there was 400ug of protein per sample per well. The amount of assay buffer added to a given sample was 90ul minus the amount of sample already in the well so that the total in the well was 90ul. The reaction was initiated with 10ul of the caspase-8 substrate made of 1.5mM Ac-IETD-AMC in DMSO that was also diluted 10-fold with the assay buffer prior to the experiment. The reaction is defined by the hydrolysis of the caspase-8 substrate by the caspase-8 in the samples resulting in the release of AMC. The AMC released can then be measured by fluorimetric detection by using 360nm as the excitation wavelength and 440nm as the emission wavelength. A calibration curve using known amounts of AMC was run with each test and used to determine the amount of nmols of AMC that was released per minute, per milligram of protein in each sample.

A kinetic measurement was obtained by allowing the reaction to carry on for 70 minutes. At that point the difference between the starting relative fluorescence and the ending fluorescence was used to determine the amount of nmols of AMC that were released from the sample by using the calibration curve. That value was divided by the duration of the experiment and the total amount of protein to get the activity value, defined as nmols of AMC released per minute per milligram of protein.

To validate that the reaction that was occurring with the samples was indeed the
release of AMC by caspase-8, a test was run to verify this. Confirmation was obtained by running samples in duplicate where one sample was run as described before and the other contained 2ul of the inhibitor of caspase-8. A positive result would show a significant decrease in the activity in the sample containing the inhibitor when compared directly to the same sample run simultaneously without any inhibitor.

**Superoxide Dismutase Activity Assay**

Tissues were thawed and then homogenized with 200 µl of 1X cell extraction buffer provided in Assay Designs kit for superoxide dismutase (SOD), which contained 10X SOD buffer, 20% (v/v) Triton X-100, distilled water, and 200mM PMSF. The resulting homogenate was then centrifuged at 11,000 x g for 10 minutes at 4°C. The supernatant was recovered from the homogenate and the protein concentrations of the supernatant were determined by using the Bradford method (BioRad Laboratories, Hercules, and CA).

The SOD reaction was carried out in a 96-well plate. Each well was designated as a blank, standard control, or sample. The blank well contained 25ul of 1X SOD buffer, 150ul of the master mix (containing 10X SOD Buffer, WST-1 reagent, xanthine oxidase, and distilled water), and 25ul of 1X xanthine solution (containing 10X xanthine solution and 1X SOD buffer) to get a total volume of 200ul. The standard control was prepared according to the design laid out by the insert in the kit. The fifty micrograms of heart extract were loaded per well and the samples were diluted using master mix so that there was a total volume of 25ul per well. After 150ul of master mix was added to each sample well, the reaction was started by adding 25ul of 1X xanthine solution to each
well. The plate was mixed by orbital shaking for 10 seconds and then read at one minute increments for twenty minutes at room temperature. The activity was measured by the absorbance levels at 450nm.

A kinetic measurement was obtained by allowing the reaction to carry on for 20 minutes. At that point the percent inhibition was determined by subtracting the slope of the sample from the slope of the 1X SOD buffer control, multiplying that by 100 and then dividing by the slope of the 1X SOD buffer control. The percent inhibition is found due to the idea that the more SOD present in a sample, the lower the absorbance rate will be. Since the 1X SOD buffer control contains no SOD it is used as the maximal rate of absorbance and is considered 100%.

**MMP-9 Activity Assay**

Tissues samples were thawed and then homogenized in assay buffer (Anaspec) containing 0.1% Triton-X 100, and then centrifuged for 15 min at 10000x g at 4°C. The supernatant was recovered from the homogenate and the protein concentrations of the supernatant were determined by using the Bradford method (BioRad Laboratories, Hercules, CA) (138).

The MMP-9 activity measurement was carried out in a 96-well plate using 1mM 4-aminophenylmercuric acetate as a substrate in the assay buffer for 2 hours at 37°C prior to adding the sample. Each sample was loaded at 200ug of protein per 50uL of solution. This was added to each well to start the reaction and the plate was incubated at room temperature for 10 minutes. The enzymatic reaction was initiated by adding 50ul of MMP-9 substrate (Anaspec) to each well and then the plate was shaken for 30 seconds
before measuring the fluorescence intensity using excitation at 340nm and emission at 490nm, continuous recording every 5 minutes for 60 minutes total. Rates were determined by taking the slope of the reactions and correcting for time.

**MMP-9 Zymography**

Heat tissues were homogenized in an extraction buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol) for use in protease analysis. Various amounts of total protein, ranging from 5ug-50ug, of each extract dissolved in a loading buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% Bromophenol Blue, BioRad) provided by the manufacture. Gels were electrophoresed through a 10% zymogram gel (BioRad) with gelatin in a running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS with deionized water at pH 8.3) for 90 minutes. The gels were washed with Zymogram Renaturation Buffer (2.5% Triton X-100, BioRad) 3 times for 10 minutes each then incubated for 24 hours, 36 hours, or 1 week at 37°C in Zymogram Development Buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl2, BioRad). The gels were stained in Zymogram Staining solution of: 0.5% Coomassie R250, 10% Acetic Acid, 40% methanol. Gels were then destained in a BioRad solution (destaining solution containing 40% methanol and 10% acetic acid) by washing 3 times for 7 minutes. Activity was identified as clear bands on a blue background.
Statistical Analysis

Results are presented as means ± S.E.M and compared between sham, heart failure and DMVA using an unpaired t-test performed with GraphPad Prism 5 software. Grubb’s test was used to assess for outliers. A difference of P<0.05 was considered significant.
III. Results

We tested our working hypothesis that the non-blood contacting ventricular assist device (VAD) will improve the overall function of the failing rabbit heart while attenuating maladaptive related cellular signaling using immunoblotting assays with well known cardiac apoptotic and stress related markers. Staining of these blots enabled us to determine the effects of heart failure and direct mechanical ventricular actuation (DMVA) had on the cardiac tissue. We measured other enzyme markers, such as superoxide dismutase (SOD) and matrix metalloprotease 9 (MMP-9) to assess both mechanical and chemical initiators of apoptosis in the cardiac tissues receiving heart failure and DMVA assisted heart failure. Summary tables of all the quantified results can be found in Appendix A.

Analysis of Protein Loading and Transfer for Immunoblotting

Before measuring the level of marker proteins in cell extracts it was necessary to determine the sensitivity of our detection method and the kinetics of protein transfer. The concentration dependence for each marker protein was determined, including the minimum level of detection and at which concentration was observed. Quantitation of all marker proteins was run in a corresponding linear range of optical density. The results showed that proteins such as glyceraldehyde 3 phosphate dehydrogenase (GAPDH), Cytochrome-C, and tumor necrosis factor receptor (TNFR) were visible at lower load.
amounts of protein, approximately 25ug of protein as determined by the Bradford Method. The other proteins; Caspase-8, Caspase-9 and Hsp-70 required a larger load amount to be visible, approximately 40ug of protein as determined by the Bradford Method (data not shown).

To determine the appropriate time for transfer, a single gel was used for four different time points, before the transfer, at 1 hour, at 1.5 hours and at 2 hours. Each resulting gel slice was stained with Coomasie Blue dye to visualize the amount of protein remaining in the gel at the different time points. The results showed good transfer at all time points, with increasing amounts of protein transfer as the duration increased. To confirm these results, the transfer membrane also was stained with Ponceau-S stain to visualize the amount of protein that had adhered to the membrane. These results together showed that at 1.5 hours there was sufficient amount of transfer to the membrane.

**Tumor Necrosis Factor Receptor Immunoblotting**

Tumor Necrosis Factor Receptor (TNFR) is a marker that is seen at the initial stages of apoptosis. This receptor is activated in the initial stages in the extrinsic pathway of apoptosis and thus is expected to be a good indicator for an acute heart failure model. As more receptors reach this activated state the likelihood of a cell to undergo apoptosis is increased. A rise in TNFR content in the acute model was seen in both the HF and DMVA groups of the left ventricle when compared to the sham group to a significant level, suggesting that apoptosis was being initiated in both groups. In the extended support/failure model, there was a significant increase of approximately 200% from the 60 to the 120 minute groups (Figs. 6-10). There was no difference at the 60 minute point,
however, at 120 minutes with heart failure there was significantly higher TNFR content than that in the 120 minute group with DMVA assistance (1.74 vs. 1.29, p=0.02). The 45% decrease of TNFR content in heart failure compared to TNFR content in the DMVA assisted heart failure indicates that the use of DMVA reduces the rate at which pro-apoptotic signaling is initiating during heart failure in a statistically significant (p=0.03) manner.

Similar results using anti-TNFR were observed in the right ventricle. There was a significant increase from both 30 minute groups when compared to the sham group. The trend in the extended support/failure model showed a steady increase in the TNFR content as the duration was increased (Figs. 11-15). Within each time point, there was no significant difference between the heart failure group and the DMVA group at any point. There was, however, a steady increase in the difference between the heart failure group and the DMVA group as the time of failure increased. The lack of significant change between the DMVA and heart failure groups is an indication that the use of DMVA did not reduce or accentuate the initial signaling for the extrinsic pathway compared to the heart failure group.
Figure 6- Immunoblotting Representation of TNFR Content in the Left Ventricle of Sham Acute Hearts. Immunoblots were performed using 25µg of protein with anti-TNFR antibody at a 1:2000 dilution to measure the relative protein content as described in the Methods section. Six sham acute hearts are represented in lanes 1-6, each normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 7- Immunoblotting Representation of TNFR Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 30 Minute Acute Hearts. Western Immunoblots were performed using 25µg of protein with anti-TNFR antibody at a 1:2000 dilution to measure the relative protein content as described in the Methods section. In part A, five acute hearts represented in lanes 1-5 had heart failure induced for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six acute hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 8- Immunoblotting Representation of TNFR Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 60 Minute Extended Support/Failure Hearts. Immunoblots were performed using 25ug of protein with anti-TNFR antibody at a 1:2000 dilution to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 60 minutes and were normalized to GAPDH at a 1:5000 dilution or quantification purposes. In (B), 7 extended support/failure hearts represented in lanes 6-12 had heart failure induced while receiving direct mechanical ventricular actuation support for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 9- Immunoblotting Representation of TNFR Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 120 Minute Extended Support/Failure Hearts. Immunoblots were performed using 25ug of protein with anti-TNFR antibody at a 1:2000 dilution to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six extended support/failure hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 10- Relative TNFR Content in the Left Ventricle in Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of relative protein amount, and * P<0.0002 30 HF vs. sham, **P<0.0001 30 DMVA vs. sham, †P<0.0001 120 HF vs. 60 HF, and ††P<0.0003 120 DMVA vs. 60HF and P<0.02 120 DMVA vs. 120 HF. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
**Figure A**

TNFR in the Acute Left Ventricle

- Sham
- 30 HF
- 30 DMVA

**Figure B**

TNFR in the Extended Support/Failure Left Ventricle

- 60 HF
- 60 DMVA
- 120 HF
- 120 DMVA

* Statistical significance
** Highly significant
†† Significantly different
Figure 11- Immunoblotting Representation of TNFR Content in the Right Ventricle of Sham Acute Hearts. Immunoblots were performed using 25μg of protein with anti-TNFR antibody at a 1:2000 dilution to measure the relative protein content as described in the Methods section. Six sham acute hearts are represented in lanes 1-6, each normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 12- Immunoblotting Representation of TNFR Content in the Right Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 30 Minute Acute Hearts.

Immunoblots were performed using 25ug of protein with anti-TNFR antibody at a 1:2000 dilution to measure the relative protein content as described in the Methods section. In (A), five acute hearts represented in lanes 1-5 had heart failure induced for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six acute hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 13- Immunoblotting Representation of TNFR Content in the Right Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 60 Minute Extended Support/Failure Hearts. Immunoblots were performed using 25μg of protein with anti-TNFR antibody at a 1:2000 dilution to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), seven extended support/failure hearts represented in lanes 6-12 had heart failure induced while receiving direct mechanical ventricular actuation support for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 14- Immunoblotting Representation of TNFR Content in the Right Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 120 Minute Extended Support/Failure Hearts. Immunoblots were performed using 25ug of protein with anti-TNFR antibody at a 1:2000 dilution to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six extended support/failure hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 15- Relative TNFR Content in the Right Ventricle in Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of relative protein amount, and the * P<0.02 30 HF vs. sham, **P<0.03 30 HF vs. sham, and †P<0.01 120 HF and 120 DMVA vs. 60 HF. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
A

TNFR in the Acute Right Ventricle

B

TNFR in the Extended Support/Failure Right Ventricle
Measurement of Heat Shock Protein 70 Content in Hearts

Heat Shock Protein 70 (Hsp70) is a marker related to mechanical stretch of myocardial cells that is seen in cardiac failure. Since Hsp70 is an early indicator of stress from cardiac tissue stretch it can be used to monitor initial stages of failure and also assess if the DMVA assistance decreased heart muscle stretch. There was an increase in the Hsp70 content in the left ventricle in both 30 minute groups as compared to the sham group. A significant trend occurred in each group as the duration of the treatment became longer. Within the 30 minute period, there was no significant difference seen in Hsp70 content between the 30 minute period heart failure and the 30 minute DMVA. At the 60 minute point, there was a significant difference between the groups with heart failure exhibiting a 61.5% increase in Hsp70 content as compared to DMVA (0.52 vs. 0.32, p=0.02). The content of Hsp70 in the 120 minute group also showed an increase with heart failure having a higher content than DMVA by about 46.6% (1.54 vs. 1.05, p=0.04) (Figs. 16-20). The increases in the longer time periods of the studies strongly suggest that the use of DMVA assistance is effective in reducing myocardial stretch during heart failure. In turn, this is a positive sign that the tissues are more likely to remain intact and be better suited to recover from the heart failure when supported by DMVA.

The right ventricle has lower tensile strength and muscle volume when compared to the left ventricle and is thus more susceptible to problems arising due to mechanical stress. These characteristics created a greater challenge for the DMVA device to overcome and exhibit noticeable benefits during heart failure. In the right ventricle there was no change in the content of Hsp70 between both 30 minute groups and the sham
group. The 60 minute time point also had no significant differences between HF and DMVA. However, there was a significant increase in both 120 minute groups when compared to the 60 HF group, increases of 100% in DMVA (0.34 vs. 0.17, p=0.02) and 124% in the HF group (0.38 vs. 0.17, p=0.001) (Figs. 21-25). No single time point exhibited a significant difference between the Hsp70 content of the heart failure group versus the DMVA group. The right ventricle showed signs of increased HSP70 activation in both groups as the duration increased. Although the use of DMVA did not attenuate the activation of Hsp70 in the right ventricle, it also did not show any signs of increasing the activation of Hsp70 either.
Figure 16- Immunoblotting Representation of Hsp70 Content in the Left Ventricle of Sham Acute Hearts. Immunoblots were performed using 40μg of protein with anti-Hsp70 antibody at a dilution of 1:2000 to measure the relative protein content as described in the Methods section. Six sham acute hearts are represented in lanes 1-6, each normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 17- Immunoblotting Representation of Hsp70 Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 30 Minute Acute Hearts.

Immunoblots were performed using 40ug of protein with anti-Hsp70 antibody at a dilution of 1:2000 to measure the relative protein content as described in the Methods section. In (A), five acute hearts represented in lanes 1-5 had heart failure induced for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six acute hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 18- Immunoblotting Representation of Hsp70 Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 60 Minute Extended Support/Failure Hearts. Immunoblots were performed using 40ug of protein with anti-Hsp70 antibody at a dilution of 1:2000 to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), seven extended support/failure hearts represented in lanes 6-12 had heart failure induced while receiving direct mechanical ventricular actuation support for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 19- Immunoblotting Representation of Hsp70 Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 120 Minute Extended Support/Failure Hearts.

Support/Failure Hearts. Immunoblots were performed using 40μg of protein with anti-Hsp70 antibody at a dilution of 1:2000 to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six extended support/failure hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 20- Relative Hsp70 Content in the Left Ventricle in Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of relative protein amount, and *P<0.001 30 HF vs. Sham, **P<0.005 30 DMVA vs. Sham, †P<0.02 60 DMVA vs. 60 HF, ††P<0.001 120 HF vs. 60 HF, and ‡P<0.04 120 DMVA vs. 120 HF and PP<0.001 120 DMVA vs. 60 HF. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
**A**

Hsp70 in the Acute Left Ventricle

- **Sham**
- **30 HF**
- **30 DMVA**

**B**

Hsp70 in the Extended Support/Failure Left Ventricle

- **60 HF**
- **60 DMVA**
- **120 HF**
- **120 DMVA**
Figure 21- Immunoblotting Representation of Hsp70 Content in the Right Ventricle of Sham Acute Hearts. Immunoblots were performed using 40ug of protein with anti-Hsp70 antibody at a dilution of 1:2000 to measure the relative protein content as described in the Methods section. Six sham acute hearts are represented in lanes 1-6, each normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 22- Immunoblotting Representation of Hsp70 Content in the Right Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 30 Minute Acute Hearts.

Immunoblots were performed using 40ug of protein with anti-Hsp70 antibody at a dilution of 1:2000 to measure the relative protein content as described in the Methods section. In (A), five acute hearts represented in lanes 1-5 had heart failure induced for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six acute hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 23- Immunoblotting Representation of Hsp70 Content in the Right Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 60 Minute Extended Support/Failure Hearts. Immunoblots were performed using 40ug of protein with anti-Hsp70 antibody at a dilution of 1:2000 to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), seven extended support/failure hearts represented in lanes 6-12 had heart failure induced while receiving direct mechanical ventricular actuation support for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 24- Immunoblotting Representation of Hsp70 Content in the Right Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 120 Minute Extended Support/Failure Hearts.

**Support/Failure Hearts.** Immunoblots were performed using 40μg of protein with anti-Hsp70 antibody at a dilution of 1:2000 to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six extended support/failure hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 25- Relative Hsp70 Content in the Right Ventricle in Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of relative protein amount, and *P<0.001 120 HF vs. 60 HF and **P<0.02 120 DMVA vs. 60 HF. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
**A**

Hsp70 in the Acute Right Ventricle

- **Sham**
- **30 HF**
- **30 DMVA**

**B**

Hsp70 in Extended Support/Failure Right Ventricle

- **60 HF**
- **60 DMVA**
- **120 HF**
- **120 DMVA**
Caspase-8 Immunoblotting in the Left Ventricle

Caspase-8 is another marker for the extrinsic pathway of apoptosis. Caspase-8 was used as a marker to examine how the activation of the TNFR was being translated downstream in the signaling cascade (Fig. 3). Caspase-8 is activated after the appropriate amount of TNFR is activated causing it to auto-cleave from the inactive pro-caspase form. The activation of caspase-8 would further show if pro-apoptotic signaling is continuing to be activated along the extrinsic pathway. Immunoblotting was not run on right ventricle samples due to the discontinuation of the caspase-8 antibody being used. Since caspase-8 activation is a process where different segments are cleaved from the inactive pro-form, the activated segment at about 45kds is commonly observed as the marker for caspase-8 activation. In the acute model, there was no difference between the sham and both 30 minute groups (heart failure and DMVA assisted). In the extended support/failure model, there was no difference between both 60 minute groups and the 120 minute DMVA group (Figs. 26-30). However, the 120 minute heart failure exhibited a significant increase from both 120 minute DMVA supported tissue by 71.9% (0.98 vs. 0.57, p=0.02) and the 60 minute HF group by 88.5% (0.98 vs. 0.52, p=0.0006). These increases indicate that pre-apoptotic events are occurring in the heart failure model, but the DMVA assistance slows the activation of caspase-8. Thus, it can be concluded from these results that the use of DMVA is attenuating the left ventricle from continuing along the extrinsic pathway of pro-apoptotic events.
Figure 26- Immunoblotting Representation of Caspase-8 Content in the Left Ventricle of Sham Acute Hearts. Immunoblots were performed using 40ug of protein with anti-Caspase-8 antibody at a dilution of 1:500 to measure the relative protein content as described in the Methods section. Six sham acute hearts are represented in lanes 1-6, each normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 27- Immunoblotting Representation of Caspase-8 Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 30 Minute Acute Hearts.

Immunoblots were performed using 40µg of protein with anti-Caspase-8 antibody at a dilution of 1:500 to measure the relative protein content as described in the Methods section. In (A), five acute hearts represented in lanes 1-5 had heart failure induced for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six acute hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 28- Immunoblotting Representation of Caspase-8 Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 60 Minute Extended Support/Failure Hearts. Immunoblots were performed using 40ug of protein with anti-Caspase-8 antibody at a dilution of 1:500 to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), seven extended support/failure hearts represented in lanes 6-12 had heart failure induced while receiving direct mechanical ventricular actuation support for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 29- Immunoblotting Representation of Caspase-8 Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 120 Minute Extended Support/Failure Hearts.

**Support/Failure Hearts.** Immunoblots were performed using 40μg of protein with anti-Caspase-8 antibody at a dilution of 1:500 to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six extended support/failure hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 30- Relative Caspase-8 Content in the Left Ventricle in Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of relative protein amount, and *P<0.0006 120 HF vs. 60 HF and **P<0.02 120 DMVA vs. 120 HF. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
A

Caspase-8 in the Acute Left Ventricle

B

Caspase-8 in the Extended Support/Failure Left Ventricle
**Caspase-8 Enzyme Activity Assay**

An enzymatic assay specifically for caspase-8 was used to substitute the results shown in the immunoblotting assays. The benefit of this assay is that it showed only the enzymatically active caspase-8. The left ventricle exhibited an initial decrease in the caspase-8 enzymatic activity for both 30 minute groups when compared to sham, the 30 minute DMVA was significantly less (0.005 nmol/min/mg vs. 0.002 nmol/min/mg, p=0.02). At 60 minutes, DMVA had slightly higher caspase-8 activity when compared to heart failure, but not to a significant level. By 120 minute treatment time, the heart failure group exhibited an increased activity that was significantly higher than the DMVA group by 48% (0.008 nmol/min/mg vs. 0.005 nmol/min/mg, p=0.01) (Fig 31). These results confirmed the immunoblotting assay (Figs 26-30); in that caspase-8 levels were increasing consistently with time in heart failure, but not with DMVA assistance. This enzyme assay shows that the use of DMVA assistance attenuates the extrinsic pathway of pro-apoptotic signaling in the left ventricle.

The right ventricle was also assayed for caspase-8 activity, giving a more downstream look at the extrinsic pathway. The 30 minute heart failure group exhibited a 37% increase in caspase-8 activity in heart failure when compared to heart failure with DMVA (0.005 nmol/min/mg vs. 0.004 nmol/min/mg, p=0.14), but not significant when compared to the sham group. Within the different treatment time points there was no significant difference seen, but there was a consistent pattern of the DMVA group showing slightly less amounts of active caspase-8 activity (Fig 32). 120 minute DMVA treated tissue did exhibit a significant increase in activity when compared to the 60 minute heart failure (0.004 nmol/min/mg vs. 0.005 nmol/min/mg, p=0.03).
Figure 31- Relative Caspase-8 Activity in the Left Ventricle in an Acute (A) and Extended Support/Failure (B) Hearts.

Values as mean ± SEM of caspase-8 activity), and * P<0.02 30 DMVA vs. sham and P<0.03 30 DMVA vs. 30 HF, ** P<0.008 120 DMVA vs. 120 HF, †P<0.01 120 HF vs. 60HF. A Caspase-8 activity assay kit was run twice to get the average of the relative activity as described in the Methods section. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
**Caspase-8 Activity in the Acute Left Ventricle (nmol/min/mg)**

- **A**
- **B**
Figure 32- Relative Caspase-8 Activity in the Right Ventricle in an Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of relative protein amount, and * P<0.03 DMVA vs. 60 HF. A Caspase-8 activity assay kit was run twice to get the average of the relative activity as described in the Methods section. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trials.
A

Caspase-8 Activity in the Acute Right Ventricle (nmol/min/mg)

B

Caspase-8 Activity in the Extended Support/Failure Right Ventricle (nmol/min/mg)
Caspase-9 Immunoblotting

Caspase-9 assesses the rate of apoptosis through the intrinsic pathway during heart failure. Since it is a more downstream marker in the intrinsic pathway (Fig. 2), caspase-9 represents a late stage initiator in the apoptotic pathway. The left ventricle saw no change in caspase-9 content in both 30 minute groups as compared to the sham group. At 60 minutes there was no difference between heart failure and DMVA assistance. By 120 minutes, caspase-9 content in both groups had increased drastically and was significantly higher in content than the 60 minute heart failure group, about a 78% increase in the 120 minute heart failure (0.09 vs. 0.16, p=0.01) and 67% increase in the 120 minute with DMVA assistance (0.09 vs. 0.15, p=0.02) (Fig 33-37). Again, there was strong evidence pro-apoptotic events were continuing at a steady rate throughout the time points measured. Additionally, there was no evidence that the use of DMVA was able to attenuate the advancement of pro-apoptotic events in the left ventricle through the intrinsic pathway as measured by caspase-9 content.

In the right ventricle the sham group saw no change in the caspase-9 content between the 30 minute heart failure or DMVA assisted groups. There were also no differences between the 60 minute groups or between the 120 minute groups (Fig 38-42). There was, however, an 83% increase measured from 60 minutes to 120 minutes in both groups, but neither was to a statistically significant level. The right ventricle showed the same trend as the left ventricle as far as caspase-9 content over the various time points. For the right ventricle the increase from 60 minute HF to both 120 minute groups gave a p-value of about 0.08, showing the proximity to statistical significance. The results from both ventricles was also able to show the effectiveness of the model to induce pro-
apoptotic events during heart failure. Although DMVA did not show any benefits in this assay, it is also important to note that the use of DMVA assistance did not cause any further increase in pro-apoptotic events as measured by caspase-9 content.
Figure 33- Immunoblotting Representation of Caspase-9 Content in the Left Ventricle of Sham Acute Hearts. Immunoblots were performed using 40ug of protein with anti-Caspase-9 antibody to measure the relative protein content as described in the Methods section. Six sham acute hearts are represented in lanes 1-6, each normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Caspase-9

GAPDH
Figure 34- Immunoblotting Representation of Caspase-9 Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 30 Minute Acute Hearts.

Immunoblots were performed using 40ug of protein with anti-Caspase-9 antibody to measure the relative protein content as described in the Methods section. In (A), five acute hearts represented in lanes 1-5 had heart failure induced for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six acute hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 35- Immunoblotting Representation of Caspase-9 Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 60 Minute Extended Support/Failure Hearts. Immunoblots were performed using 40ug of protein with anti-Caspase-9 antibody to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), seven extended support/failure hearts represented in lanes 6-12 had heart failure induced while receiving direct mechanical ventricular actuation support for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 36- Immunoblotting Representation of Caspase-9 Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 120 Minute Extended Support/Failure Hearts. Immunoblots were performed using 40μg of protein with anti-Caspase-9 antibody to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six extended support/failure hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
**Figure 37- Relative Caspase-9 Content in the Left Ventricle in Acute (A) and Extended Support/Failure (B) Hearts.** Values as mean ± SEM of relative protein amount, and *P<0.01 120 HF vs. 60 HF and ** P<0.02 120 DMVA vs. 60 HF. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
A Caspase-9 in the Acute Left Ventricle

B Caspase-9 in the Extended Support/Failure Left Ventricle
Figure 38- Immunoblotting Representation of Caspase-9 Content in the Right Ventricle of Sham Acute Hearts. Immunoblots were performed using 40ug of protein with anti-Caspase-9 antibody to measure the relative protein content as described in the Methods section. Six sham acute hearts are represented in lanes 1-6, each normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 39- Immunoblotting Representation of Caspase-9 Content in the Right Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 30 Minute Acute Hearts.

Immunoblots were performed using 40ug of protein with anti-Caspase-9 antibody to measure the relative protein content as described in the Methods section. In (A), five acute hearts represented in lanes 1-5 had heart failure induced for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six acute hearts represented in lanes 6-11 heart failure induced while receiving direct mechanical ventricular actuation support for 30 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 40- Immunoblotting Representation of Caspase-9 Content in the Right Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 60 Minute Extended Support/Failure Hearts. Immunoblots were performed using 40ug of protein with anti-Caspase-9 antibody to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), seven extended support/failure hearts represented in lanes 6-12 had heart failure induced while receiving direct mechanical ventricular actuation support for 60 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 41- Immunoblotting Representation of Caspase-9 Content in the Right Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 120 Minute Extended Support/Failure Hearts. Immunoblots were performed using 40ug of protein with anti-Caspase-9 antibody to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six extended support/failure hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 42- Relative Caspase-9 Content in the Right Ventricle in Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of relative protein amount. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
A

Caspase-9 in the Acute Right Ventricle

B

Caspase-9 in the Extended Support/Failure Right Ventricle
Cytochrome-C Immunoblotting

Cytochrome-C was used as a marker to measure cell viability. The levels should have remained constant over all groups as long as there was no cell death occurring at a greater than normal rate. Immunoblots of cytochrome-C content in the left ventricle showed that there was a large amount of cytochrome-c present in all total cell extracts. In the sham group the total content varied within the group, but the variance within the group did not create an outlier. The amount of cytochrome-c did not change over the duration of the experiment in either the heart failure or the DMVA groups (Fig 43-44). These trials were only run once to determine quickly if there was any cell death occurring, which would have been seen by a decrease in cytochrome-c protein content per lane. The fact that no change was observed suggests that to a large extent cell death had not occurred in either model.

Immunoblots of cytochrome-C content was also done in the right ventricular extracts, again showing a large amount being present in total cell samples. The amount of cytochrome-c did not change significantly over the duration of the experiment in either the heart failure or the DMVA groups. Figures of the sham group and 120 minute groups are shown (Fig 45-46). The 120 minute group had one outlier in the 120 minute heart failure group (Fig. 46A, lane 5), other than that the relative densities between the heart failure and the DMVA groups were no significantly different from each other or the sham group. Our results suggest that there was little or no necrosis in the tissues during our experimental procedure.
Figure 43- Immunoblotting Representation of Cytochrome-C Content in the Left Ventricle of Sham Acute Hearts. Immunoblots were performed using 25µg of protein with anti-Cytochrome-C antibody at a dilution of 1:5000 to measure the relative protein content as described in the Methods section. Six sham acute hearts are represented in lanes 1-6, each normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 44- Immunoblotting Representation of Cytochrome-C Content in the Left Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 120 Minute Extended Support/Failure Hearts. Immunoblots were performed using 25ug of protein with anti-Cytochrome-C antibody at a dilution of 1:5000 to measure the relative protein content as described in the Methods section. In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes. In (B), six extended support/failure hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 45- Immunoblotting Representation of Cytochrome-C Content in the Right Ventricle of Sham Acute Hearts. Immunoblots were performed using 25ug of protein with anti-Cytochrome-C antibody at a dilution of 1:5000 to measure the relative protein content as described in the Methods section. Six sham acute hearts are represented in lanes 1-6, each normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Figure 46- Immunoblotting Representation of Cytochrome-C Content in the Right Ventricle During Heart Failure (A) and Direct Mechanical Ventricular Actuation (B) in 120 Minute Extended Support/Failure Hearts.  Immunoblots were performed using 25ug of protein with anti-Cytochrome-C antibody at a dilution of 1:5000 to measure the relative protein content as described in the Methods section.

In (A), five extended support/failure hearts represented in lanes 1-5 had heart failure induced for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.  In (B), six extended support/failure hearts represented in lanes 6-11 had heart failure induced while receiving direct mechanical ventricular actuation support for 120 minutes and were normalized to GAPDH at a 1:5000 dilution for quantification purposes.
Superoxide Dismutase Enzyme Activity Assay

Superoxide ions (SOD) measure the buildup of chemical stress ions in cells. It has also been suggested that SOD plays a role in helping to initiate apoptosis through the extrinsic pathway. The enzymatic activity of SOD in the left ventricle increased at both 30 minute groups when compared to the sham group, although none of the increases were considered significant (having a p value less than or equal to 0.05). In the groups that had DMVA assistance, there was little or no change in the SOD activity, with the exception of an increase in the 30 minute DMVA group, which was not considered significant. The differences between all groups became apparent once the duration reached 60 minutes. SOD specific enzyme activity in the 60 minute heart failure group was significantly higher than the 60 minute group with DMVA assistance (88% vs. 86%, p=0.03) and the difference became larger at the 120 minute point (89% vs. 86%, p<0.01). The difference between the 60 minute heart failure group and the 120 minute DMVA group was also significant with a p value of 0.01 (Fig. 47). It can be concluded that the use of DMVA attenuated the production of superoxide ions, suggesting that the upregulation of SOD by heart failure was attenuated. In the left ventricle the use of DMVA decreased the amount of chemical stress observed when compared to the group exposed to only heart failure.

In the right ventricle the difference between the sham group and the 30 minute HF group were considered significant. When individual groups where compared with each other at each time point, a difference was observed at the 30 minute time point between heart failure and DMVA (84% vs. 80%, p=0.03). There was no difference at 60 minutes and only a slight increase in SOD activity in the heart failure group at 120 minutes.
There was little difference seen between the 60 and 120 minute time points (Fig. 48). The benefits of DMVA in the right ventricle were less pronounced than in the left ventricle, with only an improvement exhibited at the 30 minute time point, but not within the group at the 30 minute point. The rest of the time points showed no difference between the normal rate of SOD expression in heart failure by itself or with DMVA assistance. This trend throughout all the time point did show that DMVA was always slightly lower in SOD than heart failure.
Figure 47- Relative SOD Activity in the Left Ventricle in Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of percent inhibition by SOD (%), and *P<0.03 60 vs. 60 HF and †P<0.02 120 DMVA vs. 60 HF and P<0.003 120 DMVA vs. 120 HF. A SOD activity assay kit was used to get the relative content as described in the Methods section. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
A

SOD in the Acute Left Ventricle (%)

B

SOD in the Extended Support/Failure Left Ventricle (%)

Sham  30 HF  30 DMVA

60 HF  60 DMVA  120 HF  120 DMVA
Figure 48- Relative SOD Activity in the Right Ventricle in Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of percent inhibition by SOD (%), and * P<0.0001 30 HF vs. Sham and †P<0.03 30 DMVA vs. 30 HF. A SOD activity assay kit was used to get the relative content as described in the Methods section. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
MMP-9 Enzyme Activity Assay

Matrix metalloprotease 9 (MMP-9) is an important marker for the overall structure of cardiac tissue. The increase in MMP-9 activity was observed in hearts that are breaking down the cellular matrix due to the enlarging of the heart. This occurs because as the cells enlarge MMP-9 is released to breakdown the extra cellular matrix to allow this growth of the cells to occur and the dilation of the ventricles ensues. In the left ventricle, there was a significant increase in both 30 minute groups when compared to the sham group, an increase of 118% (0.80 vs. 1.74, p=0.0001) in the 30 minute heart failure group and an increase of 94% (0.80 vs. 1.55, p=0.003) in the 30 minute DMVA group (Fig. 49). Within each time point there were no groups that showed any difference between heart failure and DMVA. The effect of heart failure was only observed initially in the 30 minute time point and after 30 minutes there was no change observed. This suggested that there was no great increase in MMP-9 activity.

The right ventricle showed similar results as the left ventricle in that there was a significant increase from the sham group to both 30 minute groups and then a slight increase from 60 minutes to 120 minutes, but not within time points (Fig. 50). The increase in MMP-9 enzyme activity showed that there was the general breakdown in the matrix and overall structure of the heart being compromised due to the failure. It was unusual to see no difference between heart failure and heart failure with DMVA assistance, since the DMVA cup should be able to protect the heart from enlargement.
Figure 49- Relative MMP-9 Activity in the Left Ventricle in Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of arbitrary units, and * P<0.0001 30 HF vs. sham, †P<0.003 30 DMVA vs. sham. A MMP-9 activity assay kit was used to get the relative content as described in the Methods section. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
A

MMP-9 Activity in the Acute Left Ventricle

Sham  30 HF  30 DMVA

B

MMP-9 Activity in the Extended Support/Failure Left Ventricle

60 HF  60 DMVA  120 HF  120 DMVA
Figure 50- Relative MMP-9 Activity in the Right Ventricle in Acute (A) and Extended Support/Failure (B) Hearts. Values as mean ± SEM of arbitrary units, and * P<0.002 30 DMVA vs. sham and †P<0.002 30 HF vs. sham. A MMP-9 activity assay kit was used to get the relative content as described in the Methods section. In (A), data are presented from six acute hearts for the direct mechanical ventricular actuation (DMVA) trial and sham trial, and five acute hearts for the heart failure (HF) trial. In (B), data are presented from five extended support/failure hearts for each HF trial, seven extended support/failure hearts for the 60 DMVA trial and six extended support/failure hearts for the 120 DMVA trial.
A

MMP-9 Activity in the Acute Right Ventricle

B

MMP-9 Activity in the Extended Support/Failure Right Ventricle
MMP-9 Zymography

This test for MMP-9 was meant to see the overall expression of MMP-9; however the method was less sensitive than the enzymatic assay. Multiple trials using various load amounts, tissue preparation protocols, and incubation periods were run on a zymogram gel (data not shown) to determine if there was a significant amount of MMP-9 present. All trials produced the same result of either no MMP-9 protein present or as the load became higher only excess protein was present with no increase in MMP-9. These tests were done using both left ventricle and right ventricle homogenates.
Discussion

The results of the study demonstrate significant findings that support the study hypothesis that DMVA can improve ventricular pump function of the failing rabbit heart while attenuating maladaptive cell signaling that characterizes heart failure. Multiple markers of heart failure were looked at to build an overall picture of how heart failure was progressing with and without DMVA assistance. The data showed that DMVA assistance was able to improve the heart’s ability to attenuate the molecular signaling involved in pro-apoptotic signaling and markers associated with mechanical stretch. A table of these results (Table 1) summarizes the types of markers that were examined and the trends seen in this study. The hearts were also split into an acute model and an extended support/failure model to account for the duration of heart failure used as the control and 30 minute time points and the extended durations of the 60 minute and 120 minute time points. Intrinsic and extrinsic early signaling pathways of apoptosis were looked at to analyze how pro-apoptotic events were initializing. Mechanical and oxidative stress markers were used to evaluate how the structure of the heart was being affected during heart failure. Lastly both pathways could be linked together to better understand how they interacted to induce heart failure and what affects using DMVA had on that system (Fig. 51).

The model used in this study proved to be a valid one for testing the effects of heart failure. It clearly demonstrated the upregulation of multiple markers associated with heart failure, to be discussed later. This was an important finding to verify the
Table 1- Summary of results for the left and right ventricles in both the Acute and Extended support/failure models for heart failure. The general conclusions from each marker tested are summarized to give a quick overview of all the results obtained in this study.
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Acute</th>
<th>Extended support/failure</th>
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<tbody>
<tr>
<td></td>
<td>LV</td>
<td>RV</td>
</tr>
<tr>
<td><strong>Extrinsic Pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significant increase in both</td>
<td>LV</td>
<td>RV</td>
</tr>
<tr>
<td>groups</td>
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<tr>
<td>Slight increase in both</td>
<td>LV</td>
<td>RV</td>
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<td>groups</td>
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<tr>
<td>Caspase-8: Immunoblotting</td>
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<tr>
<td>Slight increase at 30</td>
<td>LV</td>
<td>RV</td>
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<tr>
<td>minutes for both</td>
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<td>groups</td>
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<tr>
<td>Caspase-8: Enzymatic Assay</td>
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<tr>
<td>Decrease in both</td>
<td>LV</td>
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<td>groups from sham.</td>
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<tr>
<td>Significant difference</td>
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<td>at 30 minutes</td>
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<tr>
<td>Intrinsic Pathway</td>
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<tr>
<td>Caspase-9</td>
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<tr>
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<td>RV</td>
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<tr>
<td>Cytochrome-C</td>
<td></td>
<td></td>
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<tr>
<td>No differences</td>
<td>LV</td>
<td>RV</td>
</tr>
<tr>
<td>Stress Markers</td>
<td></td>
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<tr>
<td>Hsp70</td>
<td></td>
<td></td>
</tr>
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<td>Significant increase in both</td>
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<td>RV</td>
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<td>groups</td>
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<tr>
<td>MMP-9: Enzymatic Assay</td>
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</tr>
<tr>
<td>Significant increase in both</td>
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<td>RV</td>
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<td>groups</td>
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<td>Slight increase in both</td>
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<td>RV</td>
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<td>groups</td>
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</table>
**Figure 51- The Apoptotic Pathway.** An overall diagram to show the proposed pathways looked at in this study. It shows a collaboration of multiple apoptotic diagrams to illustrate how the markers in this study can be related to each other.
validity of the model and its use in future studies. The model was ideal due to its relatively easy administration and the ability to monitor and alter the dosing rates of the drugs made it possible to adjust the severity of failure to make it consistent from one trial to the next.

**Extrinsic Pathway of Apoptosis**

For the extrinsic pathway (Fig. 3), we first determined the tumor necrosis factor receptor (TNFR) content in the heart extracts to determine if more TNF-alpha was being recruited to initiate the caspase cascade. The overall trend in the data suggested that in both ventricles there was a steady increase in the concentration of activated receptors. In the acute model increases were observed at 30 minutes in both ventricles. The left ventricle saw a slight trend that DMVA was attenuating the rise in TNFR at 30 minutes. The extended support/failure model showed an increase in TNFR content from the 60 minute time point into the 120 minute time point, further exhibiting the signs of pro-apoptotic signaling in this model of heart failure. The use of DMVA assistance attenuated the rise in TNFR activation in the 60 and 120 minute time point in the left ventricle. In the left ventricle, the DMVA device was able to attenuate significantly the amount of TNFR that was being activated; an indication that heart failure with DMVA support attenuated the signaling for pro-apoptotic events that occur through TNFR. The right ventricle showed similar trends as in the left ventricle, however, there were no significant differences between the heart failure and the DMVA assisted groups at any time point. The possible reason for the right ventricle not exhibiting an impediment of TNFR activation is not
completely known, but some theories as to why the left ventricle and the right ventricle reacted differently will be discussed later.

We next tested at an apoptotic marker that the TNFR is directly linked to, caspase-8. We used immunoblotting to show all the forms of caspase-8 in the extracts and an enzymatic assay to examine the amount of activated caspase-8 present in the differently treated hearts. The immunoblotting evidence was limited to the left ventricle due to the discontinuation of the antibody being used. Our results showed little difference in the activation of caspase-8 in the acute left ventricle. There was a rise in both 30 minute groups when compared to the sham group. However, the extended support/failure model did provide evidence that in the 120 minute heart failure group there was an 88.5% increase when compared to the 60 minute heart failure group. This was additional evidence that the use of DMVA was able to attenuate progression along the extrinsic pathway by limiting the activation of caspase-8.

Since the immunoblots only determined caspase-8 in the left ventricle we used an enzymatic assay for caspase-8 to correlate with immunoblotting results and to in addition examine caspase-8 in the right ventricle. In the left ventricle we further confirmed the results from the immunoblots. There was evidence that the left ventricle with DMVA assistance exhibited attenuation of pro-apoptotic signaling by the activation of caspase-8. In the right ventricle there were no changes in caspase-8 activity in the acute groups when compared to each other or the sham group. However, the extended support/failure hearts showed both 120 minute groups having an increase in caspase-8 activation, with the 120 minute DMVA group showing a significant increase from the 60 minute heart failure. The average values for 120 HF were actually greater that 120 DMVA for
caspase-8, but the range for 120 HF were also greater. The fact that there is no difference in the activation levels of caspase-8 between heart failure and DMVA assistance is of interest in the right ventricle since the left ventricle is seeing a significant difference. One possible explanation is that the right ventricle is less capable of withstanding the force created by the DMVA cup during contraction and therefore is less likely to benefit from the actions of DMVA.

**Intrinsic Pathway of Apoptosis**

Caspase-9 was a main focus of how the intrinsic pathway was affected in this model (Fig. 2). We were uncertain whether we would see changes in caspase-9 due to the idea that the intrinsic pathway is more related to chemical stress and DNA damage (Crow, 2004), which was not expected to be present in this model or these shorter durations. The acute model saw no change in caspase-9 in either the left or the right ventricle. The extended support/failure model did show signs of increased activation over time, but no changes were observed between heart failure and DMVA in either the left or the right ventricle (Appendix A). The left ventricle had a significant increase in both 120 minute groups compared to the 60 minute heart failure. The right ventricle showed similar trends, but not to a significant level. The results showed that caspase-9 and the intrinsic pathway were neither attenuated nor aggravated by DMVA assistance in heart failure. Our results, although limited, suggest that DMVA did not have any effect on the intrinsic pathway through caspase-9.
Stress Markers of Heart Failure

There are two different stress related markers for heart failure that we examined. The first was based on mechanical stress markers which are initiated by the stretching of the myocardial cells and the overloading of the ventricles. Heat shock protein 70 (Hsp70) is a common protein that is activated in response to the stretching of the myocardium to help preserve the native structure of proteins being damaged. In the acute left ventricle, there was little effect of DMVA on Hsp70 at the 30 minute time point. During heart failure, there was a significant increase in the amount of activated Hsp70 measured in both the left and the right ventricles of the extended support/failure. At 60 minutes and carrying into 120 minute experimental duration, DMVA assistance attenuated the amount of Hsp70 that was being recruited. The left ventricle saw a significant difference between heart failure and DMVA at 120 minutes. At that same time point the right ventricle had a similar trend, but not significant. This could be related to the DMVA device’s ability to limit the amount of left ventricle overloading that can be responsible for much of the myocardial stretch during heart failure (Fedak, Part I, 2005). Since it has been shown that stretch alone can lead to Hsp70 recruitment, we can theorize that DMVA limited the amount of mechanical stretch placed on the left ventricle (Knowlton, 1991).

In the right ventricle, DMVA assistance during heart failure did not attenuate Hsp70 recruitment as well as the left ventricle. Both groups showed similar increases in the amount of Hsp70 being recruited over time. The use of DMVA assistance did not attenuate that level significantly at any time point. One possible reason behind this could be that the right ventricle is much thinner and therefore, it has weaker contractility as
compared to the left ventricle. The cells in the right ventricle stretched more readily and even with some support the tissue was being damaged, as indicated by increased Hsp70 content. Another possible cause could be in the action of the cup itself on the heart tissue in the right ventricle. Since the heart rate of a rabbit is high to begin with, the cup was contracting and expanding at a relatively fast rate. While the heart function may have been kept normal from a mechanical sense, the actual force of the cup on the right ventricle might have been too much and thus causing some damage of its own. In a few echocardiograph images made during our experiments, we observed that the DMVA cup could at times over compress the ventricles. In contrast, the left ventricle is a much stronger muscle and was physically more capable of handling this over compression.

Matrix metalloproteinase 9 (MMP-9) is another marker that is commonly used in heart failure to assess mechanical stress from ventricle overloading (Fedak, Part II, 2005). Unlike Hsp70, which reacts in response to cellular events, MMP-9 is found in the interstitial and is recruited when the extracellular matrix is being broken down. We used zymography to assess the levels of MMP-9 in the ventricles. Our results were unable to measure any MMP-9 using the zymograms. A previous study done by Dr. Anstadt also used zymography and did find significant differences of MMP-9 levels between heart failure and DMVA assistance. Due to those previous findings and our inability to get results from zymography we used an enzymatic assay to evaluate MMP-9. We observed an increase in MMP-9 relatively from the sham group compared to the acute samples at 30 minutes in both the left and right ventricles. Extended support/failure extracts from both ventricles did not show an increase of MMP-9 content over time of heart failure or between groups. The initial increase observed (Appendix A) did not continue with
increasing experiment times possibly due to an initial overload of MMP-9 in the system and no further recruitment. We expected to see the rise in MMP-9 levels, but there was no attenuation like seen in the previous study.

Superoxide dismutase (SOD) was observed to determine if there was an increase in harmful free radicals created during the heart failure process. The most widely used model for heart failure relies on ischemia/reperfusion, which restricts blood flow to the heart and causes a shortage of oxygen. During reperfusion the blood flow is restored, but the sudden reintroduction of oxygen causes the production of free radicals. Our model hoped to eliminate those confounding effects cause by ischemia, monitored in this case by SOD. Increases in SOD were thus expected to be caused from the stretch occurring from heart failure and the ventricular overloading. The enzymatic assay that was run showed that the SOD levels in the left ventricle were increased significantly as the duration of heart failure increased. There was a difference in the heart failure groups compared to the DMVA assisted groups at the 60 minute and 120 minute time points, where DMVA significantly attenuated the production of SOD. This trend showed that while heart failure induced a steady increase in the amount of SOD, the use of DMVA showed an attenuation of the amount of SOD present.

In the right ventricle there was a significant increase in SOD activity of the 30 minute heart failure compared to both the sham and 30 minute DMVA groups. At the other time points, there were no differences between heart failure and heart failure with DMVA assistance. At 120 minutes there was a slight attenuation of SOD in the DMVA group. The trend in the right ventricle shows some promise that at 120 minutes the levels of SOD appear to be rising in the heart failure group, but DMVA shows no similar rise
when compared to 60 minutes. These results in both ventricles lead to the conclusion that the use of DMVA assistance was attenuating the rise in SOD, likely due to the stretch occurring in the ventricles during heart failure. A rise in the free radicals would be a possible precursor for future cell damage, leading to cell death.

This study showed many positive trends as far as DMVA’s ability to retard the onset of various markers of heart failure. At points these differences were significant, but just as importantly there was a consistent pattern of some benefit of using the DMVA device during heart failure. There were a few examples where at 120 minutes the differences were not significant, but were showing trends towards DMVA attenuation of pro-apoptotic markers. It is important to note that this model is for an acute heart failure, where it may be less likely to see many significant changes in the short time period used. The benefits shown by this study prove that is prudent to look further into the benefits this device would have on a chronic heart failure model at longer time periods.

There were some limitations to this study. The use of immunoblots creates a good picture of the protein content in each different pathway, but in general, the results are limited by having an error of around 10% when quantitated. Multiple trials were used to limit this error. The use of enzyme assays was able to confirm the effectiveness of the immunoblots in the case of caspase-8, helping to provide stronger evidence that the use of immunoblotting was valid. The pathways examined were routine markers commonly examined in other studies analyzing the effects of heart failure: future studies should focus more on each pathway and the additional markers that play a role in apoptosis during heart failure. One final limitation was in the use of the rabbit heart itself. While it is a commonly used model, the high heart rate could have a negative affect when using
the DMVA cup. The problem is that the high rate of compression with the cup itself could be a problem by creating additional trauma to the failing heart. However, if that were the case, then DMVA assistance would have exhibited more negative effects in the results from the markers being studied. Our results showed that this was not the case and the data presented here provides a good sign for human trials in which the heart rate is slower.

**Concluding Remarks**

Our results give a general view of how apoptosis and stress damage propagate during heart failure, with and without DMVA assistance. The findings provided some positive results for the use of DMVA assistance to attenuate the negative cellular responses that occur during heart failure. It was a positive sign that there were no negative effects on the heart tissues from the use of DMVA assistance. Furthermore, there were many positive results that as the duration of heart failure increased, the markers of apoptosis and cellular stress were either unaffected or they were attenuated. Although more tests are required to confirm these results, it was shown by our study that the use of DMVA assistance may attenuate the degradation of heart tissue by pro-apoptotic signaling and cellular stress during heart failure.

Another finding from this study concerns the model of heart failure being used. Before the results could be used it had to be determined that we were inducing a heart failure state by causing ventricular overloading with esmolol. There was a consistent increase in the levels of the markers being looked at for heart failure that provided the best evidence that heart failure was occurring as duration increased. Those increases in
the markers for heart failure showed strong evidence that we were able to induce heart failure by using esmolol to cause ventricular overloading and enlargement of the heart.
### Left Ventricle Acute Heart Failure

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>30 HF</th>
<th>30 DMVA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extrinsic Pathway</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFR</td>
<td>0.193 ± 0.006</td>
<td>0.340 ± 0.026*</td>
<td>0.308 ± 0.016**</td>
</tr>
<tr>
<td>Caspase-8 (Immunoblotting)</td>
<td>0.572 ± 0.085</td>
<td>0.750 ± 0.065</td>
<td>0.753 ± 0.089</td>
</tr>
<tr>
<td>Caspase-8 (nmols/min/mg)</td>
<td>0.0052 ± 0.0006</td>
<td>0.0045 ± 0.0002***</td>
<td>0.0032 ± 0.0005†</td>
</tr>
<tr>
<td><strong>Intrinsic Pathway</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-9</td>
<td>0.060 ± 0.011</td>
<td>0.064 ± 0.006</td>
<td>0.085 ± 0.019</td>
</tr>
<tr>
<td>Cytochrome-C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Stress Markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp-70</td>
<td>0.058 ± 0.012</td>
<td>0.152 ± 0.017††</td>
<td>0.145 ± 0.021‡</td>
</tr>
<tr>
<td>SOD (%)</td>
<td>84.9 ± 1.67</td>
<td>87.9 ± 0.73</td>
<td>87.8 ± 0.27</td>
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<tr>
<td>MMP-9</td>
<td>0.797 ± 0.079</td>
<td>1.741 ± 0.145‡‡</td>
<td>1.546 ± 0.208#</td>
</tr>
</tbody>
</table>

*P<0.0002 30 HF vs. Sham  
**P<0.0001 30 DMVA vs. Sham  
***P<0.03 30 HF vs. 30 DMVA  
†P<0.02 30 DMVA vs. Sham  
††P<0.001 30 HF vs. Sham  
‡‡P<0.005 30 DMVA vs. Sham  
‡‡‡P<0.0001 30 HF vs. Sham  
#P<0.003 30 DMVA vs. Sham
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<th>60 DMVA Mean ± SEM</th>
<th>120 HF Mean ± SEM</th>
<th>120 DMVA Mean ± SEM</th>
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<tr>
<td>TNFR</td>
<td>0.460 ± 0.040</td>
<td>0.631 ± 0.086</td>
<td>1.738 ± 0.084*</td>
<td>1.292 ± 0.127**†</td>
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<tr>
<td>Caspase-8 (Immunoblotting)</td>
<td>0.518 ± 0.042</td>
<td>0.556 ± 0.057</td>
<td>0.988 ± 0.076***</td>
<td>0.567 ± 0.119†</td>
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<tr>
<td>Caspase-8 (nmols/min/mg)</td>
<td>0.0047 ± 0.0007</td>
<td>0.0058 ± 0.0004</td>
<td>0.0080 ± 0.0098††</td>
<td>0.0055 ± 0.0003‡‡</td>
</tr>
<tr>
<td><strong>Intrinsic Pathway</strong></td>
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</tr>
<tr>
<td>Caspase-9</td>
<td>0.092 ± 0.015</td>
<td>0.094 ± 0.013</td>
<td>0.158 ± 0.014††</td>
<td>0.148 ± 0.013‡‡</td>
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<td>Cytochrome-C</td>
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<td>-</td>
<td>-</td>
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<td><strong>Stress Markers</strong></td>
<td></td>
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<tr>
<td>Hsp-70</td>
<td>0.518 ± 0.066</td>
<td>0.315 ± 0.041#</td>
<td>1.536 ± 0.191##</td>
<td>1.048 ± 0.088∞∆</td>
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<tr>
<td>SOD (%)</td>
<td>88.1 ± 0.61</td>
<td>85.8 ± 0.66∞∞</td>
<td>88.9 ± 0.70</td>
<td>85.5 ± 0.70††∆∆</td>
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<tr>
<td>MMP-9</td>
<td>1.776 ± 0.170</td>
<td>1.812 ± 0.132</td>
<td>1.956 ± 0.284</td>
<td>2.160 ± 0.170</td>
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*P<0.0001 120 HF vs. 60 HF
**P<0.0003 120 DMVA vs. 60 HF
†P<0.02 120 DMVA vs. 120 HF
***P<0.0006 120 HF vs. 60 HF
†P<0.02 120 DMVA vs. 120 HF
††P<0.01 120 HF vs. 60 HF
‡P<0.008 120 DMVA vs. 120 HF
‡‡P<0.02 120 DMVA vs. 60 HF
#P<0.02 60 DMVA vs. 60 HF
##P<0.001 120 HF vs. 60 HF
∞P<0.04 120 DMVA vs. 120 HF
∆P<0.001 120 DMVA vs. 60 HF
∞∞P<0.03 60 DMVA vs. 60 HF
∆∆P<0.003 120 DMVA vs. 120 HF
### Right Ventricle Acute Heart Failure

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<th>30 DMVA Mean ± SEM</th>
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<tr>
<td>TNFR</td>
<td>0.148 ± 0.001</td>
<td>0.246 ± 0.034*</td>
<td>0.218 ± 0.025**</td>
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<td>Caspase-8 (Immunoblotting)</td>
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<tr>
<td>Caspase-8 (nmols/min/mg)</td>
<td>0.0049 ± 0.0004</td>
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<td>0.0038 ± 0.0007</td>
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<tr>
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<td>0.086 ± 0.010</td>
<td>0.108 ± 0.016</td>
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<tr>
<td>Cytochrome-C</td>
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</tr>
<tr>
<td><strong>Stress Markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp-70</td>
<td>0.080 ± 0.008</td>
<td>0.090 ± 0.023</td>
<td>0.0583 ± 0.0202</td>
</tr>
<tr>
<td>SOD (%)</td>
<td>79.1 ± 0.57</td>
<td>83.9 ± 0.62***</td>
<td>80.4 ± 1.28†</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.899 ± 0.0515</td>
<td>1.389 ± 0.105††</td>
<td>1.276 ± 0.091‡</td>
</tr>
</tbody>
</table>

*P<0.02 30 HF vs. Sham
**P<0.03 30 DMVA vs. Sham
***P<0.0001 30 HF vs. Sham
†P<0.03 30 DMVA vs. 30 HF
‡P<0.0003 30 HF vs. Sham
‡‡P<0.002 30 DMVA vs. Sham
## Right Ventricle Extended/Support Heart Failure

<table>
<thead>
<tr>
<th></th>
<th>60 HF</th>
<th>60 DMVA</th>
<th>120 HF</th>
<th>120 DMVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td><strong>Extrinsic Pathway</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TNFR</td>
<td>0.302 ± 0.043</td>
<td>0.364 ± 0.026</td>
<td>0.552 ± 0.056*</td>
<td>0.457 ± 0.028**</td>
</tr>
<tr>
<td>Caspase-8 (Immunoblotting)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Caspase-8 (nmols/min/mg)</td>
<td>0.0039 ± 0.0004</td>
<td>0.0033 ± 0.0002</td>
<td>0.0059 ± 0.0010</td>
<td>0.0048 ± 0.0002***</td>
</tr>
<tr>
<td><strong>Intrinsic Pathway</strong></td>
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<tr>
<td>Caspase-9</td>
<td>0.064 ± 0.021</td>
<td>0.054 ± 0.010</td>
<td>0.112 ± 0.013</td>
<td>0.105 ± 0.008</td>
</tr>
<tr>
<td>Cytochrome-C</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Stress Markers</strong></td>
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<tr>
<td>Hsp-70</td>
<td>0.172 ± 0.016</td>
<td>0.191 ± 0.027</td>
<td>0.376 ± 0.038†</td>
<td>0.337 ± 0.0520‡‡</td>
</tr>
<tr>
<td>SOD (%)</td>
<td>84.4 ± 1.01</td>
<td>84.3 ± 1.20</td>
<td>86.3 ± 1.46</td>
<td>84.2 ± 0.98</td>
</tr>
<tr>
<td>MMP-9</td>
<td>1.488 ± 0.148</td>
<td>1.488 ± 0.103</td>
<td>1.704 ± 0.157</td>
<td>1.652 ± 0.156</td>
</tr>
</tbody>
</table>

*P<0.01 120 HF vs. 60 HF
**P<0.01 120 DMVA vs. 60 HF
***P<0.03 120 DMVA vs. 60 HF
†P<0.001 120 HF vs. 60 HF
‡‡P<0.02 120 DMVA vs. 60 HF
References


Blom, A., et al. (2005). “Infarct size reduction and attenuation of global left ventricular remodeling with the CorCap(TM) cardiac support device following acute myocardial infarction in sheep.” Heart Failure Review, 10, 125-139.


Fedak, PW., et al. (2005). "Cardiac remodeling and failure: from molecules to man (Part II)." Cardiovascular Pathology, 14(1), 49-60.


Hypertrophic and Apoptotic Responses to Mechanical Stretch in Cardiac Myocytes.” Circulation Research, 89(5), 453-60.


