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Treatment of Aortic Heart Valve Conduit with Glutamine and Heat Shock as a Means to Deter the Constituent Cellular Population From Becoming Apoptotic

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TREATMENT OF AORTIC HEART VALVE CONDUIT WITH

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CONSTITUENT CELLULAR POPULATION FROM BECOMING

APOPTOTIC

by

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ABSTRACT

TREATMENT OF AORTIC HEART VALVE CONDUIT WITH GLUTAMINE AND
HEAT SHOCK AS A MEANS TO DETER THE CONSTITUENT CELLULAR
POPULATION FROM BECOMING APOPTOTIC

Alyce Marie Linthurst Jones
Old Dominion University, 2012
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Cryopreserved allograft heart valves represent the best solution for a patient with a failing heart valve. However, the constituent cells become apoptotic and within months of transplant the heart valve becomes acellular and the recipient's cells do not repopulate the allograft (3, 51). A strategy to prevent this situation would be to minimize or prevent apoptosis from occurring by strategically altering steps during heart valve processing. Recently it has been demonstrated that: 1) Heat shock protein 70 is a negative modulator of the apoptotic cascade; 2) Cells in culture exposed to hypothermic conditions produce heat shock protein 70 upon rewarming; and 3) Glutamine can induce heat shock protein 70 production. The purpose of the current research is to apply these concepts to allograft heart valve tissues and begin to understand the mode(s) of action by investigating the resultant intracellular heat shock protein 70 concentration, presence of caspase 3, presence of cytochrome c outside of the mitochondria and compromised DNA.

Allograft heart valve conduit tissue was processed traditionally at 4°C and 37°C with and without glutamine and assessed prior to and after cyropreservation. Intracellular heat shock protein 70 and cytochrome c concentration inside and outside of the mitochondria was assessed utilizing enzyme-linked immunoassay; the presence of caspase 3 was assessed utilizing immunohistochemistry and histomorphometry; and the presence of compromised DNA was assessed utilizing the TUNEL assay and histomorphometry. It was found that treatment of allograft heart valve conduit tissue at 37°C plus glutamine

lead to a statistically significant increase in heat shock protein 70 concentration, maintenance of the mitochondrial cytochrome c concentration, less caspase 3 positive cells and less TUNEL positive cells relative to the other three treatment groups immediately prior to cyropreservation and after thawing and subsequent incubation at 37°C for seventy-two hours.

Therefore it is concluded that allograft heart valves can be processed at, 37°C with glutamine, which will facilitate increased amounts of heat shock protein 70 thereby reducing the amount of apoptotic cells. The presence of heat shock protein 70 appears to modulate apoptosis at or upstream of cytochrome c release from the mitochondria.

This Dissertation is lovingly dedicated to the memory of my father and my mother who always put my needs, wants, desires and ambitions head of their own and sacrificed much so I would have an opportunity to succeed. Successful completion of my doctoral degree would be the most meaningful way to honor them and their devotion to their only child. Utilizing my knowledge to advance and develop new treatment options for patients with cardiovascular disease will best utilize the skills and knowledge I have obtained and continue to honor their memory and their love for me.

ACKNOWLEDGMENTS

I would like to thank Dr. Lloyd Wolfinbarger for his faith in me during the course of my doctoral work. Without his guidance, love and patience I would not have developed the skills necessary to complete my degree nor to be successful outside the walls of academia. Wolf, the lessons you have taught me over the last thirteen years will serve me well and contribute substantially to whatever degree of success I attain. I would also like to thank my husband Derek and son Ian for their love, patience and understanding over the years as I continued to work to complete my degree. Without the support of some very dear friends, this process would have been much more difficult to endure and not nearly as much fun – Katrina Crouch Ruth, Mary Hayward, Dada Softic, Lisa Williams and Xiao fei Qin. Perry Lange has played a significant role in my personnel and professional development over the last eleven years. He has taught me about the realities of making scientific decisions in industry, but always conducted his mentoring in a supportive manner. I would like to thank my Dissertation Committee, new and old for supporting me through the process (Dr. Lloyd Wolfinbarger, Dr. Chris Osgood, Dr. Emilia Oleszak and Dr. Ralph Powers). I would also like to acknowledge my LifeNet Health family as well. Many of them have inquired frequently over the years about how my Dissertation was going and it felt good that so many people cared enough to ask and offer their support. Additionally, it should be noted that LifeNet Health supported the research presented in this Dissertation. Finally, this research would not have been possible without the selfless decision of the individual's who's donated tissues were used during the course of this research.

Thank you everyone for believing in me, supporting me, encouraging me and not giving up on me. I will do my best to ensure all of your efforts to help me were not in vain.

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CHAPTER 1

INTRODUCTION

Each year, approximately 2600 allograft heart valves are transplanted into adults and children with congenitally malformed or degenerating native or bioprosthetic valves (1). Allografts are human tissues, which are procured from a deceased donor who has previously consented for their organs and tissues to be removed with the intent of them being transplanted into another human being. Surviving family members may also consent to organ and/or tissue donation. Cryopreserved allograft heart valves are the best replacement for failing heart valves for three reasons: 1) allograft heart valves provide the best hemodynamic support due to their geometry; 2) allograft heart valves are the least thrombogeneic option available; and 3) allograft heart valves best resist calcification relative to bioprosthetic (porcine or bovine) valves. However, cryopreserved allograft valves have inherent problems that prevent them from being the perfect replacement heart valve. These problems include the inability to support cellular in-growth or remodel once implanted *in vivo,* and the propensity to calcify, which is often accentuated in pediatric recipients. All of these weaknesses can potentially be attributed to cryopreserved valves becoming acellular within months of transplant (2, 3, 4, and 5). The rapid *in vivo* decellularization of the allograft heart valve is attributed to the cells being apoptotic, having undergone programmed cell death, either at the time of transplant or shortly thereafter. Without a viable cell population, the valves cannot renew their collagen and elastin matrix which would allow them to grow with a pediatric recipient and resist normal wear and tear over a lifetime *in vivo.* Additionally, the presence of apoptotic cells provides the necessary reactants (calcium and phosphate) to initiate calcification. Conversely, the presence of a viable cell population would allow for both the continuous renewal of the extracellular matrix, thus resisting degradation, and production of factors to aid in

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combating calcium formation in the arterial wall (6). Therefore, there is a need to develop a mechanism to reduce the propensity of the constitutive cells in allograft heart valves to become apoptotic, which should improve the long-term durability of allograft heart valves. To successfully do this requires improved understanding of the cellular events occurring between procurement and cryopreservation and the ability to manipulate these events to halt the constitutive cells in the allograft valves from progressing to apoptosis. It has been reported in the literature that heat shock protein 70 (Hsp70) is capable of halting the progression of apoptotic cascade and is inducible via glutamine (7). Therefore a potential strategy to prevent the constitutive cells in an allograft heart valve from becoming apoptotic might be to engage the heat shock response and Hsp70 expression through the use of heat and/or by supplementing the disinfection media with additional glutamine during processing.

To date, researchers have not attempted to manipulate the heat shock response (any of the heat shock protein family members) during the processing phase for allograft heart valves destined for cryopreservation. Successful application of this strategy might be utilized to improve allograft heart valve processing methods, thus resulting in the maintenance and long term viability of the primary constitutive cell population, vascular smooth muscles cells, in heart valves once transplanted.

1.1 PURPOSE AND HYPOTHESES

It has been demonstrated that cells exposed to hypothermic temperatures and subsequently returned to physiological temperature can be stimulated to produce the anti-apoptotic protein, Hsp70, when returned to physiological temperature, 37°C (8). A second strategy to chemically stimulate Hsp70, reported in the literature, is addition of glutamine beyond what is present in traditional tissue culture media (7, 9). These results suggest the possibility that the cryopreserved allograft heart valve cell population can be preconditioned during processing (Chapter 2.1) to minimize the potentially pro-apoptotic effects

of hypothermic insult that occurs during transport and processing. As a result of preconditioning (hypothermia followed by return to physiological temperature in the presence of supplemental glutamine), when the valve is thawed and transplanted, it may have a reserve of protective heat shock proteins to aid in correctly refolding proteins that may be denatured during the cryopreservation process. Another potential scenario is that the allograft heart valve cell population may be preconditioned to efficiently produce these heat shock proteins upon rewarming the graft prior to implantation, consequently minimizing the prevalence of apoptotic cells and thus preventing the valve from becoming acellular *in vitro*. Based upon these observations from the literature, the following hypotheses were proposed: 1) Returning the heart valve to physiological temperature during processing will result in an increase in Hsp70 in the constitutive cells, thereby minimizing the presence of pro-apoptotic proteins and apoptotic cells; 2) Returning the heart valve to physiological temperature in the presence of glutamine during processing will result in an increase in Hsp70 in the constitutive cells, thereby minimizing the presence of pro-apoptotic proteins and apoptotic cells; and 3) Upon thawing the allograft heart valve post-cryopreservation and incubating it under physiological conditions, increased amounts of Hsp70 in the constitutive cells will minimize the presence of pro-apoptotic proteins and apoptotic cells in conduit tissue. Information gained from exploring these hypotheses may provide important information needed to improve the cell viability, and hence long term durability and utility, of implanted cryopreserved allograft heart valves. The end result of exploring and beginning to understand the cellular events that occur during allograft heart valve processing could have a positive impact on the thousands of adults and children who receive these life saving allograft heart valves every year.

CHAPTER 2

BACKGROUND OF THE STUDY

2.1 STANDARD ALLOGRAFT HEART VALVE PROCESSING

A discussion of the potential cellular mechanisms involved in cryopreserved allograft heart valves becoming acellular *in situ,* requires understanding how an allograft heart valve is processed and what is meant by the term "processed." Once an individual dies and consent is given for organ and/or tissue donation, the heart is aseptically removed from the donor, within 24-hours of asystole and washed repeatedly with cold (1-10°C) sterile isotonic solution to remove all of the blood. The heart is then transported to a tissue bank within 24-hours of procurement on wet ice (1°C - 10°C) in sterile isotonic solution. Once at the tissue bank, the aortic and pulmonary valves are dissected free from the heart in cold (1-10°C) sterile isotonic solution and placed into a cocktail of antibiotics and tissue culture media for 24 +/- 2 hours at 1-10°C. The final step is to cryopreserve the valves in a solution comprised of 10% dimethylsulfoxide (DMSO), 10% fetal calf serum and RPMI 1640 tissue culture media. The cryopreservation process is performed using a control rate freezer that facilitates cooling the tissue at 1°C per minute to control ice crystal formation. One the tissue reaches -40°C it is transferred to the vapor phase of liquid nitrogen (-120 to -195°C) for storage until shipment in liquid nitrogen vapor for transplantation. Obviously, the valves spend a considerable time during processing at hypothermic conditions.

2.2 HEAT SHOCK PROTEIN 70

Ferruccio Ritossa first described the heat shock phenomenon in 1962 after a laboratory worker accidently increased the temperature in the *Drosophila* incubator (10). When the fly's salivary glands were microscopically examined, it

was noted that the polytene chromosomes had a puffed band (10). This indicated that the gene represented by that band was experiencing elevated rates of transcriptional activity. In 1974, Tissieres *et al.* first reported the presence of unique proteins in *Drosophila melanogaster* exposed to elevated temperatures (11). These proteins were aptly named heat shock proteins due to their mode of expression. Since then, a multitude of heat shock proteins have been discovered in all organisms from bacteria to humans and characterized into families according to their relative molecular weights, cellular compartment and distribution (82, 123). The Heat Shock Protein 70 Family can be found in the cytosol, nucleus, mitochondria and the endoplasmic reticulum (82). Heat shock proteins have also been demonstrated to be highly conserved; more specifically, the Hsp70 family demonstrates 60-80% sequence homology among eukaryotes (12).

One of the most interesting and unique properties of heat shock proteins is they are an integral part of normal cell physiology and they are an integral part of cell/organism survival during periods of extreme physiological stress (45, 63, 124, 122). During protein translation, when the organism is euthermic (~37°C), Hsp70 ensures nascent peptide chains with exposed hydrophobic residues do not inappropriately aggregate with one another rendering them inactive. Hsp70 also functions as an intracellular protein chaperone to transport peptides to desired locations (86, 87, 88, 89, 90). However, during periods of extreme physiological stress, such as changes in pH, cell oxidative state, nitrogen reactive species, superoxide, hypoxia, ischemia, inflammation and accumulation of malfolded or denatured proteins, Hsp70 has been demonstrated to be essential and integral to cell and organism survival (85, 91, 102, 103, 127). The Hsp70 binding event may prevent further unfolding of the target protein, may refold the protein or may continue to fold the protein into a functional configuration within a stressed cell (12). Another function of Hsp70 is it may shuttle malformed proteins to the ubiquitin and proteolysis degradation pathways as the accumulation of misfolded proteins is a stimulus for apoptosis a subsequent cell death (12, 111)

A brief understanding of the Hsp70 gene, its inducible transcription and translation and protein structure will facilitate putting the results of the research described herein into perspective. The Hsp70 gene, as found in the genomic DNA, is comprised of 2,440 base pairs with a 212-base pair leader sequence and 242-base pair 3' untranslated region (14,110). The 5' region contains at least two regulatory elements that interact with heat shock transcription factors (HSFs) (99, 100, 110). The efficiency of the Hsp70 transcriptional process is attributed to the constitutive presence of inducible HSFs monomers, the lack of introns in the Hsp70 gene, the presence of an internal ribosome entry site in the 5' untranslated region of the Hsp70 mRNA, alternative Hsp70 mRNA splicing and heat-induced stabilization of Hsp70 mRNAs (15, 16, 17, 74, 110, 128).

During periods of stress, HSFs separate from Hsp70 in the cytosol, are phosphorylated by protein kinase, oligomerize as homotrimers in the cytosol, enter the nucleus and bind to heat shock elements (HSE) in the hsp70 gene promoter and cause transcription to be initiated (Figure 1) (92). The increase in hsp70 is attributable to the activation of heat shock transcription factor 1 (HSF1). Interestingly, HSF1 is hyperphosphorylated when activated by heat treatment of cells. In contrast, HSF1 activated by cold shock (2-3°C) followed by rewarming to room temperature (22°C), is not hyperphosphorylated and this difference may point to a different mechanism for mediating the response to cold and heat induced cellular stress responses (18). This lack of phosphorylation may affect how tightly HSF1 binds to its target DNA and what target genes it comes into contact with and thereby activate or down regulate these genes. During induction of the heat shock response, the resultant protein products can account for 15% to 25% of the total intracellular proteins within minutes after physiological stress (19).

From a structural perspective, heat shock proteins are ATP binding proteins and consist of three major functional domains. Figure 2 (page 8) is a ribbon crystal structure of the three Hsp70 functional regions. The N-terminal ATPase domain is 44kDa, binds adenosine triphosphate (ATP), hydrolyzes it to adenosine diphosphate (ADP) and is characterized by having four domains that
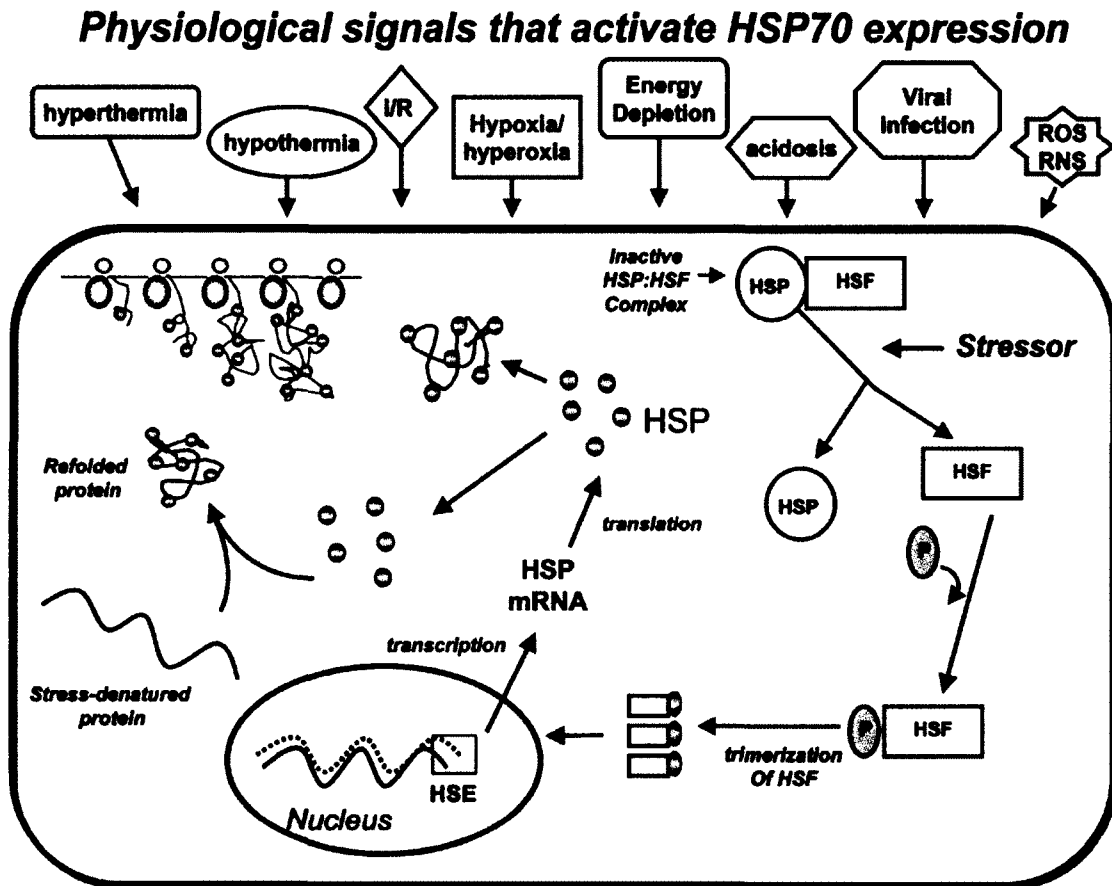
FIGURE 1. Hsp70 Inductive Signals, Hsp70 Gene Activation and Hsp70 Synthesis. A myriad of extracellular stimuli can result in activation of the heat shock response. Inducible Hsp70 transcription factors (HSFs) are bound to Hsp70 in the cytosol and maintained in an inactive state. However, upon application of an external stressor the HSFs separate from Hsp70 are phosphorylated (P) by protein kinases and form trimer complexes in the cytosol. The complexes proceed to the nucleus, enter and bind to heat shock elements (HSE) in the promoter region of the Hsp70 gene. Transcription of Hsp70 mRNA commences and exits the nucleus and enters the cytosol, where new Hsp70 is synthesized (21).
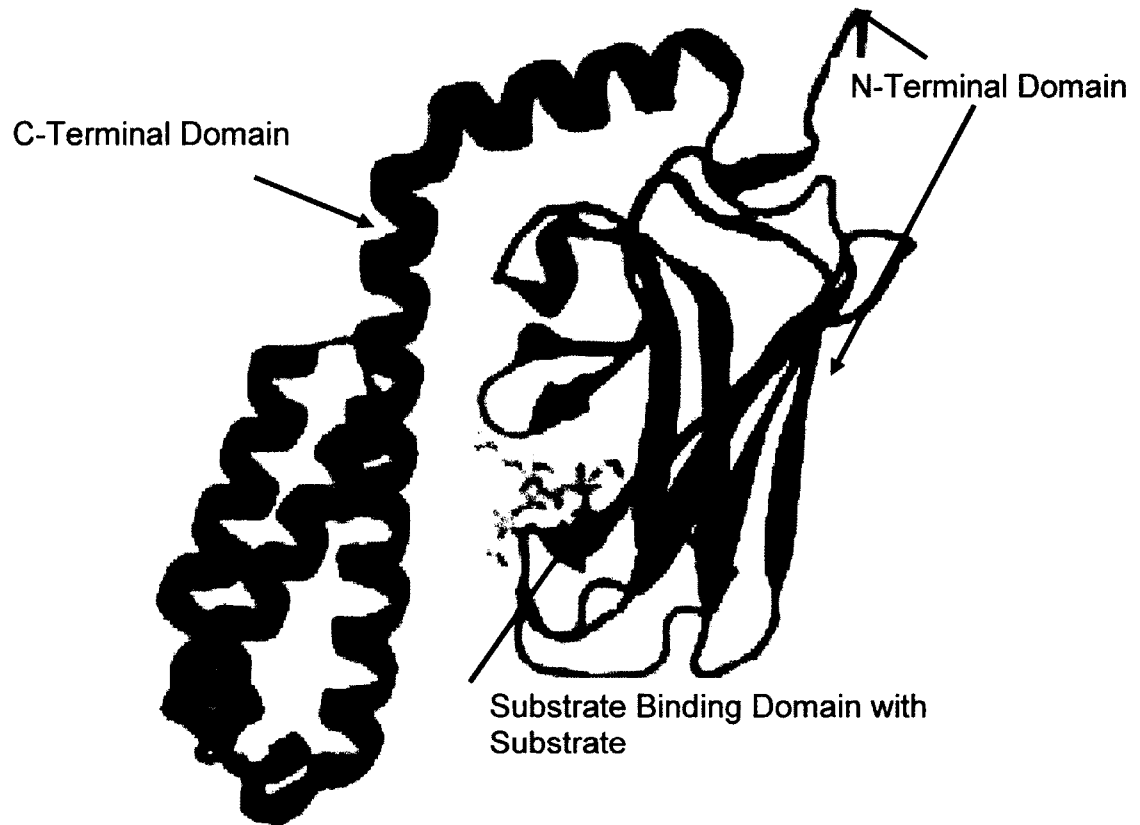
FIGURE 2. Crystal Structure of Hsp70 with Bound Substrate. The 44kDa N-terminal region contains the ATPase domain, the 18 kDa substrate binding domain and the 10kDa C-terminal region that closes in the presence of tightly bound protein (23).

form two lobes with a cleft between them (20). The hydrolysis of ATP to ADP fuels the conformational changes for the remaining two domains. The substrate binding domain is 18 kDa and interacts with hydrophobic regions on target peptide and proteins, but favors peptides with basic residues over those with acidic residues. It is characterized by having four anti-parallel beta pleated sheets and one alpha helix (22). The C-terminal domain is 10 kDa and aids in regulating how tightly a target protein is bound in the substrate binding domain. It is characterized by an alpha helix followed by a glycine-proline rich aperiodic segment next to a highly conserved segment containing glutamic acid, valine and

aspartic acid (23). This conserved sequence affects the amount of mRNA translated during heat shock (25).

The strength of the target peptide binding is directly related to whether Hsp70 is bound to ATP (loose target peptide interactions) or ADP (tight target peptide interactions). The hydrolysis of the phosphate group from the ATP molecule results in Hsp70 undergoing an allosteric change that sequesters the bound peptide. The strength of binding is dictated by the target peptide constitutive residues and is a likely control point for Hsp70 activity. Peptides containing large hydrophobic residues and aromatic residues stimulate the ATPase activity of Hsp70; conversely, peptides containing hydrophobic and basic residues are poor ATPase stimulators. The amino acid composition of the target peptide chain is used to distinguish native versus unfolded proteins. Native proteins merely need to be chaperoned to their destination, whereas Hsp70 prevents aggregation of newly forming nascent peptide chains and production of misfolded proteins that could initiate apoptosis (26). Once the protein is completely translated, a nucleotide exchange factor causes ADP to be released from Hsp70 and a new ATP to bind to Hsp70 which results in Hsp70 undergoing a conformational change and the new properly folded protein is released.

In summary, Hsp70 is ubiquitously expressed across phyla and cellular compartments. It is constitutively expressed to prevent aggregation of newly formed nascent peptides and refold improperly folded proteins. Upon application of physiological stress to the cell, Hsp70 transcription is significantly upregulated and highly efficient. During these periods of stress, Hsp70 contributes to rescuing the cells through refolding denatured proteins, at a minimum. Therefore, it is reasonable to suggest that Hsp70 is a key element in the maintenance of intracellular and organism homeostasis.

2.3 HYPOTHERMIA AND THE HEAT SHOCK RESPONSE

Hypothermia is often used as a tool to arrest metabolic activity within cells, thereby minimizing autolysis, free radical formation, reactive oxygen species

formation and other postmortem changes that are not compatible with cellular viability (13). Several roles for hypothermia to protect cells from becoming apoptotic or necrotic have been reported in the literature (27, 28, 116, 119). Some examples are performing cardiac bypass and spinal surgery under mild hypothermia, approximately 33°C, treating stroke patients with hypothermia and the well documented use of hypothermia to preserve whole organs for transplant. However, prolonged exposure to hypothermia has its own inherent risks such as lipid peroxidation, lipid membrane phase transition and lipid membrane leakiness. Compromising the integrity of cell and organelle membranes can potentially result in apoptosis or necrosis (29). *In vitro* experimentation with mammalian cells exposed to hypothermia have demonstrated increased populations of misfolded proteins that could result from denaturation during hypothermic insult or from nascent proteins that did not complete the folding process (30).

Hypothermia has also been utilized in nature as a successful survival strategy. Hibernating animals undergo a process called hypothermic preconditioning where they cycle between euthermic and various degrees of hypothermia to survive during winter by maximizing their food stores when sources are scant. They do this by reducing their body temperature to 15°C for 7 days and then rewarming to 37°C for several days. This is followed by 20 days at approximately 5°C (31). This strategy is hypothesized to increase the efficiency of Hsp70 induction and production during each subsequent hibernation cycle to protect animals from the detrimental cellular effects of prolonged exposure to hypothermic conditions and aid in their recovery. This model from nature points to a mild hypothermic preconditioning followed by rewarming to 37°C followed by a deep hypothermic episode from which the animal successfully recovers. This finding may provide a parallel to one of the key experimental groups investigated during the research described herein.

Other researchers have utilized cell culture systems as well as small animal models to better understand how the Hsp70 response can be manipulated using hypothermia to benefit cell survival for potential human clinical

applications. Research by Currie *et al* demonstrated that hearts subjected to cold ischemia for 17 hours at 4°C exhibited significant Hsp70 production upon reperfusion at 37°C (32). This same experiment carried out with mammalian cells in culture demonstrated that when incubated at 4°C and subsequently rewarmed to 37°C the cells had high levels of Hsp70 (33). Importantly, Laios *et al* conducted research to investigate the chronology of Hsp70 induction post-hypothermic exposure upon return to 37°C. An *in vitro* model of cardiac myocytes demonstrated that after one hour of hypothermia at 4°C, Hsp70 production peaked after 4-6 hours of rewarming at 37°C (27).

Liu *et al*, investigated incubating human IMR-90 diploid fibroblasts at 4°C for 0, 1, 1.5, 2, 2.5, 3, 3.5 or 4 hours followed by incubation at 37°C for 5 hours to assess for differential production of Hsp70 protein (33). Pulse labeling of the cells with ^{35}S-labeled amino acids during the last hour of incubation at 37°C demonstrated that the longer the cells were held at 4°C, the more Hsp70 was produced by the cells upon return to 37°C. In particular, the cells incubated at 4°C for 4 hours prior to their incubation at 37°C produced similar amounts of Hsp 70 to the control cells that were traditionally heat shocked by placing them at 42°C for 5 hours.

Another finding of the Liu study was that those cells that were cold stressed for the longest period of time, produced Hsp70 for the longest period of time (greater than 8 hours) upon rewarming, relative to the other experimental groups (33). To further investigate their findings, an electromobility gel shift assay (EMSA) was performed. The purpose of the EMSA was to demonstrate if the HSF binding to the heat shock element (HSE) in the hsp70 gene in the cells that were rewarmed was equivalent to HSF binding to the HSE in the positive control cells (traditionally heat shocked at 42°C). Their results demonstrated that an increase in the amount of HSF binding to the HSE was proportional to the length of time the cells were held at 4°C. Those cells with the longest incubations at 4°C had EMSA results equivalent to the positive control cells that were traditionally heat shocked. Liu *et al*. interpreted the data to mean that there was an increase in the transcriptional activity associated with the hsp70 gene. Their conclusion

was that the magnitude and the duration of Hsp70 production were directly related to the severity of the cold stress. In addition, cells that were maintained at 4°C without rewarming had no change in the amount of Hsp70 present in the cells or heat shock element (HSE) binding.

Fujita *et al* subjected cardiomyocytes to hypothermic treatment and found that Hsp70 expression increased as the cells were returned to physiological temperature, rather than as they were being cooled. They also determined the amount of Hsp70 produced was proportional to the duration of hypothermia. After rewarming, the cardiomyocytes retained their functionality as assessed by electromechanical activity (43).

In support of the data reported by Fujita *et al*, Cullen *et al* exposed one group of rats to 2-3°C for 8 hours and a second group of rats to 2-3°C for 8 hours plus an additional incubation at room temperature (22°C) for one hour. At the conclusion of their respective incubations the animals were sacrificed and the hearts, kidneys, livers and lungs were removed and analyzed for hsp70 mRNA production. It was determined that hsp70 mRNA was present in all the organs from the hypothermic treatment group (group one) but at very low amounts. However, the same organs from the animals that underwent hypothermic treatment followed by rewarming to 22°C (second group) had at least doubled the amount of hsp70 mRNA in their hearts and kidneys and increased it by six-fold in the liver (18).

Thus the research conducted to date on Hsp70 indicates: 1) Hsp70 can be induced in response to a wide variety of physiological insults such as hypothermia and denatured intracellular proteins, not just heat, 2) Hsp70 can be induced to be transcribed and translated in response to changing the environment from 4°C to 37°C and 3) the amount of Hsp70 formed is directly related to the time at hypothermic temperatures and impacts the chronology of Hsp70 formation. It is intriguing to consider the possibility that cells incubated at 4°C would perceive returning to physiological temperature, 37°C, as heat shock. Therefore the hypothesized impact of transferring allograft heart valves from 1-10°C to 37°C for a prescribed period of time is that the constitutive cell

population will have an increased amount of Hsp70 present at the conclusion of the disinfection incubation and be pre-conditioned for the second hypothermic insult that occurs during cryopreservation. The benefit of heat shocking the cells to produce Hsp70 during antibiotic disinfection may be to promote the refolding of proteins which were denatured and/or misfolded during hypothermic transport. This "proteotoxicity," is a potent stimulator of the intrinsic apoptotic cascade (35). The initial heat shock may also precondition the cells and aid in their recovery after cryopreservation. Therefore, reducing the proteotoxicty of the constitutive allograft heart valve cells through augmenting the amount of Hsp70 present would be expected to prevent the apoptotic cascade from proceeding from this type of pro-apoptotic signal.

2.4 GLUTAMINE AND HEAT SHOCK PROTEIN INDUCTION

L(+)-Glutamine (Gln) is the most abundant amino acid in plasma and many tissues (7). Gln is a neutral amino acid with a molecular weight of 147 Da. It can enter the cell through sodium-dependent systems and sodium independent systems (36). Gln is not considered an essential amino acid; however, when the body is under physiological stress, it may be essential for survival (120). Recently published data on a meta-analysis of all human clinical trials using Gln as the sole agent in surgical and critical illness demonstrated a strong trend toward reduction of infection complications post-surgically and a reduction in complications and mortality rates in the critically ill (37). Interestingly, Gln is known to stimulate the production of Hsp70 in the gut and retina when under physiological stress (7). This stimulation is advantageous because, as discussed in the previous chapter, Hsp70 protects cells under stress by binding to the hydrophobic sites on intracellular proteins that may have become denatured during the physiological stress.

There are several precedents for adding Gln to cells or tissue to induce Hsp70 production. Gln has been shown to enhance the synthesis of Hsp70 in *Drosophila* cells and Chinese hamster ovary cells (13, 106). Additionally, it has

been demonstrated that other neutral, small amino acids such as glycine and alanine protect renal tubule cells from lethal stress and injury caused by ischemia, hypoxia, anoxia and toxic chemicals (39, 108). These amino acids were also cytoprotective for hepatocytes exposed to cold storage conditions (40). Kojima et al. tested the efficacy of administering various Gln dosages to rats. The rats were anesthetized 1, 2, 4 or 6 hours after Gln administration and their hearts were harvested, stored at 4°C in University of Wisconsin solution for 24 hours, heterotopically transplanted to the neck of syngeneic recipient rats and monitored daily for function by palpation. Several hearts were not transplanted but sacrificed for immunoblotting for the presence of Hsp70. The immunoblotting demonstrated Hsp70 at 1 hour and maximally 2 to 4 hours post Gln administration (9). The results of the study demonstrated that the more Hsp70 detected in the surrogate tissue, the longer the heterotopically transplanted heart beat in the rat. This research dealt specifically with the transplant of the entire heart and a hypothermic incubation for 24 hours prior to transplant. In contrast, the current research deals with the excision of the aortic valve, hypothermic incubation for 24 to 48 hours and heat shock followed by cryopreservation.

The research described thus far has focused on inducing Hsp70 through addition of Gln to the culture medium or administering it to a test animal. Research conducted in Wischmeyer's lab has begun to elucidate the mechanisms involved (38). The experimentation was performed by heat shocking mouse embryonic fibroblasts in a water bath at 44°C for 50 min after administration of 0-16 mM Gln (8 mM was optimal). From this set of experiments they concluded (Figure 1 is a helpful reference to follow their conclusions): 1) Gln increased binding of HSF-1 to the HSE, and 2) Gln enhanced phosphorylation of nuclear HSF-1 which is the final step in the HSF-1 transactivation pathway leading to transcription of Hsp70. Another group (Ropeleski et al) examined the mechanism by which Hsp70 transcription is potentiated by Gln using a similar model system as Wischmeyer. They concluded that: 1) Gln is able to augment the amount of Hsp70 transcript produced, 2) Gln is able to increase HSF-1 DNA binding during heat shock and, 3) no Gln dependent differences in HSF-1

phosphorylation, trimerization, nuclear localization, or HSF-1 promoter activity were demonstrated as a result of administration of Gln. They hypothesize that Gln is able to: 1) interact with HSF-1 and the core transcriptional machinery, 2) affect the chromatin organization of the 5' flanking regions of the Hsp70 gene, and 3) affect other trans-factors upstream in the Hsp70 promoter. In spite of this progress, the actual mechanism of how Gln positively affects transcription and/or translation of Hsp70 remains to be conclusively demonstrated (41, 112).

Based upon the Gln research discussed above, it seems plausible that Gln will successfully induce Hsp70 in aortic conduit cells subjected to the aforementioned regime. Therefore, pre-conditioning the constitutive cells to produce Hsp70 may protect the viability of the constitutive smooth muscle cell population and thus positively impact the durability of the valve so it functions for a clinically significantly longer period of time *in situ*.

2.5 APOPTOSIS

Research performed during the last twenty years has transformed apoptosis from a field defined by morphological changes and genetic demise to a well characterized deconstruction of the cellular machinery that has many modes of regulation. As a result, the literature review in this and subsequent chapters will be limited to apoptosis and the associated factors as they relate to the research to be reported herein. The term apoptosis appeared in the peer-reviewed literature in 1972 to describe a structurally distinct mode of cell death within living tissues (42). The hallmark changes an apoptotic cell undergoes are chromatin condensation, DNA laddering, cell shrinkage, phosphatidylserine exposure on the exterior of the phospholipid bilayer and membrane blebbing. Apoptosis is a catabolic process, requiring ATP, which ensures that the regulated cascade of events proceeds in an orderly fashion so as not to elicit an inflammatory response. An alternative pathway for cell death is necrosis, a non-ordered process by which the contents of damaged cells are released inside the organism and result in an inflammatory response.

Apoptosis is a necessary part of normal cell function. It is involved in embryological development, normal tissue turnover, atrophy, negative selection of T-cells in the thymus and T-cell mediated killing. Apoptosis is also a necessary and important part of organism survival after exposure to cytotoxic compounds, hypoxia or viral infection. As a result, the organism's affected cells are efficiently removed and new healthy cells can replace them so that the organism might survive the physiological insult. To underscore the importance of apoptosis, cells that lose their ability to undergo apoptosis are transformed into neoplastic cells.

Apoptosis can proceed by two main pathways: the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway. The mitochondria serves multiple functions during the progression of the intrinsic pathway: 1) as an integrator of the multiple signals traveling along the proapoptotic cascade, 2) as a coordinator of the catabolic reactions leading to apoptosis and 3) as a gate keeper of proapoptotic proteins (BAX). Therefore upon permeabilization of the outer mitochondrial membrane and disregulation of Bcl-2 and Bcl-2 related anti-apoptotic proteins, cytochrome c is released from the intermembrane space, and the caspase cascade is activated (42). Figure 3 on page 18 is a schematic representation of the intrinsic apoptotic pathway. The extrinsic apoptotic pathway is activated by cross-linking specific receptors on the surface of the cell. These receptors are often referred to as death receptors and include TNFR1, CD95/APO-1, Fas and TRAIL-R1. The structural feature that endows these receptors with their death domain status is their intracytoplasmic domain called the death domain. The death domain on the receptors interacts with the death domains of the cytosolic proteins TRADD and FADD. In turn, their death effecter domains interact with the death effecter domains of pro-caspase-8 thereby causing its proteolytic autoactivation with the end result being active caspase-8 and thereby activation of the extrinsic apoptotic cascade. However, the intrinsic pathway is the most likely progression for the constitutive cells in allograft heart valves and thus will be given more attention throughout this Dissertation.

2.6 THE MITOCHONDRIA AND CYTOCHROME C

Mitochondria are intracellular organelles whose primary role is to manufacture ATP that fuels daily activities in the cell. Mitochondria are 0.5 – 1.0 μm in diameter and comprised of an outer membrane, an inner membrane, an intermembrane space and the cristae formed from the folding of the inner membrane and the matrix (97). The intermembrane space houses cytochrome c, a water-soluble protein that is a pivotal molecule in the apoptotic cascade (56). Cytochrome c is a highly conserved protein across species, has a molecular weight of 12 kDa, has a net positive charge and is loosely bound to the mitochondrial intermembrane space (56). Cytochrome c normally functions in the respiratory chain by transferring electrons between Complexes III and IV; however, in response to apoptotic stimuli, the mitochondrial permeability transition pore is activated and opened by BAX, a pro-apoptotic Bcl-2 family member, and cytochrome c is released from the mitochondrial intermembrane space into the cytosol Once in the cytosol, cytochrome c complexes with apoptosis protease activating factor (Apaf-1), procaspase-9 and dATP which results in pro-caspase 9 being cleaved into its active form, caspase-9. Procaspase-3 is activated by caspase-9 and commits the cell to completing the apoptotic program (Figure 3 page 18), (42, 58 83).

Another key role for the mitochondria is maintenance of cell homeostasis. Fundamental to this role, the mitochondria serves as a calcium buffer/reservoir for the cell and plays a critical role, along with the endoplasmic reticulum in storing and releasing calcium as needed by the cell to activate necessary intracellular signaling pathways (98, 114). The mitochondria are also the cell's sensor for abnormal changes in temperature, pH, osmolality, accumulation of denatured proteins and other aberrant physiological events (49). Four examples of these changes, which are relevant to cryopreserved allograft heart valves, are: 1) abnormal temperature fluctuations (37°C to 4°C to -195°C), 2) osmolality changes (10% DMSO has an approximate osmolality of 2000 mOsm/kg HOH
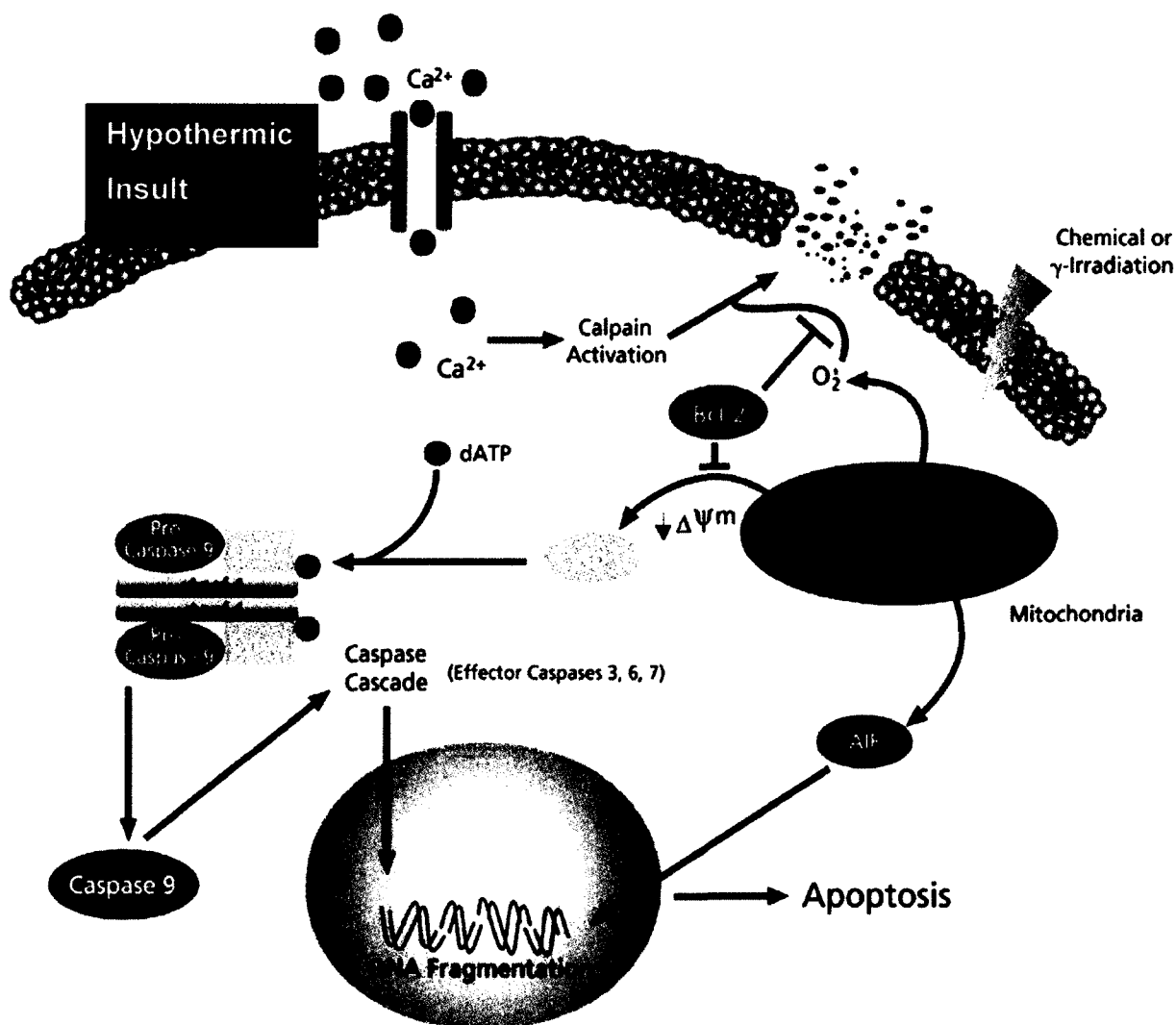
FIGURE 3. Schematic Representation of the Apoptotic Cascade.

Hypothermia causes changes in the permeability of the cell membrane allowing extracellular calcium to enter the cell. The mitochondrion attempts to sequester the calcium but is ultimately unsuccessful and results in increased permeability of the mitochondrial outer membrane. This results in cytochrome c translocating from the mitochondria intermembrane space into the cytosol. Cytochrome c, an essential element in electron transport, forms a complex with Apaf-1, a protease, which in turn activates procaspase 9, and initiates a cascade of downstream caspases (caspase-3). The proteolytic activity of the caspases eventually results in DNA fragmentation and cell death. Bcl-2 and Bcl-X can block the release of

cytochrome c from the mitochondria; prevent activation of the caspase cascade and ultimately apoptosis (44).

versus physiologic osmolality of 280-300 mOsm/kg), 3) accumulation of denatured proteins as a result of the hypothermic insult and 4) influx of extracellular calcium as a result of cell membrane damage from prolonged hypothermic exposure.

Perturbations in normal physiology can cause the mitochondria to activate the intrinsic apoptotic pathway (49, 56). The exact physiochemical manner in which mitochondria detect internal abnormalities and connect them to executing the apoptotic program is not understood. However, there is speculation that the mitochondria may initiate the apoptotic program after detecting increases in intracellular calcium (114,115). This apoptotic triggering mechanism may be applicable to the proposed experimental system. Zachariassen *et al* demonstrated that when cells experience prolonged hypothermic exposure it results in increased membrane fluidity and increased permeability to extracellular calcium as depicted in Figure 3 on page 18 (29).

Calcium influx, as a result of hypothermia, occurs as the phospholipid membrane cools and its fluidity changes such that it becomes more permeable to extracellular calcium. The concentration gradient favors calcium diffusing from the extracellular to intracellular environment. There is a millimolar to micromolar calcium gradient, extracellular to intracellular (29, 114). As a result of calcium influx, the mitochondria functions to sequester the excess calcium and at certain concentrations, pro-apoptotic signals begin within the mitochondria that initiate the intrinsic apoptotic pathway through activation of the mitochondrial permeability transition pore (MPTp). This pore allows for the free diffusion of ions and cytochrome c into and out of the mitochondria (114). This release of cytochrome c initiates a cascade of events that concludes in the execution of the apoptotic program.

Newmeyer *et al.* first described that the mitochondria played a key role in apoptosis in a cell-free model (104). This research was followed by that of Liu *et al* by using cell extracts, where it was demonstrated that cytochrome c could activate the apoptotic-signaling cascade *in vitro* (33). They also demonstrated an increase in cytochrome c concentration in the cytosol of apoptotic cells, which supports the results of the research described herein. A more detailed examination of how cytochrome c is release from the intermembrane space is as follows and is focused on the anti-apoptotic proteins under investigation in the research reported herein. The release of cytochrome c likely occurs due to the removal or inactivation of the Bcl-2 family of anti-apoptotic proteins (Bcl-2 and Bcl-X_L), which are housed in the inner mitochondrial membrane (54, 83, 96). The Bcl-2 proteins form a pore that remains closed and regulates the opening of BAX which covers and regulates another pore, termed the mitochondrial permeability transition pore (MTPp). As long as both pores remain closed, cytochrome c cannot escape (47).

A proposed mechanism is centered upon the balance of Bcl-2 homodimers versus BAX homodimers and the prevailing pair determines whether the cell resists apoptosis or succumbs to apoptosis, respectively (94, 95, 96, 105). The exact mechanism of how this occurs, whether there is an upstream protease that facilitates their inactivation, or another mechanism, is still to be determined. Once cytochrome c is released it binds to the endoplasmic reticulum causing stored calcium to be released into the cytosol and thus establishing a positive feedback loop with the mitochondria to drive the apoptotic cascade forward. Additionally, once in the cytosol cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1), and in the presence of dATP/ATP promotes assembly of the apoptotasome. This large complex subsequently activates pro-caspase-9. Procaspase-9 contains a caspase recruitment domain (CARD) that is utilized to moderate specific interactions with the CARD domain in Apaf-1 (48). Consequently, procaspase-9 is cleaved, by an unknown mechanism, into its active form caspase-9, and it cleaves and activates the key executioner caspase,

caspase-3. Once caspase-3 is activated the cell has reached a point of no return and is committed to programmed cell death.

An interesting phenomenon that impacts the research conducted for this Dissertation is that, several studies have demonstrated that cells going through apoptosis do not experience damage to their mitochondrial structures when proteins are released from the mitochondrial intermembrane space. It was further found that when cytochrome c was released from the mitochondria, the mitochondria shrank rather than swelled (93). If caspase inhibitors were added to the reaction, the cells were rescued and after growth factors were added the mitochondria returned to their original size and cytochrome c content (49). Another conclusion drawn from the Martinou research was that no irreversible damage had occurred, such as rupture of the outer membrane. A subsequent study to investigate the integrity of the inner mitochondrial membrane was conducted by von Ahsen *et al* (50). They isolated mitochondria treated with BAX or Bid and cells treated with UV radiation or staurosporin and depleted them of their cytochrome c. The mitochondria retained a fully intact protein import mechanism, which indicates that the mitochondrial transmembrane potential ($\Delta\Psi$m), ATP and the inner membrane were fully intact and functional. Only the outer membrane was breached thereby allowing the passage of cytochrome c from the mitochondria (50).

Therefore, it becomes readily apparent that mitochondria play key roles in maintaining intracellular homeostasis and responding to physiological insults by activating the apoptotic cascade. These functions are being performed in addition to maintaining the capacity to produce the ATP necessary to successfully drive the apoptotic program through to a successful conclusion to support the long term viability of the organism.

2.7 BCL-2

Bcl-2 is an anti-apoptotic member of the larger family of Bcl-2 proteins that regulate the permeability of the mitochondrial outer membrane. The family is

coded by no less than 25 genes and contains anti-apoptotic and pro-apoptotic members. *Bcl-2* is a proto-oncogene encoded by a 230 kb gene that results in a 24-26 kDa protein which is localized to the mitochondrial, microsomal and nuclear membranes within a diverse number of cell types, inclusive of aortic smooth muscle cells (54). The mammalian gene, *bcl-2*, is a homologue to the ced-9 gene in the nematode worm *Caenorhadbitis elegans* (52). The human *bcl-2* gene can function in *C. elegans*, thereby indicating that control of apoptosis is a highly evolutionarily conserved process (52).

Bcl-2 is a protein that prevents the apoptotic program from proceeding forward by preventing the release of cytochrome c from the mitochondria (109). Bcl-2 is theorized to inhibit apoptosis by binding to other Bcl-2 family of proteins via their BH1/BH2/BH3 domains (53, 83, 84). These domains are comprised of α helices in close proximity, which create hydrophobic pockets that are presumptive areas where Bcl-2 family members interact with each other via intercalation (54). The pro-apoptotic proteins, BAX and BAK, can stoichometrically overwhelm Bcl-2 molecules by binding to them via their own BH1/BH2/BH3 domains in the mitochondrial membrane thereby allowing cytochrome c to escape from the mitochondria (55). Therefore, the group of proteins with the most molecules present prevails. The expression of Bcl-2 can promote cell survival by countering some but not all apoptotic stimuli (56). In addition, *bcl-2* is well known to be deregulated in tumor cells as a result of translocation into the immunoglobulin heavy chain locus and as a result is constitutively activated in B cell malignancies (57). Also, Bcl-2 has been demonstrated to protect mammalian cells from DNA, RNA, and protein synthesis inhibitors, and sodium azide, colchicines, steroids, heat shock and irradiation (58).

Research has demonstrated that hypothermia enhances *Bcl-2* expression and protects against apoptotic death in Chinese hamster ovary cells (13). The importance of these results to the research described herein is that it demonstrates a link between hypothermic treatment and subsequent induction of

the anti-apoptotic gene Bcl-2 which has the potential to prevent progression to apoptosis by blocking release of cytochrome c from the mitochondria.

In summary, Bcl-2 like many of the other key elements in the apoptotic cascade is highly conserved across species speaking to its importance in contributing to cell homeostasis. Bcl-2 is essential in regulating the escape of cytochrome c from the mitochondrial intermembrane space into the cytosol and, as such, Bcl-2 is a key regulatory protein in the apoptotic cascade. Previous research has indicated Bcl-2 is enhanced by hypothermic exposure which is analogous to the test articles used during the research reported herein.

2.8 CASPASES

Caspases (cysteinyl-aspartic-acid- proteinases) are a family of cysteine proteases that specifically cleave their substrates after aspartate residues. Their targets are other pro-caspases in the apoptotic cascade as well as pro-apoptotic proteins such as Bid. Two other pro-apoptotic proteins that caspases cleave are poly (ADP-ribose) polymerase (PARP), a protein essential for DNA repair, and gelsolin, whose active proteolytic products mediate some of the morphological changes that occur during apoptosis and activation of the anti-apoptotic proteins in the Bcl-2 family (58). The end result of this cascade is the characteristic chromatin condensation, DNA laddering, cell shrinkage and formation of apoptotic bodies that are associated with apoptotic cells.

Genetic analysis of the nematode *Caenorhadbitis elegans (C. elegans)* has provided much data regarding apoptosis, the proteins that regulate programmed cell death and how highly conserved this process has been throughout evolution. Activation of two genes in particular, *ced-3* and *ced-4*, have been deemed essential for apoptosis in this nematode. *Ced 3* was found to be homologous to the human protein, 1β-converting enzyme (ICE). A protein resembling ICE is found in apoptotic cell extracts, prICE, and it cleaves substrates known to be targeted during apoptosis, but it does not cleave pro-interleukin-1β. Later it was determined that prICE was caspase-3 and it is

responsible for the cleavage of PARP and the catalytic subunit of DNA-dependent protein kinases. Both of these substrates have an aspartate (ASP) residue in the P_1 and P_4 positions of the caspase-3 catalytic domain as their recognition site. Mosser *et al* found that Hsp70 inhibits the transition from the zymogen to active caspase-3, thereby inhibiting the progression of the apoptotic program (59).

One of the hallmarks of caspases is their high degree of regulation, which is necessary given their extraordinary capabilities. To that end, caspases exist in most cell types as zymogens, an inactive form of an enzyme. Caspases are cysteine proteases that have a minimum of 100 target substrates during apoptosis (60). They cleave on the carboxyl side of the ASP residue and their basic structure is similar. Each active caspases has an $\alpha_2\beta_2$ tetramer comprising two identical large subunits (17-22 kDa) and two identical small subunits (20–12 kDa). The zymogen form has an N-terminal prodomain, a large subunit and a small subunit. Of the 12 main caspases, 7 are felt to be involved in apoptosis. Caspases 3, 6 and 7 have been classified as effector caspases. Upon activation, by being cleaved between their large and small subunits, they cleave most known apoptotic substrates (60). Caspases 2, 8, 9 and 10 have been classified as initiator caspases for the apoptotic program. The main difference between the activator and the effecter caspases is effecter caspases have a homotypic protein-protein interaction motif and a caspase activation and recruitment domain (CARD) which contribute to the transduction of various signals into proteolytic activity. Some of the basic regulatory strategies for caspases include selective use of contact between the interacting protein domains which may take advantage of hydrophobic interactions and allosteric changes in protein conformation.

Procaspase-9 is specifically activated by the release of cytochrome c from the mitochondria. Procaspase-9 is cleaved and activated which in turn cleaves the zymogen form of caspase-3, and activates caspase-3 (61). Caspase-3 production is a milestone in the commitment of the cell to become apoptotic and the junction between the intrinsic and extrinsic apoptotic pathways. In contrast,

the external pathway is activated when the Fas receptor is cross linked, thereby activating the death domains which result in the cleavage of the zymogen form of caspase-8, activating it and allowing it to cleave procaspase-3 into active caspase-3 (62). The activation of caspase-3 results in the commencement of DNA cleavage by endogenous nucleases that result in cell shrinkage and the formation of apoptotic bodies (62).

In summary, caspases are a highly conserved family of proteins across species whose activities are tightly regulated post-transcriptionally, which is indicative of how important and powerful they are. Left unchecked, they are capable of bringing about the demise of their host. Hsp70 may be capable of inhibiting pro-caspases from progressing to their active forms. Thus augmentation of the Hsp70 response may help avert activation of the caspases in allograft heart valve cells, thereby preventing progression of the apoptotic cascade.

2.9 THE HEAT SHOCK RESPONSE AND APOPTOSIS

It has been experimentally demonstrated that constitutive over expression of Hsp70 yields sustained protection from apoptosis (64). This is somewhat paradoxical, as heat shock protein accumulation in ischemic cells has been considered a hallmark of cell injury and viewed as a negative finding. Rather, their presence should perhaps be considered indicative of the cells attempting to survive the physiological insult. To support this, it has been demonstrated that when *Xenopus* are microinjected with denatured proteins, heat shock protein production is stimulated in response to the stress of accumulating denatured proteins (65). In addition, oxidation of thiol groups in proteins as the result of exposure to oxidizers and ischemia has been demonstrated to lead to protein denaturation (107). Therefore, whether or not a cell goes through apoptosis after being physiologically insulted may be a race between the ability of heat shock proteins to repair the damaged intracellular proteins and the apoptotic program to progress as the cells continue to receive apoptotic stimuli.

Hsp70 has been demonstrated to inhibit the progression of the apoptotic cascade (59). Polla and coworkers reported that the mitochondria might be the target for heat shock induced protection against apoptosis from stress (66). Recent research has demonstrated several points within the intrinsic apoptotic cascade where Hsp70 acts as a negative regulator (Figure 4, page 27). Those points of negative regulation are as follows: 1) inhibition of stress- activated protein kinase pathway (SAPK/JNK) phosphorylation and subsequent activation of transcription factors and caspases, 2) inhibition of pro-caspase-3 transformation into caspase-3 and committing the cell to apoptosis, 3) prevention of cytochrome c release from the mitochondria by increasing the amount of Bcl-2, 4) competition for Apaf-1 binding sites on the apoptosome during recruitment of pro-caspase-9 to the apoptosome, 5) interference with conversion of pro-caspase-3 to active caspase-3 and 4) inhibition of downstream of caspase-3, potentially (53). In support of these potential regulatory points within the apoptotic cascade, Li *et al* found that Hsp70 inhibited apoptosis downstream of the release of cytochrome c and upstream of the activation of caspase-3 (42). These data would point to caspase-9 being the Hsp70 target.

There is evidence that Hsp70 induction can be correlated with protection against stress induced apoptosis (74, 75, 76, 77, 78, 79, 80, 81, 82). An assertion made in this dissertation's hypotheses is that hypothermic preconditioning (1-10°C incubation followed by 37°C incubation) prior to cryopreservation will result in a significant increase in heat shock protein in the cells. The presence of Hsp70 is hypothesized to decrease the quantity of pro-apoptotic proteins and the number of cell that are irreversibly apoptotic compared to constitutive cells in tissues that do not undergo the preconditioning treatment. Research conducted by Mosser *et al.* used an *in vitro* model to demonstrate that cells, in which the expression of Hsp70 was either constitutively elevated or inducibly elevated by a tetracycline-regulated transactivator, were resistant to heat stress-induced apoptosis. They determined that cells induced to produce elevated Hsp70 inhibited activation of stress-activated protein kinase (SAPK/JNK). This nuclear pathway is implicated in apoptosis induction after

ceramide treatment of cells. They were able to further elucidate that cleavage of pro-caspase-3 to the active form, caspase-3, is inhibited in cells expressing elevated Hsp70 and may at least partially account for inhibition of apoptosis, (59).

Interestingly, Beere *et al.* demonstrated that Hsp70 is able to directly inhibit caspase-9 processing by interacting with Apaf-1 to prevent the recruitment
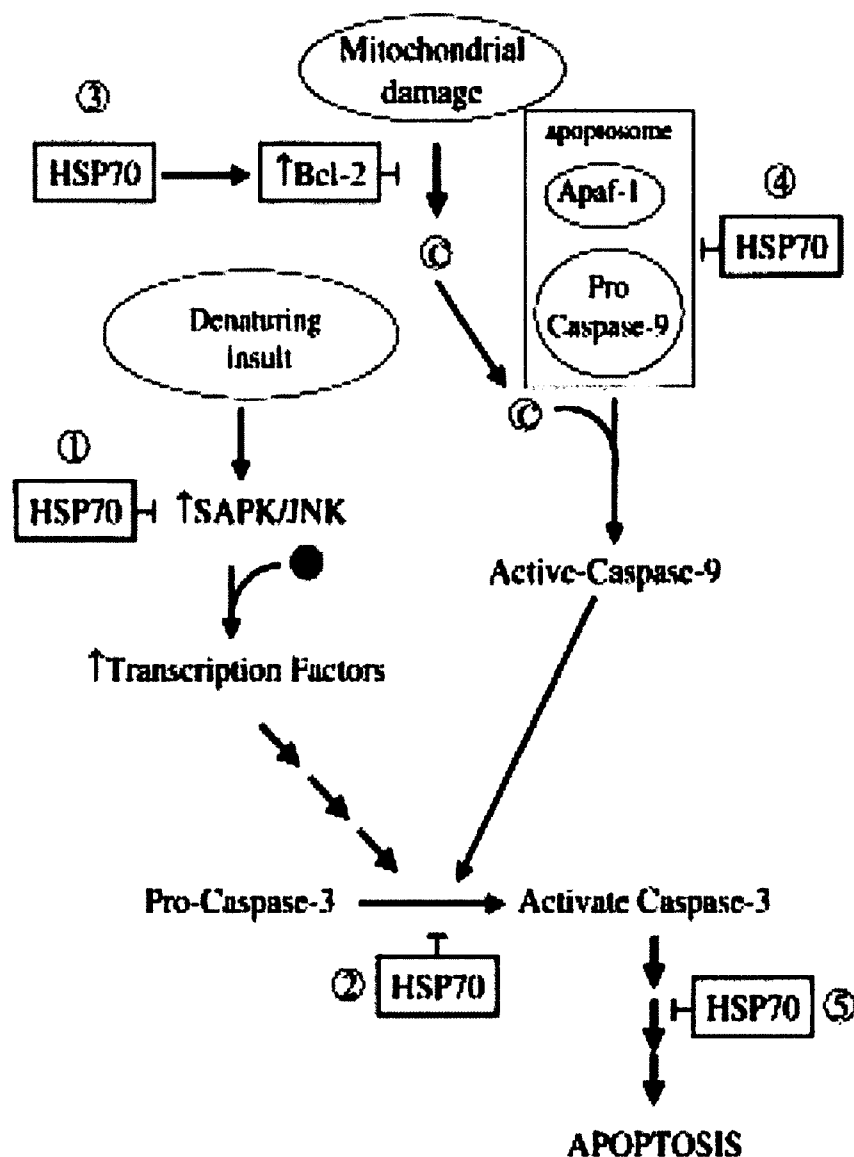
FIGURE 4. Multiple Negative Control Points in the Apoptotic Cascade that Hsp70 May Modulate. The potential points where Hsp70 may negatively

regulate the apoptotic cascade are as follows: 1) Inhibition of stress-activated protein kinase pathway (SAPK/JNK) phosphorylation and activation of transcription factors and caspases, 2) Inhibition of pro-caspase-3 transformation into caspase-3 and committing the cell to apoptosis, 3) Prevention of cytochrome c release from the mitochondria by increasing the amount of Bcl-2, 4) Competition for Apaf-1 binding sites on the apoptosome during recruitment of pro-caspase-9 to the apoptosome, 5) Inhibition downstream of caspase-3 activation potentially. (53)

of procaspase-9 to the apoptosome, (67,68). The apoptosome is a large protein triggered to assemble upon release of cytochrome c from the mitochondria as a result of cellular insult. The apoptosome is comprised of cytochrome c that binds Apaf-1 (apoptotic activating factor 1) along with dATP. This complex then recruits and transforms procaspase 9 from a zymogen to an active enzyme able to active procaspase-3 and the apoptotic cascade (48).

Collectively, these data demonstrate multiple places where Hsp70 may act to regulate the progression of the apoptotic cascade. It is most probable that Hsp70 acts simultaneously at the multiple control points. Our current understanding of cell physiology teaches that most systems have redundancy and there is every reason the regulation of the apoptotic cascade should not be an exception to the rule.

2.10 Summary

Apoptosis, and many of the key components in programmed cell death such mitochondria, cytochrome c and caspases are highly conserved elements across species and evolution. Hsp70 is also highly conserved across species and time. The linkage between the two systems is being experimentally established by many investigators along with the chronology of emergence of each element in relation to the other. Hsp70 has been demonstrated to

negatively regulate the apoptotic cascade in no less than four points. Glutamine has been demonstrated to have a positive effect on the rate of Hsp70 transcription during heat shock. The research described herein seeks to build upon the knowledge already gained in the field and add to it in a meaningful manner.

It was a hypothesis of the current research that addition of Gln to allograft heart valve disinfection media and subsequently heat shocking the mixture by returning it to euthermic conditions (37°C) during disinfection would augment the amount of Hsp70 in the constitutive cells. Elevation in the amount of Hsp70 present in the cells would prevent them from progressing to apoptosis as a result of prolonged hypothermic insult and thus be viable at the time of implantation and undergo normal cell turn over once *in situ*. The long term benefit to the patient would be that the allograft heart valve would contain a viable cell population that can renew and repair the extracellular matrix for prolonged durability and resistance to calcification.

The results from the research described herein demonstrated that supplementing the disinfection media with a final concentration of 10 mM Gln and heat shocking the heart valve at 37°C during disinfection resulted in significant increases in the amount of Hsp70 present in the cell, cytochrome c remained localized in the mitochondria, Bcl-2 levels were augmented, caspase-3 levels remained low and the constitutive cell population had reduced amounts of apoptotic cells relative to the 4°C controls. There was no increase in Hsp70 observed when Gln supplementation was added to the 4°C disinfection treatment group and thus it performed identically to the untreated 4°C control in that all of the pro-apoptotic markers were elevated and anti-apoptotic markers were not discernable. While there were positive results obtained when the tissues were heat shocked without Gln, the optimal results, with respect to reducing the number of apoptotic indices, were obtained when the cells were heat shocked in the presence of Gln.

These results make it evident Gln and heat shock act synergistically to halt the progression of the apoptotic cascade in constitutive allograft heart valve

cells treated according to this regime by augmenting the amount of Hsp70 present. This could have a significant impact to the long term durability of allograft heart valves that are used to save the lives of thousands of adults and children annually.

CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents and Suppliers

Table 1. Reagents and Suppliers

Reagent	Supplier	Catalog Number
RPMI 1640	Hyclone	TXSH3A146502LN
Cryosolution	Hyclone	TXSH3A146702LN
Dimethylsulfoxide	Sigma	D2438
Lactated Ringers	B.Braun	R5410-01
Polymixin B	Bedford Laboratories	NDC55390-139-01
Cefoxitin	Merck	NDCC0006-3356-45
Vancomycin	Eli Lilly	NDCC0002-7297-01
Lincomycin	Pharmacia & Upjohn	NDCC0009-0555-02
Sterile water for injection	American Pharmaceutical Partners, Inc.	NDCC63323-185-10
Glutamine	Sigma	G7513
Glutamine Quantitation Kit	Sigma	GLN2
10% Buffered formalin	Sigma	HT501640
Liquid Nitrogen	Roberts Oxygen	RLN240
F12K Medium	ATCC	30-2004
HEPES	Sigma	H4034
TES	Sigma	T5691
Ascorbic acid	Sigma	A4403
Insulin/Transferrin/Sodium Selenite Cocktail	Sigma	I1884
Endothelial Cell Growth Supplement	Sigma	E9640

Table 1. Continued

Reagent	Supplier	Catalog Number
BCA Protein Assay	Pierce Chemical	23235
Ethanol	Fisher	HC-800
CitriSolve	Fisher	22-143-975
Paraffin	Fisher	P31-500
Hematoxylin	Richard Allen	7231
Eosin	Richard Allen	7111
Ultrmount	Dako	S1964
Function ELISA™ Cytochrome c Kit	Active Motif	48506
Mitochondrial Fractionation Kit	Active Motif	40015
Caspase-3 antibody	R and D Systems	AF835
Retrievit-8	Biogenex	BS-1008-00
Envision Immunohistochemistry Kit	DAKO	K4008
Universal Blocking Solution	Dako	X-9090
CardioTacs TUNEL Kit	Trevigen	4827-30-K
Bcl-2 ELISA Kit	Oncogene	QIA23
Hsp70 ELISA Kit	Stressgen Biotechnologies Corp.	EKS-700

3.2 Aortic Heart Valve Conduit

Human aortic heart valve conduit was obtained from a tissue bank in accordance with their standards for providing human tissue for scientific research. All donor tissues had consent for research. All donors, ischemic times associated with the donor tissue and handling of the tissue met the criteria set forth by the American Association of Tissue Banks (AATB) and therefore the

tissue utilized in this research mirrored the handling and acceptance criteria applied to tissue deemed acceptable for clinical transplant. The tissue was procured from tissue donors whose bodies were refrigerated within at least 15 hours of asystole, the heart aseptically recovered from the donor within 24 hours of asystole and shipped to the tissue bank on sterile isotonic saline that was maintained at 1-10°C. The aortic valves were dissected and placed into RMPI 1640 and held at 1-10°C until obtained by the researcher, 0.5 – 2.5 hours post-dissection. The tissues were allocated to their respective treatment regime within 48 hours of asystole per AATB guidelines with respect to time. The tissue was randomly assigned to an experimental group. The researcher was unaware of the tissues' ischemic times, donor genders and donor ages until all of the analyses were completed. This was done to avoid biasing the results.

The demographics of the donors are as follows: 5 females and 7 males and the mean age of these donors are 44.3 years +/- 8.4. The mean ages for each experimental group are listed in Table 2 below.

3.3 EXPERIMENTAL GROUPS

Three valve experimental treatment groups were followed throughout the processing protocol to determine the effect of the experimental treatment on the amount of heat shock protein present in the cells and apoptotic indices of the cells over time, pre and post cryopreservation, relative to the standard processing protocol, fourth group. The three experimental groups were traditional antibiotic incubation method at 4°C supplemented with Gln; antibiotic incubation method at 37°C; and antibiotic incubation method at 37°C supplemented with Gln. The control group was the traditional antibiotic incubation method at 4°C. The experimental system was human aortic conduit tissue.

Table 2. Donor Demographics and Ischemic Times

Treatment Group	Average Age (years) n = 3 per group	Average Warm Ischemic Times (hours) [a]	Average Cold Ischemic Times (hours) [b]	Average Total Ischemic Time (hours)
4°C	38.3 +/- 8.7	13.8 +/- 3.0	12.8 +/-3.3	26.5 +/- 6.4
37°C	43.0 +/- 5.9	11.5 +/-4.7	13.3 +/- 3.5	24.5 +/- 8.0
4°C + Gln	51.0 +/- 7.3	10.0 +/- 4.1	18.8 +/- 4.0	28.8 +/- 5.7
37°C + Gln	45.0 +/- 8.5	11.3 +/- 4.3	16.3 +/- 3.5	27.5 +/- 3.5

[a] Warm ischemic time is the period of time from asystole until the heart is removed from the donor and placed into cold, 1 -10°C, isotonic solution.

[b] Cold ischemic time is the period of time from the heart being placed into cold, 1 -10°C, isotonic solution at procurement until placement of dissected heart valve tissues in antibiotics.

3.4 EXPERIMENTAL TIME COURSE

The target proteins, Hsp70, Bcl-2, cytochrome C, caspase-3 and apoptotic state of the cells were quantitated during the 24 hour antibiotic incubation phase and during the rewarming phase post-cryopreservation. During the antibiotic incubation, samples were assayed at time zero and after 1, 2, 3, 4, 6 and 24 hours of incubation in the antibiotic solution. During the post-cryopreservation incubation, samples were assayed at zero time post thawing and after 1, 2, 6, 24, 48 and 72 hours of *in vitro* incubation at 37°C. The entire experimental time course is outlined in the flow diagram below.

Flow Diagram of Experimental Scheme and Time Course

1. Donor asystole, initiation of warm ischemic time.

↓

2. Donor placed in refrigerated morgue, initiation of cold ischemic time.

↓

3. Heart and other tissues aseptically recovered in an operating room and transported to tissue bank on wet ice in isotonic solution.

↓

4. Heart valves dissected, distal aortic conduit placed in RMPI 1640 and transported on wet ice to laboratory for testing.

↓

5. Tissue incubated aseptically in antibiotic solution under conditions the tissue was randomized to (4°C or 37°C and with or without Gln) which stops cold ischemic time.

↓

6. Tissue removed from antibiotic solution after 0, 1, 2, 3, 4, 6 and 24 hours of incubation and placed in formalin or on dry ice waiting to be assessed, see box 9 below.

↓

7. Remaining tissue cryopreserved and stored at $\leq$120 °C in vapor phase nitrogen for a minimum of 72-hours.

↓

8. Tissue aseptically thawed and diluted of cryoprotectant and placed in vascular smooth muscle cell culture media at 37°C and 5% CO_2.

↓

7. Tissue removed after 0, 1, 2, 6, 24, 48 and 72 hours of incubation for testing and placed in formalin or on dry ice.

↓

9. Assess the Hsp 70, cytochrome c, Bcl-2, caspase-3 content and TUNEL positivity of the cells in addition to the protein content of the tissue extracts.

↓

10. Samples for Hsp70, cytochrome c and Bcl-2 were crushed under liquid nitrogen and the samples for caspase-3 and TUNEL staining were histologically prepared.

3.5 ANTIBIOTIC SOLUTION PREPARATION

Polymyxin B Sulfate was prepared to a final concentration of 100 μg/ml, Cefoxitin was prepared to a final concentration of 240 μg/ml, Vancomycin was prepared to a final concentration of 50 μg/ml and Lincomycin HCl was prepared to a final concentration of 120 μg/ml in RPMI 1640. The target Gln concentration for the two Gln treatment groups was 10.5 mM, therefore an additional 8.5 mmoles of Gln was added (Kojima, 1998).

3.6 CRYOPRESERVATION

At the conclusion of the 24-hour antibiotic incubation the tissue was placed in Teflon pouches with Cryosolution (RPMI 1640 and 10% fetal calf serum) such that the total volume was 100 mL. Subsequently dimethylsulfoxide (DMSO) cryoprotectant was added at a rate of 1% per 2 minutes, to a final concentration of 10% to minimize osmotic shock in the aortic tissue constitutive cellular population. The Cryosolution equilibrated in the tissue for two hours at 1-10°C. The Teflon pouches containing individual allograft conduits were placed into individual CryoSink® devices and placed into the freezing chamber so that the long axis of the CryoSink was aligned with the liquid nitrogen fan diffuser. A qualified thermocouple probe was inserted into the control pack, the control pack was placed into a CryoSink device and the control pack was placed into the freezing chamber. The double lead end of the thermocouple was plugged into the freezing chamber receptacle and the door was closed and latched. A liquid nitrogen line was securely attached to the CryoMed, the main liquid nitrogen valve was opened and the cryopreservation process was initiated. The tissue was cryopreserved by decreasing the temperature in the CryoMed by 1-2°C per minute. Immediately following the conclusion of the cryopreservation process, the CryoSinks containing the aortic conduits were placed into a liquid nitrogen freezer in the vapor phase. The grafts were then removed from the CryoSinks and placed into pre-conditioned fiberboard boxes for storage. The conduit tissue

was stored in the LN_2 vapor phase for at least 72 hours prior to further experimentation.

3.7 THAWING AND DILUTIONAL REMOVAL OF CRYOPROTECTANT FROM TISSUES

Each box of tissue was removed from the vapor phase of liquid nitrogen and placed on the counter for seven minutes to slowly warm through the glass transition phase. Subsequently, the tissue was removed from the fiberboard box, and placed in a basin of sterile 37°C water and incubated for seven minutes. The bath temperature was monitored and addition warm solution added as needed to maintain the temperature between 37-42°C. In a laminar flow hood, the thawed graft and Cryosolution were aseptically poured into the dilution basin, an AlloFlow® chamber. A 1 L bag of room temperature Lactated Ringers was suspended on an intravenous fluid pole located adjacent to the hood. Sterile tubing connected the bag of Lactated Ringers to the AlloFlow chamber. The fluid entered the chamber and initiated the dilutional removal of cryoprotectant from the tissue. Once 250 ml of fluid entered the AlloFlow chamber, the chamber began to drain into a sterile basin. The Lactated Ringers flowed into the chamber over a period of seven minutes. Once the bag was empty and the AlloFlow chamber stop draining, the tissue was removed from the AlloFlow chamber, aseptically aliquoted into seven pieces for each time point. The zero time point was portioned into a section that was placed into 10% buffered formalin and the remainder was frozen on dry ice. The remaining six pieces were individually placed into tissue culture flasks with 30 mls of media and stored at 37°C and 5 % CO_2.

3.8 MEDIA FORMULATION FOR *IN VITRO* INCUBATION

RPMI 1640 was supplemented with the following components as prescribed by the American Type Culture Collection (ATCC) for the maintenance of vascular smooth muscle cell line, CRL1999: 2mM glutamine, 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 10mM 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid (TES), 0.05 mg/ml ascorbic acid, 0.01 mg/ml insulin, 0.01 mg/ml transferrin, 10ng/ml sodium selenite, 0.03mg/ml endothelial cell growth supplement and 10% fetal calf serum. This media formulation was utilized to provide the vascular smooth muscle cells, the primary resident cell population of the aortic conduit, with all the necessary nutrients to maintain their viability. Therefore, if cells died during the 72 hour incubation, it was a result of the cells not being able to recover from the hypothermic transport, antibiotic disinfection process and/or the cryopreservation process, rather than due to a lack of necessary nutrients. The media was not changed during the 72-hour incubation as ATCC recommends changing the media every 48-72-hours. The ratio of media to tissue surface area was held constant.

3.9 GLUTAMINE QUANTITATION

Glutamine concentration in the RMPI 1640 disinfection media was assessed using a glutamine quantitation kit from Sigma. The assay is based on the reductive deamination of L-glutamine by Sigma's proprietary enzyme. The assessment of L-glutamine is accomplished by linking a dye directly to the reductive reaction. The reaction is specific for L-glutamine and does not cross-react with other amino acids or ammonia. The dynamic range of the assay is 0.25 mM to 6.0 mM L-glutamine; however, the sensitivity of the assay can be increased beyond 0.25 mM by extending the incubation time. An internal standard of L-glutamine was included in the assay to account for positive and negative interference from the media components. The manufacture's

instructions were followed to assay the amount of Gln in the antibiotic incubation media.

Briefly, a 7-point Gln standard curve was prepared over the range of 0 mM to 6.0 mM Gln. The internal standard was prepared by adding an aliquot of undiluted standard to an aliquot of RPMI1640. The test samples were prepared by adding reaction buffer, sample and enzyme. The tubes were incubated for 1 hour in a 37°C waterbath. Subsequently color reagent was added to the tubes, mixed and the samples were analyzed spectrophotometrically at 550 nm.

The L-glutamine concentration was calculated as follows. The slope of the regression line from the standards was used to calculate the uncorrected L-glutamine concentration of the samples. The recovery of the internal standard was used to correct the data obtained in the assay for deviations caused by the interaction of media components with the test. The following formula was used to calculate the recovery value:

1. Recovery = D = (A-B)/C
2. [L-glutamine] = mM concentration of L-glutamine
3. A = [L-glutamine] in sample with internal standard (spiked sample)
4. B = [L-glutamine] in sample without internal standard (unspiked sample)
5. C = [L-glutamine] added as internal standard.

The concentration of L-glutamine in the samples was corrected for any inhibition/enhancement using the recovery value of the internal standard calculated above according to the following formula:

1. [L-glutamine] Corrected = E/D
2. D = Recovery value previously calculated
3. E = [L-glutamine] uncorrected in unspiked sample calculated from the standard curve.

3.10 PIERCE BCA PROTEIN ASSAY

The protein concentration in the extracts for all the ELISAs was determined using the Pierce BCA Assay. A standard curve ranging from 0.02 mg/ml to 2.0 mg/ml was utilized to calculate the concentration of protein in the experimental samples. The assays were performed in a microplate, therefore the sample and standard volumes were 20 μl and the working reagent (1 part reagent A and 49 parts reagent B) volume was 200 μl per sample. Once the samples, standards and working reagent were added to the microplate, it incubated at 37°C for 30 minutes and then the optical density of the solution was assessed on an ELISA plate reader at 570 nm. A standard curve was generated by performing a linear regression analysis of the bovine serum albumin (BSA) standards to obtain the slope equation and the correlation coefficient. The BSA standards were made in the buffer or solution that the samples were in for the specific assay. The resultant optical densities of the standards diluted with buffer from each kit was compared to the optical density of standards diluted with ultrapure water to assure there was no positive or negative interference with protein quantitation. The coefficient of variation was calculated for all the slopes and the correlation coefficients are listed in the applicable results section.

3.11 PREPARATION OF AORTIC CONDUIT TISSUE FOR HISTOLOGICAL ANALYSIS

The aortic conduit samples were fixed in 10% neutral buffered formalin (1:10 w/v) for a minimum of 48 hours at 4°C prior to processing. The samples were dehydrated, cleared and infiltrated with paraffin as follows. Each piece of tissue was placed in a cassette and the cassette was labeled with a pencil. The tissue cassettes were placed in 70% ethanol for 30 minutes (1-30 cassettes, 500 ml; 30-60 cassettes, 1.0 L), in a second bath of 70% ethanol for 30 minutes, in 95% ethanol for 30 minutes, in a second bath of 95% ethanol for 30 minutes, in 100% ethanol for 30 minutes, in a second bath of 100% ethanol for 30 minutes,

in a third bath of 100% ethanol for 30 minutes, in paraffin: CitriSolve (1:3) for 60 minutes at 60°C, in paraffin: CitriSolve (1:1) for 60 minutes at 60°C, paraffin: CitriSolve (3:1) for 60 minutes at 60°C and in 100% paraffin overnight at 60°C. Subsequently they were embedded according to the following protocol.

Tissue cassettes were placed in the cassette warmer on the Tissue Tek embedding center (or in a 60°C water bath) to prevent the infiltrated paraffin from solidifying. An appropriately labeled (using pencil) embedding cassette was placed on top of a base mold. The mold/cassette unit was placed on the warm portion of the Tissue Tek embedding center. The mold/cassette unit was filled with paraffin and a pair of forceps was used to remove the tissue from its cassette and place it at the bottom of the mold on the thin edge such that cross sectional (the adventitia, media and intima) samples would be obtained. The mold was transferred to the cold plate and the cold forceps were used to hold the tissue in the desired orientation until solidification began (approximately 10 seconds). After the paraffin began to solidify in the mold the unit was transferred to an ice bath to complete the solidification process. The base molds were separated from the paraffin blocks for sectioning. The paraffin blocks were stored at 4°C and were sectioned a minimum of 24 hours post embedding according to the following protocol.

The block was secured in the microtome chuck and a disposable blade was placed in the blade holder. The position of the block was positioned to ensure that it cleared the knife. Thick sections, 20 μm, were initially cut to ensure the blade was cutting the paraffin evenly and to travel quickly into the block to obtain representative samples. Once the blade was sufficiently advanced into the specimen, the microtome was set to cut 9 μm sections. The resultant ribbons were placed in a 50°C flotation bath using a warmed metal probe. The sections were allowed to remain in the water bath until they were flat and devoid of wrinkles and air bubbles. Then a positively charged glass microscope slide was slid under the ribbon and raised from the water bath and placed on a slide warmer to properly affix to the slide. Heat fixation was crucial as no gelatin was

used in the bath to minimize background staining for the immunohistochemical procedures.

To deparaffinize the slides prior to staining, the slides were placed in CitriSolve for 3 minutes and repeated two more times, in 100% ethanol for 3 minutes, then 95% ethanol for 3 minutes, 70% ethanol for 3 minutes and water for three minutes and repeated two more times.

3.12 CYTOCHROME C QUANTITATION

To assess cytochrome c content of mitochondria in aortic conduit cells, a cytochrome c sandwich ELISA kit was used. Extraction of cytochrome c from aortic conduit tissue was performed using a Mitochondrial Fractionation Kit and subsequently quantitated using a FunctionELISA™ Cytochrome c kit, both obtained from Active Motif North America. The Mitochondrial Fractionation kit separates the mitochondria from the cytosol by chemical means and differential centrifugation. The FunctionELISA™ Cytochrome c ELISA plate is pre-coated with anti-cytochrome c antibody and thereby facilitates the detection of cytochrome c in the mitochondrial and cytosolic fractions obtained during the fractionation process. The kit has a dynamic range of 0.781 to 50 ng/ml of cytochrome c and is able to detect human cytochrome c.

Sample preparation for the Mitochondrial Fraction kit was performed according to manufacturer specifications for tissues. What follows is the tissue protocol to separate the tissue into cytosolic and mitochondrial fractions using the Mitochondrial Fractionation Kit. Tissue which had previously been snap frozen was crushed using liquid nitrogen in a motor and pestle suited for liquid nitrogen. The tissue was ground into a fine powder and collected into a cold microcentrifuge tube in 50 mg aliquots, labeled and held at -80°C until extraction.

The extraction process was as follows. Ice cold cytosolic buffer, a detergent based kit component, supplemented with a protease inhibitor cocktail supplied with the kit, was added to each microcentrifuge tube at a ratio of 1 ml per 50 mg of crushed tissue. The tubes were incubated on ice for 15 minutes to

facilitate cell extraction and lysis. The samples were subsequently centrifuged at 800 x g, at 4°C for 20 minutes to separate the cytosolic lysate from the tissue remnants. The supernatants were transferred to fresh pre-chilled microcentrifuge tubes and centrifuged a second time at 800 x g, at 4°C for 10 minutes to remove any residual nuclear debris. The supernatants were transferred to a fresh pre-chilled microcentrifuge tubes and centrifuged at 10,000 x g, at 4°C for 20 minutes to separate the mitochondria from the cytosol. The supernatant, cytosol, was transferred to fresh pre-chilled microcentrifuge tubes and held at 4°C until needed for the BCA assay. The remaining pellets were washed with 100 μl Complete Cytosolic Buffer by centrifuging them at 10,000 x g for 10 minutes at 4°C. The resulting supernatant was discarded. The mitochondrial pellet was lysed by the addition of 100 μl of Complete Mitochondria Buffer; a detergent based kit component supplemented with dithiothreotol (DTT) and protease inhibitors, and subsequently incubated on ice for 15 minutes. After the incubation was complete, the samples were vigorously vortexed for 10-15 seconds to rupture the mitochondria. The protein concentration of the cytosolic and mitochondrial fractions was then obtained using the Pierce BCA assay, section 3.10. The remaining cytosolic and mitochondrial samples were frozen and held at -80°C awaiting the ELISA.

To determine the amount of cytochrome c in the cytosolic and mitochondrial fractions, the Function ELISA™ Cytochrome c kit was utilized. The manufacturer's protocol was followed as described herein. The samples did not need to be diluted as their protein concentration was within the range specified for the assay, 20 – 100 mg/ml. One hundred microliter aliquots of the well mixed mitochondrial and cytosolic extracts were added to the anti-cytochrome c pre-coated microplates in triplicate. The samples were incubated for two hours at room temperature with slow rocking. The plates were washed 3 times with 250 μl of room temperature wash buffer, a detergent based solution supplied with the kit. The cytochrome c antibody supplied with the kit was diluted 1:1000 with room temperature blocking solution and 100 μl was added to each well. The plate was incubated for one hour at room temperature with rocking. At the conclusion of

the incubation the plate was washed 3 times with 250 µl of room temperature wash buffer. Subsequently, the streptavidin horseradish peroxidase conjugate, supplied with kit, was diluted 1:1000 in room temperature blocking buffer and 100 µl was added to each well. The plate was incubated at room temperature with rocking for 1 hour. Cytochrome c was visualized upon addition of 100 µl of room temperature tetra-methylbenzidine (TMB) substrate, supplied with the kit, to each well. The plates were incubated statically in the dark for 10 minutes at room temperature. The reaction was halted after 10 minutes by the addition of 50 µl 0.5 N sulfuric acid, supplied with the kit. The sulfuric acid stop solution was added to the samples in the same order that the TMB reagent was added. The optical density of the solution in the wells was determined using a microplate reader and the 450 nm filter. The absorbance was proportional to the amount of cytochrome c present in the sample. The results were determined from a standard curve that was run concomitantly with the samples, 0.781 ng/ml to 50 ng/ml. The results were expressed as nanograms of cytochrome c per milligram protein relative to the standard curve. By expressing the result per mg of protein, the amount of cytochrome c was normalized for each sample.

To determine recovery and if a substance in the tissue extract was causing positive or negative interference in the assay, three samples were spiked with 12.5 ng of recombinant cytochrome c and their companion samples were not spiked. The expected value (non-spiked sample concentration + known amount of cytochrome c) minus the calculated concentration of the spiked sample divided by the expected value multiplied by 100 was the percent recovery.

3.13 CASPASE-3 IMMUNOHISTOCHEMISTRY

The presence of active Caspase-3 in the aortic tissue constitutive cells was detected immunohistochemically. To detect caspase-3 in aortic conduit tissue sections, an antibody suitable for immunohistochemistry of formalin fixed, paraffin embedded samples was obtained from R and D Systems. The antibody was rabbit anti-human/mouse active Caspase-3. Standard immunohistochemical

procedures were utilized. Briefly, tissue was fixed in 10% buffered formalin for 48 hours, dehydrated, cleared and infiltrated with paraffin, embedded and sectioned at a thickness of 9 µm and mounted on positively charged slides without the use of gelatin. Samples were taken from the first, middle and last third of the block to obtain representative samples of the tissue. The slides were then deparaffinized and rehydrated as outlined in chapter 3.11. To reveal the caspase-3 antigenic sites that may have been masked by formalin fixation, the samples were subjected to a target retrieval protocol.

The samples were placed in Retrievit-8, target retrieval solution from Innogenex, and placed in the microwave and treated on high power for 1 minute, then 20% power for 20 minutes and then cooled for 20 minutes at room temperature. The endogenous peroxidases were quenched to prevent possible false positives reactions by using the peroxidase blocking reagent supplied with the immunohistochemistry kit. The peroxidase blocking reagent, 50 µl, was applied to the samples and allowed to incubate at room temperature for 5 minutes, subsequently washed away using a room temperature phosphate buffered saline bath for 5 minutes followed by an additional 5 minute incubation at room temperature in a room temperature water bath. The slides were then incubated with 50 µl of Universal Blocking Solution (Dako Envision Immunohistochemistry Kit) for 20 minutes at room temperature. Subsequently, the slides were incubated with 50 µl of the optimal concentration of caspase-3 antibody (10 µg/ml). The slides were incubated for 30 minutes at 37°C in a humidity chamber, rinsed in a room temperature phosphate buffered saline bath at for 5 minutes followed by an additional 5 minutes at room temperature in a waterbath. This was followed by incubation at room temperature in a humidity chamber for 30 minutes with 50 µl of peroxidase labeled polymer, a kit component. The polymer was comprised of mouse and rabbit Fab' fragments conjugated together using the peroxidase. After incubation, the slides were rinsed in a phosphate buffered saline bath at room temperature for 5 minutes followed by an additional 5 minutes at room temperature in a water bath. Subsequently, the slides were incubated with 50 µl of 3-Amino-9-ethylcarbazole,

AEC, chromagen substrate, a kit component, for 10 minutes at room temperature, rinsed in a phosphate buffered saline bath at room temperature for 5 minutes followed by an additional 5 minutes at room temperature in a water bath. The samples were counterstained in Mayer's Hematoxylin (Lillie modification), for 5 minutes, rinsed, in room temperature water for 3 minutes, after which an aqueous mounting media was applied along with a coverslip and the histology slide was observed under a light microscope.

A positive and negative control slide was also carried through the immunostaining to detect any nonspecific binding of antibody to non-target antigenic sites within the histological section and ensure the antibody was detecting the target antigen. The controls were processed as described above with the exceptions detailed in the text that follows. The negative control sample was generated by omitting the primary antibody to facilitate detection of non-specific secondary antibody Fab' fragment binding. Only antibody diluent was used during this incubation step. The positive control was an aortic tissue section known to contain apoptotic cells and treated identically to the experimental samples.

The caspase-3 slides were subjected to thresholding. The number of caspase-3 positive cells was determined by using an image analysis program, Scion Image 4.0.2 software, NIH, using the thresholding function. The images were captured using Spot Advanced 4.0.1 software, Diagnostic Instruments, Inc. The image was visualized on the computer screen by double clicking the cursor on the "Live" icon on the computer screen. To capture the image, the F9 key on the keyboard of the computer was depressed. To resize the image, "Edit" was selected on the task bar the "Resize" option was selected and the image was resized to 50% of original size. Once the file was saved in TIFF format the image was ready to undergo thresholding.

The result was determination of the number of cells in every other field of the H&E slides using the 10X objective. Subsequently, the number of caspase-3 positive cells was determined in approximately the same fields of view. The thresholding was performed as follows. The image from the microscope was

captured as an uncompressed TIFF. The TIFF image was opened and the black and white image was selected for analysis. Under the Process menu, Smooth was selected to reduce the effects of background staining. Next, under the Process menu, Sharpen was selected to increase the contrast of the target components against the background. This may have been done twice, depending on the clarity of the target component within the image. In the Map window, the bottom right icon was selected. The scale labeled 'B' was used to adjust the contrast of the image so that only the target components were visible. In the Process menu, Binary was selected, to exclude the gray scale from the image and to transform all the pixels into black or white. In the Process menu, Binary, Erode was selected to separate two or more objects that appeared as one. This may have been done twice, depending on the amount of remaining non-target components. In the Analyze menu, Analyze Particles was selected. In the Particle Analysis window, the Minutes Particle Size was set to 1, and the Max. Particle Size was set to 150. Next, 'Label Particles' and 'Reset Measurement Counter' were selected followed by ok. The Info window contained 'Count= X.' Where "X" was the number of target components in the image. The number of caspase-3 positive cells was divided by the number of total cells and multiplied by 100 to provide the percentage of caspase-3 positive cells in the tissues.

These results were then statistically analyzed, see Chapter 3.18, to determine if there was a statistically significant difference in caspase-3 positive cells between the four treatment groups.

3.14 TUNEL STAINING

A common method used to analyze DNA laddering is the Terminal Deoxynucleotide Transferase uNTP Nick End Labeling assay, TUNEL. Detection of DNA laddering was performed in aortic conduit tissue sections by using the CardioTacs TUNEL kit available from Trevigen. Detection of nuclear

DNA fragmentation is a widely accepted method to assay for apoptosis and can be performed *in situ.*

Briefly, tissue was fixed in 10% buffered formalin for 48 hours, dehydrated, cleared and infiltrated with paraffin, embedded and sectioned at a thickness of 9 μm and mounted on positively charged slides without the use of gelatin. Samples were taken from the first, middle and last third of the block to obtain a true representative sample of the tissue. The slides were then deparaffinized and rehydrated as outlined in chapter 3.11. The manufacturer's instructions were followed.

The samples were washed for ten minutes in room temperature phosphate buffered saline. Subsequently the tissue was permeabilized by addition of 50 μl of Cytonin, a kit component, for ten minutes at room temperature. The slides were washed twice in an deionized water bath for two minutes each at room temperature. Following this, the samples' endogenous peroxidases were quenched by incubating the samples in a mixture of methanol and 3% hydrogen peroxide at room temperature for five minutes. The quenching step was followed by a one minute wash in deionized water at room temperature. To prepare the slides for labeling, they were incubated in 1X TdT Labeling Buffer, supplied with the kit, for 5 minutes at room temperature. Subsequently each sample had 50μl of Labeling Reaction Mixture (biotinylated deoxynucleotidylphosphates (dNTP), terminal deoxynucleotidyl transferase (TdT) and manganese, supplied with the kit) added to each of tissue, minimum of two sections per slide, and was incubated at 37°C for 60 minutes in humidity chamber. During this time, any nicks in the DNA at the 3' hydroxyl groups, characteristic of apoptotic cells, were targets for the TdT to add the biotinylated dNTPs to. Once the labeling reaction was complete it was halted by incubating the slides in room temperature 1X Stop Buffer, supplied with the kit, for five minutes. This was followed by two washes in room temperature deionized water for five minutes each. Strepavidin conjugated to horseradish peroxidase (HRP), supplied with the kit, was utilized to detect the nicked DNA in the cells via the biotin labeled dNTPs. To accomplish this, 50 μl of strepavidin-HRP conjugate

was added to each tissue section of interest. The samples were incubated at room temperature in a humidity chamber for ten minutes. At the conclusion of the incubation, the samples were washed three times for two minutes each in room temperature deionized water. Subsequently, the complex was visualized upon addition of 50 μl of TACS Blue, tetra-methylbenzidine (TMB), substrate that forms a blue precipitate upon reaction with its enzyme, HRP. At the conclusion of the ten minute incubation the samples were washed three times for two minutes each in room temperature deionized water. To visualize the nuclei in the tissue, regardless of their condition, the samples were incubated for 30 seconds in room temperature Nuclear Fast Red, a kit component. The samples were then dipped ten times in each of the following solutions: deionized water, 95% alcohol, 100% alcohol and p-xylene. At the conclusion of the incubations, the slides were cover-slipped with Ultrmount and stored in the dark until analysis as the TACSBlue is light labile.

The positive and negative controls were tissues that had received no pretreatment prior to assaying them. To generate the positive and negative controls, 50 μl of nuclease was added to induce nicks in the 3' hydroxyl groups of the cells' DNA and the slides were incubated for 15 minutes in a humidity chamber at 37°C. At the conclusion of the incubation the samples were washed for 5 minutes in a waterbath at room temperature. The positive control slide was then processed identically to the experimental samples to ensure the assay was working properly. The negative control slide received similar treatment with the exception that the TdT was omitted to prevent the addition of the dNTPs and thus the colorimetric response. The purpose of this slide is to ascertain nonspecific interaction of the strepavidin with the tissue.

The number of TUNEL positive cells was determined by using an image analysis program, Scion Image 4.0.2 software, NIH, and the thresholding function. The images were captured using Spot Advanced 4.0.1 software, Diagnostic Instruments, Inc. The result was determination of the number of cells that were TUNEL positive relative to the number of cells on the companion H&E slide. To accomplish this task the following steps were taken. The image was

visualized on the computer screen by double clicking the cursor on the "Live" icon on the computer screen. To capture the image, the F9 key on the keyboard of the computer was depressed. To resize the image, "Edit" was selected on the task bar the "Resize" option was selected and the image was resized to 50% of original size. Once the file was saved in TIFF format the image was ready to undergo thresholding.

To initiate the thresholding, the number of TUNEL positive cells was determined in approximately the same fields of view as the companion H&E slide preparations. The image from the microscope was captured as uncompressed TIFF using Spot Advanced software. The TIFF image was opened and the black and white image was selected for analysis. Under the Process menu, Smooth was selected to reduce the effects of background staining. Next, under the Process menu, Sharpen was selected to increase the contrast of the target components against the background. This may have been done twice, depending on the clarity of the target component within the image. In the Map window, the bottom right icon was selected. The scale labeled 'B' was used to adjust the contrast of the image so that only the target components were visible. In the Process menu, Binary was selected, to exclude the gray scale from the image and to transform all the pixels into black or white. In the Process menu, Binary, Erode was selected to separate two or more objects that are appear as one. This may have been done twice, depending on the amount of remaining non-target components. In the Analyze menu, Analyze Particles was selected. In the Particle Analysis window, the Minutes Particle Size was set to 1, and the Max. Particle Size was set to 150. Next, 'Label Particles' and 'Reset Measurement Counter' were selected followed by ok. The Info window contained 'Count= X.'. This was the number of target components in the image. The number of caspase-3 positive cells was divided by the number of total cells and multiplied by 100 to provide the percentage of TUNEL positive tissues.

These results were then statistically analyzed; see Chapter 3.18, to determine if there is a statistically significant difference in TUNEL positive cells between the four treatment groups.

3.15 IMAGES

Images were obtained using an Olympus BX41 microscope, SPOT insight color camera and their size reduced by using SPOT Advanced image analysis software, version 4.0.1 by Diagnostic Instruments, Inc.. The images were obtained using the 4x and 10x objectives and reduced in size by 50 percent using the resize function in the software.

3.16 BCL-2 QUANTITATION

Bcl-2 ELISA kits were obtained from Oncogene to detect the presence of Bcl-2 in the conduit tissue. The kit is designed to detect Bcl-2 in tissue extracts and has a dynamic range of 0 – 200 U Bcl-2/ml. One unit of Bcl-2 equals the Bcl-2 activity in 5.6×10^4 HL-60 cells.

Sample preparation was according to manufacturer's instructions and is briefly described. Tissue that was flash frozen during the experimental time course was subsequently ground in the presence of liquid nitrogen using a mortar and pestle. Once a fine powder was achieved it was removed and placed into a pre-chilled microcentrifuge tube and stored at -80°C until needed for extraction. To extract Bcl-2 from the tissue, antigen extraction solution included in the kit was added at a ratio of 1 ml per 50 mg tissue. The tissue was incubated on ice for 30 minutes, vortexed every 10 minutes, and centrifuged at 800 x g at 4°C for 10 minutes to pellet the tissue debris. The protein concentration of the cell extracts (supernatant) was determined using the Pierce BCA protein assay (chapter 3.10) prior to freezing the extract.

The ELISA was performed as follows. The samples were diluted 1:25 (w/v) in sample diluent, kit component, and mixed well. Subsequently 50 µl of diluted sample was added to the plate in triplicate along with 50 µl of Bcl-2 detector antibody (FITC-conjugated monoclonal anti-human Bcl-2 antibody), standards (5.12 – 200 U/ml) and blanks. The plates were incubated for two hours with rocking at room temperature. Subsequently they were washed 3 times with

250 μl of phosphate buffered saline at room temperature. Conjugate (rabbit anti-FITC peroxidase conjugate, kit component) was diluted 1:200 using Conjugate Diluent, kit component, and 100 μl of diluted sample was added to the plate. The plates were incubated for 30 minutes at room temperature with rocking. After the incubation was complete, the plates were washed 3 times with 250 μl of room temperature phosphate buffered saline. An additional wash was performed by flooding the plate with room temperature deionized water. One hundred microliters of tetra-methylbenzidine (TMB) substrate, kit component, was added to each sample and the plates were statically incubated for 30 minutes in the dark at room temperature. The reaction was halted upon addition of 100 μl of 2.5 N sulfuric acid, kit component, and subsequently the samples were read at 450 nm on a plate reader. The sulfuric acid was added to the samples in the same order that the TMB reagent was added. The results were expressed as Units of Bcl-2 per milligram protein as calculated from the Bcl-2 and protein standard curves.

To determine percent recovery and if a substance in the tissue extract was causing positive or negative interference in the assay, three samples were spiked with 100 U of recombinant Bcl-2 and their companion samples were not spiked. The expected value (non-spiked sample concentration + known amount of Bcl-2) minus the calculated concentration of the spiked sample divided by the expected value multiplied by 100 was the percent recovery.

3.17 HEAT SHOCK PROTEIN 70 QUANTITATION

Hsp70 ELISA kits were obtained from Stressgen Biotechnologies Corporation to detect the inducible form of Hsp70 present in the human aortic conduit tissue samples. The kit is designed to detect Hsp70 in tissue extracts and has a dynamic range of 0.78 to 50 ng/ml.

Sample preparation and assay performance was executed according to manufacturer instructions. Tissue that was flash frozen during the experimental time course was subsequently ground in the presence of liquid nitrogen in a

mortar with a pestle. Once a fine powder was achieved it was removed from the mortar. Extraction buffer and protease inhibitor cocktail, kit components, were added (0.1 g powder /1 ml of extraction buffer supplemented with protease inhibitor), and the tissues were vigorously vortexed and incubated on ice for 30 minutes with occasional vortexing. Subsequently the tissue extract was centrifuged at 21,000 x g for 10 minutes at 4°C. The supernatant was collected and the supernatant protein concentration was determined using the commercially available Pierce, BCA protein assay kit and the remainder of the sample was frozen at minus 80 awaiting quantitation.

The quantitative ELISA utilizes a mouse monoclonal antibody specific for inducible Hsp70. The 96-well plate was pre-coated by the manufacturer with inducible anti-Hsp70. One hundred microliters of samples, in triplicate, and standards (0.78 – 50 ng/mL) were added to the 96-well plate and incubated for two hours at room temperature with rocking. Subsequently the plate was washed 6 times with 250 μl room temperature phosphate buffered saline. The antigen was detected by addition of 100 μl biotinylated rabbit anti-inducible Hsp70 polyclonal antibody diluted 1:500 in biotin anti-Hsp 70 diluent, all kit components, according to manufacturer's instructions. The plates were incubated 1 hour at room temperature with rocking, and subsequently washed 6 times with 250 μl of phosphate buffered saline at room temperature. Next, 100 μl of avidin-horseradish peroxidase conjugate, diluted 1:11,000 in avidin-horseradish peroxidase conjugate diluent, both kit components, was added and the plate was incubated for 1 hour at room temperature. The plate was washed 6 times with 250 μl of room temperature phosphate buffered saline. The plate was developed by addition of 100 μl of tetramethylbenzidine substrate (TBM) and a blue color developed in proportion to the amount of Hsp70 that was present after incubating for 10 minutes at room temperature. The color development was stopped by addition of 100 μl of sulfuric acid stop solution, causing the color to become yellow. The amount of Hsp70 detected was determined by analyzing the solution in the wells spectrophotometrically at 450 nm. The results were expressed as

nanograms of Hsp70 per milligram protein relative to the Hsp70 and protein standard curves.

To determine recovery and if a substance in the tissue extract was causing positive or negative interference in the assay, three samples were spiked with 12.5 ng of recombinant Hsp70 and their companion samples were not spiked. The expected value (non-spiked sample concentration + known amount of Hsp70) minus the calculated concentration of the spiked sample divided by the expected value multiplied by 100 was the percent recovery.

3.18 STATISTICAL ANALYSIS

A one factor analysis of variance, ANOVA, statistical test was performed to determine if there was a statistically significant difference between the various treatments groups and their ability to induce Hsp70 and Bcl-2 production, to inhibit leakage of cytochrome C from the mitochondria, to inhibit Caspase-3 formation and to inhibit the formation of apoptotic cells. A Tukey HSD test was performed to determine which groups were statistically significantly different from the others. Lilliefors test was performed to ensure the data were normally distributed.

CHAPTER 4

RESULTS

4.1 DONOR VARIABLES

An ANOVA was performed, at the 95% confidence interval, to determine if there was a statistically significant difference between the age of the donors, the warm ischemic time of the tissue, the cold ischemic time of the tissue and the total ischemic time of the tissue. A significant difference could bias the data obtained from the donor tissues. There were no statistically significant differences between the ages of the donors ($p = 0.188$), the warm ischemic times of the donors ($p = 0.651$), cold ischemic times of the donors ($p = 0.179$) and total ischemic times of the donors ($p = 0.794$).

4.2 PROTEIN CONCENTRATION OVER TIME

Protein concentration over the experimental time course was investigated to confirm there were no fluctuations. Protein content was utilized to normalize the results for all of the ELISA studies to quantitate Hsp70, cytochrome c and Bcl-2. Therefore significant fluctuations over time in protein content could have falsely elevated or reduced the target protein concentration and impacted the validity of the conclusions derived from the data. To demonstrate that the protein content of the tissue and resultant extracts did not decrease over time, the results of the BCA protein assay were plotted and analyzed over time (Figure 5). The results for each time point in Figure 5 are the average and resultant standard deviation for all four tissue extracts from three independent experiments, each with two or three replicates. This graph demonstrates the constancy of the protein concentration in tissue over the 24 hour and 72 hour incubations as the slope of the line is approximately zero, -0.0018. Thus, the results presented herein are the result of alterations in the target protein
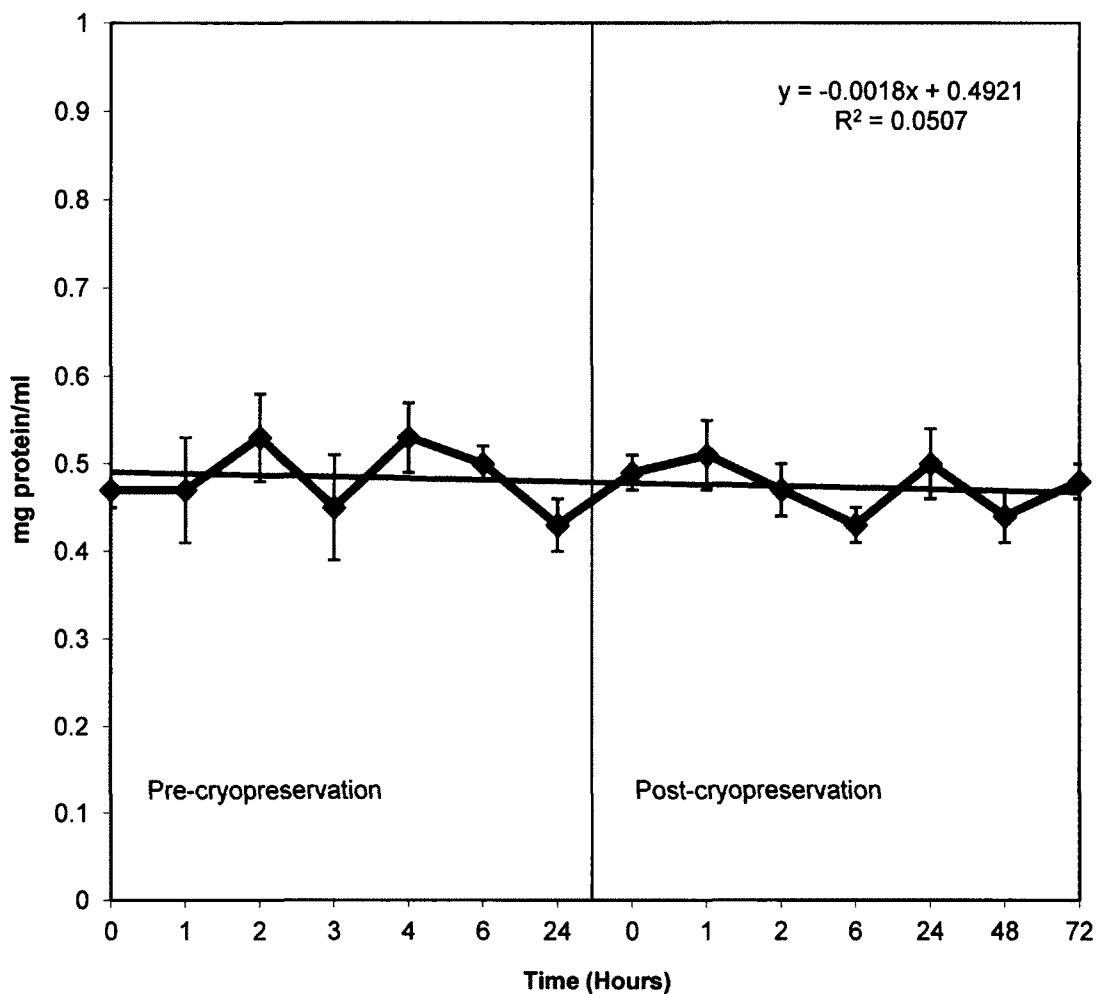
FIGURE 5. Protein concentration in aortic conduit samples over time. The linear regression analysis demonstrates the protein values approximate a straight line and therefore, there was not an appreciable variability in the protein concentration in the samples over time. The Pierce BCA assay was utilized to quantitate the amount of protein in cell and/or tissue lysates for the cytochrome c (mitochondrial and cytoplasmic extracts), Bcl-2 and Hsp70 ELISA assays. The protein values were used to normalize the target protein concentration per milligram of protein.

concentration and not due to decreased amounts of protein, due to proteolysis, in the extracts that may falsely elevate or reduce the target protein concentration.

The optical densities for BSA standards diluted with each assay buffer were compared to the optical density of BSA standards diluted with ultrapure water and found to not be statistically significantly different at each concentration, $p > 0.05$. Therefore none of the assay buffers had a positive or negative effect on the outcome of the BCA assay and thus did not affect the outcome of the Hsp70, cytochrome c and Bcl-2 ELISA assay results.

4.3 GLUTAMINE CONCENTRATION

To determine the amount of Gln in the RMPI 1640 incubation media a quantitative Gln assay was performed. The assay is based on the reductive deamination of L-glutamine by Sigma's proprietary enzyme. The assessment of L-glutamine is accomplished by linking a dye directly to the reductive reaction. The reaction is specific for L-glutamine and does not cross-react with other amino acids or ammonia. The average Gln concentration in RPMI 1640 used for the antibiotic disinfection of the 4°C and 37°C treatment groups was 2.5 +/- 0.21mM, coefficient of variation 8.4%. The average glutamine concentration in RPMI 1640 used for the antibiotic disinfection of the 4°C + Gln and 37°C + Gln was 10.36 +/- 0.81mM, coefficient of variation 7.81%

4.4 HEAT SHOCK PROTEIN 70 QUANTITATION – ANTIBIOTIC INCUBATION

A Hsp70 sandwich ELISA assay was performed to determine whether incubation at 37°C or 37°C plus Gln during disinfection would result in increased amounts of Hsp70 present in aortic conduit tissue cells over time.. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln as described in the Materials and Methods section. The data were obtained by dividing the total picograms Hsp 70 by the total milligrams of protein in the extract for each time point. Percent recovery analysis

was performed as described in chapter 3.17 of the Materials and Methods. The percent recovery of Hsp 70 was 94.5 +/-3.7%. The Hsp70 data set were determined to be normally distributed.

Figure 6 demonstrates that there was not a statistically significant difference in the amount of Hsp70 present in the cells of all the tissue treatment groups at time zero, $p > 0.05$. There was not a statistically significant change in the amount of Hsp 70 present in the tissue incubated at 4°C or 4°C + Gln over the 24 hours of incubation, $p > 0.05$. There was statistically significantly more Hsp70 present in the cells of the tissue incubated at 37°C with and without Gln from the 1 hour assessment point through the conclusion of the 24 hour incubation than in the cells of the tissue incubated at 4°C with and without Gln.

There was a statistically significant increase in the amount of Hsp70 present in the cells of the tissue incubated at 37°C between zero time and 1 hour, 1 hour and 2 hours and 2 hours and 6 hours, $p < 0.05$.

There was a statistically significant increase in the amount of Hsp70 present in the cells of the tissue incubated at 37°C + Gln between zero time and 1 hour, 1 hour and 2 hours and 3 hours and 4 hours. There was also a statistically significant decrease in the amount of Hsp70 present between 4 hours and 24 hours, $p < 0.05$. There was statistically significantly more Hsp70 present in the cells of the tissue incubated at 37°C with Gln from the 1 hour assessment point through the conclusion of the 24 hour incubation than in the cells of the tissue incubated at 37°C without Gln.

These data demonstrate that incubation of aortic conduit tissue at 37°C and 37°C plus Gln significantly increases the amount of Hsp 70 present in the cells over a 24 hour period relative to their baseline Hsp 70 content and the 4°C with and without Gln treatment groups. Additionally, 37°C plus Gln is superior to 37°C alone. These data also demonstrate that the largest increase in Hsp70 occurs between 3 and 4 hours of incubation at 37°C with Gln supplementation and then declines over the remaining 24 hours but remains well above baseline.
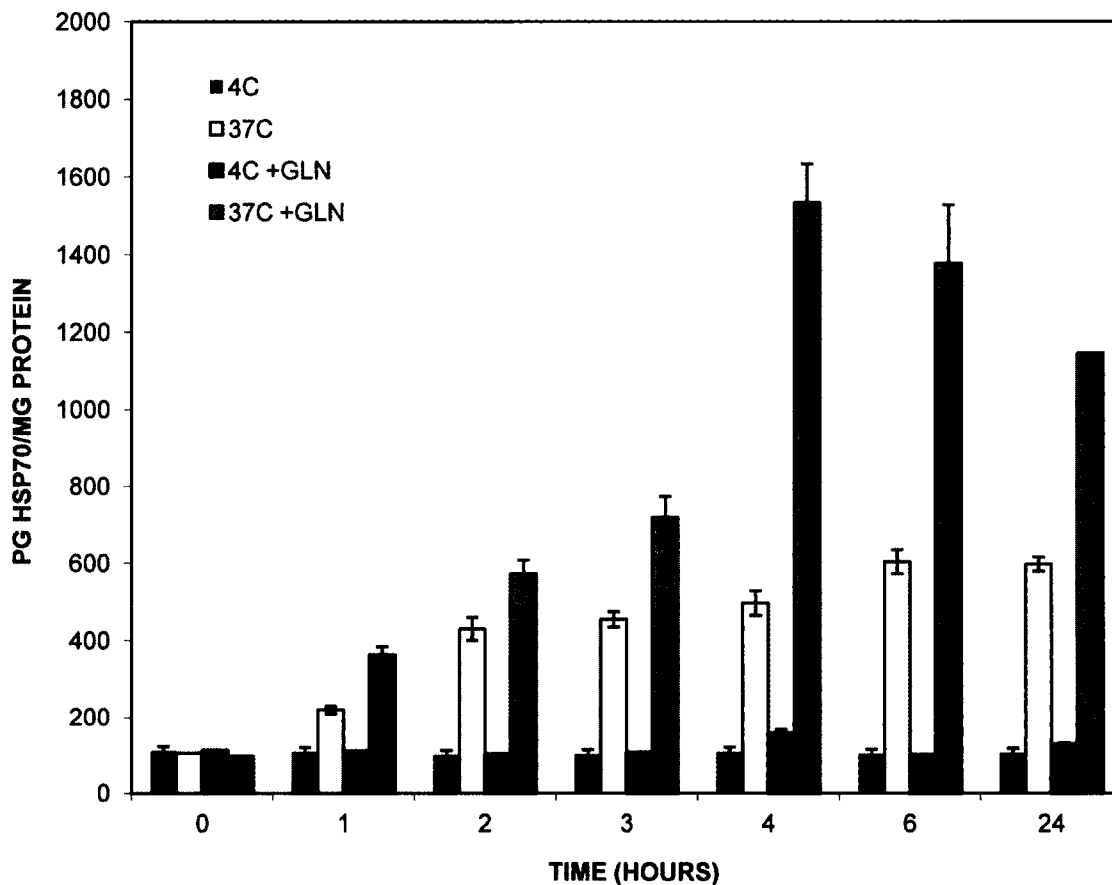
FIGURE 6. Quantitation of heat shock protein 70 in aortic conduit tissue during disinfection under various treatment conditions. Treatment of human aortic conduit at 37°C and at 37°C plus Gln, during antibiotic disinfection, the results demonstrate increased amounts of and/or stability of Hsp 70 at 37°C with and without Gln relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, pulverized in liquid nitrogen, detergent extracted, quantitated by Hsp 70 sandwich ELISA and results normalized by Pierce BCA protein assay.

4.5 HEAT SHOCK PROTEIN 70 QUANTITATION – INCUBATION POST-CRYOPRESERVATION

To determine whether incubation at 37°C or 37°C plus Gln during the antibiotic treatment phase would result in increased amounts of Hsp 70 present in aortic conduit tissue that had been subsequently cryopreserved, thawed and incubated at 37°C under 5% CO_2 for 72 hours, a Hsp70 sandwich ELISA assay was performed. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln, cryopreserved, thawed and diluted of cryoprotectant and incubated in tissue culture media optimized for the viability of vascular smooth muscle cells over 72 hours at 5% CO_2 as described in the Materials and Methods chapter. The data were obtained by dividing the total picograms Hsp 70 by the total milligrams of protein in the extract for each time point. Percent recovery analysis was performed as described in chapter 3.17 (page 52) of the Materials and Methods. The percent recovery of Hsp 70 was 94.5 +/-3.7%. The Hsp70 data set were determined to be normally distributed.

Figure 7 demonstrates that the amount of Hsp70 detected at time zero post-cryopreservation was not statistically different among any of the treatment groups than the data obtained immediately pre-cryopreservation (24-hour time point). In the tissue previously incubated at 4°C and 4°C + Gln there was no statistically significant increase in Hsp70, $p > 0.05$. There was statistically significantly more Hsp70 present in the cells of the tissue incubated at 37°C with and without Gln from the 0 hour assessment point through the conclusion of the 72-hour incubation than in the cells of the tissue incubated at 4°C with and without Gln.

There was not a statistically significant increase in the amount of Hsp70 present in the cells of the tissue incubated previously at 37°C without Gln after 72 hours *in vitro,* $p > 0.05$. However, there was a statistically significant decrease in Hsp70 between 6 hours and 72 hours, $p < 0.05$.

In the tissue previously incubated at 37°C plus Gln there was a statistically significant increase in the amount of Hsp70 detected in the cells between 0 hours

and 6 hours of incubation *in vitro.* Additionally, there was statistically significant decline in Hsp70 present in the cells between 6 hours and 24 hours, 24 and 72 hours *in vitro,* $p < 0.05$. There was statistically significantly more Hsp70 present in the cells of the tissue incubated at 37°C with Gln from the 0 hour assessment point through the conclusion of the 72 hour incubation than in the cells of the tissue incubated at 37°C without Gln.

These data demonstrate that incubation of aortic conduit tissue at 37°C plus Gln significantly increases the amount of Hsp 70 present in the cells after cryopreservation, thawing and incubation under tissue culture conditions over a 72-hour period relative to their baseline Hsp 70 content. The 4°C treatment groups did respond to the heat shock of being placed at 37°C post-cryopreservation by producing elevated amounts Hsp70 over baseline. The 37°C with Gln group had greater Hsp70 content than the 4°C with and without Gln and 37°C without Gln treatment groups. These data also demonstrate that the increase in Hsp70 occurs between 2 and 6 hours of incubation and then declines over the remaining 24 hours but remains well above baseline. These data demonstrate a difference in Hsp70 augmentation in cells relative to whether they were supplemented with Gln. The unsupplemented group was able to maintain elevated levels of Hsp70 while the supplemented group continued to augment the amount of Hsp70 present over the first 6 hours *in vitro.*
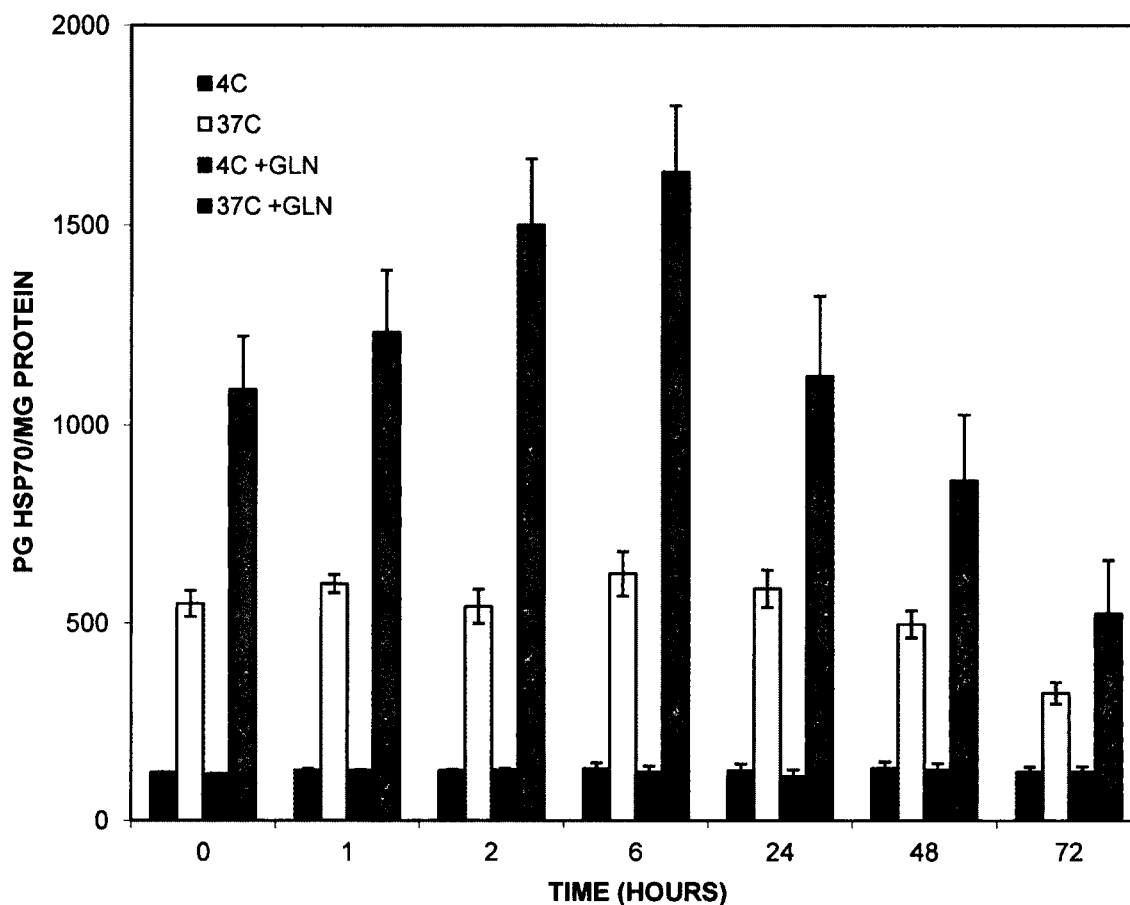
FIGURE 7. Quantitation of heat shock protein 70 in aortic conduit tissue after cryopreservation. Treatment of human aortic conduit previously incubated at 37°C and at 37°C plus Gln during antibiotic disinfection, cryopreserved, thawed and incubated under cell culture conditions resulted in increased amounts of and/or stability of Hsp 70 relative to treatment at 4°C post cryopreservation. Tissues were incubated under their respective conditions, pulverized in liquid nitrogen, detergent extracted, quantitated by Hsp 70 sandwich ELISA and results normalized by Pierce BCA protein assay.

4.6 CYTOCHROME C QUANTITATION – CYTOSOLIC ANALYSIS DURING ANTIBIOTIC INCUBATION

Cells entering or undergoing apoptosis can have increased amounts of cytochrome c in their cytosol and therefore cytochrome c is a presumptive trigger of the apoptotic cascade. To determine whether incubation at 37°C or 37°C plus Gln would result in maintenance of baseline levels of cytochrome c in the cytosol of aortic conduit tissue cells over time during antibiotic incubation, a cytochrome c sandwich ELISA assay was performed. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln as described in the Materials and Methods chapter. The tissue samples were digested and differentially centrifuged to separate the mitochondria from the cytosol and nucleus. This methodology facilitated the detection of cytochrome c in the cytosolic and mitochondrial fraction. The cytochrome c content of the mitochondrial fraction can be found in chapter 4.8. The data were obtained by dividing the total nanograms cytochrome c by the total milligrams of protein in the extract for each time point. Percent recovery analysis was performed as described in chapter 3.17 of the Materials and Methods. The percent recovery of cytochrome c was 92.7 +/-5.7%. The cytochrome c, cytosolic data set were determined to be normally distributed

Figure 8 demonstrates that in the tissue previously incubated at 4°C there was a statistically significant increase in the amount of cytochrome c present in the cytosol between 0 and 1 hour and 1 hour and 2 hours, $p < 0.05$. The amount of cytochrome c in the cytosol was relatively constant thereafter. The tissue incubated at 4°C plus Gln also had statistically significant increases in cytochrome c content in the cytosol that replicated the same trend as the 4°C samples. The increases in cytochrome c correspond to increases in capase-3 and TUNEL positive cells in chapters 4.12 and 4.14 respectively.

There was a statistically significant increase in the amount cytochrome c present in the cytosol of cells incubated at 37°C between 1 and 4 hours, $p < 0.05$, with a downward trend thereafter. In the tissue previously incubated at 37°C plus

Gln, there was not a statistically significant increase in the amount of cytochrome c detected in the cytosol of the cells between zero and 24 hours of incubation in antibiotics, $p > 0.05$.

These data demonstrate that incubation at 4°C with or without Gln results in an increase in cytosolic cytochrome c content. However, incubation of aortic conduit tissue at 37°C plus Gln maintains the baseline level of cytochrome c present in the cytosol. Treatment at 37°C without Gln results in a statistically significant increase in cytochrome c content over baseline; however, not to the degree noted in both 4°C treatment groups. Figures 9 and 10 clearly demonstrate that there is an inverse relationship between the amount of Hsp70 present in the cell and the amount of cytochrome c that is found in the cytosol. The less Hsp70 present in the cell, the more cytochrome c is found in the cytosol (Figure 9) and conversely the more Hsp70 present in the cell, the less cytochrome c is found in the cytosol (Figure 10). These data begin to establish a relationship between elevated Hsp70 and an absence of pro-apoptotic conditions in the cell.
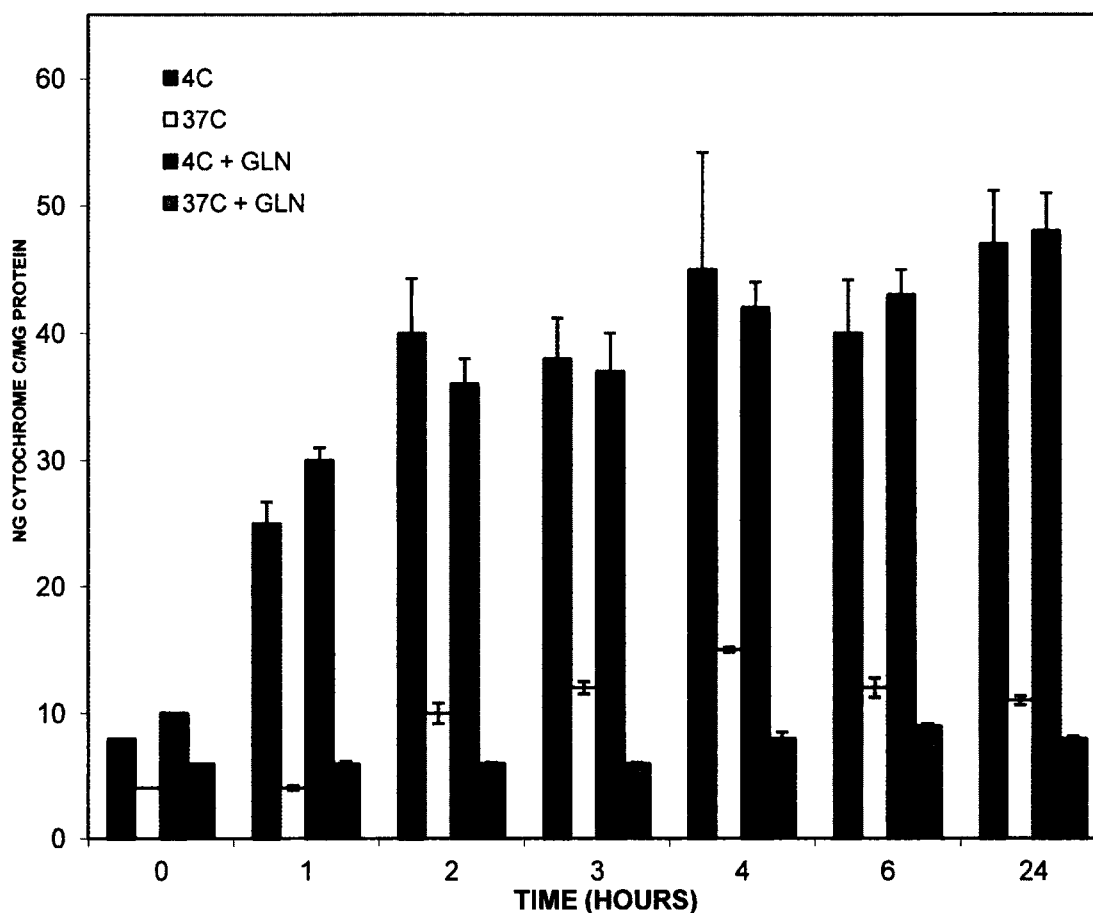
FIGURE 8. Quantitation of cytosolic cytochrome c in aortic conduit tissue during disinfection under various treatment conditions. Treatment of human aortic conduit at 37°C plus Gln, during antibiotic disinfection, results in maintenance of baseline cytosolic cytochrome c levels relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, pulverized in liquid nitrogen, detergent extracted, differentially centrifuged to isolate the cytosolic cellular fraction, quantitated by cytochrome c sandwich ELISA and results normalized by Pierce BCA protein assay.
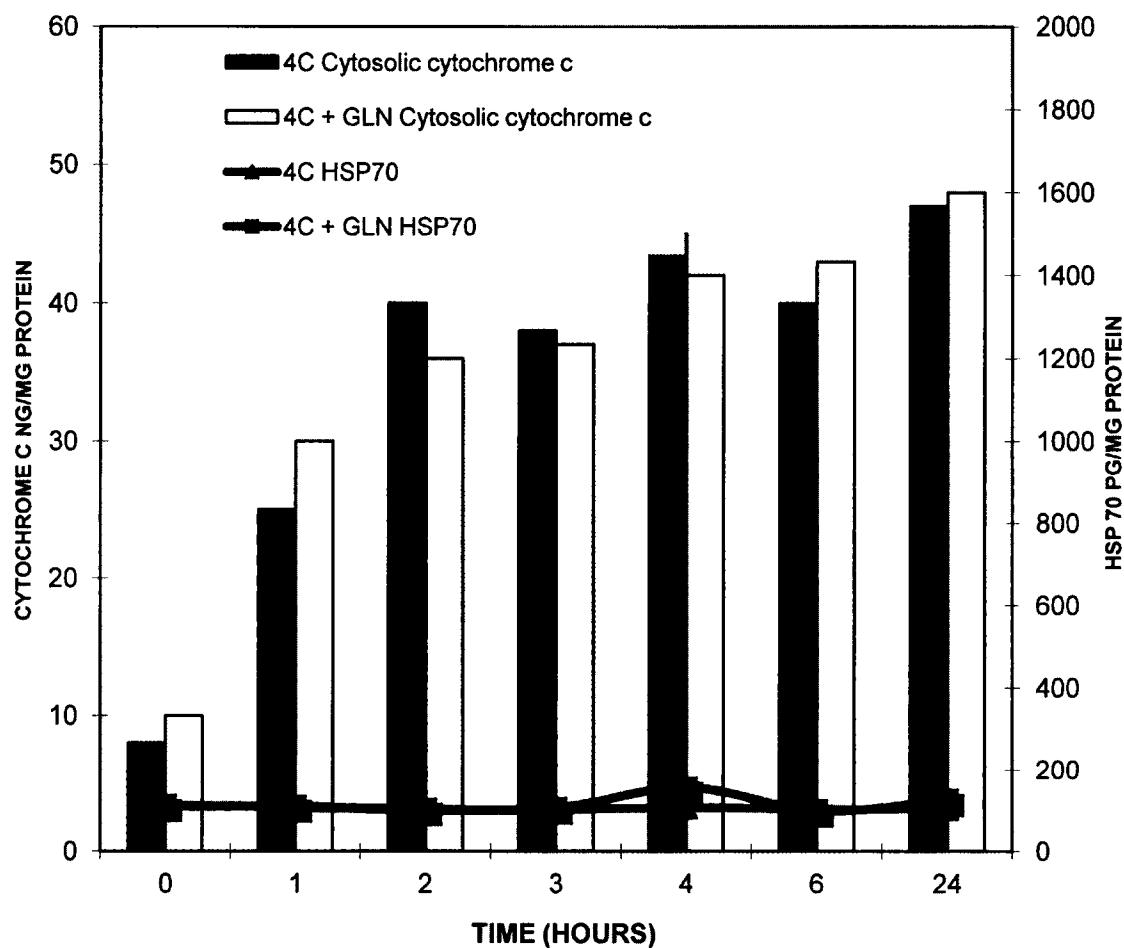
FIGURE 9. Cytosolic cytochrome c vs. Hsp70 content over time during disinfection in the 4°C and 4°C+GLN treatment groups. These data demonstrate the relationship between incubation at 4°C during disinfection, no augmentation in Hsp70 and increasing amounts of cytochrome c detected in the cytosol. The increase in cytosolic cytochrome c was similar with or without the addition of GLN to the disinfection solution at 4°C. These data are derived from the Hsp70 and cytochrome c ELISA results.
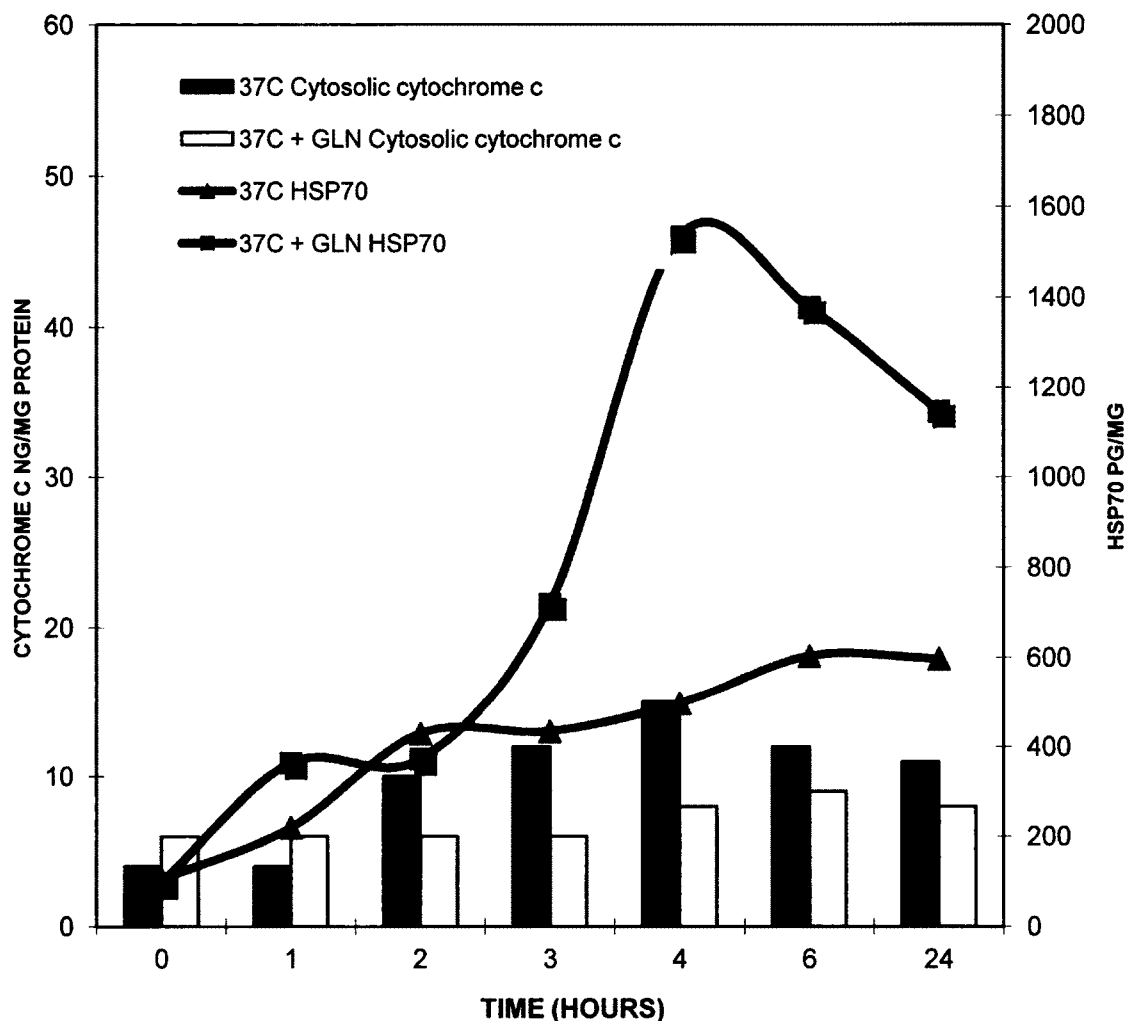
FIGURE 10. Cytosolic cytochrome c vs. Hsp70 content over time during disinfection in the 37°C and 37 °C+GLN treatment groups. These data demonstrate the relationship between incubation at 37°C during disinfection, increasing levels of Hsp70 and the amount of cytochrome c detected in the cytosol. Comparing these data with those in Figure 9, the effect of increased amounts Hsp70 on maintenance of intracellular cytochrome c levels is apparent. These data are derived from the Hsp70 and cytochrome c ELISA results.

4.7 CYTOCHROME C QUANTITATION – CYTOSOLIC ANALYSIS POST-CRYOPRESERVATION

To determine whether incubation at 37°C or 37°C plus Gln during the antibiotic treatment phase would result in increased amounts of cytochrome c present in the cytosolic fraction of aortic conduit tissue that had been subsequently cryopreserved, thawed and incubated at 37°C under 5% CO_2 for 72 hours, a cytochrome c sandwich ELISA assay was performed. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln, cryopreserved, thawed and diluted of cryoprotectant and incubated in tissue culture media optimized for the viability of vascular smooth muscle cells over 72 hours at 5% CO_2 as described in the Materials and Methods chapter. The data were obtained by dividing the total nanograms of cytochrome c by the total milligrams of protein in the extract for each time point. Percent recovery analysis was performed as described in section 3.17 of the Materials and Methods chapter. The percent recovery of cytochrome c was 92.7 +/-5.7%. The cytochrome c cytosolic data were determined to be normally distributed.

Figure 11 demonstrates that there was not a statistically significant difference in the amount of cytochrome c detected immediately prior to cryopreservation and the zero time point in the current series for any of the treatment groups. In the tissue previously incubated at 4°C and 4°C plus Gln, the amount of cytochrome c present in the cytosol during the incubation did not increase; however, there was a statistically significant decline between 6 and 24 hours, $p < 0.05$. This trend continued for the remaining incubation period.

There was a statistically significantly increase in the amount cytochrome c present in the cytosol of cells in the tissue previously incubated at 37°C, after 6 hours *in vitro*, $p < 0.05$. In the tissue previously incubated at 37°C plus Gln there was not a statistically significant increase in the amount of cytochrome c detected in the cytosol of the cells between zero and 72 hours of incubation in tissue culture media, $p > 0.05$; however, there was decrease over time. The increases in

cytochrome c correspond to increases in capase-3 and TUNEL positive cells in chapters 4.13 and 4.15 respectively.

These data demonstrate that incubation of aortic conduit tissue at 37°C plus Gln maintains the baseline level of cytochrome c present in the cytosol and that Gln is necessary to achieve the result. However, incubation at 4°C with or without Gln results in elevated levels of cytochrome c in the cytosol that decline over time potentially due to degradation. Figures 12 and 13 clearly demonstrate that there continues to be an inverse relationship between the amount of Hsp70 present in the cell and the amount of cytochrome c that is found in the cytosol. The less Hsp70 present in the cell, the more cytochrome c is found in the cytosol (Figure 12) and conversely the more Hsp70 present in the cell, the less cytochrome c is found in the cytosol (Figure 13). The data obtained during the disinfection/treatment phase of the experimentation mirror the data obtained during the *in vitro* portion of the study. These data continue to establish a relationship between elevated Hsp70 and an absence of pro-apoptotic conditions in the cell.
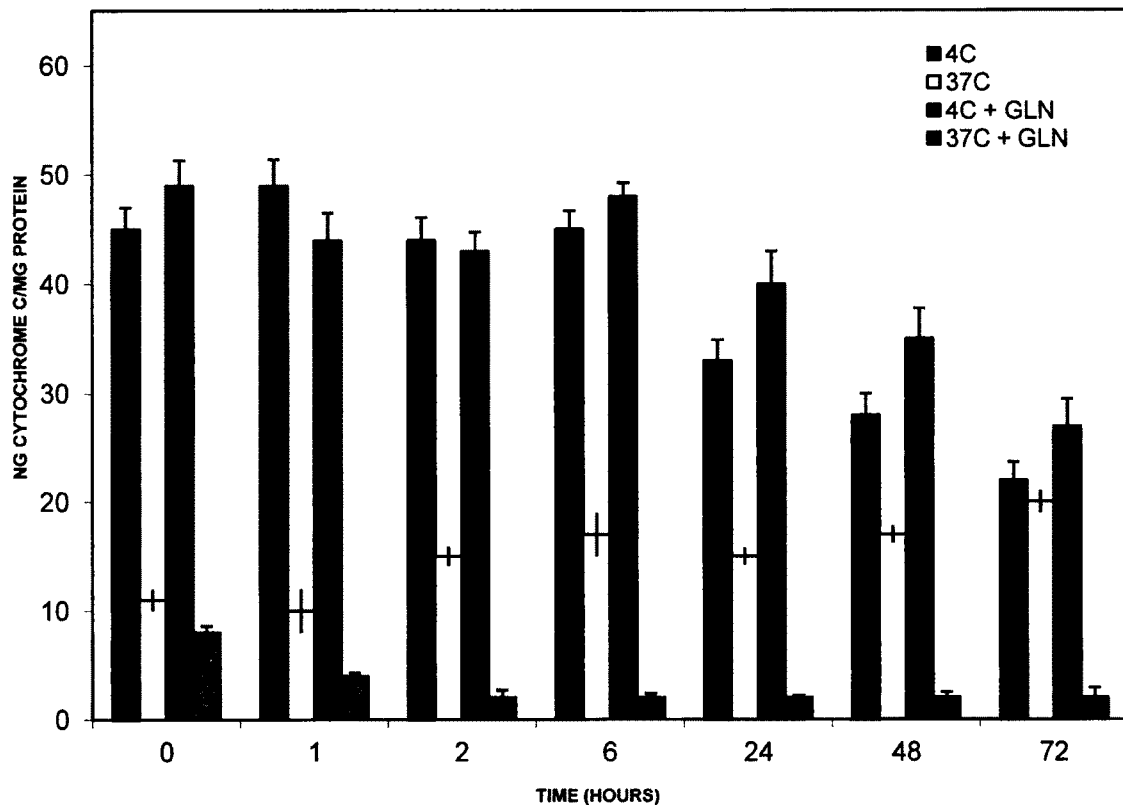
FIGURE 11. Quantitation of cytosolic cytochrome c in aortic conduit tissue after cryopreservation. Treatment of human aortic conduit previously incubated at 37°C plus Gln during antibiotic disinfection, cryopreserved, thawed and incubated under cell culture conditions results in declining amounts of cytochrome c post cryopreservation relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, pulverized in liquid nitrogen, detergent extracted, differentially centrifuged to isolate the cytosolic cellular fraction, quantitated by cytochrome c sandwich ELISA and results normalized by Pierce BCA protein assay.
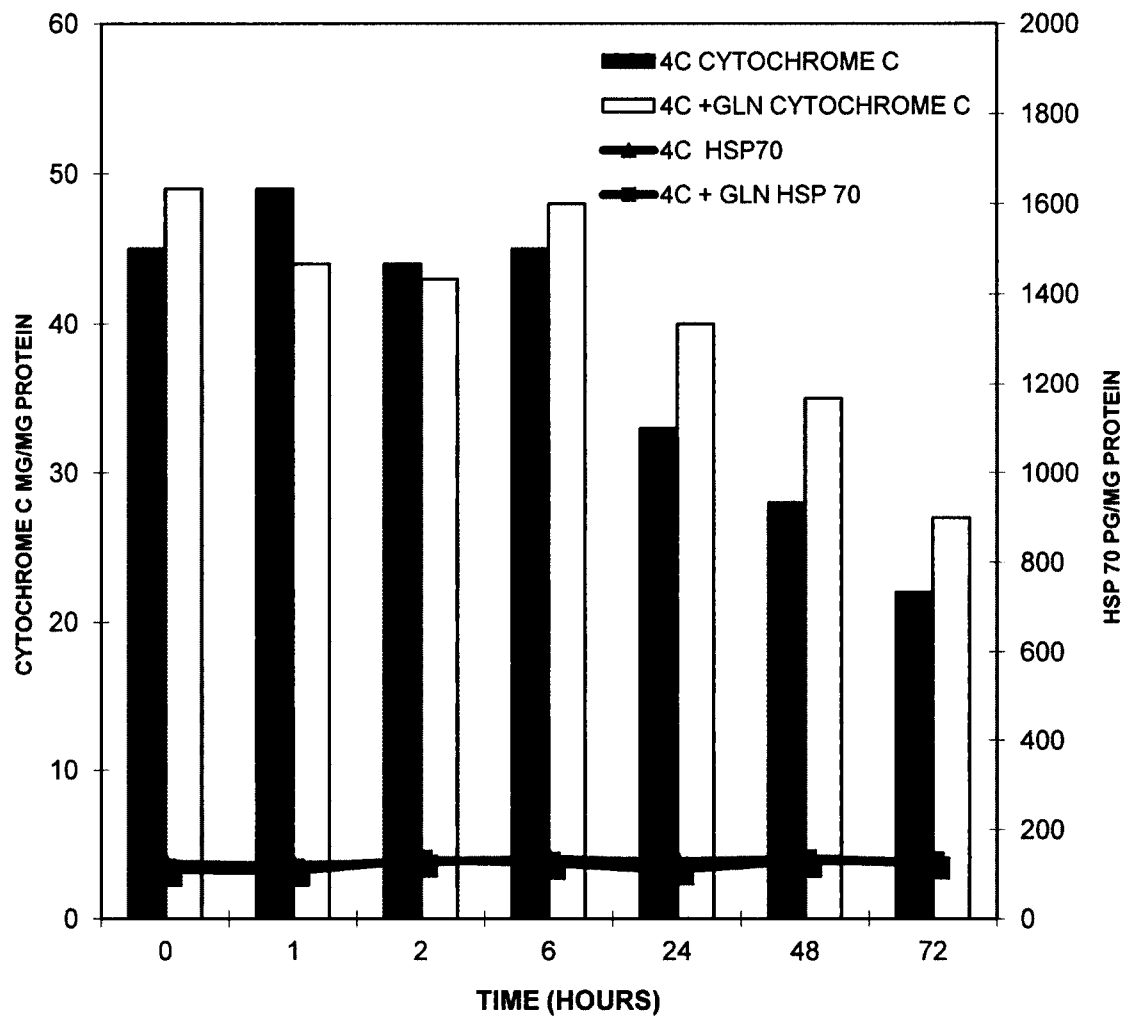
FIGURE 12. Cytosolic cytochrome c vs. Hsp70 content over time post-cryopreservation in the 4°C and 4°C+GLN treatment groups. These data demonstrate the relationship between incubation at 4°C during disinfection, no augmentation in Hsp70 and elevated amounts of cytochrome c detected in the cytosol. These data are derived from the Hsp70 and cytochrome c ELISA results.
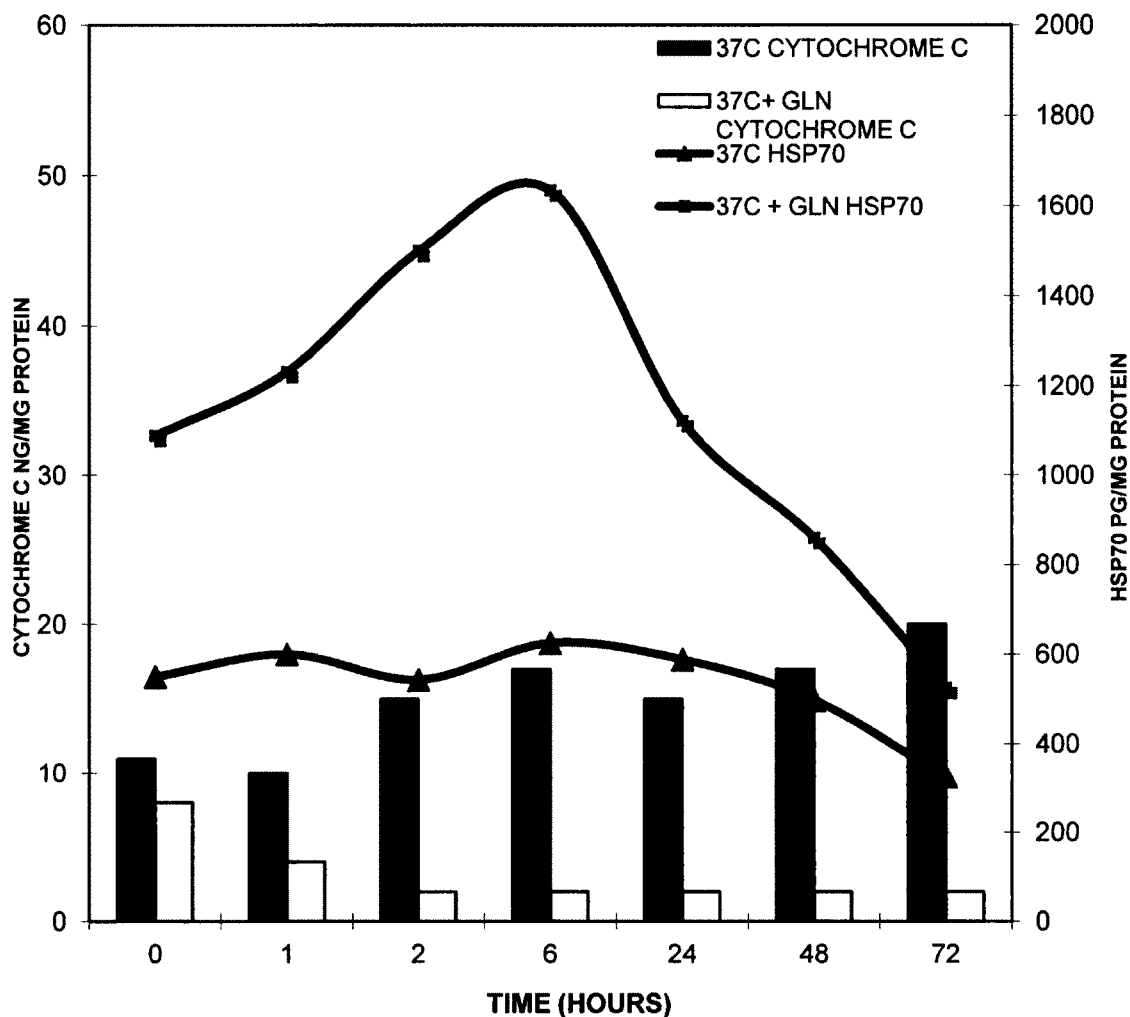
FIGURE 13. Cytosolic cytochrome c vs. Hsp70 content over time post-cryopreservation in the 37°C and 37 °C+GLN treatment groups. These data demonstrate the relationship between incubation at 37°C with Gln during disinfection, increasing levels of Hsp70 and the amount of cytochrome c detected in the cytosol. Comparing these data with those in Figure 12, the effect of increased amounts Hsp70 on maintenance of low cytoplasmic cytochrome c levels is apparent. These data are derived from the Hsp70 and cytochrome c ELISA results.

4.8 CYTOCHROME C QUANTITATION – MITOCHONDRIAL ANALYSIS DURING ANTIBIOTIC INCUBATION

Cells entering or undergoing apoptosis can have increased amounts of cytochrome c in their cytosol and therefore it is a presumptive trigger of the apoptotic cascade. To determine whether incubation at 37°C or 37°C plus Gln would result in maintenance of baseline levels of cytochrome c in the mitochondria of aortic conduit tissue cells over time during antibiotic incubation, a cytochrome c sandwich ELISA assay was performed. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln as described in the Materials and Methods chapter. The data were obtained by dividing the total nanograms cytochrome c by the total milligrams of protein in the extract for each time point. Percent recovery analysis was performed as described in chapter 3.17 of the Materials and Methods chapter. The percent recovery of cytochrome c was 92.7 +/-5.7%. The mitochondrial cytochrome c data were determined to be normally distributed

Figure 14 demonstrates that there was not a statistically significant difference in the amount of cytochrome c present in the mitochondria of the cells in all the tissue treatment groups at time zero, $p > 0.05$. The cells in the tissues incubated at 4°C with and with out Gln experienced a significant decrease in the amount of cytochrome c present in the mitochondria between zero time and three hours of incubation, $p < 05$. The decreases in cytochrome c correspond to increases in capase-3 and TUNEL positive cells in chapters 4.12 and 4.14 respectively.

In the tissue previously incubated at 37°C there was a statistically significant decrease in the amount of cytochrome c detected in the mitochondria of the cells between zero and 3 hours of incubation in antibiotics, $p<0.05$. In the tissue previously incubated at 37°C plus Gln there was not a statistically significant decrease in the amount of cytochrome c detected in the mitochondria of the cells between zero and 24 hours of incubation in antibiotics, $p>0.05$. The tissues previously incubated at 37°C with and without Gln contained statistically

significantly more cytochrome c in their mitochondria for the duration of the incubation relative to both 4°C treatment groups.

These data demonstrate that incubation of aortic conduit tissue at 37°C plus Gln maintains the baseline level of cytochrome c present in the mitochondria. Incubation at 37°C alone results in a significant decrease in mitochondrial cytochrome c but not nearly as dramatic as the decrease in the 4°C and 4°C with Gln treatment groups. Figures 15 and 16 clearly demonstrate that there is a relationship between the amount of Hsp70 present in the cell and the amount of cytochrome c that is found in the mitochondria. A lack of increased Hsp70 correlates with declining amounts of cytochrome c in the mitochondria over time when the tissue is not heat shocked (Figure 15). Conversely, augmentation of the Hsp70 levels corresponds with maintenance of mitochondrial cytochrome c levels (Figure 16). Additionally, it is apparent heat shock in the absence of Gln is not as successful at maintaining the cytochrome c in the mitochondria. These results correlate with those in Figures 8-10 that demonstrated during the disinfection/treatment phase of this study a lack of Hsp70 correlated with high levels of cytoplasmic cytochrome c.
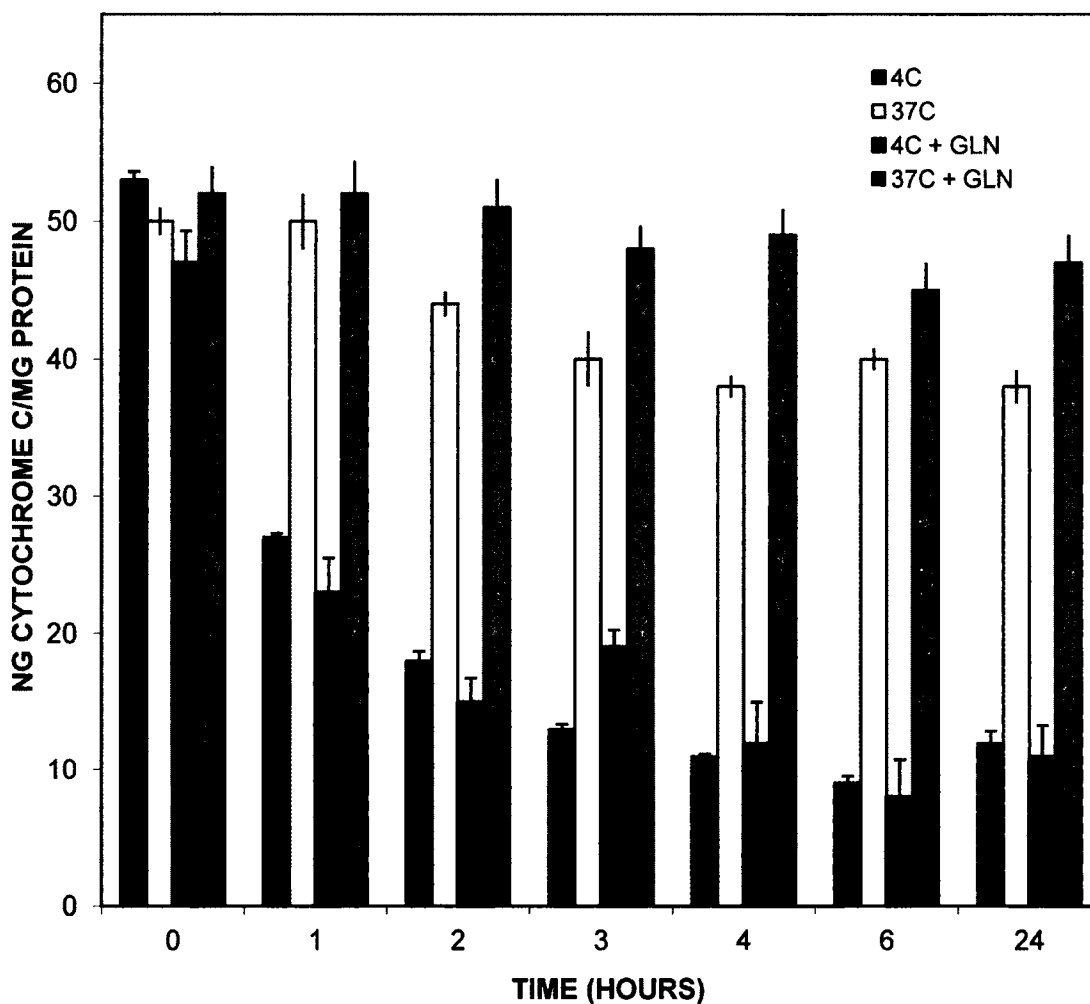
FIGURE 14. Quantitation of mitochondrial cytochrome c in aortic conduit tissue during disinfection under various treatment conditions. Treatment of human aortic conduit at 37°C plus Gln, during antibiotic disinfection, results in maintenance of baseline mitochondrial cytochrome c levels relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, pulverized in liquid nitrogen, detergent extracted, differentially centrifuged to isolate the mitochondrial fraction, quantitated by cytochrome c sandwich ELISA and results normalized by Pierce BCA protein assay.
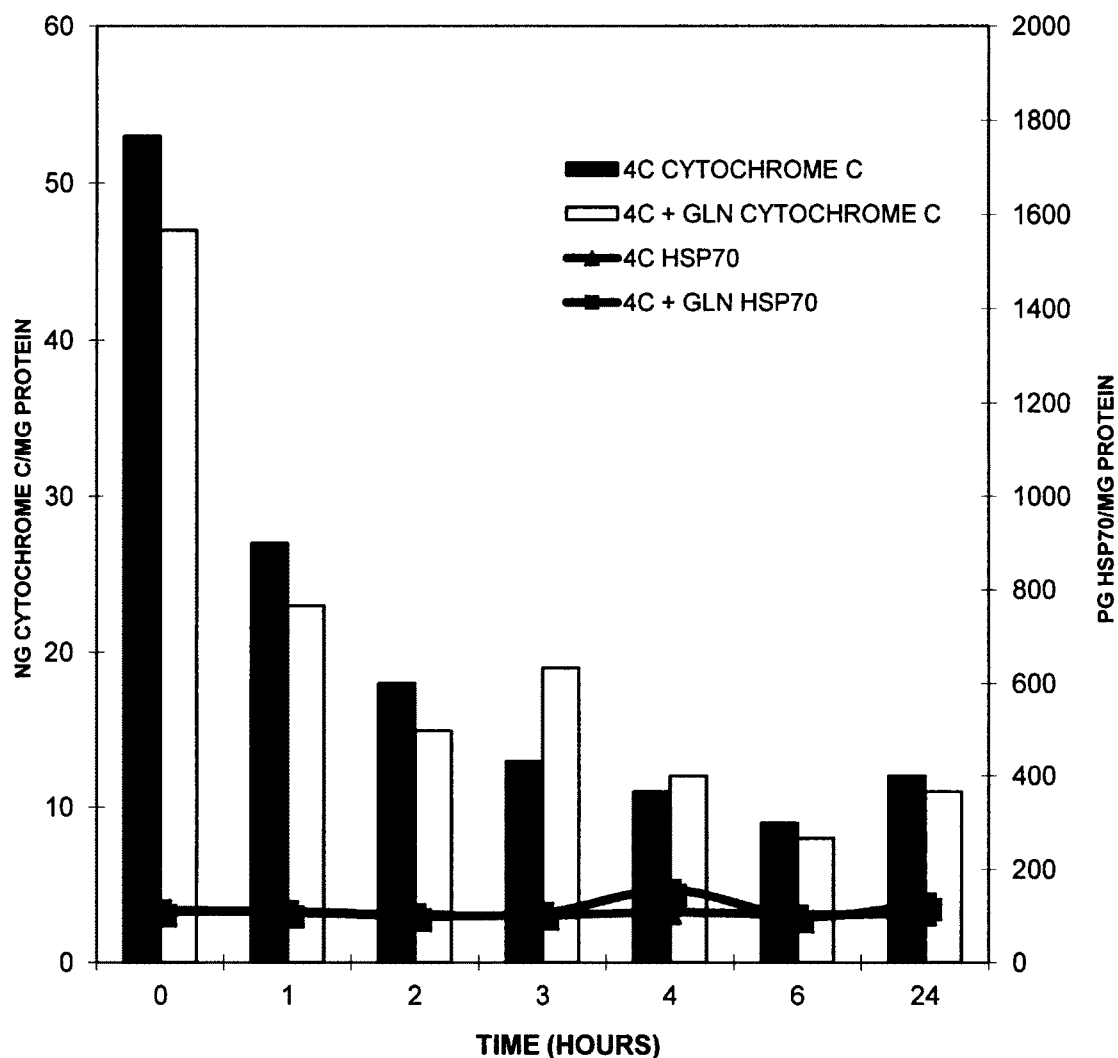
FIGURE 15. Mitochondrial cytochrome c vs. Hsp70 content over time during disinfection in the 4°C and 4°C+GLN treatment groups. These data demonstrate the relationship between incubation at 4°C during disinfection, lack of augmentation in Hsp70 levels and decreasing amounts of cytochrome c detected in the mitochondria. The decrease in mitochondrial cytochrome c was similar with or without the addition of Gln to the 4°C disinfection solution. The decrease in mitochondrial cytochrome c also corresponds with the increase in cytosolic cytochrome c demonstrated in Figure 8. These data are derived from the Hsp70 and cytochrome c ELISA results.
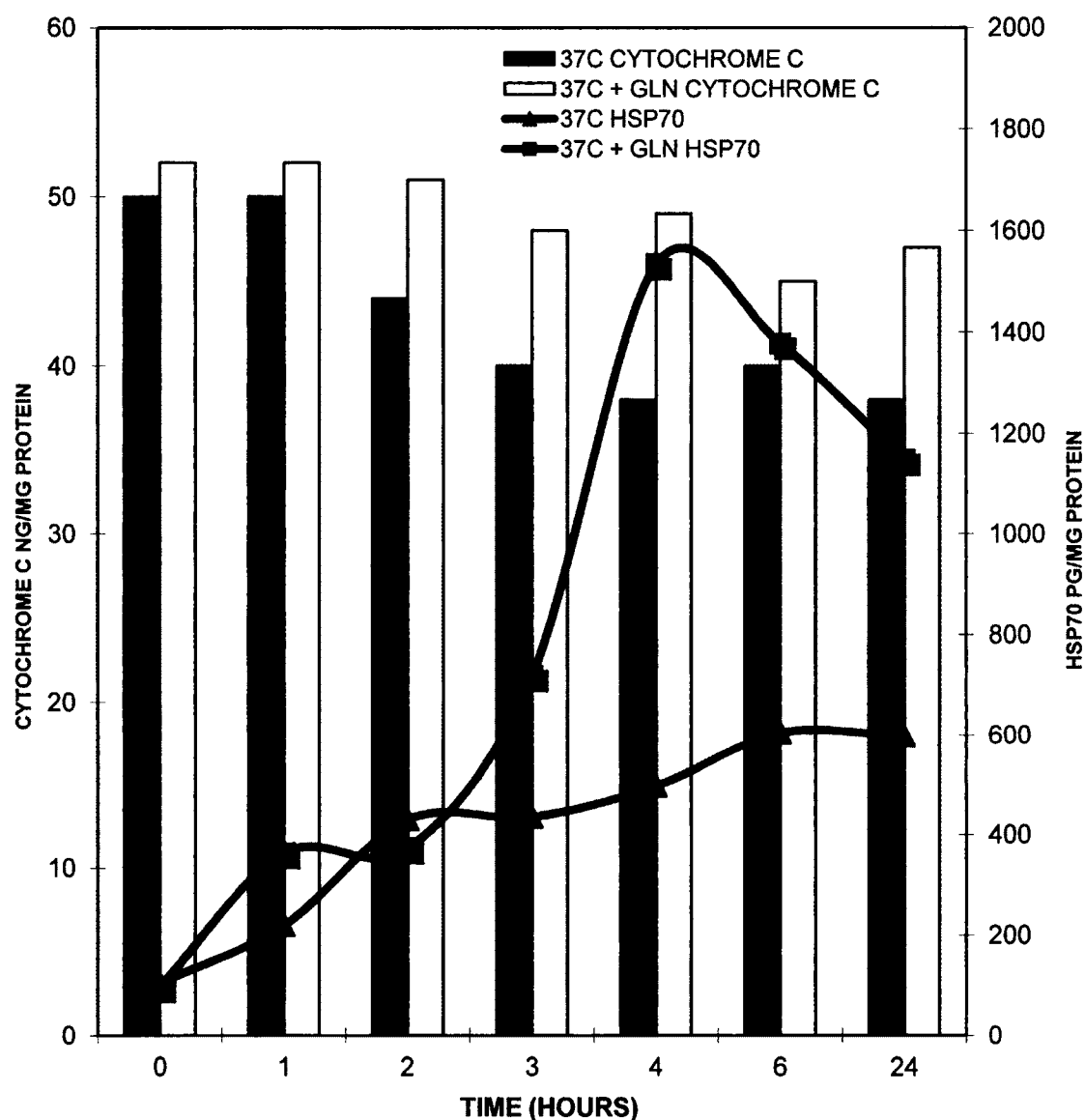
FIGURE 16. Mitochondrial cytochrome c vs. Hsp70 content over time during disinfection in the 37°C and 37°C+GLN treatment groups. These data demonstrate the relationship between incubation at 37°C during disinfection, increased levels of Hsp70 and the amount of cytochrome c detected in the mitochondria. The increased amounts of Hsp70 and steady state of mitochondrial cytochrome c demonstrate the benefit of incubating at 37°C in the presence of Gln. The elevated steady state in mitochondrial cytochrome c levels corresponds with the steady, low state of cytochrome c in the cytosol as demonstrated in Figure 8.

4.9 CYTOCHROME C QUANTITATION – MITOCHONDRIAL ANALYSIS POST-CRYOPRESERVATION

To determine whether incubation at 37°C or 37°C plus Gln during the antibiotic treatment phase would result in maintenance in the amounts of cytochrome c present in the mitochondrial fraction of aortic conduit tissue that had been subsequently cryopreserved, thawed and incubated at 37°C under 5% CO_2 for 72 hours, a cytochrome c sandwich ELISA assay was performed. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln, cryopreserved, thawed and diluted of cryoprotectant and incubated in tissue culture media optimized for the viability of vascular smooth muscle cells over 72 hours at 5% CO_2 as described in the Materials and Methods chapter. The data were obtained by dividing the total nanograms of cytochrome c by the total milligrams of protein in the extract for each time point. Percent recovery analysis was performed as described in chapter 3.17 of the Materials and Methods chapter. The percent recovery of Hsp70 was 92.7 +/-5.7%. The mitochondrial cytochrome c data set were determined to be normally distributed.

Figure 17 demonstrates that the 4°C and 4°C plus Gln did not experience a statistically significant change in the amount of mitochondrial cytochrome c, p>0.05 during the 72 hour incubation. In the tissue previously incubated at 37°C and 37°C plus Gln there was not a statistically significant change in the amount of cytochrome c detected in the mitochondria of the cells between zero and 72 hours of incubation in tissue culture media, p>0.05. However, the cells in the tissue previously incubated at 37°C with Gln had statistically significantly more mitochondrial cytochrome c than the tissue incubated at 37°C without Gln, p<0.05. The increases in cytochrome c correspond to increases in capase-3 and TUNEL positive cells in chapters 4.13 and 4.15 respectively.

Figures 18 and 19 clearly demonstrate that there is a relationship between the amount of Hsp70 present in the cell and the amount of cytochrome c that is found in the mitochondria during the *in vitro* incubation. A lack of increased

Hsp70 correlates with sustained low quantities of cytochrome c in the mitochondria over time (Figure 18). Conversely, augmentation of the Hsp70 levels corresponds with maintenance of mitochondrial cytochrome c levels (Figure 19). Additionally, it is apparent heat shock in the absence of Gln is not as successful over the time course studied. These results correlate with those in Figures 11-13, that demonstrated during the *in vitro* incubation phase of this study a lack of Hsp70 correlated with high levels of cytoplasmic cytochrome c and high levels of Hsp70 correlated with low levels of cytoplasmic cytochrome c. Figure 20 summarizes the amount of cytochrome c present in the mitochondria over both phases of the study. This figure clearly demonstrates a decline in mitochondrial cytochrome c over time for the 4°C treatment groups, even with Gln supplementation and being placed at 37°C post-cryopreservation, i.e. being heat shocked. Conversely, 37°C with Gln maintained a steady state of mitochondrial cytochrome c for the duration of the study.

These data demonstrate that incubation of aortic conduit tissue at 37°C plus Gln maintains the baseline level of cytochrome c present in the mitochondria after cryopreservation, thaw and dilution and additional incubation at 37°C. The 37°C without Gln group experienced a significant decrease in mitochondrial cytochrome c content over time and was unable to return to baseline during the *in vitro* incubation (heat shock). The 4°C and 4°C plus Gln groups sustained continuously low amounts of mitochondrial cytochrome c and these levels are significantly lower than those for the 37°C with or without Gln treatment groups.
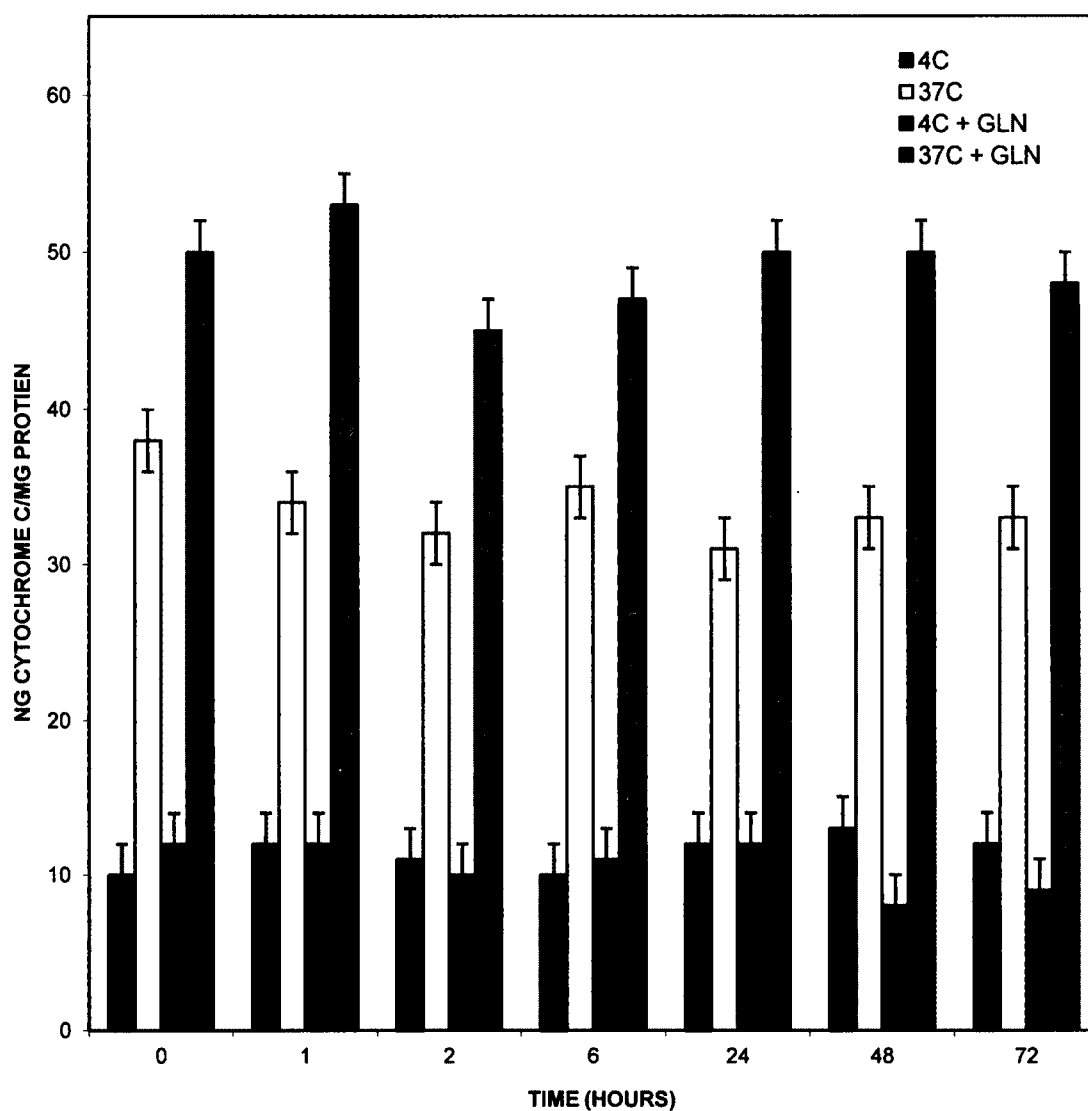
FIGURE 17. Quantitation of mitochondrial cytochrome c in aortic conduit tissue after cryopreservation. Treatment of human aortic conduit previously incubated at 37°C plus Gln during antibiotic disinfection, cryopreserved, thawed and incubated under cell culture conditions maintains the amount of cytochrome c post cryopreservation in the mitochondria relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, pulverized in liquid nitrogen, detergent extracted, differentially centrifuged to isolate the mitochondrial fraction, quantitated by cytochrome c sandwich ELISA and results normalized by Pierce BCA protein assay.
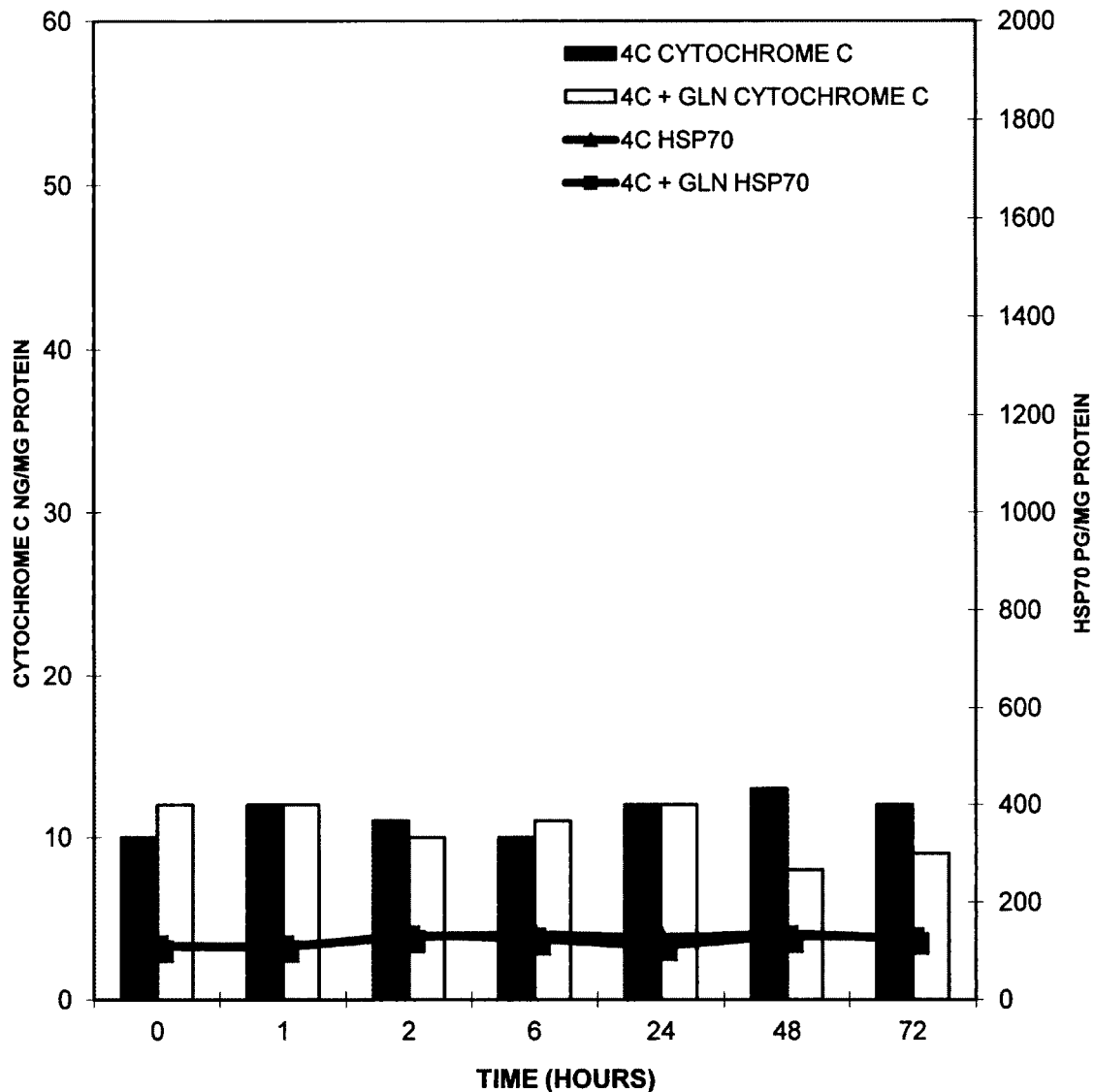
FIGURE 18. Mitochondrial cytochrome c vs. Hsp70 content over time post-cryopreservation in the 4°C and 4°C+GLN treatment groups. These data demonstrate the relationship between incubation at 4°C during disinfection, maintenance of baseline levels of Hsp70 and no increase in the amounts of cytochrome c detected in the mitochondria during the post-cryopreservation incubation at 37°C. These data demonstrate that the addition Gln during disinfection at 4°C makes no difference in the amount of Hsp70 present in the cells. These data are derived from the Hsp70 and cytochrome c ELISA results.
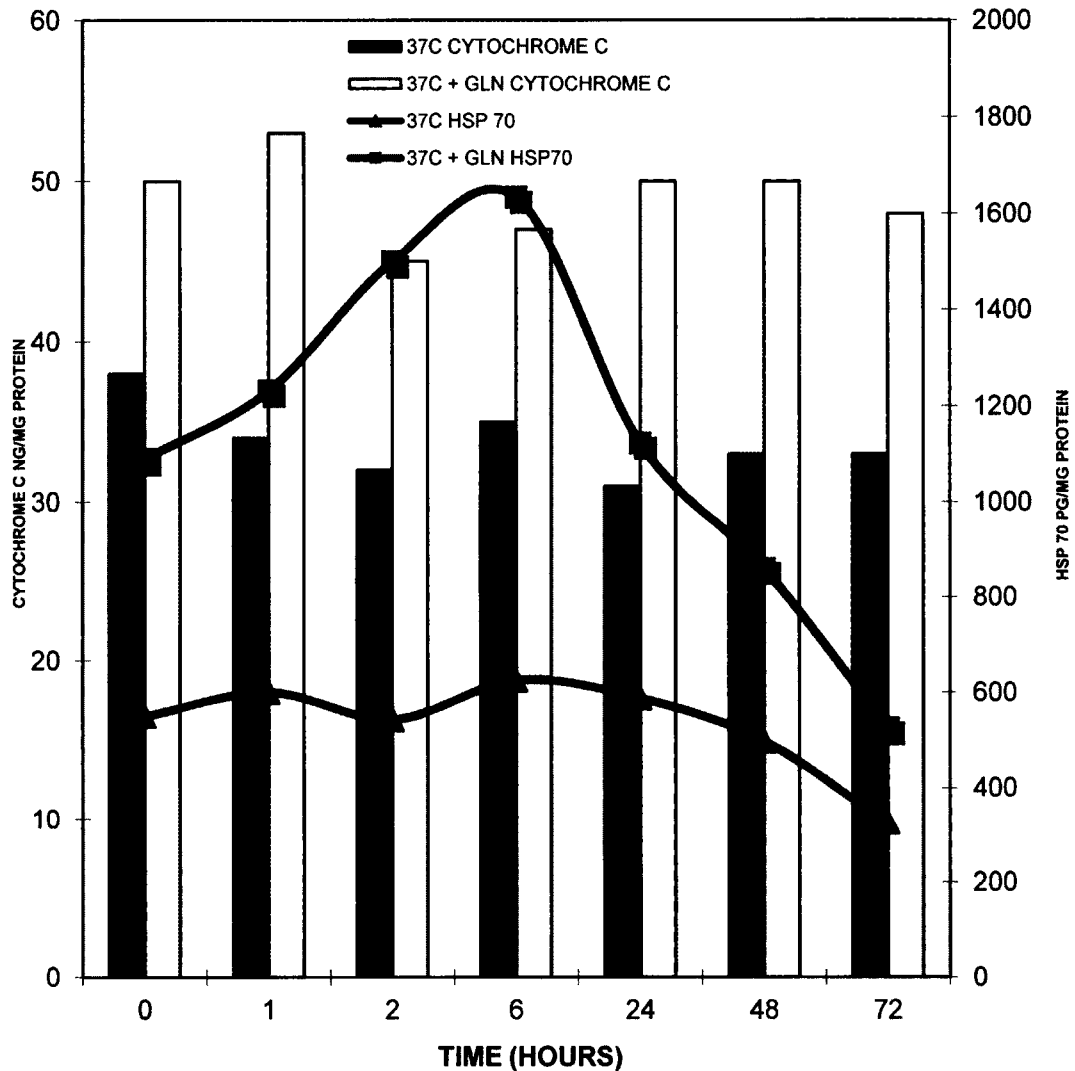
FIGURE 19. Mitochondrial cytochrome c vs. Hsp70 content in the cells post-cryopreservation in the 37°C and 37°C+GLN treatment groups. These data demonstrate the relationship between incubation at 37°C during disinfection, increased levels of Hsp70 and maintenance in the amounts of cytochrome c detected in the mitochondria during the post-cryopreservation incubation at 37°C. The group incubated during disinfection with Gln has increased levels of Hsp70 and mitochondrial cytochrome c relative to the tissues without added Gln. These data demonstrate that the addition Gln during disinfection makes a difference in the amount of Hsp70 present in the cells and mitochondrial cytochrome c. These data are derived from the Hsp70 and cytochrome c ELISA results.
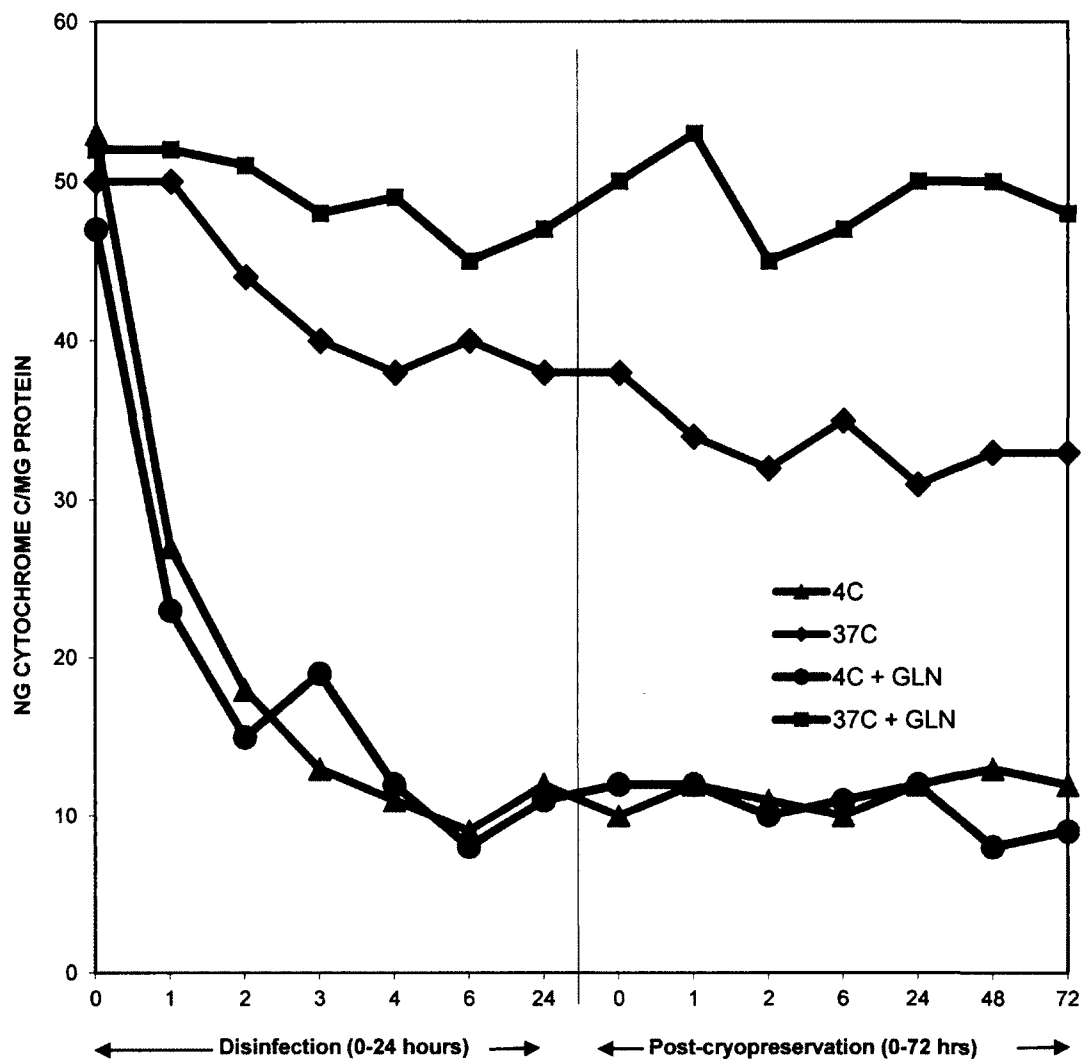
FIGURE 20. Cytochrome c content in the mitochondria for each treatment group for the duration of the study – disinfection and post-cryopreservation. These data demonstrate that the tissues incubated at 4°C during disinfection had a severe drop in mitochondrial cytochrome c that was never recovered. Tissues incubated at 37°C during disinfection experienced declining levels of cytochrome c; however, after one hour post-cryopreservation the mitochondrial cytochrome c levels approach a steady state. The tissue incubated at 37°C in the presence of glutamine demonstrate mildly fluctuating levels of cytochrome c, but over the course of the study the amount of mitochondrial cytochrome c was consistent. Increased levels of cytosolic cytochrome c are known activators of Apaf-1 and thus the apoptotic cascade.

4.10 BCL-2 QUANTITATION - PRECRYOPRESERVATION

Cells resisting entering or undergoing apoptosis can have increased amounts of Bcl-2 and therefore an increase in Bcl-2 over baseline may be indicative of a cellular population resisting entering the apoptotic cascade. To determine whether incubation at 37°C or 37°C plus Gln would result in increased levels of Bcl-2 in aortic conduit tissue cells over time during antibiotic incubation, a Bcl-2 sandwich ELISA assay was performed. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln as described in the Materials and Methods chapter. The data were obtained by dividing the total units of Bcl-2 by the total milligrams of protein in the extract for each time point. Percent recovery analysis was performed as described in chapter 3.17 of the Materials and Methods. The percent recovery of Bcl-2 was 91.3 +/-4.9%. The Bcl-2 data set were determined to be normally distributed

Figure 21 demonstrates that there was not a statistically significant difference in the amount of Bcl-2 present in the cells in all the tissue treatment groups at time zero, $p > 0.05$. There was no change in the amount of Bcl-2 detected in the 4°C and 4°C plus Gln treatment groups, $p > 0.05$. There was an increase in Bcl-2 in the 37°C group during the incubation but it was not significant. There was a statistically significant increase in Bcl-2 content in the 37°C plus Gln group between 2 and 4 hours, $p < 0.05$, and it continued to increase and plateau between 6 and 24 hours of incubation. Figures 22 and 23 demonstrate an absence of increased Bcl-2 in the 4°C treatment groups correlates with no increase in Hsp70 and an increase in Bcl-2 correlates well with an increase in Hsp70 as demonstrated by both 37°C treatment groups.

These data demonstrate that at 37°C and most significantly in the presence of Gln there is an increase in Bcl-2 which correlates with an increase in Hsp70 over time.
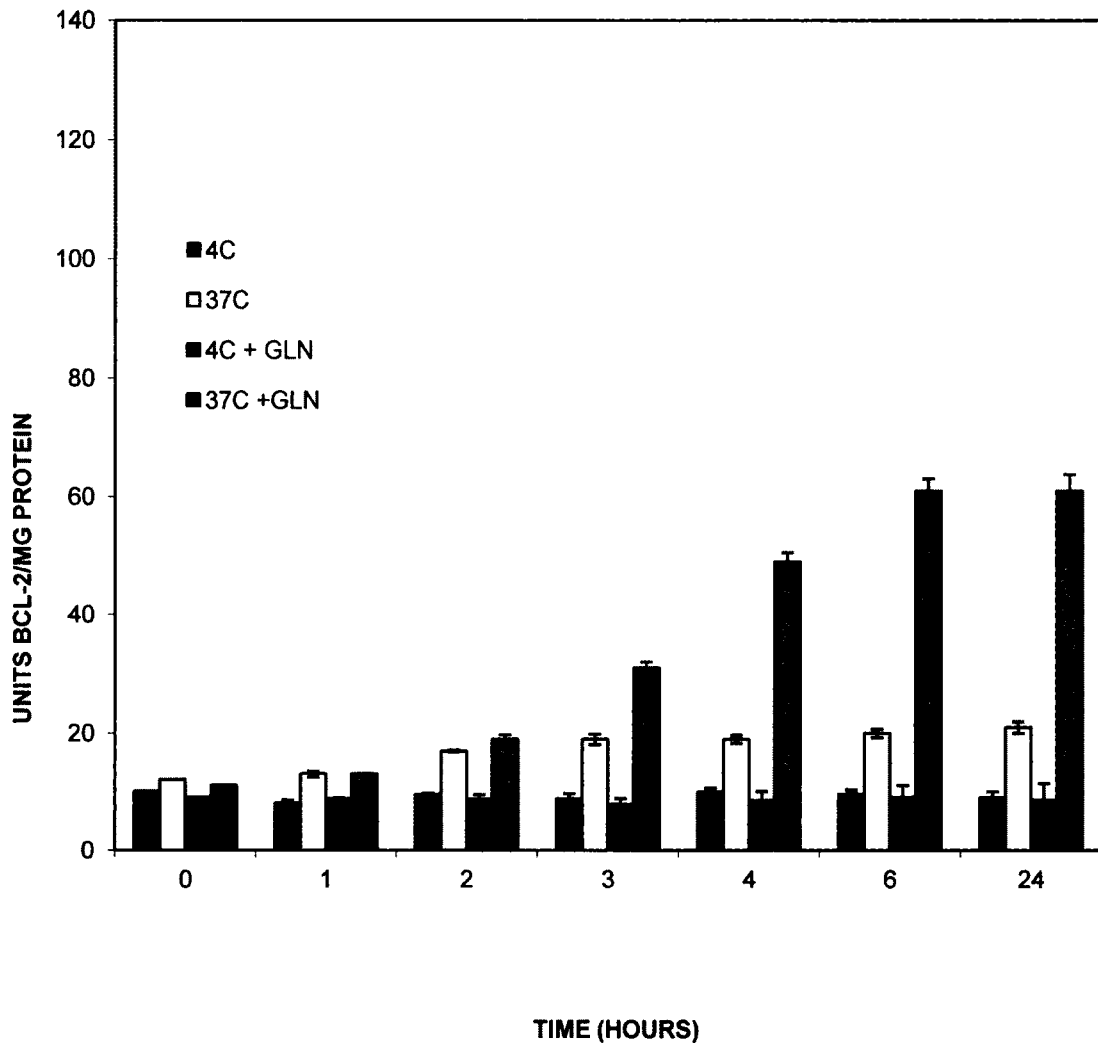
FIGURE 21. Quantitation of Bcl-2 in aortic conduit tissue during disinfection under various treatment conditions. Treatment of human aortic conduit at 37°C plus Gln, during antibiotic disinfection, results in an increase in Bcl-2 levels, relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, pulverized in liquid nitrogen, detergent extracted, quantitated by cytochrome c sandwich ELISA and results normalized by Pierce BCA protein assay.
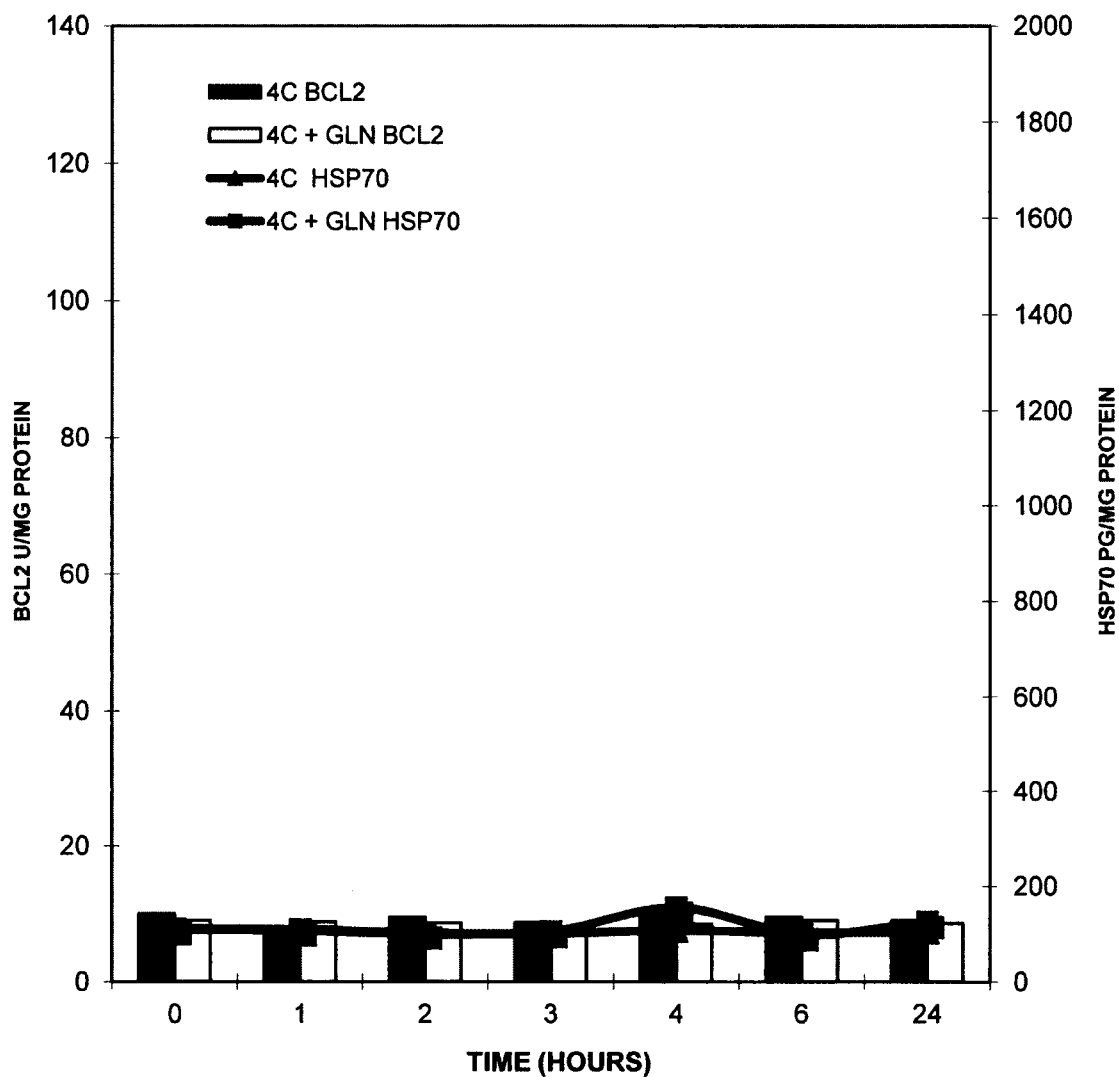
FIGURE 22. Bcl-2 vs. Hsp70 content over time during disinfection in the 4°C and 4°C+GLN treatment groups. These data demonstrate the relationship between incubation at 4°C during disinfection and maintenance of baseline levels of Hsp70 and Bcl-2. Increased levels of Bcl-2 are known to be anti-apoptotic by preventing cytochrome c from exiting the mitochondria and as such these data support the decrease in mitochondrial cytochrome c demonstrated in Figure 14. These data are derived from the Hsp70 and Bcl-2 ELISA results.
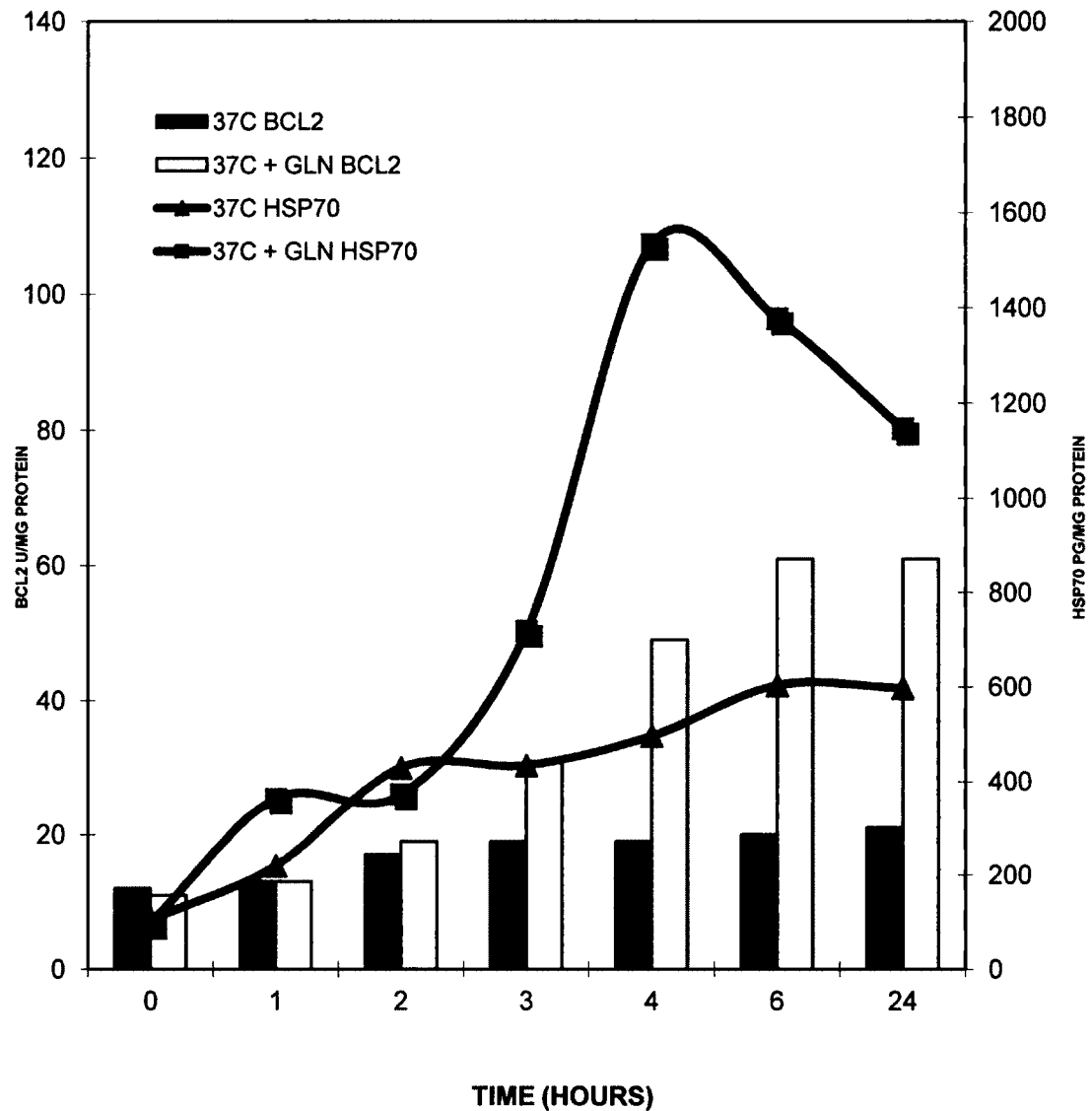
FIGURE 23. Bcl-2 vs. Hsp70 content over time during disinfection in the 37°C and 37°C+GLN treatment groups. These data demonstrate the relationship between incubation at 37°C plus Gln during disinfection, increased levels of Hsp70 and increased levels of Bcl-2. The increased amounts of Hsp70 and Bcl-2 in the 37°C plus Gln treatment group demonstrate the benefit of incubating at 37°C in the presence of Gln. Increased levels of Bcl-2 are known to be anti-apoptotic by preventing cytochrome c from exiting the mitochondria and as such these data support the maintenance in mitochondrial cytochrome c in the 37°C plus Gln treatment group demonstrated in Figure 14. These data are derived from the Hsp70 and Bcl-2 ELISA results.

4.11 BCL-2 QUANTITATION - POSTCRYOPRESERVATION

To determine whether incubation at 37°C or 37°C plus Gln during the antibiotic treatment phase would result in increased amounts of Bcl-2 in aortic conduit tissue that had been subsequently cryopreserved, thawed and incubated at 37°C under 5% CO_2 for 72 hours, a Bcl-2 sandwich ELISA assay was performed. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln, cryopreserved, thawed and diluted of cryoprotectant and incubated in tissue culture media optimized for the viability of vascular smooth muscle cells over 72 hours at 5% CO_2 as described in the Materials and Methods chapter. The data were obtained by dividing the total units of Bcl-2 by the total milligrams of protein in the extract for each time point. Percent recovery analysis was performed as described in chapter 3.17 of the Materials and Methods chapter. The percent recovery of Bcl-2 was 91.3 +/-4.9%. The Bcl-2 data set were determined to be normally distributed

Figure 24 demonstrates that there was not a statistically significant change in the amount of Bcl-2 present in the 4°C, 4 °C plus Gln and 37°C treatment groups, p>0.05. There was a statistically significant increase in Bcl-2 in the 37°C plus Gln group between 2 and 6 hours, p<0.05, and a statistically significant decline between 6 and 26 hours, 24 and 48 hours and 48 and 72 hours, p < 0.05. Figure 25 demonstrates that an absence of increased Hsp70 correlates with an absence of increased Bcl-2 over baseline for both 4°C treatment groups. Figure 26 demonstrates than an increase in Hsp70 correlates with an increase in Bcl-2 for both 37°C treatment groups; however, the 37°C plus Gln group experiences the largest increases in Bcl-2.

These data demonstrate that the 37°C treatment group supplemented with Gln during the antibiotic incubation experienced an increase in the anti-apoptotic Bcl-2 protein that correlated positively with increases in Hsp70.
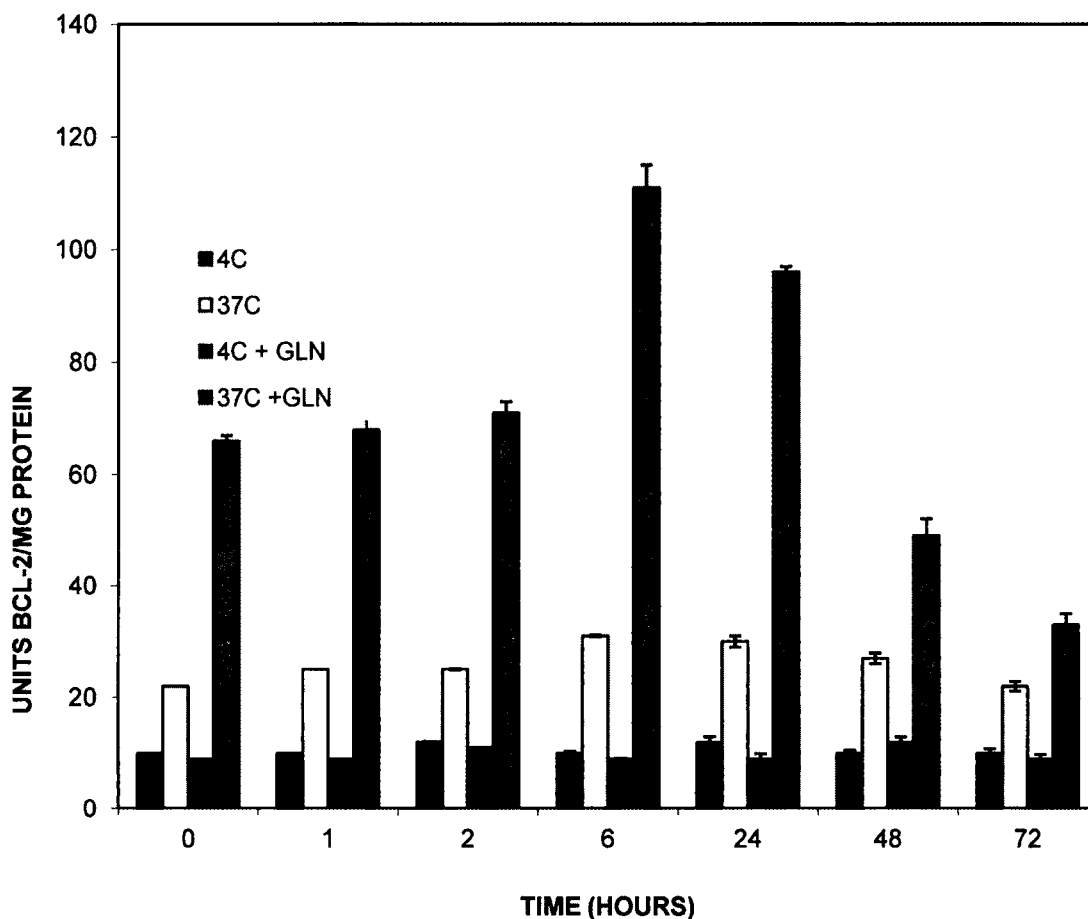
FIGURE 24. Quantitation of Bcl-2 in aortic conduit tissue after cryopreservation. Treatment of human aortic conduit previously incubated at 37°C plus Gln during antibiotic disinfection, cryopreserved, thawed and incubated under cell culture conditions results in increased amounts of Bcl-2 after 6 hours of incubation, post cryopreservation relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, pulverized in liquid nitrogen, detergent extracted, quantitated by cytochrome c sandwich ELISA and results normalized by Pierce BCA protein assay.

FIGURE 25. Bcl-2 vs. Hsp70 content over time post-cryopreservation in the 4°C and 4°C+GLN treatment groups. These data demonstrate the relationship between incubation at 4°C during disinfection, maintenance of baseline levels of Hsp70 and no increase in the amounts of Bcl-2 detected in the cells during the post-cryopreservation incubation at 37°C. These data demonstrate that the addition Gln during disinfection at 4°C makes no difference in the amount of Hsp70 present in the cells. Increased levels of Bcl-2 are known to be anti-apoptotic by preventing cytochrome c from exiting the mitochondria and as such these data support the decrease in mitochondrial cytochrome c demonstrated in Figure 14. These data are derived from the Hsp70 and Bcl-2 ELISA results.
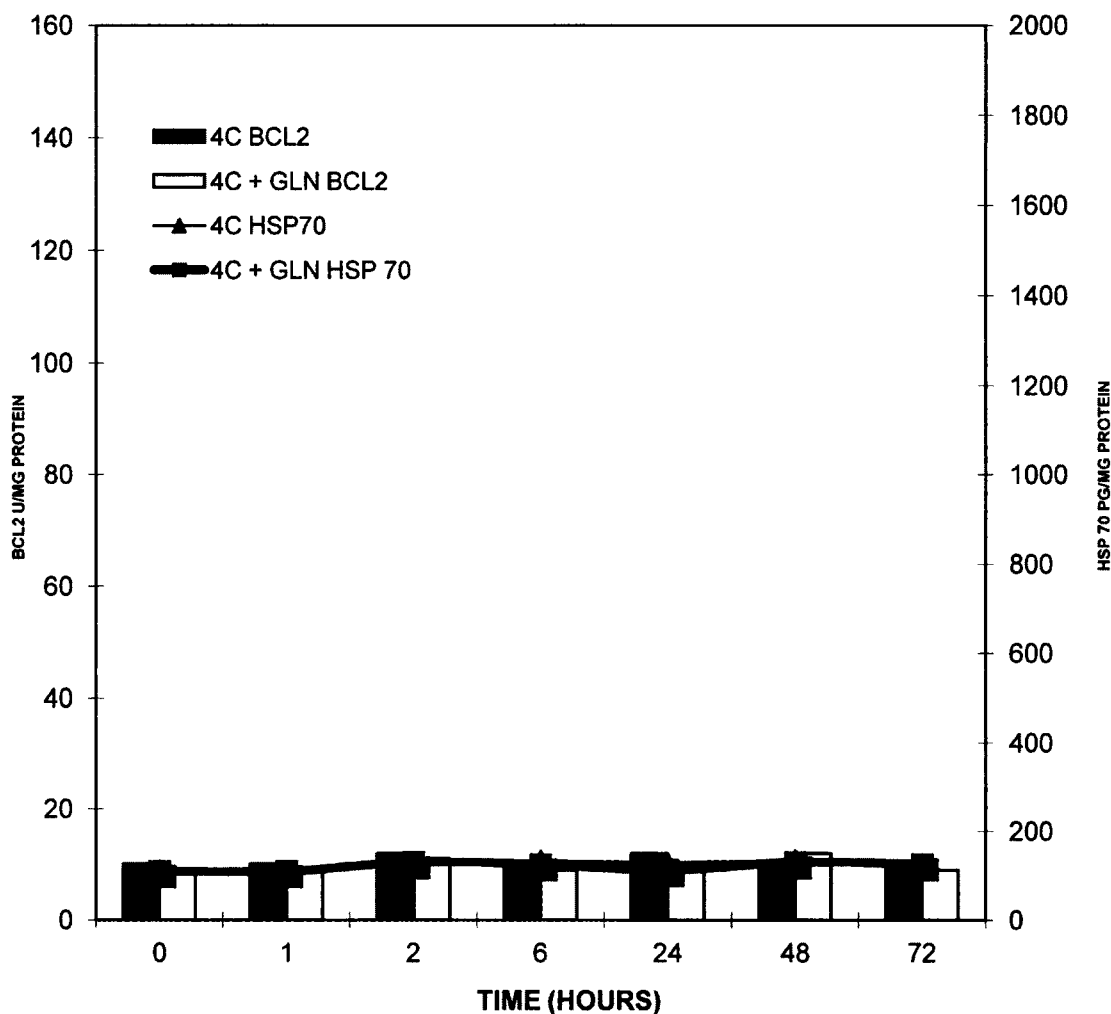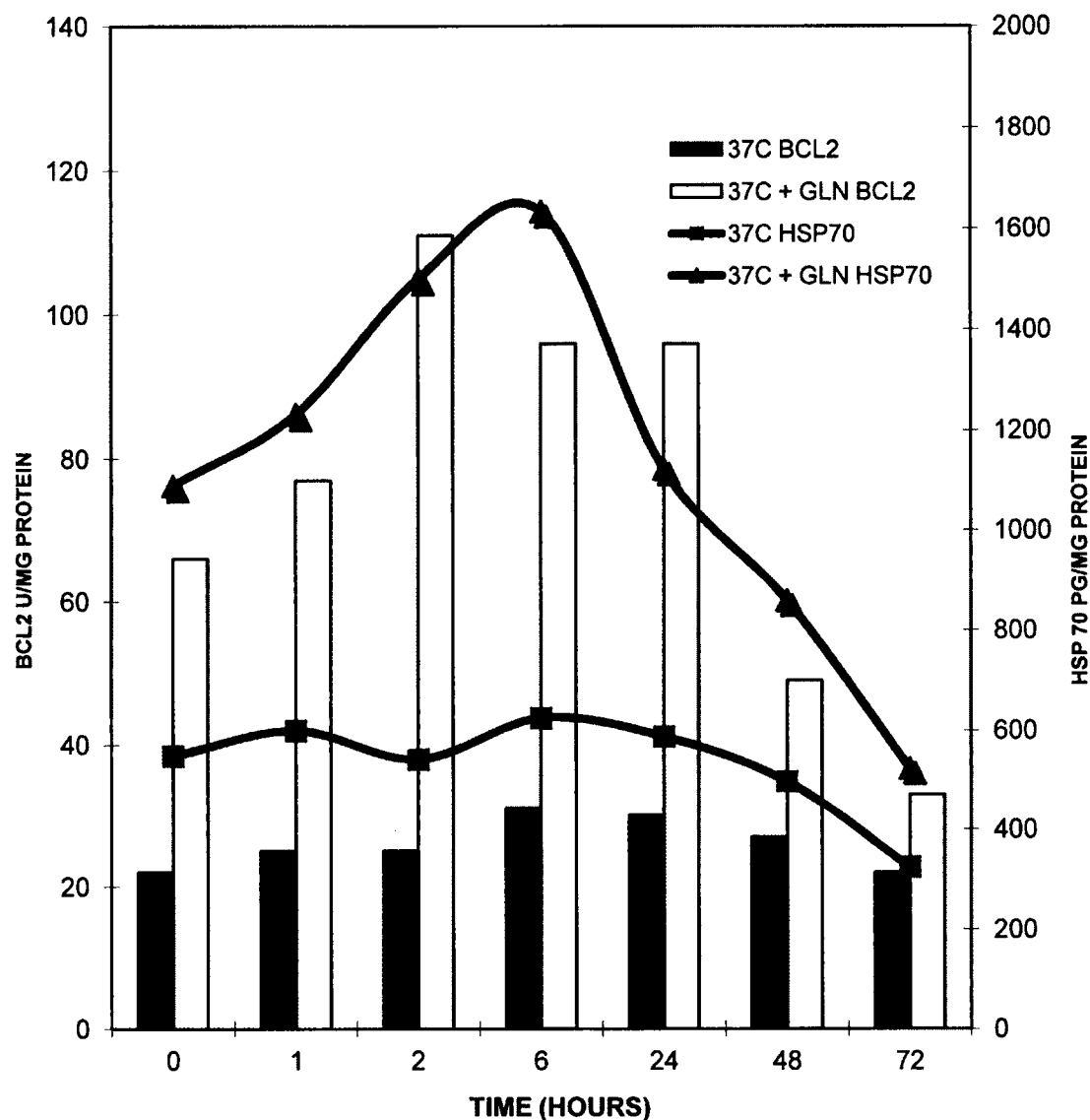
FIGURE 26. Bcl-2 vs. Hsp70 content in the cells post-cryopreservation in the 37°C and 37°C+GLN treatment groups. These data demonstrate the relationship between incubation at 37°C plus Gln during disinfection, increased levels of Hsp70 and increased levels of Bcl-2 during the post-cryopreservation incubation at 37°C. These data demonstrate that the addition Gln during disinfection makes a difference in the amount of Hsp70 and Bcl-2 present in the cells. Increased levels of Bcl-2 are known to be anti-apoptotic by preventing cytochrome c from exiting the mitochondria and as such, these data support the maintenance in mitochondrial cytochrome c levels demonstrated in Figure 14. These data are derived from the Hsp70 and Bcl-2 ELISA results.

4.12 CASPASE-3 IMMUNOHISTOCHEMISTRY - PRECRYOPRESERVATION

The presence of caspase-3 indicates a cell is committed to completion of the apoptotic cascade. Therefore to determine whether incubation at 37°C or 37°C plus Gln would result in maintenance of baseline levels of caspase-3, it was probed for using immunohistochemistry and quantitated histomorphometrically. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln as described in the Materials and Methods chapter. The data were obtained by dividing the total number of caspase-3 positive cells in the tissue section by the total number of cells present in the tissue sections on the companion Hematoxylin and Eosin stained slides and the percentage of positive cells was obtained. The caspase-3 data set were determined to be normally distributed.

Figure 27 demonstrates that at time zero there was not a statistically significant difference in the number of caspase-3 positive cells, $p > 0.05$. The 4°C, 4°C plus Gln and 37°C treatment groups experienced a significant increase in caspase-3 positive cells over baseline $p < 0.05$; however, the number of caspase-3 positive cells in the 37°C treatment group was significantly lower than both 4°C treatment groups, $p < 0.05$. The 37°C plus Gln group did not experience a statistically significant increase in the percentage of caspase-3 positive cells over baseline, $p > 0.05$. Figure 28 demonstrates that without an increase in Hsp70 there is a significant increase in the number caspase-3 positive cells both 4°C treatment groups. Conversely, Figure 29 demonstrates that with an increase in Hsp70 there is not a significant increase the number caspase-3 positive cells in the 37°C plus Gln treatment group. Representative micrographs of caspase-3 immunostained tissue sections are provided in Figures 33-40.

These data demonstrate that cells incubated at 37°C with Gln do not experience an increase in caspase-3 positive cells, relative to the other three treatment modalities. Increased amounts of Hsp70 correlate with a lack of increased amounts of caspase-3 positive cells.
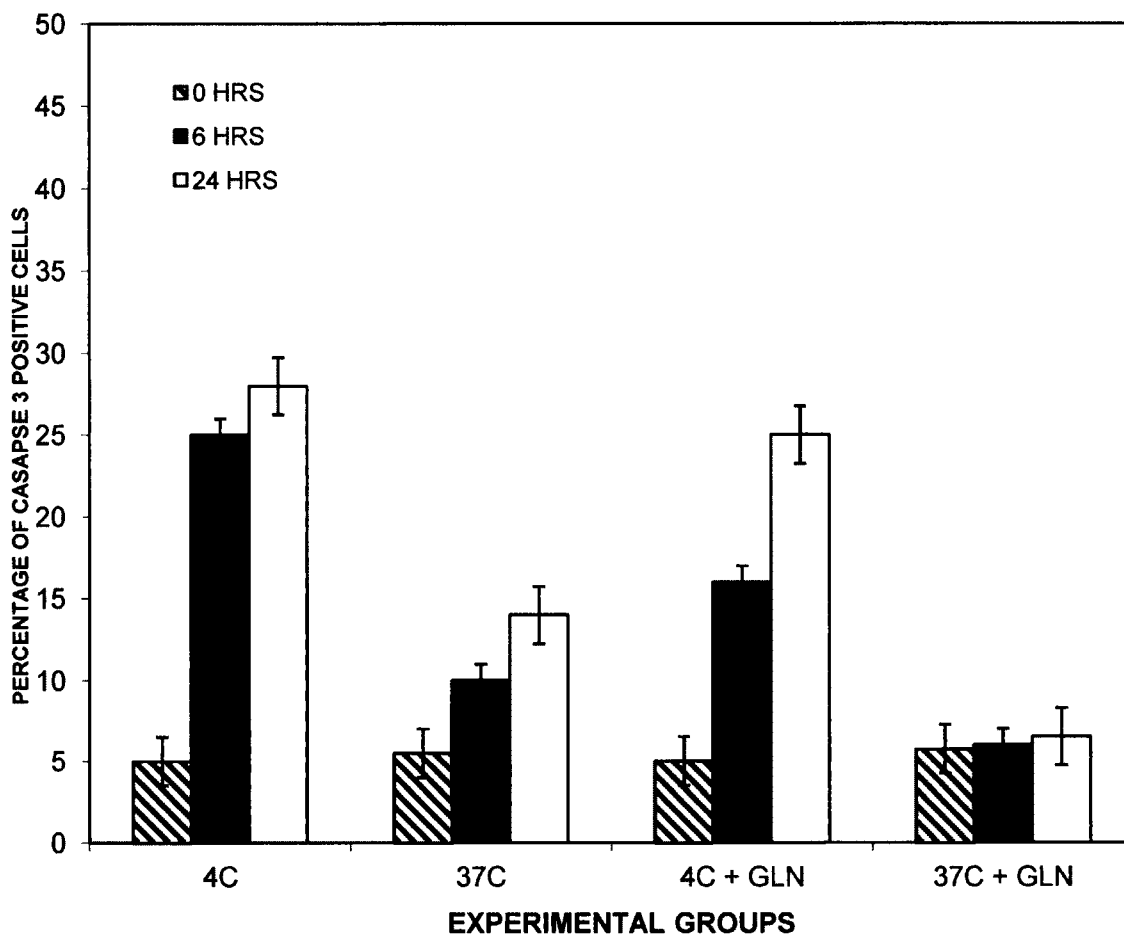
FIGURE 27. Quantitation of caspase-3 in aortic conduit tissue during disinfection under various treatment conditions. Treatment of human aortic conduit at 37°C plus Gln, during antibiotic disinfection, results in no significant increase in caspase-3 positive cells over time, relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, formalin fixed, paraffin embedded, sectioned and probed with an antibody specific for the active form of caspase-3 using standard immunohistochemical procedures.

FIGURE 28. Caspase-3 vs. Hsp70 content in the cells during disinfection in the 4°C and 4°C+GLN treatment groups. These data demonstrate the relationship between incubation at 4°C during disinfection, maintenance of baseline levels of Hsp70 and decreasing amounts of caspase-3 detected in the cell. The increased levels of caspase-3 correspond with the increased levels of cytochrome c in the cytosol and baseline levels of Bcl-2, Figures 8 and 21 respectively, as all of these biomarkers are predecessors to initiation of the apoptotic cascade. These data are derived from the Hsp70 ELISA and caspase-3 immunohistochemistry results.

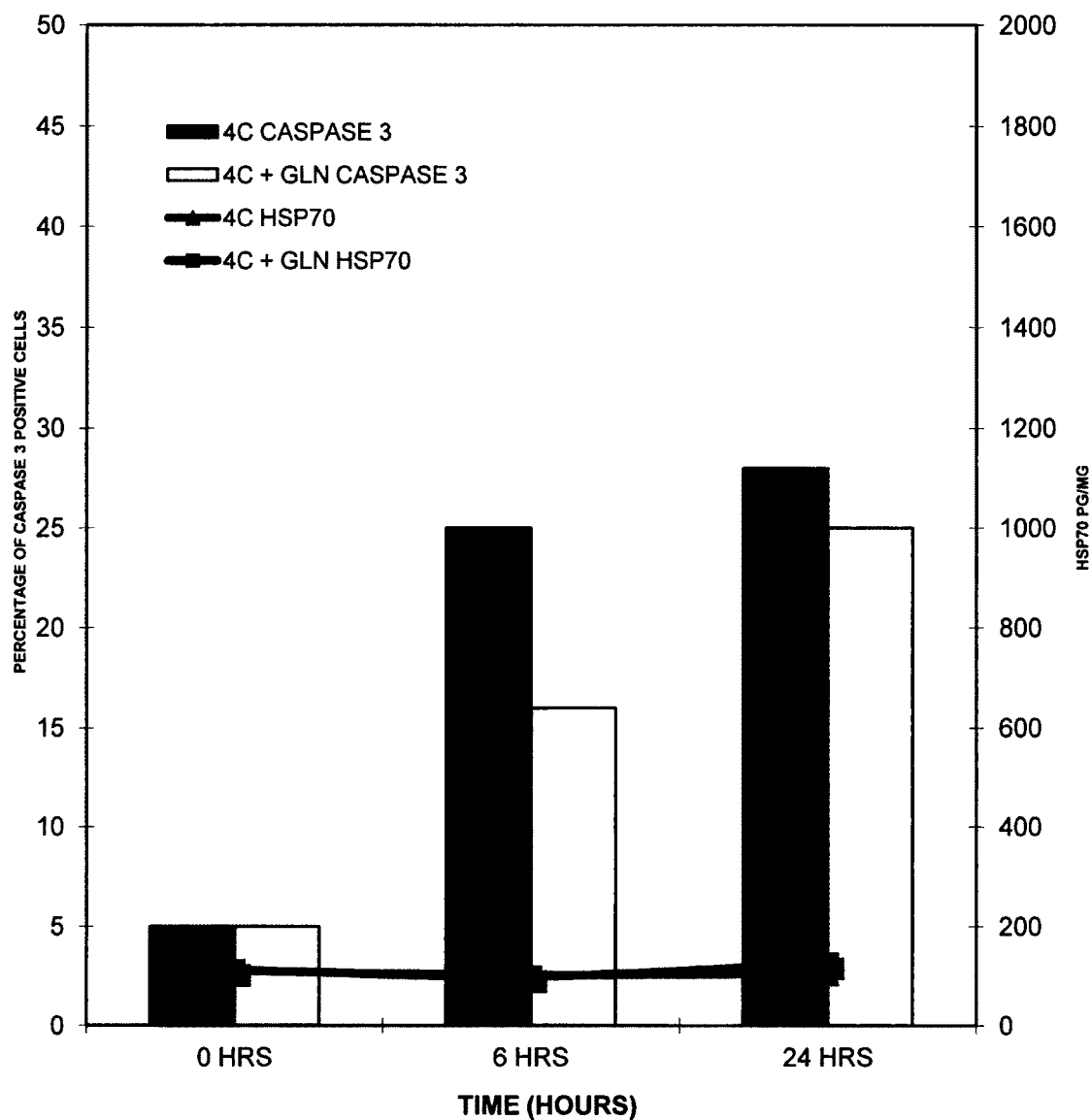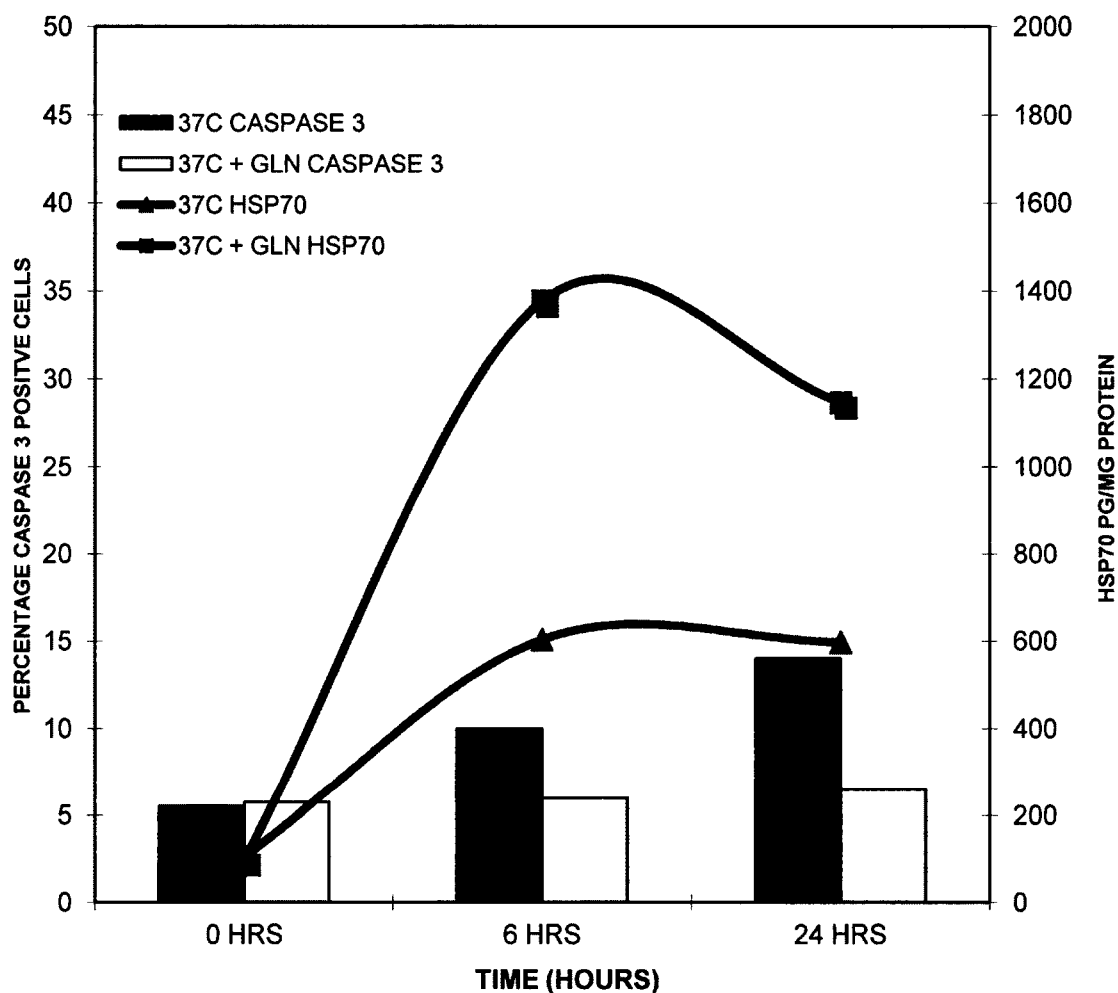**FIGURE 29. Percentage of Caspase-3 positive cells vs. Hsp70 content over time during disinfection in the 37°C and 37°C+GLN treatment groups.** These data demonstrate the relationship between incubation at 37°C during disinfection, increased levels of Hsp70 and the amount of caspase-3 detected in the cells. The increased amounts of Hsp70 and steady state of caspase-3 demonstrate the benefit of incubating at 37°C in the presence of Gln. The increased levels of caspase-3 in the 37°C treatment group corresponds with the increased levels of cytochrome c and small increase in Bcl-2, Figures 8 and 21 respectively, as all of these biomarkers are predecessors to initiation of the apoptotic cascade. Conversely, the baseline levels of caspase-3 in the 37°C plus Gln treatment group, the baseline levels of cytoplasmic cytochrome c and the increased levels

of Bcl-2 reflect the protective effects of elevated levels of Hsp70. These data are derived from the Hsp70 ELISA and caspase-3 immunohistochemistry results.

4.13 CASPASE-3 IMMUNOHISTOCHEMISTRY – POST-CRYOPRESERVATION

To determine whether incubation at 37°C or 37°C plus Gln during the antibiotic treatment phase would result in maintenance of baseline levels of caspase-3 positive cells in aortic conduit tissue that had been subsequently cryopreserved, thawed and incubated at 37°C under 5% CO_2 for 72 hours, immunohistochemistry and histomorphometry were used to detect and quantitate caspase-3. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln, cryopreserved, thawed and diluted of cryoprotectant and incubated in tissue culture media optimized for the viability of vascular smooth muscle cells over 72 hours at 5% CO_2 as described in the Materials and Methods chapter. The data were obtained by dividing the total number of caspase-3 positive cells in the tissue section by the total number of cells present in the tissue sections on the companion slides and obtaining the percentage of positive cells. The caspase-3 data set were normally distributed.

Figure 30 demonstrates that the 4°C and 4°C plus Gln treatment groups experienced a significant increase in caspase-3 positive cells over baseline, $p<0.05$, specifically between 2 and 24 hours, $p<0.05$. The 37°C treatment group also experienced a significant increase in caspase-3 positive cells between 2 and 6 hours; however, the number of caspase-3 positive cells in the 37°C treatment group was significantly lower than both 4°C treatment groups, $p< 0.05$. The 37°C plus Gln group did not experience a statistically significant increase in the percentage of caspase-3 positive cells over baseline, $p> 0.05$. Figure 31 demonstrates that without an increase in Hsp70 there is a significant increase the number caspase-3 positive cells in both 4°C treatment groups. Conversely,

Figure 32 demonstrates that with an increase in Hsp70 there is not a significant increase the number caspase-3 positive cells in the 37°C plus Gln treatment group. Representative micrographs of caspase-3 immunostained tissue sections are provided in Figures 33-40.

These data demonstrate that cells in tissue incubated at 37°C with Gln do not experience an increase in caspase-3 positive cells, relative to the other three treatment modalities that do experience an increase in caspase-3 positive cells.
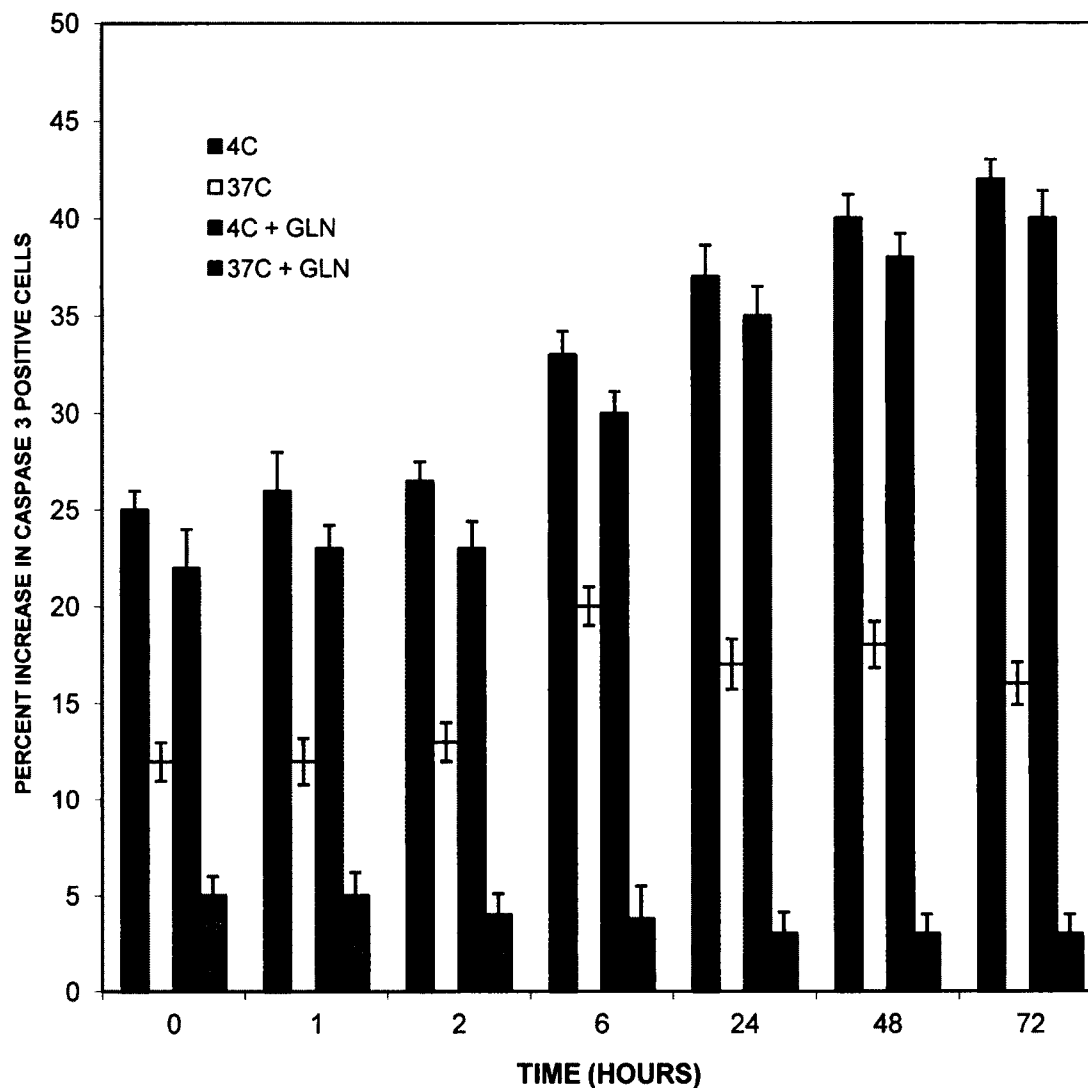
FIGURE 30. Quantitation of caspase-3 in aortic conduit tissue after cryopreservation. Treatment of human aortic conduit previously incubated at 37°C plus Gln during antibiotic disinfection, cryopreserved, thawed and incubated under cell culture conditions results in no significant increase in caspase-3 positive cells over time, post cryopreservation relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, formalin fixed and probed with an antibody specific for the active form of caspase-3 using standard immunohistochemical procedures.

FIGURE 31. Percentage of Caspase-3 positive cells vs. Hsp70 content in the cells post-cryopreservation in the 4°C and 4°C+GLN treatment groups. These data demonstrate the relationship between incubation at 4°C during disinfection, maintenance of baseline levels of Hsp70 and increased amounts of caspase-3 detected during the post-cryopreservation incubation at 37°C. These data demonstrate that the addition Gln during disinfection makes no difference in the amount of Hsp70 present in the cells. The increased levels of caspase-3 correspond with the increased levels of cytochrome c in the cytosol and baseline levels of Bcl-2, Figures 11 and 24 respectively, as all of these biomarkers are predecessors to initiation of the apoptotic cascade. These data are derived from the Hsp70 ELISA and caspase-3 immunohistochemistry results.
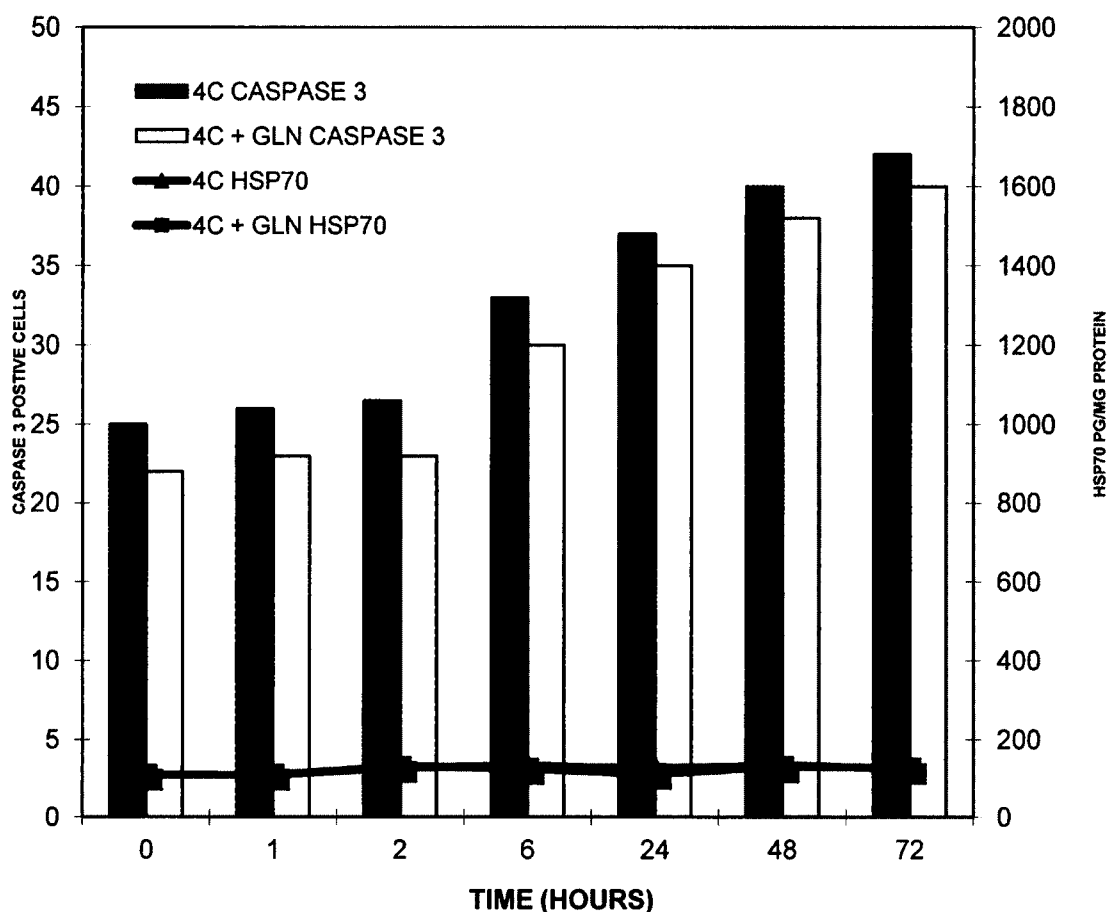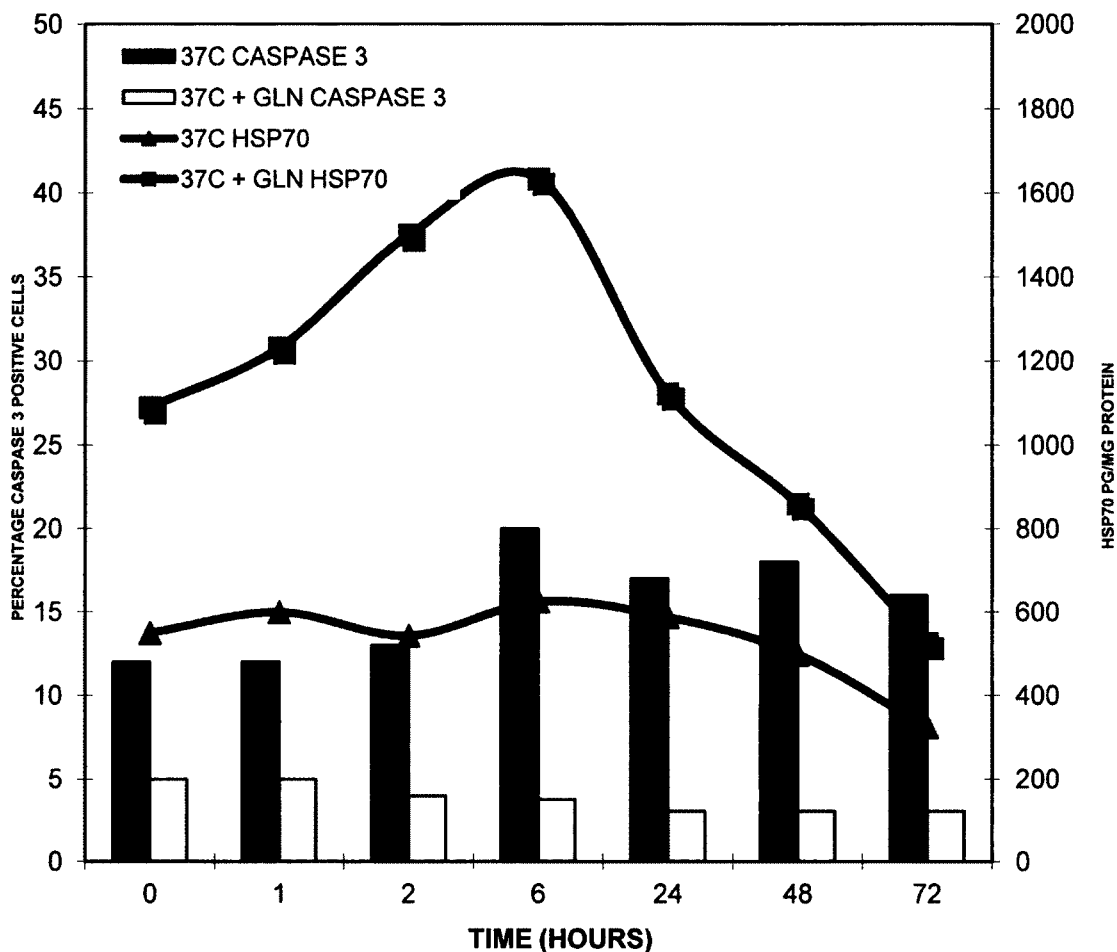
FIGURE 32. Percentage of caspase-3 positive cells vs. Hsp70 content over time post-cryopreservation in the 37°C and 37°C+GLN treatment groups. These data demonstrate the relationship between incubation at 37°C plus Gln during disinfection, increased levels of Hsp70 and maintenance of the baseline percentage of caspase-3 positive cells detected during the post-cryopreservation incubation at 37°C. The baseline level of caspase-3 in the 37°C plus Gln treatment group correspond with the baseline levels of cytochrome c in the cytosol and increased levels of Bcl-2, Figures 11 and 24 respectively. These levels of the apoptotic biomarkers in 37°C plus Gln treatment group are indicative of a cell population that should not succumb to apoptosis beyond that associated with normal cellular turn-over. The same cannot be said for the 37°C treatment group. These data are derived from the Hsp70 ELISA and caspase-3 immunohistochemistry results.

FIGURE 33. Caspase-3 Positive Control. Micrograph demonstrating blue nuclear staining and brick-red staining of caspase-3 in the cytoplasm, 100X. Areas containing numerous positive cells are indicated by the arrows. Fifteen percent of the cells in this control are positive.
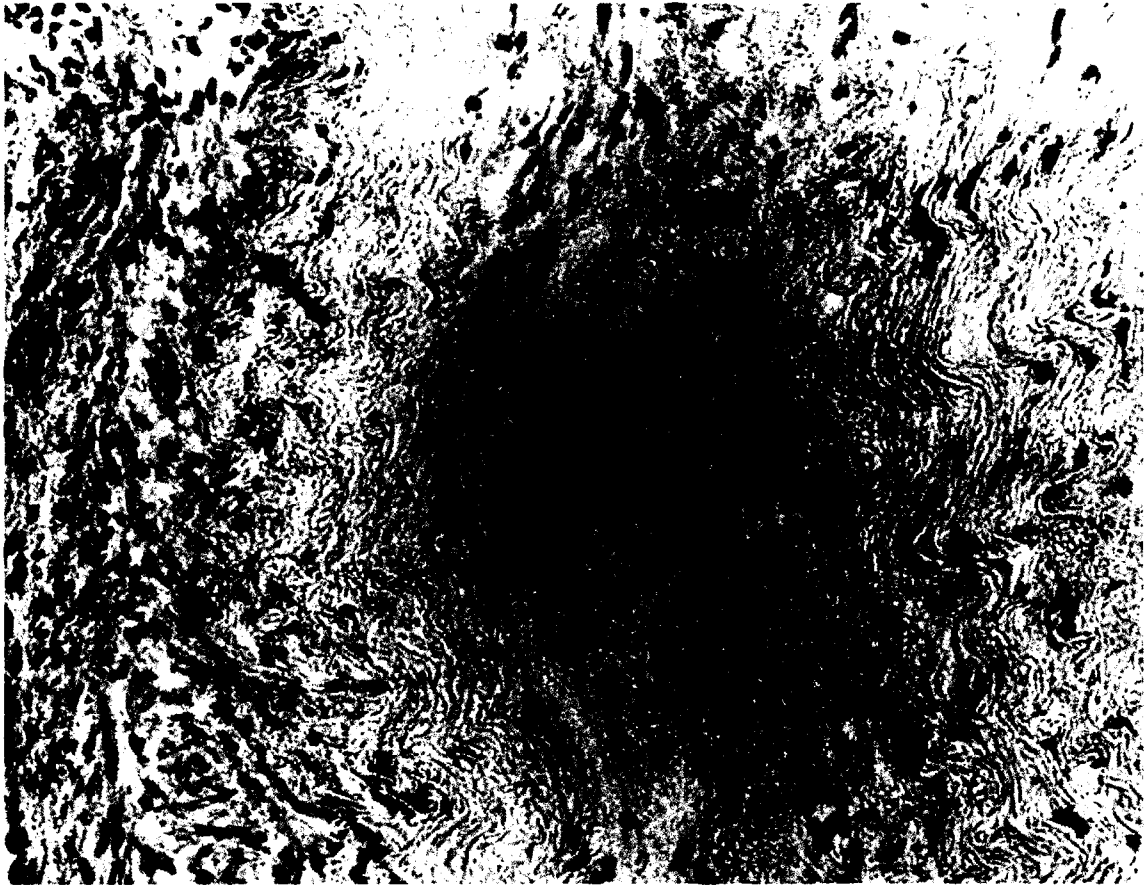
FIGURE 34. Caspase-3 Positive Control. Micrograph demonstrating blue nuclear staining and brick-red staining of caspase-3 in the cytoplasm, 200X. Areas containing numerous positive cells are indicated by the arrows.
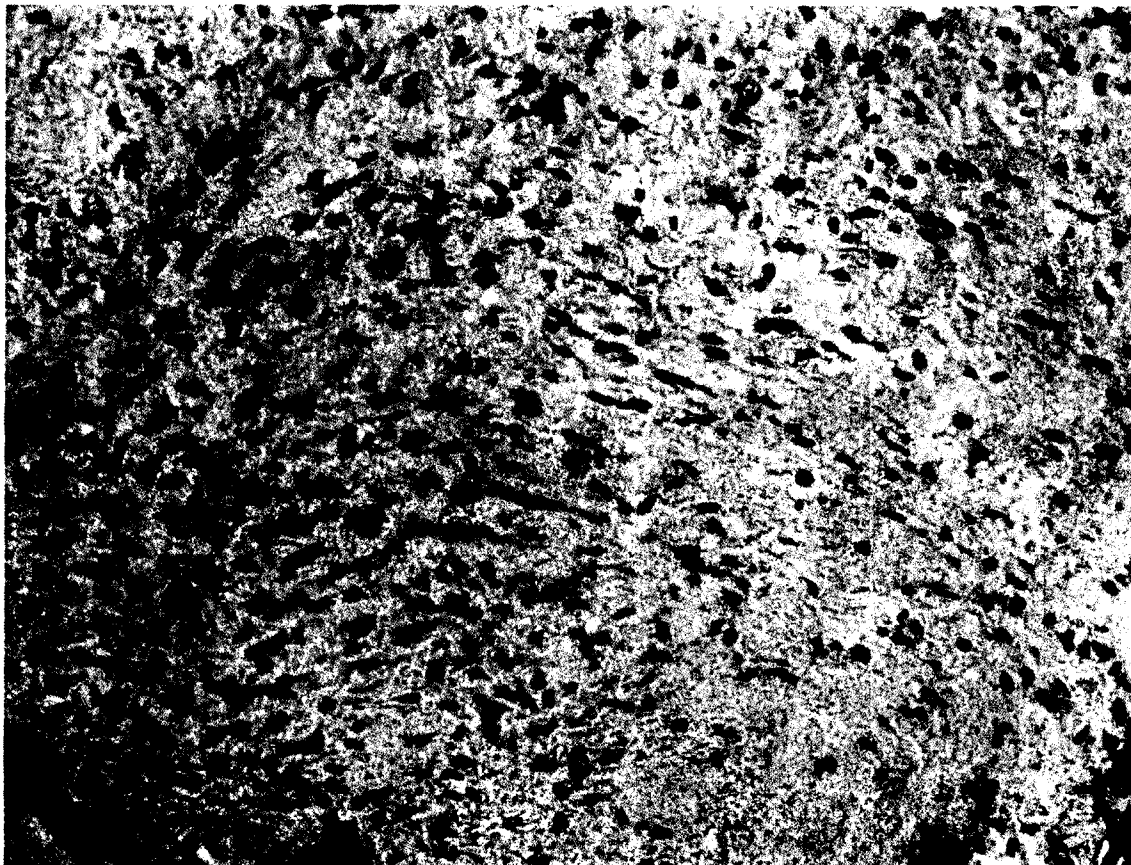
FIGURE 35. Caspase-3 Negative Control. Micrograph demonstrating blue nuclear staining and absent brick-red staining for caspase-3 in the cytoplasm, 200X. The negative control was generated from the same tissue as in Figure 34; however, the primary antibody was omitted during the staining procedure.

FIGURE 36. Baseline caspase-3 staining. This micrograph is of a tissue

section from the 37°C + Gln experimental group at 0 time post thaw to

demonstrate a sample where thresholding revealed that approximately 5% of the

cells were caspase-3 positive, 100X. The nuclei stain blue and caspase-3

positive cells stain brick red in the cytoplasm. The arrows are pointing to the

areas, which stained positively for caspase-3.

FIGURE 37. Baseline caspase-3 staining. This micrograph is of a tissue section from the 37°C + Gln experimental group at 0 time post thaw to demonstrate a sample where thresholding revealed that approximately 5% of the cells were caspase-3 positive, 200X. The nuclei stain blue and caspase-3 positive cells stain brick red in the cytoplasm. The arrows are pointing to the areas, which stained positively for caspase-3.
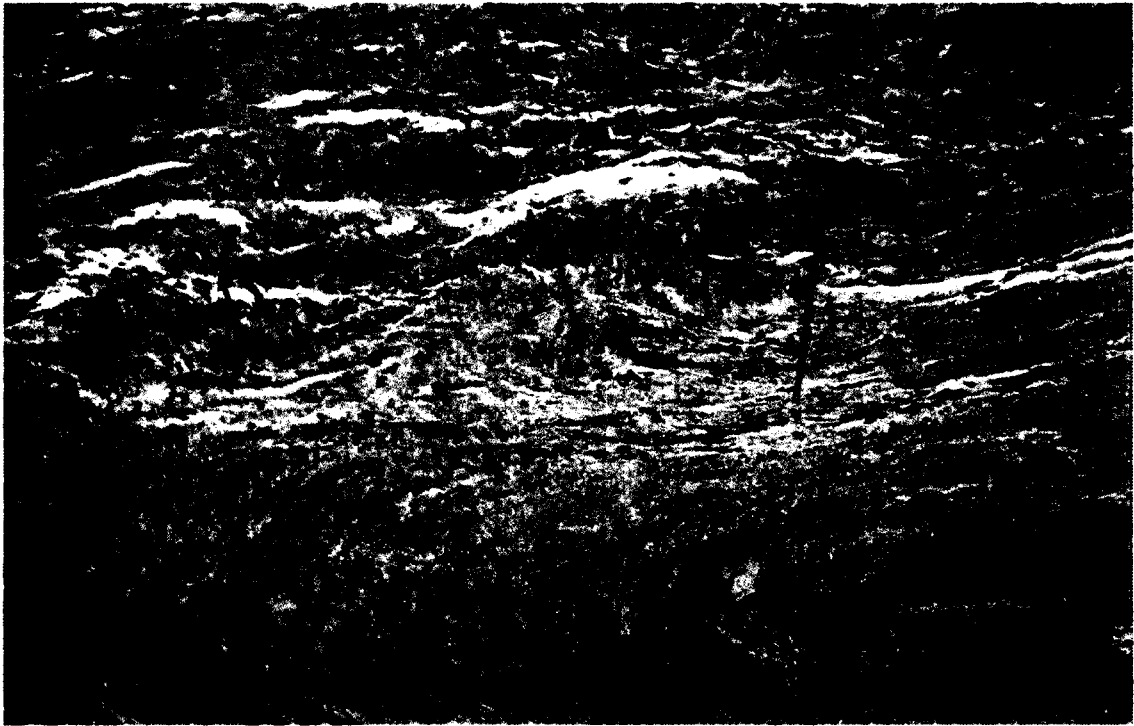
FIGURE 38. Caspase-3 micrograph, 25% positivity. This micrograph is of a tissue section from the 4°C plus Gln experimental group at 6 hours post-thaw to demonstrate a sample where thresholding revealed that approximately 25% of the cells were caspase-3 positive, 100X. The nuclei stain blue and caspase-3 positive cells stain brick red in the cytoplasm. The arrows are pointing to the areas, which stained positively for caspase-3.
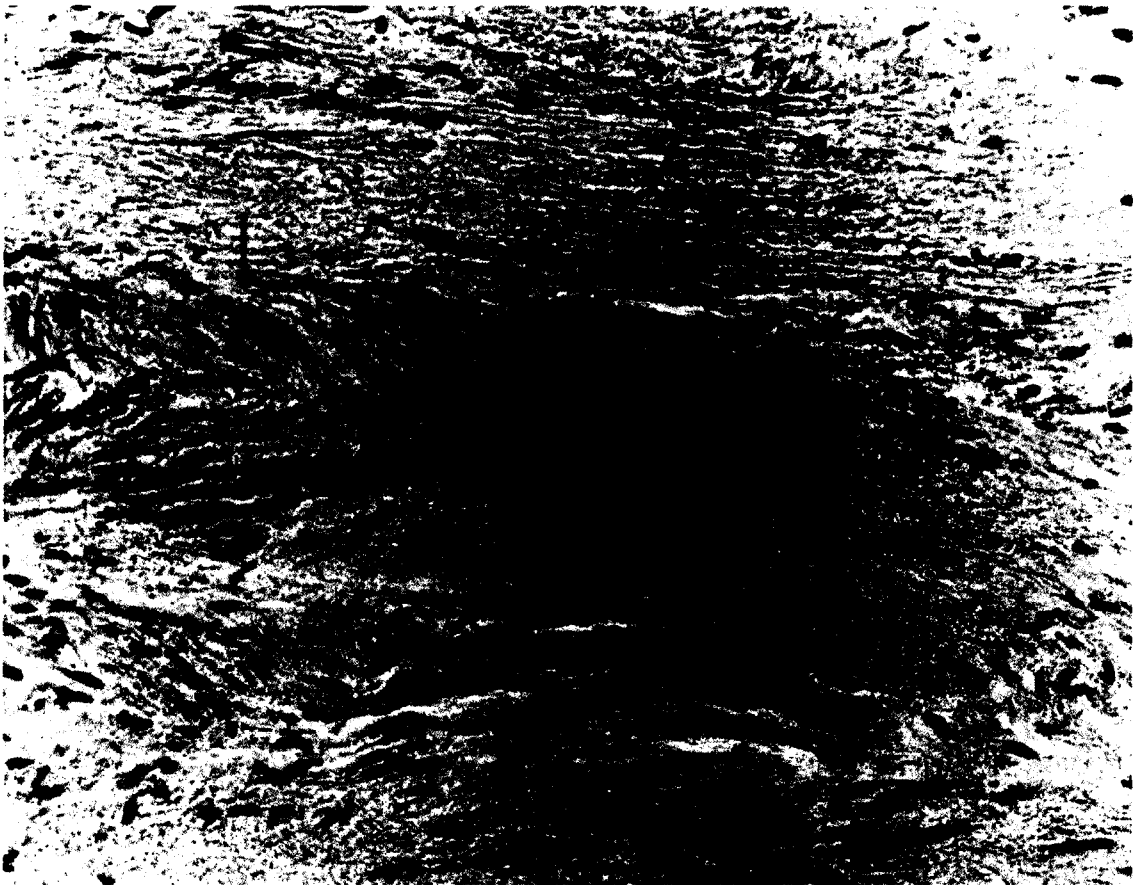
FIGURE 39. Caspase-3 micrograph, 25% positivity. This micrograph is of a tissue section from the 4°C plus Gln experimental group at 6-hours post-thaw to demonstrate a sample where thresholding revealed that approximately 25% of the cells were caspase-3 positive, 200X. The nuclei stain blue and caspase-3 positive cells stain brick red in the cytoplasm. The arrows are pointing to the areas, which stained positively for caspase-3.

FIGURE 40. Caspase-3 micrograph, 45% positivity. This micrograph is of a tissue section from the 4°C experimental group at 48-hours post-thaw to demonstrate a sample where thresholding revealed that approximately 45% of the cells were caspase-3 positive, 100X. The nuclei stain blue and caspase-3 positive cells stain brick red in the cytoplasm. The arrows are pointing to the areas, which stained positively for caspase-3.
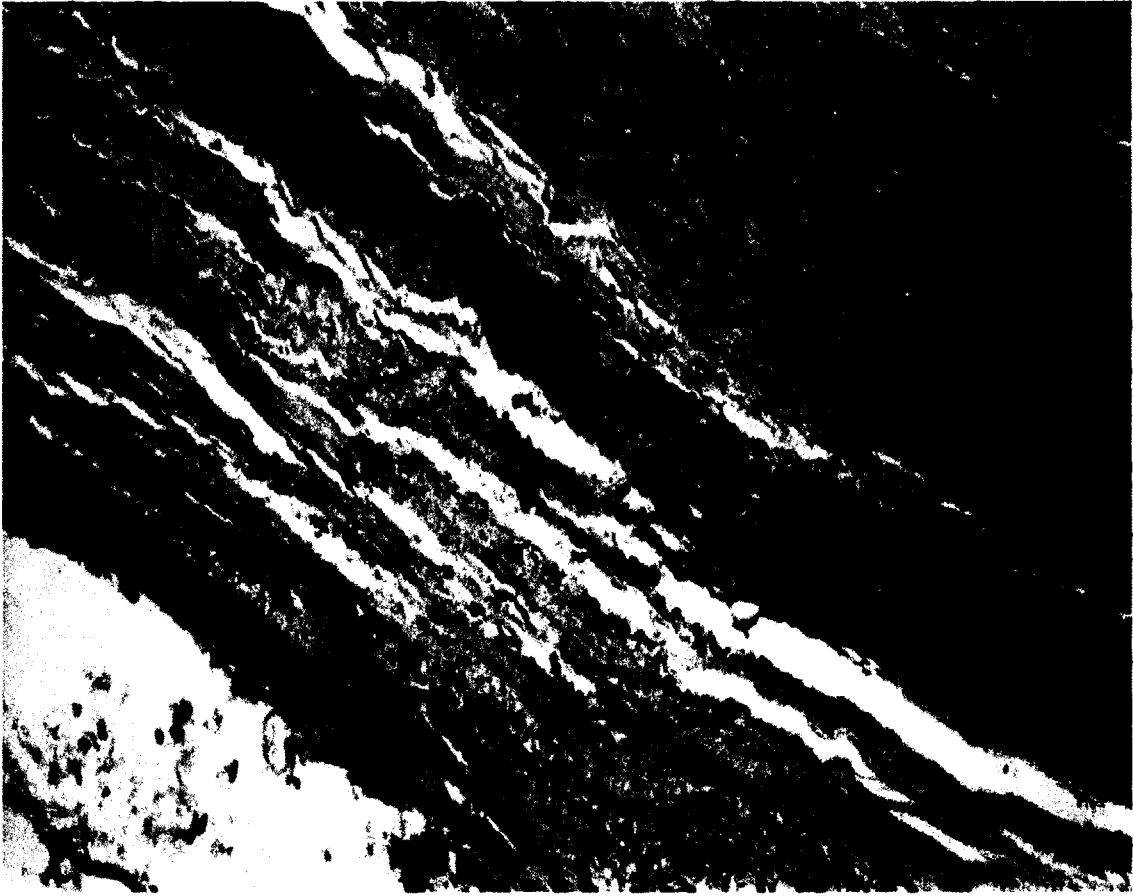
4.14 VISUALIZATION OF APOPTOTIC CELLS - PRECRYOPRESERVATION

The presence of TUNEL positive cells indicates the cell has initiated the classical cellular apoptotic changes that are discernable under the microscope. To determine whether incubation at 37°C or 37°C plus Gln would result in maintenance of baseline levels of apoptotic in aortic conduit tissue cells over time during antibiotic incubation, apoptotic cells were detected using the TUNEL assay and histomorphometrically quantitated. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln as described in the Materials and Methods chapter. The data were obtained by dividing the total number of TUNEL positive cells in the tissue section by the total number of cells present in the tissue sections on the companion Hematoxylin and Eosin slides and obtaining the percentage of positive cells. The TUNEL data are normally distributed

Figure 41, demonstrates that at time zero there was not a statistically significant difference in the number of TUNEL positive cells, $p>0.05$. The 4°C and 4°C plus Gln treatment groups experienced a significant increase in TUNEL positive cells over baseline, $p<0.05$, specifically between 6 and 24 hours, $p<0.05$. The 37°C treatment group had significantly less TUNEL positive cells than both 4°C treatment groups after 6 and 24-hours of incubation, $p< 0.05$. However, the 24-hour 37°C samples had significantly more apoptotic cells over baseline relative to the 37°C plus Gln treatment group. The 37°C plus Gln group did not experience a statistically significant increase in the percentage of TUNEL positive cells over baseline, $p> 0.05$. Figure 42 demonstrates that incubation at 4°C, regardless of Gln supplementation, results in increased numbers of TUNEL positive cells and correlates with a lack of Hsp70 production. Figure 43 demonstrates that incubation at 37°C, especially in the presence of Gln results in no significant increase in the number of TUNEL positive cells and correlates with elevated Hsp70 levels. Without Gln supplementation, there is an increase in TUNEL positive cells in the 37°C group in spite of an increase in Hsp70.

Representative micrographs of TUNEL stained tissue sections are provided in Figures 47-51.

These data demonstrate that cells incubated at 37°C with Gln do not experience an increase in TUNEL positive cells, relative to the other three treatment modalities that do experience an increase in TUNEL positive cells.

EXPERIMENTAL GROUPS
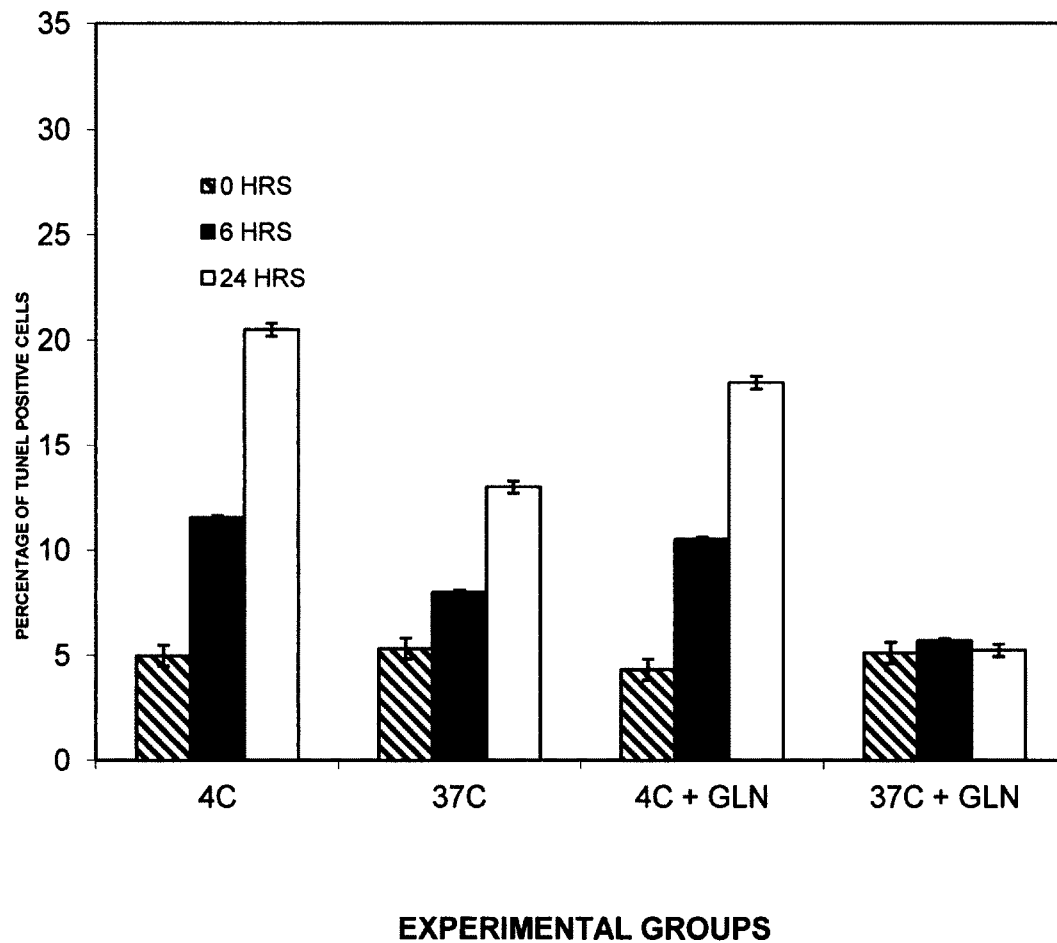
FIGURE 41. Quantitation of TUNEL positive cells in aortic conduit tissue during disinfection under various treatment conditions. Treatment of human aortic conduit at 37°C plus Gln, during antibiotic disinfection, results in no significant increase in TUNEL positive cells over time, relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, formalin fixed; paraffin embedded, sectioned and investigated using the TUNEL assay. Positive control slides were 42% positive.
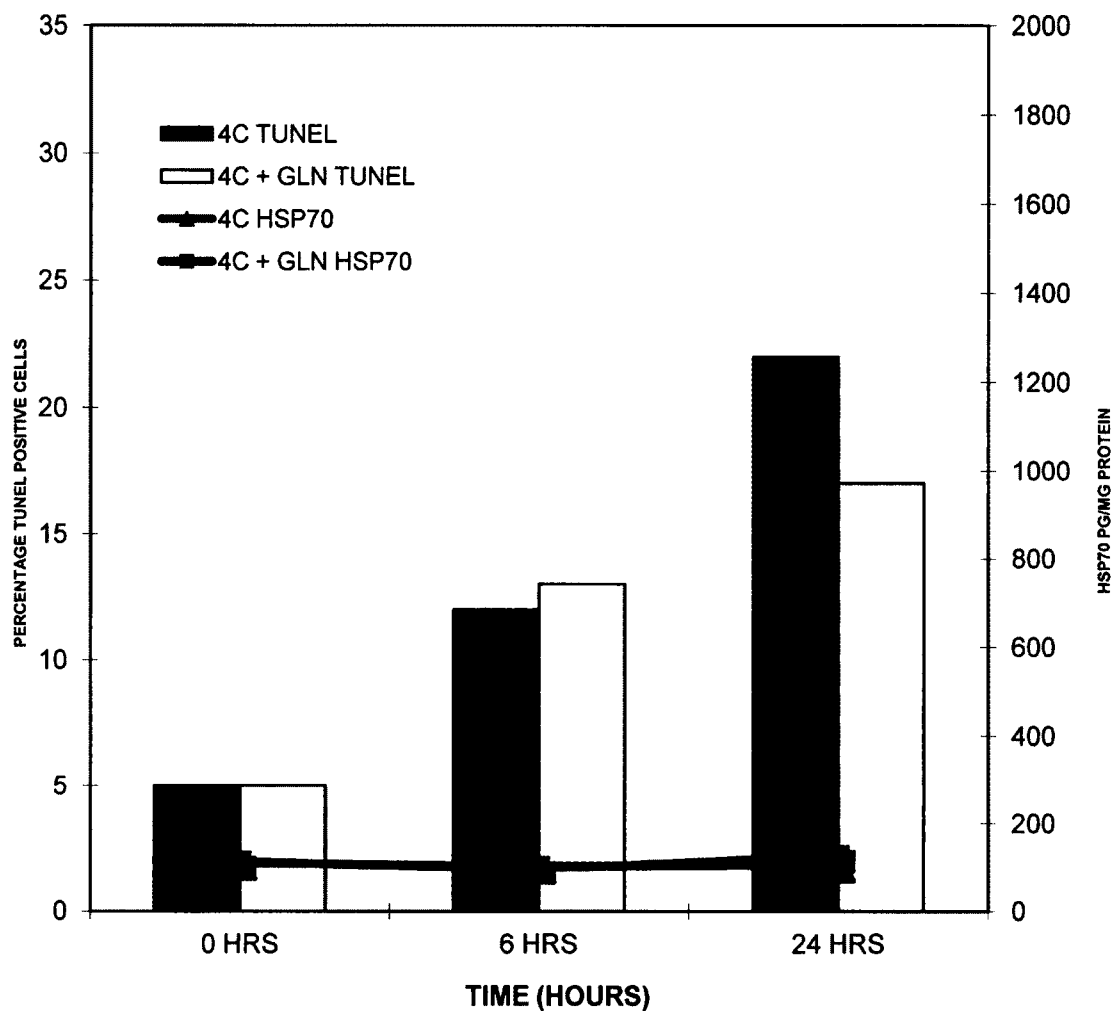
FIGURE 42. Percentage of TUNEL positive cells vs. Hsp70 content over time during disinfection in the 4°C and 4°C+GLN treatment groups. These data demonstrate the relationship between incubation at 4°C during disinfection, maintenance of baseline levels of Hsp70 and increasing percentage of TUNEL positive cells detected in the tissue. The increase in percentage of TUNEL positive cells was similar with or without the addition of Gln to the 4°C disinfection solution. The increased percentage of TUNEL positive cells is supported by the apoptotic cascade biomarker data presented earlier in this Dissertation. The cells in these two treatment groups experienced decreased amounts of mitochondrial cytochrome c, corresponding increased amounts of cytochrome c in the cytosol, no increase in Bcl-2 and increased percentage of caspase-3 positive cells all

culminating in an increased percentage of apoptotic cells as demonstrated in this figure. These data are derived from the Hsp70 ELISA and TUNEL staining.

Fig 43. Percentage of TUNEL positive cells vs. Hsp70 content in the cells during disinfection in the 37°C and 37°C+GLN treatment groups. These data demonstrate the relationship between incubation at 37°C during disinfection, increased levels of Hsp70 and the percentage of TUNEL positive cells detected in the tissue. The increased percentage of TUNEL positive cells in the 37°C treatment group is supported by the apoptotic cascade biomarker data presented earlier in this Dissertation. The cells in the 37°C treatment group experienced decreased amounts of mitochondrial cytochrome c, corresponding increased

amounts of cytochrome c in the cytosol, a small increase in Bcl-2 and increased percentage of caspase-3 positive cells all culminating in an increased percentage of apoptotic cells as demonstrated in this figure. However, the 37°C plus Gln treatment group demonstrated nearly an opposite outcome in pro-apoptotic and anti-apoptotic biomarkers thereby resulting in tissue that did not have elevated levels of apoptotic cells as demonstrated by TUNEL staining.

4.15 VISUALIZATION OF APOPTOTIC CELLS – POST-CRYOPRESERVATION

To determine whether incubation at 37°C or 37°C plus Gln during the antibiotic treatment phase would result in maintenance of baseline levels of TUNEL positive cells in aortic conduit tissue that had been subsequently cryopreserved, thawed and incubated at 37°C under 5% CO_2 for 72 hours, TUNEL staining and histomorphometry were used to detect and quantitate apoptotic cells. The tissue was incubated for 24 hours in antibiotics at the experimental temperatures, 4°C and 37°C, with or without Gln, cryopreserved, thawed and diluted of cryoprotectant and incubated in tissue culture media optimized for the viability of vascular smooth muscle cells over 72 hours at 5% CO_2 as described in the Materials and Methods chapter. The data were obtained by dividing the total number of TUNEL positive cells in the tissue section by the total number of cells present in the tissue sections on the companion slides and obtaining the percentage of positive cells. The TUNEL data were normally distributed

Figure 44 demonstrates that the 4°C and 4°C plus Gln treatment groups experienced a significant increase in TUNEL positive cells over baseline, $p<0.05$, specifically between 0 and 48 hours, $p<0.05$. The number of TUNEL positive cells in the 37°C treatment group was significantly lower than the 4°C and 4°C plus Gln treatment groups, $p< 0.05$. However, the 37°C treatment experienced a statistically significant increase in TUNEL positive cells between 6 and 72 hours of incubation, $p < 0.05$. The 37°C plus Gln group did not experience a statistically

significant increase in the percentage of TUNEL positive cells over baseline, p> 0.05. Figure 45 demonstrates that a lack of Hsp70 production correlates with increased rate of TUNEL positive cells in both 4°C treatment groups. Figure 46 demonstrates that increased amounts of Hsp70 correlate with no increase in TUNEL positive cells in the 37°C with Gln treatment group. Representative micrographs of TUNEL stained tissue sections are provided in Figures 47-51.

These data demonstrate that cells incubated at 37°C with Gln do not experience an increase in TUNEL positive cells, relative to the other three treatment modalities. The data also demonstrate the advantage conferred to the cells in the 37°C plus Gln treatment over those in the 37°C without Gln treatment group.
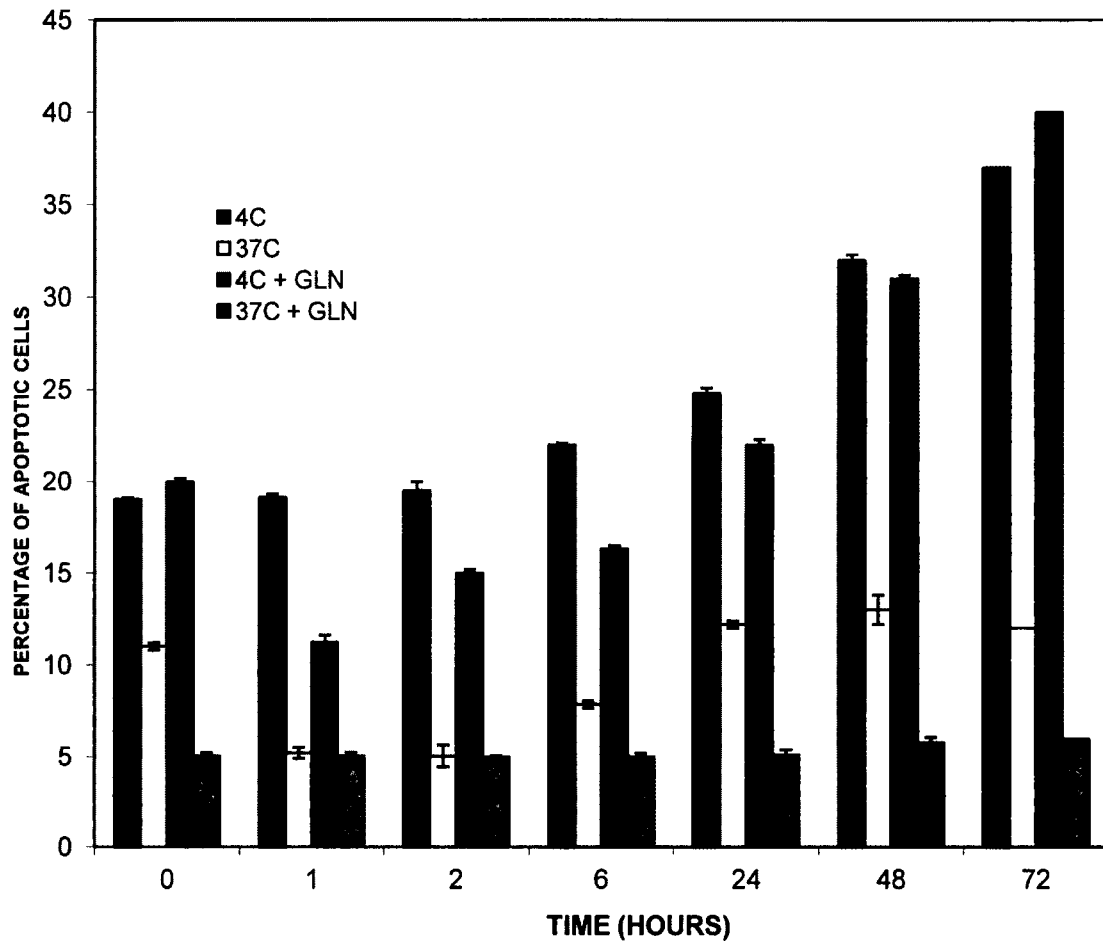
FIGURE 44. Quantitation of TUNEL positive cells in aortic conduit tissue after cryopreservation. Treatment of human aortic conduit previously incubated at 37°C plus Gln during antibiotic disinfection, cryopreserved, thawed and incubated under cell culture conditions results in no significant increase in TUNEL positive cells over time, post cryopreservation relative to treatment at 4°C prior to cryopreservation. Tissues were incubated under their respective conditions, formalin fixed; paraffin embedded, sectioned and investigated using the TUNEL assay. Positive control slides were 42% positive.
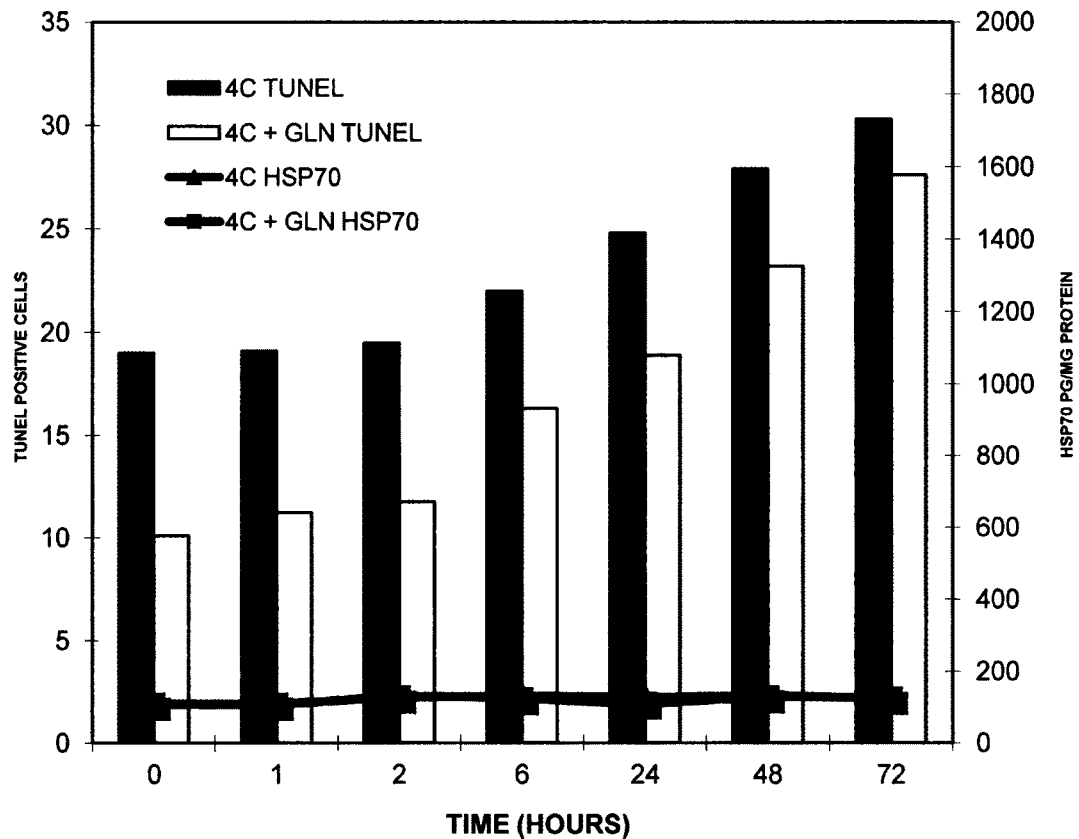
FIGURE 45. Percentage of TUNEL positive cells vs. Hsp70 content in the cells post-cryopreservation in the 4°C and 4°C+GLN treatment groups. These data demonstrate the relationship between incubation at 4°C during disinfection, maintenance of baseline levels of Hsp70 and increasing percentage of TUNEL positive cells detected in the tissue post-cryopreservation. The increase in percentage of TUNEL positive cells was similar with or without the addition of Gln to the 4°C disinfection solution. The increased percentage of TUNEL positive cells is supported by the apoptotic cascade biomarker data presented earlier in this Dissertation. The cells in these two treatment groups experienced decreased amounts of mitochondrial cytochrome c, corresponding increased amounts of cytochrome c in the cytosol, no increase in Bcl-2 and increased percentage of caspase-3 positive cells all culminating in an increased percentage of apoptotic cells as demonstrated in this figure. These data are derived from the Hsp70 ELISA and TUNEL staining results.
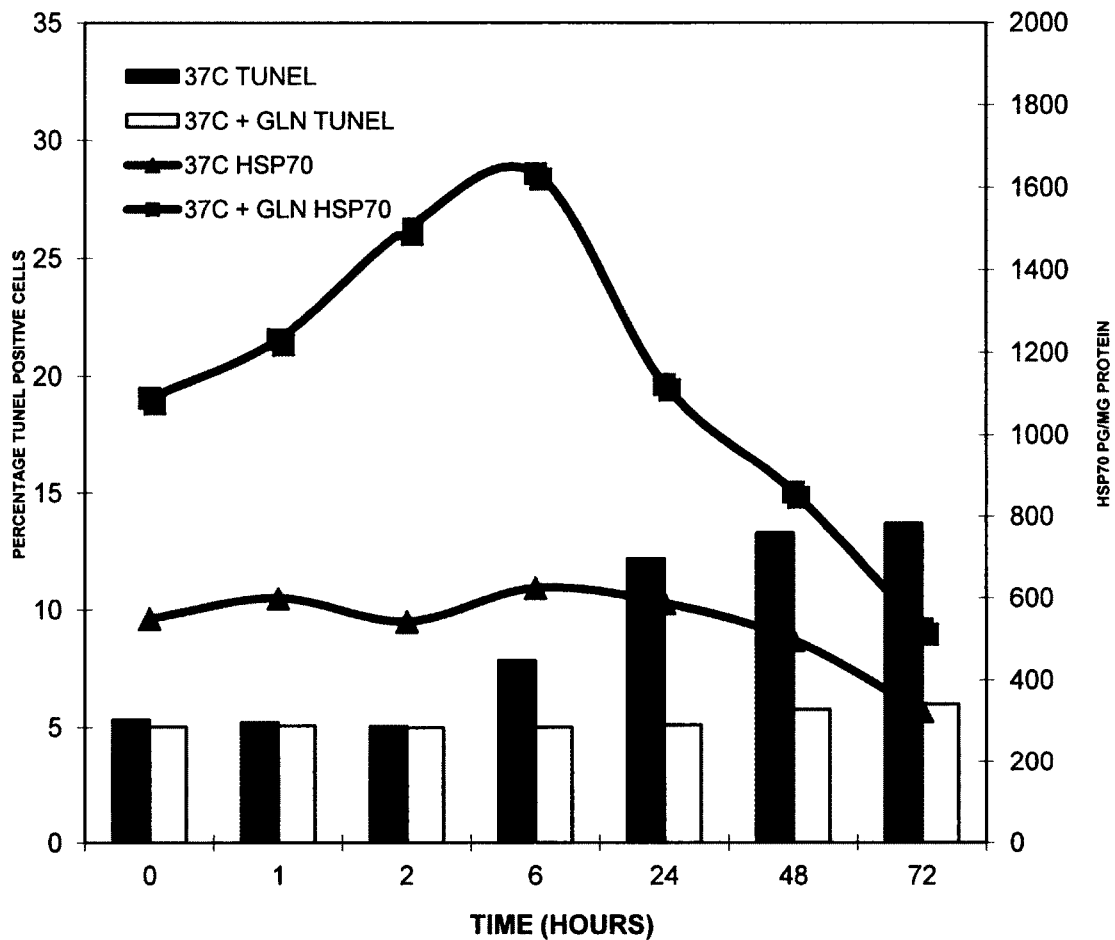
FIGURE 46. Percentage of TUNEL positive cells vs. Hsp70 content in the cells post-cryopreservation in the 37°C and 37°C+GLN treatment groups.
These data demonstrate the relationship between incubation at 37°C during disinfection, increased levels of Hsp70 and the percentage of TUNEL positive cells detected in the tissue post-cryopreservation. The increased percentage of TUNEL positive cells in the 37°C treatment group is supported by the apoptotic cascade biomarker data presented earlier in this Dissertation. The cells in the 37°C treatment group experienced decreased amounts of mitochondrial cytochrome c, corresponding increased amounts of cytochrome c in the cytosol, a small increase in Bcl-2 and increased percentage of caspase-3 positive cells all culminating in an increased percentage of apoptotic cells as demonstrated in this figure. However, the 37°C plus Gln treatment group demonstrated nearly an

opposite outcome in pro-apoptotic and anti-apoptotic biomarkers thereby
resulting in tissue that did not have elevated levels of apoptotic cells as
demonstrated by TUNEL staining.

FIGURE 47. TUNEL Positive Control. Micrograph demonstrating pink nuclear
staining and blue staining of TUNEL positive nuclei, 200X. Examples of TUNEL
positive cells are indicated by the arrows. Forty-two percent of the cells are
TUNEL positive.

FIGURE 48. TUNEL negative control. Micrograph demonstrating pink nuclear staining and absent blue staining for TUNEL positive cells, 200X. The negative control was generated by adding nuclease to the tissue to nick the DNA but omitting the TdT from the reaction mixture to prevent labeling of the nicked DNA on the 3' hydroxyl groups.

FIGURE 49. Baseline TUNEL staining. This micrograph is of a tissue section from the 37°C + Gln experimental group at 0 time post thaw to demonstrate a sample where thresholding revealed that approximately 5% of the cells were TUNEL positive, 100X. The nuclei stain pink and TUNEL positive cells stain blue. The arrows are pointing to the areas, which were TUNEL positive.
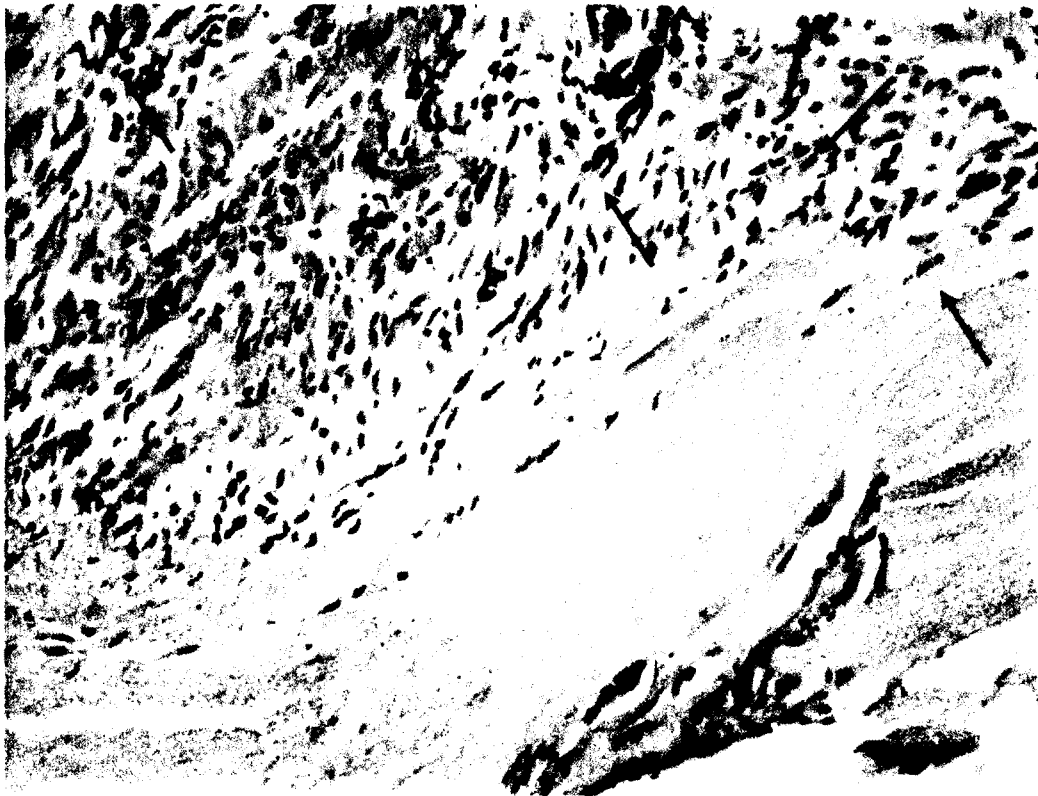
FIGURE 50. TUNEL micrograph, 25% positivity. This micrograph is of a tissue section from the 4°C plus Gln experimental group at 6-hours post-thaw to demonstrate a sample where thresholding revealed that approximately 25% of the cells were TUNEL, 100X. The nuclei stain pink and TUNEL positive cells stain blue. The arrows are pointing to the areas, which were TUNEL positive.

FIGURE 51. TUNEL micrograph, 45% positivity. This micrograph is of a tissue section from the 4°C experimental group at 48-hours post-thaw to demonstrate a sample where thresholding revealed that approximately 45% of the cells were caspase-3 positive, 100X. The nuclei stain pink and TUNEL positive cells stain blue. The arrows are pointing to the areas, which were TUNEL positive.

CHAPTER 5

DISCUSSION

It is well established in the peer-reviewed literature that the constitutive cells in allograft heart valves are apoptotic upon implantation (8, 68, 69, 70, 71). As a result, the graft calcifies, decellularizes *in situ* and ultimately will fail due to stenosis (lumen narrowing) caused by the calcification (8). Therefore, developing a methodology to avert the apoptotic cascade would be clinically relevant and benefit the thousands of adults and children who receive lifesaving allograft heart valves annually. Hence, the specific aims of this research were three fold: 1) to ascertain if the constitutive cell population in allograft heart valves would perceive being transferred from a 1-10°C transport solution to a 37°C disinfection solution as heat shock; augmenting the amount of Hsp70 in the cells as well specific anti-apoptotic proteins, 2) to ascertain if addition of Gln during disinfection would augment the amount of Hsp70 in the cells as well as specific anti-apoptotic proteins and 3) to ascertain if the initial heat shock during disinfection would pre-condition the cells such that, in response to subsequent hypothermic storage and heat shock the amount of Hsp70 would be augmented in a shorter period of time and increase specific anti-apoptotic proteins. To investigate these hypotheses, generously donated human aortic conduit tissue was subjected to one of four treatment regimes during disinfection: 1) 4°C, 2) 4°C plus Gln, 3) 37°C and 4) 37°C plus Gln. To determine the ability of the four treatment regimes to augment the Hsp70 response and where the anti-apoptotic effect could be detected, the amount of Hsp70, cytoplasmic and mitochondrial cytochrome c and Bcl-2 were quantitated using ELISA and the number of caspase-3 and TUNEL positive cells were quantitated histomorphometrically. Tissue from each of the four groups was then cryopreserved, stored in nitrogen vapor, thawed and diluted of cryoprotectant, placed in smooth muscle cell nutrient media and incubated at 37°C under 5% CO_2 for 72 hours. During this second heat shock, the tissues were assessed for the same target proteins as before. This second incubation

was performed to simulate the *in situ* situation post-transplantation (specific aim three).

The current research produced three significant outcomes. They are: that the constitutive cellular population in allograft heart valves perceived going from 1-10°C to 37°C as heat shock; they can be pre-conditioned to respond to subsequent heat shock; and the magnitude and rate of Hsp70 augmentation was potentiated by Gln. These observations led to an important discovery on the mechanism of apoptosis related to heat shock of allograft cells during processing. It is that the amount, rate and the timing of the heat shock response are critical to prevent the apoptotic cascade from proceeding through to completion. This evidence is supported in part by maintenance of baseline amounts of cytochrome c in the mitochondria and cytosol, significant augmentation in Bcl-2 levels and no increase in caspase-3 and TUNEL positive cells in the 37°C plus Gln group. Therefore, a significant augmentation in Hsp70 levels in a time dependent manner was able to halt the progression of the apoptotic cascade in allograft constitutive heart valve cells.

The Hsp70 data demonstrated several phenomena. As anticipated the constitutive heart valve cells were unresponsive to the effects of Gln when the tissue was disinfected at 4°C. This result is attributed the cell's metabolic processes being significantly hindered at 4°C. However, the 4°C and 4°C plus Gln provided data on the upper limit on how long tissue could be stored at 4°C and still perceive being incubated at 37°C as heat shock. After cryopreservation these tissues were placed at 37°C for 72-hours; however, they demonstrated no evidence of augmented Hsp70 or the presence of any other anti-apoptotic biomarkers. It is thought this occurred because a significant portion the constitutive cells in both groups were already committed to the apoptotic cascade as evidenced by the data in Figures 8, 9, 11, 14, 17, 21, 24, 27, 30, 41 and 44. The cells could not respond to heat shock as that would have required ATP metabolism and commitment to transcription and translation by the cells; however, no metabolic assay was executed at the time to confirm this hypothesis. Therefore, based upon these observations, the maximum amount of

time that the constitutive cells in allograft heart valves can be at stored at 4°, and still perceive being returned to 37°C as shock, is approximately 23 hours.

The Hsp70 data coupled with the performance of the other apoptotic biomarkers clearly demonstrate that augmentation of Hsp70 is not sufficient to prevent progression through the apoptotic cascade. The 37°C and 37°C with Gln data supports this conclusion. The levels of expression of the anti-apoptotic biomarker Bcl-2 in these groups is a good example of this conclusion. Figure 23, that overlays the Hsp70 augmentation data on the Bcl-2 augmentation data demonstrates that even though the Hsp70 levels in the 37°C group increased approximately six-fold over the 24-hour disinfection period, the amount of Bcl-2 present barely doubled. Whereas, the 37°C plus Gln group had significant increases in Hsp70, sixteen-fold, between one and six hours. This magnitude and time course correlates with a six-fold increase in Bcl-2 that began after three hours of incubation at 37°C. Between two and four hours of incubation Hsp70 increases dramatically and is accompanied by a five-fold increase in the amount of Bcl-2 present. During the second phase of the research, 72-hour incubation *in vitro*, the 37°C group maintained a steady state of Hsp70. This could have been the result of extended mRNA half-life; extended protein half life and/or a modest amount of hsp70 transcription and translation at the same rate it was being degraded. This lack of augmentation in Hsp70 correlated to little or no increase in Bcl-2. Conversely, Hsp70 increased markedly in the 37°C plus Gln group as did the Bcl-2 levels. These observations, correlate with the temporal appearance of pro-apoptotic biomarkers. All three specific aims were achieved with respect to increased Hsp70 in response to 37°C incubation, increase in a pro-apoptotic protein and that the constitutive cells can be pre-conditioned to produce Hsp70 more efficiently after a second hypothermic-euthermic cycle.

The cytochrome c results also provide an excellent example of the importance of having augmented amounts of Hsp70, but more importantly having it present early in the disinfection phase and at significant quantities. Paired Figures 9/15 and 10/16 provide a stark contrast to one another and support this assertion. Figures 9/15 (4°C and 4°C plus Gln) demonstrate that in the absence

of augmented Hsp70, cytochrome c exits the mitochondria and enters the cytosol and the mitochondrial fraction is never restored. However, in Figures 10/16 the presence of increased amounts of Hsp70 modulates the amount of cytochrome c that exits the mitochondria and enters the cytosol. This figure also demonstrates the significance of having Hsp70 present early and in significant amounts. This observation provides, perhaps, a glimpse inside the cells. The data are consistent with the hypothesis that there are significant pro-apoptotic conditions present inside the cell such as large amounts of denatured proteins as a result of the hypothermic transport. In addition to the physiologic insult of hypothermic transport, there is the period of time that the donor valves are still *in situ* awaiting procurement (10.0 − 13.8 hours for the study tissues) that likely contributes to the milieu of denatured proteins that can activate the intrinsic apoptotic cascade. During this period of time the donor is usually placed into a morgue where the body is held at 1-10°C but the body temperature does not equilibrate prior to procurement. There, are of course, other potential activators of the apoptotic cascade present such as hypoxia, acidosis and increased calcium content, to name a few.

The caspase-3 and TUNEL immunohistochemistry results obtained further corroborate the pro-apoptotic conditions present during routine disinfection at 4°C and the effect of heat shock in the presence of Gln during disinfection. It is important to recall that all of the groups contained apoptotic cells at the initiation of the study and as such it was expected to observe them throughout the course of the study in all treatment groups at the established (time zero) baseline levels. Paired Figures 28/42 and 29/43 (caspase-3 immunohistochemistry and TUNEL staining, respectively) illustrate that the lack of augmented amounts of Hsp70 correlate to an increasingly large population of the constitutive cells in the allograft heart valves being committed to the apoptotic cascade. Specifically, after 24-hours of disinfection at 4°C both groups contain 27-30% caspase-3 positive cells and 17-23% TUNEL positive cells (Figures 28 and 42). This correlates well to the previous data that demonstrate the pro-apoptotic protein cytochrome c entering the cytosol in large amounts and no augmentation in the

anti-apoptotic protein Bcl-2. Conversely, Figures 29 and 43 demonstrate that augmentation in Hsp70 levels modulates the apoptotic cascade. The constitutive cell population in the 37°C group was approximately 15% caspase-3 and TUNEL positive. This is reduced relative to the 4°C treatment groups; however, the 37°C plus Gln group experienced no increase in caspase-3 and TUNEL positive cells. Again, the importance of Gln is clear. Without Gln supplementation there is a threefold increase in the percentage of cells committed to the apoptotic cascade that will be unable to contribute to the renewal of the extracellular matrix once the heart valve is transplanted and *in situ*.

The data obtained during this study are easily contextualized with the results reported by others and it becomes possible to say that Hsp70 was induced in the 37°C and 37°C plus Gln experimental groups even in the absence of Hsp70 mRNA quantitation (113). This is reasonable due to the work of Liu and Cullen (33,18). Liu demonstrated a direct correlation between the amount of time that human fibroblasts (IMR-90) were at held at hypothermic conditions (4°C) and the amount of Hsp70 present after heat shock at 37°C. These data were derived through a compilation of Western blot/densitometry, pulse labeling the cells with Trans [35] S-label, gel mobility shift assay for HSF-1 binding to the HSE and quantitation of the hsp70 mRNA. They demonstrated a 7.7 fold increase in mRNA the corresponded to a similar increase in Hsp70 after heat shock. Cullen demonstrated that animals held at 2-8°C and returned to room temperature experienced an eight fold increase in the amount of hsp70 mRNA. This correlates well with the six to seven-fold increase in Hsp70 measured in the 37°C group. Additionally, this group demonstrated the longer the animals were exposed to hypothermic conditions the more hsp70 mRNA was detected (18). The additional amounts of Hsp70 detected during the current research could be due to the extensive amount of hypothermic insult (18-20 hours) and the potentation of Hsp70 transcription by Gln. Therefore, put into context the data reported herein indicate that the constitutive cells in allograft heart valves cells can be induced to produce significant amounts of Hsp70, especially in the presence of Gln. Further, the data support that induction of Hsp70 correlates

significantly with an increase in the anti-apoptotic protein Bcl-2 and prevents the appearance of pro-apoptotic proteins such as cytochrome c and caspase-3.

To continue to contextualize the results presented herein, Mosser *et al* demonstrated that hsp70 transfected cell lines that over expressed Hsp70 protected the cells from becoming apoptotic when subjected to physiological stress by blocking the release of cytochrome c from the mitochondria (59). This correlates with the results presented herein that induction of Hsp70 (37°C treatment group) modulates the amount of cytochrome c released after physiological stress. However, Mosser's data most closely correlated with the 37°C plus Gln group where the amount of Hsp70 present was 16-fold greater than baseline and prevented cytochrome c from exiting the mitochondria.

The effects of Gln previously reported in the literature also substantiate the 37°C plus Gln results reported herein. Both Wischmeyer and Ropeleski have demonstrated that Gln potentiates the transcription of Hsp70 during heat shock through an indirect mechanism (7, 72). They postulate that Gln increases binding of HSF-1 to the HSE, affects the chromatin organization of the 5' flanking regions of the Hsp70 gene and that Gln enhances phosphorylation of nuclear HSF-1. This step is the final part of the HSF-1 transactivation pathway leading to transcription of Hsp70. The effect on the 5' untranslated region is corroborated by Rubtsova team that has demonstrated Hsp70 has an internal ribosomal entry site to potentiate translation (17). Ropelenski also determined that there are no Gln dependent differences in HSF-1 phosphorylation, trimerization, nuclear localization, or HSF-1 promoter activity (121). These results support the differences observed during this research between the 37°C and 37°C plus Gln experimental groups. The 37°C resulted in Hsp70 induction though, the amount and timing was insufficient to halt the progression of the apoptotic cascade. However, in the presence of Gln the amount and time course of Hsp70 production was significantly augmented and this benefit was realized by the constitutive cells in the tissue as they expressed Bcl-2, had no loss of mitochondrial cytochrome c and where free of increased amounts of caspase-3 and TUNEL positive cells.

Based upon these results, it is possible to propose a mechanism of action for how the constitutive cells in allograft heart valves are rescued by disinfecting them at 37°C in the presence of Gln. This proposed mechanism also demonstrates that the specific aims of this research were successfully achieved. It should be noted, that this mechanism of action involves some level of speculation and therefore it should be viewed as a hypothesis. The mechanism can be based upon Figures 1 and 5 and they are provided again below as Figures 52 and 53 (pages 128 and 129 respectively) for ease of reference.

The physiological insults to the cells begin after death and are accentuated when the heart is placed in 1-10°C transport solution. During transport the intracellular proteins are denaturing. When tissues in the 37°C plus Gln, as demonstrated in Figure 52, are placed in disinfection media, and supplemented to contain 10 mM Gln, they experience an increase in hsp70 transcription and translation. This increase is likely due to Gln acting as a trans-activator of the process by perhaps increasing the efficiency of HSF-1 binding the HSE. It is also highly probably that the integrity of the lipid membrane is not intact and calcium from the RPMI1640 disinfection media is diffusing into the cells. This diffusion of calcium can be pro-apoptotic. The swift increase in Hsp70 facilitates efficient refolding of the significant number of denatured proteins to decrease the pro-apoptotic signals. Additionally, the integrity of cell membrane has likely been restored. The remaining Hsp70 is acting at various points in the apoptotic cascade to negatively regulate the progression, Figure 53 on page 129. The most downstream point of interference, supported by the data presented herein, is preventing activation of procaspase-3 by caspase-9 perhaps by steric hindrance of the cleavage site by Hsp70. The next point of regulation would be inhibition of Apaf-1 activation by cytochrome c. Again, this could be accomplished through steric hindrance of the cytochrome c binding site on

Physiological signals that activate HSP70 expression
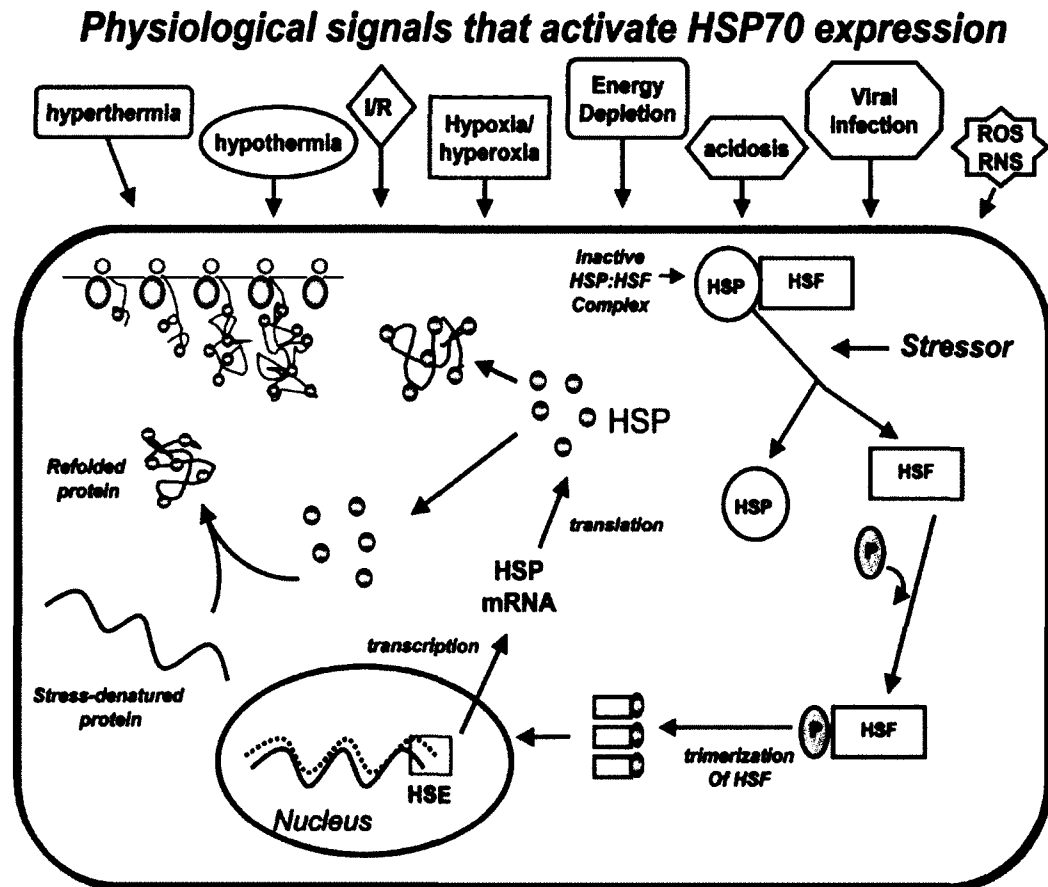
FIGURE 52. Hsp70 Inductive Signals, Hsp70 Gene Activation and Hsp70 Synthesis. A myriad of extracellular stimuli can result in activation of the heat shock response. Inducible Hsp70 transcription factors (HSFs) are bound to Hsp70 in the cytosol and maintained in an inactive state. However, upon application of an external stressor the HSFs separate from Hsp70 are phosphorylated (P) by protein kinases and form trimer complexes in the cytosol. The complexes proceed to the nucleus, enter and bind to heat shock elements (HSE) in the promoter region of the Hsp70 gene. Transcription of Hsp70 mRNA commences and exits the nucleus and enters the cytosol, where new Hsp70 is synthesized (21).

Mitochondrial damage

③

HSP70 ⟶ ↑Bcl-2

apoptosome

Apaf-1

Pro Caspase-9

④ ⊣ HSP70

©

Denaturing Insult

①

HSP70 ⊣ ↑SAPK/JNK

●

↑Transcription Factors

©

Active-Caspase-9

Pro-Caspase-3 ⟶ Activate Caspase-3

② HSP70

⊣ HSP70 ⑤

APOPTOSIS
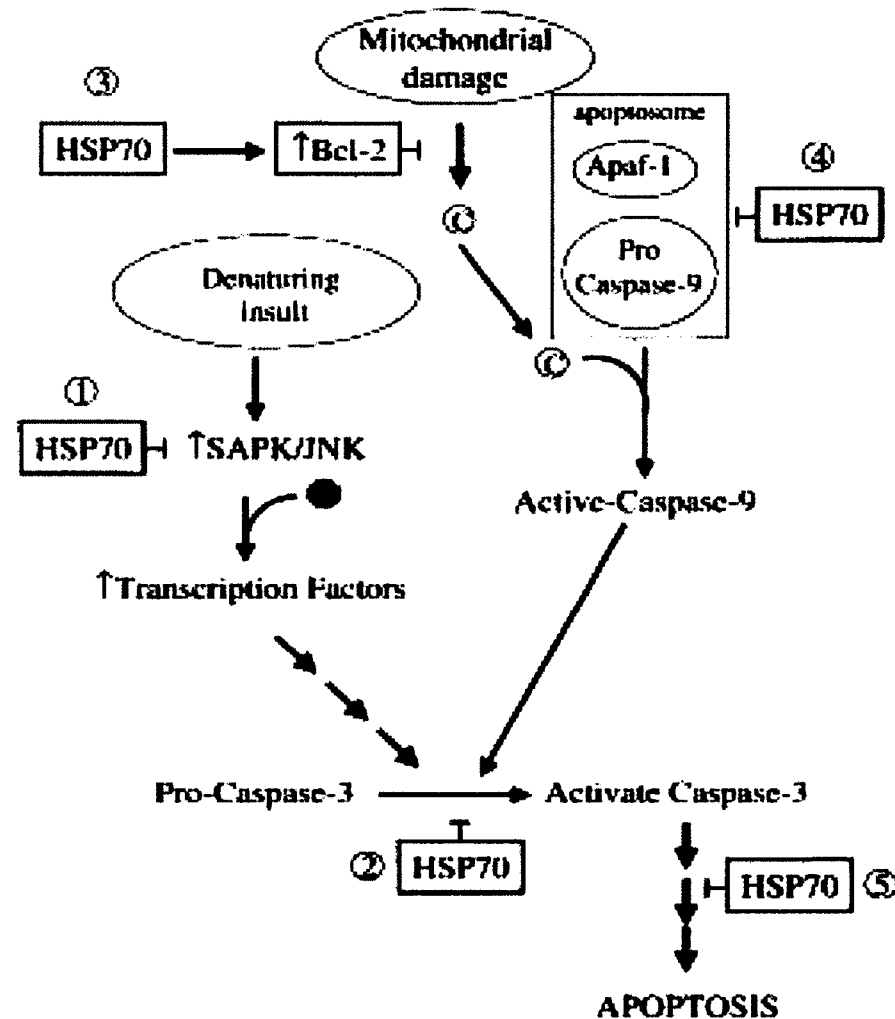
FIGURE 53. Multiple Negative Control Points in the Apoptotic Cascade that Hsp70 May Modulate. The potential points where Hsp70 may negatively regulate the apoptotic cascade are as follows: 1) Inhibition of stress-activated protein kinase pathway (SAPK/JNK) phosphorylation and activation of transcription factors and caspases, 2) Inhibition of pro-caspase-3 transformation into caspase-3 and committing the cell to apoptosis, 3) Prevention of cytochrome c release from the mitochondria by increasing the amount of Bcl-2, 4) Competition for Apaf-1 binding sites on the apoptosome during recruitment of pro-caspase-9 to the apoptosome, 5) Inhibition downstream of caspase-3 activation potentially. (53)

Apaf-1 or physical competition for the site. A point in the cascade where cytochrome c is the target protein would be Hsp70 potentiating the transcription and translation of Bcl-2. This would prevent cytochrome c from exiting mitochondrial intermembrane space, binding to Apaf-1, activating caspase-9 and subsequently caspase-3 which commits the cells to the apoptotic cascade. The other points of regulation detailed in the drawing are not supported by the research presented herein and thus will not be pondered.

From this proposed mechanism it is possible to understand that not producing enough Hsp70 in a prescribed timeframe would result in a portion of the cells progressing through the apoptotic cascade. This is hypothesized to be the explanation for why the 37°C group was only partially successful in rescuing the cells from becoming apoptotic.

There are several experiments that could have been performed or performed differently that would strengthen the conclusions. It would have been beneficial to have an experimental group that was classically heat shocked. This was not possible with human tissue because of the logistics of the donation process. It can take up to 24-hours for tissue to be procured because of the time involved to gain consent, assemble the recovery team, travel time and gaining access to an operating room. However, a classically heat shocked group could have been accomplished through use of an animal model but the potential issue raised by using animal tissues is the applicability of the data to human tissues. If an animal model had been pursued, the heart could have been procured at asystole, a control tissue sample obtained at the time of death and the remaining tissue classically heat shocked at 44°C for approximately 50 minutes with no previous hypothermic storage. The information derived from this control group would have been the amount of Hsp70 and Bcl-2 at baseline and the amounts produced through classical heat shocking versus hypothermic-euthermic induced heat shock. This would have allowed the Hsp70 and Bcl-2 results to be framed against the results from the classically heat shocked group. Another control group that only could have been obtained using an animal model would be to pharmacologically induce the intrinsic apoptotic cascade and frame the 4°C and

4°C plus Gln caspase-3 and TUNEL results in the context of this group. A pharmacologically induced group would have provided a different view of heat shock in the absence and presence of Gln to more fully characterize the ability of Gln to induce sufficient quantities of Hsp70 to avert progression of the apoptotic cascade in that group as well.

Another approach to understand the importance of Hsp70 in halting the progression of the apoptotic cascade would be to utilize RNAi to block translation of the Hsp70 mRNA or Hsp70 knockout mice. Neither of these models was available at the time this research was conducted. The data that would have been gleaned from these approaches would be whether or not other heat shock proteins are capable of performing the same job as Hsp70 in modulating the apoptotic cascade. Current literature points to the importance of Hsp70 plays in regulating progression through apoptosis using both of these model systems (72, 73). The RNAi researchers were using this molecular technique to inhibit Hsp70 to treat gastric cancer, as it is over expressed in this disease process. They demonstrated RNAi to hsp70 mRNA successfully down-regulated the presence of Hsp70 in the cells and consequently the cancerous cells preceded through the apoptotic cascade – a desirable outcome in this instance (72). Lee *et al* investigated the effects of Hsp70.1 gene knockout on the mitochondrial apoptotic pathway after focal cerebral ischemia in the mouse model (73). Much like the research conducted in the course of this Dissertation they investigated cytochrome c release from the mitochondria, caspase-3 activation and the presence of TUNEL positive cells. Their functional results indicate the knockout mice had significantly larger infarct areas relative to wild type mice. After eight hours of ischemia they observed markedly increased amounts of cytochrome c in the cytoplasm of the knockout versus the wild type mice who also were expressing significantly increased amount of Hsp70 (73). The capsase-3 results also align with the observations in this Dissertation, physiological insult without subsequent Hsp70 induction results in a significant portion of the cell population to be caspase-3 positive and as such committed to the completion of the apoptotic program.

The data presented herein, coupled with data in the peer reviewed literature in the field allow the following conclusions to be drawn: 1) the constitutive cell population in allograft heart valves perceive being transferred from a 1-10°C transport solution to a 37°C disinfection solution as heat shock; and augments the amount of Hsp70 in the cells as well specific anti-apoptotic proteins, 2) Gln supplementation of the disinfection solution augments the amount of Hsp70 in the cells as well as specific anti-apoptotic proteins and 3) the initial heat shock during disinfection pre-conditions the cells such that, in response to subsequent hypothermic storage and heat shock the amount of Hsp70 is augmented in a shorter period of time and specific anti-apoptotic proteins are increased. Therefore the proposed change to the disinfection solution composition and incubation temperature has the strong potential to improve current allograft heart valve processing, and that of other allograft cells, organs and tissues, such that durability of the heart valve and resistance to calcification improve the many physiological and functional benefits of allograft heart valves for the thousands of adults and children annually that need these life saving allografts.

CHAPTER 6

CONCLUSIONS AND FUTURE RESEARCH

This research has conclusively demonstrated the benefits of incubating aortic conduit tissue at 37°C with Gln supplementation during the disinfection process prior to cryopreservation. The experimental treatment modality heat shocked the tissue as a result of returning the tissue to physiological temperature after hypothermic transport. Hsp70 formation however cannot be fully attributed to heat shock, Gln also likely contributed to the increased amount of Hsp70 detected. The data also demonstrated the constitutive cells in the heart valve tissue can respond to additional hypothermic – euthermic temperature cycling in a more efficient manner as measured by Hsp70 formation over time and resistance to apoptosis. The amounts of Hsp70 produced are able to stimulate production of other anti-apoptotic proteins such as Bcl-2. The production of Bcl-2 ensures little to no leakage of cytochrome c into the cytosol that can stimulate the intrinsic apoptotic cascade through activation of the apoptosome.

This research has conclusively demonstrated that only elevating the temperature of the tissue to 37°C or incubating the tissue at 4°C with Gln is not sufficient to induce adequate production of protective levels of Hsp70 to ensure a cell population with minimal apoptosis. Rather, both approaches, 37°C and Gln, should be employed.

The results of this Dissertation research support the findings of previous researchers and expand the scientific knowledge base by providing a practical application for this naturally occurring phenomenon. The biological response of the cells to hypothermia and subsequent return to physiological condition as heat shock is elegant. It is not surprising that there is an evolutionarily conserved mechanism to support the cell during extremes in temperature. The ability of cells to exploit the heat shock response from a cold perspective is an excellent example of key survival mechanism being evolutionarily conserved. Rather than the heat shock response being a utilitarian response to increased temperature,

the molecular biology is in place for the cells to perceive their return to physiological conditions as heat shock after hypothermic exposures. The benefits of hypothermia for surgeries augment these findings. One could easily imagine Hsp70 aiding the body in the recovery process postoperatively and protecting the cells from the trauma of surgery. The balance seen in the 37°C plus Gln experimental group's cells ability to produce high levels of Hsp70 and Bcl-2 coupled with increased resistance to cytochrome c leakage from the cells, increased resistance to caspase-3 formation and subsequent resistance to apoptosis speaks to the benefits of exploiting these outcomes to benefit the patients receiving cryopreserved allografts and thus overcoming their main cause of failure apoptotic cells. As the apoptotic cells serve as nidi for calcification which leads to stenosis thus impairing the performance of the heart as it is pumping against a pressure gradient and may hypertrophy.

The future of this research should involve quantitation of Hsp70 mRNA in parallel with the Hsp70 ELISA to confirm that Hsp70 was actually being transcribed during the incubation. Quantification of the intracellular calcium concentration during the course of the incubation would determine if a rise in intracellular calcium is contributing to initiating the apoptotic cascade. Executing the animal models described earlier would further solidify the validity of the observation made during the current research. Additionally treating hearts per normal protocol, 4°C without Gln and at 37°C with Gln and implanting the resultant heart valves in a female juvenile sheep model for 20-weeks would allow one to ascertain if there is actual physiological benefit to the 37°C plus Gln treatment.

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