Addition of ischemic preconditioning does not afford added protection to neonatal rabbit myocardium protected by hypothermic cardioplegia

by

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Abstract

Background- Each year in British Columbia, approximately 500 children are born with congenital heart disease; more than a third of which require surgical intervention. During surgery, the neonatal myocardium does not tolerate ischemia (decreased perfusion/metabolism ratio) or perfusion with cold cardioplegic solutions as well as the adult myocardium does. In addition to such exogenous measures taken to protect the heart, there is an endogenous phenomenon— ischemic preconditioning (IPC)—that acts as a mechanism to protect the myocardium from ischemic damage. It is unknown whether IPC can further protect the neonatal myocardium beyond that which it receives with hypothermic cardioplegic arrest.

Methods and Results - Isolated immature (10 - 11 days old) rabbit hearts were subjected to either one 5 minute ischemic challenge with 10 minutes of reperfusion or one 1 minute ischemic challenge with 10 minutes of reperfusion on a working heart apparatus. Hearts were then administered cold St. Thomas' cardioplegia, kept in hypothermic arrest for 120 minutes and then reperfused for 40 minutes. Hemodynamic parameters and biochemical markers were collected for analysis. The percentage of baseline positive derivative of maximal peak systolic pressure ($+dP/dt_{max}$) at 30 minutes working reperfusion for the 1 minute IPC group differed with its time matched control (45.43 ± 4.25% vs 60.70 ± 4.08%, $p=0.018$) respectively.

Conclusions - There was no significant additional protection to hearts with the combination of IPC and hypothermic cardioplegic arrest then with hypothermic cardioplegic arrest alone.

Dedication

This thesis is dedicated to my family, especially to my parents, for without their considerable support and sacrifice this work would have never been completed.

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There is only one good, knowledge, and one evil, ignorance.

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1.0 Introduction

In British Columbia, approximately 500 children every year are born with congenital heart and/or circulatory defects (B.C. Health Status Registry). These congenital defects : 1) obstruct blood flow in the heart or vessels near the heart, 2) cause blood to flow through the heart in an abnormal pattern, 3) lead to less oxygenated blood being pumped into the systemic circulation. In about a third of these cases, surgery is necessary to correct the defect and allow the newborn a better quality of life. Unfortunately, cardiothoracic surgery poses a threat to the newborn. For the surgeon to have a quiescent workspace for the corrective procedure, s/he must stop the flow of blood to the heart and put the patient on cardiopulmonary bypass for the duration of the surgery. The heart is usually arrested with a cardioplegic solution and the corrective procedure is performed. Stopping the heart for the duration of the surgery poses a threat to the viability of the organ. Ischemia is an inadequate of blood flow relative to the metabolic demand of the tissue. The longer the heart is stopped and blood flow ceases, the longer the duration of cardiac ischemia. In the ischemic state, the supply of oxygen and metabolites are insufficient to meet the tissues needs; while catabolites are present in large quantities. Under normal flow, the excess of catabolites can follow their respective methods of removal and not affect the myocardium. It is well documented that

prolonged ischemia is harmful to the organ (52). With the reduced method of removal during ischemia, the accumulation of the catabolites can alter the normal homeostasis of the cell and alter function in deleterious ways. Reperfusion of the heart after the corrective procedures also poses a threat to the survival of the patient (52).

The development of specialized cardioprotective techniques, such as modified cardioplegic solutions that are lower in calcium and hypothermic conditions that serve to slow down metabolism, have greatly increased the prognosis of a positive outcome for the patient undergoing cardiac surgery. Neonates are less able to tolerate the ischemic bout put upon them by the surgical process. They are more vulnerable to functional and histological damage during cardiac surgery, examples of which are myofibrillar disruption, calcium deposition and leakage of proteins into the extra cellular fluid due to damage of the membranes (9).

The response of the immature myocardium to the ischemia / reperfusion (IR) cycle is not well understood and is in need of further investigation. An endogenous technique of myocardial protection, which has been shown to be present in adult and younger cardiac tissue, is ischemic preconditioning (IPC).

1.1 Ischemic preconditioning

The first documentation of the phenomenon coined "ischemic preconditioning" was by Murray et al (89). The group was investigating the different contributions of ATP depletion from catabolite accumulation in the genesis of lethal ischemic injury. The experiment attempted to show that a series of brief ischemic episodes would deplete ATP in adult canine myocardial tissue and that a series of reperfusions would wash out the accumulation of catabolites. Their observations did not support the null hypothesis that the drop in ATP would correlate with the amount of successive IR cycles. They observed that after the initial IR cycle, the levels of ATP did not drop significantly. The protective effect to the myocardium was noted in the fact that approximately 85% of the dogs did not suffer from a myocardial infarction stemming from the ischemia compared to the matched control. This observation was also counterintuitive, because it was hypothesized that the repeated ischemic episodes would exacerbate the chances of an infarct due to the expected increase of necrotic tissue. Further experimentation involved the administration of 4 cycles of 5 minutes of ischemia followed by 5 minutes of reperfusion prior to a later 40 minute ischemic bout. The preconditioned animals suffered a 25% decrease in infarcted tissue when compared to control.

Experiments using the working heart (WH) and Langendorff apparatus, cardiomyocytes and different species *in vivo* have been carried out to elucidate the mechanisms by which the phenomenon exerts its protection (19).

The cascade of events that lead up to the final protection of the myocardium is complex. The breakdown of the sequence of events at which the preconditioning is afforded can be labeled as follows:

1) the generation of the signal to initiate preconditioning;
2) the generation of the intracellular message from that signal;
3) effectors that afford the protection (proteins which may have modifications to their structure or function); and
4) the second window of protection.

See Figure 1 for a schematic representation of the events in the cascade.

Preconditioning is based on the premise that repeated IR cycles of short duration can afford a certain degree of protection for a longer period of ischemia. One must bear in mind that the protection is of a finite duration. Preconditioning allows the myocardium to have an extended period at which the ischemic tissue can readily be returned to normal functioning (reversible injury). Figure 2 is a modified graphical representation of the concept of reversible injury from King and Opie (55).

Without reperfusion, the cell will ultimately die and no amount of preconditioning will be able to save it. Many different experimental protocols have been used to precondition the heart. The preconditioning bout lasts from 1 to 20 minutes and the sustained ischemic bout can last from as little as 30 minutes to over 2 hours.

1.2 Generation of the signal

Logically, the first place for the elucidation of the protective mechanism would be at the generation of the signal. In order to initiate the cascade of events, there must be an underlying release of a chemical which may or may not be present in the cell or its surroundings under normal physiological conditions.

1.21 Adenosine

Adenosine has long been the choice for exploration into the signal generation of preconditioning in the ischemic myocardium. Post ischemia, there is a higher than basal level of adenosine in the tissue, as reported by Belardinelli et al (7). This observation seems logical because the levels of ATP in the cell should fall drastically in the cell for the duration of the ischemia. The cell should be using the ATP for survival and since adenosine and a phosphate group are intermediates in the breakdown of ATP, it seems intuitive that the levels of adenosine accumulate during the ischemic phase.

Experimental evidence that adenosine may be implicated in preconditioning can be seen in the isolated rabbit heart (64). The use of RPIA (R-phenyl-isopropyl-adenosine) an A1 receptor agonist, was able to limit the infarct size of hearts to 10 percent of the area at risk while control hearts had infarcts closer to 34 percent. Functionally, the use of the adenosine agonist is superior to the control hearts and even to those which received a bout of preconditioning. The hearts which were administered the A1 agonist recovered to 86 percent of baseline levels using coronary flow and percentage recovery of left ventricular diastolic pressure as indices of recovery. The preconditioned hearts only recovered to 65 percent of basal levels. The use of an A1 receptor antagonist DPCPX (8-Cyclopentyl-1,3-dipropylxanthine), successfully

blocked the protection afforded to the heart by the agonist. However, the administration of the blocker did not affect the functional recovery of the ischemically preconditioned hearts, suggesting that there must be another mechanism whereby the heart can become preconditioned. Nonetheless, other experiments with the administration of various of the A1 adenosine receptor agonists and antagonists have shown that adenosine is involved to a degree in the preconditioning process in rabbits, dogs and pigs (32, 57, 71, 72, 87, 88, 104, 125). Studies involving rat hearts and adenosine have come to various conclusions. In human atrial muscle strips, Walker et al were able to demonstrate that the use of the A1 agonist RPIA mimicked the effect of preconditioning (126). As in the previous examples of other species, the use of an antagonist for the A1 receptor blocked the protection of the agonist.

The addition of adenosine to one cycle of IPC is termed adenosine-enhanced preconditioning. In this method of protection, a bolus of adenosine is given during the preconditioning ischemia to enhance the protection afforded by IPC alone. The rationale behind the addition is that the extra adenosine would make sure that there was a sufficient amount of 'trigger' molecules to reach the threshold necessary to start the protection cascade. The strategy has been effective in sheep and rabbit myocardium (80-82, 120). In crystalloid perfused rabbit hearts, adenosine-enhanced ischemic preconditioning is superior to normal preconditioning alone and equally effective in minimizing the area of

infarcted tissue (82). In sheep hearts perfused with blood, the strategy of adding a bolus of adenosine to IPC provided better protection from infarction than from IPC alone (120). Both the A_1 and A_3 adenosine receptors have been implicated in the protection afforded by adenosine-enhanced IPC (24, 58, 88). Recent evidence using mice genetically manipulated to lack the A_3 adenosine receptor, has challenged the role of the receptor as an initiator of protection (40). Hearts that lacked the receptor had less injury when comparing area of infarct after an ischemic bout to normal hearts. It is postulated that the increased injury may be due to the A_3 receptors role in inducing the inflammatory response.

1.22 NorEpinephrine

Another pathway for signal generation could occur via the sympathetic nervous system. Nor epinephrine (NE) has been shown to be released in appreciable levels during transient ischemia. In rats, the administration of NE in lieu of preconditioning afforded the same protection (124). The use of reserpine, which depletes the cells of catecholamines such as NE, tended to decrease the infarct size with respect to controls (25% controls vs. 19% reserpine treated) but the difference was not statistically significant. The hearts that were preconditioned had a decrease of almost 20% in the area of infarction whereas the reserpine treated hearts only had a decrease of 4% when a

bout of IR was given which would indicate that NE was involved in the protection of the myocardium.

1.23 Other triggers

Other factors, such as the ones derived from the surrounding endothelium, may also play a role in the generation of the signal for protection. Goto et al examined the role of bradykinin in the protection afforded by preconditioning (37). They looked at both isolated rabbit hearts and rabbit hearts *in situ*. Administration of icatibant (HOE 140), a selective blocker for the bradykinin receptor, to the hearts of preconditioned rabbits prior to the preconditioning stimulus abolished protection. If the blocker was administered in the reperfusion phase of, the preconditioning effects were uninhibited. In the second part of the study, infusion of the isolated rabbit hearts with bradykinin mimicked the protective effect of preconditioning while the administration of polymyxin B or staurosporine (protein kinase C inhibitors) or HOE 140 completely blocked the preconditioning protection. An interesting observation was made in the *in situ* part of the experiment. It was noted that the increase in the intensity of the preconditioning stimulus could overcome the blockage of the protection that was previously afforded by the addition of HOE 140. This observation led to the theory that the increase of the stimulus allowed for the accumulation of another molecule that stimulated the activity of protein kinase C. Kositrapa et al

have demonstrated that bradykinin is also involved in the second window of protection (60). Rabbits injected with bradykinin 24 hours prior to I/R displayed similar protection to that of IPC when the area of infarction was measured. The protective effect was blocked by the administration of HOE 140.

Free radical generation has been reported as a possible trigger for ischemic preconditioning. Pain et al using either global ischemia or 10 micromol/L diazoxide were able to precondition the rabbit heart, thus decreasing the area of infarcted tissue in the heart. They determined that the opening of the channel was a trigger for IPC by the selective blockage of the channel at different periods of time throught the experiment. When they bracketed either the PC ischemia or the diazoxide treatment with 5-HD, there was an ablation of protection seen with the two treatments. Administration of the blocker prior to the index ischemia did not decrease the protection seen by IPC or diazoxide. The administration of free radical scavengers, N:-(2-mercaptopropionyl) glycine (MPG) 300 micromol/L or Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (7 micromol/L), bracketing the diazoxide infusion abrogated the protection seen with the K_{ATP} channel opener.

Therefore, it seems there is a redundancy in the mechanism of signal generation. This may be seen as an advantage over the course of evolution whereby it would be safer to have many routes to protection.

1.3 Generation of the intracellular message

The second step in the preconditioning cascade is the generation of the intracellular message. Once the stimulus for the preconditioning is generated, a means of getting the message to the effector is necessary. Downey was the first to suggest that the intracellular messenger was the enzyme protein kinase C (PKC)(25). In this section, the structure of the enzyme is described first, followed by some functional experimental data. The last part of the section deals with the problems of interpreting the many PKC experiments.

1.31 Protein Kinase C

It has been shown that various receptors are linked to PKC via a GTP-binding protein phospholipase C and, more recently, by a phospholipase D cascade (119). Ten different isoforms of phospholipase C have been identified and separated into three subtypes; PLC-β, PLC-γ and PLC-δ (123). The beta subtype is modulated by G proteins while the gamma subtype is modulated by tyrosine phosphorylated receptors.

Nishizuka et al in 1977 were the first to identify the existence of PKC (115). Following the initial discovery, others were able to ascertain some of the diverse regulatory roles of the protein in development, memory, and carcinogenesis (2, 96). The PKC family of phosphorylating

polypeptides is the largest serine / threonine specific kinase subfamily known to date (66). The structure and various isoforms of the protein illustrate the interesting role of evolution whereby all of the isoforms contain a conserved region and yet are slightly dissimilar with respect to amino acid sequences, calcium activation and species.

The primary function of PKC is as a cellular transducer involved in many cellular regulatory cascades. The pathway in which the enzyme is involved is as follows: A ligand such as adenosine or catecholamines such as epinephrine, binds to its receptor on the cell membrane. This then facilitates a reaction in the membrane whereby PLC breaks down PIP_2 to DAG and IP_3. It is the presence of the DAG in the membrane that causes the PKC enzyme to translocate from the cytosol to the membrane. Once it is associated to the membrane it can then phosphorylate its effector.

1.32 Structure of PKC

In mammals, researchers have been able to isolate 12 isoforms of PKC (α, β_1, β_2, γ, δ, ε, ζ, η, θ, ι, κ, λ, μ) (66, 101, 105). An interesting phenomenon lies in the fact that the individual PKC gene products are more conserved between different species than between genes from the same species (66). The isoforms are divided into three groups: conventional, novel and atypical. The conventional isoforms are α, β_1, β_2 and γ. This subfamily needs both calcium and lipids to be activated. The novel isoforms, δ, ε, η, θ do not need calcium present to become activated but still require the presence of phospholipids. The last subfamily of isoforms is the atypical PKCs which include the ζ, ι, λ and μ isoforms. They lack the Zn^{2+} finger motif needed for binding of diacylglycerol (DAG) and phorbol esters. PKC ζ needs phosphatidyl serine for its activation.

1.33 Activation characteristics

When calcium is present in the medium, the calcium dependant isoforms can make non-productive associations with the membrane (62, 66). This fact points out the role of calcium as a regulator of these enzymes. It seems to indicate for at least these isoforms there is a two part physiological regulatory role in the cell. It is known that it is

necessary for DAG to be present for the enzyme to translocate from the cytosol and associate with the membrane. Conformational changes occur in two stages; first with the association in the membrane and second with the binding of DAG or phorbol esters.

Experimentally, it has been shown that in cases of chronic activation with phorbol esters, there is a decrease in steady state activation in some isoforms (62, 66). The change of the activation rate is not due to changes in the mRNA levels or changes in the rate of synthesis but rather to the increase in the rate of proteolysis.

There is a good correlation between the DAG produced from physiological hydrolysis of inositol lipids and PKC membrane translocation (association) whereas the DAG that comes from other sources does not correlate well with membrane association (62, 66). This lack of correlation may be explained by the measurement of the phosphorylation of possible effectors of the specific PKC isoform. For instance one of the PKC isoforms could associate itself with a phospholipid membrane and could target other effectors which may not be measurable under certain experimental protocols.

Experiments have been done using a myriad of direct and indirect approaches using agonists and inhibitors of the kinase, agonists and antagonists of G-protein coupled receptors which are postulated to have direct relations with the enzyme and the use of inhibitors and agonists on one of the possible effectors of the kinase, the K_{ATP} channel.

The controversy stems from the fact that there is no proof that PKC is involved in ischemic preconditioning in all species. Many studies using activators and inhibitors have been carried out in a wide variety of mammalian and avian species both *in situ* and *in vivo* (1, 11, 14, 16-18, 26, 49, 50, 56, 65, 68-70, 84, 86, 103). If the phenomenon is a naturally occurring endogenous mechanism then it would seem counterintuitive for PKC to be involved only in some species and not others. For example, in a study by Vahlhaus et al in porcine hearts, the blocker staurosporine was unable to block the protective effect of preconditioning even at doses which were able to block the effect of the PKC-activating phorbol ester (121). Other studies support this observation in other species, such as rabbit and rat (103, 104, 112, 129).

There are also numerous studies which support the hypothesis that PKC isoforms are translocated from the cytosol into the sarcolemma. Mizumura et al indirectly were able to show involvement using the agonist of the A1 receptor in a canine model (88). The compound used to bind to the receptor allowed for a shorter time needed for the protection of the myocardium with preconditioning. Light et al, using inside out vesicles, were able to use purified PKC to increase the protective effect afforded by the decrease in the action potential duration from the increased conductance of the inward rectifying K_{ATP} channel (70). The protective effect was blocked by a specific peptide inhibitor of PKC targeted to amino acids 19-31.

1.34 Questioning the involvement of PKC

There are four main factors which contribute to the uncertainty of the kinases involvement (70):

1. dangers of indirect or inappropriate measurement of PKC activity;
2. the use and abuse of activators and inhibitors;
3. the distinct isoforms of the enzyme; and
4. models, species and endpoints used.

Many studies make the leap of faith when assuming that the administration of agonists and antagonists of receptors which can be linked to PKC, truly stimulate the enzyme and that it somehow modifies the end effector. One can not be certain that the stimulation and translocation of the signal is done solely by PKC. Again the same type of error can be made when the activators or inhibitors are used for PKC itself. Hard biochemical evidence is lacking in these studies to prove that the PKC molecule is activated under the experimental conditions.

The use of activators and inhibitors also poses a problem with respect to indiscriminant binding of the drugs to other cellular contents. This non specific binding can lead to other effects which may mask or enhance the desired protective effect of preconditioning. Staurosporine, a potent and popular PKC blocker, is a potent blocker for all isoforms of the enzyme. It also can block other protein kinases if it is present in sufficient concentrations. Its low specificity with respect to binding of

the molecule is due to the fact that it competes with ATP for the catalytic binding site (70). Another blocker which is used frequently in experiments for the blockage of the enzyme is polymyxin B. The problem that arises with the use of polymyxin B is that it is not a specific blocker of PKC and it can block the inward rectifying K_{ATP} channel. Chelerythrine, a highly specific blocker of PKC, has a poor solubility in aqueous solutions, which makes the delivery of the blocker and the effects of the vehicle an issue. To date, 12 different isoforms of PKC have been isolated from the heart (101). Another problem is that the possibility exists that different isoforms can react differently to the different blockers, although this has not been documented thus far. Antibodies specific to the various isoforms have been raised but difficulty surrounds the identification of the activated vs. the inactivated state of the enzyme. Ping et al were able to demonstrate that with the use of antibodies directed to the various isoforms, PKC ε and η were selectively translocated without increasing the overall activity of PKC (101). Wang et al demonstrated using immunocytochemistry that PKC δ was translocated to the mitochondria after diazoxide treatment (127). Diazoxide is a potent opener of the mK_{ATP} and it has been show that PKC can modulate the activity in mK_{ATP} channels (109). The prolonged administration of PMA (24 hours), an antagonist of PKC, inhibited the opening of the mK_{ATP} channel and resulted in no protection of rat hearts from I/R injury (127).

The last area of concern is that there is a myriad of different protocols used for the various experiments which make it difficult to compare studies to ascertain validity. Studies use varying time duration and frequencies of ascetic treatments, different types of blockers / activators, different species and different indices to measure success. Even when the same protocol is used, two laboratories can come to different conclusions which really makes the elucidation of the "correct" interpretation of the phenomenon difficult.

1.35 Other Kinase involvement

Although the PKC hypothesis has more or less been shown to be valid, other kinase cascades seem to be just as important in classical preconditioning and in the second window of protection. Both tyrosine kinase (TK) and mitogen activated protein kinases (MAPK) have been shown to be directly involved (34, 79, 128). One of the targets of PKC in the heart is the myristolated alanine rich C kinase substrate MARCKS *(41, 128)*. PKC feeds into the MAPK pathway, which is known as a convergence point for several signaling pathways. PKC interacts with the MAPK pathway via the Ras-Raf-MAPKkinase-MAPK cascade. c-Raf acts as a phosphorylator of MAPKkinase. This cascade is important because MAPK phosphorylates transcription factors like c-Jun and Elk-1 which then enhance the early transcription of the c-fos gene (41, 128). A PKC-

independent MAPK pathway is the receptor activated kinase pathway or the p38/RK pathway. This kinase pathway phosphorylates (MAPK activated protein kinase 2) which then has heat shock protein 27 (HSP27) as a substrate (31). HSP27 is involved in the stabilization of the actin cytoskeleton which aids in the survival of the cell after ischemia (39). Overexpression of HSP27 and alpha B crystalline, also a structural protein, has been demonstrated in the adult rat myocyte model to protect the cell from simulated ischemia. Martin et al used an antisense oligonucleotide sequence inserted into an adenovirus to decrease the level of HSP27 and HSP25, a native HSP in neonatal rat myocytes, increasing the severity of injury to the cell (78). It has been shown that pre-treatment of rat hearts with a tyrosine kinase inhibitor can attenuate the preconditioning effect. Fryer et al were successful in showing that lavedustin A, a specific tyrosine kinase inhibitor, was able to block the preconditioning effect if administered during the pre ischemia cycles. The earlier reports of success with tyrosine kinase inhibition are held in less favour after it was discovered that the blocker, genistein, was not specific at the concentrations it was used. Paillart et al were able to show that there was direct blockage of the voltage sensitive Na^+ channels by genistein in isolated myocytes at the doses used in these experiments (97). Weinbrenner et al used a phosphorylation assay detection in order to prove that it was the phosphorylation of tyrosine 128 on the p38 MAPK, that induced the preconditioning effect (128). The use of 8-SPT,

an adenosine antagonist, blocked the phosphorylation to the p38 MAPK and in parallel, lost the protective effect of preconditioning. The administration of asinomycin, a p38 MAPK agonist, was found to be as protective as preconditioning in the rat heart using the Langendorff preparation. In the rat model, evidence presented by Fryer et al suggests that there is an additive effect of PKC inhibition and tyrosine kinase inhibition. Pre-treatment with genistein only partially attenuated the PC effect in male Wistar rats and administration of the drug during the longer bout of ischemia had no significant effect on PC.

1.4 Possible Effectors of IPC

The third level of the cascade of preconditioning is the effector mechanism. The sarcolemmal K_{ATP} (sK_{ATP}) channel has been the focus for many of the experiments to date, although recently some groups have focused on the mitochondrial K_{ATP} (mK_{ATP}) as the primary effector for the preconditioning protection.

1.41 Sarcolemmal K_{ATP} channel

The K_{ATP} channels are defined by their single channel properties, pattern of regulation of nucleotides, such as ATP, and by their reaction to inhibitors such as glibenclamide. The sK_{ATP} channels are weakly inward rectifiers. Application of ATP independent of magnesium results in a reduction of the mean open time by prolonging the interburst interval

and shortening the open time duration of the channel (51). The channel's normal physiological role is not clear. Under normal levels of ATP in the cell, the channels are in a closed state. It is when the cell is metabolically challenged such as in ischemia, that the role of the channel may become more evident. The inward rectification property of the channel would allow for the quicker return of the cell to the resting membrane potential. The shortening of the action potential duration could serve as a protective measure by decreasing the flux of Ca^{2+} through the DHP receptor. The lesser amount of Ca^{2+} could have an energy sparing effect due to the fact that the myofibrillar apparatus of the heart uses up approximately 70 % of the ATP generated (94). The channels are heteromultimers made up of two distinct regions, the K_{IR}6.2 is the channel protein responsible for the movement of K^+ and the SUR2A portion that is sensitive to sulphonyl ureas like glibenclamide (45, 46, 111). Both sections of the channel are important for the proper function of the channel. Expression of the K_{IR}6.2 subunit alone did not give any functional channel activity , where as co-expression with the SUR subunit did (46).

The sarcolemmal K_{ATP} channel was first described by Noma in 1983 (93). Using the patch clamp technique, he was able to detect an outward injury current. This outward efflux of K^+ correlated well with the falling of intracellular ATP. Conversely, he was able to observe the closing of the channel by high levels of ATP (half maximal closure at 20 to 100 μM).

The shortening of the action potential seen in ischemia correlates well with the opening of this channel.

The membrane density of the K_{ATP} channel is relatively high, ten channels per μm^2, with respect to other potassium channels whereas the other inward rectifiers and the delayed rectifier are less than 1.25 channels per μm^2 (15). The channels also have a very large unitary conductance, 80 pS using a physiological asymmetrical K^+, which again is 3-4 times the unitary conductance of the other potassium channels (92). The properties allow for a huge current with even the slightest open probability. For example, at an open probability of 0.01% approximately 45 channels are open per cell which generate a current of 16 nanoamperes at –60 V. This coupled to the fact that at open probabilities of 30 and 60 percent, the inward rectifier generates about 2 and 4 nA while the voltage gated outward delayed rectifier produces 8 and 16 nA, respectively (15).

The sheer numbers of the K_{ATP} channels along with their higher conductance allow for the channel to "take over" in times of decreased high energy phosphates. Other factors such as increased intracellular concentrations of adenosine and a decrease in the cytosolic pH act to increase the open probability of the channel even if the intracellular concentration of ATP is not at critical levels (15). ATP acts not as a plug, but as an allosteric inhibitor of the channel. Activation of the channel has proven to protect the myocardium across all species tested.

A deleterious effect of the activation of the channels is that the decrease in the action potential duration may cause arrythmogenicity of the region and possibly cause the tissue to go into fibrillation which may cause the ultimate death of the tissue (15).

Many experiments have been carried out with the hopes of proving that the K_{ATP} channel is the effector, or the final partner in the cascade of events which ultimately leads to the protection of the myocardium. Various potassium channel openers (PCO) have been used, some of which are nicorandil, pinacidil, cromakalim, and aprikalim. Benefits with the usage of the PCO include the decrease of infarcted tissue relative to the area at risk, an improvement of the mechanical functioning of the heart during the final reperfusion after the ischemic event, a reduction in the loss of adenine nucleotides, a decrease in the elution of cellular markers of injury such as lactate dehydrogenase and the decrease of the ultrastructural damage with a bout of global ischemia (11, 12, 15, 20, 38, 42, 70, 113, 129). Experiments focused on the K_{ATP} channel usually involve the use of one of the potassium channel openers and one or both of the blockers. In attempts to link the K_{ATP} channels to the preconditioning cascade, agonists and antagonists of the adenosine receptor or another receptor, or drugs which inhibit or activate PKC are used. For example, Mei et al used monophosphoryl lipid a (MLA), an endotoxin derivative, to stimulate the second window of preconditioning effect in dogs (83). They administered the MLA 24 hours before the

ischemic event and found that the protective effect in the dog was blocked by the administration of the potassium channel blockers 5-HD and glibenclamide. Elliot et al showed the same protection in the rabbit using an almost identical protocol and found that the administration of glibenclamide blocked the infarction limiting effects of preconditioning (27). Schultz et al used the rat myocardium to explore the effects of the glibenclamide blocking of preconditioning (110). They found that the glibenclamide could block preconditioning as long as it was administered 30 minutes prior to the ischemic episode. If the blocker was administered 5 minutes before the ischemic episode, it failed to show any blocking effect whatsoever. Their results showed that there is a time dependence in the effect of the blocker. One factor to take into consideration is that the use of sodium pentobarbital as an anesthetic can interfere with the effectiveness of K_{ATP} channel blockers. To overcome this fact, a combination of ketamine and xylazine should be used (19).

1.42 Mitochondrial K_{ATP} channel

More recent developments have challenged the assumption that it is the sarcolemmal K_{ATP} channel which afforded the protection. Garlid et al were first to foray into the area of the mitochondrial K_{ATP} channel as a target for the conventional PCO (36). They noticed, like other researchers, that there was a problem linking the shortening of the action potential

duration and the protective effect of preconditioning. The use of agents which did not have and effect on the action potential duration, like verapamil, did not stop the cardioprotective effects of preconditioning. Sato et al have put forth the idea that it is the mitochondrial K_{ATP} channel which is responsible for the protection of the myocardium (108). Using the potassium channel opener diazoxide, which they were able to demonstrate has 2000 times more affinity for the mitochondrial K_{ATP} channel than the sarcolemmal K_{ATP} channel, they were able to show that PKC activation had an effect on the oxidation of flavoproteins by the myocardium. The lack of an outward potassium current and the return of functioning of the myocytes suggests an alternate method of protection. Garlid's group has also shown that the use of the mitochondrial selective PCO and blockers do indeed effect the recovery of the myocardium (48). They hypothesize that the sole function of the mitochondrial K_{ATP} channel is to regulate the volume of the matrix of the mitochondria by keeping the membrane bound proteins in their normal geometry. The maintenance of the inner membranes normal geometry by ischemic preconditioning has also been demonstrated to maintain the functional coupling of the adenine nucleotide translocase and the mitochondrial creatine kinase shunt. This allows for the maintenance of energy transfer from the mitochondria to the cell (63). The increase in the volume may play a role in the activation of the electron transport and stimulation of fatty acid oxidation (48).

It would seem that both channels may be operating congruently in affording the protection to the myocardium. The decrease in the action potential duration has been seen and as discussed earlier, seems beneficial to the myocardium for its energy sparing effects. The mitochondrial channel activity is intriguing because of the metabolic effects it may have for the heart. Sanada et al demonstrated in the canine heart that the use of non specific openers and inhibitors of K_{ATP} channels, fully mimicked or abrogated protection as measured by infarcted tissue (107). The use of specific inhibitors of either the mK_{ATP} or the sK_{ATP} only partially blocked the protection of decreased infarcted tissue. This implicated both sets of channels in the protection of the heart.

The mK_{ATP} has also been linked to the generation of free radicals during the ischemic preconditioning and reperfusion of hearts (30, 35, 67, 98, 131). Yue et al demonstrated that the administration of menadione (3 micromol/L), a free radical generator, instead of IPC reduced the infarct size in buffer perfused rat hearts (131). MPG blocked the protection that was afforded by the free radical generator as did myxothiazol, and inhibitor of site III respiration, when using area of infarct as an index of damage. Using the p38 MAPK blocker SB203580, they were also able to attenuate the protection afforded by menadione which suggests a link between the generation of free radicals and the phosphorylation and activation of the p38 MAPK pathway. Forbes et al

hypothesized that the swelling and enhanced respiration of the mitochondria due to the opening of the channel may generate free radicals (30). Using dichlorofluorescein, a dye that fluoresces when its acetate group is cleaved and is oxidized by reactive oxygen species, cells loaded with diazoxide (50 micromol/L) increased the generation of free radicals by 173% compared to baseline. The use of 5-HD blocked the increase in free radical generation. In the rat heart, the use of the antioxidant N-acetylcysteine (NAC) decreased the protective effect of diazoxide (100 micromol/L) from 84% to 54% recovery of left ventricular developed pressure. The fact that there wasn't a complete blockage of protection indicates that free radicals may be only one trigger for protection and mentioned earlier, the redundancy of protective mechanisms help to ensure protection. Fryer et al noted that the generation of free radicals in the PC ischemia may act as a trigger but the the mK_{ATP} channel must also be open in the reperfusion phase to protect the heart (33). Using a rat heart model, the administration of 5-HD during the index ischemia and reperfusion phases attenuated the protection seen with IPC alone. This implicates the mK_{ATP} as a mediator of protection and not just a trigger of protection as demonstrated in the rabbit heart by Pain et al.

1.5 Second Window of Protection

It has been shown that there are two phases in the effects of preconditioning. Much attention in the literature has been given to the acute phase: n cycles of preconditioning prior to a major bout of ischemia prolong the viability of the tissue for approximately one to three hours. In addition, there have been studies which show that there are protective mechanisms which come into play approximately 24 hours after the ischemic episode, which has been called the second window of protection. The most plausible explanation for the protective effects seen in the second window are changes in gene expression, given its time frame. Two main candidates for altered expression are HSP and antioxidant enzymes such as superoxide dismutase (SOD) (6, 21, 22, 77).

Heat stress can protect the myocardium against oxidative stress by increasing the amount of a 70 kilodalton protein 24 to 72 hours post insult (90, 132). Experiments with mice over expressing HSP 70 demonstrated better tolerance to six hours of hypoxia and 2 hours of heat stress than mice that were producing normal levels of HSPs (77, 102).

A product of IR injury is the generation of free radicals in the tissue (28, 29). Damage as a result of the oxidizing agents can be extensive to the regular homeostasis of the cell. The free radicals can

attack and damage proteins, altering their folding and therefore their function, they can oxidize lipids in the sarcolemmal or other membranes which may cause the membranes to loose their barrier properties and affect their channels which may cause alterations in the ionic handling of the cell. With the addition of heat stress and IR cycles, activity and protein levels of SOD were shown to increase in the mitochondria 24 hours post insult (43). Li et al used gene therapy to introduce a vector for the extracellular isoform of SOD into hepatocytes of rabbits (67). Heparin was used two hours before the occlusion of the coronary vessel to release the extracellular isoform of SOD from the liver to the other organs of the rabbit, especially the heart. Over expression of SOD protected the myocardium in both the acute and SWOP phases of IPC compared to control hearts when the area of infarct was measured. These proteins could be a part of a larger wave of protein level up-regulation in the myocardium caused by the PKC cascade or other phosphorylating cascade such as the tyrosine kinases or the p38 MAPK pathway (13, 22, 23, 44, 123).

1.6 Protection in neonates?

Three studies have examined the possibility of the IPC phenomenon in immature myocardium. Awad et al attempted to determine the age at which the neonatal rat myocardium develops the

ability to precondition (3). Using a Langendorff preparation, they tried to precondition the hearts of 4, 7, 14, 21 and 50 day old rats. The hearts of the four and seven day old rats were not protected by ischemic preconditioning irrespective of changes in the duration of the preconditioning bout or the time of the sustained ischemia. The older age groups (14, 21 and ~50) were protected by the preconditioning protocols, as assessed using the left ventricular developed pressure as an index of recovery. The second study by Ostadalova et al also looked at the neonatal rat heart with respect to its ability to become preconditioned (95). Using a standard protocol for the preconditioning ischemic bout, 2 cycles of 3 minute ischemia and 5 minute reperfusion), they too were not able to see a significant difference in the recovery of developed force for rat hearts of 1 and 4 days of age. However, seven day old hearts showed a significant difference in the recovery of developed force, from 40% ± 2% versus 33% ± 2%. This finding is contrary to Awad et al in that protection was seen in the 7 day old myocardium. Two differences in these studies that may explain the apparent age at which the myocardium can be preconditioned include the indices of function and the protocol that was used in the experiments. Awad et al used LVDP measured with an intraventricular balloon whereas Ostadalova et al used developed force measured from an exogenous isometric force transducer attached to the apex of the heart. Both transducers are measuring force but the developed force should not be taken as an index of contractile ability of

the left ventricle. Subtle changes in the developed pressures are best seen at the source, meaning, measured from the ventricle itself. The protocols differed in that the hearts were paced at only 200 b.p.m. in the Ostadalova study while the unpaced hearts in the Awad study had a much higher heart rate. The higher the heart rate, the greater cardiac output, which is often used as an index of function.

The third study looked at the ability of chronically hypoxic neonatal myocardium to ischemically precondition (4). Using 7-10 day old rabbits Baker et al were able to demonstrate that litters that were normoxic from birth had the ability to be preconditioned (70% recovery of LVDP in the preconditioned group versus 43% in the control group). The second part of the study focused on the memory effect of preconditioning. The time between the PC test and the sustained ischemia was increased from 10 to 20 to 30 minutes. A waning of the protection afforded to the myocardium was evident at the 30 minute mark. This time period is much shorter than that reported for adults from various species (upwards of 3 hours). This study used a time of only 30 minutes for the prolonged ischemia while the Awad study used a time of 90 minutes. Again, the differences in protocols may account for the PC seen in the 7-10 day old rabbit hearts. Species differences also may account for the different onsets but the overall trend seems to be that the immature myocardium develops the ability to be preconditioned a couple of days after the first week of life.

2.0 Objectives of the thesis

Clearly, after the first week of life, the myocardium has the ability to precondition. In the clinical setting, the use of cardioplegia is wide spread and the clinical outcome of its administration in children is less successful when compared to adult recovery. To date, no studies have been carried out that examine the additive role of ischemic preconditioning with cold cardioplegia in the immature myocardium. We hypothesized that there is an additive benefit of IPC when administered prior to cold cardioplegia.

3.0 Experimental design

3.1 Working heart apparatus

To test the effect of ischemic preconditioning on the neonate, the Langendorff-working heart preparation was used. The apparatus used in this set of experiments was a left heart perfusion system. Oscar Langendorff first published a description of the heart apparatus in 1895 (114). The perfusate to the heart and the perfusion pressure are controlled using this system. The Langendorff system falls short in its attempts to model the *in vivo* state because the heart does not pump, but it is good for controlling the perfusion pressure and the composition of the perfusate (85). In our experiments the Langendorff preparation is used to equilibrate the heart back to its normal state after the trauma of surgery. Coronary flow is provided from the retrograde perfusion of the aortic root. To represent better the *in vivo* state of the heart, the working heart apparatus is incorporated into the system, allowing for the introduction of afterload. Preload is the load put upon the ventricles from the end diastolic volume while afterload is the pressure the heart must overcome to eject blood into the aorta. See figure 3 for a schematic representation of the perfusion system.

3.2 Rational for using free fatty acids in the perfusate

In the case of the 10 day old rabbit, the use of free fatty acids in the perfusate keeps the model as close to the *in vivo* state as possible. The new-born, until approximately 7 days of age uses glycolysis and lactate oxidation as its main sources of energy production with β oxidation responsible for about 10% of the overall ATP produced (5). By the seventh day, the neonate begins to use the β oxidation pathway as the primary source for ATP (73). As such when needed the perfusate will contain the normal Krebs-Henseleit buffer with 0.4 mM palmitic acid added. Studies that fail to use the preferred substrate of the heart must interpret the results carefully as it may not be accurate to compare findings to the *in vivo* case where the proper substrate is available.

3.3 Rational for the use of cardioplegia in the experiments

Addition of cold cardioplegic solutions used to stop the heart in the proposed experiments closely follows the actual protocol of cardiac surgery in today's hospitals. If the preconditioning phenomenon is not seen with cardioplegia then the better of the two should be adopted in the common practice of surgery. Valen et al found that in the rat heart, that PC did not afford any protection after the administration of St. Thomas' II cardioplegic solution (122). It is imperative that this problem

be addressed for the fact that the cardioplegic techniques have proven their effectiveness and if preconditioning cannot work with this proven mechanism then it will difficult to apply any of the findings of preconditioning to the hospital setting.

4.0 Methods

4.1 Animals

Neonatal New Zealand white rabbits (10 and 11 days old) of either sex were used in this study. All animals were handled humanely according to the guidelines set forth by the University Animal Care Facility. The rabbits were anaesthetized intraperitoneally with the administration of sodium pentobarbitol (Somnotol, 0.1ml/100g body weight) and premedicated with the anticoagulant heparin (50 units/kg).

4.2 Heart Isolation and Perfusion

Once anaesthetized, a tracheostomy was performed and the rabbit was connected to a Harvard rodent ventilator (Harvard Apparatus Co., Inc., S. Natik, MA.). The excess mediastinal and pericardial tissue was removed and the heart was quickly excised and placed in ice cold Krebs-Henseleit buffer (KHB) and transferred to the working heart apparatus (WHA). The aorta was cannulated with a straight cannula. The diameter of the cannula depended on the age of the rabbit; and was determined/confirmed with the isolation of the heart. The aorta was secured to the cannula and a small hole was cut into the nearby pulmonary artery to assist the flow of coronary drainage. The heart was immediately perfused in the Langendorff mode at a constant perfusion pressure of 55 cm H_2O to minimize the time that the heart was ischemic.

The average time of cannulation of the hearts was 59 ± 4 s. The left atrium was then cannulated via the pulmonary veins in preparation for the working heart mode as described by Neely (91) and modified by Bove and Stammers (10) and modified in our lab. To test the proper positioning of the curved cannula, the right atrium was inspected and if any bulging occurred the cannula was repositioned. All hearts underwent an equilibration period of aerobic perfusion for 10 minutes in the Langendorff mode.

4.3 Exclusion Criteria

Hearts that did not satisfy pre-assigned exclusion criteria at either the onset of the working mode or for the duration of the reperfusion phase were excluded from the study. The acceptable ranges of aortic flow (ml/min), coronary flow (ml/min), systolic pressure (mmHg) and heart rate (beats/min) for the onset of the working mode were > 3 ml/min, > 2 ml/min, 40 mmHg and 150 beats/min, respectively. In the reperfusion phase, the aortic flow was allowed to drop to zero as long as the heart rate was greater than or equal to 60 beats/min.

4.4 Perfusion medium

The composition of the KHB buffer was in mM: NaCl 118.0, $NaHCO_3$ 25.0, KH_2PO_4 1.2, CaCl 2.0, KCl 4.7, $MgSO_4$ 1.2, and glucose 11.1. The buffer was prepared daily and was filtered through a 5 µm filter before use and was continuously gassed with 95% O_2 and 5% CO_2 to give a pH of 7.4 at 37°C. This KHB was used in the Langendorff mode. The working heart solution used in this experiment was a modified KHB solution identical except for the addition of palmitate (0.4 mM) bound to 3% bovine serum albumin (BSA). To bind the palmitate to the BSA, the palmitate (0.5-0.6 g Na_2CO_3/g palmitate) was dissolved in a 25 ml beaker with 15 ml of distilled H_2O and 10 ml of ethanol. The solution was heated with constant stirring until the ethanol was completely removed. The KHB with 3% BSA was brought up to approximately the same temperature as the palmitate solution and then the two were added together. Rapid stirring ensured the proper mixing of the two. The new solution was dialyzed overnight against 1 L of KHB using 8-12,000 mol wt cut off dialysis tubing.

4.5 Ischemic Preconditioning Protocol

Please see figure 4 for a graphical representation of the following protocol.

After the 10 minute equilibration period the hearts were subjected to one of the two protocols shown in figure 4. Throughout the experiment the hearts were thermally regulated with a temperature controlled water jacketed heart chamber. The hearts were exposed to the room air inside the chamber and were bathed in their coronary effluent which was either collected at the stated intervals or recirculated.

4.51 Study one- 5 minute IPC in addition to hypothermic cardioplegic arrest

After a ten-minute time lapse from the onset of the Langendorff mode, the apparatus was switched to the working heart mode. The heart was now perfused with a modified Krebs-Henseleit buffer with 0.4 mM palmitate. The heart was kept in the working heart mode for 10 minutes. At the 9th minute, the coronary effluent and the aortic effluent were collected to measure the rate of their respective flows. At this same time, the heart rate, pressures and their derivatives were stored. These data were used as the baseline data for the study. The control group continued to be in the working heart mode for an additional 15 minutes to act as a time match group for the experimental group. The

experimental group underwent a 5-minute global ischemic bout with a subsequent 10-minute reperfusion period. One minute prior to the end of the reperfusion, collection of the coronary effluent, aortic effluent and transducer data made up the second point for the data collection.

The hearts were then arrested using the modified St. Thomas' Hospital cardioplegic solution. The composition of the solution adjusted to pH of 7.8 and maintained at 10°C was (in mM): NaCl 110.0, $MgCl_2$ 16.0, $NaHCO_3$ 10.0, KCl 16.0 and $CaCl_2$ 0.4. The cardioplegic solution was delivered for 3 minutes at a hydrostatic pressure of 55 mm H_2O. The heart was then stored in a water-cooled jacket (10-12 °C) for the 2 hours of cold ischemia.

After 2 hours of cold ischemia the heart was reperfused in the Langendorff mode under the same protocol as before. Measurements of coronary and aortic effluent were made 15 and 30 minutes after the initiation of the working heart mode. All samples taken were immediately frozen in liquid nitrogen and stored at -80°C until they were tested for their cTnI content using an enzyme linked immunosorbent assay (ELISA) developed in the laboratory.

4.52 Study two- 1 minute IPC in addition to hypothermic cardioplegic arrest

It was observed that many of the hearts in the previous study were not able to last for the full 30 minutes of the reperfusion phase. It was then postulated that the 5 minute ischemic challenge may have been too severe and that the hearts were irreversibly injured. This second study used a one minute global ischemic insult instead of the five minute ischemic insult used in study one. All other procedures and protocols were identical.

4.6 Indices of function

Coronary and aortic flow rates were measured at certain time points from the 1-minute sample collected into a graduated syringe. Systolic and diastolic pressures along with heart rate were collected from a pressure transducer (Baxter) attached to a sidearm of the aortic line. The analogue signal was digitized using a BNC-2090 (National Instruments) data acquisition board and Labview (National Instruments) was used to collect data and calculate the derivative of pressure with respect to time using a sampling rate of 2000 Hz. The cTnI from the coronary effluent was analyzed using a sandwich ELISA developed in the laboratory with antibodies (polyclonal capture with monoclonal signaling) from Spectral Diagnostics, Toronto.

4.7 Statistical analysis

The experiment is set up as a Group (2) x Time (4) mixed model multivariate ANOVA. The power of the experiments was set as 0.8. The three neonatal experiments discussed in the section1.6 included groups of 10 or less animals. Power calculation was based on the following formula, $\delta = \gamma (N/2)^{-1/2}$, where δ= index of power, γ= the difference or the effect size (determined by the difference of the two means divided by the standard deviation of the population), and N is the number of animals per group. Using data from the previous papers, an N of 10 gave power values no lower than .8 and as such, an N of 10 was used for the experiments. Comparisons were made using ANOVA on SPSS (SPSS Inc., Chicago, IL) with the level of significance set at $p \leq 0.05$. The Bonferroni correction for multiple comparisons was applied a priori. Data are reported as mean ± S.E.

5.0 Results

Table 1 shows the baseline data for both the five minute and one minute groups.

Three variables were statistically significantly different between the groups: weight of rabbits, heart rate and baseline temperature. The 21 rabbits in the 5 minute group were heavier than the 21 rabbits in the one minute group (171.7 ± 8.2 g vs. 139.1 ± 6.51g; p=0.003). This may have been a cause of concern due to the fact that the hearts of the heavier rabbits may have been significantly larger than the hearts of those rabbits that were lighter. Further analysis revealed that the heart dry weight did not differ significantly between the two groups. Heart rate differed significantly between the groups. The five minute groups' mean heart rate was faster than the one minute group (246 ± 8 beats/min vs 218 ± 7 beats/min; p=0.01). The baseline temperature (37.5 ± 0.2 °C vs 37.0 ± .2 °C; p=0.031) was also greater for the 5 minute group.

5.1 Study one- 5 minute IPC in addition to hypothermic cardioplegic arrest

For this study 21 rabbits were used in total, (control=11, experimental =10). There were no significant differences in the age, weight, heart dry weight and cannulation time.

Table 3 displays the mean and standard errors for all variables in the study. Graphical representations can be found in the figures section of the thesis. When there is a statistical significance present, its p value is listed on the graph itself.

5.11 Functional Recovery

Four indices showed significant differences: aortic flow, coronary flow, cardiac output and troponin I.

Both aortic and coronary flows showed a significant difference in their percentage of PC vs baseline. Aortic flow percentage was higher in the experimental group versus the control group (103 ±12% vs 71 ±7%; $p<0.036$) as was the coronary flow's percentages (98 ±6% vs 73 ±5%; $p<0.008$), respectively. The sum of these variables creates a value of cardiac output (ml/min), which was also significantly different at the PC

time point. In the reperfusion phase, specifically at the fifteen minute of reperfusion, the mean values of aortic flow, coronary flow and thus cardiac output displayed a difference that may have been significant but the variance in the values was too great. The preconditioned group displayed a trend of protection in the indecies mentioned above.

The other variable that displayed significance was the release of cTnI. Percentages of baseline were not calculated due to difficulties in the interpretation of the results. If there was no significant amount of cTnI in the baseline reading then the percentage of baseline would not be calculable. The difference between the baseline and the other time point was then taken and analyzed. Significance was found in the difference between baseline and the PC value. The mean difference for the control group was 0.70 ±.45 ng/ml while the mean difference for the experimental group was 3.45 ± 1.11 ng/ml (p=.042). Removal of one of the data points that was greater than 3 standard deviations from the mean still maintained the statistical significance (0.70 ±.45 ng/ml control vs 2.47 ± 0.58 ng/ml experimental (p=.028))

5.2 Study two- 1 minute IPC in addition to hypothermic cardioplegic arrest

For this study 21 rabbits were used in total (control=10, experimental =11). There were no significant differences in the age, heart

dry weight and cannulation time for the two groups. There was a significant difference in the weights of the control rabbits and the experimental ones, (153.9 ± 8.4 g vs 125.7 ± 8.2 g) respectively.

5.21 Functional Recovery

Three indices were significantly different: contractility, heart rate and temperature.

The percentage of baseline contractility for the 30 minute reperfusion point was significantly lower in the experimental group versus the control group, (45 ± 4 % vs 61 ± 4 %, p=0.018). Note that the measured values did not significantly differ. Heart rate at baseline was also different. The control group had a lower heart rate than that of the experimental group; (203 ± 7 vs 232 ± 10 beats/min; p=0.035). The temperature of the hearts differed not only at baseline but also at the PC sampling point. At baseline, the control group's hearts had an average temperature of 36.5 ± 0.2 °C while the experimental group's hearts were significantly warmer at 37.5 ± 0.1 °C (p=0.001). The hearts also differed in the fact that after the application of the preconditioning ischemia, the experimental hearts were significantly warmer than the control hearts (36.6 ± 0.2 °C vs 37.5 ± 0.2 °C), respectively.

6.0 Discussion

The two studies were unable to prove that there is statistically significant additive protection to neonatal myocardium when ischemic preconditioning is added to hypothermic cardioplegia, regardless of the duration of the preconditioning ischemia.

6.1 Study one

Study one used a 5 minute global ischemic period for the preconditioning ischemia. No significant protection was seen for the 2 measurement times of 15 and 30 minutes of reperfusion in any of the indices that were measured and calculated.
Differences were found, however, in the second sampling point. The preconditioned group exhibited a higher percentage of baseline aortic flow, coronary flow and therefore cardiac output. The finding is puzzling because it is contrary to the intuitive expectation that the flows be lesser in a heart that has undergone a period of ischemia, albeit a non lethal bout. These flow data did not differ in their raw scores, only in the percentage of baseline recovery. Interestingly, the increase in these percentages occurred in the presence of an increase in the differences of cTnI released for that period. It is notable that the preconditioned group

had a significant increase in the difference of TnI released into the coronary effluent.

6.2 Study two

An observation was made that hearts in the experimental group were having difficulty in surviving for the full duration of 30 minutes reperfusion. It was then postulated that decreasing the ischemic insult time to 1 minute may allow for the preconditioning to occur without the possibility of creating an excess of irreversible injury.

Only one variable demonstrated a significant difference in the reperfusion phase. There was a significant decrease in the percentage of the positive maximal derivative of pressure at 30 minutes reperfusion for the preconditioned group versus the control group. The control group had a lower heart rate and temperature at baseline and had a lower temperature at the PC point also. Percentages of the variables did not differ significantly. The difference in heart rate can have an effect on the flow rates of the heart. Assuming a constant stroke volume, decreases or increases of heart rate can then increase or decrease the cardiac output of the heart. No differences were seen in the cardiac output which would lend itself to the assumption that the stroke volume of the heart then compensated for the decrease in heart rate. The decrease in the stroke

volume could be attributed to the decreased perfusate entering the ventricles or to the decrease in contractility of the ventricles which would decrease the force generated per beat and thus, the amount of blood expelled from the ventricle. The temperature difference at baseline and at the PC sampling point could be due to the decreased heart rate. There is a correlation between heart rate and temperature. The less the heart is working, the less energy that will be dissipated as heat.

The results in this thesis can be explained in one of two ways; either the addition of protection was there and it was not observed or that there truly is no additive benefit of ischemic preconditioning to cold cardioplegia in the neonate.

6.3 The addition of IPC afforded an increase in protection but went unseen

One must compare what indices are used when looking at claims of protection. Murray et al used the area of infarction and preservation of high energy phosphates to first describe the protective effect of PC (89). In the three studies which have dealt with the preconditioning of the neonatal myocardium, heart rate, coronary flow, left ventricular developed pressure, left ventricular end diastolic pressure, time to peak

contracture and developed force were used as measurements (3, 4, 95). In our study, cTnI was the only measure that we used to assess necrosis. It is assumed that the leakage of this molecule is an indicator of the irreversible injury of the cell. This method of measuring necrosis is secondary to the more established and accepted measurement using 2,3,5-triphenyltetrazolium chloride (TTC) to directly measure the area at risk and the amount of necrotic cells in that region. There is also no definite correlation to the elution of biochemical markers and impaired functional performance of the heart. Takeshima et al using a model of ischemic preconditioning and cardioplegia found that IPC caused a significant difference in the release of TnT, another biochemical marker, with the incorporation hypothermic cardioplegia (23°C) but not with the incorporation of deep hypothermic cardioplegic arrest (4°C). Other researchers have also found that IPC may or may not attenuate the release of TnT from the isolated rat heart (106, 130).

Comparisons of heart rate and peak systolic pressure data from neonatal rabbit hearts of approximately the same age yielded similar results with other previous studies. Baseline data from Toshiyuki et al using 7 day old rabbit hearts perfused with a modified KHB with 0.4 mM palmitate exhibited a heart rate of 203 ±4 beats per minute and a peak systolic pressure of 45.6 ± 1.2 mmHg where hearts in this study exhibited a grouped baseline heart rate of 232 ± 6 beats per minute and

53.3 ± .7 mmHg (47). However, the study mentioned used a biventricular setup and may have had some ischemic injury in the longer cannulation process. Therefore, it seems that the hearts in our setup at least at baseline conditions, were normal with respect to other published work.

A main difference in our set up in comparison to other setups is that we used an off line (aortic) pressure transducer for the measurement of systolic and diastolic pressures. While the frequency response of the transducer was well above the maximal heart rate of the rabbits, 320 beats·min^{-1} or 5.33 Hz, one must take care in comparing the results to studies that have used and intraventricular balloon attached to a catheter/transducer. Signal dampening due to the plastic tubing may have somewhat altered the pressure wave sensed by the transducer.

Two studies (Awad et al and Baker et al) used an intraventricular balloon to assess the pressures seen in the heart. Baker et al used a working heart set up while Awad et al used a Langendorff set up (3, 4). The intraventricular placement of the transducer allows for a more accurate reading of the ventricular pressure trace than placement outside the ventricle. The possibility exists that the transducer set up that we used was not as sensitive in picking up the subtle changes of the ventricular pressure trace and may have impacted calculation of the derivative of peak systolic pressure. The distance of the sidearm of the transducer was not very large but there is no substitute for having the

transducer in the ventricle to decrease the possibility of dampening of the signal.

6.4 The addition of IPC did not afford an increase in protection

Various studies are in agreement with the results presented in this thesis (53, 54, 59, 75, 99, 100, 118, 122). However problems comparing these conclusions lie in the fact that there is no standard measure that these studies use to assess protection. Coupled to the fact that different species and protocols are used, make it difficult to assess whether or not the results of these studies can be compared.

Kaukoranta et al looked at the addition of ischemic preconditioning (5 minute ischemia) to humans undergoing coronary artery bypass grafting (53). They found that there was no difference in the oxygen utilization, ATP content, lactate release and cytosolic pH of hearts undergoing aortic cross clamping. A possible caveat in the study is that they did not reperfuse the myocardium with normal blood after the PC challenge. They perfused the heart immediately with normothermic cardioplegia, which may have altered the effect of the preconditioning cascade.

Takeshima et al used a Langendorff system to evaluate the effects of temperature on the addition of IPC to cardioplegia (116). They found that a 2 minute occlusion with a reperfusion time of 5 minutes was able

to afford additive protection to hearts that were already protected with cardioplegia (normothermic) and hypothermia (23°C) but not in deeply hypothermic cardioplegia (4°C). They measured left ventricular developed pressure, left ventricular end diastolic pressure and heart rate with an intraventricular balloon catheter/transducer. Troponin T from coronary effluent was collected and analyzed at baseline and at different time points during reperfusion. It seems that the temperature of the cardioplegic stage may prove to be the limiting factor whether or not there is additive protection. Our hearts were administered St. Thomas' cardioplegia and kept at approximately 10°C and stored in a cold chamber also set to 10°C. It is possible that the temperature used may have been cold enough to stifle the preconditioning response.

Thourani et al used the adenosine A_3 receptor agonist Cl-IB-MECA (2-chloro-N6-(3-iodobenzyl) adenosine 5'-N-methuluranamide) to pre-treat hearts prior to cardioplegic arrest (117). Using the Langendorff mode, they gave the hearts a 30 minute normothermic ischemic insult followed by a 1 hour multi dose cardioplegic arrest and then reperfused for 2 hours. Hearts that were treated with the agonist prior to the cardioplegia exhibited an increased protection as measured during reperfusion. Hearts that were treated with a cardioplegic solution including the agonist did not become further protected than hearts that were administered the cardioplegia alone. A fourth group of hearts received the pretreatment with the agonist and protection with a

cardioplegic solution that included the agonist also. There was no difference in the protection between the group that received the agonist and the group that received the agonist and cardioplegia/agonist combination. This set of experiments provides further credence to the hypothesis that preconditioning, or one of its triggers, fails to give additional protection under colder conditions.

Lu et al have furthered the argument in that they were able to demonstrate that there is an additive protection afforded to normothermic cardioplegic arrest if the preconditioning ischemia is of sufficient duration to maximally activate the signaling cascade (76). The administration of sub maximal ischemia translocated PKC α to the membrane fraction whereas maximal preconditioning ischemia caused the translocation of isoforms α, δ and ε. The use of chelerythrine blocked the translocation of all the PKCs when administered during the aerobic perfusion prior to the administration of the cardioplegia. This too points to the involvement of temperature due to the fact that PKC activation decreases with temperature and thus under hypothermic cardioplegia, the enzymes activity will slow down according to the enzymes Q_{10}. PKC ε has been shown to translocate to the membrane fractions with preconditioning in the rabbit, which may explain why in our experiment, the 2 hours of cold cardioplegia blocked the possibility of activation of the enzyme (105).

Baker et al used 10 day old neonatal rabbit hearts for their study of ischemic preconditioning (4). In this work, rabbit hearts were administered a 5 minute ischemic challenge followed by a 10 minute reperfusion prior to 30 minute normothermic global ischemia and 35 minute reperfusion. Using these results, we used one cycle of 5 minutes of ischemia and 10 minutes reperfusion. One caveat of our work is that we did not redo these experiments in our own laboratory, especially because we were using a different buffer solution. It would have been helpful in the interpretation of the results of our work. The lack of palmitic acid in their perfusion media may have created and unphysiological environment in which the hearts metabolic and contractile activity may have been altered.

It may be the case that the addition of palmitate in our solution altered the metabolic pathways in some way as to decrease the amount of high energy phosphates or that the free radical generation from the challenged mitochondria were able to attack the palmitate and create further free radical generation. Kudo et al demonstrated that reperfusion with a buffer free of fatty acids performed better than hearts that were reperfused with a buffer containing high concentrations of fatty acids (61). The loss of efficiency in the metabolism of fuels led to a decrease in the peak systolic pressure and heart rate recovery (74). In other words, the rat hearts resumed overall Krebs cycle and O_2 consumption but were unable to convert the energy produces to pre ischemic cardiac work

levels. The free calcium in the palmitate buffer could also have been an issue (8). The albumin in the solution has a tendency to bind Ca^{2+} and this could have an impact on the contractility of the hearts. If the free calcium in the solution was less than optimal, then there would be less calcium available for calcium induced – calcium release in the heart.

6.5 Conclusions and future directions

As mentioned previously the first task in the elucidation of whether or not there is added protection afforded by ischemic preconditioning is to reproduce the results of the Baker study to confirm that in fact the use of fatty acid buffer does not hinder the ability of preconditioning to occur. The next set of experiments could then determine if it is the temperature or the cardioplegic solution which then interferes with the added protection, if it exists at all.

The rig itself could stand to have some modifications made. The use of inline filters was terminated in the middle of our experiments as we saw no use for them. No visible filtration was seen after the removal of the filters at the end of the experiments. There was often a loss of perfusate upon the changing of the filters that in instances made the completion of the experiment difficult. This was an error and may have contributed to the quick downgrading of functional parameters seen in the hearts. Other laboratories have used the 0.4 mM palmitic acid in

their buffers and perfused hearts for 2 hours or more (47). It is true that the duration of our experiments was much longer, 198 minutes and 194 minutes for study one and two, 120 of those minutes was in cold cardioplegic arrest and the hearts should have been able to work for a much longer reperfusion period. The hearts should be sealed in a thermoregulated chamber to ensure less temperature variation. Small modifications made to the glassware would allow for the collection of coronary flow and still have the chamber sealed. A thermocouple could then be fixed to the heart such that the measurement of its temperature would be better regulated, instead of removing the heart from the chamber and inserting a temperature probe manually, as was done in both studies. The rig should incorporate an intraventricular pressure transducer assembly. The placement of the transducer needle in the ventricle itself would allow for more accurate measurement of ventricular pressure measurements and their derivatives. It is better to use this type of transducer versus a balloon transducer in the sense that the insertion of the balloon transducer could damage the heart and that the lack of fluids in the heart could somehow alter the mechanics. The balloon method of pressure measurement creates an unphysiological environment because it does not allow for the contraction of the ventricle. This lack of ventricular kinesis could alter the hearts movement of fluids in and through and as such this altered state could

make the interpretation of data collected somewhat less applicable to the *in vivo* state.

One of the more costly changes that may be invaluable would be to not use a re circulating buffer. If three hearts are hung a day, as was the case during these studies, the amount of catabolites and biochemical markers could provide to be a confounding variable that is unaccounted for.

The age of the rabbits is another point that should be controlled for. There were instances when 6 animals were collected and thus 3 of the rabbits were kept overnight for the following days experiments. This could have biased the results due to the fact that the nutritional state of the animal could have been in question. At 10 days of age the neonate will only suckle for nutrition and thus if the animal was collected before it had a chance to suckle, the condition may have arisen whereby the animal was in a mode of starvation. This could then challenge the myocardium's stores of glycogen and therefore could alter the metabolic activity of the hearts.

Ultimately, protection is assessed by looking at the recovery of the indices in the reperfusion phase of buffer perfused working heart preparations. In the two studies performed for this thesis, none of the indices used indicated that there was additional protection afforded to the myocardium by IPC. The only significant difference that was found was a lesser recovery with respect to baseline for the IPC group(study

one) for $+dP/dt_{max}$. This result would indicate that IPC was decreasing the protection afforded by the hypothermic cardioplegia. There were no other indices that showed a better recovery with the IPC added. Replication of these studies with some of the modifications of the rig that were suggested in the previous sections may help to confirm the finding of "no added protection" in these studies.

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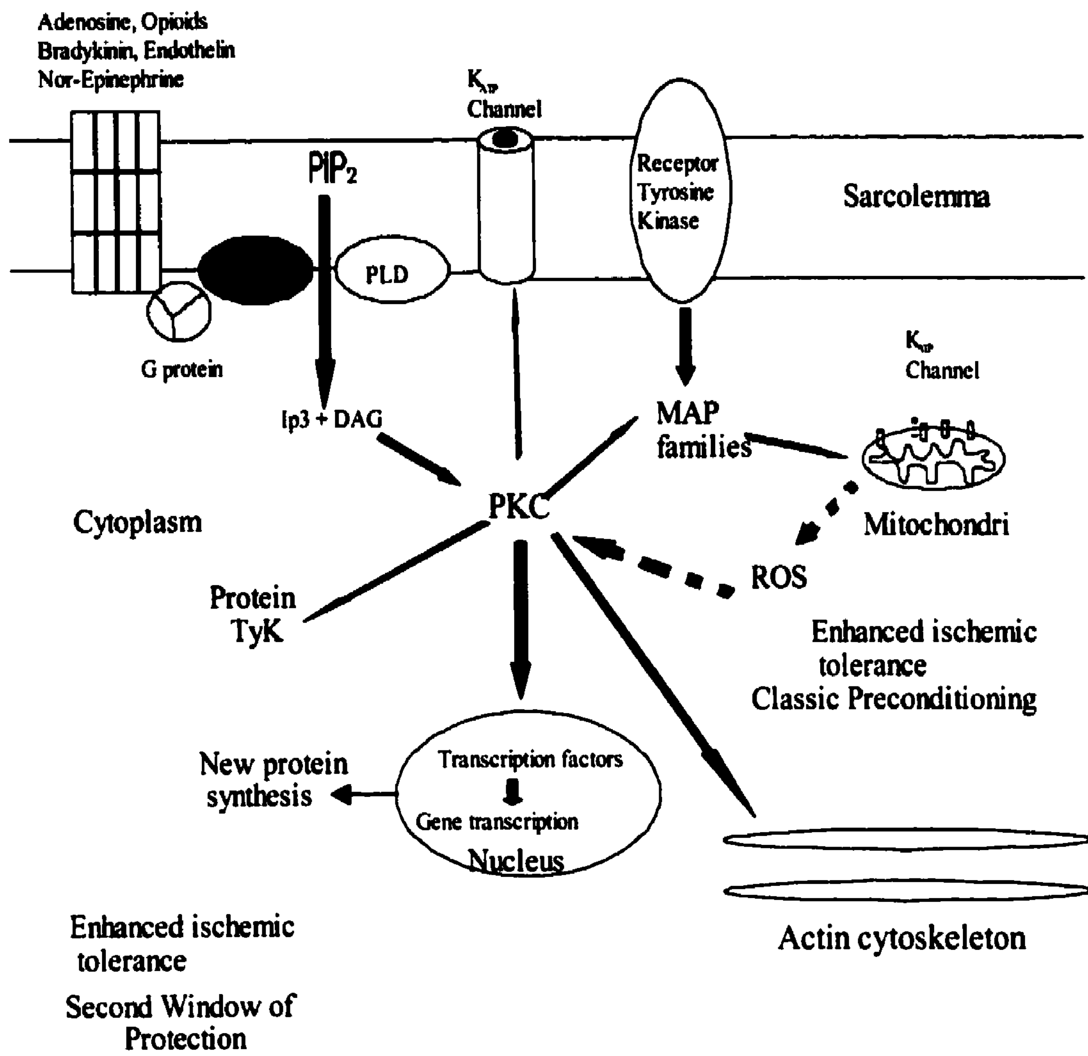


Figure 1. The cascade of events in ischemic preconditioning (adapted from D.M. Yellon et al, Cardiovascular Research. Vol. 37 (1998) Pg 21-33)

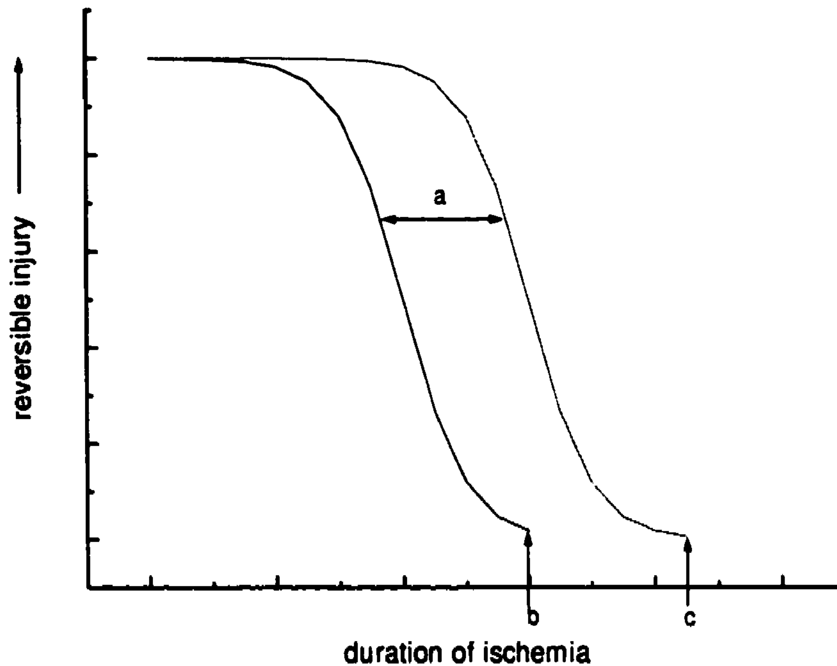
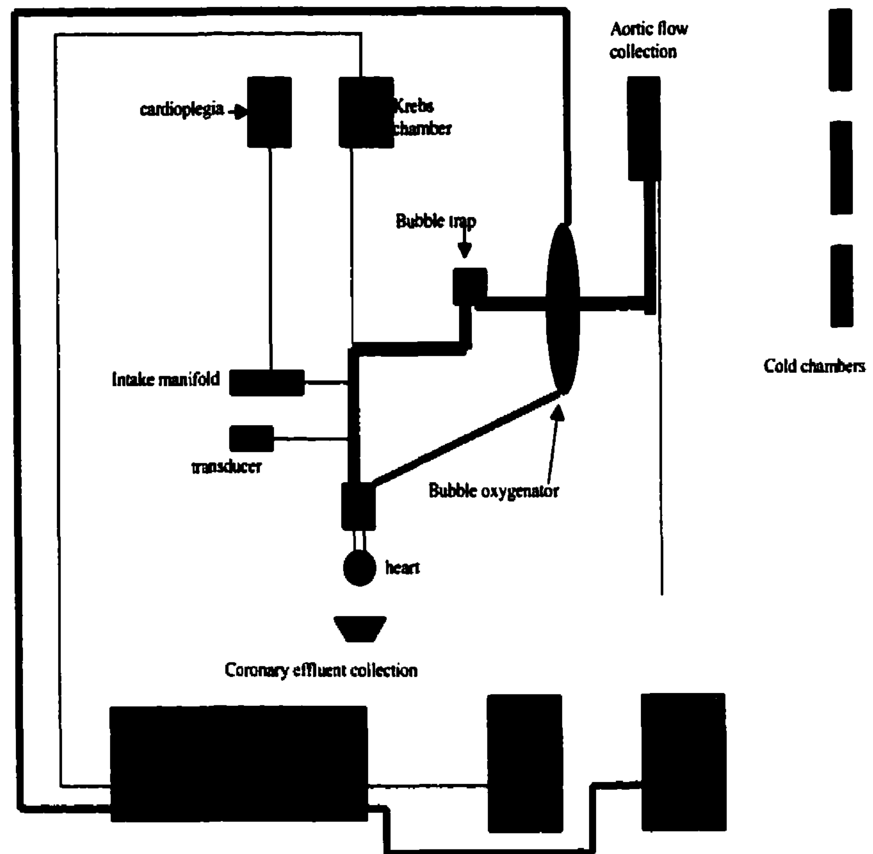


Figure 2. Effect of PC on residual function of the heart with the increase in the duration of ischemia. Part a refers to the rightward shift of the curve thus allowing a greater amount of reversible injury for an increase in the duration of ischemia. Points b and c show that both hearts will end in total infarct if the duration of the ischemia is long enough.



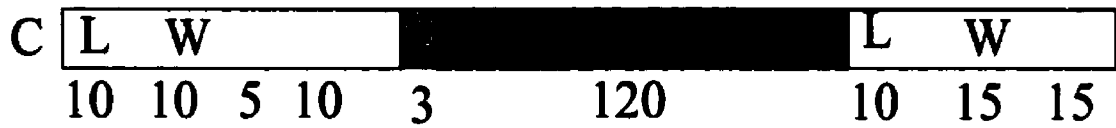
—— corresponds to the Krebs movement through the system

▬ corresponds to the movement of the free fatty acid buffer through the system

█ corresponds to common tubing used by both perfusates at different times

Figure 3. Schematic representation of the working heart apparatus

Study 1



Global Ischemia

Study 2

Global Ischemia

Figure 4. Protocols for the two studies. White bars represent aerobic perfusion while black bars represent global ischemia. Gray bars represent the administration of cold cardioplegia. L represents the Langedorff mode while W represents the working heart mode.

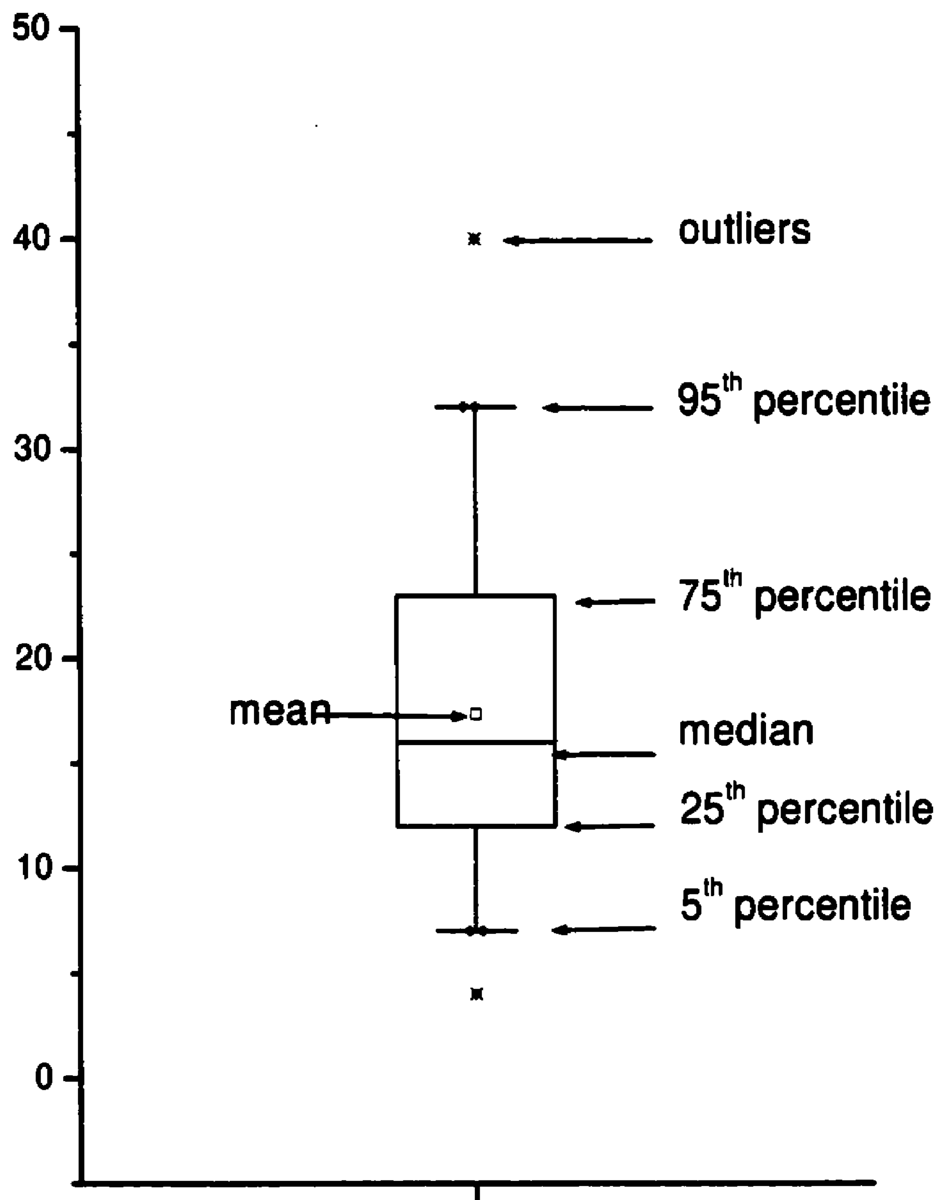


Figure 5. Breakdown of the box plot used in the results section for both studies one and two.

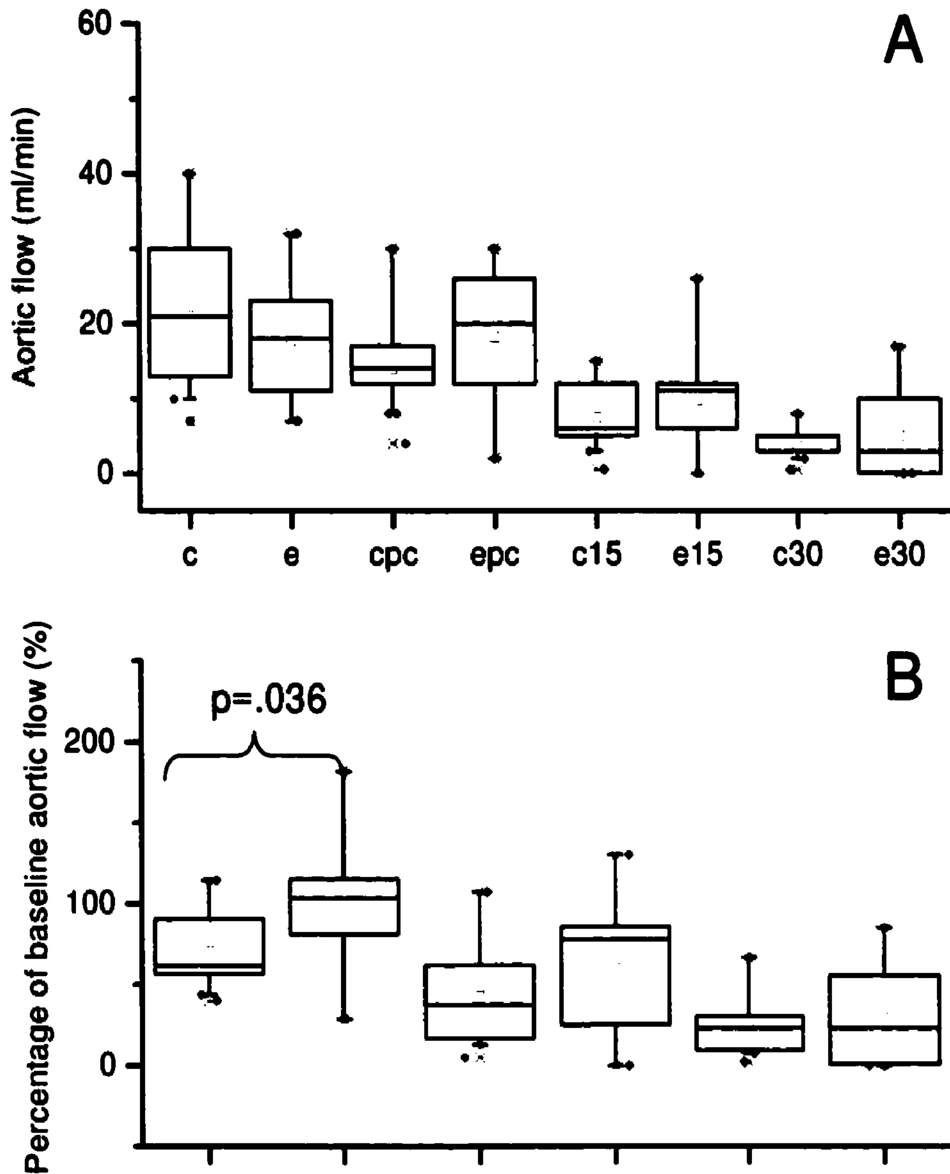


Figure 6. Aortic flow graphs for study one. Aortic flow raw data (A) and calculated percentages (B). Statistical significance indicated with p value. Please see table 5 for the graphical legend.

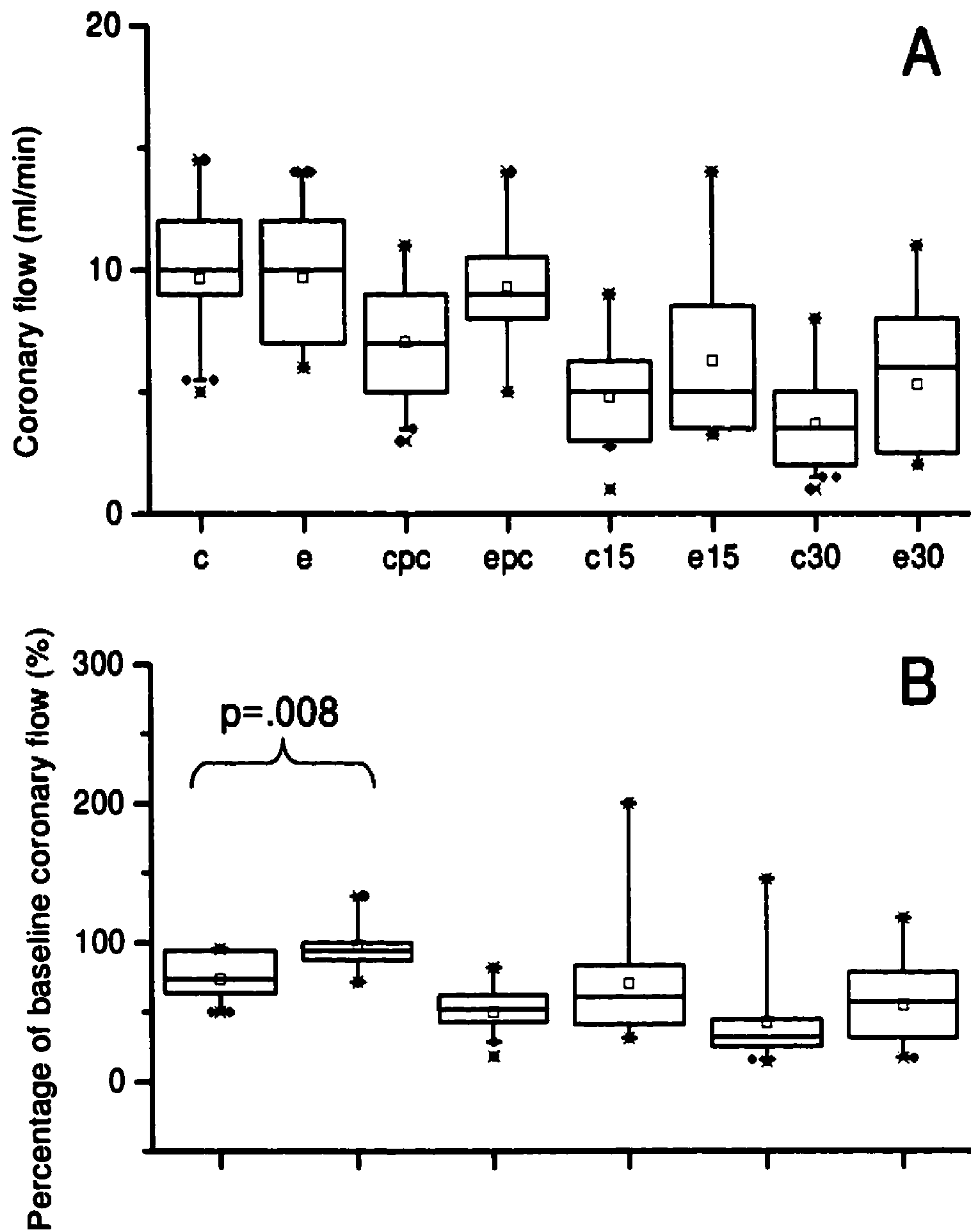


Figure 7. Coronary flow graphs for study one. Box plots of raw data (A) and calculated percentages (B) of coronary flow for rabbit hearts. Statistical significance indicated with p value.

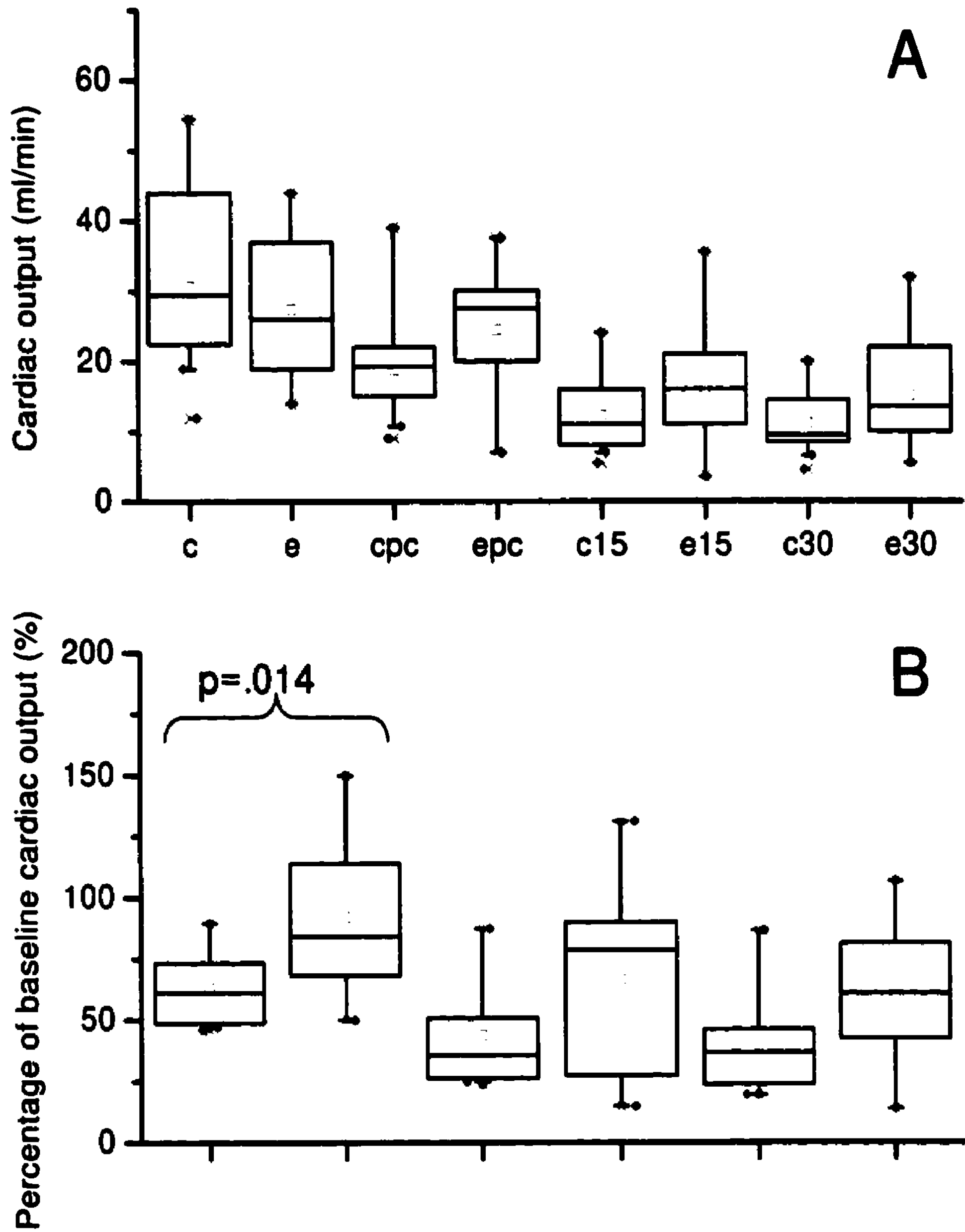


Figure 8. Cardiac output graphs for study one. Box plot of cardiac output raw data (A) and percentage of baseline calculations (B). Statistical significance indicated with p value.

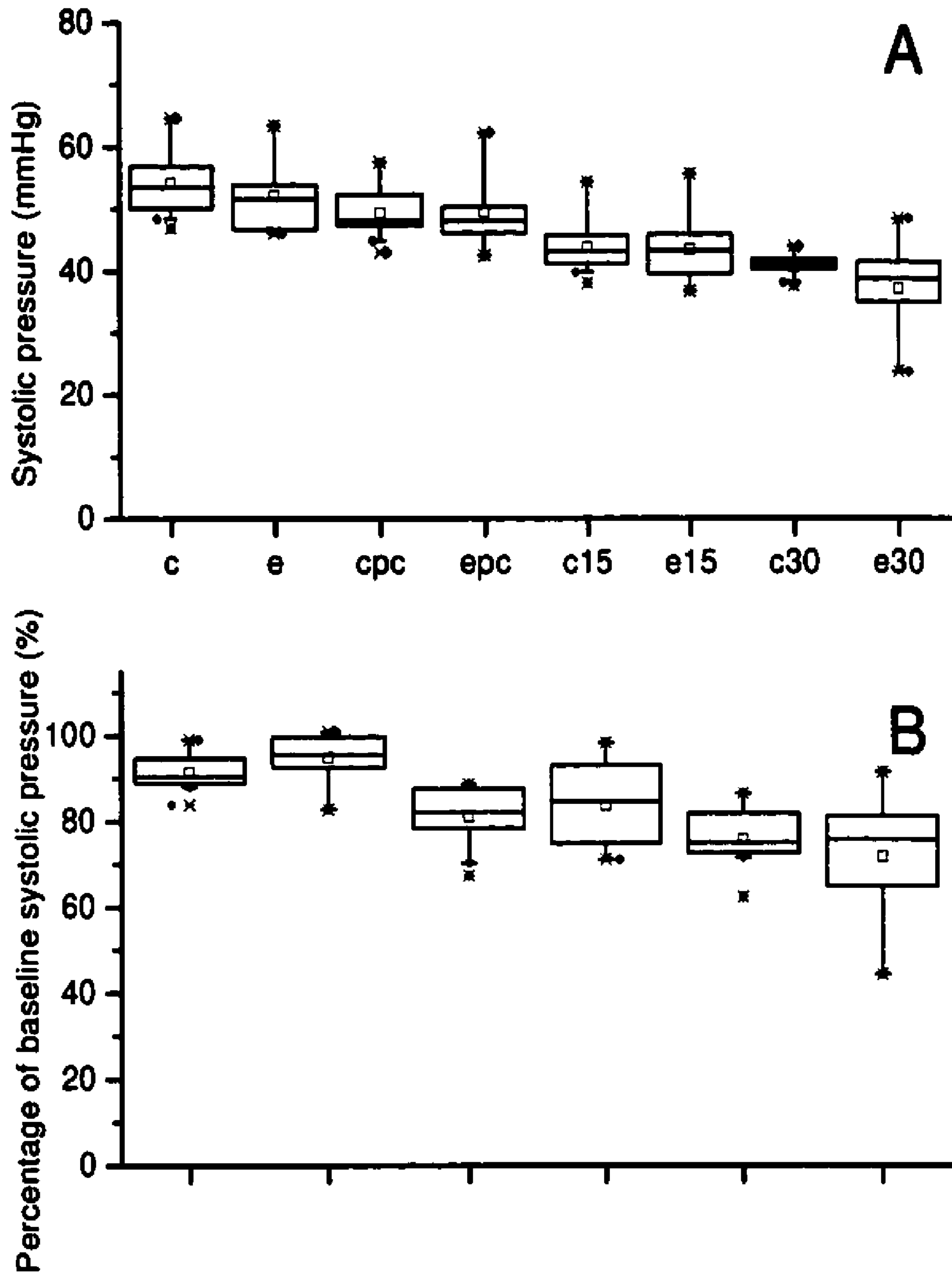


Figure 9. Systolic pressure graphs for study one. Box plot of systolic pressure raw data values (A) and calculated percentage of baseline values (B).

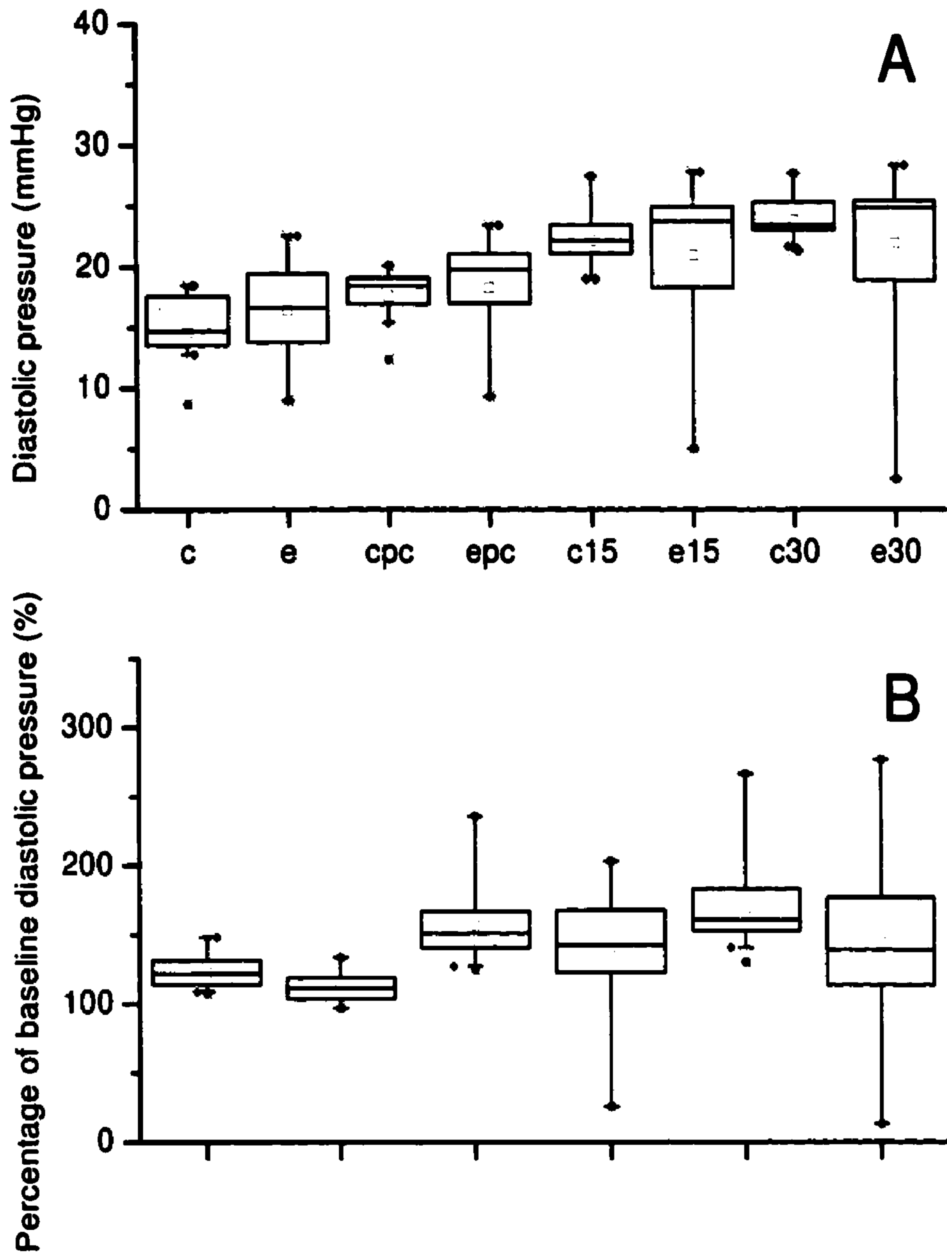


Figure 10. Diastolic pressure graphs for study one. Box plot of diastolic pressure raw data values (A) and calculated percentage of baseline (B).

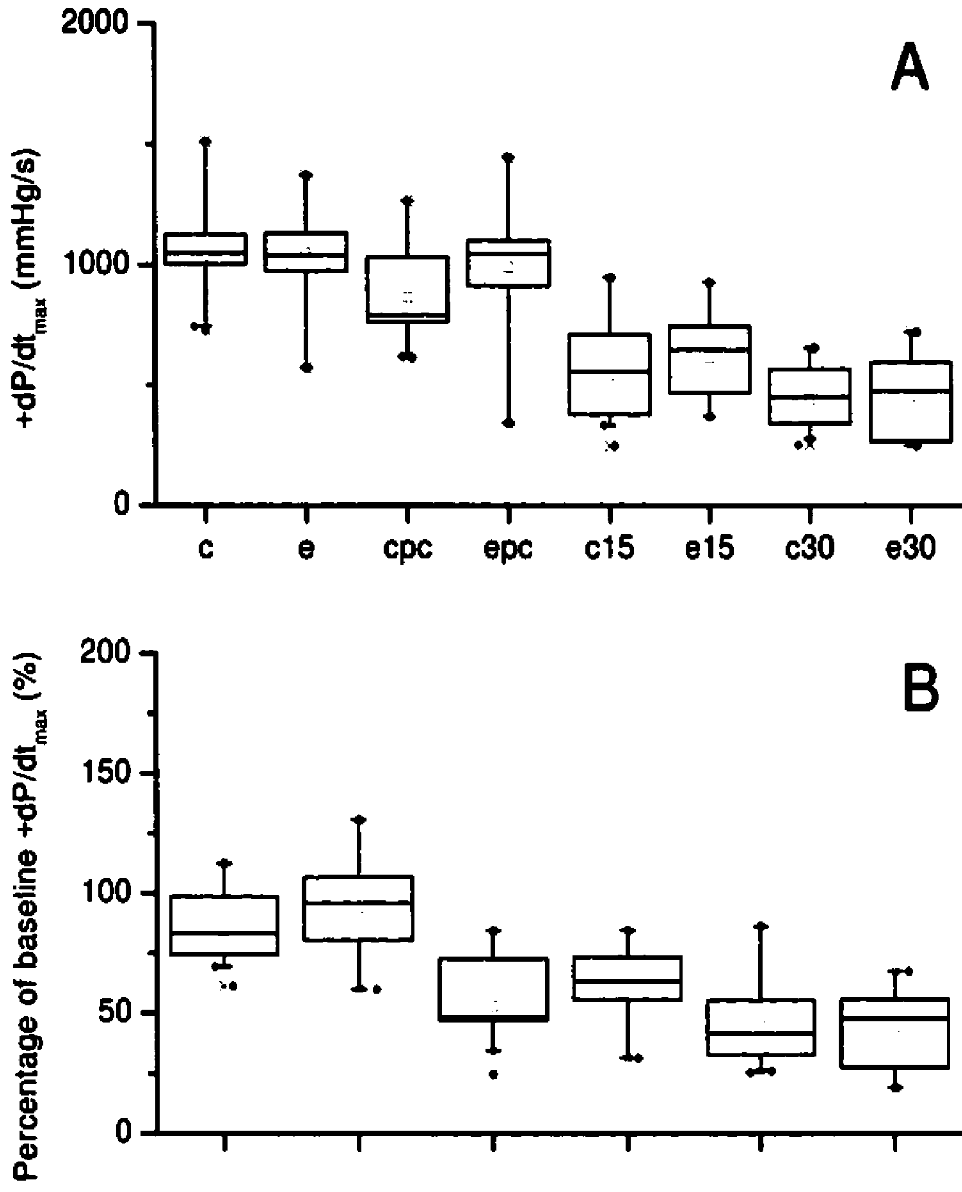


Figure 11. Positive dP/dt$_{max}$ graphs for study one. Box plot of the positive derivative of pressure maximum raw values (A) and the calculated percentage of baseline (B).

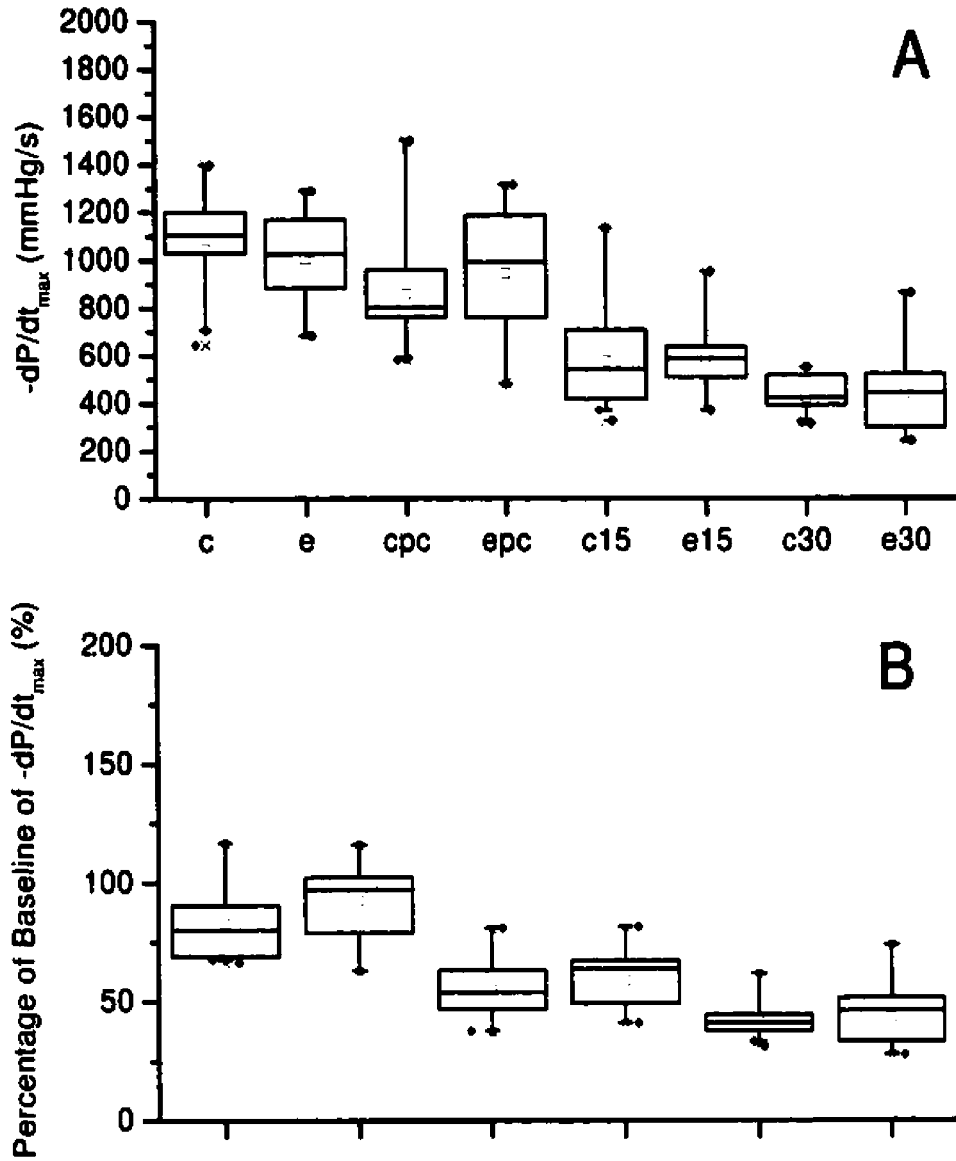


Figure 12. Negative dP/dt$_{max}$ graphs for study one. Box plot of the negative derivative of pressure maximum raw values (A) and the calculated percentage of baseline (B).

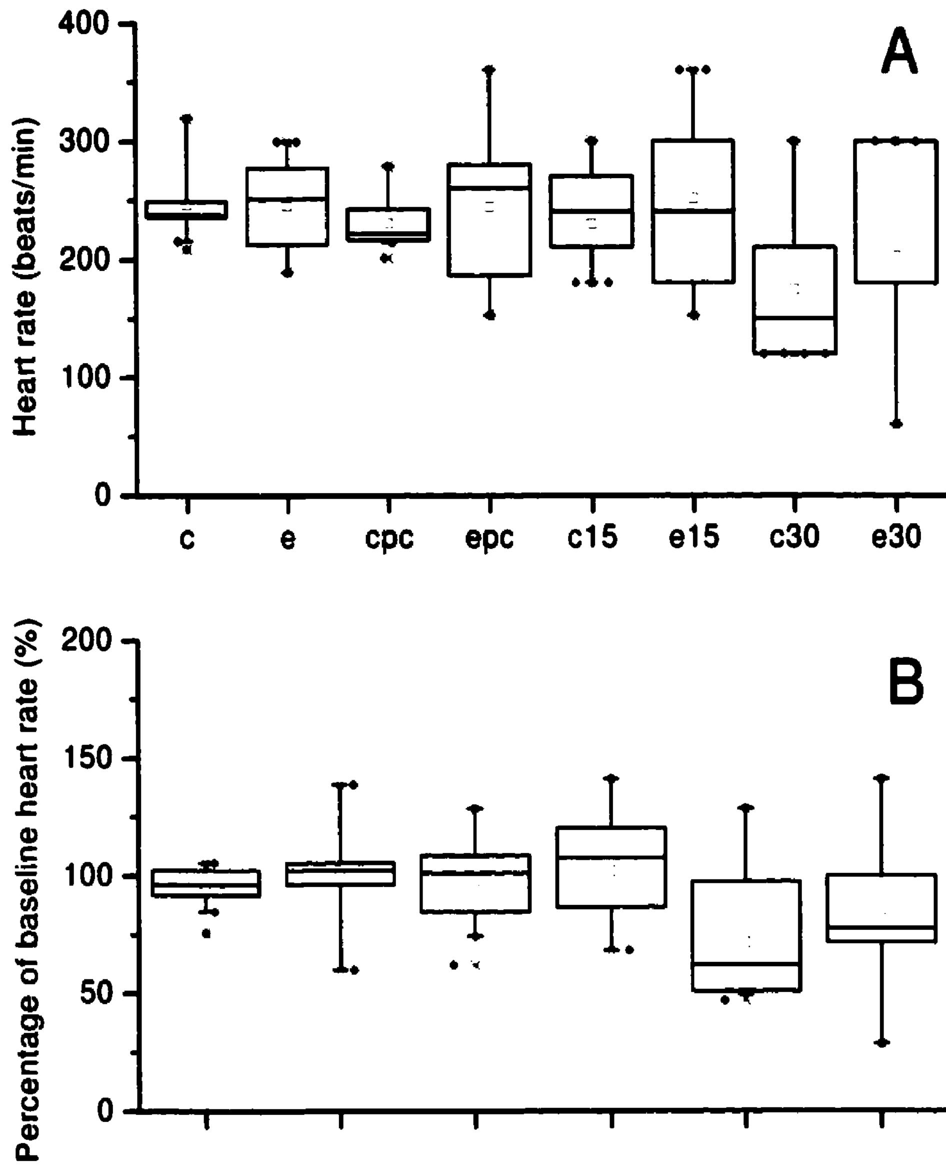


Figure13. Heart rate graphs for study one. Box plot of heart rate raw values (A) and calculated percentages of baseline (B).

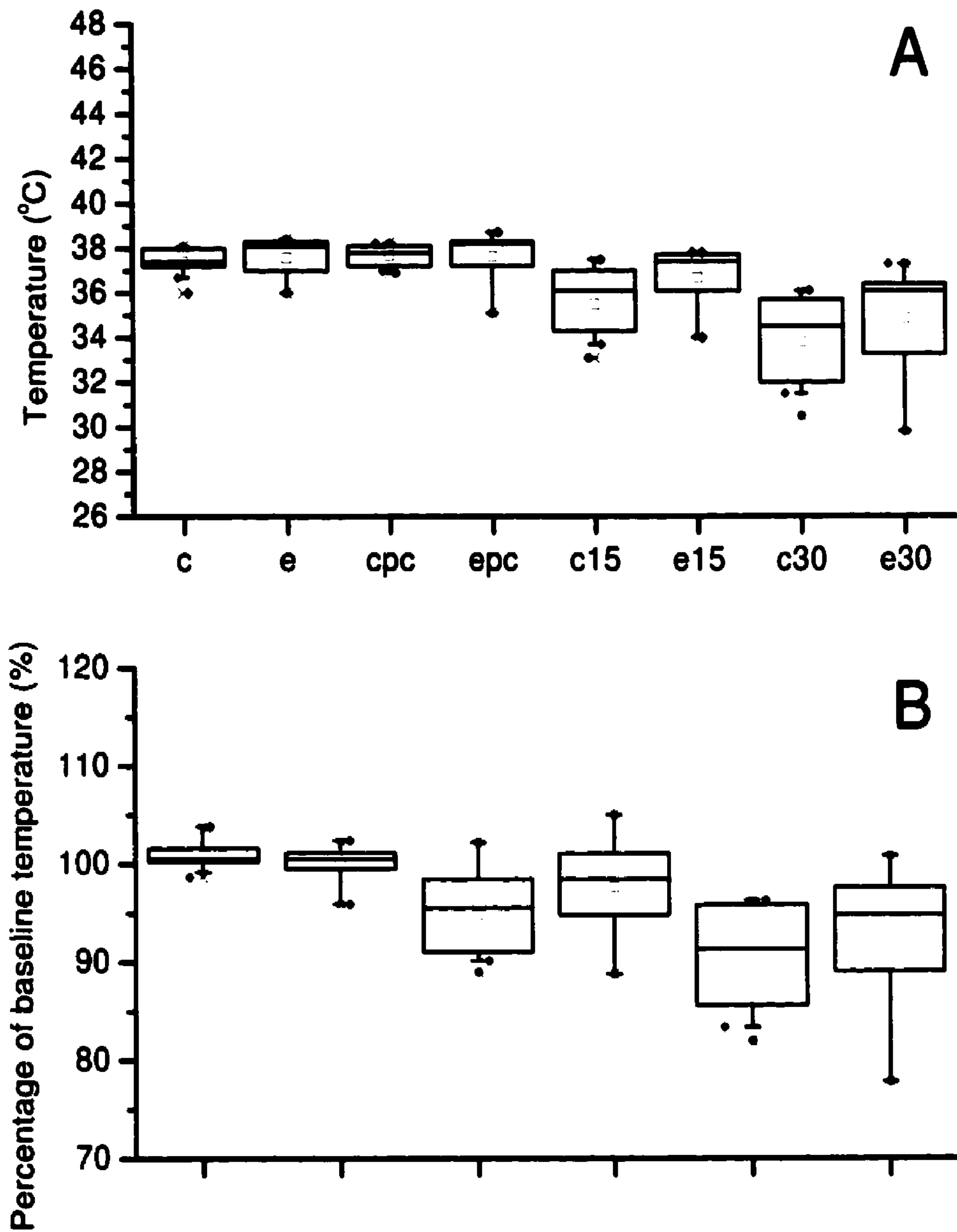


Figure 14. Temperature graphs for study one. Box plot of temperature raw data values (A) and calculated percentages of baseline values (B).

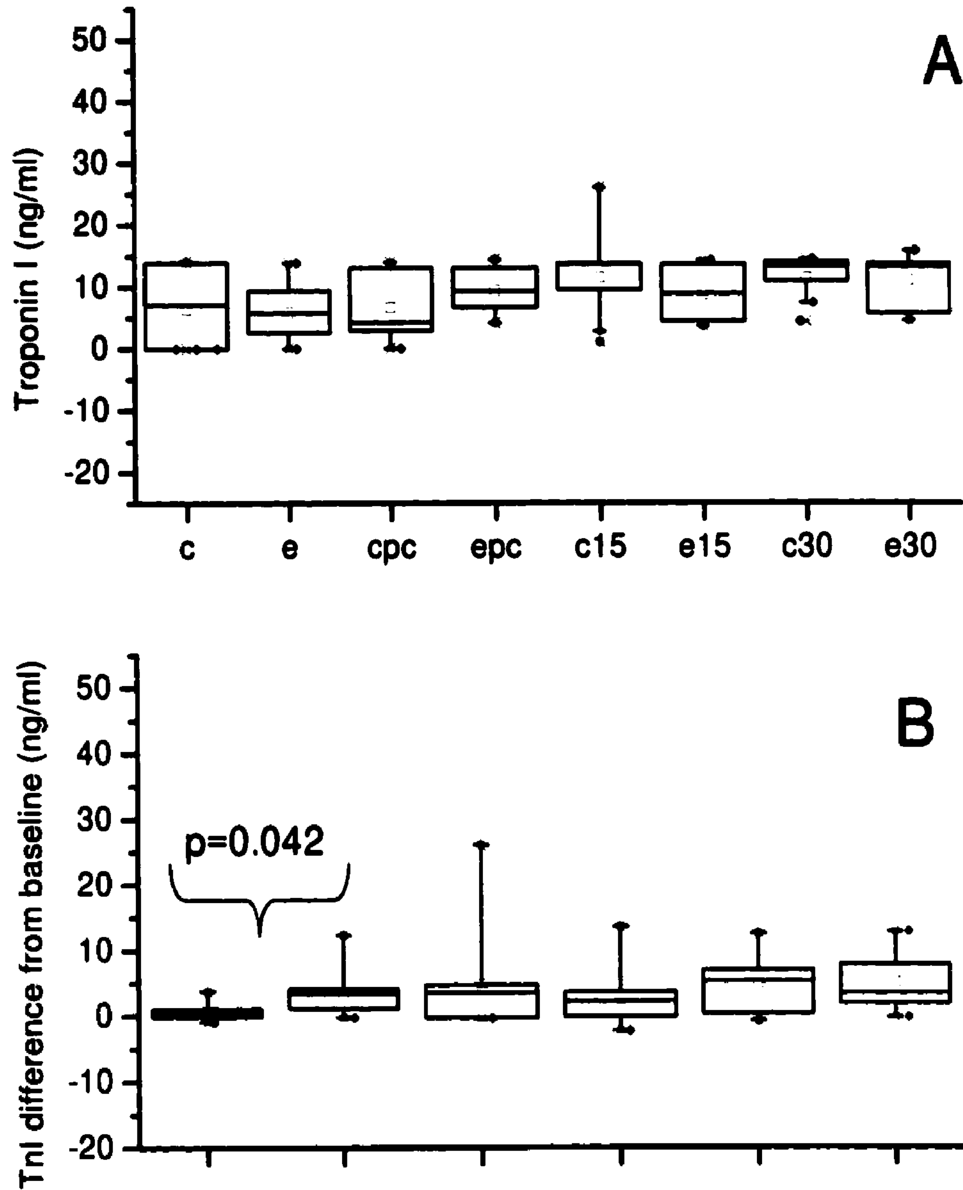


Figure 15. Cardiac troponin I graphs for study one. Box plot of cardiac troponin I raw data values (A) and calculated percentages of baseline values (B).Statistical significance indicated with p value.

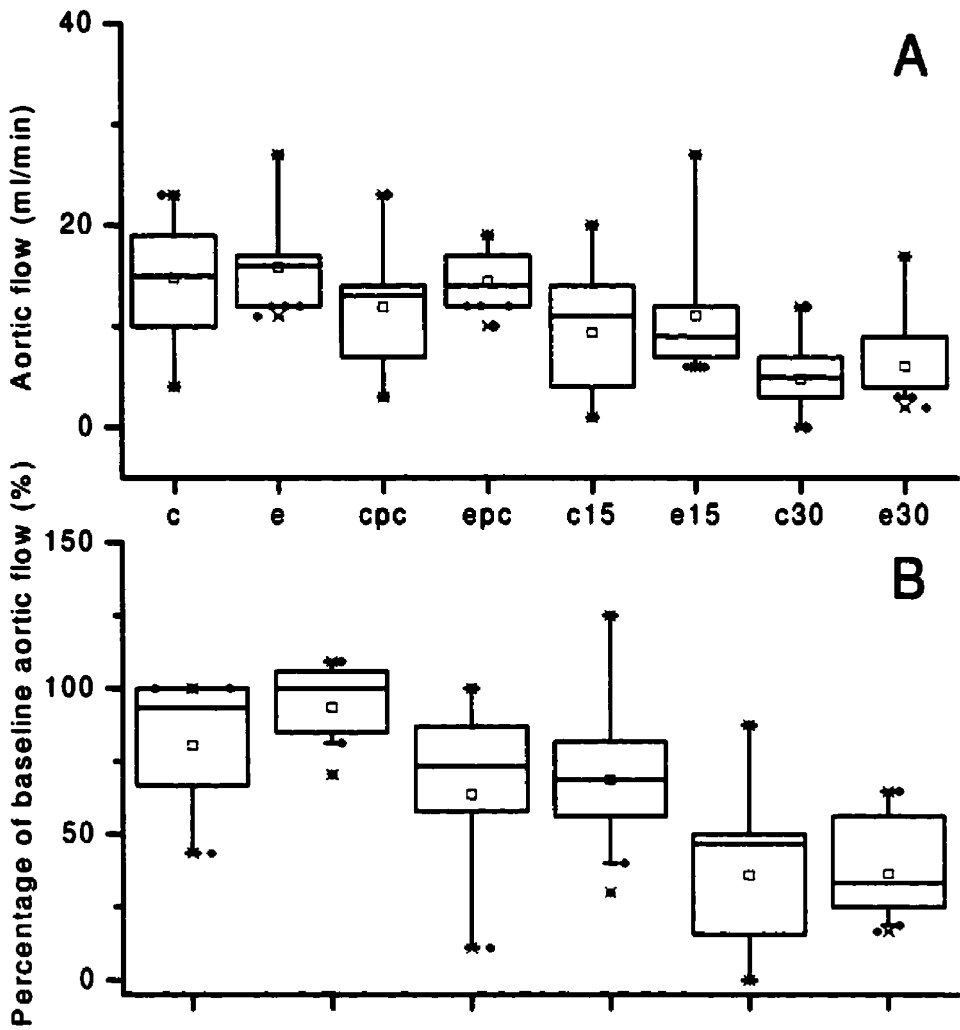


Figure 16. Aortic flow graphs for study two. Box plot of aortic flow raw data values (A) and calculated percentages of baseline values (B).

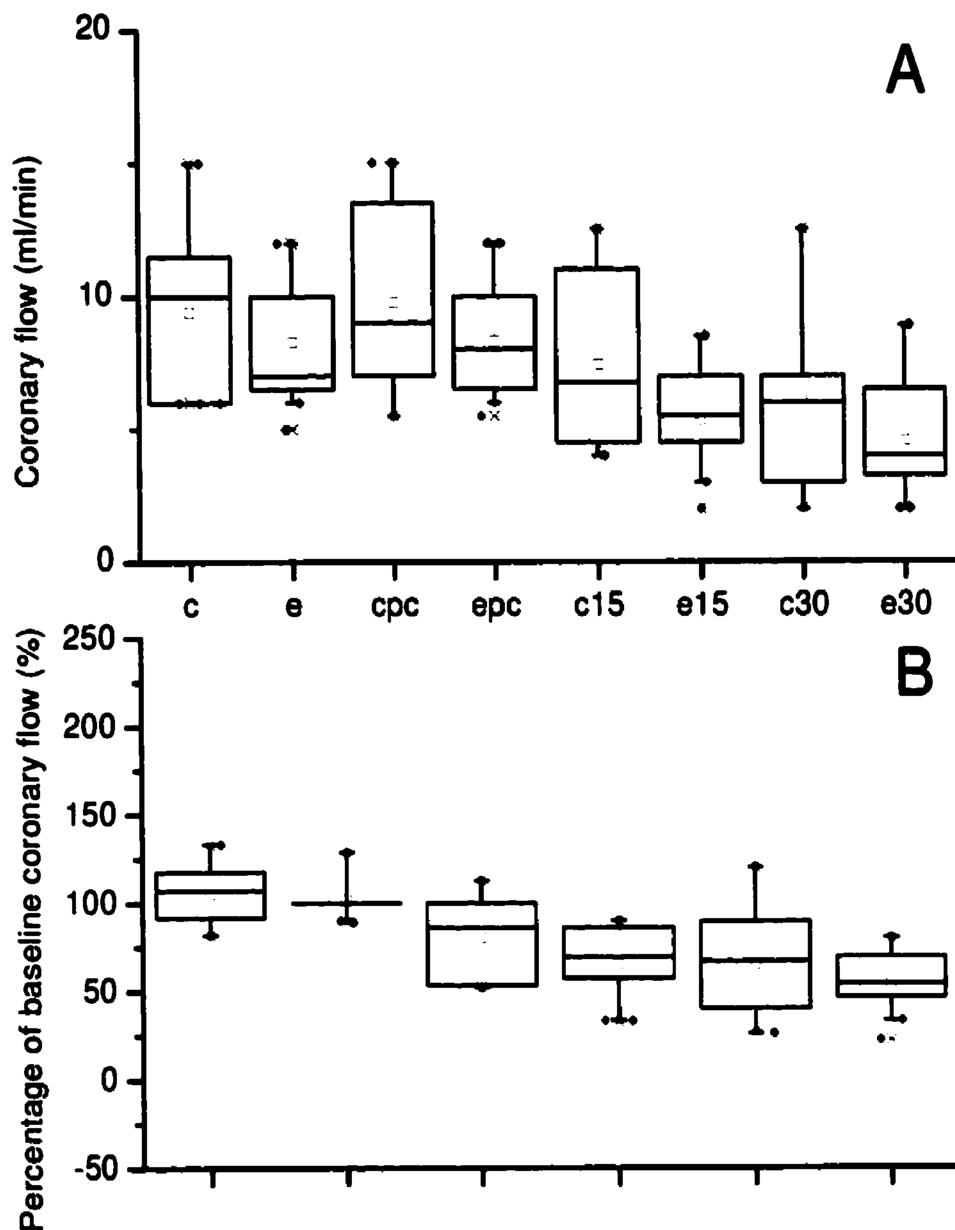


Figure 17. Coronary flow graphs for study two. Box plot for coronary flow raw data values (A) and calculated percentages of baseline values (B).

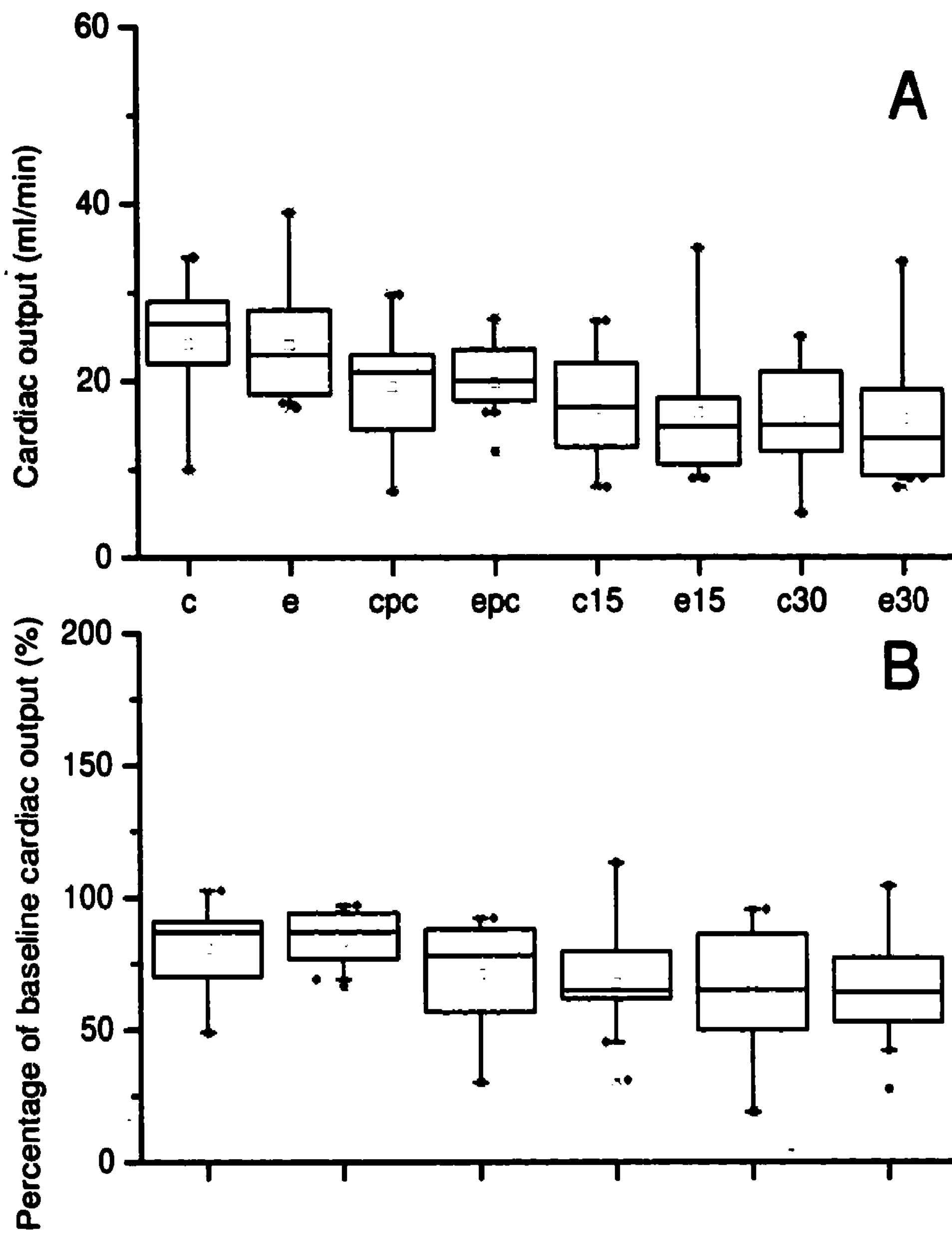


Figure 18. Cardiac output graphs for study two. Box plot of cardiac output raw values (A) and calculated percentage of baseline values (B).

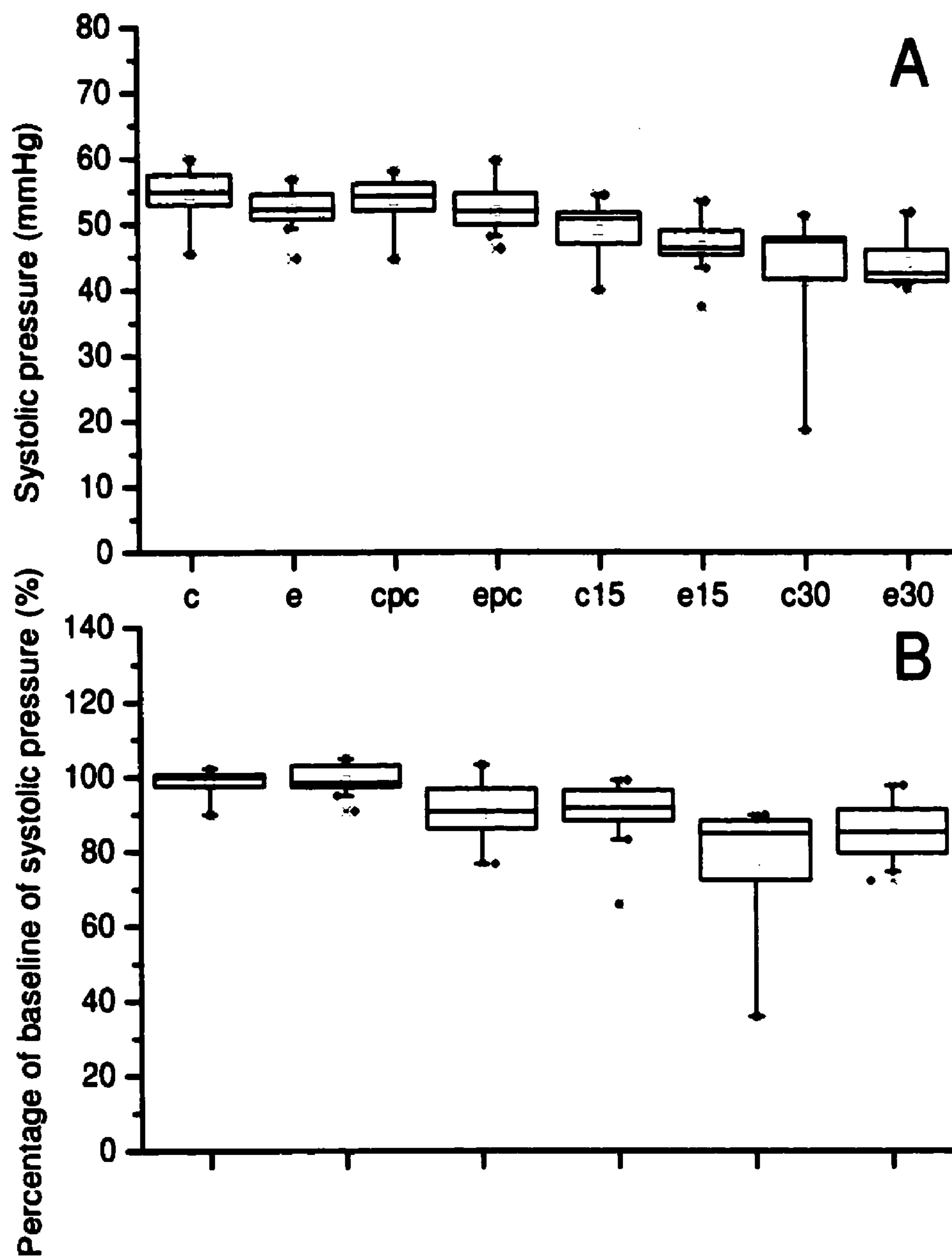


Figure 19. Systolic pressure graphs for study two. Box plot of systolic pressure raw data values (A) and calculated percentage of baseline values (B).

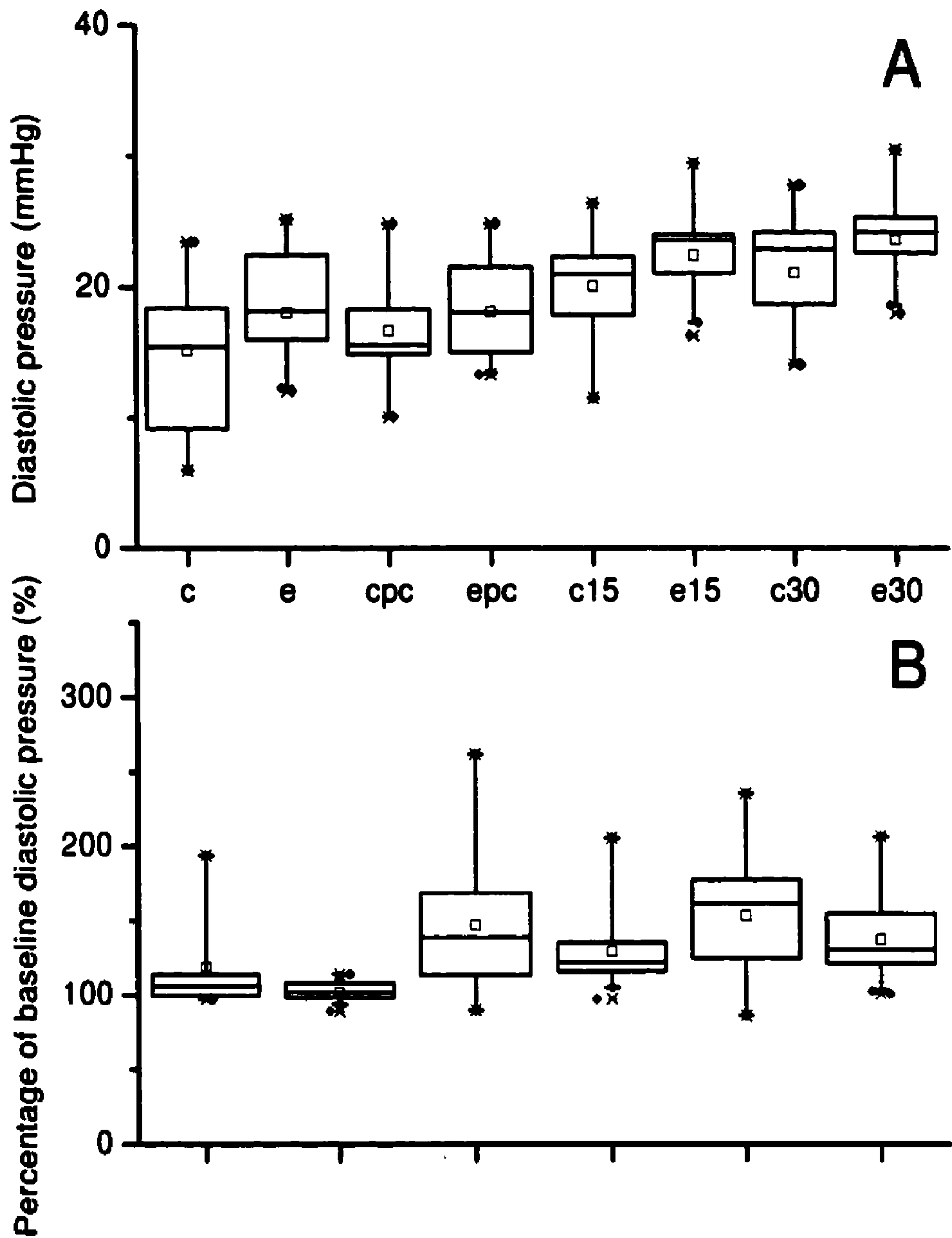


Figure 20. Diastolic pressure graphs for study two. Box plot of diastolic pressure raw data values (A) and calculated percentage of baseline values (B).

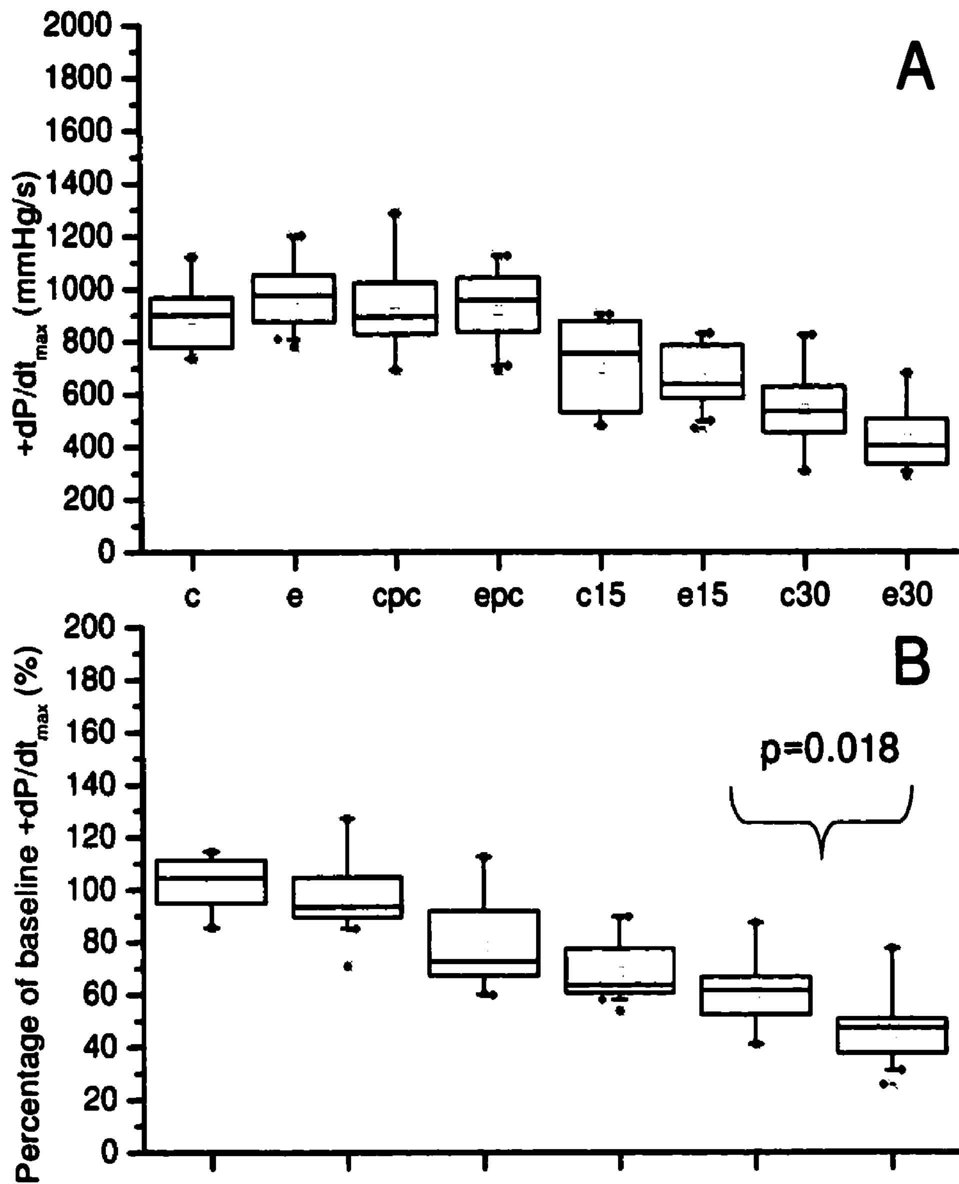


Figure 21. Positive dP/dt$_{max}$ graphs for study two. Box plot of the positive derivative of pressure maximum raw values (A) and the calculated percentage of baseline (B). Statistical significance indicated with p value.

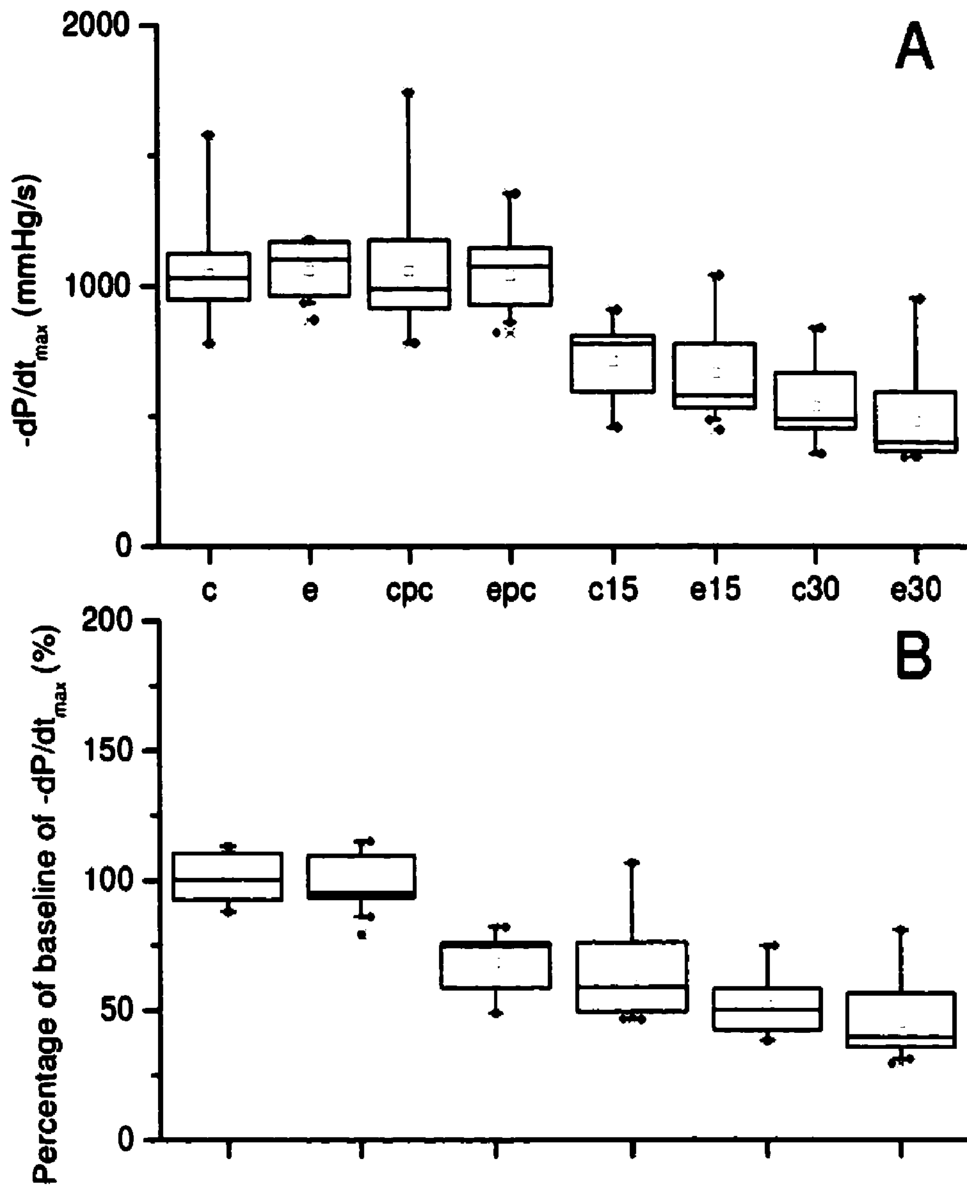


Figure 22. Negative dP/dt_{max} graphs for study two. Box plot of the negative derivative of pressure maximum raw values (A) and the calculated percentage of baseline (B).

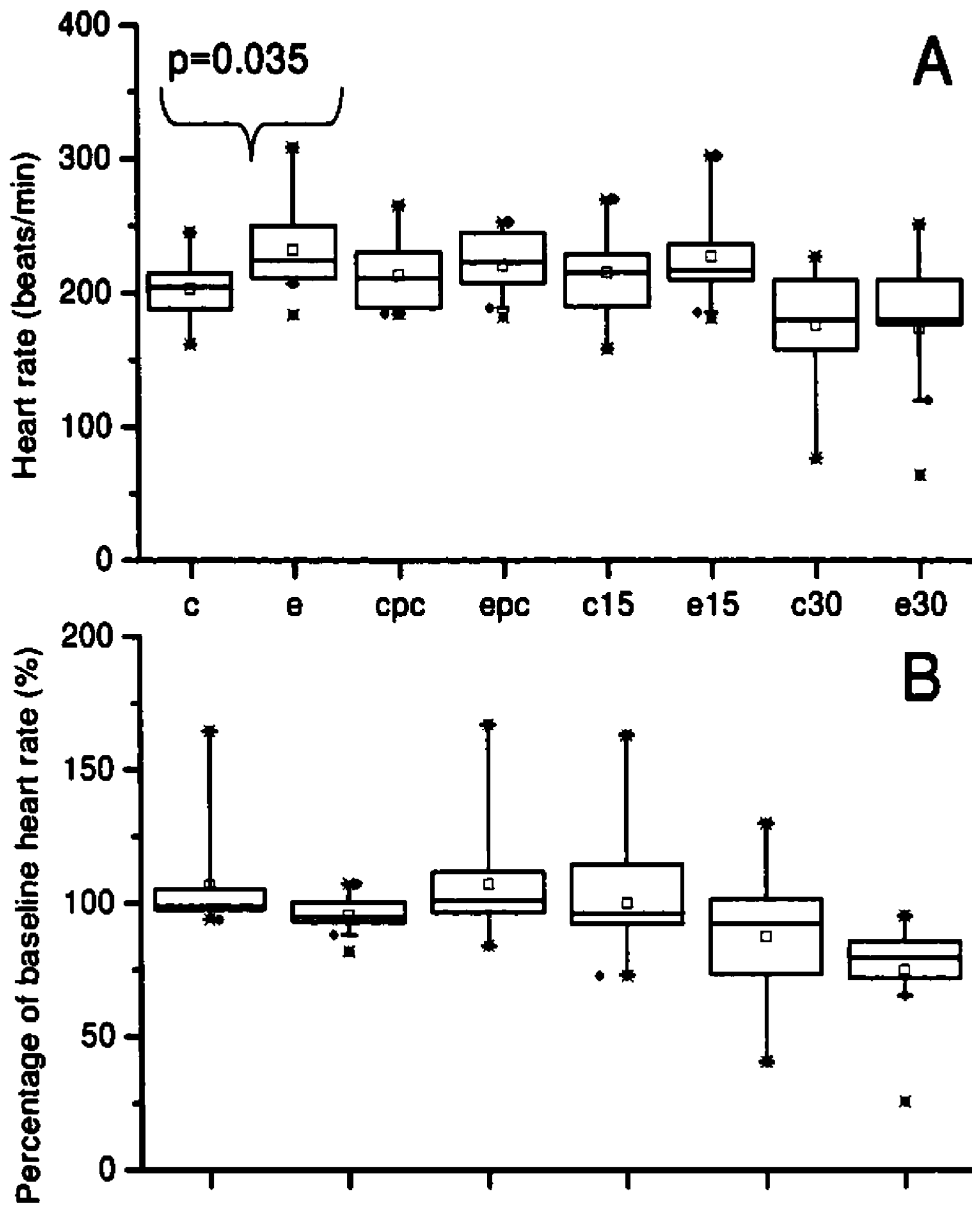


Figure 23. Heart rate graphs for study two. Box plot of heart rate raw values (A) and calculated percentages of baseline (B). Statistical significance indicated with p value

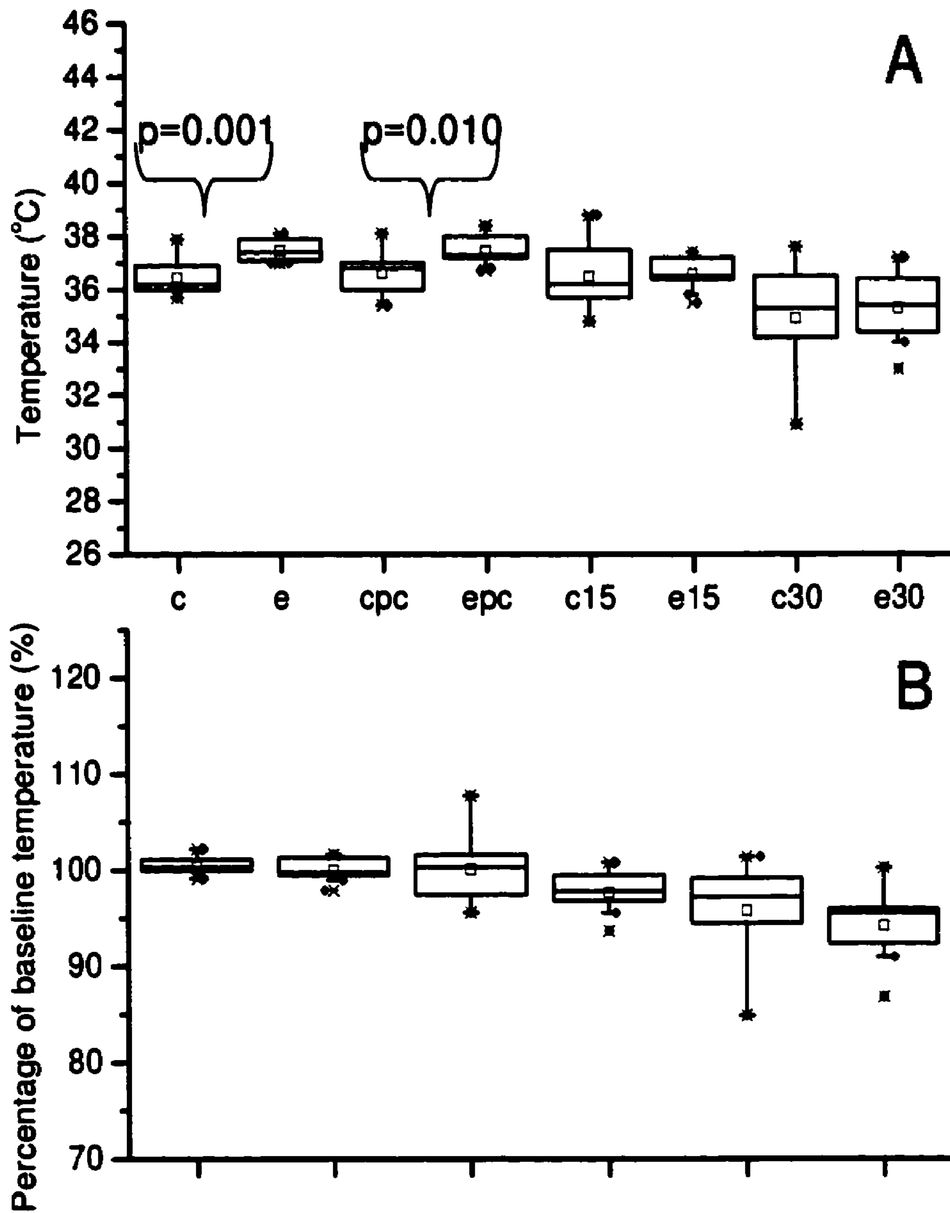


Figure 24. Temperature graphs for study two. Box plot of temperature raw data values (A) and calculated percentages of baseline values (B). Statistical significance indicated with p value.

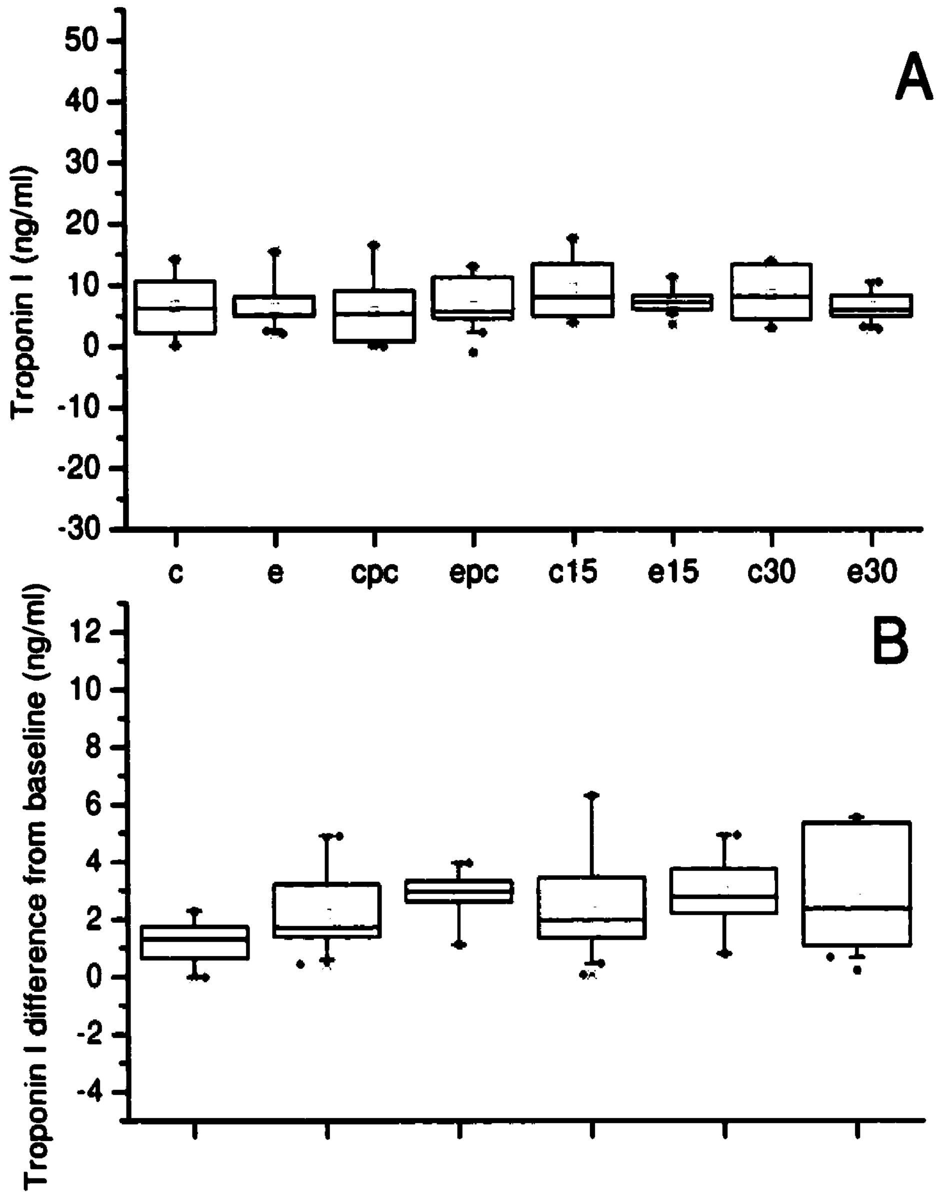


Figure 25. Cardiac troponin I graphs for study two. Box plot of cardiac troponin I raw data values (A) and calculated percentages of baseline values (B).

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Table 1. Baseline comparisons for both groups

1a. Weight, heart dry weight, temperature, heart rate and biochemical data

	Time to cannulation seconds	Weight, grams	Heart dry weight, milligrams	Heart rate, beats/min	Temperature, °C	cTnI, ng/ml
5 min	60.2 ± 5.9	171.7 ± 8.2	136.2 ± 8.8	246 ± 8	37.5 ± .2	6.04 ± 1.22
1 min	57.0 ± 4.9	139.1 ± 6.5*	121.4 ± 4.7	218± 7*	37.0 ± .2*	6.44 ± .94

1b. Baseline hemodynamic data for the five minute and one minute group

	Aortic flow, ml/min	Coronary flow, ml/min	Cardiac output, ml/min	Systolic pressure, mmHg	Diastolic pressure, mmHg	$+dP/dt_{max}$, mmHg/sec	$-dP/dt_{max}$, mmHg/sec
5min	19.4 ± 2.0	9.7 ± 0.7	29.1 ± 2.5	53.2 ± 1.2	15.45 ± 0.76	1039.8 ± 49.2	1027.2 ± 48.9
1min	15.3 ± 1.1	8.8 ± 0.6	24.1 ± 1.5	53.4 ± 0.8	16.65 ± 1.7	926.0 ± 28.3	1053.8 ± 36.8

Values represent means ± S.E. of 21 animals in each group* Values significantly different at $p<0.05$.

Table 2. Symbol legend

Symbols

Con	control group
PC	preconditioning group
baseline	measurement taken at baseline
PC	measurement taken after the PC administration (or time matched control)
15	measurement taken at 15 minutes of aerobic perfusion in working mode
30	measurement taken at 30 minutes of aerobic perfusion in working mode
%PC	percentage of PC value vs baseline
%15	percentage of 15 value vs baseline
%30	percentage of 30 value vs baseline
diffPC	value of the difference in [cTnI] at PC vs baseline
diff15	value of the difference in [cTnI] at 15 vs baseline
diff30	value of the difference in [cTnI] at 30 vs baseline

Table 3 – Mean and standard errors for data collected and calculated for study one. $p<0.05$ compared with the control group

3a. Hemodynamic Parameters

		baseline	PC	15	30	%PC	%15	%30
Aortic flow,	Con	21.0 ± 3.1	14.0 ± 2.1	7.5 ± 1.4	3.7 ± 0.6	71 ± 7	43 ± 10	23 ± 6
ml/min	PC	17.7 ± 2.7	18.2 ± 2.8	9.8 ± 2.3	5.1 ± 1.9	103 ± 13*	66 ± 14	29 ± 11
Coronary flow,	Con	9.8 ± 1.0	7.1 ± 0.8	4.8 ± 0.7	3.7 ± 0.7	74 ± 5	50 ± 6	43 ± 11
ml/min	PC	9.7 ± .9	9.3 ± .9	6.3 ± 1.1	5.3 ± 1.1	98 ± 6*	71 ± 16	55 ± 10
Cardiac output,	Con	30.7 ± 3.8	18.8 ± 2.5	12.3 ± 1.7	11.1 ± 1.5	63 ± 5	42 ± 7	41 ± 7
ml/min	PC	27.4 ± 3.2	24.5 ± 2.8	16.1 ± 2.9	15.1 ± 2.5	92 ± 10*	67 ± 13	60 ± 9

3b. Systolic and diastolic pressures with their derivatives

		baseline	PC	15	30	%PC	%15	%30
Systolic pressure,	Con	54.1 ± 1.7	49.4 ± 1.3	43.7 ± 1.4	40.7 ± 0.6	91 ± 1	81 ± 2	76 ±2
mmHg	PC	52.1 ± 1.8	49.4 ± 2.1	43.5 ± 1.7	37.2 ± 2.3	95 ± 2	84 ± 3	72 ± 4
Diastolic pressure,	Con	14.6 ± 0.8	17.6 ± 0.7	22.2 ± 0.7	23.9 ± 0.6	123 ± 4	157 ± 9	169 ± 11
mmHg	PC	16.4 ± 1.3	18.3 ± 1.4	21.0 ± 2.1	22.0 ± 2.4	111 ± 4	135 ±15	145 ± 21
$+dP/dt_{max}$,	Con	1033.4 ± 70.0	864.1 ± 66.8	542.1 ± 61.9	438.3 ± 39.6	85 ± 5	53 ± 5	45 ± 5
mmHg/s	PC	1046.8 ± 72.6	988.4 ± 96.1	613.6 ± 54.1	455.8 ± 57.6	94 ± 7	61 ± 6	45 ± 5
-dP/dtmax,	Con	1046.6 ± 75.7	857.9 ± 79.3	575.4 ± 68.8	423.4 ± 24.8	83 ± 5	55 ± 4	42 ± 3
mmHg/s	PC	1005.9 ± 63.6	943.1 ± 89.3	585.0 ± 49.0	438.4 ± 57.6	93 ± 5	59 ± 4	44 ± 5

3c. Heart rate and temperature data

		baseline	PC	15	30	%PC	%15	%30
Heart rate, beats/min	Con	247 ± 10	230 ± 6	230 ± 12	175 ± 18	94 ± 3	94 ± 6	72 ± 8
	PC	246 ± 12	244 ± 20	252 ± 23	203 ± 25	100 ± 7	102 ± 7	82 ± 9
Temp., °C	Con	37.4 ± 0.2	37.7 ± 0.2	35.5 ± 0.5	33.8 ± 0.6	101 ± .0.5	95 ± 1	90 ± 2
	PC	37.6 ± 0.3	37.6 ± 0.4	36.7 ± 0.4	34.9 ± 0.8	100 ± 1	98 ± 1	93 ± 2

3d. Cardiac troponin I data

		baseline	PC	15	30	diffPC	diff15	diff30
cTnI, ng/ml	Con	6.40 ± 1.92	6.63 ± 1.76	11.47 ± 2.00	11.48 ± 1.01	0.70 ± 0.45	4.85 ± 2.48	4.87 ± 1.42
	PC	5.90 ± 1.51	9.35 ± 1.13	8.70 ± 1.45	10.97 ± 1.35	3.45 ± 1.11*	2.80 ± 1.47	5.07 ± 1.50

Table 4 – Mean and standard errors for data collected and calculated for study two. $p<0.05$ compared with the control group

4a. Hemodynamic parameters

		baseline	PC	15	30	%PC	%15	%30
Aortic flow,	Con	14.8 ± 1.9	11.9 ± 1.9	9.4 ± 1.9	4.8 ± 1.1	81 ± 7	64 ± 10	36 ± 8
ml/min	PC	15.8 ± 1.4	14.5 ± .09	11.1 ± 2.0	6.1 ± 1.4	94 ± 4	69 ± 8	36 ± 5
Coronary flow,	Con	9.4 ± 1.1	9.6 ± 1.1	7.4 ± .1.0	6.0 ± 1.2	105 ± 5	81 ± 7	65 ± 10
ml/min	PC	8.3 ± 0.7	8.3 ± 0.7	5.3 ± 0.6	4.6 ± 0.7	102 ± 3	65 ± 6	54 ± 5
Cardiac output,	Con	24.2 ± 2.2	19.3 ± 2.0	16.8 ± 2.1	15.4 ± 2.1	81 ± 5	71 ± 6	65± 7
ml/min	PC	24.1 ± 2.0	19.8 ± 1.3	16.4 ± 2.4	15.7 ± 2.4	84 ± 3	68 ± 7	64 ± 6

4b. Systolic and diastolic pressures with their derivatives* indicates $p<0.05$ compared with the control group

		Baseline	PC	15	30	%PC	%15	%30
Systolic pressure,	Con	54.6 ± 1.3	53.6 ± 1.2	49.1 ± 1.4	41.4 ± 3.7	98 ± 1	90 ± 3	75 ± 6
mmHg	PC	52.4 ± 1.1	52.0 ± 1.1	46.6 ± 1.3	44.2 ± 1.2	99 ± 1	89 ± 3	84 ± 2
Diastolic pressure,	Con	15.2 ± 1.9	16.7 ± 1.5	20.1 ± 1.4	21.1 ± 1.4	119 ± 11	147 ± 16	153 ± 14
mmHg	PC	18.0 ± 1.4	18.1 ± 1.2	22.4 ± 1.1	23.7 ± 1.1	102 ± 2	129 ± 9	137 ± 10
$+dP/dt_{max}$,	Con	886.6 ±38.6	909.0 ± 54.2	697.2 ± 52.2	541.1 ± 47.0	103 ± 3	79 ± 5	61 ± 4
mmHg/s	PC	961.9 ±39.6	917.7 ± 47.6	659.2 ± 37.6	430.0 ± 37.3	96 ± 4	69 ± 4	45 ± 4*
$-dP/dt_{max}$,	Con	1047.0 ±69.7	1058.7 ± 90.8	710.1 ± 49.7	538.6 ± 47.6	100 ± 3	68 ± 4	52 ± 4
mmHg/s	PC	1060.0 ±34.3	1040.6 ± 50.8	666.5 ± 61.8	480.3 ± 55.8	98 ± 3	63 ± 6	45 ± 5

4c. Heart rate and temperature data * indicates $p<0.05$ compared with the control group

		baseline	PC	15	30	%PC	%15	%30
Heart rate,	Con	203 ±7	214 ± 8	215 ± 11	176. ± 14	107 ±7	107 ± 7	88 ± 8
beats/min	PC	232 ± 10*	221 ±8	227 ± 12	174 ± 16	96 ± 2	100 ± 8	75 ± 6
Temp.,	Con	36.5 ± 0.2	36.6 ± 0.2	36.5 ± 0.4	34.9 ± 0.7	100 ± 0.3	100 ± 1	96 ± 2
°C	PC	37.5 ± 0.1*	37.5 ± 0.2*	36.6 ± 0.2	35.3 ± 0.4	100 ± 0.4	98 ± 1	94 ± 1

4d. Cardiac troponin I data

		Baseline	PC	15	30	adiffPC	adiff15	adiff30
cTnI,	Con	6.60 ± 1.64	6.40 ± 2.05	9.41 ± 1.67	8.50 ± 1.46	1.36 ± 0.25	2.81 ± 0.30	2.96 ± 0.45
ng/ml	PC	6.32 ± 1.14	6.44 ± 1.33	7.15 ± 0.63	6.48 ± 0.77	2.20 ± 0.44	2.27 ± 0.53	2.71 ± 0.60

Table 5 Symbols used in all graphs

Symbol	
c	Control baseline value
e	Experimental baseline value
cpc	Time point one minute prior to cardioplegia for control hearts
epc	Time point one minute prior to cardioplegia for experimental hearts
c15	Sample taken at the 14th minute of working mode reperfusion for control animals
e15	Sample taken at the 14th minute of working mode reperfusion for experimental animals
c30	Sample taken at the 29th minute of working mode reperfusion for control animals
e30	Sample taken at the 14th minute of working mode reperfusion for experimental animals

9.0 Appendix

Preparation of microtitre plate for cTnI ELISA

1.) Immobilization of primary Ab to plate wells:

a.) prepare solution of :

19.37 µL goat polyclonal anti-human cTnI in 20 mL sensitizing buffer (bicarbonate pH 9.6, 0.1M)

b.) add 200 µl of this solution to each well to be used

c.) incubate overnight at 4^0 C, covered in parafilm

d.) remove solution via inverting the plate over the sink

e.) wash wells 2 times with PBS (phosphate buffered saline):

PBS: 0.1 M phosphate buffer in a solution of 0.15 M NaCl at pH 6.8

f.) add 250 µl of blocking buffer (1% BSA in PBS 0.1 M, pH 7.0) to each well and incubate for 60 minutes at room temperature on shaker at 200 rpm.

g.) wash wells 2 times with PBS-T (0.1 M phosphate, 0.15 M NaCl, pH 6.8, 0.03 % Tween-20) + wash wells once with PBS (as in step 1e.)

2.) Addition of Standards, Unknowns, and Antibodies

a.) add 50 µL of standard (recombinant cTnI) or unknown to the appropriate well: standards as such: 16 ng/ml--- 1.6 µL std in 25 ml distilled water. the 8,4,2,1 ng/ml standards are prepared via ½ dilutions in distilled water. i.e. 1 mL of 16

ng/ml and 1 ml water yields the 8 ng/ml std. note that the standards are prepared in 10 mL glass beakers.

b.) with no delay add 100 µl of primary antibody solution 7.77 µL 2I-14 and 5.35 µL 8I-7in 11mL of PBS-T-1° (0.1 M phosphate, 0.15 M NaCl, 0.05 % Tween-20, 1 % BSA, pH 6.0)

c.) the plate is then immediately incubated at room temp for 45 minutes on a shaker (200 rpm)

d.) wash wells four times with PBS-T buffer(as described in step1g.)

e.) wash wells once with the PBS (as described in step 1e.)

f.) add 150 µL of secondary Ab solution (3.2 µL goat antimouse Fc fragment specific anti-immunoglobin conjugated to HRP stored in 50/50 glycerol/water) in 16 mL P-T-HRP (0.02 M phosphate, 0.05% Tween-20) dilution 1:10,000.

g.) the plate is them incubated at room temp for 1 hour 20 minutes on shaker (200 rpm)

h.) wash four times with PBS-T

i.) wash once with PBS

3.) Addition of Substrate

a.) add 150 µl of OPD substrate solution (25mg OPD, 25 ml 0.1 M citrate buffer pH 5.0, 62.5 µl of 3% H_2O_2)

b.) incubate the plate at 25 °C for 20 minutes in the dark

c.) reaction should be stopped with 50 µL of 3 M H_2SO_4

d.) after this time the Softmax Pro should have the "READ" function initiated and measurements completed at 490 nm.

Buffers, Dilutents and Washes

Sensitization Buffer: Bicarbonate

-2.12 g of Na_2CO_3 (0.04 M)

-2.52 g of $NaHCO_3$ (0.06 M)

-500 mL of distilled H_2O

-titrate to pH of 9.6

-store in autoclaved 500mL container at $4^{\circ}C$

Blocking Buffer: PBS

-3.55 g of Na_2HPO_4 (0.05 M)

-3.45 g of $NaH_2PO_4 \cdot H_2O$ (0.05 M)

-4.39 g of NaCl (0.15 M)

-500 mL of distilled H_2O

-titrate to pH of 7.0

-store in autoclaved container at $4^{\circ}C$

-250 mg of BSA is added to 25 ml of this PBS prior to use on that day makes a 1% BSA blocker

Primary incubation buffer

-3.55 g of Na_2HPO_4 (0.01 M)

-3.45 g of $NaH_2PO_4 \cdot H_2O$ (0.01 M)

-4.39 g of NaCl (0.15 M)

-500 mL of distilled H_2O

-250 µL of Tween-20 (measured with a positive displacement pipette)

-titrate to a pH of 6.0

-store in autoclaved container at $4^{\circ}C$

-110 mg of BSA is added to 11mL of the buffer to yield 1% BSA on the day used

Secondary incubation buffer

-0.71g of Na_2HPO_4 (0.01 M)

-0.69g of $NaH_2PO_4 \cdot H_2O$ (0.01 M)

-500 mL of distilled H_2O

-250 µL of Tween-20 (measured with a positive displacement pipette)

-titrate to a pH of 7.0

-store in autoclaved container at 4^0C

Substrate Buffer

-10.5g of $C_6H_8O_7 \cdot H_2O$ (citric acid) (0.05 M)

-7.1g of Na_2HPO_4 (0.1 M)

-500 mL of distilled water

-titrate to pH 5.0

-store in autoclaved container at 4^0C

-25mg of OPD is added to 25mL of this solution just prior to use

-62.5µL of 3% H_2O_2 is added after the OPD is dissolved as close to actual time of use as possible

Wash Buffers

-7.1g of Na_2HPO_4 (0.1 M)

-6.9g of $NaH_2PO_4 \cdot H_2O$ (0.1 M)

-8.76g of NaCl (0.15 M)

-1 L of distilled water

-titrate after mixing to pH of 6.9

-to 500 mL of this add 150 µL of Tween-20 to make PBS-T (0.03% Tween)

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