

***ISCHAEMIC PRECONDITIONING OF THE HUMAN HEART:
MECHANISMS OF MYOCARDIAL PROTECTION.***

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To my father for his unstinting academic influence and to my wife and daughter for their love and support.

ABSTRACT

Ischaemic preconditioning (PC) describes the increased resistance to myocardial infarction that follows short sublethal periods of ischaemia. It has been shown to exist in all mammalian species investigated to date. In the early 90's, some evidence started to evolve which reported that this powerful cardioprotective phenomenon exists in man. After ischaemia that triggers preconditioning, there are 2 phases of protection: an early phase (short-lived) termed early or classic preconditioning and a late (more prolonged) phase termed a second window of protection. The evidence for this has primarily come from work on the rabbit and rat isolated hearts. There is no information related to the characteristics of this preconditioning phenomenon in humans.

The study commenced with the development of an *in vitro* model of human myocardium to study the effects of ischaemia and reperfusion injury. The data provided evidence that the *in vitro* incubation of human atrial tissue is stable and slices are viable for at least 24 hr and that this permits the study of early and delayed consequences of ischaemia and reperfusion in the human myocardium. This model was also used to study the preconditioning phenomenon fully. There were some unanswered questions pertaining to the characteristics of the PC phenomenon. The results showed that maximal protection can be achieved with 4-6 min of ischaemic stimulus/5min reperfusion and beyond that, protection was abolished. It also reported that this early phase was protective for only 2 hours after the trigger and that in the atrium, a second window of protection existed although much less potent.

Attention was then focussed on the mechanisms underlying this powerful event. The mechanisms have only been partly characterised in humans. It would appear that a trigger (e.g. adenosine, bradykinin, adrenaline) activates a receptor-mediated Gi protein which activates a signalling pathway (Protein kinase C + Mitogen activated Protein Kinase systems) which in turn open ATP-dependent K⁺ channels (end-effector), either at the sarcolemmal or mitochondrial membrane, which when activated leads to protection. The initial sets of experiments were performed to elucidate this effector via pharmacological means primarily. It showed that this putative effector in human preconditioning was the mitochondrial K_{ATP} channel and not the sarcolemmal channel, as previously described.

There has been conflicting evidence suggesting that this phenomenon is a healthy heart phenomenon. In parallel studies, experiments were conducted to explore the impact of cardiac function and diabetes mellitus on the protection induced by PC. The data revealed that cardioprotection induced PC is abolished in the failing myocardium and also in diabetic hearts. This is a novel finding and the implications are far-reaching. It also demonstrated that the failure to precondition the diabetic heart is due to dysfunction of the mitochondrial K_{ATP} channels and the mechanism of failure in the failing heart lies in other elements of the signal transduction pathway different from the mitochondrial K_{ATP} channels.

Despite intensive investigation into the phenomenon, clinical application is still controversial. Whether this powerful cardioprotective phenomenon has a role in clinical practice is still not established. The apparent discrepancy between clinical studies in the literature could be reconciled if one takes into account one possible hypothesis - that preconditioning and its salutary effects are only observed in situations of unprotected ischaemia. As a result, this led to the design of a prospective

randomised clinical study with the following aims: (i) to investigate whether ischaemic preconditioning with 5 min ischaemia followed by 5 min reperfusion is protective in patients undergoing coronary artery bypass graft (CABG) surgery with cardiopulmonary bypass using cardioplegia and ventricular fibrillation techniques and in patients undergoing CABG on the beating heart without cardiopulmonary bypass, and (ii) to elucidate the underlying cause of any protection. The results of the studies suggested that PC is protective in patients undergoing coronary artery surgery on the beating heart without the use of CPB but offers no additional benefit when associated to CPB regardless of the mode of cardioprotection used and the reason for this being that CPB per se induces preconditioning.

What is clear, however, is that the unravelling of the mechanisms and phenomenon of PC may permit the development of methods to delay the onset of irreversible ischaemic injury, so providing a greater time window during which reperfusion may be achieved.

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Presentations

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Abbreviations

An attempt has been made to avoid the use of abbreviations. In certain circumstances, due to the size of a technical term or the frequency of its repetition, use of abbreviations was considered prudent. Consequently, the following abbreviations have been used.

DCD; Diet controlled diabetes

NIDDM; Non insulin dependent diabetes

IDDM; Insulin dependent diabetes

LVEF; Left ventricular ejection fraction

LDH; Lactate dehydrogenase

CK; creatine kinase

OD OA; optical density absorbance

MTT; 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide

PKC; Protein kinase C

K_{ATP}; ATP-dependent potassium channels

MAP; Mitogen activated protein kinase

IP; Ischaemic preconditioning

PC; Preconditioning

CPB; Cardiopulmonary bypass

HR; Heart rate

TnT; troponin T

BCP; blood cardioplegia

X-clamp; cross clamp

CI; cardiac index

CVP; central venous pressure

MAP; mean arterial pressure

PAP; pulmonary artery pressure

PCWP; pulmonary capillary wedge pressure

PVRI; pulmonary vascular resistance index

SVRI; systemic vascular resistance index

CHAPTER I

INTRODUCTION

1.1 Introduction

Ischaemic heart disease remains the most common cause of death in the Western World today. In 1996, the total mortality rate from coronary heart disease was 160, 000 in the UK [183]. Symptoms of ischaemic heart disease include angina pectoris, myocardial infarction with its associated complications: left ventricular aneurysm, ventricular septal defect, acute mitral regurgitation, arrhythmias and cardiac failure.

Myocardial ischaemia results when coronary blood flow is reduced to such an extent that the supply of oxygen and nutrient substrates, required for energy metabolism, does not adequately meet demand. This ultimately leads to a reduction in energy production via aerobic metabolism, with a substantial increase in the anaerobic metabolic rate. Almost immediately after the onset of ischaemia, as a consequence of the loss of normal energy production, a profound contractile dysfunction becomes evident [36]. In addition to the biochemical changes that mark the onset of ischaemia, there are specific cellular and structural changes that can be discerned. As the duration of the severity of ischaemia increases, the resulting cellular injury becomes more severe, and reperfusion may then fail to effect an immediate return of function and/or salvage of the myocardial tissue. Simply, there comes a point of “no return”, beyond which, the myocytes are destined to necrose irrespective of reperfusion.

Investigators have sought and continue to seek new approaches to myocardial protection with the aim of preventing or limiting ischaemic injury. This could theoretically be accomplished by either reducing the metabolic rate of the myocytes, thus conserving energy, or by slowing down and delaying the occurrence of the biochemical changes associated with ischaemia. However, in the clinical setting of acute myocardial

infarction the only technique amenable to manipulation is the re-establishment of the coronary blood flow to the myocardium at risk.

Once the coronary occlusion is established, the use of therapies designed to limit the extent of acute myocardial infarction are hampered by the inherent time delay that often occurs between the onset of occlusion, and the diagnosis and institution of treatment. Apart from the early restoration of blood flow to the ischaemic myocardium using intravenous thrombolytics or emergency coronary artery angioplasty or bypass grafting (CABG), results have mainly been disappointing.

Early restoration of coronary blood flow (reperfusion) to the ischaemic myocardium, by use of thrombolytics or early CABG, may be thought of as “exogenous” means of myocardial salvage/protection. The success of these exogenous means of myocardial protection depends on how quickly they can be administered to the patient.

It is important to note here that the heart has its own inherent protective mechanisms designed to diminish the injury sustained during ischaemia, this being independent of restoration of coronary blood flow. One of these “endogenous” protective mechanisms is known as “ischaemic preconditioning”. It was described by Murry [224] and is triggered by a short (reversible) burst of ischaemia followed by reperfusion. This renders the myocardium more resistant to ischaemic injury. The importance and the potential mechanisms underlying this phenomenon are discussed in more detail later in this chapter.

1.2 Myocardial Ischaemia

Myocardial ischaemia refers to a pathophysiological state brought about by stopping or severely reducing coronary blood flow. As a result of reduced coronary blood flow, a state of “supply-demand imbalance” rapidly develops in which the blood supply is inadequate to meet the energy needs of the heart. This imbalance is brought about when the supply of oxygen and metabolic substrates is diminished thus causing a drastic reduction in mitochondrial oxidative phosphorylation [175,251]. In order to maintain a degree of energy production during ischaemia, the myocyte reverts to anaerobic metabolism with almost complete reliance on anaerobic glycolysis for energy production [224,257]. Although the glycolytic rate increases many fold, it fails to meet the overwhelming energy requirements of the myocyte for contractile and biochemical functions. To make matters worse glycolysis becomes self-limiting due to accumulation of glycolytic metabolites such as lactate and H^+ ions, which in turn inhibit glycolysis [155,251]. In addition to the acidotic load on the cell, H^+ ion accumulation leads to a Ca^{2+} accumulation in the cytosol and the mitochondrion thus contributing further to the ischaemic damage [286]. Acidosis also causes inhibition of specific calcium channels with an initial decrease in available free calcium for excitation-contraction coupling [89,105,130], an event which may cause a reduction in contractile force. Acidosis also renders the contractile process less sensitive to Ca^{2+} , thus further reducing the force of contraction. In essence ischaemia is an in vivo state characterised by severely diminished coronary blood flow, reduction of oxygen and nutrients, depressed or absent

mitochondrial respiration, and a many fold increase in the rate of glycolysis associated with an accumulation of anaerobic metabolites.

Different cell types withstand ischaemia for varying periods of time; skeletal muscle can still recover after several hours of ischaemia, whereas minutes of ischaemia are all that is required to cause irreversible neuronal injury [50]. Currently it is only possible to state that myocardial damage which occurs after 3 hours of ischaemic insult[155] is unlikely to recover at all. Some recovery of contractile function may occur, upon reperfusion, following 60 minutes of ischaemia, however, full recovery may require up to several hours to days of reperfusion.

Over the past decade, advances in research have furthered our understanding of myocardial ischaemia and the important relationship between ischaemia and contractile function. It is apparent that when the myocardium is subjected to an ischaemic insult, it undergoes a whole host of changes which can be categorised as reversible biochemical and structural changes, contractile dysfunction (stunning and/or hibernation) with its specific biochemical and ultrastructural changes, and irreversible myocardial injury (cell death and infarction). The different types of myocardial injury are described in detail below.

1.2.1 Reversible Injury - Following a short period of global or regional ischaemia myocardial tissue invariably undergoes biochemical [148,153] and structural changes [158,250,267] that are associated with anaerobic metabolism and the deprivation of oxygen and substrates. These changes are reversible in the majority of cases where the ischaemic episodes are limited to short periods [156,252]. Return of ischaemic tissue to both biochemical and structural normality, following reperfusion, may take from a few

hours up to several days to complete [155,251]. However, the point of no return, i.e. the point at which reversible injury becomes irreversible (cell death) is poorly understood [151,155].

1.2.2 Myocardial Stunning - This condition occurring during reperfusion and following short periods of ischaemia consists of myocardial contractile dysfunction, that, as pointed out as above, may last for hours or days before complete recovery [34,36]. It is of importance to note that cell death is not considered an underlying mechanism for the mechanical dysfunction in this setting, although some researchers do suggest that cell death may occur in some cases of stunning. Stunning also has been recognised in 5-10% of patients undergoing cardiac surgery [80,322] but it may be undetected in a great proportion of patients. The mechanisms underlying myocardial stunning remain unknown. Although the pathogenesis of myocardial stunning has not been definitively established, the two major hypotheses are that it is caused by the generation of oxygen-derived free radicals (oxyradical hypothesis) and by a transient calcium overload (calcium hypothesis) on reperfusion. The final lesion responsible for the contractile depression appears to be a decreased responsiveness of contractile filaments to calcium. Recent evidence [34] suggests that calcium overload may activate calpains, resulting in selective proteolysis of myofibrils; the time required for re-synthesis of damaged proteins would explain in part the delayed recovery of function in stunned myocardium. The oxyradical and calcium hypotheses are not mutually exclusive and are likely to represent different facets of the same pathophysiological cascade. For example, increased free radical formation could cause cellular calcium overload, which would damage the contractile apparatus of the myocytes. Free radical generation could also directly alter

contractile filaments in a manner that renders them less responsive to calcium (e.g., oxidation of critical thiol groups). However, it remains unknown whether oxyradicals play a role in all forms of stunning and whether the calcium hypothesis is applicable to stunning in vivo. Nevertheless, it is clear that the lesion responsible for myocardial stunning occurs, at least in part, after reperfusion so that this contractile dysfunction can be viewed, in part, as a form of "reperfusion injury." An important implication of the phenomenon of myocardial stunning is that so-called chronic hibernation may in fact be the result of repetitive episodes of stunning, which have a cumulative effect and cause protracted post-ischaemic dysfunction. A better understanding of myocardial stunning will expand our knowledge of the pathophysiology of myocardial ischemia and provide a rationale for developing new therapeutic strategies designed to prevent post-ischaemic dysfunction in patients.

1.2.3. Irreversible Injury - For decades researchers have attempted to establish the specific biochemical changes in the myocyte, that determine the point of onset of the irreversibility of ischaemic damage. Although this has remained elusive, various biochemical parameters such as the depletion of high energy phosphates, accumulation of Ca^{2+} , severe tissue acidosis, depletion of glycogen stores and many others have been linked to the onset of cell death [153,155,251]. It is likely that the various changes due to severe ischaemia all contribute to the demise of the myocyte, and that no single factor is wholly responsible for cell death. That the various biochemical changes may be interrelated has been well established. The relationship between hydrogen ion accumulation and calcium accumulation, and its effect on cell viability, has been highlighted by a number of investigators [54,89,105,130]. Depletion of high energy

phosphates and its influence on hydrogen ion and lactate accumulation, and subsequently calcium accumulation, has been vigorously tested[227,229,286] using models of ischaemia/reperfusion.

Myocardial infarction is the clinical expression of irreversible myocardial ischaemic injury. When ischaemia persists for increasing lengths of time cell death will inevitably occur, becoming clinically manifest as a myocardial infarction (necrosis). In this new setting of ischaemic injury the myocytes fail to contract, or to generate an action potential, and energy production ceases completely [153]. This may be associated with the clinical consequences of myocardial infarction which include chest pain, hypotension, arrhythmias, and pulmonary oedema. Myocardial infarction also leads to specific electrocardiographic and serum biochemical changes that can be detected within minutes-hours of its onset. The return of blood to the ischaemic heart may have an important role in the development of irreversible injury and this is discussed in more detail below.

1.3 Reperfusion

In experiments performed on animals, early restoration of coronary blood flow has been shown to limit the size of the infarct [129,149,156]. In man this may be accomplished by the use of thrombolytic therapy, percutaneous transluminal coronary angioplasty (PTCA) or by emergency CABG surgery.

Although early reperfusion of the ischaemic myocardium is probably the most important intervention in limiting the size of the evolving myocardial infarction, it may have serious consequences and the damage associated with this period is known as “reperfusion injury” [129]. Reperfusion injury can be manifested by contractile (eg.

stunning) and electrical events [33,119,199,200] that may place in jeopardy the life of the patient. The contribution of ischaemia and of reperfusion to tissue injury has been the subject of exhaustive debate for more than 3 decades.

Reperfusion at an early stage causes massive cell swelling with the formation of sarcolemmal blebs, contraction band necrosis with rupture of cell membranes [148,152]. This is also linked with calcium accumulation in the mitochondrial granules and release of cytosolic enzymes into the extracellular space[185]. Severe cellular changes have also been described following reperfusion late in the reversible phase of ischaemia [156]. Irreversible ischaemia seems to render cells, ion and energy depleted with complete disorganisation of the enzymatic systems causing mechanical and osmotic fragility of the myocytes [251]. Due to the loss of ionic homeostasis, once reperfusion occurs, the final blow is then delivered: the myocytes rupture with loss of integrity of the cell membrane. Needless to say that the role played by reperfusion in either accelerating the death of severely ischaemic myocytes, or actually causing their death remains unresolved and can only add to the complexity of the situation.

The consequences of ischaemia-reperfusion may be local, but tissue destruction may also occur at sites distant from critical injury. The “no-reflow” phenomenon associated with reperfusion may also be considered a form of reperfusion induced injury [37]. In this patho-physiological process progressive microcirculatory obstruction due to clumping of blood cells may lead to failure to establish coronary blood flow to the ischaemic regions [185]. The phenomenon remains poorly understood, but microscopic examination of the infarcted regions which suffered relatively short periods of ischaemia showed sludged red and white cells and oedema of the endothelial cell lining of the microcirculation [185].

1.4 Myocardial Protection

This refers to experimental and clinical means that may be employed in order to reduce the degree of ischaemic damage and ultimately reduce or even eliminate myocardial infarction.

Methods of myocardial protection may be applicable in patients with ischaemic heart disease (the use of pharmacological drugs), or in patients undergoing open heart operations where elective ischaemia is used to enable the completion of the surgical procedure. Examples of pharmacological approaches to myocardial protection include nitrates, calcium antagonists and β -blockers. Recently a new class of drugs, the ATP-sensitive potassium (K_{ATP}) channel openers, have been shown to be of tremendous benefit in the setting of both animal experiments and human trials of angina pectoris. The advent and use of thrombolytic therapy post-infarction has lead to increased incidence of myocardial salvage through reperfusion and improved patient survival.

Other forms of myocardial protection which are applicable to cardiac surgery include hypothermia, chemical cardioplegia, intermittent ischaemia and also cardiopulmonary bypass. Hypothermia can be produced by use of cold cardioplegia solutions, topical cooling with ice slush and total body cooling. Cardioplegia is the infusion of a protective solution through the coronary vasculature, resulting in rapid diastolic arrest, the slowing of degradative cellular reactions, and allows for the use of specific protective agents such as magnesium and procaine [39]. Intermittent ischaemia involves subjecting the heart to short episodes of ischaemia with adequate periods of reperfusion, allowing for recovery of metabolic pathways [317,318].

The tolerable duration of unmodified ischaemic arrest is considered to be under 20 minutes. If this length of time is exceeded irreversible myocardial injury may occur. There is evidence that by modifying the metabolic state of the myocardium during ischaemia it may be possible to delay or reduce irreversible damage.

The principle of myocardial protection using cardioplegia solutions is based on the fact that energy expenditure by the myocytes is almost totally devoted to contraction. Thus eliminating the contractile function, by arresting the heart in diastole (using high K⁺ concentrations), the energy utilisation is reduced by almost 90%. This enables the surgeon to subject the heart to otherwise intolerably prolonged ischaemic periods, which may be extendible to 2-3 hours in complicated procedures. During prolonged periods of ischaemia in order to improve protection of the myocardium even further, cooling of the heart to 4°C, as well as body cooling to 22-28°C adds greatly to cardioplegic solution [21,30,179].

There are potential problems with the use of cardioplegia: due to critical coronary arterial stenoses the distribution of the cardioplegia may be heterogeneous leaving areas of myocardium at greater risk of ischaemic damage. Also the cold solutions can cause damage to both the myocardium and the endothelium [51].

In addition to the use of chemical cardioplegia solutions, the application of brief intermittent ischaemic episodes to the heart enables the performance of aorto-coronary bypass surgery. This is performed while on cardiopulmonary bypass and combined with cooling to 32°C. During aortic cross-clamping (ischaemia) the distal coronary anastomosis is performed, which is followed by removal of the cross-clamp and reperfusion, during which time the proximal anastomosis of the graft to the aorta is

performed. This process is repeated until all the grafts are completed. It was believed that the repeated global ischaemia associated with this technique, might cause cumulative myocardial ischaemic damage. Geft et al [101] have shown that myocardial infarction may develop following several (18) periods of reversible ischaemia. This level of ischaemic insult is never reached in aorto-coronary bypass surgery.

Methods of protecting the myocardium as described above rely on either re-establishing blood flow or reducing the metabolic rate of the myocardium using cardioplegia or pharmacological means. In contrast, there exists a unique form of myocardial protection, of an endogenous nature, that does not rely on external influences. As mentioned before, this form of protection, which is triggered by short bursts of ischaemia and reperfusion, is termed ischaemic preconditioning.

1.5 Ischaemic Preconditioning

When the heart is subjected to brief periods of ischaemia separated by reperfusion it becomes tolerant to infarction from a more prolonged ischaemic insult. Murry et al have shown that four 5 minute coronary artery occlusions, separated by 5 minutes of reperfusion just prior to a sustained 40 minute ischaemic insult, resulted in a 75% reduction in infarct size when compared to sham control dog hearts [224]. This protective effect appears to occur in all animal species studied [11,83,167,226] including man [242,307,346] although the evidence for the latter is controversial.

Preconditioning protocols vary widely between experiments and species but with a similar degree of protection. It would appear that preconditioning protection is a graded response[274]. Preconditioning protocols range from a single ischaemic episode[186,321]

to multiple cycles [57,224] of ischaemia and reperfusion. There is no evidence to date investigating the optimal preconditioning protocol in man, but it may be possible that the optimal ischaemic preconditioning protocol depends on the species studied and the model used.

Ischaemic preconditioning has been shown to cause specific and favourable metabolic changes in the myocardium during the prolonged ischaemic insult. These include preservation of adenosine triphosphate (ATP) [226], reduced lactate production [11,167,226,287] and decreased intra-myocardial acidosis [11]. These findings have been confirmed in various species [88,150], and have been used in elucidating preconditioning in man [3]. Preconditioning has been shown to delay cell death, but does not completely abolish it. It has been shown that extending the ischaemic insult will eventually overwhelm preconditioning with loss of protection [224]. Ischaemic preconditioning has now been shown to protect the heart against infarction, reperfusion-induced arrhythmias, contractile dysfunction and ischaemic contracture [11,56,224,277].

There appears to be a bimodal phase to the cardioprotective nature of this phenomenon. There is an early phase (described by many investigators as classical) although very potent in terms of protection is short lived and decreases with time, lasting for 1-2 hours in most species [189,321]. Recently there has been evidence to suggest that there may be the reappearance of a delayed protective effect 24 hours later (second window or delayed protection) [177,201,291,323]. This delayed effect has also been shown to last for 72 hours [24]. There is no evidence to date that this delayed phase exists in man.

1.6 Possible Mechanisms of Ischaemic Preconditioning Protection

Several theories were initially proposed as possible explanations for ischaemic preconditioning, some of which have now been discredited.

1.6.1 Collateral Circulation - One of the earlier mechanisms proposed for ischaemic preconditioning protection was that of increased collateral blood flow to the ischaemic zone. Using a dog model (known to have high collateral flow), Murry et al [224] found that infarct size was reduced at all levels of collateral blood flow in the preconditioned hearts when compared to the control non-preconditioned hearts. Other researchers have been able to precondition certain animal species which are deficient in collateral blood flow [88]. The role of coronary collateral circulation in limiting ischemia and infarction has been studied prospectively. In humans, transient occlusion of a coronary artery angioplasty has provided evidence that collateral circulation decreases wall motion abnormalities, ST segment changes and lactate production [78]. Patients who have collateral flow also have a better outcome after coronary artery dissection and acute closure than patients without collateral flow [66]. Collateral circulation also limits infarct size during acute myocardial infarction with and without thrombolysis [234]. Although collateral flow may decrease coronary artery bypass graft patency in certain subgroups of patients, the perioperative infarct rate and mortality is decreased [48]. However, other researchers [88] have shown that in the presence of no collateral circulation, protection induced by ischaemic preconditioning is as effective, thereby calling into question the role played by recruiting collateral circulation within the myocardium.

1.6.2 Stunning - As seen before, this is the myocardial contractile dysfunction that occurs on reperfusion of the reversibly ischaemic myocardium[34]. This myocardial

contractile dysfunction was initially proposed as the underlying mechanism of ischaemic preconditioning protection, since reduced contractile activity may lead to energy conservation [34].

The lack of temporal relation between the induction and elimination of stunning, and of ischaemic preconditioning has suggested that the two processes are not inter-dependent. Thus, it has been shown that prolonging the reperfusion part of the ischaemic preconditioning stimulus results in loss of protection prior to elimination of myocardial stunning [225]. Also the time course of stunning is much more prolonged than that of preconditioning and can persist for hours to days [34], also the degree of stunning does not correlate with the limitation of infarct size [326].

Since in the setting of stunning myocardial contractility is depressed but is expected to undergo full recovery, myocardial contractility could be improved to pre-ischaemic levels. The use of inotropes such as adrenaline and isoprenaline is a potent method of improving contractility of the stunned myocardium. The potency of these β adrenoreceptor agonists has been established both experimentally and clinically. In animal experiments isoprenaline, for example, has been used to the so called inotropic reserve of stunned myocardium, which is probably independent of the size and extent of myocardial infarction/necrosis [271-272].

1.6.3 Heat Shock Proteins – Increasing whole body temperature causes the heart to become more resistant to subsequent ischaemia [344]. This increased myocardial tolerance has been associated with an increase in heat shock protein synthesis (a family of proteins synthesized upon stress, to protect from stress) [68-69,344]. Ischaemia itself can also cause an increase in the synthesis of these stress proteins, in particular the 72

kilodalton stress proteins. These proteins become raised following 2 hours of reperfusion and remain raised for at least 24 hours [172]. These findings contrast sharply with the time course of ischaemic preconditioning which tends to wane within 2 hours of reperfusion [321]. The mechanisms by which stress proteins exert their protective effects remain unknown, but it is most likely to be independent of classical ischaemic preconditioning [345].

Marber et al [201] found that cardiac stress proteins remain elevated 24 hours following a brief ischaemic insult or heat stress, and this was associated with limitation of infarct size. This work lead to the concept of the 'second window of protection'. Therefore the generation of heat-shock proteins may play a part in the delayed phase (second window) of ischaemic preconditioning rather than the classical phase [201].

1.7 Specific mechanisms shown to be involved in Classical Ischaemic Preconditioning

The underlying mechanisms of ischaemic preconditioning protection have now been partly characterised. It would appear that involvement of specific membrane receptor (adenosine, noradrenaline, bradykinin and possibly others) activation, protein kinase C (PKC) and mitogen activated protein kinase (MAPK) mediation and the possible opening of the ATP-sensitive potassium (K_{ATP}) channel may play an integral role in the protection conferred by ischaemic preconditioning.

1.7.1 Adenosine Receptor Stimulation

It has been shown that stimulation of the adenosine A₁ receptor mimics ischaemic preconditioning protection [192]. Also blockade of the receptors during a typical ischaemic preconditioning protocol abolishes cardioprotection in the rabbit heart [192] and in other species [15,52]. These data and others established the involvement of the adenosine receptor as a mediator of ischaemic preconditioning protection. There is also evidence that preconditioning involves a subtype of adenosine receptor, the A₃ receptor [191].

Adenosine is released from ischaemic myocytes (due to high energy phosphate breakdown), and is thought to act as a local regulator for the modulation of cellular function. Adenosine may also be released from the endothelium during ischaemia [29]. It appears to act on adenosine receptors, subsequently causing protection by modulation of a G_i protein. Also, during ischaemia, numerous other metabolites are released locally by the myocardium. These include catecholamines and bradykinin that have been proposed as triggers of ischemic preconditioning [104]. The role of adenosine in ischaemic preconditioning has been extensively examined in various animal models [15,52,192,281].

Adenosine A₁ receptors couple to PKC [138] with activation of PKC, appearing to be necessary for preconditioning protection to occur in the rabbit [349], the rat [281], and possibly the human myocardium [282]. Activation of PKC causes phosphorylation of a secondary effector protein, possibly the MAPK system [46], which may bring about protection by triggering a sequence of biochemical reactions in the cytosol. G_i proteins are also involved in the signal transduction pathway of ischemic preconditioning [306],

and these proteins have been suggested, in rat ventricular myocytes, to link adenosine receptors to an end-effector, initially thought to be the K_{ATP} channel [169]. The coupling to the G_i protein may explain why activation by other trigger receptors coupled to G proteins can also mimic preconditioning. Liu et al [191] have suggested that it may be necessary for multiple receptors to be populated in order to trigger preconditioning protection, and that although ischaemia may result in many receptors being occupied, the actual importance of individual receptors may vary amongst species. It may be necessary to exceed a certain threshold of receptor occupancy in order to stimulate the rest of the pathway leading to protection [191].

1.7.2 ATP-sensitive potassium (K_{ATP}) channel

It has been suggested that the ATP-sensitive potassium (K_{ATP}) channel may be the final end-effector of the mechanism of ischaemic preconditioning protection, since opening of these channels may cause an efflux of potassium which would shorten the cardiac action potential and so limit ATP depletion and calcium accumulation [14]. Experimental evidence for involvement of the K_{ATP} channel in preconditioning exists for the pig [273], and the dog [14,107], but studies in the rat [113,193] and the rabbit [305,309] have been conflicting.

Ischaemic preconditioning may therefore release adenosine which may activate PKC leading to protein phosphorylation ultimately altering the K_{ATP} channel sensitivity to ATP, leading to an earlier or greater channel opening during sustained ischaemia. As much of the thesis involves investigation into the role played by the ATP-sensitive K^+ channel (K_{ATP} channel) in protection of human atrium, the biology, the pharmacology

and relationship of this channel to myocardial protection will be discussed in detail below.

Ion channels are proteins that span cell membranes and make them permeable to physiological ions, with channel proteins being selective to particular ions. An aqueous pore runs through the protein from inside to the outside of the membrane, through which ions can flow passively, down the electrochemical gradient. This mode of passive flow through the K^+ channel is in common with that of other types of ion channel. Electrical current is carried when there is a flow of ions through the channel, and high rate of ion allows currents to be measured even from a single channel, as well as from whole cells by the electrophysiological technique of patch clamping.

The pore of most K^+ channels can be opened or closed, that is to say they are gated, by a conformational change in the protein molecule. Potassium channels are specific for K^+ , but differ by the factors which regulate gate opening or closing, or in the rate of K^+ flow through an open channel. Channels can be further subdivided depending on whether gating is dependent primarily on membrane potential, voltage gated channels, or whether it is linked to binding of a specific chemical ligand, ligand-gated channels; although ligand-gated channels often show some voltage sensitivity as well. ATP-sensitive K^+ channels have a specific K^+ selective pore, and intracellular ATP plays an important role in their gating. These channels provide a unique link between potassium permeability and action potential to cellular metabolism. K_{ATP} channels are found in excitable tissues such as the heart, skeletal and smooth muscle, neurones and pancreatic β -cells, and link the excitability of the cells to the metabolic state [9].

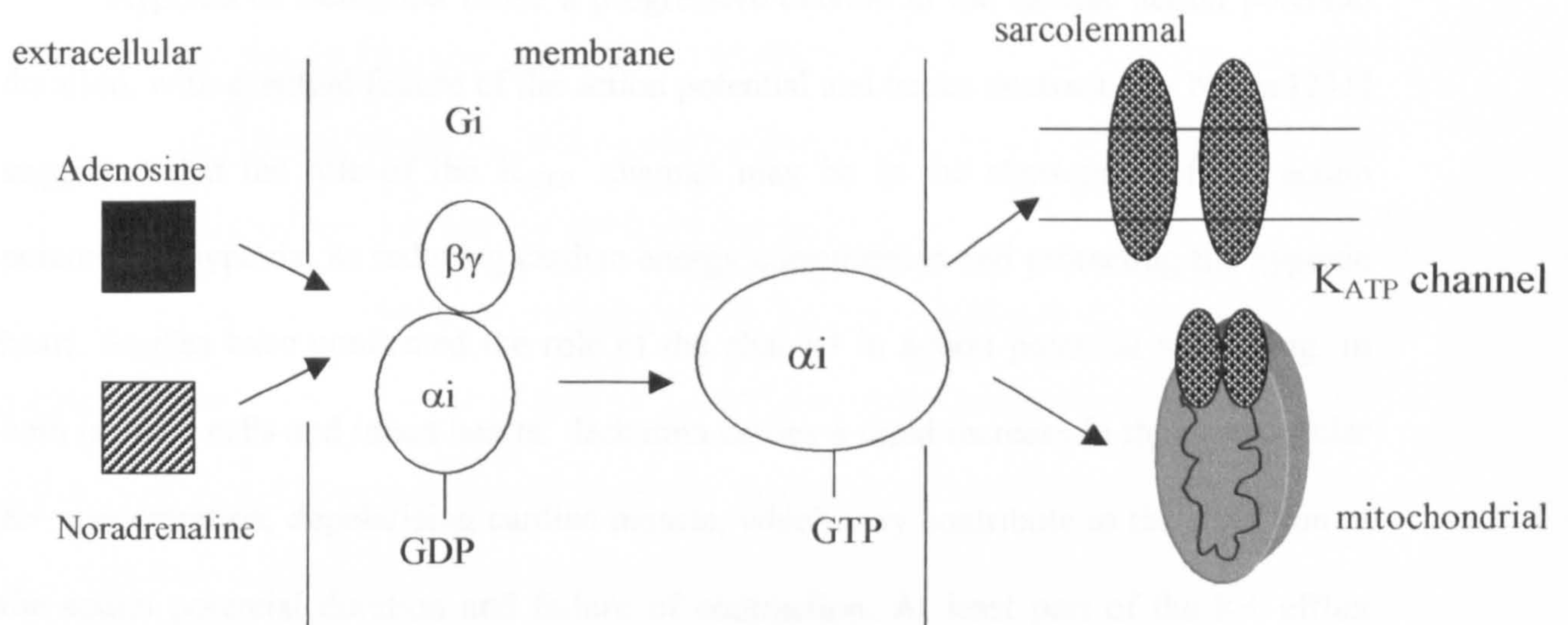
K_{ATP} channels were initially found in cardiac muscle in 1983 by Noma [231], but have since been identified in other tissues eg. skeletal and smooth muscle, pancreatic β -cells, certain neurones and epithelial cells. It is interesting to note that the properties of K_{ATP} channels differ between these tissues.

ATP probably binds to the sites on the protein to cause closure of the channel. The ATP binding site is known to be intracellular. Since ATP is ineffectual when outside the cell [72], the K_{ATP} channel provides a mechanism for linking membrane potassium permeability to cellular metabolism [231]. Intracellular factors affecting cardiac K_{ATP} channels include adenosine diphosphate (ADP), lactate and intracellular pH. It has been shown that it is the ADP/ATP ratio, rather than the ATP alone, that may exert a more sensitive control on the K_{ATP} channel [79]. Intracellular pH changes rapidly during hypoxia and ischaemia and this has been shown to affect these channels, with a fall in pH causing a decrease in the sensitivity to ATP, and so to channel inactivation by ATP [174]. Intracellular lactate also activates cardiac K_{ATP} channels [166]. These regulating factors change more rapidly than ATP itself during ischaemia/hypoxia, and all cause a shift in the ATP-inhibition curve, such that they reduce channel inhibition by ATP; ie. they cause an increase in K_{ATP} channel open probability at a fixed ATP concentration [79,174]. Activation of the K_{ATP} channel is inhibited (closed) at physiological levels of intracellular ATP and open when the intracellular ATP concentration falls.

In addition to regulation by metabolic factors acting within the cytosol, K_{ATP} channels maybe modulated by transmitters that activate membrane receptors linked to G-proteins. In ventricular myocytes, adenosine extracellularly binding to an adenosine receptor (A1) appears to activate K_{ATP} channels by way of the alpha-subunit of the pertussis toxin

sensitive G-protein (G_i , see Figure below) [169]. The G protein activation pathway may modulate the sensitivity of the K_{ATP} channel to inhibition by ATP, so acting via a similar manner to the metabolic regulators [143]. The activity of K_{ATP} channels can also be modulated by phosphorylation, which might come about by activation of protein kinase C (PKC) [9].

Other factors that influence the state of the K_{ATP} channel include the binding of Mg^{2+} salts of nucleoside diphosphates (NDP), such as Mg ADP and Mg GDP to an activating site on the channel. The activating site is different from the inhibitory ATP site. Therefore the opening of K_{ATP} channels is regulated by the quotient of ATP/NDP [9].



The physiological role of the K_{ATP} channels depends on the tissue. Those found in pancreatic β -cells determine the resting membrane potential and regulate insulin release in response to the plasma glucose levels. When glucose is taken up by the pancreatic β -cells, this causes an increase in ATP concentration near the channel, so closing the K_{ATP} channel which depolarises the β -cell and leads to Ca^{2+} entry into the cell via voltage-gated calcium channels, so inducing insulin secretion [9]. In many

tissues the K_{ATP} channels are closed under physiological conditions, only opening when the tissue becomes metabolically compromised.

1.7.3 K_{ATP} channels in Cardiac Muscle

The proposed function of these channels relates to the response of the myocardium to hypoxia and ischemia. Noma[231] originally hypothesised that this channel coupled myocardial metabolism to membrane electrical activity and suggested that opening of the K_{ATP} channel may serve as an endogenous cardioprotective mechanism.

Hypoxia or ischaemia cause a progressive decline in the cardiac action potential duration, with eventual failure of the action potential and hence contraction. Noma [231] suggested that the role of the K_{ATP} channel may be in the shortening of the action potential in hypoxia, so reducing cardiac energy consumption and protecting the hypoxic heart. Studies have confirmed the role of the channel in action potential shortening, in both isolated cells and intact hearts. Ischemia causes a rapid increase in the extracellular K^+ concentration, depolarising cardiac muscle, which may contribute to the reduction in the action potential duration and failure of contraction. At least part of the K^+ efflux occurs through K_{ATP} channels: K_{ATP} channel blockade reduces extracellular K^+ accumulation [100], while activation with nicorandil can increase the rise in extracellular K^+ seen during ischemia [215].

K_{ATP} channels are composed of 2 proteins, an inwardly rectifying potassium channel (Kir6.x) and a sulphonylurea receptor (SUR) sub-unit [302]. The channel pore region has been shown to be surrounded by 4 Kir6.x subunits, and each subunit requires a SUR subunit to form a tetradimeric channel [278]. At least 2 inwardly rectifying

subunits, Kir6.1 and Kir6.2, and 3 sulphonylurea subunits, SUR1, SUR2A and SUR2B have been identified and found to form channels with specific characteristics and tissue sites. K_{ATP} channels are present at high density (several thousand channels per cell) in the sarcolemmal and mitochondrial membranes of myocardial cells. It has been suggested that SUR1 and Kir6.2 are found in pancreatic islets, SUR2A and Kir6.2 in cardiac and skeletal muscle, and SUR2B and Kir6.1 in vascular smooth muscle. Unfortunately, the mito K_{ATP} channel has not been cloned as yet.

Noma [231] originally hypothesized that opening of the surface or sarc K_{ATP} channel produced by hypoxia, ischemia, or pharmacological K_{ATP} openers would enhance the shortening of the cardiac action potential duration (APD) by accelerating phase 3 repolarization. This enhanced phase 3 repolarization would inhibit calcium entry into the cell via L-type channels and prevent calcium overload.

In addition, membrane hyperpolarization or the slowing of depolarization would also inhibit calcium entry and slow or prevent the reversal of the sodium-calcium exchanger that normally extrudes calcium in exchange for sodium. The result of these actions would be a reduction in calcium overload during ischaemia and possibly early reperfusion and subsequent increased cell viability. Indeed, a number of early studies seemed to support this theory.

Inoue et al [141] first identified an ATP-sensitive K channel in the inner mitochondrial membrane (mito K_{ATP}) in rat liver by patch clamping giant mitoplasts prepared from rat liver mitochondria. These authors found that the mito K_{ATP} channel had several characteristics similar to those of the sarc K_{ATP} in that the channel was reversibly inactivated by ATP applied to the matrix side and inhibited by glibenclamide.

Subsequently, Jaburek et al [145] in Garlid's laboratory, isolated and partially purified a mito K_{ATP} channel from beef heart mitochondria that had several characteristics similar to those of the sarc K_{ATP} channel. However, the function of these channels appears to be intimately involved in matrix volume control as opposed to electrical activity for sarc K_{ATP} . In this regard, opening of mito K_{ATP} leads to membrane depolarization, matrix swelling, slowing of ATP synthesis, and accelerated respiration. Interestingly, these mito K_{ATP} channels are only sensitive to inhibitors of the channel such as 5-HD or glibenclamide when Mg, ATP, and a pharmacological or physiological K_{ATP} opener such as diazoxide or GTP are present [99].

1.7.4 Pharmacological K_{ATP} channel Openers

K_{ATP} channel openers have been divided into seven structural classes as shown below:

<i>Structural type</i>	<i>Prototype compound</i>
Benzopyrans	levcromakalim
Thioureas/cyanoguanidines	pinacidil
Pyridines	nicorandil
Pyrimidines/triazines	minoxidil
Benzothiadiazines	diazoxide
Thioformamides	aprikalim
Dihydropyridines	niguldipine

K_{ATP} channels are opened by a chemically diverse group of drugs, and in smooth muscle they generally act at low (nM to low μ M) concentrations, being potent vasodilators, whereas higher concentrations appear to be required to act in cardiac tissue (10-1000 μ M). K_{ATP} channel openers appear to be more effective under conditions where

K_{ATP} activation is already enhanced, eg. reduced intracellular ATP caused by ischaemia/hypoxia, and thereby appear to have useful property of targeting ischemic tissues. The consequences of the interaction of K_{ATP} channel openers, the ATP concentration and other metabolic regulators, is that K_{ATP} channel openers are much more potent during periods of ischemia or hypoxia. It is not yet known whether the drug receptor that K_{ATP} channel openers act upon is the K_{ATP} channel itself or a separate protein.

The first generation of these compounds lacked selectivity [13], but the newer compounds being developed are more likely to be tissue selective, and therefore have greater therapeutic potential. The profound vasodilatory effect induced by the early K_{ATP} channel openers may be due to their almost exclusive action on vascular smooth muscle, thereby limiting their use in patients. Newer agents have been proposed, eg BMS 180884, which appear to be cardioselective but have been shown to cause profound side effects and thus their clinical application has not succeeded.

At present in the UK, the only licensed K_{ATP} channel opener for patient use is nicorandil. Nicorandil is commercially available for the treatment of angina pectoris, but has not been shown to be superior to placebo when used as monotherapy [292]. At present, there is an on-going multi-center clinical trial investigating nicorandil as a treatment for angina pectoris as a second line drug (IONA Study) and this is expected to yield its interim analysis in the next year. The K_{ATP} channel opener activity of nicorandil causes an indirect blockade of calcium channels, and dilatation of arterial resistance vessels. In addition, the nitrate group activates guanylate cyclase, which causes an increase in intracellular cyclic guanosine monophosphate (cGMP) leading to dilatation of

venous capacitance vessels. At therapeutic doses nicorandil has little effect on blood pressure, with minimal increase in heart rate. Headache is the most frequently reported side-effect, with the majority reported in the early stages of treatment [91]. Nicorandil not only has beneficial vascular effects for patients with coronary artery disease, but since various animal experiments have shown it to be directly cardioprotective [106,178,220-221], it may well protect cardiomyocytes from the detrimental effects of ischaemia.

1.7.5 Pharmacological K_{ATP} channel Blockers

In common with the structural diversity of drugs that open potassium channels, the blockers are also chemically different. K_{ATP} channel blockers fall into three clinical categories:

1. Oral hypoglycaemic agents e.g. glibenclamide, tolbutamide
2. Antiarrhythmic agents e.g. sotalol
3. Bradycardic agents e.g. tedisamil

K_{ATP} channels are blocked by anti-diabetic sulphonylurea drugs [9] with glibenclamide, the most widely used for this purpose [84]. Glibenclamide has been reported to be effective in blocking K_{ATP} channels in the heart at a concentration of 0.1-10 μ M [90]. Glibenclamide has not been reported to block other types of K^+ channels in cardiovascular tissues, suggesting that it is selective for K_{ATP} channels in these tissues at concentrations up to about 10 μ M. Other sulphonylureas can also block K_{ATP} channels, but tolbutamide is much less potent than glibenclamide. Chemically different blockers that are site selective for K_{ATP} channels are 5-hydroxydecanoate for mitochondrial sites [232] and HMR 1883 for sarcolemmal channels [102]. Glibenclamide, 5-HD and HMR

1883 abolish the cardioprotective effects K_{ATP} channel openers but do not affect the activity of other classes of anti-anginal agents [265].

There is a growing body of evidence to support the thesis that the activation of the mitochondrial K_{ATP} channels may be the protein effector responsible for the protection observed in ischaemic preconditioning [99,196].

1.8 Human Models of Preconditioning

Despite the evidence suggesting the involvement of a number of potential theories for ischaemic preconditioning protection there remains a lack of a unifying hypothesis or pathway that may lead to protection. This makes it more interesting which mediator (s) may turn out to be involved in protection by ischaemic preconditioning in man. In contrast to the animals used for experiments with their normal myocardium and coronary blood flow, human subjects often suffer from anatomical coronary artery disease with its associated haemodynamic consequences.

Although a limited number of human studies have been performed, it remains difficult to adapt these models to investigate the underlying mechanisms of protection by ischaemic preconditioning. Therefore in vitro human muscle preparations have been developed which may be more easily adaptable for the purpose of investigating the involvement of individual mechanisms.

1.8.1 In Vitro Models

A. Human Atrial Trabeculae

An in vitro human model of preconditioning has been developed in Yellon's laboratory using isolated human right atrial trabeculae. This model allows ischaemic

preconditioning to be investigated using a functional end-point. In this model, preconditioned trabeculae had a significantly improved post-ischaemic recovery of contractile function compared to non-preconditioned trabeculae [285,325]. Using this model, Walker et al [325] showed that the adenosine A₁ receptor agonist R-phenyl-isopropyl-adenosine (R-PIA) protects human atrial trabeculae to a similar extent as ischaemic preconditioning. Speechly –Dick et al [325] from the same group showed that the non selective K_{ATP} channel opener cromakalim, conferred a similar degree of protection to that of ischaemic preconditioning and that the protection against contractile dysfunction can be induced by activation of PKC.

An in vitro model of ischaemia/reperfusion has been developed in our laboratory using slices of human right atrial trabeculae. As this model has been fundamental to this thesis, it will be discussed in greater detail in chapter II.

B. Cell Culture

Ikonomidis et al [140] have demonstrated that it is possible to directly precondition isolated human cardiomyocytes with ischaemia. After stabilisation, cells were exposed to simulated ischaemia for 90 minutes followed by re-oxygenation for 30 minutes. The cells were then subjected to an ischaemic preconditioning protocol consisting of 20 minutes of simulated ischaemia followed by 20 minutes of re-oxygenation. Cellular injury was assessed by the exclusion of uptake of trypan blue dye, and survival was assessed by culturing the cells for 24 hours post-intervention. Preconditioned cells showed reduced cell injury following the longer 90 minutes of simulated ischaemia: preconditioned cells had less uptake of trypan blue and increased survival rate. Ikonomidis' group also

examined the supernatant from cells, and found that the preconditioned cells had lower hydrogen ion, lactate and lactic dehydrogenase concentration.

Using this isolated cardiomyocyte model it has been shown that the exogenous administration of adenosine can mimic ischaemic preconditioning protection. In addition, administration of the supernatant from preconditioned cells was shown to protect other cardiomyocytes that did not undergo a preconditioning protocol, and that this protection could be blocked by the administration of the adenosine receptor antagonist 8-p-sulfophenyltheophylline (SPT) [139].

1.8.2 In Vivo Models

A. Angioplasty

Percutaneous transluminal coronary angioplasty (PTCA) provides an opportunity to study the human heart with respect to controlled regional ischaemia and reperfusion. It has been shown that following the first balloon inflation subsequent balloon inflations causes less chest pain, less ST segment shifts and less lactate production [79]. However, these studies must be interpreted with some caution because of the short periods of ischaemia involved (less than 2 minutes) and the possible role in the recruitment of collateral circulation. However, the same investigators [78] went on to report that the adaptation to ischaemia was probably not due to an increase in coronary collateral blood flow. Although Cribier et al [66] showed similar reduction in severity of angina and degree of ST-segment elevation, but acute recruitment of coronary collateral flow occurred and may have led to the improvements observed.

In the angioplasty model, Tomai et al [308] have also shown that the selective adenosine A₁ receptor antagonist bamiphylline was able to abolish the reduction in anginal pain and ST elevation normally seen with the second balloon inflation. This may suggest that the adaptation to ischaemia in man may involve adenosine A₁ receptor stimulation.

Tomai et al [307] also demonstrated that glibenclamide prevents the preconditioning effect seen during repeat balloon inflations suggesting that the K_{ATP} channels are involved in ischaemic preconditioning.

B. Prodromal Angina

Studies of prodromal angina (angina occurring prior to myocardial infarction) 24-48 hours before an acute myocardial infarction have provided evidence for both a positive and negative effect on the outcome following myocardial infarction. Ottani et al [234] showed that infarct size, as assessed by peak creatinine kinase (CK) MB fraction release was lower in patients with previous angina (within the preceding 24 hours) compared to those without. Also the number of hypokinetic segments was smaller in the angina group. Angiography also showed no evidence of increased collateral blood flow in the prodromal angina group [234]. Data from the Thrombolysis in Myocardial Infarction (TIMI) 4 study, showed that patients with angina preceding infarction by 48 hours, or with a history of angina, had a lower in-hospital death rate, decreased frequency of severe congestive cardiac failure and smaller infarct size using serial serum CK measurements. There was also no increase in collateral blood flow in these patients [171]. However, it should be mentioned that patients with a history of angina preceding myocardial infarction are more likely to have multi-vessel coronary artery disease and some studies

have shown worse long-term prognosis in these patients [67]. Most of the studies showing a negative effect of prodromal angina on outcome are from the period prior to the use of thrombolytic therapy, however, as reperfusion is necessary for the preconditioning phenomenon, these findings should be considered with caution. However, there are recent studies from the thrombolytic era which fail to show benefit [23].

C. 'Warm-Up' Angina

This situation describes acute tolerance to angina developing following repeated anginal episodes. In this model an initial effort induces angina, followed by a subsequent, equivalent effort which produces no angina with increased exercise tolerance [157]. It has been suggested that this could be due to an increase in collateral blood flow, although Okaszaki and colleagues [233] found no evidence for this. Wayne and colleagues [328] also found that the second effort reproducibly exceeded the first effort provided that these two were separated by a rest of at least 2-5 minutes but not more than 30-60 minutes. These time intervals are consistent with the well established biological nature of ischaemic preconditioning in animals [319]. Small collateral vessels which cannot be visualised by coronary angiography might 'open-up' between the two exercise periods and cannot be totally eliminated as a possible reason for the improvement of symptoms.

D. Cardiac Surgery

The first in vivo study using a model of global ischaemia, thereby eliminating the possible influence of collateral blood flow, was performed by Yellon's group [346]. They examined whether a preconditioning protocol prior to cross clamp fibrillation protects the myocardium from prolonged ischaemia during coronary artery bypass surgery. Twenty

patients were randomized into two groups. Patients randomized to preconditioning received a preconditioning stimulus of two 3 minute periods of cross-clamping separated by 2 minutes of reperfusion prior to the long 10 minute ischaemic insult. The control patients received 10 minutes cross-clamping with fibrillation only. During the 10 minutes cross-clamping the first distal aorto-coronary anastomosis was performed. Myocardial ATP was determined from biopsy specimens taken at the onset of cardiopulmonary bypass, at the end of preconditioning, and at the end of the 10 minute ischaemic insult. Preconditioning resulted in a significant depletion of the myocardial ATP content. The ten minutes of ischaemia resulted in a significant depletion of ATP in the controls. Ischaemic preconditioning appeared to slow down the rate of myocardial ATP depletion in the preconditioned group to such an extent that at the end of the 10 minutes ischaemic insult, preconditioned hearts had a significantly higher ATP content than the controls. This was in keeping with the metabolic changes that have already been demonstrated in animal models [252]. Yellon's group has also investigated whether preconditioning alters the release of troponin T, a reliable and sensitive marker of myocardial damage, in patients undergoing coronary artery bypass surgery using the same preconditioning protocol. They found that the preconditioned patients released significantly less troponin T into the blood at 72 hours post surgery [146]. However, subsequent investigators [161,242] have failed to show any benefit from preconditioning when used in similar models in the presence of cardioplegia. Therefore the evidence that ischaemic preconditioning protects the human heart still remains scant and conflicting.

1.9 Aims of the thesis

The aims of the thesis are to investigate the optimal preconditioning protocol in the human myocardium and the specific role played by K_{ATP} channels as an effector of ischaemic preconditioning protection. These experiments are based on an *in vitro* model of ischaemic preconditioning using human right atrial appendage myocardium.

1. To characterise the short and prolonged effects of ischaemia and reoxygenation in this model.
2. To characterise the ischaemic preconditioning phenomenon in the human myocardium.
3. To investigate the role of K_{ATP} channels as an effector in the protection induced by ischaemic preconditioning in the human myocardium
4. To investigate the effects of age and common pathological conditions i.e. diabetes mellitus, poor cardiac function on the protection induced by ischaemic preconditioning.
5. To investigate the protection of the human heart against myocardial tissue damage by ischaemic preconditioning in an *in vivo* clinical setting.

CHAPTER II

THE HUMAN RIGHT ATRIAL TRABECULAE MODEL

2.1 Introduction

Over the last two decades, a great deal has been learnt about the pathophysiology of myocardial ischaemia, the consequences of reperfusion and how to combat their adverse effects. Most of our knowledge has been gained by using in-vivo and in-vitro experimental animal models, and the extrapolation of this information to the human heart has resulted in the implementation of novel therapeutic approaches and in a progressive decrease in the death rate attributed to cardiac ischemic events.

Studies on cardiac ischaemia and reperfusion in man are difficult because of the presence and potential influence of a whole host of clinical factors. Several models of ischaemia/reperfusion have been utilised for the purposes of investigating mechanisms of ischaemic/reperfusion damage and also for targeting specific agents. The utilisation of human isolated myocytes [140,243], papillary muscles [52,237] and atrial myocardium [53,258-259,325] has provided the possibility to directly investigate the effects and mechanisms of ischaemia and reperfusion in man without the need to resort to assumptions from animal studies and to safely test interventions intended to be used clinically. Thus, for example use of cultured myocytes [55,140] and the right atrium [53,282] has served to identify some of the mechanisms involved in ischaemic preconditioning in the human myocardium, which compared with those seen in other animal species [17,107,187,273,324] has opened the door for its clinical application [346].

In this chapter, I describe an isolated atrial trabeculae model that has unique advantages. The right atrium preparation is of particular interest because the tissue is easily obtainable from patients undergoing open-heart surgery, it is simple to prepare and it is largely

inexpensive. Briefly in this model, right atrial tissue is sliced and then nourished and oxygenated in an organ bath using the principles of superfusion. The model has yet to be characterised and the aim of this chapter is to investigate the stability of the human right atrium when incubated in a buffered media, its response to various degrees of ischaemic insult and the short and prolonged effects of reoxygenation.

2.2 Tissue slices

Cardiac muscle cells are relatively small compared to skeletal muscle cells, so it is possible to slice myocardium without cutting more than a small proportion of them, especially if the slices are cut parallel to the long axis of the myocytes. With thin slices (0.2 –0.5 mm), vascular integrity are not required to support viability and metabolic function [237]. However, because oxygen and substrate supplies are derived from diffusion, the thickness of the slice is critical. It must be sufficiently thin to allow O₂ to diffuse to its center at the rate required to maintain aerobic metabolism.

2.3 Preparation of atrial slices

Specimens of human right atrium appendage were obtained from patients undergoing elective heart surgery. During surgery, the right atrial tissue is routinely removed for venous cannulation and establishment of cardiopulmonary bypass. Samples were quickly immersed in cold (4°C) Krebs-Henseleit-HEPES medium which comprised (in mM): NaCl (118), KCl (4.8), NaHCO₃ (27.2), KH₂PO₄ (1), MgCl₂ (1.2), CaCl₂ (1.25), Glucose (10), HEPES (20). The medium was pre-bubbled with 95% O₂/5% CO₂ to attain a pO₂ of 25-30kPa and pH 7.4. The atrial appendage was immediately sliced free hand with

Swann-Morton skin graft blades (Swann-Morton Ltd, Sheffield, UK) to a thickness of 0.5 mm and a weight 5-10 mg each as originally described for the preparation of rat renal slices [351]. The slices were weighed on a torsion balance (AND Ltd, Model HR 120, Bristol, UK). Briefly, the tissue was placed with its epicardial surface faced down on filter paper fixed to a rectangular glass base (5 x 25cm). A ground glass slide (2.5 x 7.5 cm) was then pressed against the tissue and the blade was drawn between slide and the tissue. The slicing apparatus and the tissue was kept wet at all times with medium which was stored on ice (4-10°C).

2.4 Experimental time course

After preparation, the slices (3-5 slices per specimen) were blotted with wet filter paper and loaded into glass conical flasks (25 ml Erlenmeyer flasks, Duran, Astell Scientific, Kent, UK), followed by addition of 5 ml of medium continuously bubbled with 95% O₂/5% CO₂ to maintain a pO₂ of 25 kPa and a pH of 7.4. The slices were then placed in a shaking water bath (100 cycles/minute) at 37°C for 30 min equilibration period (Figure 2.1).

Following this, the slices were rinsed with the medium, blotted and added to new flasks which also contained 5 ml of oxygenated medium for various periods of time to serve as time-matched aerobic controls. For the induction of simulated ischaemia, the slices were washed with one rinse of medium bubbled with 95% N₂/5% CO₂ at a pH of 6.8. In this solution, glucose was removed and replaced with 2-deoxy-D-glucose (grade II, 10mM) to maintain iso-osmolarity. The slices were then added into new flasks containing 5 ml of the same medium which was continuously bubbled with 95% N₂/5% CO₂ and maintained at 37°C during the entire ischemic period. Monitoring of pO₂ with an oxygen detector

of each ischemic period, slices were reoxygenated by removing the non-oxygenated medium, they were then rinsed with oxygenated medium and further incubated in 5 ml of oxygenated medium at 37°C with added glucose for another 120 min.

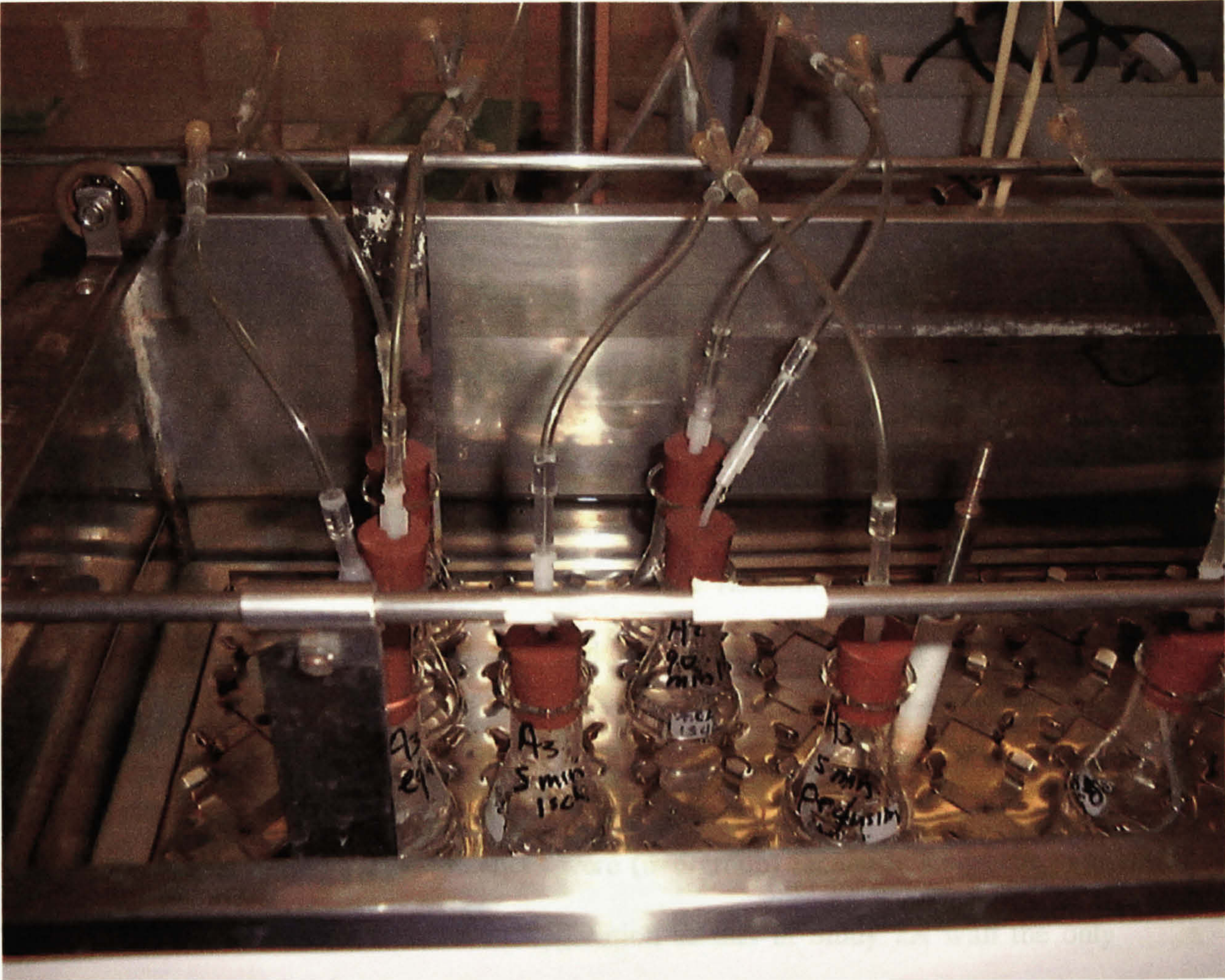


Figure 2.1. Photograph of the shaking water bath and the supply of oxygen and nitrogen/carbon dioxide

2.5 Study groups

Three different studies were performed to investigate: (i) the stability of the preparation (Study 1), (ii) the effect of the severity of ischaemia (Study 2), and (iii) the effect of the duration of reoxygenation (Study 3).

In Study 1 (Figure 2.2), atrial slices (n=6/group) were subjected to various periods of aerobic incubation after an initial 30 min equilibration period. At the end of the experimental time, samples from the incubation media were taken for the assessment of LDH leakage and the slices were removed for the determination of oxygen consumption, tissue viability, nucleotide metabolite analysis and morphological examination.

Study 2 was divided into two parts. In study 2A (Figure 2.3A), slices (n=6/group) were initially equilibrated for 30 min and then they were randomized to be subjected to various periods of ischaemia (30, 60 and 120 min) followed by 120 min of reoxygenation.

In Study 2B (see Figure 2.3B), the slices were (n=6/group) randomly subjected to an identical protocol of ischaemia and reoxygenation as that in Study 2A with the only exception being that they were incubated aerobically for 24 hr before they were subjected to ischaemia and reoxygenation. At the end of the experimental time, samples from the incubation media were taken for the measurement of LDH leakage and the slices were taken for the determination of tissue viability in both Study 2A and Study 2B. In addition, oxygen consumption of the slices was assessed in study 2A only.

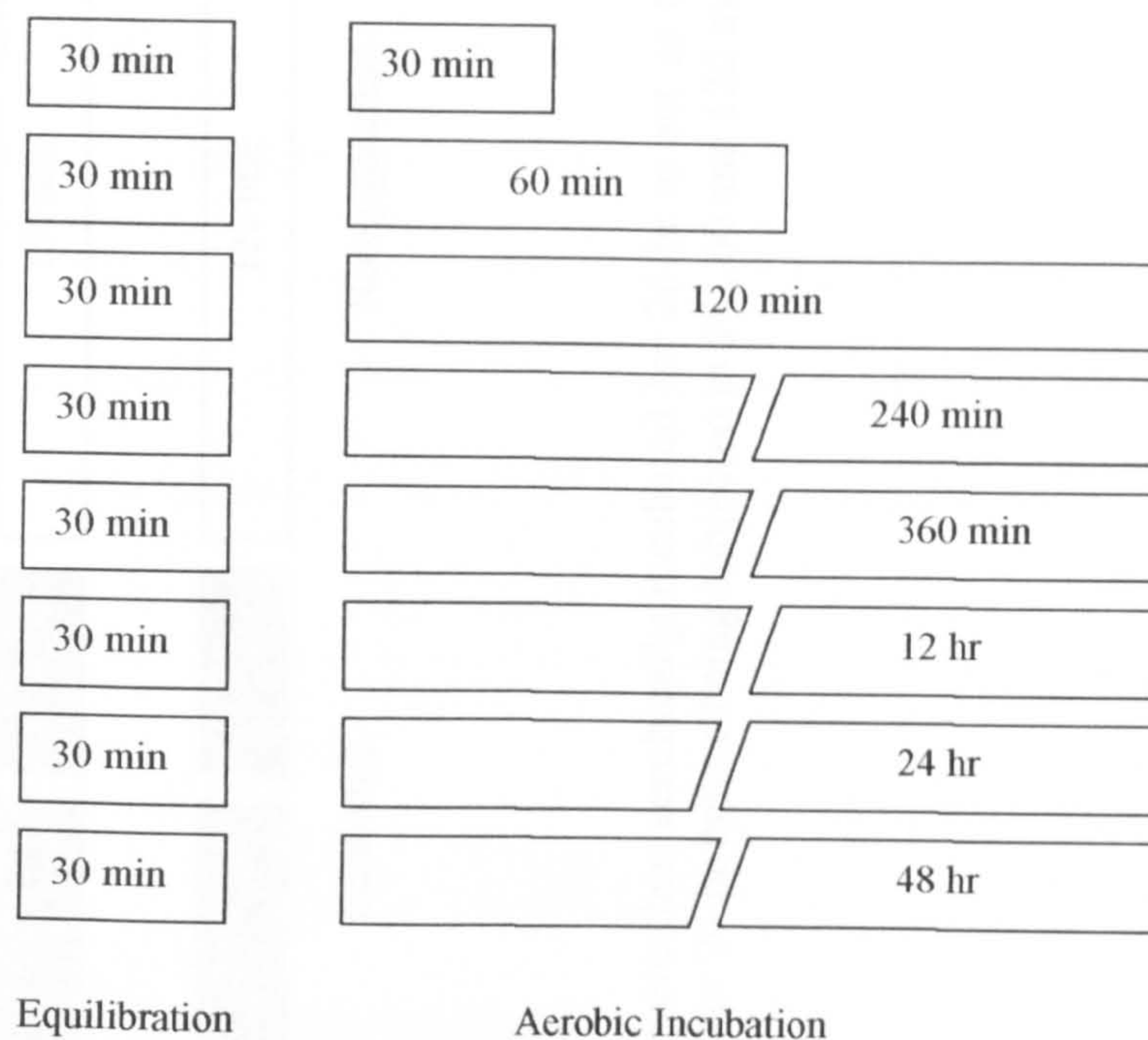


Figure 2.2. Experimental protocols in Study 1. All groups were equilibrated for 30 min in aerobic conditions (37°C). Following this, the right atrial slices (n=6/group) were further incubated aerobically for various time periods.

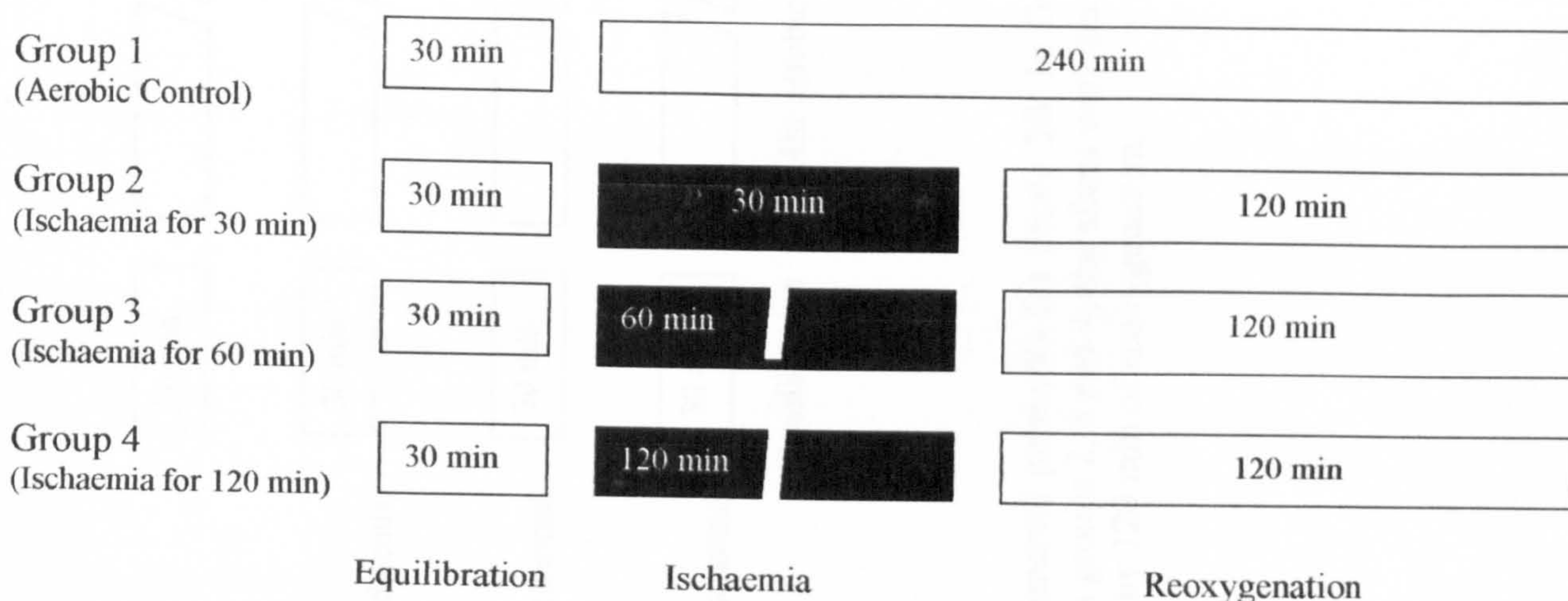


Figure 2.3A. Experimental protocols for Study 2A. In Group 1, the slices were then incubated aerobically for 240 min. After 120 min, the incubation medium was changed and the slices were incubated for further 120 min. In Group 2, 3 and 4, after equilibration, the slices were subjected to 30, 60 and 120 min of ischaemia respectively and then subjected to 120 min reoxygenation.

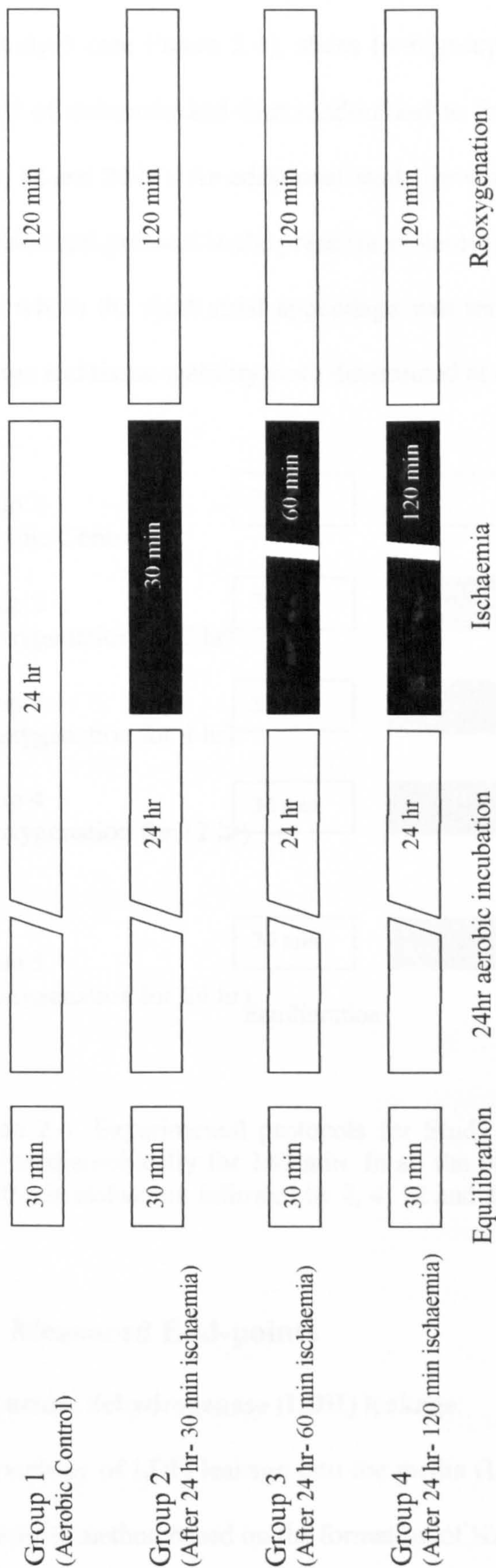


Figure 2.3B. Experimental protocols for Study 2B. In Group 1, the slices were then aerobically incubated for 26 hr to act as time-matched controls. In groups 2, 3 and 4, the slices were aerobically incubated for 24 hr before being subjected to 30 , 60 and 120 min of ischaemia followed by 120 min of reoxygenation.

In Study 3 (see Figure 2.4), slices (n=6/group) were subjected to a fixed 60 min period of ischaemia and then randomized to follow various times of reoxygenation (2, 4, 12 and 24 hr). An additional study (n=4/group) was performed using the same experimental protocol in the presence of neutrophils obtained from the same patients from whom the right atrial appendage was removed. As in previous studies, LDH leakage and tissue viability were determined at the end of the experimental period.

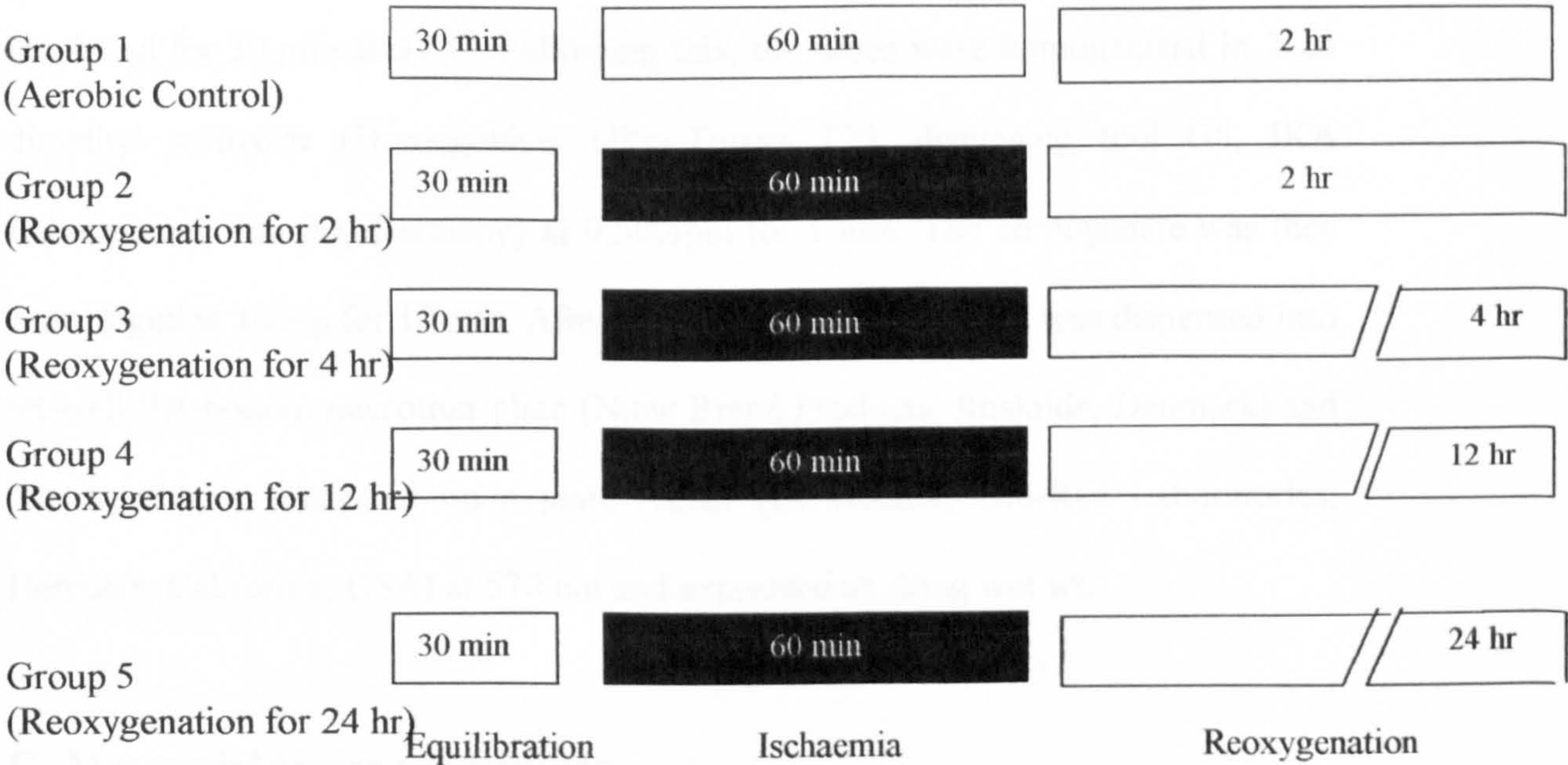


Figure 2.4. Experimental protocols for Study 3. In Group 1 the slices were then incubated aerobically for 180 min. In all the other groups the slices were subjected to 60 min ischaemia followed by 2, 4, 12 and 24 hr of reoxygenation.

2.6 Measured End-points

A. Lactate dehydrogenase (LDH) leakage:

The activity of LDH leakage into the media (U/g wet wt) was assayed by a kinetic ultraviolet method based on the formation of NAD (Sigma Catalogue No. 1340-K).

B. Tissue Viability:

The 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay was used to quantitate tissue viability. In this assay, the yellow MTT is reduced to a blue formazan product by the mitochondria of viable tissue. Briefly, at the end of the experiment, the slices were loaded into a Falcon conical tube (15ml, Becton Dickinson Labware, Cowley, UK) and 2 ml of PBS (0.05 M) containing MTT (1.25 mg/ml, 3 mM at final concentration) was added. The specimens were incubated for 30 min at 37°C. Following this, the slices were homogenised in 2 ml dimethyl sulfoxide (Homogeniser Ultra-Turrax T25, dispersing tool G8, IKA Laboratories, Staufen, Germany) at 9,500rpm for 1 min. The homogenate was then centrifuged at 1000g for 10 min. After this, 0.2 ml of supernatant was dispensed into 98-well flat-bottom microtiter plate (Nunc Brand Products, Roskilde, Denmark) and the absorbance measured on a plate reader (Benchmark, Bio-Rad Laboratories, Hercules, California, USA) at 570 nm and expressed as *A*/mg wet wt.

C. Myocardial oxygen consumption:

Oxygen consumption by the slices was measured by a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK). The electrode contained 1mL of air-saturated incubation medium (pH 7.4) and was set up at 37°C. The slices were loaded into the chamber with care to avoid the formation of bubbles and oxygen consumption was recorded for 5-8 min. Tissue respiration was calculated as the decrease rate of oxygen concentration after the addition of the slices and expressed as nmol O₂/g wet wt.

D. Metabolite analysis

Samples were taken and frozen in liquid nitrogen at the end of 0.5, 4, 24 and 48 hrs of aerobic perfusion. For tissue metabolite analysis, the dried slices were extracted into perchloric acid (0.6 mol/l, 25 µl/mg dry tissue). The extract was then centrifuged (11000 g for 5min at 4°C) before removal of the supernatant and neutralization with an appropriate volume of potassium hydroxide (2mol/l). Aliquots (20 µl) were taken for analysis by high performance liquid chromatography [280]. The values obtained (µmol/ g dry wt) were used to calculate the following index of myocardial energy status:

$$\text{Total adenylate pool} = [\text{ATP} + \text{ADP} + \text{AMP}]$$

(where ATP= adenosine triphosphate; ADP = adenosine diphosphate; and AMP – adenosine monophosphate).

E. Morphological Examination

Samples were taken at the end of 0.5, 4, 24 and 48 hrs of aerobic perfusion and fixed in 3% glutaraldehyde, post fixed in 2% aqueous osmium tetroxide, dehydrated in ethanol and embedded in Araldite resin. A semiquantitative estimate of cell damage was performed on 60-nM-thick-sections. Randomly chosen blocks from each tissue sample were examined for quantification of cell damage without prior knowledge of the protocol groups. 1 of 3 degrees of cell damage was assigned to each cell. Cell morphology was assessed according to following classification [10,219]: grade 1 (normal): compact myofibers with uniform staining of nucleoplasm, well defined rows of mitochondria between myofibrils, and the non-separation of opposing intercalated disks; grade 2 (mild damage): same as above,

except some vacuoles were present adjacent to the mitochondria; and grade 3 (severe damage): reduced staining of cytoplasmal organelles, clumped chromatin material, wavy myofibrils and granularity of cytoplasm or cells with contraction band necrosis.

F. Preparation of neutrophils

Heparinized, venous blood (4 ml) was obtained from patients the day before surgery in accordance with a protocol approved by our local ethics committee. Polymorphoneutrophils (PMNs) were isolated as described previously using Hypaque-Fico density-gradient dextran sedimentation [82]. Purified PMNs were resuspended in PBS and kept on ice until use. PMNs ($1-10 \times 10^6$ cells/ml) were added to the slices during the experimental protocol.

2.7 Statistical Analysis

Data was entered into SPSS 9.0 spreadsheet (Chicago, IL, USA). Data were expressed as means \pm SEM. One way analysis of variance (ANOVA) was used for the comparisons of more than two means. ANOVA for repeated measurements was used to test the significance of mean values overtime. A p value less than 0.05 was considered to be statistically significant.

2.8 RESULTS:

A. Stability of the preparation (Study 1)

LDH leakage: The leakage of LDH into the medium during the incubation period, as shown in Figure 2.5, represents the accumulation of the enzyme overtime. LDH leakage steadily increased during the first 12 hr of incubation, so that it rose from 2.8 ± 0.2 U/g wet wt at 0.5 hr to 10.4 ± 0.7 U/g wet wt at 12 hr. However, if the

leakage is calculated as the net LDH leakage (the difference in the enzyme leakage between two adjacent time points divided by the period of incubation in hours), it may be observed (Figure 2.6) that in fact the greatest leakage occurs during the first 0.5 hr (2.96 ± 0.31 U/g wet wt/hr) with a continuous decrease in the leakage during the ensuing 12 hr. Interestingly, irrespective of the way the results are expressed, there was hardly any LDH leakage between 12 hr and 24 hr of incubation but the leakage was massive by 48 hr of incubation. The logarithmic regression analysis of values revealed (inset to Figure 2.5) that the profile of LDH leakage for the first 24 hr of incubation was linear, this suggesting a single process of continuous enzyme leakage into the media. These results suggest that the preparation does not sustain significant tissue injury for the first 24 hr of incubation but that it has deteriorated by 48 hr.

Tissue viability: The reduction of MTT by the slices (see Figure 2.7), an index of tissue viability, was 0.89 ± 0.05 OA/mg wet wt after 30 min of incubation. These values were not significantly decreased after either 4 hr (0.84 ± 0.02 A/mg wet wt) or 24 hr (0.84 ± 0.03 A/mg wet wt). However, by 48 hr these values were significantly reduced to only 10% (0.09 ± 0.01 A/mg wet wt; $p < 0.05$) of the values seen in the 30 min incubation group. These results also indicate that the preparation is stable for at least the first 24 hours of incubation but that tissue viability cannot be maintained for 48 hr.

Myocardial Oxygen Consumption: As shown in Figure 2.8, the oxygen consumption by the slices was approximately 907 ± 39 nmol O₂/g wet wt/min after 30 min of incubation. Similar values were obtained after 4 hr (827 ± 21 nmol O₂/g

wet wt/min; $p = \text{NS}$) and 24 hr ($876 \pm 40 \text{ nmol O}_2/\text{g wet wt/min}$) of incubation, but again by 48 hr oxygen consumption dramatically decreased to 20% of the initial values ($184 \pm 12 \text{ nmol O}_2/\text{g wet wt/min}$; $p < 0.05$). These findings further support that this human atrium preparation is viable and stable for at least 24 hr of incubation but not for 48 hr.

Tissue metabolites and myocardial energy status: Table 2.1 shows the high-energy phosphate content of the slices following the various time periods of aerobic incubation. The ATP, ADP and AMP contents, ADP/ATP ratio and the total adenylate pool were all similar in the slices aerobically incubated for 0.5, 4 and 24 hr. However, in line with previous results there was a significant reduction in all metabolites after 48 hr of aerobic incubation.

Morphological Assessment

The results of the semi-quantitative morphological examination are shown in Figures 2.9. Figures 2.10a, b and c show representative high resolution electron transmission micrographs of grade 1 (normal), grade 2 (mild damage) and grade 3 (severe damage). The morphological appearance of specimens aerobically perfused for 0.5, 4 and 24 hr was similar; however, significant damage was observed in specimens aerobically perfused for 48 hr.

B. Effect of the severity of ischaemia: (Study 2)

LDH leakage: As shown in Figure 2.11, ischaemia caused a duration-related increase in enzyme leakage and this leakage was exacerbated when the preparation was incubated for 24 hr before being subjected to ischaemia.

Tissue Viability: The level of injury observed by the LDH leakage was mirrored by the MTT reduction results. As shown in Figure 2.12, ischaemia sustained for 30 min significantly decreased the ability of the slices to reduce MTT to 75% of the aerobic control mean values with further decrease when the period of ischaemia was extended to 60 and 120 min. Again, viability was further reduced when the tissue was incubated for 24 hr prior to ischaemia.

Myocardial Oxygen Consumption: As shown in Figure 2.13, oxygen consumption was significantly reduced by 30 min of ischaemia to almost 50% of the aerobic control values. However, in contrast with the pattern observed with LDH leakage and MTT reduction, the increase of the ischaemic time was not accompanied by a commensurate decrease in oxygen consumption and the mean values after 60 and 120 min of ischaemia were not significantly different from those observed after 30 min ischaemia.

2.10 Effect of the duration of reoxygenation (Study 3)

In this study, the period of ischaemia was 60 min in all groups but the muscles were reoxygenated for 2, 4, 12 and 24 hr.

LDH Leakage: Figure 2.14 shows that the enzyme leakage was gradually increased with the extension of the duration of reoxygenation; however, as shown in Study 1, this elevation may represent the overtime accumulation of the enzyme rather than net release of the enzyme. Thus, the regression analysis of the values (inset to Figure 2.14) revealed a LDH leakage profile similar to that seen in Study 1. Also a similar LDH profile was observed in the presence of neutrophils for the first 12 hr of reoxygenation, however, after 24 hr of reperfusion, LDH leakage was massive when compared with the group without neutrophils (20.32 ± 0.64 vs 11.02 ± 0.5 U/g wet wt respectively; $p < 0.05$).

Tissue viability: As shown in Figure 2.15 and seen before in Study 2, 60 min of ischaemia followed by 120 min reoxygenation significantly decreased MTT reduction. Interestingly, and in contrast with the LDH leakage results, extension of the reperfusion period beyond 2 hr revealed a delayed reoxygenation injury with three possible phases. The first corresponding to the initial 2 hr, the second observed between 4 and 12 hr of reperfusion and the third manifesting after 24 hr where the mean MTT reduction values decreased to 50% of the aerobic control values. Again, an identical profile of MTT reduction was observed for the first 12 hr of reoxygenation in the presence of neutrophils but a more substantial decrease following 24 hr of reoxygenation than that observed in the group without neutrophils (0.102 ± 0.04 vs 0.401 ± 0.02 A/mg wet wt, respectively; $p < 0.05$).

2.11 DISCUSSION:

The present studies have demonstrated that the incubation of human atrium slices in a buffered media is a useful preparation for the investigation of the mechanisms of injury underlying myocardial ischaemia and reperfusion in man. The preparation can be maintained stable and viable for at least 24 hr, the severity of ischaemia can be readily evaluated, and the possibility of attaining a long reperfusion period allows to distinguish between early and delayed reperfusion injury. These notable features of the preparation are of value for the investigation of ischaemic syndromes, for pharmacological and toxicological studies, and, potentially, for the evaluation of the effects of genetic manipulation of the human myocardium. A number of aspects of our studies warrant further discussion.

Stability of the preparation:

The in vitro model of human right atrium characterised in the present studies has the major advantage of being stable for at least 24 hr and it does not exhibit the rapid and gradual deterioration observed in other in vitro preparations during this period [96]. Thus, for the first 24 hr of incubation tissue viability is not decreased and myocardial oxygen consumption and high energy phosphates are maintained within the starting point value range. However, by 48 hr of incubation the preparation has deteriorated significantly, so that viability of the tissue has been reduced to almost only 10%, myocardial oxygen consumption decreased to less than 20% of the starting values and the total high energy phosphates decreased to 30% of the starting values. Morphological examination of the tissue also supports this thesis.

The stability of the preparation is also reflected by the profile of LDH leakage, an index widely accepted as a marker of tissue damage [126]. In both instances there was a small but gradual leakage into the incubation media that at first sight could be interpreted as indication of some on-going tissue injury, however, albeit this cannot be completely ruled out, it is most probably due to a physiological transmembrane movement of proteins and enzymes [128,210]. The massive LDH leakage observed at the end of 48 hr of incubation supports the argument that significant tissue damage has occurred by this time.

A number of factors including the temperature of the media in which the atrial tissue is collected and processed, the slice thickness and the pO_2 of the incubation media may affect the viability of the preparation. In preliminary studies (data not shown) we observed that all these factors play an important role in maintaining the stability of the preparation and that for this to be achieved the temperature of the media for the collection of the specimen should be 4-10°C, recovered and processed at about 37°C, the thickness of the slices should not be more than 0.5 mm and the pO_2 of the media should be 25-30 kPa. These findings are supported by Paradise *et al.* [237] and Prasad *et al.* [246] who report that the thickness of the muscle and the pO_2 of the media are determinants of the oxygen diffusion rate and the viability of the preparation. The small and not statistically significant reduction in tissue viability seen after the initial 30 min equilibration period associated with an elevated LDH leakage is possibly the result of mechanical injury sustained during the sectioning of the tissue. The possibility that ischaemic injury maybe a contributor to this phenomenon is unlikely because the procedure is carried out under hypothermic conditions and the time spent in processing the tissue samples is under 2 min, a time

clearly insufficient to induce myocardial injury. Furthermore, during this short period of sample processing the muscles are not preconditioned when subjected to a long period of ischaemia.

Studies on Ischaemia:

To the best of my knowledge, the present studies are the first in characterising the response of the human myocardium to various degrees of ischaemic insult. I have demonstrated that a period of simulated ischaemia of only 30 min already induces significant tissue injury, as measured by MTT reduction and LDH leakage, and that lengthening the ischaemic time to 2 hr results in a loss of viable tissue of more than 75% and massive LDH leakage. It is worth noting that the reduction in oxygen consumption observed after 30 min of ischaemia was not further decreased by 60 or 120 min of ischaemia. This suggests that in fact the remaining viable tissue augments oxygen consumption when compared to aerobically perfused tissue or to tissue subjected to shorter periods of ischaemia. This phenomenon of oxygen sparing effect after ischaemia has been described in various animal preparations [43,73,162,184] suggesting that it may not be a reliable index of tissue damage.

It is evident from these studies and, not unexpected, that although the atrial myocardium aerobically incubated for 24 hr is still viable it becomes more susceptible to ischaemic injury. This observation is of particular relevance when investigating the delayed effects of ischaemic syndromes such as the second window of ischaemic preconditioning.

Studies on Reoxygenation:

My findings show that two different pictures can emerge from the injury sustained during reperfusion depending on whether LDH leakage or MTT reduction are examined. In one hand, the absence of significant net increase in LDH leakage over the 24 hr reoxygenation period to that seen during the first 2 hr of reperfusion (results in Figure 2.14 as compared to those in Figure 2.5) suggests that in this preparation tissue injury is limited to the initial 2 hr reoxygenation period. On the other hand, the results of the MTT reduction support the view that reperfusion injury is a progressive process throughout the 24hr reoxygenation period. A possible explanation for this apparent discrepancy may be that LDH leakage represents the loss of enzyme from necrotic tissue whereas MTT reduction reflects the loss of tissue viability via both necrosis and apoptosis. If this is the case, then one may be tempted to conclude that necrosis is confined to the early reperfusion period ($\leq 2h$) and that apoptosis is the main mechanism for the loss of tissue viability during the late reperfusion period. Support that apoptosis may play a role in the injury sustained during ischaemia and reperfusion has been recently reported in different experimental preparations and animal species [103,206,244]. Certainly, more studies are required to clarify the role played by apoptosis in the ischaemia/reperfusion injury of the human heart. It is worth noting that the presence of blood components may influence the response and the degree of injury sustained during ischaemia/reperfusion. These studies have shown that neutrophils may exacerbate the late reoxygenation injury and therefore the presence or absence of blood components should be taken into account at the time of designing a study and when interpreting the results.

The present model may facilitate the investigation of the underlying mechanisms of injury during early and delayed reperfusion in the human myocardium. This model will also allow us to elucidate the true effects of therapeutical interventions. By looking exclusively to the early reoxygenation period, it may be possible that many interventions proved to be beneficial in the past may have in fact limited therapeutic value if their action is delaying rather than reducing myocardial injury.

Comparison with other preparations:

The use of either in vivo and in vitro experimental models have advantages and disadvantages of their own but both are regarded as necessary and complementary. Thus, for example, in vivo models are useful to study the physiological relevance and long term effects of the processes under investigation, however, they are complex and the influence of factors such as blood elements, neuroendocrine system, and even animal welfare handling and seasonal variations cannot be ruled out. By contrast, in vitro models are not exposed to internal and external effects as living animals, but they are limited by their short duration (usually a few hours) and stability.

Studies on myocardial ischaemia in the clinical setting are difficult to carry out and to interpret and frequently they are ethically unacceptable. An alternative to overcome these difficulties is the use of the right atrium in preparations like the one presented here. Certainly, this right atrium preparation is relatively easy to use, the tissue is readily available and inexpensive since it is regarded as “surgical waste” in open-heart procedures, and more importantly, it provides meaningful information on the human myocardium. The use of atrial tissue for studies with ischaemia may also

offer advantages over isolated and cultured myocytes since these are more difficult to obtain, they do not retain the normal cell-to cell contact, ischemia is more difficult to accomplish and they can be maintained viable for short periods of time [42,315]. A notable benefit derived from the prolonged stability of our preparation is that effects of reperfusion can be examined for a period that extends beyond the first few hours that usually is not possible with in-vitro preparations.

Limitations of the preparation:

My study has several limitations which need to be mentioned. First, the preparation is superfused (“simulated ischaemia”) as opposed to being arterially perfused. However, the removal of the vasculature as the natural pathway for the provision of substrate may also be advantageous by separating the confounding effects of the vascular changes and collateral flow induced by ischaemia and reperfusion.

The present study used atrial tissue to characterize the effects of ischaemia and reperfusion in the human myocardium. However, atrial and ventricular myocardium possess characteristics of their own that may influence the susceptibility to ischaemia/reperfusion injury and as a consequence results from one may not be applicable to the other. Thus, for example, the reported differences in the distribution of potassium channels [132], which contribute to the characteristic differences between atrial and ventricular action potentials, may determine a different response to ischaemia/reperfusion.

Albeit, the fact that atrial tissue is generally stable and disease free, individual biological variation between patients may result in different susceptibility to ischaemia/reperfusion injury and this must be accounted for when interpreting results from any human study.

2.12 Conclusion:

I have characterised a model of ischaemia and reoxygenation of human myocardium using right atrium appendage obtained from patients undergoing cardiac surgery. The tissue is readily available and the preparation is inexpensive. The preparation is stable for at least 24 hr and this permits the study of the early and delayed consequences of ischaemia and reperfusion. In addition, the extended stability of the model may be potentially used for genetic manipulation to investigate the pathophysiological mechanisms underlying the injury sustained during ischaemia and reperfusion, and to develop new therapeutical strategies to combat their undesirable effects. Once, the model was characterised, I will be able to use it to investigate the phenomenon of ischemic preconditioning in the human myocardium. The characterisation of the early and delayed windows of protection has not been explored in full and is the subject of the third chapter.

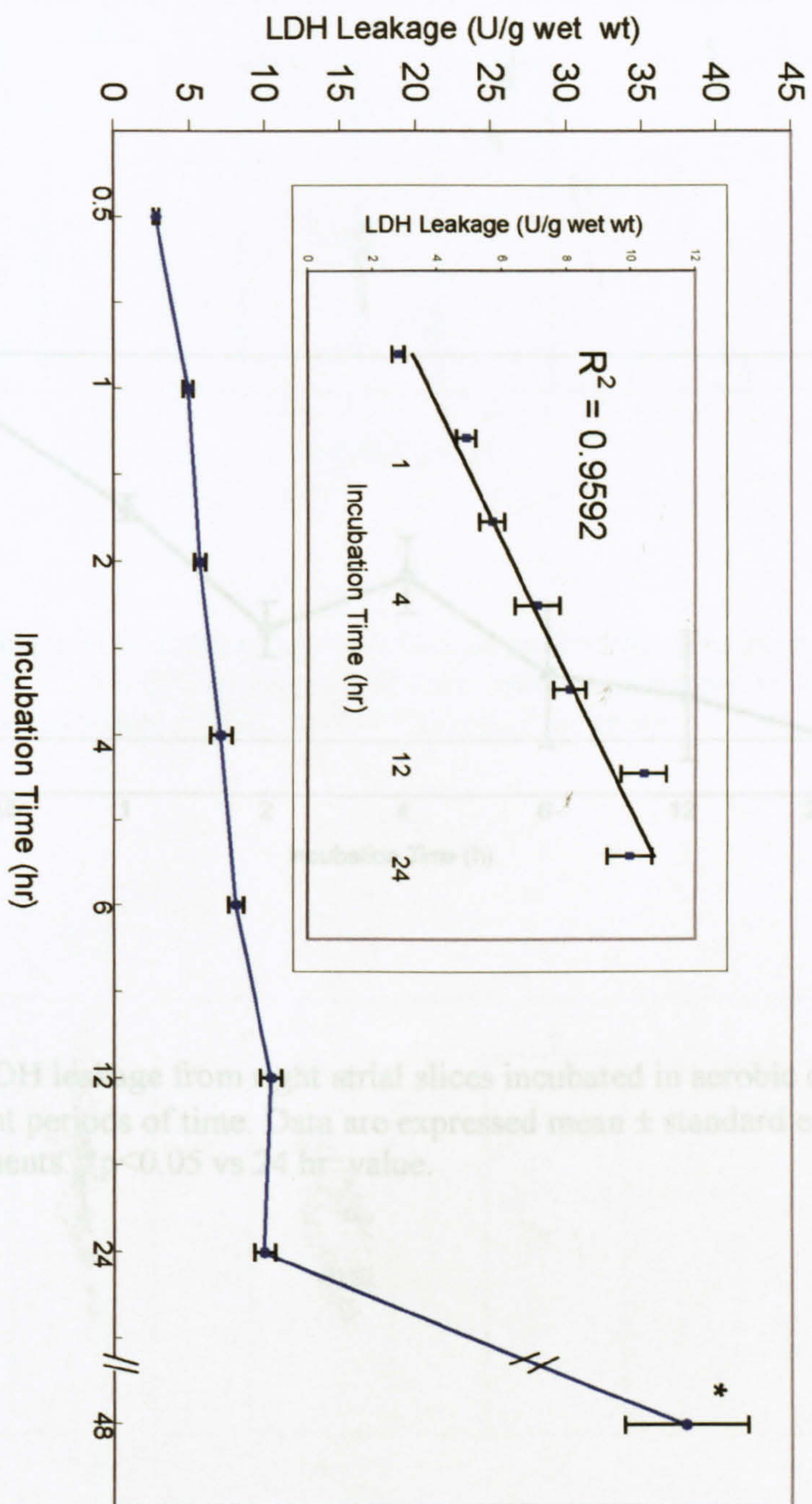


Figure 2.5. Leakage of lactate dehydrogenase (LDH) from right atrial slices incubated in aerobic conditions (37°C) for different periods of time. Data are expressed as mean value. Inset: Log linear regression analysis of the data over the 24 hr period.

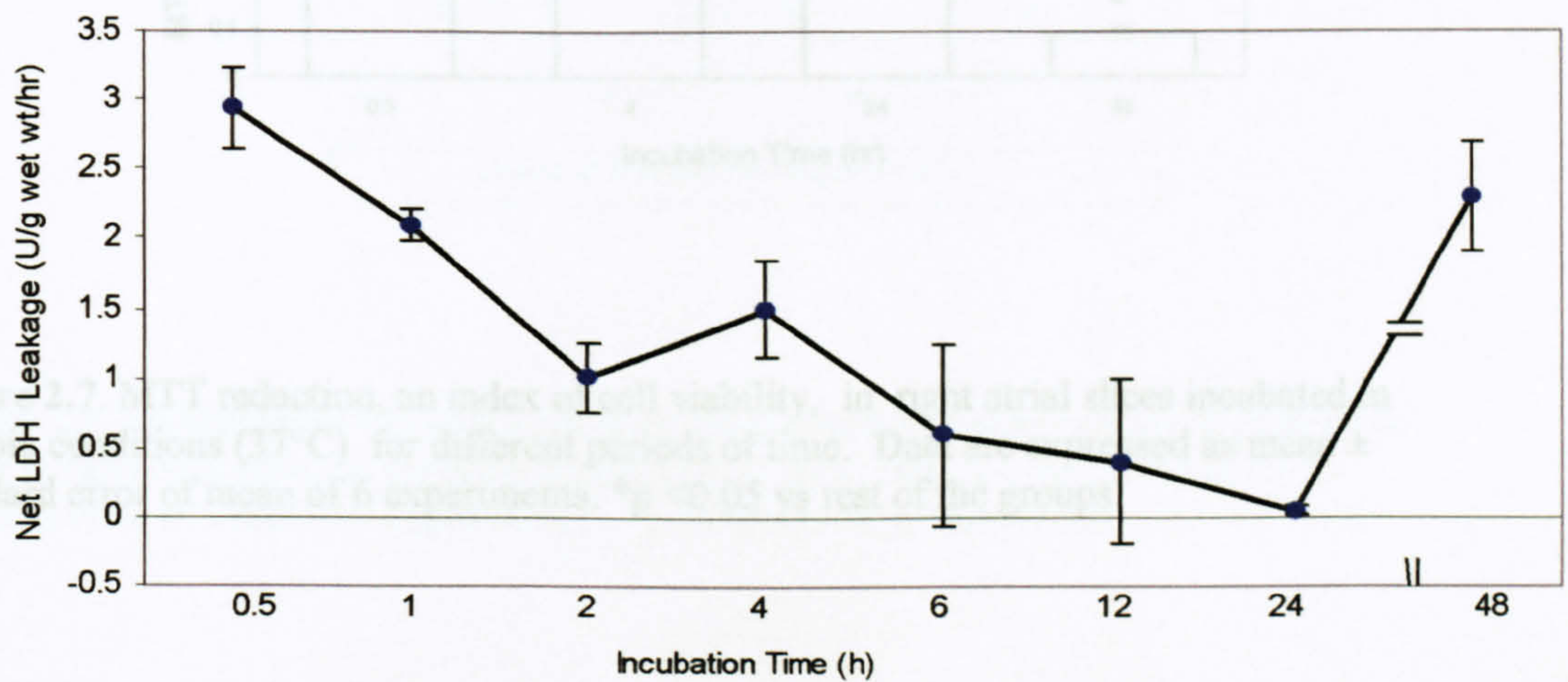


Figure 2.6. Net LDH leakage from right atrial slices incubated in aerobic conditions (37°C) for different periods of time. Data are expressed mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs 24 hr value.

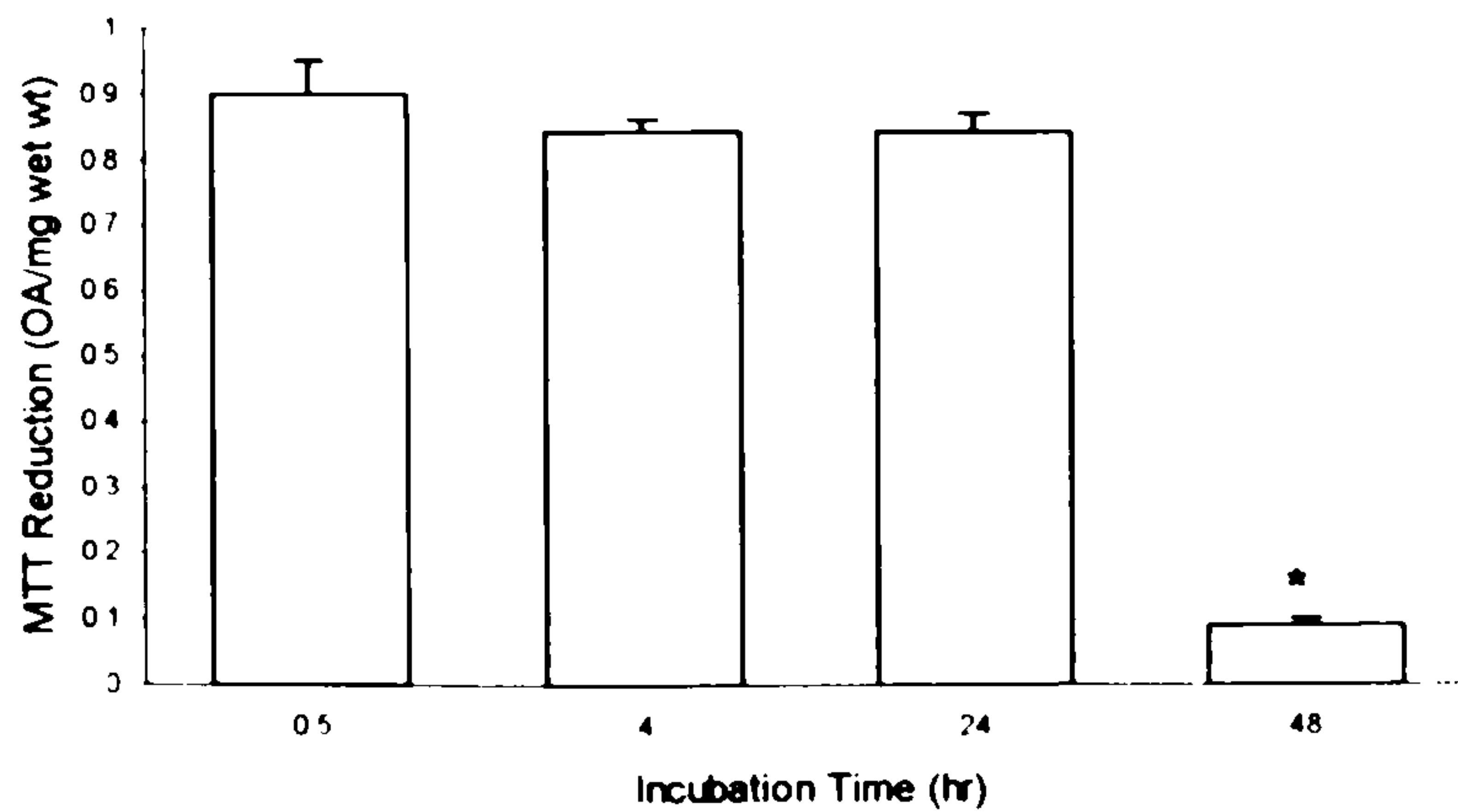


Figure 2.7. MTT reduction, an index of cell viability, in right atrial slices incubated in aerobic conditions (37°C) for different periods of time. Data are expressed as mean \pm standard error of mean of 6 experiments. *p < 0.05 vs rest of the groups.

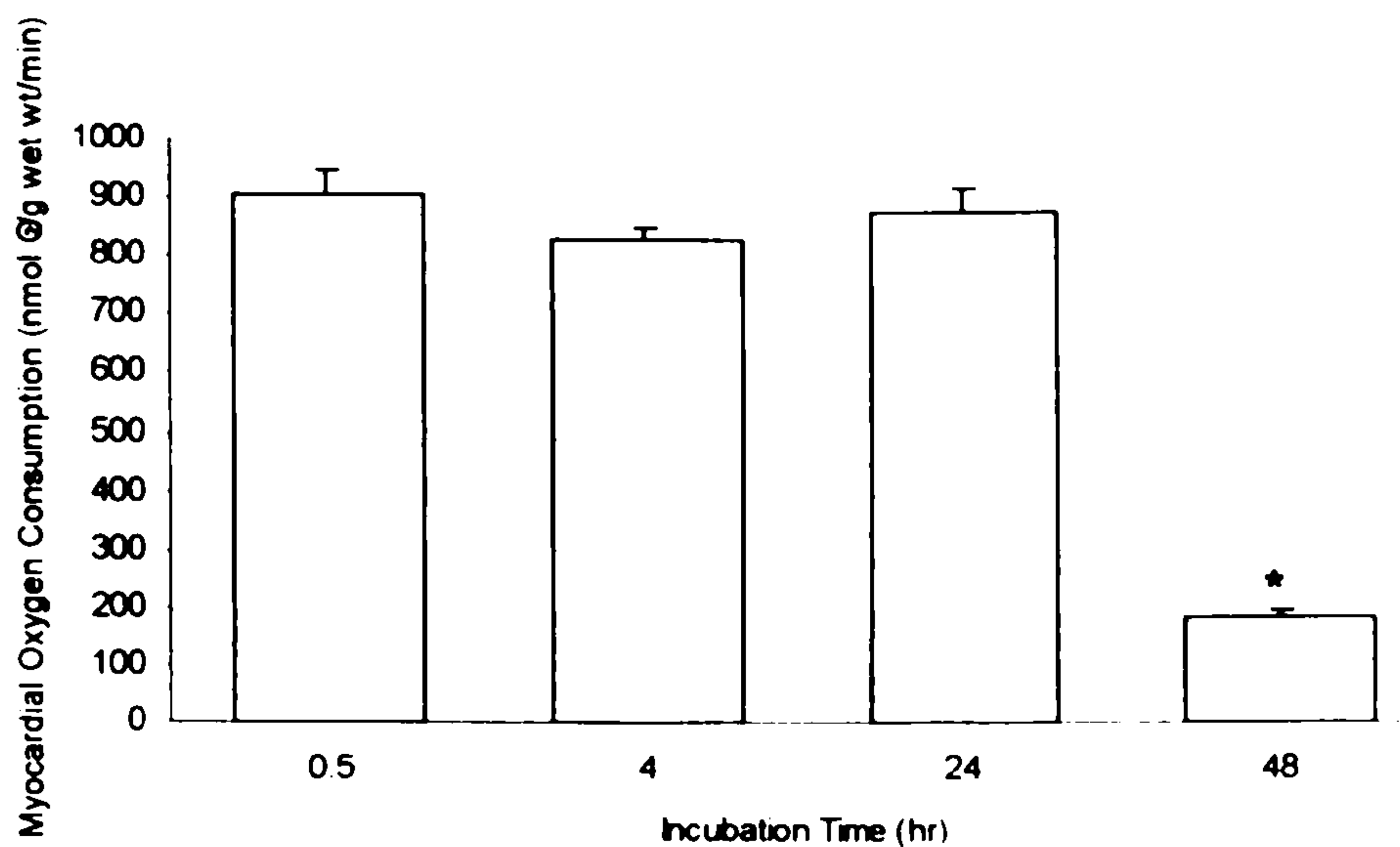


Figure 2.8. Myocardial oxygen consumption of right atrial slices in aerobic conditions (37°C) for different periods of time. Data are expressed as mean \pm standard error of mean of 6 experiments. *p < 0.05 vs the rest of the groups.

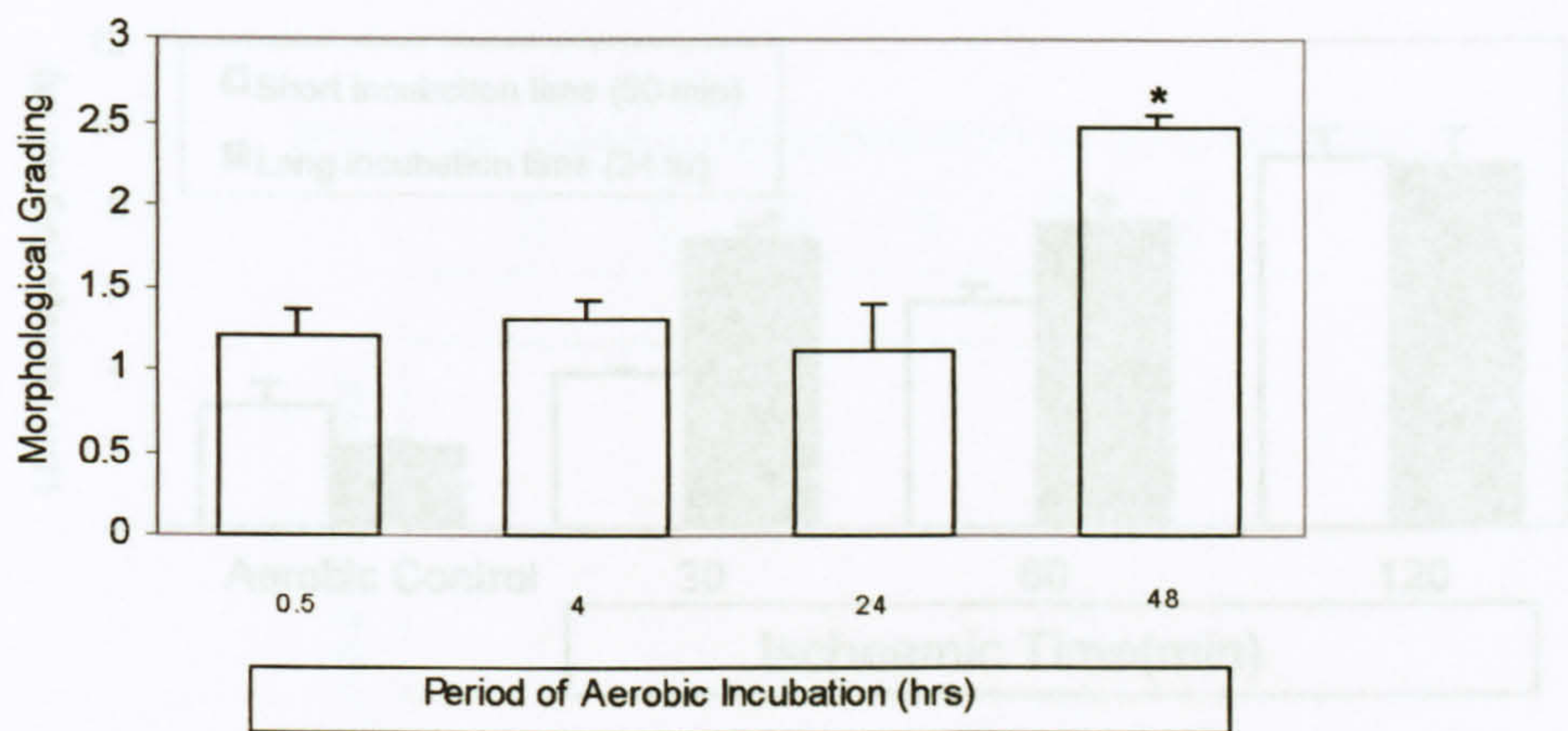


Figure 2.9. Transmission Electron micrograph morphological examination of right atrial slices in aerobic conditions for different periods of time. Data are expressed as mean ± standard error of mean of 6 experiments. *p<0.05 vs rest of the groups.

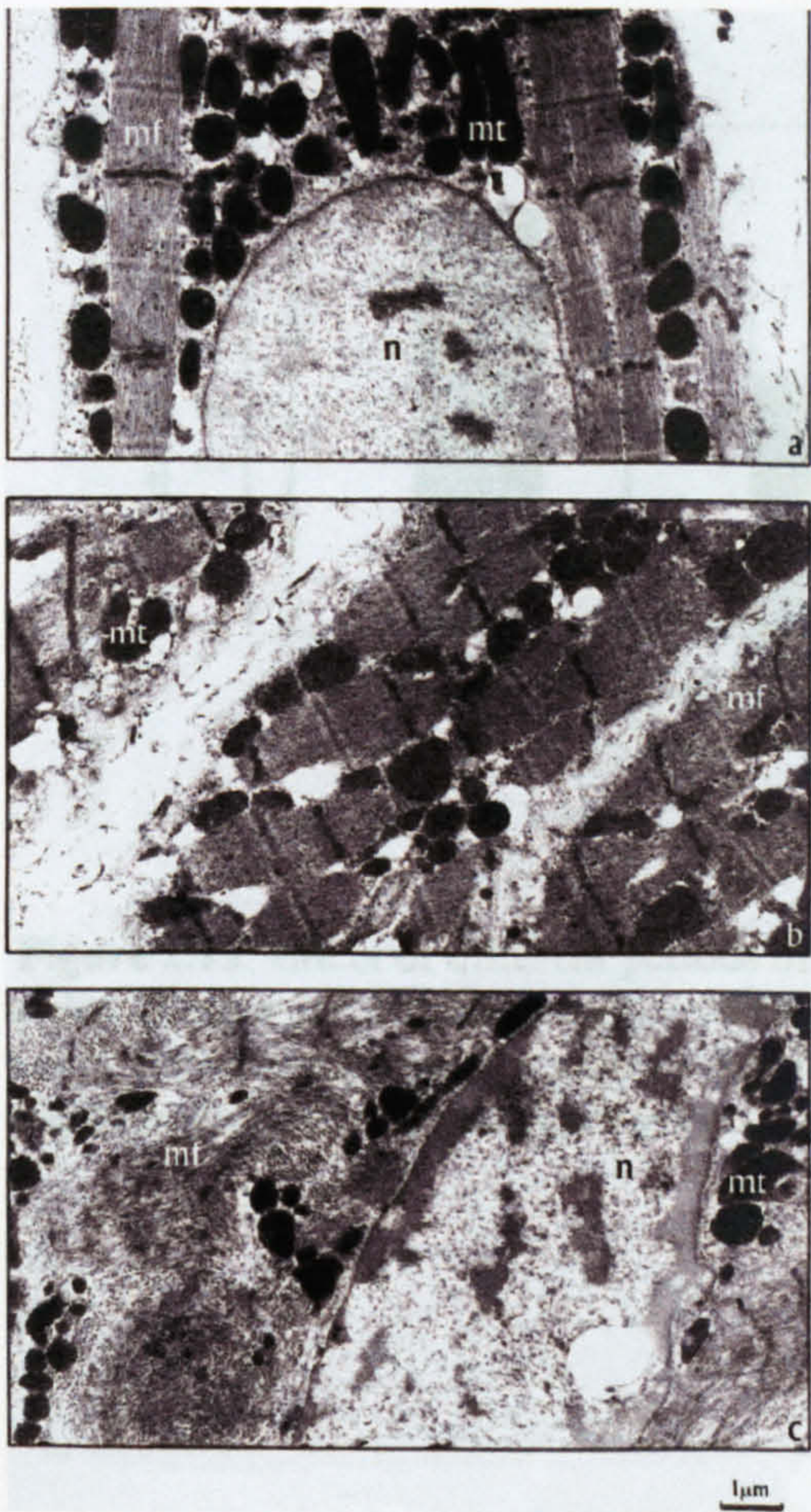


Figure 2.10. Representative transmission electron micrographs of the graded morphological changes: grade 1 – normal; grade 2 – mild damage; grade 3 – severe damage (see text for details) nucleus (n); myofibril (mf); mitochondria (mt), Scale bar = 1 micron and applies to all panels.

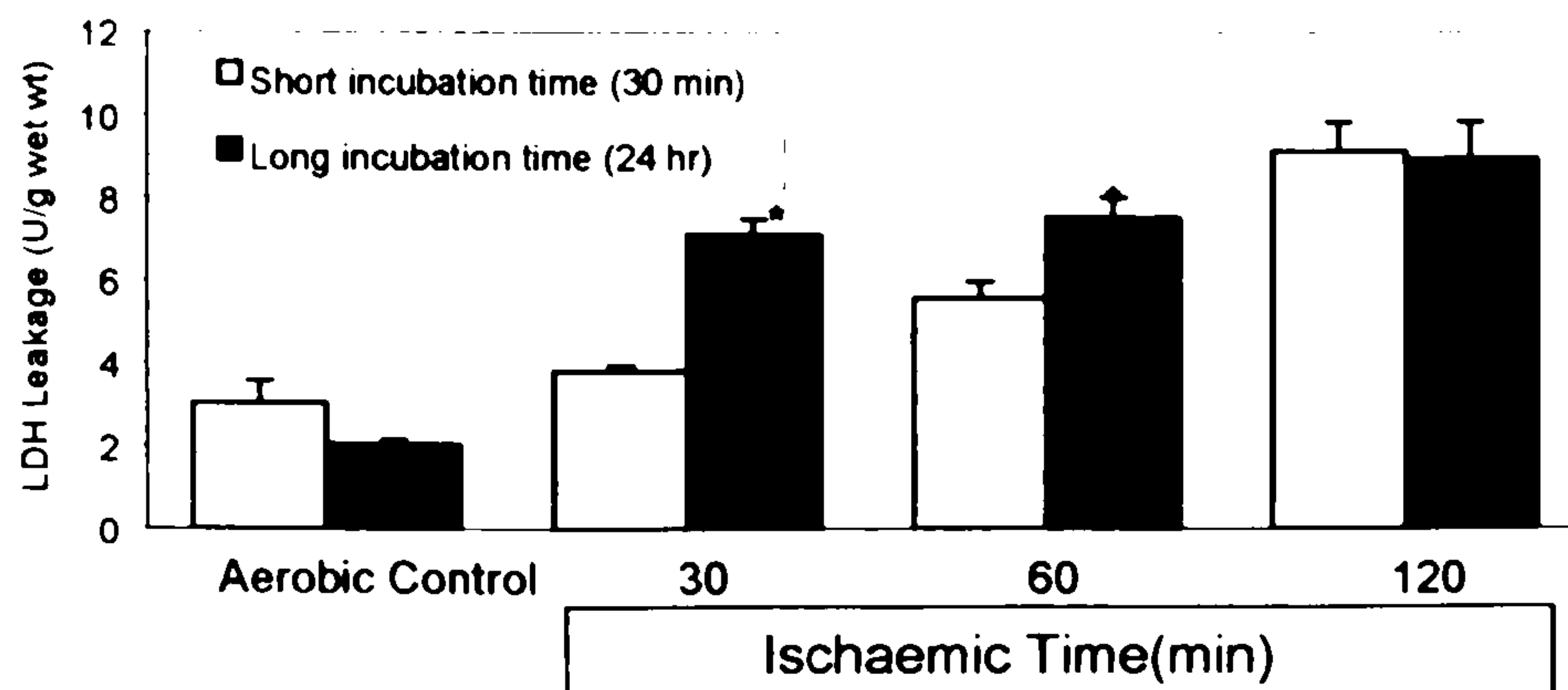


Figure 2.11. Effect of different periods of ischaemia followed by 120 min of reperfusion on the leakage of LDH from the right atrial slices after short (30 min) and long (24 hr) incubation times. Data are expressed as mean \pm standard error of mean of 6 experiments. *p < 0.05 vs the corresponding short incubation time group

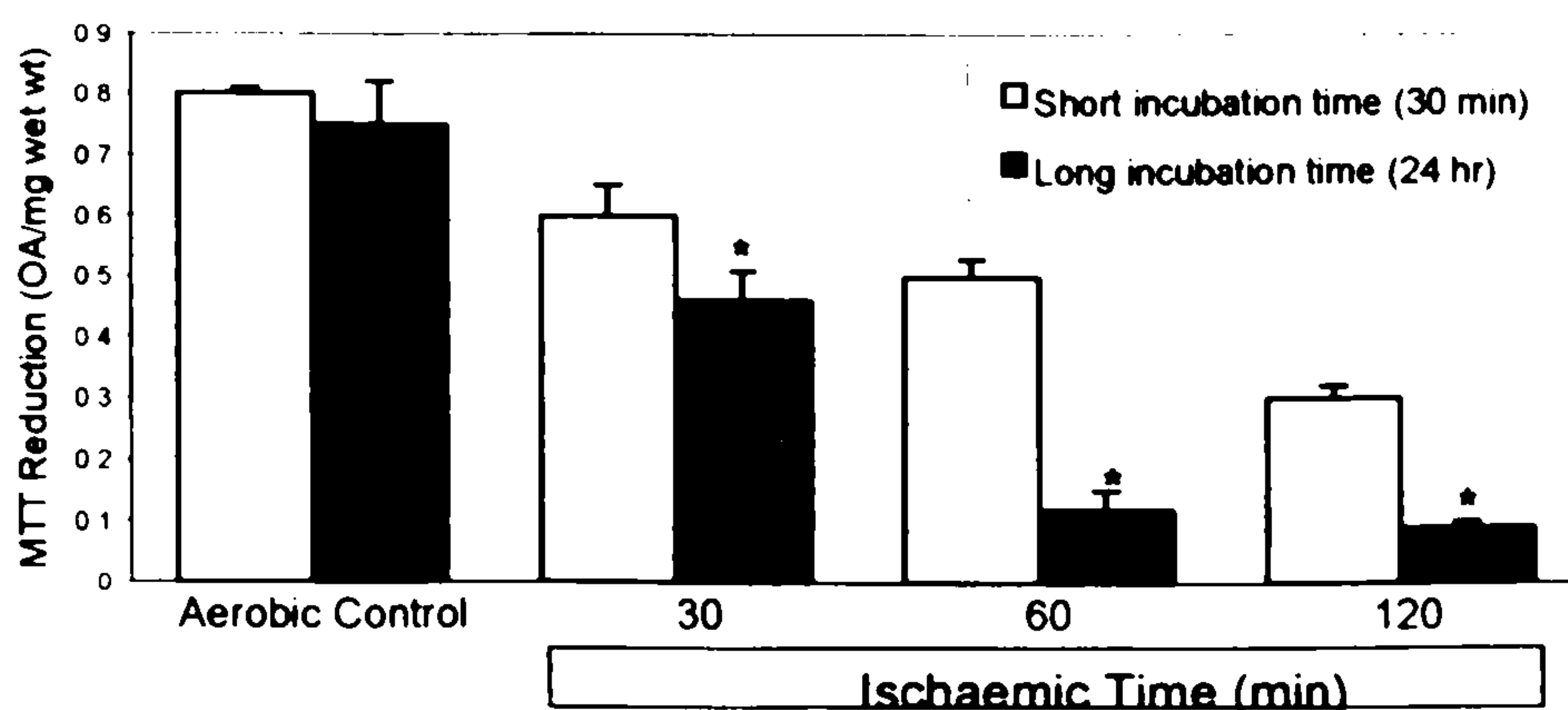


Figure 2.12. Effect of different periods of ischaemia followed by 120 min reperfusion on the ability of right atrial slices to reduce MTT after short (30 min) and long (24 hr) incubation times. Data are expressed as mean \pm standard error of mean of 6 experiments. *p < 0.05 vs the corresponding short incubation time group.

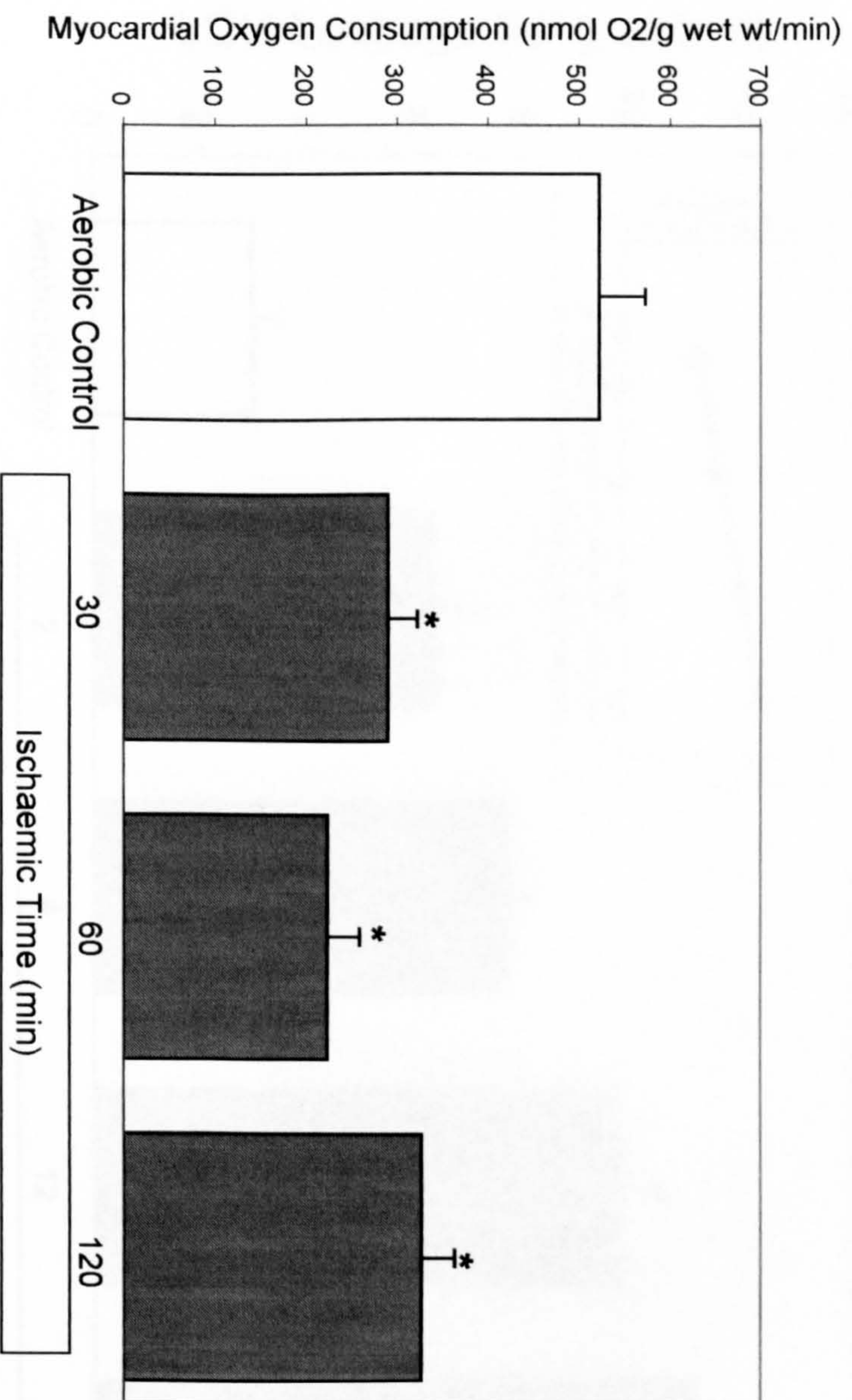


Figure 2.13. Effect of different periods of ischaemia followed by 120 min reperfusion on myocardial oxygen consumption of right atrial slices after short incubation time (30min). Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs aerobic control.

Figure 2.14. Effect of different periods of reoxygenation after 60 min ischaemia on the LDH leakage from right atrial slices. Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs aerobic control. Inset: Linear regression analysis of the LDH values during the different periods of reoxygenation.

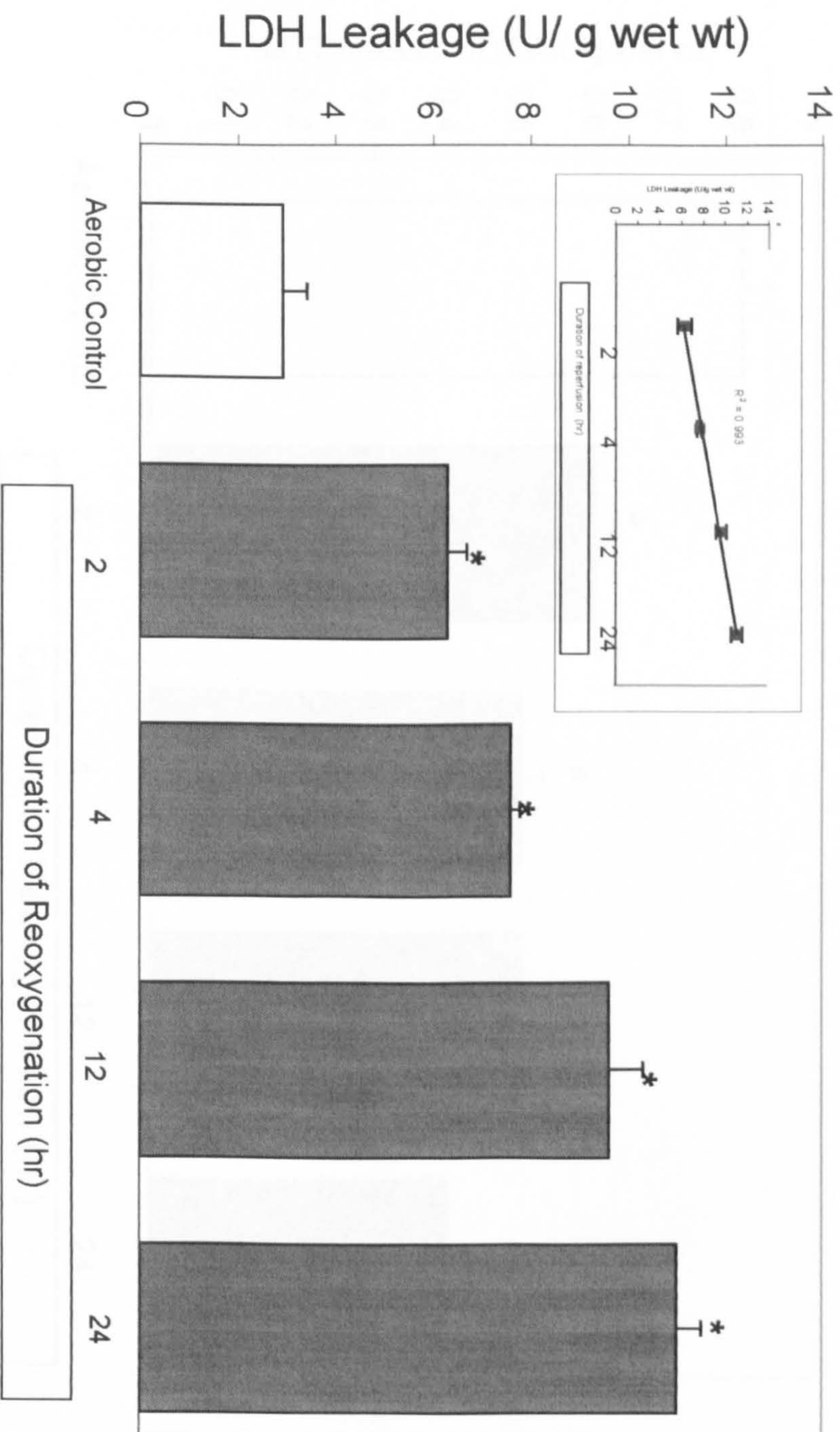


Figure 2.15. Effect of different periods of reoxygenation after 60 min of ischaemia on the ability of right atrial slices to reduce MTT. Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs aerobic control; † $p < 0.05$ vs rest of the groups.

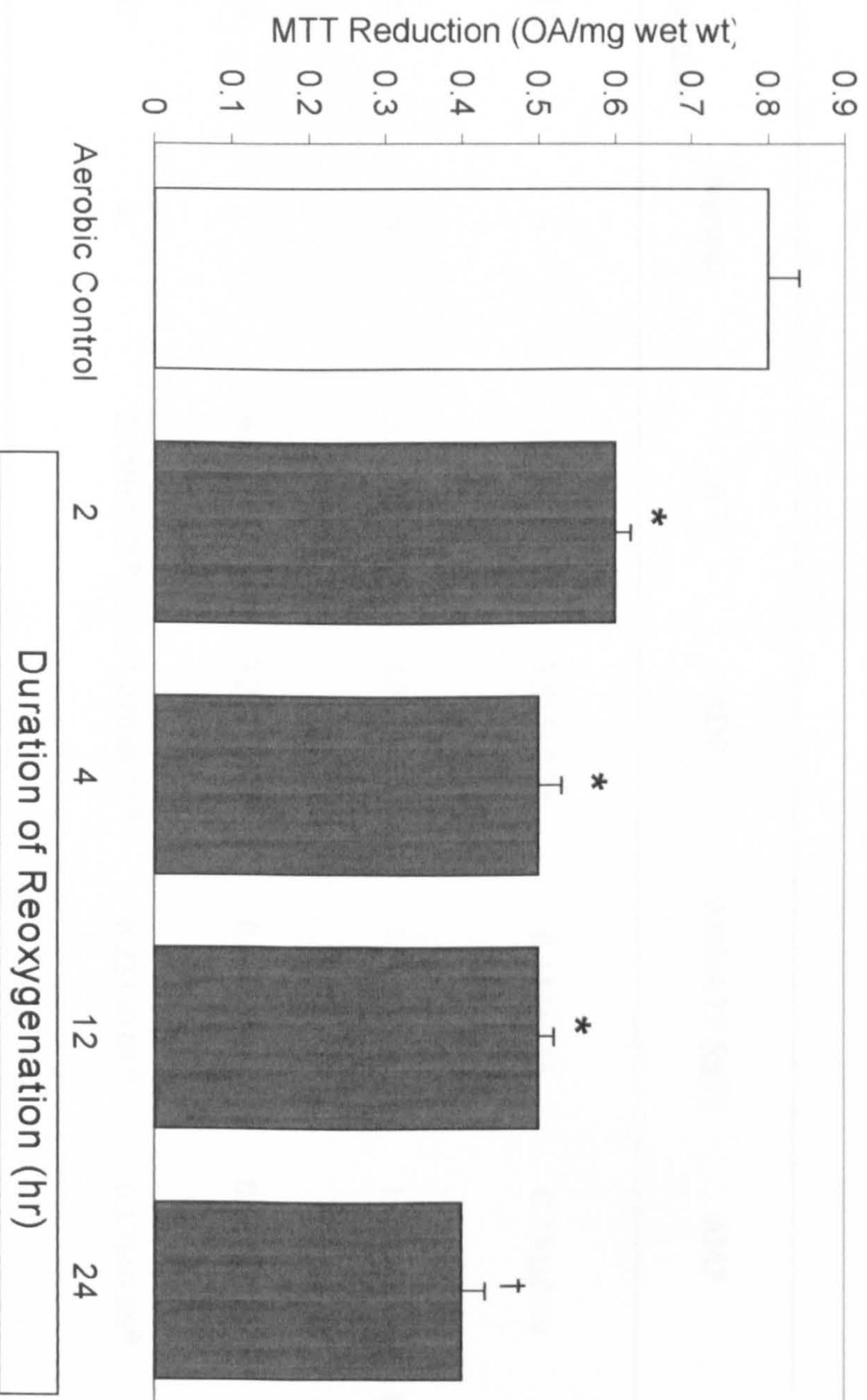


Table 2.1: Tissue high energy phosphate contents ($\mu\text{mol/g}$ dry wt) in right atrial slices subjected to aerobic perfusion (37°C) for different periods of time. * $p<0.05$ vs 0.5 hr aerobic incubation group

Period of Aerobic Incubation	n/group	ATP	ADP	ADP/ATP Ratio	AMP	TAN
0.5 hr	6	5.559 \pm 0.80	3.515 \pm 0.35	0.449 \pm 0.15	0.730 \pm 0.19	9.804 \pm 1.97
4 hr	6	5.496 \pm 0.55	4.541 \pm 0.27	0.516 \pm 0.14	1.031 \pm 0.33	10.649 \pm 1.05
24 hr	6	4.300 \pm 0.42	3.383 \pm 0.27	0.460 \pm 0.11	0.614 \pm 0.12	8.165 \pm 0.89
48 hr	6	2.159 \pm 0.73*	1.070 \pm 0.15*	0.233 \pm 0.04*	0.175 \pm 0.06*	3.400 \pm 1.36*

CHAPTER III

**PRECONDITIONING THE HUMAN MYOCARDIUM WITH
ISCHAEMIA**

3.1 INTRODUCTION:

Brief periods of ischaemia and reperfusion appear to protect the myocardium from a subsequent lethal ischaemic injury. This phenomenon of ischaemic preconditioning, originally described by Reimer et al , 1986 [252], has been shown to exist in all animal species studied to date. There is now compelling evidence that it exists in humans. This evidence arises from in-vitro experiments with human atrial trabeculae[325], ventricular trabeculae [52] and cultured ventricular myocytes[140], studies of patients undergoing planned procedures which invariably involve brief periods of ischemia such as percutaneous transluminal coronary angioplasty [78]and coronary bypass graft surgery[346]. Despite the wealth of information generated by these human studies, the most effective ischemic preconditioning protocol in man remains unknown.

In rats, rabbits, dogs and pigs, separation of the brief preconditioning ischemic episodes from the long occlusion by 60 to 120 min results in complete or nearly complete loss of protection. However, if the duration of this separation is extended to 24 to 72 hr, the infarct size will be reduced again. Hence there appears to be a distinct first (early) as well as a second (delayed) phase of protection. There is no evidence that this biphasic mode of protection exists in humans.

Although studies during angioplasty have given evidence for ischaemic preconditioning in man, clearly there are practical and ethical limitations on the extent to which such situations can be used to investigate the characteristics of preconditioning in the human myocardium. In contrast, *in vitro* preparations, allow a wide range of experimental manipulations. The aims of the studies in this chapter were to investigate the most effective preconditioning protocol in human myocardium and also the existence and potency of a second window of protection. To achieve this, I subjected to simulated ischaemia isolated, right atrial trabeculae slices obtained from patients undergoing elective cardiac surgery.

3.2 MATERIALS AND METHODS:

3.2.1 Experimental Preparation

Experiments were performed as described in section 2.3 (Chapter 2).

3.2.2 Solutions

The incubation medium was prepared daily as described in section 2.3 (Chapter 2).

3.2.3 Experimental Protocols

After sectioning the atrium, the preparations were allowed to stabilise for 30min and then randomly allocated to various protocols. In most studies simulated ischemia was induced for a period of 90min followed by 120min of reoxygenation.

Study 1: In this study, the effect of the duration of the preconditioning ischemic period was investigated. The preparations (n=6/group) were preconditioned with 2, 3, 5 or 10min of ischaemia followed by 5min of reoxygenation before the 90min long ischaemic insult. Figure 3.1 shows the time course for the six study groups.

Study 2: In this study, the effect of the number of cycles of ischemia/reoxygenation for preconditioning was investigated. In study 2A, preconditioning was induced by 1 to 4 cycles of 2 min ischemia/5min reoxygenation (n=6/group), whereas in study 2B, preconditioning was induced by 1 to 3 cycles of 5min ischaemia/5min reoxygenation (n=6/group). Figures 3.2A and 3.2B display the time course for the two study groups.

Study 3: In this study, the duration of the initial protective effect of preconditioning (“early protection” or “first window of protection”) was investigated. The preparations (n=6/group) were

preconditioned with the protocol attaining the greatest protection in studies 1 and 2; this was one single cycle of 5min ischaemia. Then the tissues were reoxygenated for 1, 2, 3, or 4 hr before the 90min of ischaemia. Figure 3.3 shows the experimental time course for the six study groups.

Study 4: In this study, the “delayed protection” or “second window of protection” was investigated. I have demonstrated in the previous chapter that the human right atrial preparation used in the present studies remains viable for at least 24 hr but is more sensitive to ischaemia following 24 hr aerobic incubation. For this reason, two periods of ischaemia, 30 min (Study 4A) and 90 min (Study 4B) were studied (n=6/group). Again the preconditioning protocol consisted of a single cycle of 5min ischaemia/5min reoxygenation. Figures 3.4A and 3.4B show the experimental time course.

Assessment of tissue injury and viability:

At the end of each experimental protocol, tissue injury was determined by measuring the leakage of creatinine kinase (CK) into the incubation medium and tissue viability by the reduction of 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) to blue formazan product. In the following chapters in the thesis, LDH was replaced by release of CK because of technical problems relating to acquiring reagents from distributors.

CK Leakage: The activity of CK leakage into the media during the reoxygenation period (U/g wet wt) was assayed by a kinetic ultraviolet method based on the formation of NAD (Sigma Catalogue No. 1340-K).

MTT reduction: At the end of the experimental time, MTT analysis was performed as described in section 2.6B (Chapter 2) and the results expressed as OD/mg wet wt.

2.5 Statistical Analysis

All data are presented as mean \pm SEM. All values were compared by ANOVA with application of a post hoc Tukey's test. Statistical significance was assumed at the $p < 0.05$ level.

Figure 3.1 Experimental protocols for Study 1. All groups were equilibrated for 30 min in aerobic conditions (37°C). Following this, the right atrial slices (n=6/group) were preconditioned (IP) with various periods of ischaemia followed by 5min reoxygenation. The study groups were matched with an aerobic control and ischaemia alone group.

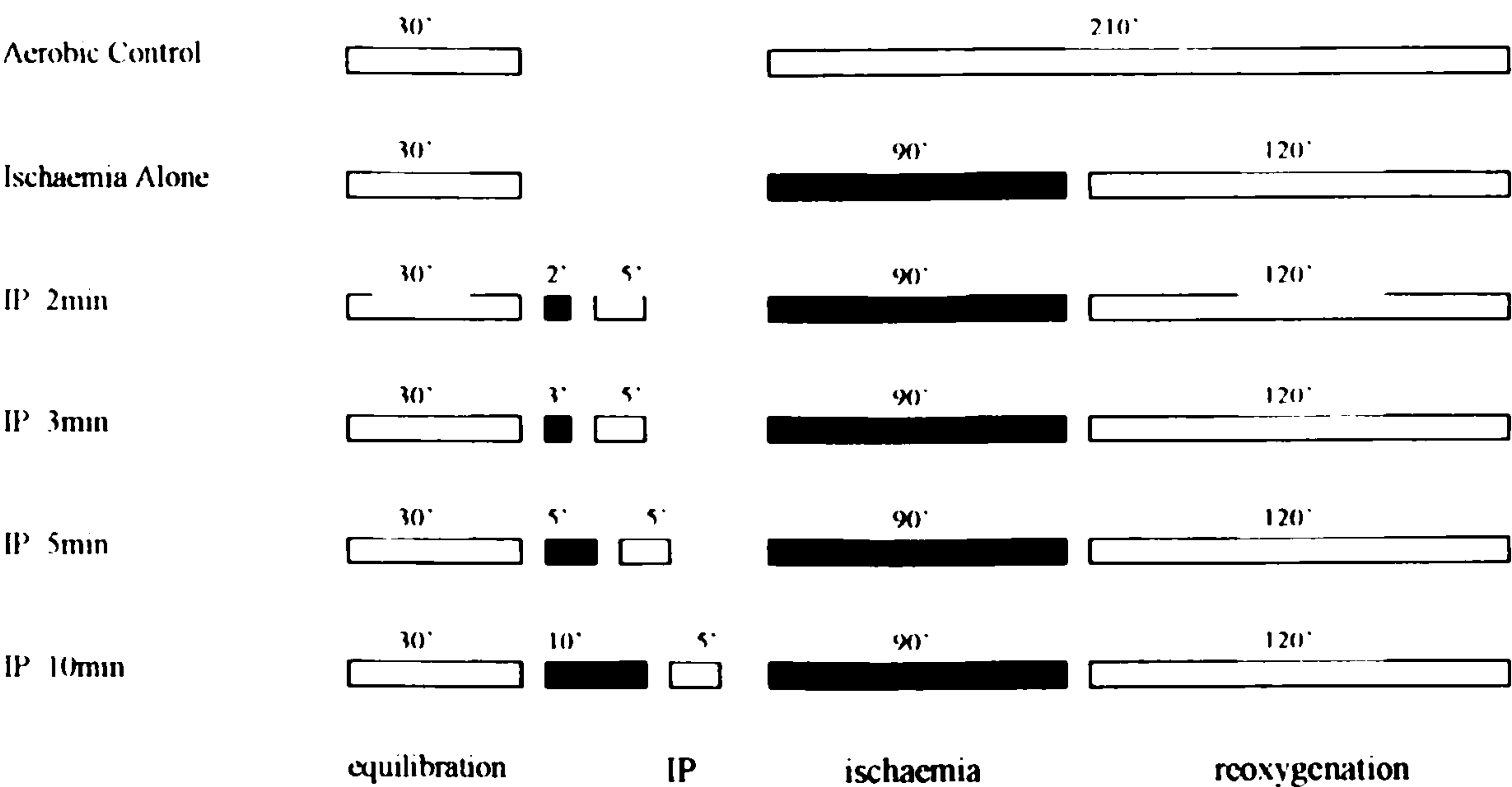


Figure 3.2. Experimental protocols for Study 2A and 2B. Right atrial slices in all groups (n=6/group) were equilibrated for 30 min. In Study 2A, the slices were then preconditioned(IP) with 1-4 cycles of 2min ischaemia/5min reoxygenation before being subjected to 90min of ischaemia/120min reoxygenation. In Study 2B, after equilibration, the slices were subjected to 1-3 cycles of 5min ischaemia/5min reoxygenation preconditioning before the 90min ischaemic period.

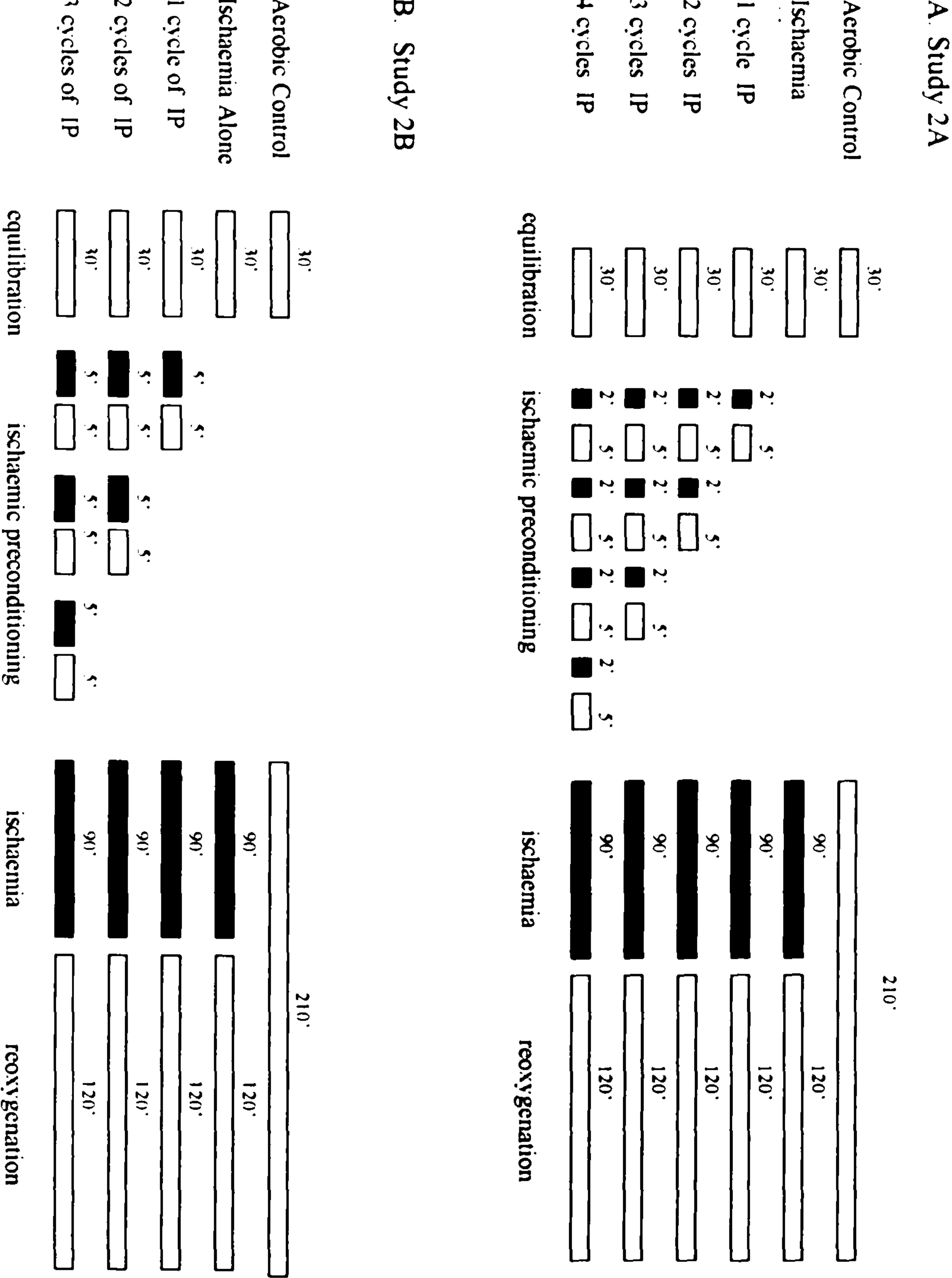


Figure 3.3 Experimental protocols for Study 3. Right atrial slices in all groups (n=6/group) were equilibrated for 30 min. The right atrial slices (n=6/group) were then subjected to 5min preconditioning(IP). Following this, the study groups were reoxygenated for various time intervals before the 90min ischaemia/120min reoxygenation

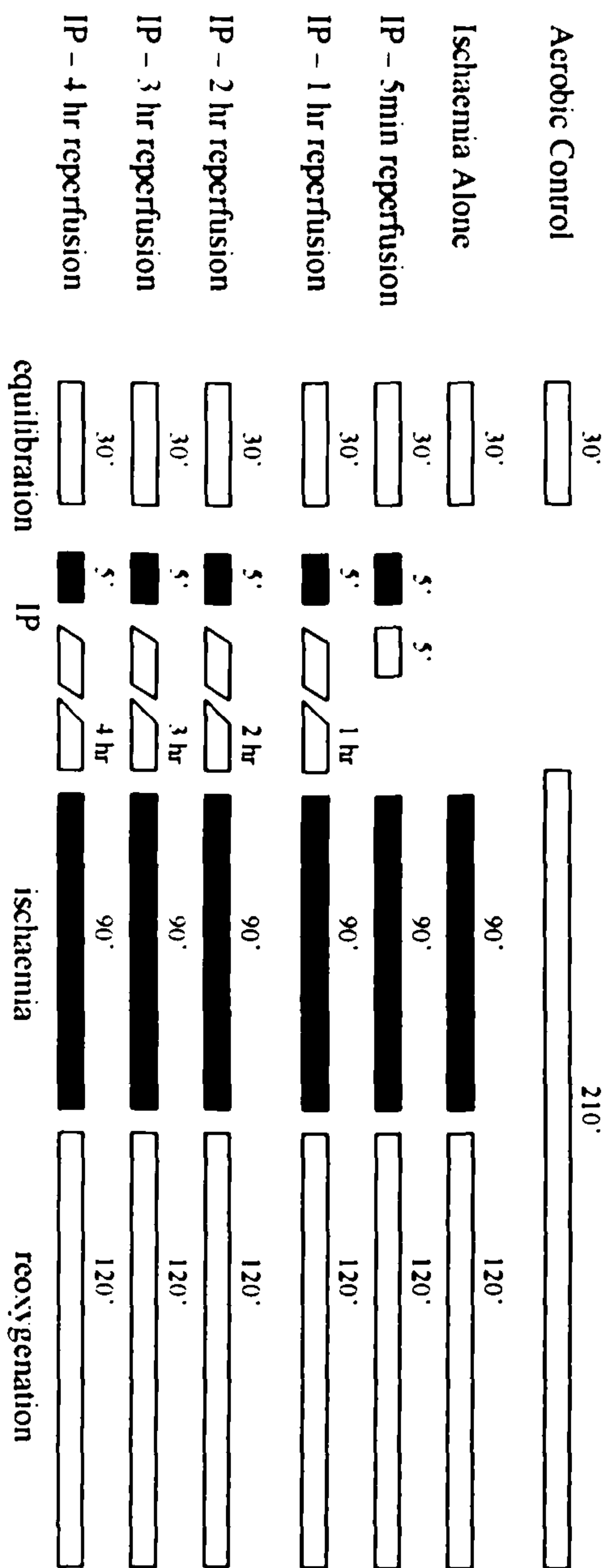
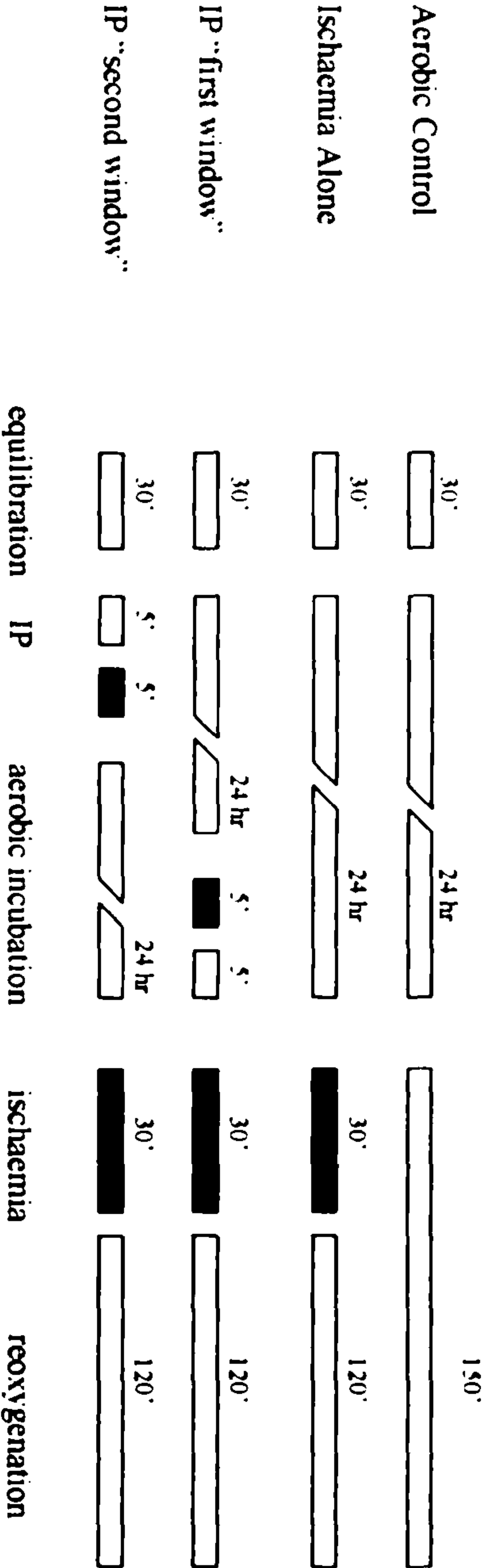
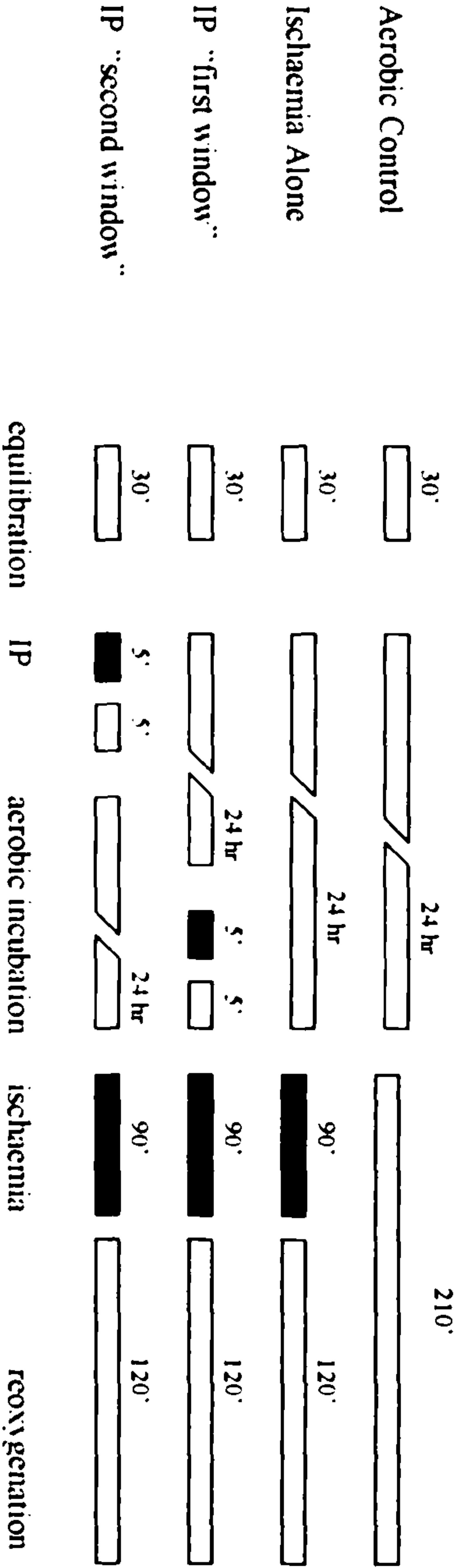


Figure 3.4 Experimental protocols for Study 4. Right atrial slices in all groups (n=6/group) were equilibrated for 30 min. In study 4A, after equilibration, the slices were aerobically incubated for 24 hr. The 'first window' group were then preconditioned(IP) with 5 min ischaemia/5 min reoxygenation before being subjected to 30 min ischaemia/120 min reoxygenation. The 'second window' group were preconditioned initially with 5 min ischaemia/5min reoxygenation before the 24 hr incubation and then immediately subjected to 30 min ischaemia/120 min reoxygenation. In study 4B, identical protocols were applied except the ischemic time was extended to 90 min.

A. Study 4A (with 30min ischaemia)



B. Study 4B (with 90min ischaemia)



3.4 RESULTS:

All samples entering the studies completed the applied experimental protocol and were included in the analysis.

Study 1 – Effect of the duration of the preconditioning ischaemic stimulus

As shown in Figures 3.5A and 3.5B, 90min of ischaemia resulted in significant increase in CK leakage and decrease in MTT reduction. An inverted bell shape curve was observed for CK leakage when various periods of ischaemic preconditioning were applied. 2min of ischaemia was not protective; 3min of ischaemia was the minimum period required to achieve a significant reduction in CK leakage but maximal protection was obtained with 5min ischaemic preconditioning with mean CK leakage values not significantly different from those in the aerobic control group. Interestingly, protection was lost when ischaemic preconditioning was extended to 10min.

A mirror image to that seen for CK leakage was observed for MTT reduction. Thus, 5min of ischaemic preconditioning afforded maximal protection such that MTT reduction values were similar to those seen in the aerobic control group, and again, protection was lost when the duration of ischaemia was less than 3min or increased to 10min.

Study 2 – Effect of the number of cycles of preconditioning

Figures 3.6A and 3.6B show the results of preconditioning with increasing cycles of 2min ischaemia/5 min reoxygenation. The results from CK leakage and MTT reduction show that maximal protection was obtained with two cycles of 2min ischaemia. Interestingly, in this study preconditioning with one cycle of 2 min ischaemia resulted in a small but statistically significant decrease in CK leakage. This result contrasts with that observed in study 1 where the enzyme

leakage resulting from preconditioning with 2 min ischaemia was similar to the mean values of the ischaemia alone group. This apparent contradictory result may be attributed, at least in part, to the greater CK leakage seen in the ischemia alone group in study 2, since the CK leakage mean values in the 2 min ischaemic preconditioning groups were similar in both studies. Alternatively, it may suggest that CK leakage is more sensitive than MTT reduction in assessing tissue injury and that 2 min ischaemia is in the threshold of protection by ischaemic preconditioning, a possibility that would be supported by the absence of protection in terms of MTT reduction with 2 min of ischaemic preconditioning in the two studies. As shown above, increasing the number of cycles and the total ischaemic preconditioning period beyond 5 min reduced or abolished the protection seen in the results with CK leakage and MTT reduction.

Figures 3.7A and 3.7B show the results on CK leakage and MTT reduction of preconditioning with increasing numbers of cycles of 5min ischaemia/5 min reoxygenation. Both CK leakage and MTT reduction demonstrate clearly that protection is lost beyond 5min of ischaemic preconditioning. Overall these studies show that to precondition the human atrial myocardium the most important factor is the total ischaemic stimulus and not the number of cycles.

Study 3 – First window of protection

Figures 3.8A and 3.8B show the results on CK leakage and MTT reduction when ischaemic preconditioning of atrial myocardium is followed by various reoxygenation periods before the 90 min ischaemia and 120 min reoxygenation. The results show that the protection induced by preconditioning with only 5 min of reoxygenation is maintained when the interval between the preconditioning and ischaemia is within 2 hours and that the beneficial effect is lost when that interval is extended to 3 or more hours.

Study 4 – Second window of protection

In Chapter 2, I showed that the right atrial preparation used in the present experiments is viable for at least 24 hours; however, after this time the preparation is more susceptible to ischemia/reperfusion injury than when incubated for shorter periods. For this reason, in this study two different periods of ischaemia, 30 (moderate ischaemia) and 90 min (severe ischaemia), were used to investigate the late or second window of protection of ischaemic preconditioning.

Figures 3.9A and 3.9B show the results with 30 min ischaemia. Ischaemia alone caused a significant increase in CK leakage and decrease in MTT reduction when compared with the aerobic control group. Both the first and second window of protection gave a similar decrease in CK leakage and amelioration of MTT reduction.

As shown in Figures 3.10A and 3.10B, extension of the period of ischaemia to 90 min resulted in greater CK leakage and lower MTT reduction than with 30 min ischaemia. As expected, the first window of preconditioning significantly improved CK leakage and MTT reduction, however, this beneficial effect was not seen with the second window of preconditioning and values were similar to those observed in the ischaemia alone group.

3.5 DISCUSSION:

The present studies have characterised the ischaemic preconditioning phenomenon in the human myocardium and have disclosed the following important results: (i) there is a graded narrow window of protection by preconditioning with 4-5 min of ischaemia being the most effective period, (ii) the number of preconditioning cycles in itself does not influence protection, and (iii) as shown in other animal species, there are two windows of protection, the first (≤ 2 hr) being more protective than the second (≈ 24 hr). These results have significant clinical implications and warrant further discussion.

Intensity of the preconditioning stimulus

These studies are the first to demonstrate that maximal protection of the human myocardium by ischaemic preconditioning is obtained with an ischaemic stimulus of 4-5 min. It was not surprising that shorter periods of ischaemia resulted in a decrease or loss protection since studies in animals have shown that the ischaemic period should be greater than 2 min to achieve protection [218,321]. However, the loss of protection with ischaemic periods beyond 6 min was unexpected since several investigators have reported that 10 min of ischaemia preconditions the heart of a number of animal species [26,104,285].

The findings that 4-5 min of ischaemia is the optimal time for preconditioning is supported by studies performed in the course of percutaneous transluminal coronary angioplasty (PTCA) [75,307]. In these studies, coronary arteries are typically occluded for 2 min by balloon inflation with 5 min apart. Consistently in all studies the severity of myocardial ischaemia, assessed by changes in S-T segment shifts and angina symptoms, are less during the second and third balloon inflation than during the first inflation. This suggests that a total of 4-6 min of ischaemic preconditioning also confers maximal protection in the clinical setting.

The present studies have also shown a dose-response effect in preconditioning the human myocardium so that the phenomenon should be identified as a graded rather than an all-or-nothing event. It is worth noting that the time window of protection was confined to a limited period, between 3 and 6 min of ischaemia, and therefore studies involving few ischaemic times may give the false impression that preconditioning is an all-or-nothing phenomenon. This thesis is supported by studies on anaesthetized pigs[274] and rabbits[264] where graded ischaemia determined the extent of infarct size reduction. It is also worth noting that my findings are in agreement with those of Downey et al [321] that preconditioning ischaemia less than 2 min did not confer protection, indicating that preconditioning has a threshold somewhere between 2 and 5 minutes. Their explanation was that the threshold of protection reflects the duration of ischaemia required to build up adenosine levels to the point where adenosine receptors are adequately populated. It should be mentioned however that preconditioning by repeated short periods of ischaemia and reperfusion may have resulted in wash-out of tissue adenosine and that in fact adenosine may not have been raised sufficiently to reach the threshold of protection. If this is the case then the mechanism of protection induced by repeated short ischaemic cycles should involve the stimulation of receptors other than or in addition to adenosine receptors. Indeed Downey [104] has previously suggested that the threshold of protection by preconditioning can be obtained by the additive effect of the stimulation of several membrane receptors (i.e. adenosine receptors, α_1 adrenoreceptors, bradykinin and opioid receptors).

Another finding of my study that may have clinical implications is the loss of protection when the preconditioning ischaemic stimulus was extended to 10 min, a time that has been reported to elicit protection in several animal species. Thus, if my results are extrapolated to clinical situations it is possible that repeated occlusions of a coronary artery during PTCA or of the ascending aorta

during cardiac surgery totalling 10 or more min of ischemia may result inadvertently in loss of protection.

The number of preconditioning cycles

The results in Figures 3.6 and 3.7 clearly demonstrate that the number of preconditioning cycles *per se* do not influence the outcome and that in fact protection is determined by the intensity of the ischaemic stimulus. Clinical studies on preconditioning in the course of PTCA where changes in S-T segment shift and the severity of angina are reduced during the second and third coronary occlusion [78] may be the reflection of fulfilling the optimal ischaemic stimulus (i.e. 4-6 min) rather than an effect directly promoted by the increasing number of ischaemic cycles. Animal studies in which preconditioning was elicited by increasing the number of cycles but using cycles of one ischaemic duration time only [57,224,341] cannot separate the effects of the number of preconditioning cycles from those corresponding to the total duration of ischaemia and hence, do not support or refute the above suggestion. Therefore, my results argue the conventional wisdom that preconditioning can be made more effective by increasing the number of ischaemic cycles. It should be emphasised however that this argument may apply to this model of ischaemia/reoxygenation used in this thesis and that it may not necessarily be valid for shorter or longer periods of ischaemia or different degrees of severity of tissue injury.

First Window of Protection

To the best of my knowledge, our studies are also the first to demonstrate that classical preconditioning of the human myocardium, also known as the early or first window of preconditioning, is restricted to the initial 2 hr following its application, and this has obvious clinical implications. A similar response has been reported in a variety of animal species [218,225,321] supporting the view that the underlying mechanisms of the first window of

preconditioning may be identical in all species. Certainly, the stimulation of membrane receptors such as A_1/A_3 and α_1 -adrenoreceptors and the activation of protein kinase C and K_{ATP} channels have been shown to be involved in the majority of the animal species studied [15,187,273,275] and in man [52,60]. My finding that preconditioning is a graded phenomenon is also compatible with the notion that a number of triggers could be activated to achieve protection.

The realization that preconditioning is a phenomenon probably shared by all mammalian species studied including man and the evidence that it may be elicited through identical molecular pathways, it makes possible that the results obtained from laboratory based studies may be extrapolated to the clinical setting with a high degree of confidence.

Second Window of Protection

My studies are again the first to demonstrate the existence of a second window of protection in the human myocardium. However, I have shown that the second window is not as protective as the first window, a result that is consistent with other reports in anaesthetized rabbits and dogs [177,201,335]. The issue is not without controversy and some investigators [249,298] have reported that in anaesthetized rabbits preconditioning does not result in infarct size reduction if the ischaemic insult is applied in the following 24 or 48 hr. The reasons for these discrepancies are not entirely clear, but differences in the experimental preparations and protocols should be taken into account. Preliminary studies conducted in the laboratory showed that the extent of protection obtained in the second window of preconditioning was similar with one cycle and with repeated cycles of ischaemia as long as the total ischaemic stimulus was between 4 and 5 min (data not shown).

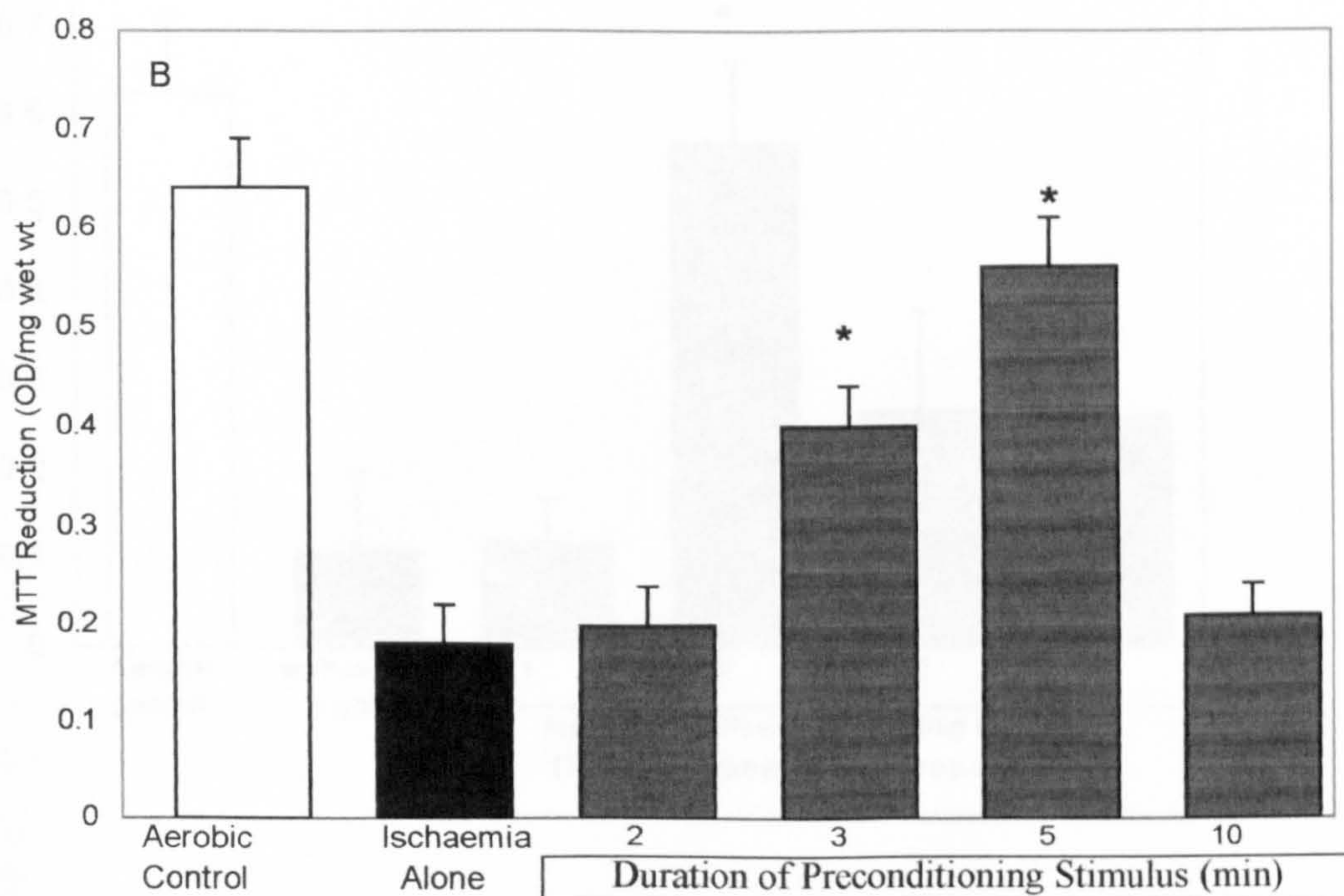
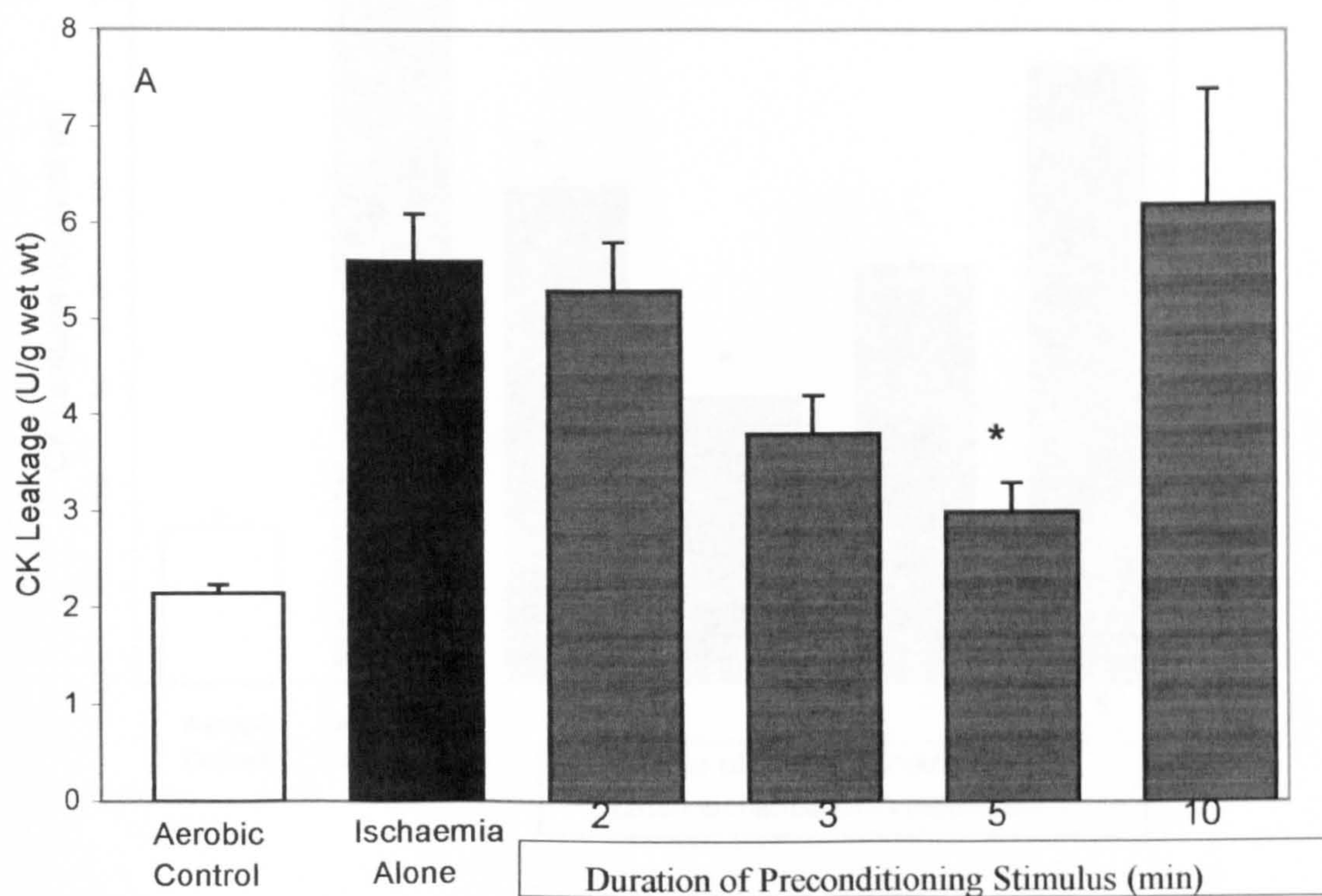
The contrast between the universal presence of the first window of protection and the controversial second window may suggest that the mechanism underlying the two windows are

different. The cellular mechanisms underlying the second window are not fully understood at present. Some experimental evidence for the involvement of adenosine receptor stimulation and activation of protein kinase C (PKC) in the development of delayed protection has been reported in rabbits [24-25]. However, PKC could influence a host of other signal transduction pathways and it is possible that other protein kinase events play a role in the mechanism. Certainly, other end-effectors have been implicated in the second window. These include the intracellular antioxidant superoxide dismutase (SOD) [336], heat shock proteins [347] and nitric oxide synthetase [238]. At present, there is limited evidence to suggest that K_{ATP} channels are involved in the delayed phase of protection [94]. It is quite clear that further research is needed in this area.

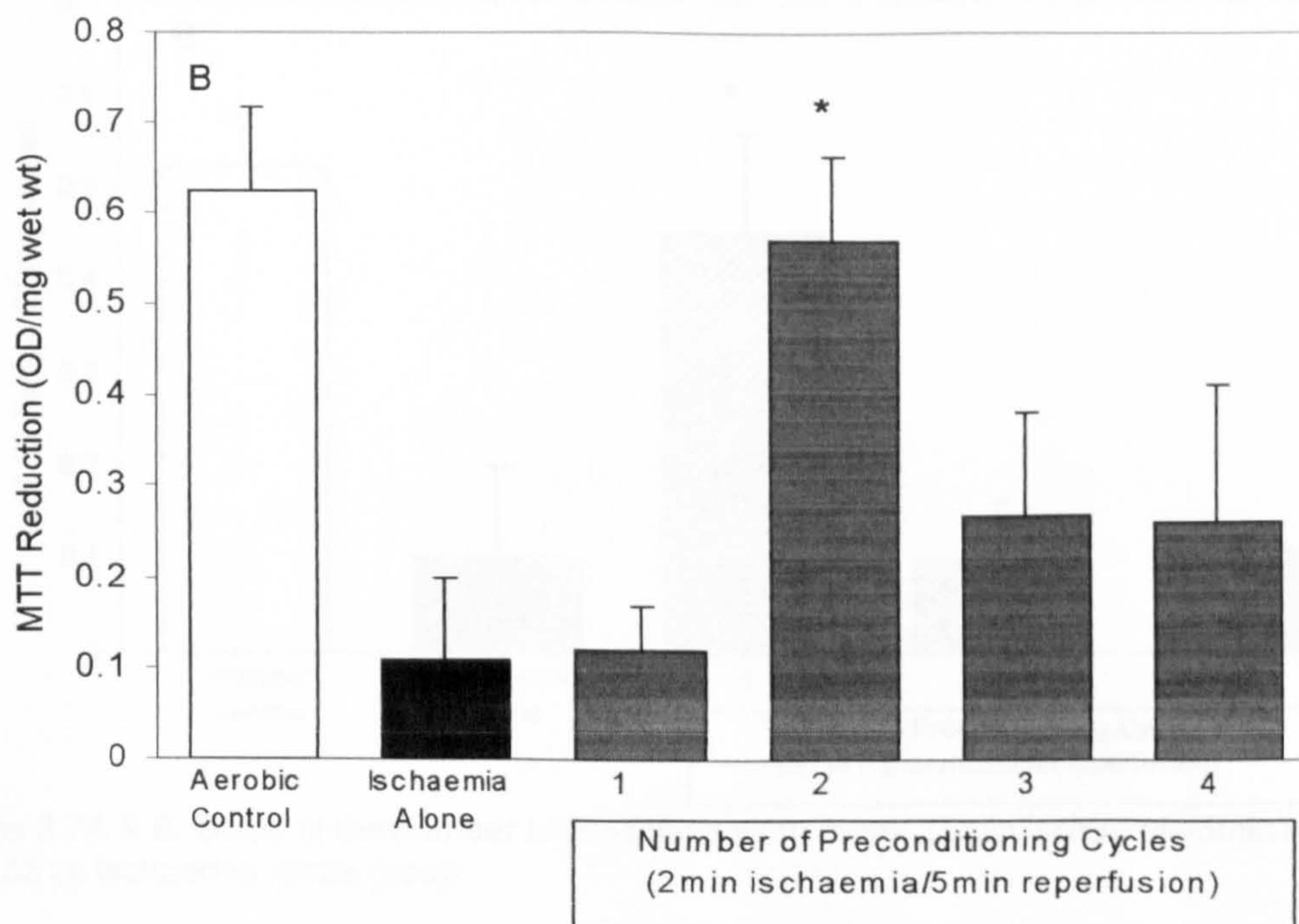
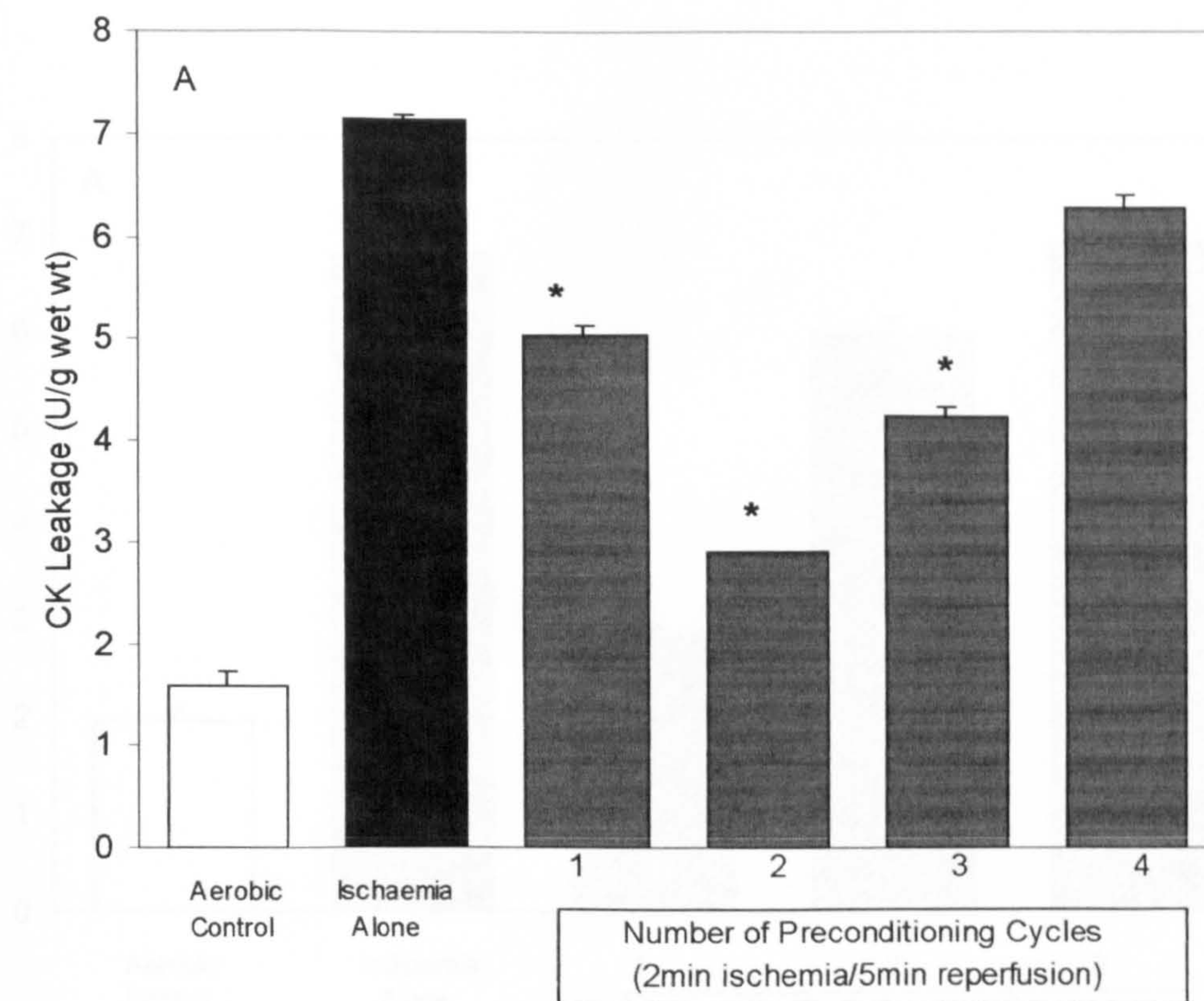
Limitations of the Study and Clinical Implications

The present work has several limitations. First, in our preparation ischaemia was induced by removing O_2 and nutrient substrate but toxic metabolites, usually accumulated during ischaemia, freely diffused into the incubation media ("simulated ischaemia"). I accept that there are important differences between this model and true ischaemia, particularly in respect to the washout of ischaemic metabolites and pH changes. Second, I used atrial tissue and any extrapolation to ventricular myocardium must be conducted with caution; however, Yellon's group [325] has suggested that identical protection can be obtained by preconditioning in both tissues. Third, right atrial specimens were obtained from patients subjected to medical treatments (e.g. nitrates, β -blockers, calcium antagonists) that potentially may themselves influence ischaemia/reoxygenation injury and the protection induced by preconditioning. Fourth, this model is an *in-vitro* preparation and the results may not completely apply to the clinical setting, although the findings during coronary artery occlusion in the course of PTCA may suggest that protection by preconditioning can be achieved with similar protocols in both situations.

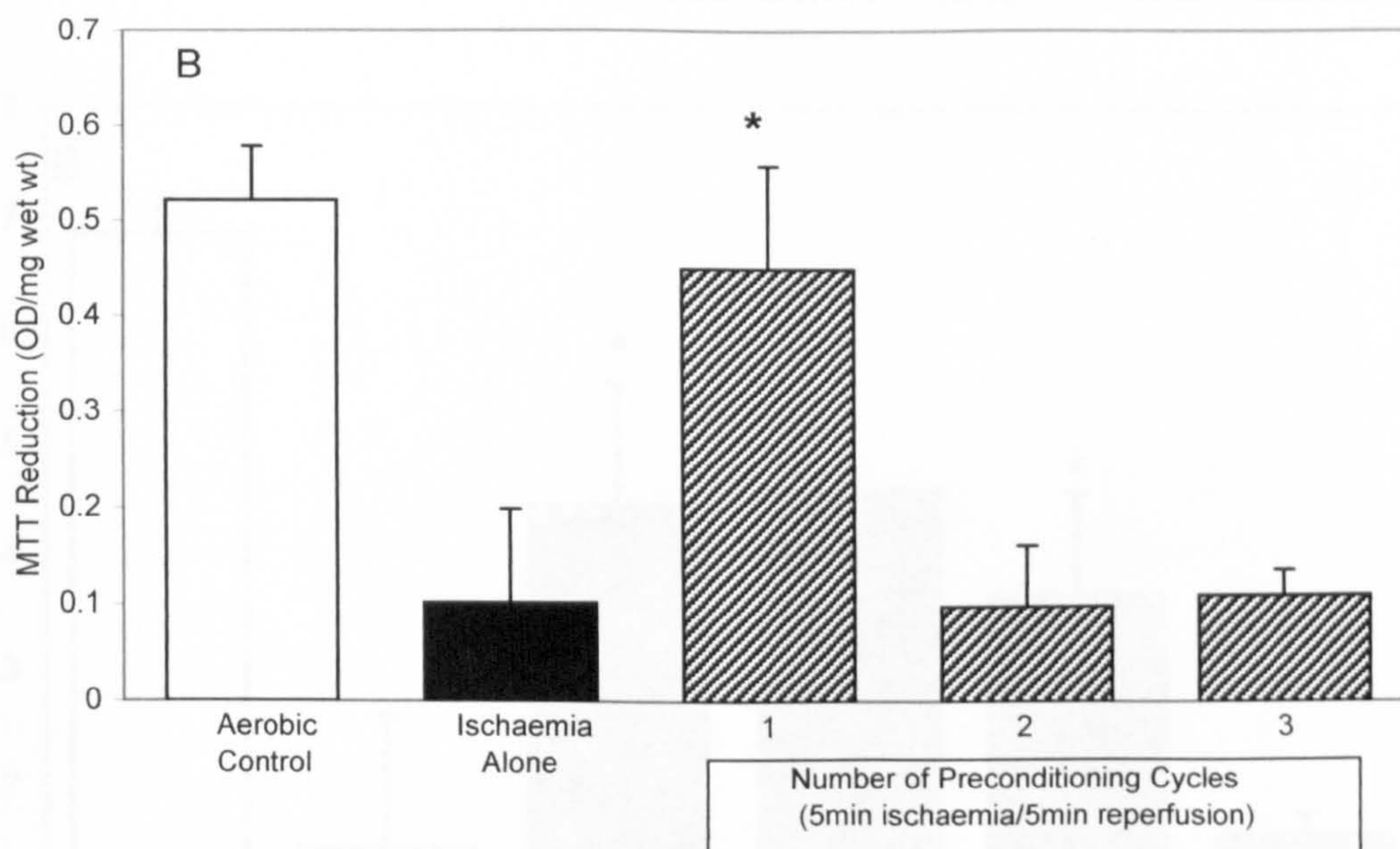
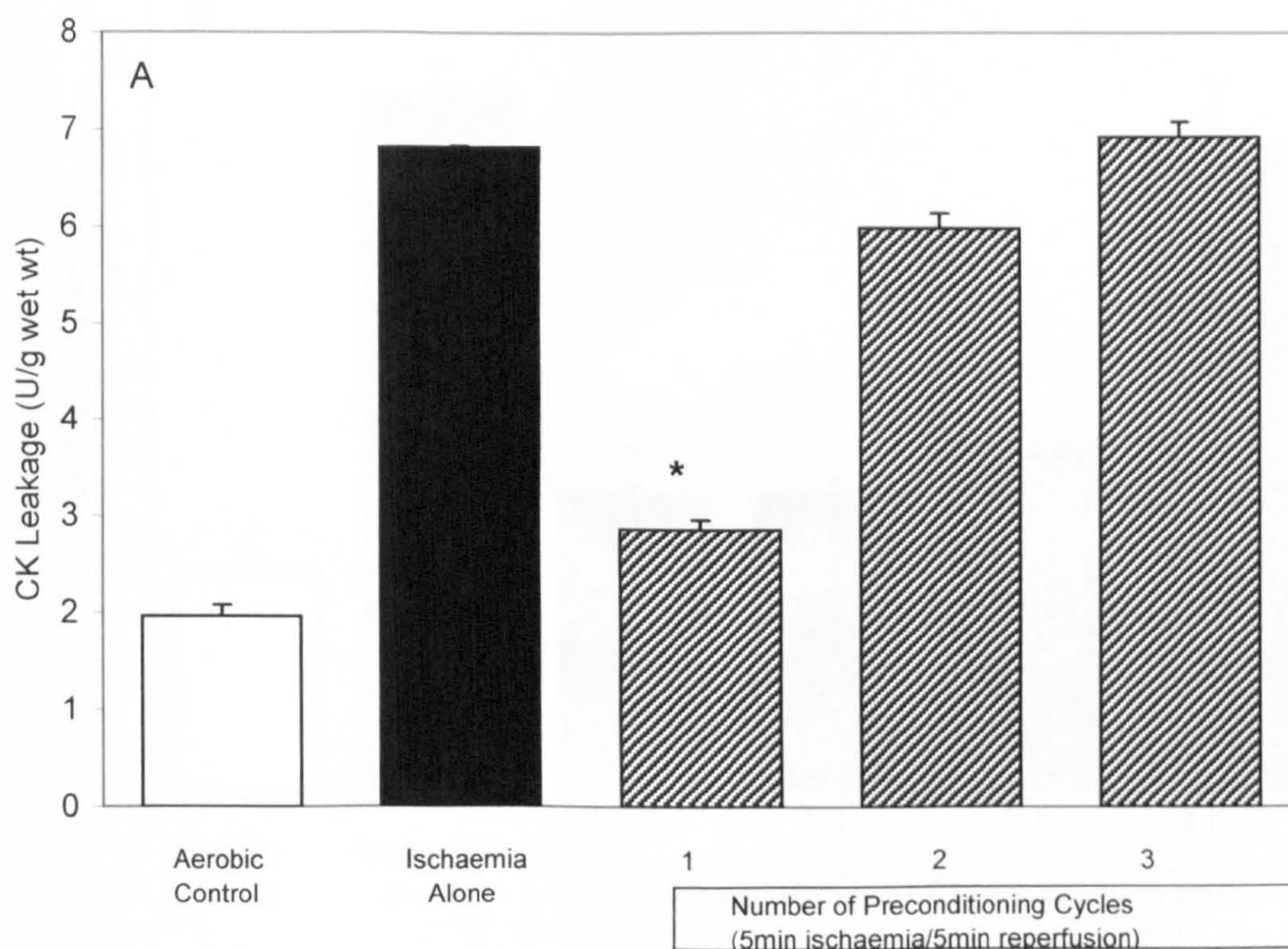
My results have important clinical implications by revealing that the duration of the ischaemic stimulus rather than the number of cycles is the most important element influencing myocardial protection by preconditioning. Furthermore, this protection is a graded phenomenon with maximal benefit with 4-5 min ischaemic duration and reduction or loss of protection if the ischaemic stimulus is extended beyond 5 min. I have also shown that the second window of protection by preconditioning is not as effective as the first window and that this may lessen its relevance as a potential therapeutical intervention. However, further studies may be required to confirm this latter finding. With the phenomenon of preconditioning investigated, I turned my attention to the cellular mechanisms involved in the cascade that leads to the protection induced by ischaemic preconditioning. In particular, I focused on the role of ATP-dependent potassium channels in this cascade. The chapters describe in detail the experimental work involved in elucidating the role played by these channels in this model of preconditioning.



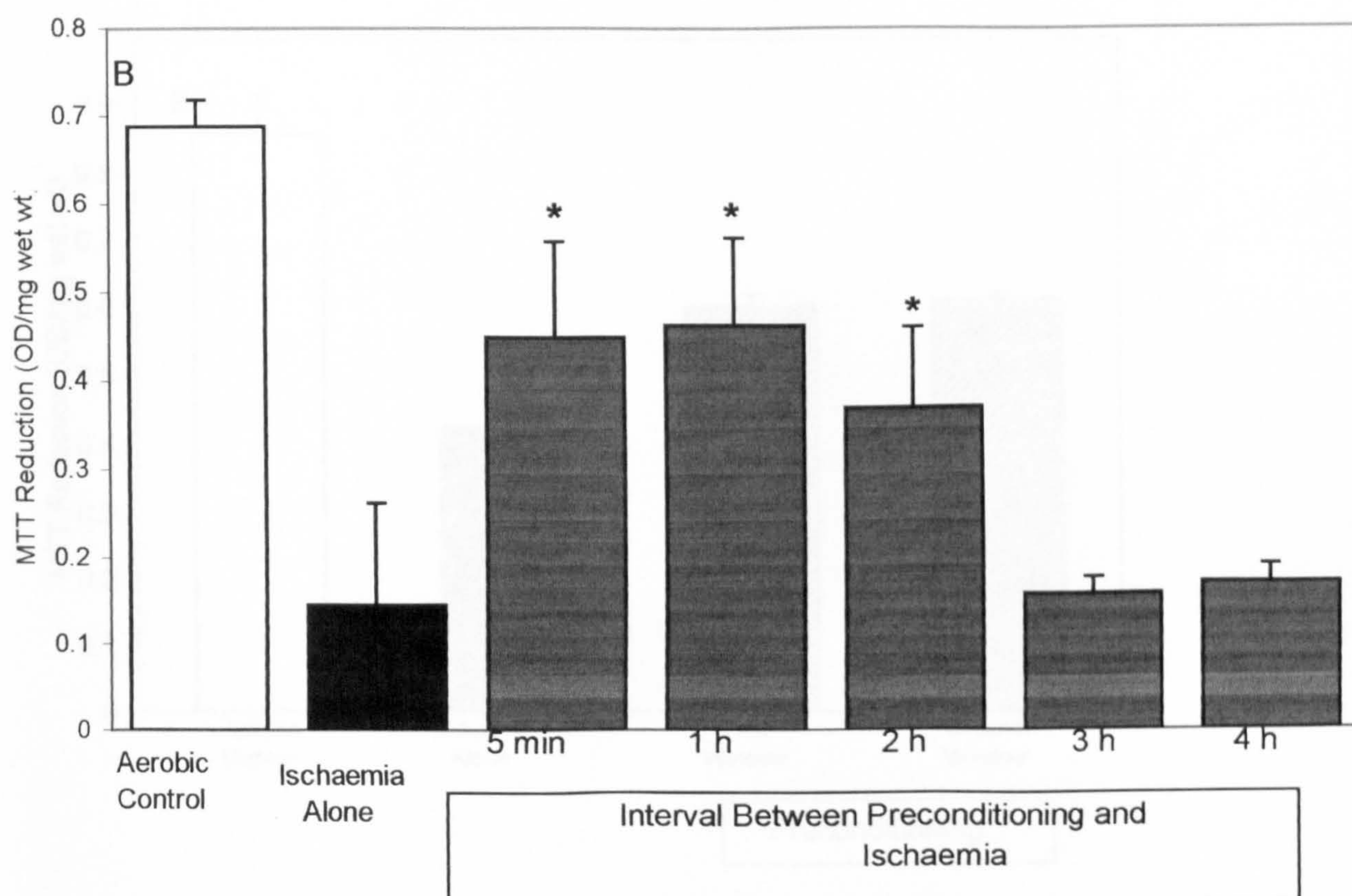
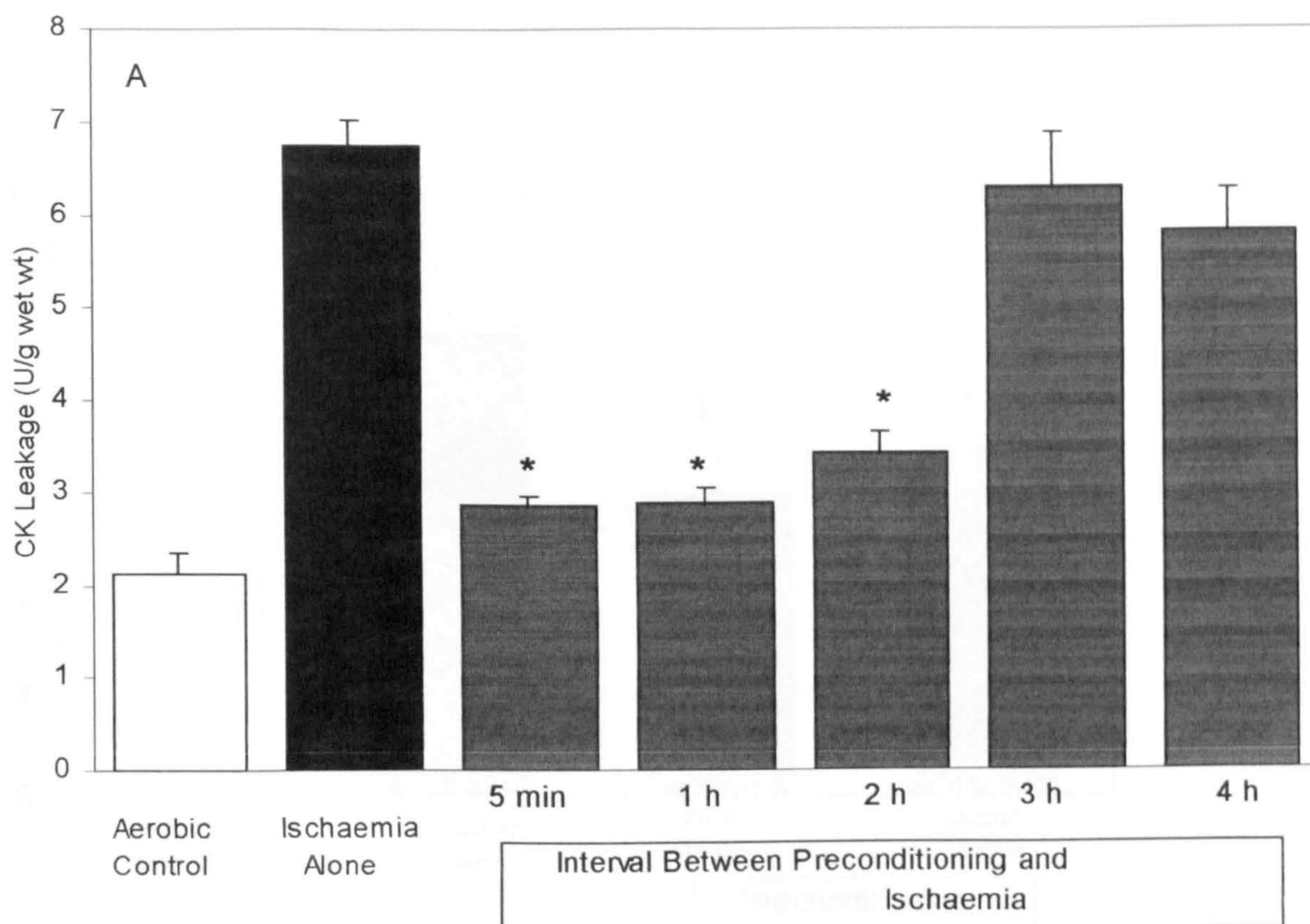
Figures 3.5A & B Study of the effect of the duration of the preconditioning ischaemic stimulus *p < 0.05 vs Ischaemia Alone group



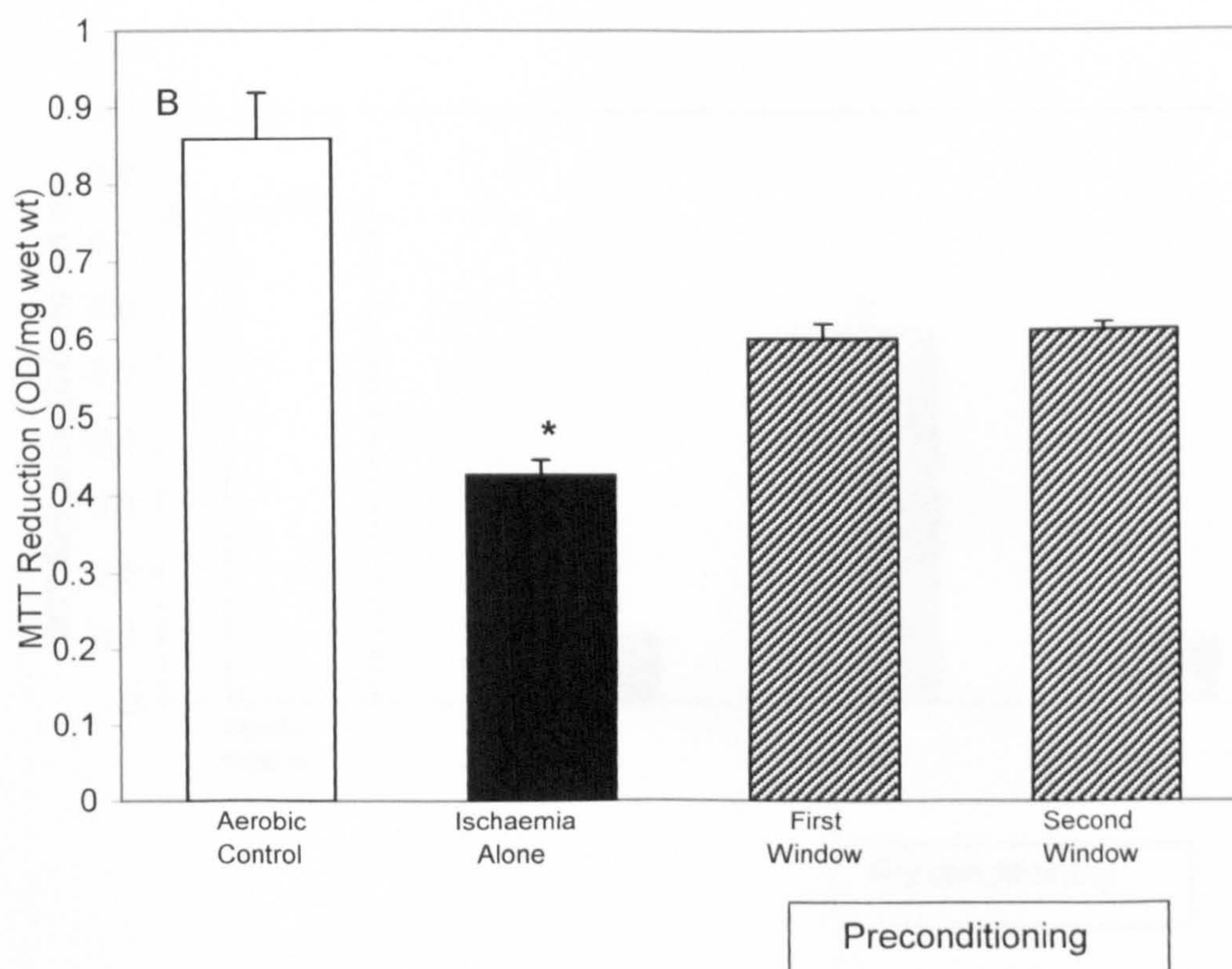
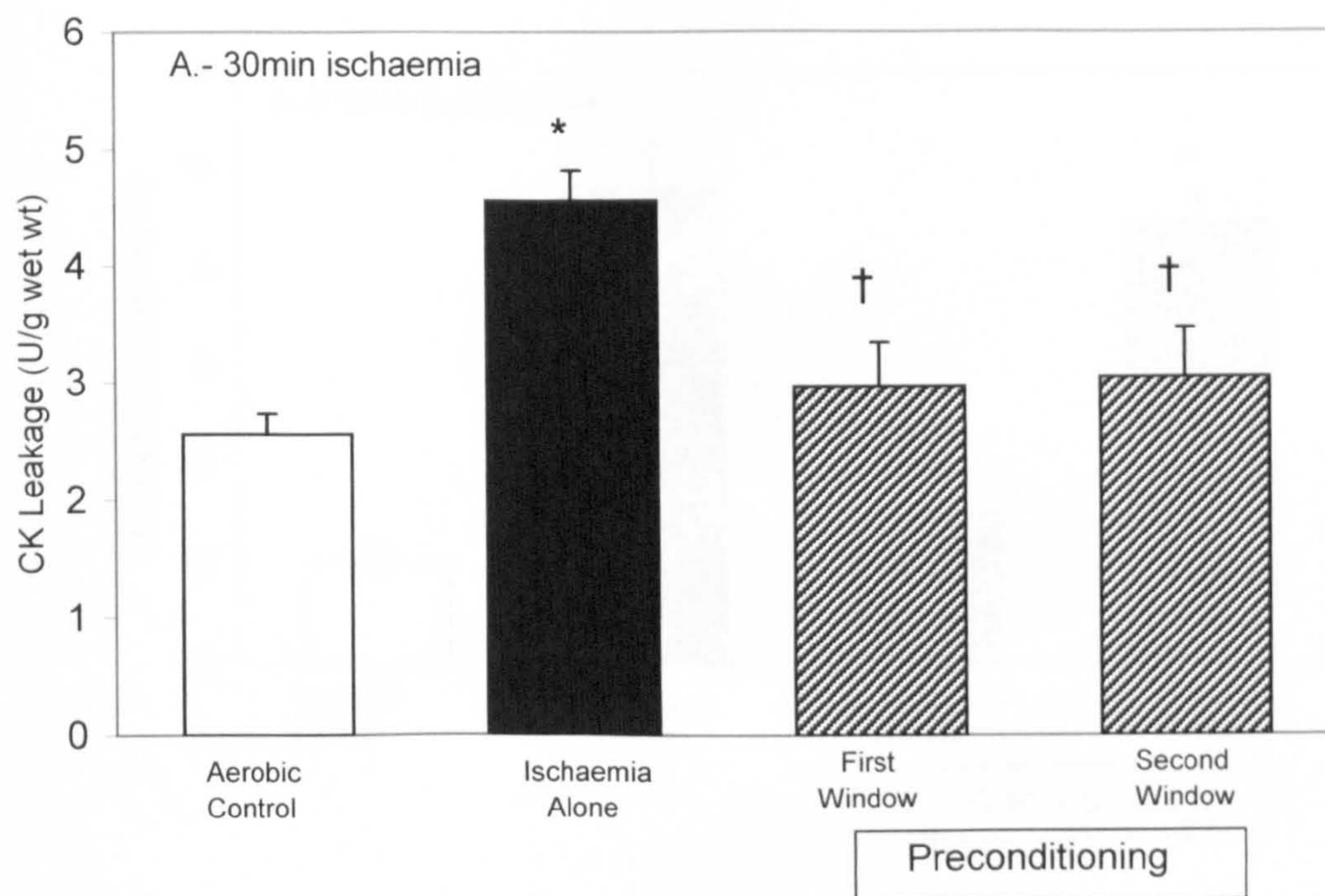
Figures 3.6A & B. Study of the number of preconditioning cycles (2min ischaemia/5min reperfusion). *p < 0.05 vs Ischaemia Alone group.



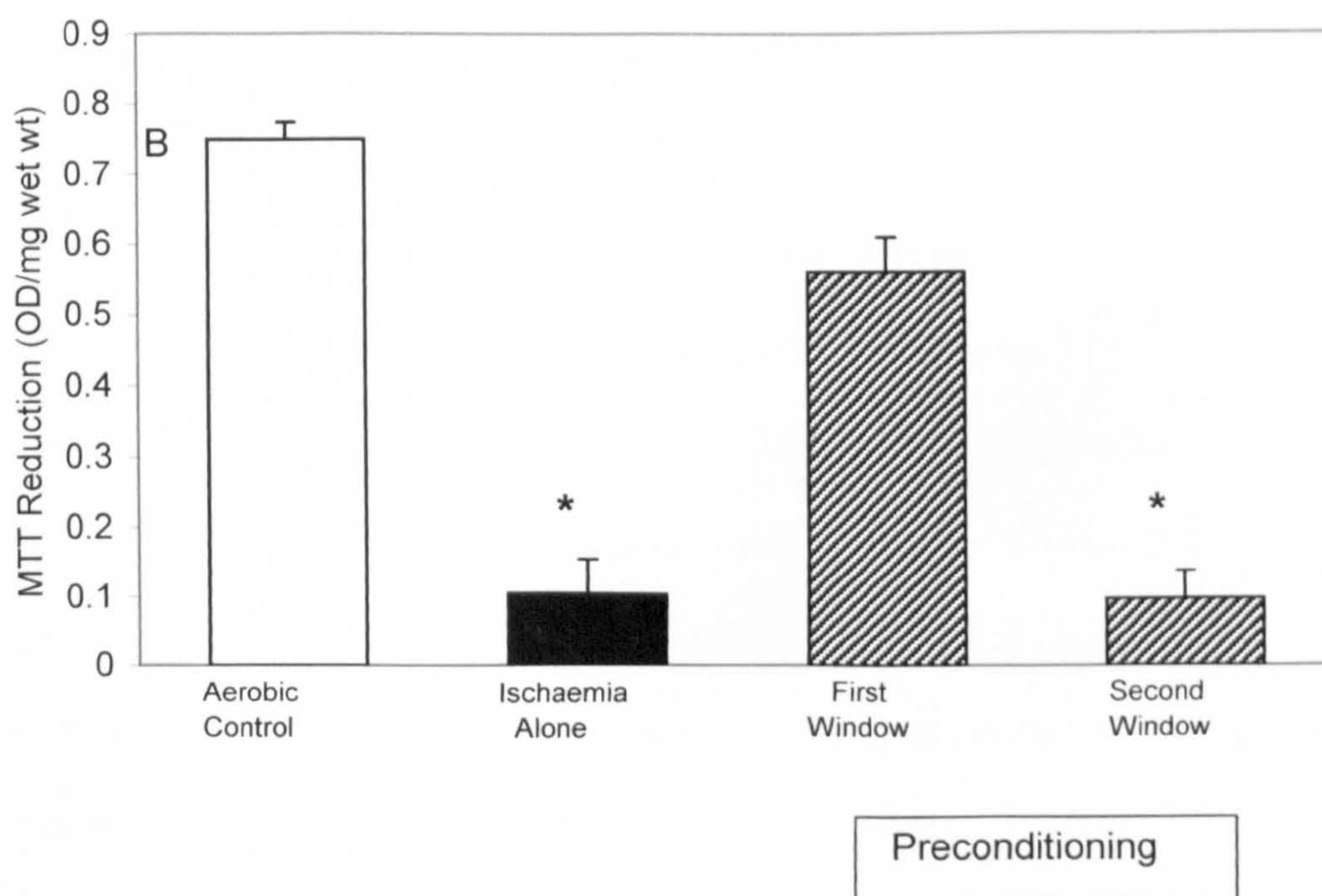
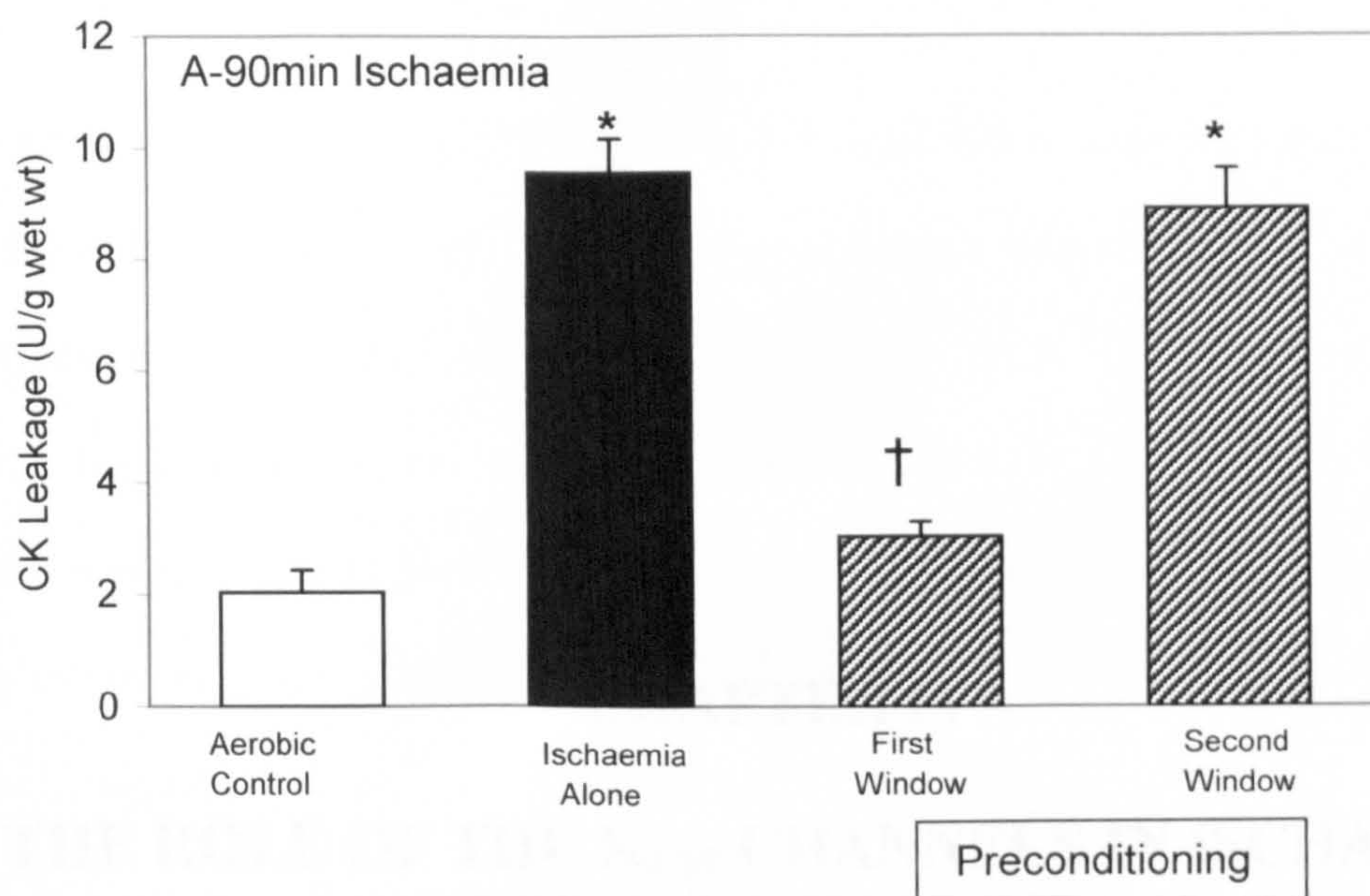
Figures 3.7A & B. Study of the number of preconditioning cycles (5min ischaemia/5min reperfusion)
*p < 0.05 vs Ischaemia Alone group



Figures 3.8A & B. Study of the duration of the initial protective effect of preconditioning ('first window'). * $p < 0.05$ vs Ischaemia Alone group.



Figures 3.9A & B. Study of the effect of the 'first and second window' of preconditioning
 * $p < 0.05$ vs Aerobic control group. † $p < 0.05$ vs Ischaemia Alone group.



Figures 3.10A & B. Study of the effect of the 'first and second window' of preconditioning
 * $p < 0.05$ vs Aerobic control group. † $p < 0.05$ vs Ischaemia Alone group.

CHAPTER IV

THE ROLE OF THE K_{ATP} CHANNELS IN ISCHAEMIC PRECONDITIONING

4.1 INTRODUCTION

The basis of cardioprotection by ischaemic preconditioning is not fully elucidated despite intensive investigation. The most favoured current hypothesis for preconditioning suggests that a variety of endogenous ligands such as adenosine, bradykinin, catecholamines and opioids activate receptors linked to protein kinase C to initiate an intracellular signal transduction pathway. PKC may activate a tyrosine kinase, which in turn activates MAP or JUN kinases leading finally to phosphorylation of an effector protein, which ultimately leads to protection [46].

The ATP-sensitive K⁺ channel (K_{ATP}) has been suggested as a possible end-effector in the mechanism of ischaemic preconditioning [118]. This evidence arises primarily from pharmacological studies [8,107,273]. K_{ATP} channels exist in the sarcolemma and in mitochondria as described in Chapter 1. However, since the effects of sarcolemmal K_{ATP} channels [109,111,115] on excitability cannot alone account for the protection of preconditioning, it has been suggested that mitochondrial K_{ATP} channels may be the true effectors mediating the beneficial action of preconditioning. This hypothesis is supported by recent reports by Garlid et al [99] showing that diazoxide opens reconstituted mitochondrial K_{ATP} channels at cardioprotective concentrations but much less potent on sarcolemmal channels and by Liu et al [196], who showed that diazoxide caused oxidation of mitochondrial flavoproteins in isolated cardiac myocytes, consistent with activation of mitochondrial K_{ATP}.

It has been reported that K_{ATP} channels are also participating in the protection of preconditioning in man [282,307], however, to the best of my knowledge, there is no evidence as yet that mitochondrial K_{ATP} channels are involved in human preconditioning. The aim of the present chapter was to investigate whether the protection induced by preconditioning in the human myocardium is mediated by mitochondrial K_{ATP} channels. To achieve this, I used our model of simulated ischaemia with right atrial trabeculae obtained from patients undergoing elective cardiac surgery and investigated the effects of the mito K_{ATP} channel opener diazoxide, the blockers 5-hydroxydecanoate(5-HD) and glibenclamide, and the sarcolemmal K_{ATP} blocker HMR 1883.

4.2 MATERIALS AND METHODS:

4.2.1 *Experimental Preparation*

Experiments were performed on trabeculae as described in section 2.3 (Chapter 2).

4.2.2 *Solutions and drugs*

Solutions were prepared daily as described in section 2.3 (Chapter 2). The K_{ATP} channel blockers glibenclamide and HMR 1883 were made up to a concentration of 10 μ mol in 100 mL K-H solution and sodium 5-hydroxy decanoic acid (5-HD), was made up to a concentration of 1 mmol in 500 mL K-H solution. The K_{ATP} openers pinacidil and diazoxide, were dissolved in DMSO immediately before being added into experimental solutions. The final concentration of DMSO was <0.1 %. Pinacidil and 5-HD were purchased from Research Biochemical Int. Glibenclamide and diazoxide were purchased

from Sigma Chemicals and HMR 1883 was a gift from Hoechst Marion Roussel, Frankfurt.

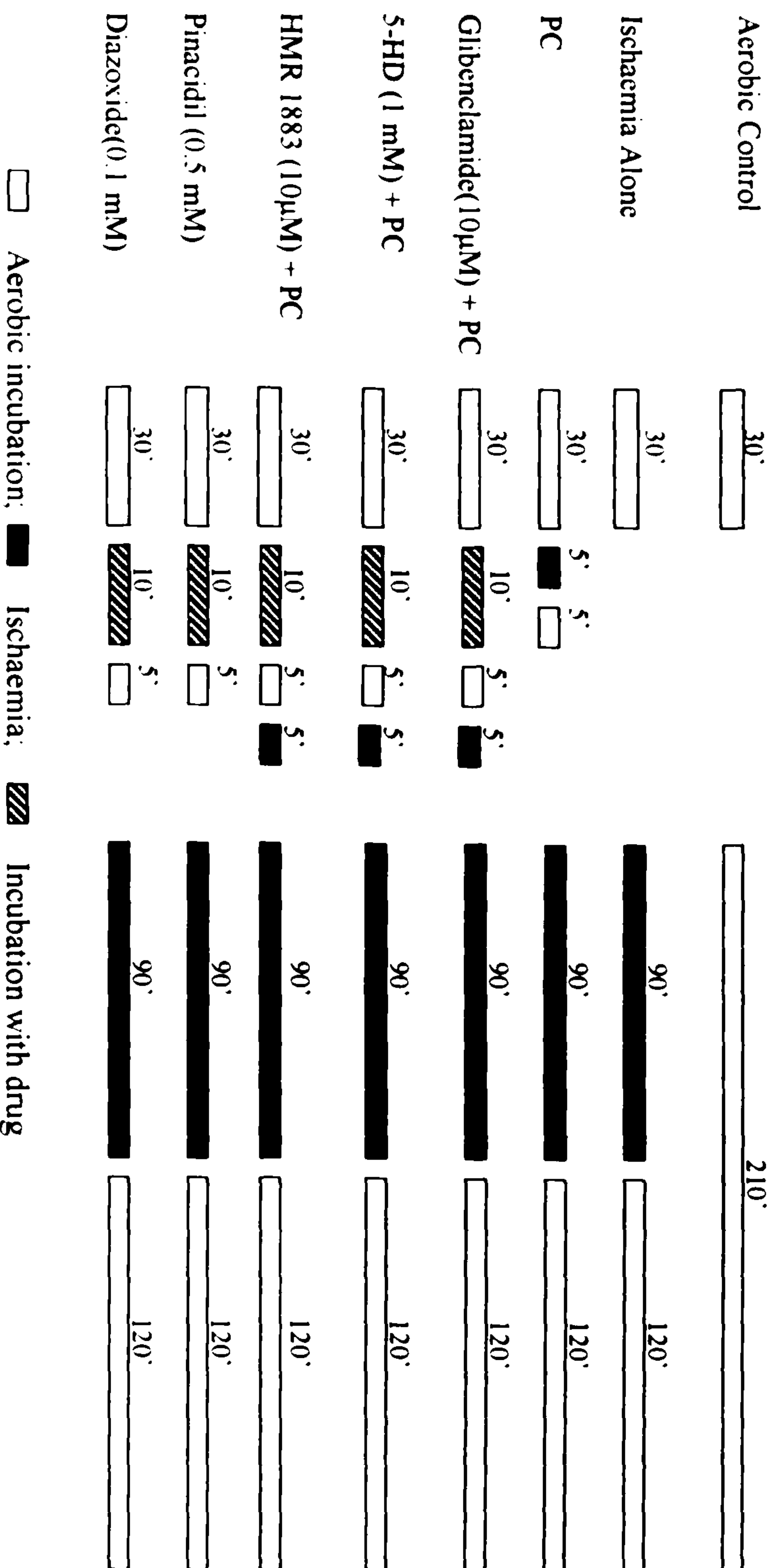
4.2.3 *Experimental Protocols*

Initially, a dose-response analysis was undertaken with various doses for each of the five different drugs used in the experimental protocols. The drugs were applied to the sections for 10min and then washed out after 30min of stabilisation, followed by prolonged ischaemia and reoxygenation. Once the optimal dose for each drug was determined, the preparations were randomly allocated to various protocols (n=6/group). In the groups subjected to simulated ischemia, this was induced for a period of 90min and then it was followed by 120min of reperfusion. The time course and the incubation times with the various agents are shown in Figure 4.1

4.2.4 Assessment of tissue injury and viability:

Tissue injury was analysed as described in section 3.2.4 (Chapter 3).

Figure 4.1: Experimental protocols for Study 1. All groups were equilibrated for 30min in aerobic conditions (37°C). Following this, the right atrial slices (n=6/group) were randomly assigned to the study groups. **Aerobic control:** slices were aerobically incubated for the entire experimental time course. **Ischaemia alone:** the slices underwent 90min simulated ischaemia followed by 120min reoxygenation. **PC:** the slices were preconditioned with 5min ischaemia followed by 5min reoxygenation before undergoing 90min ischaemia. **Glibenclamide:** the slices were pre-treated in 10µM of the drug for 10min before 5-min ischaemia followed by 5min reoxygenation before undergoing 90min ischaemia. **5-HD:** the slices were pre-treated in 1mM of the drug for 10min before preconditioning. **HMR 1883:** the slices were pre-treated in 10µM of the drug for 10min before preconditioning. **Pinacidil:** the slices were incubated in 0.5 mM of the drug for 10min before exposure to 90min ischaemia. **Diazoxide:** the slices were incubated in 0.1mM of the drug for 10min before exposure to 90min ischaemia.



4.2.5 Statistical Analysis

All data are presented as mean \pm SEM. All values were compared by ANOVA with application of a post hoc Tukey's test. A p value of < 0.05 was considered statistically significant.

4.3 RESULTS

All samples entering the studies completed the applied experimental protocol and were included in the analysis. A dose-response analysis (0 to 5 mM) based on both CK leakage and MTT reduction, revealed that diazoxide and pinacidil were found to be most protective at a dose of 100 μ M and 0.5 mM respectively. Diazoxide lost its protective effect at doses ≥ 500 μ M and pinacidil at doses ≥ 1 mM (see Figures 4.2 and 4.3). At the above optimal doses, the greatest degree of protection was afforded when the K_{ATP} openers was applied for 10 min before ischemia (pretreatment) followed by wash-out.

Glibenclamide abolished the protective effects of preconditioning at doses > 10 μ M (see Figure 4.4). A dose response analysis for 5-HD (Figure 4.5) was performed between concentrations of 0-10 mM. The minimal effective concentration for 5-HD which abolished protection afforded by preconditioning was 1 mM. The dose-response analysis for HMR 1883 (Figure 4.6) revealed that concentrations between the ranges 0 to 100 μ M had no effect on the protection afforded by preconditioning. Again pretreatment with these drugs for 10min before ischemia was the most effective protocol.

Figure 4.7 shows that ischaemia alone resulted in a significant increase in CK leakage and that preconditioning completely reversed the effect of ischaemia so that CK leakage was similar to that seen in the aerobic control group. It also shows that glibenclamide (10 μ M), which blocks K_{ATP} channels both in the sarcolemma and the mitochondrial inner membrane, partially blocked the beneficial effect of preconditioning on CK leakage.

5-HD is a K_{ATP} channel blocker which appears to show selectivity for mito K_{ATP} channels over sarcolemmal K_{ATP} .

Thus 5-HD has been shown to block K_{ATP} channels in isolated mitochondria [145] but did not affect sarcolemmal K_{ATP} currents activated by cromakalim [207]. In isolated rabbit cardiac myocytes, Sato et al [266] have recently shown that 5-HD inhibited oxidation in response to the opener pinacidil, but did not block sarcolemmal K_{ATP} current activated by the same opener, consistent with 5-HD showing selectivity for blocking of mito K_{ATP} over sarcolemmal K_{ATP} . Figure 4.7 also shows that 5-HD (1 mM) abolished the protective effect of PC on CK leakage in human myocardium. We also used the novel sulphonylthiourea HMR 1883, which is thought to have the reciprocal selectivity to 5-HD, preferentially blocking the sarcolemmal K_{ATP} channel [102,190]. HMR 1883 did not block the protective effect of preconditioning.

The results in Figure 4.7 also show the effects of pretreatment with K_{ATP} channel openers in the absence of an ischemic preconditioning stimulus. Both the non-selective K_{ATP} channel opener pinacidil and the selective opener of mito K_{ATP} channels diazoxide were protective; with diazoxide reducing CK leakage to levels not significantly different from those obtained with PC itself and pinacidil exhibiting a less potent effect than PC. In this

connection, it is worth noting that Garlid et al [refs] found that diazoxide was around 2000-fold more potent at opening mitoK_{ATP} than cardiac sarcolemmal K_{ATP} channels.

Figure 4.8 shows the results of MTT reduction. In essence, it reveals a mirror image of the results with CK leakage in the aerobic control, ischemia alone and preconditioning groups. Thus, ischemia alone caused a five-fold decrease in MTT reduction values to those seen in the aerobic control group, and this effect was significantly prevented by preconditioning. Both glibenclamide and 5-HD similarly abolished the protective effect of preconditioning. However, HMR 1883 decreased MTT reduction values to a similar degree as that seen in PC group. Interestingly, diazoxide was as effective as preconditioning in that the MTT reduction values were similar in the two groups, however, pinacidil was less effective and the MTT reduction values in this group were only one half of those seen in the preconditioning and diazoxide groups.

Figure 4.2 Dose-response experiments of diazoxide on leakage of creatinine kinase (CK) into the media during the 120min reoxygenation period (last 120min in the aerobic control period) and MTT reduction. Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs Aerobic control group, # $p < 0.05$ vs Ischaemia Alone group.

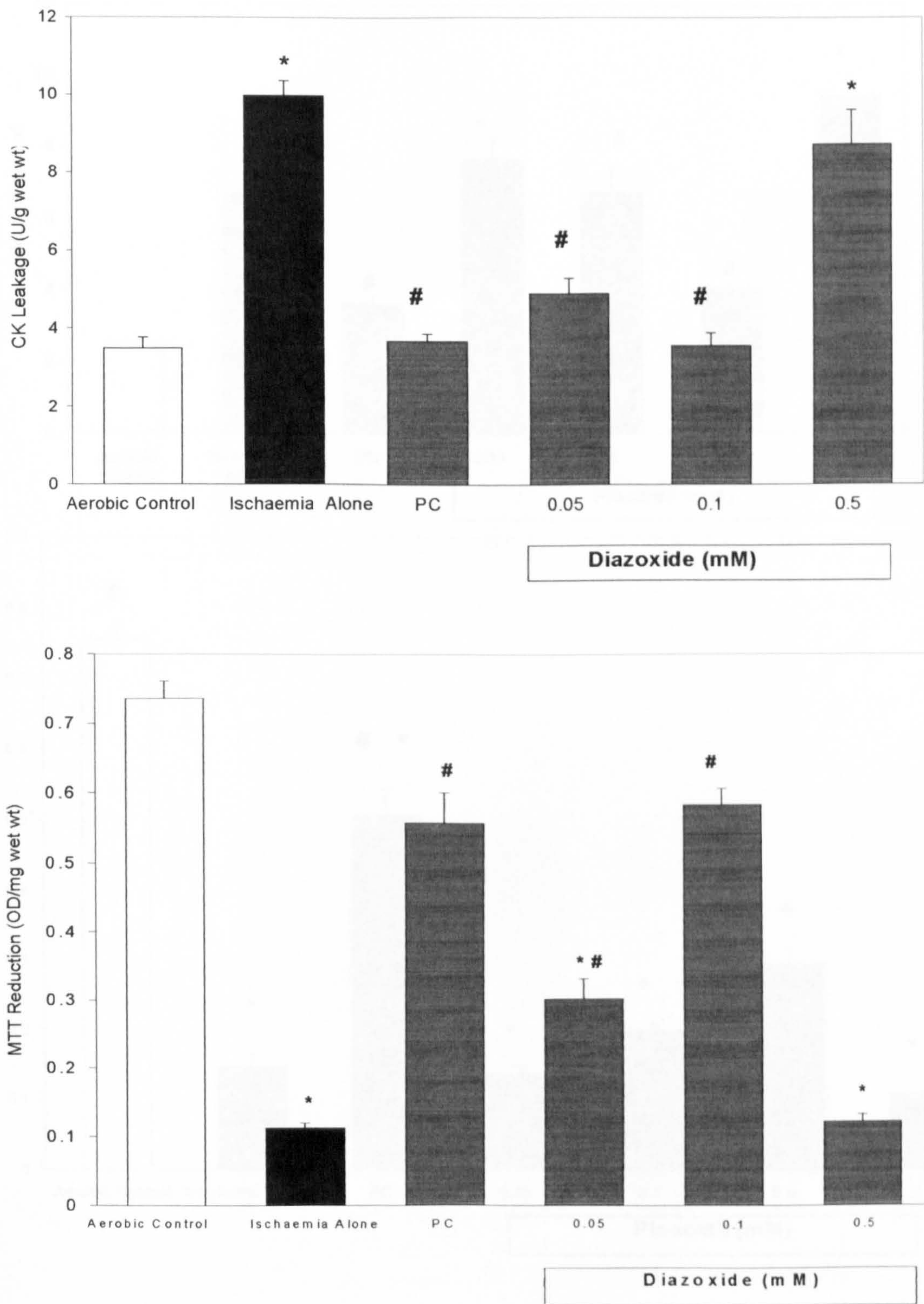


Figure 4.3 Dose-response experiments of pinacidil on leakage of creatinine kinase (CK) into the media during the 120min reoxygenation period (last 120min in the aerobic control period) and MTT reduction. Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs Aerobic control group, # $p < 0.05$ vs Ischaemia Alone group.

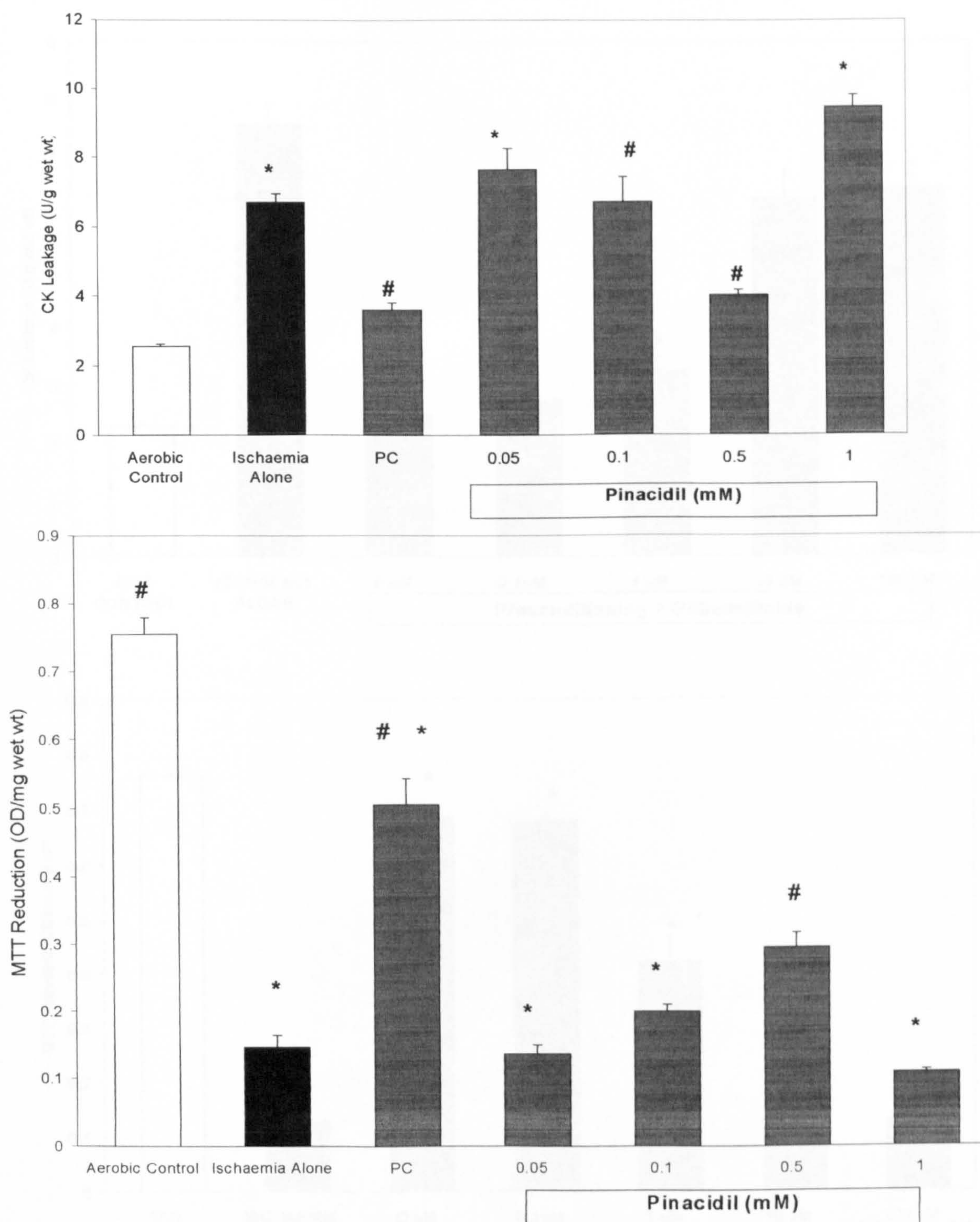


Figure 4.4 Dose-response experiments of glibenclamide on leakage of creatinine kinase (CK) into the media during the 120min reoxygenation period (last 120min in the aerobic control period) and MTT reduction. Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs Ischaemia Alone group.

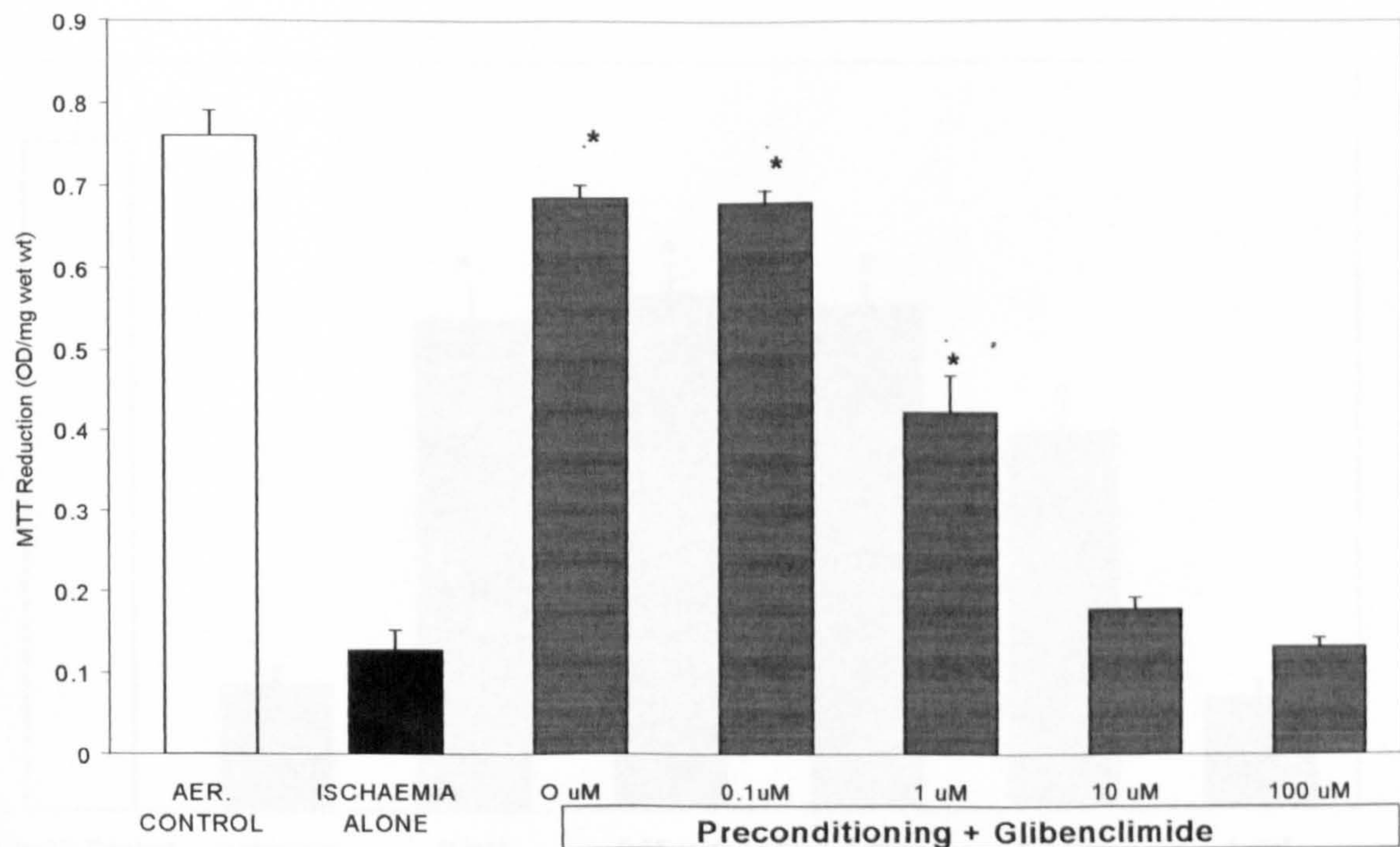
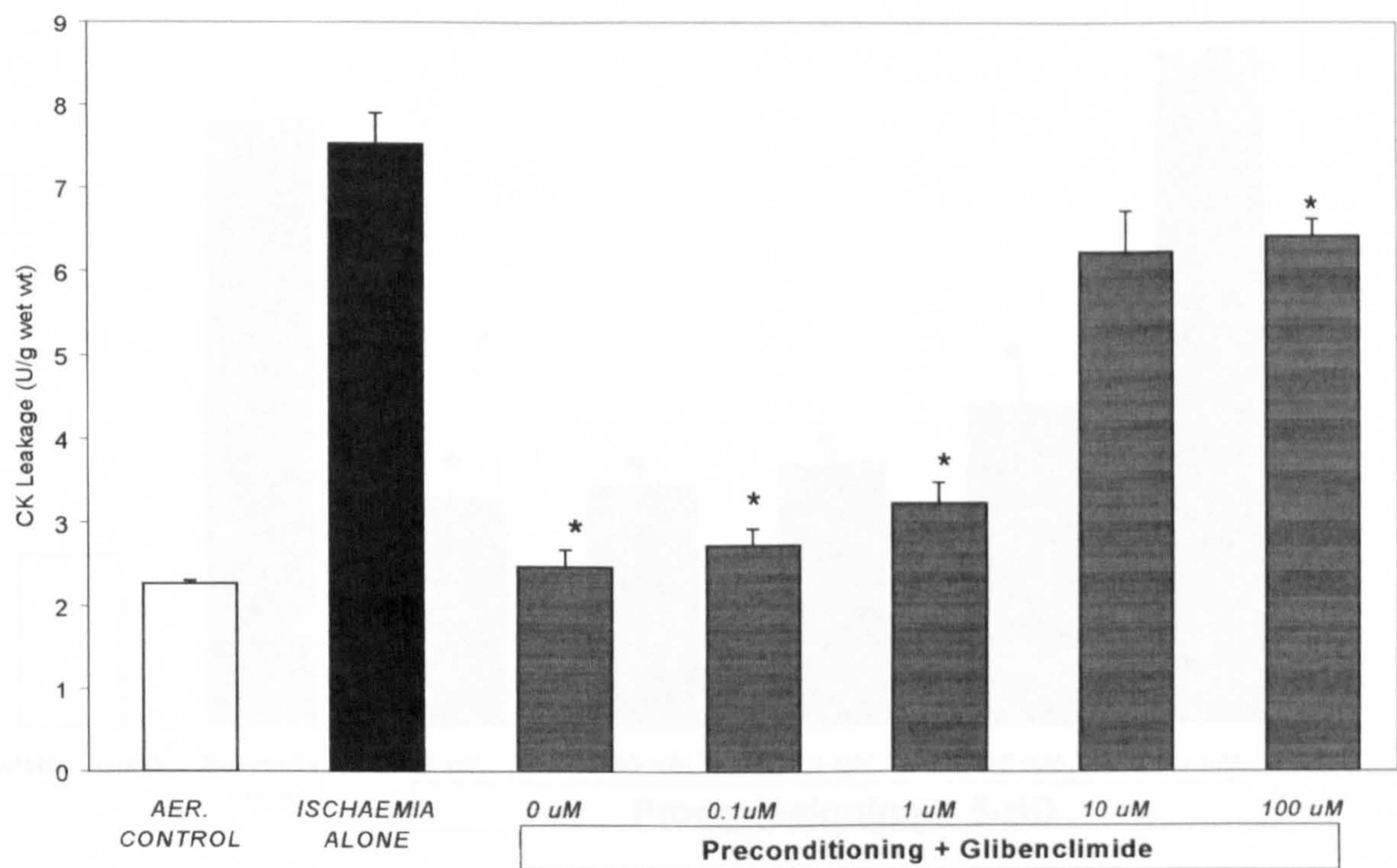


Figure 4.5 Dose-response experiments of 5-HD on leakage of creatinine kinase (CK) into the media during the 120min reoxygenation period (last 120min in the aerobic control period) and MTT reduction. Data are expressed as mean \pm standard error of mean of 6 experiments. *p <0.05 vs Ischaemia Alone group.

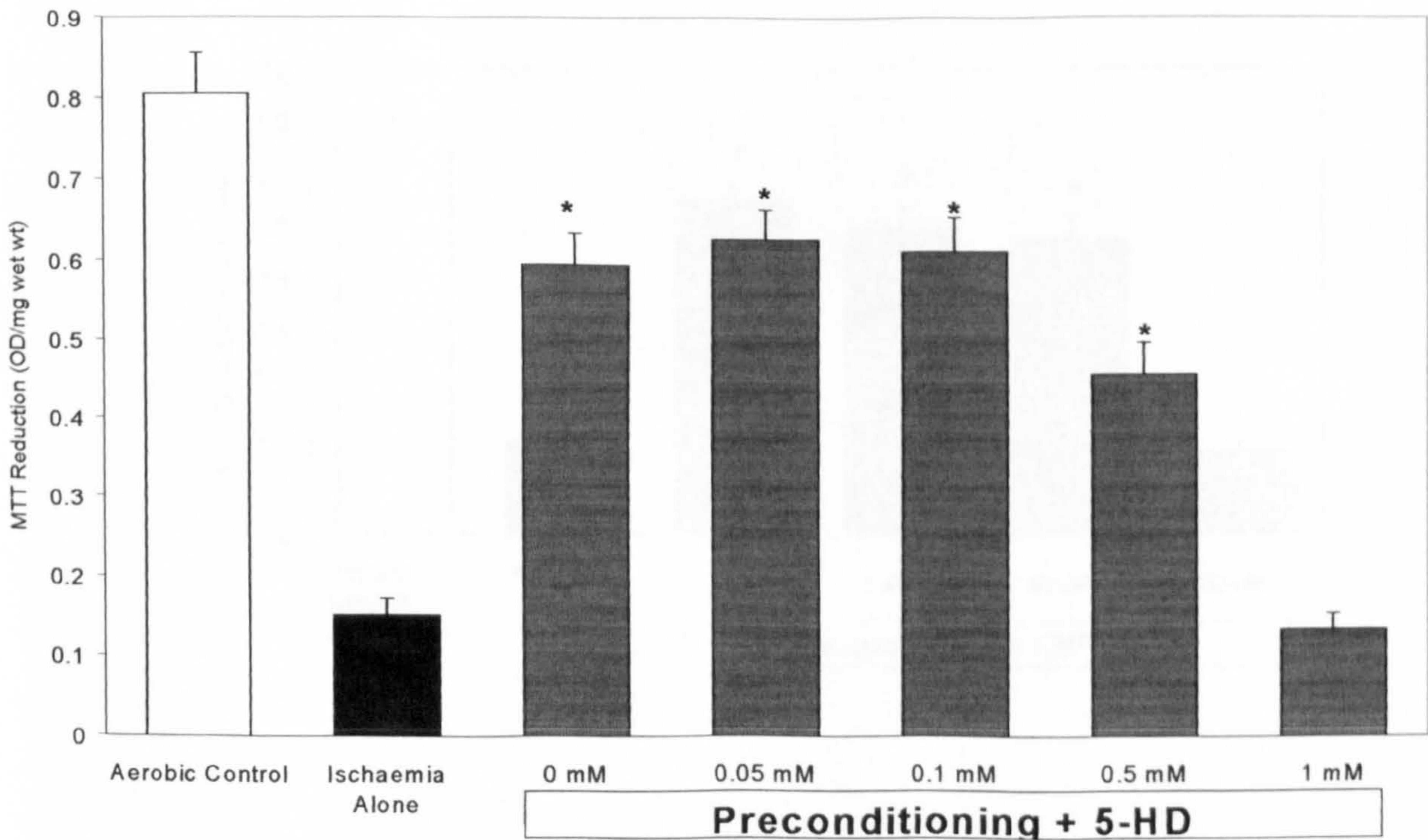
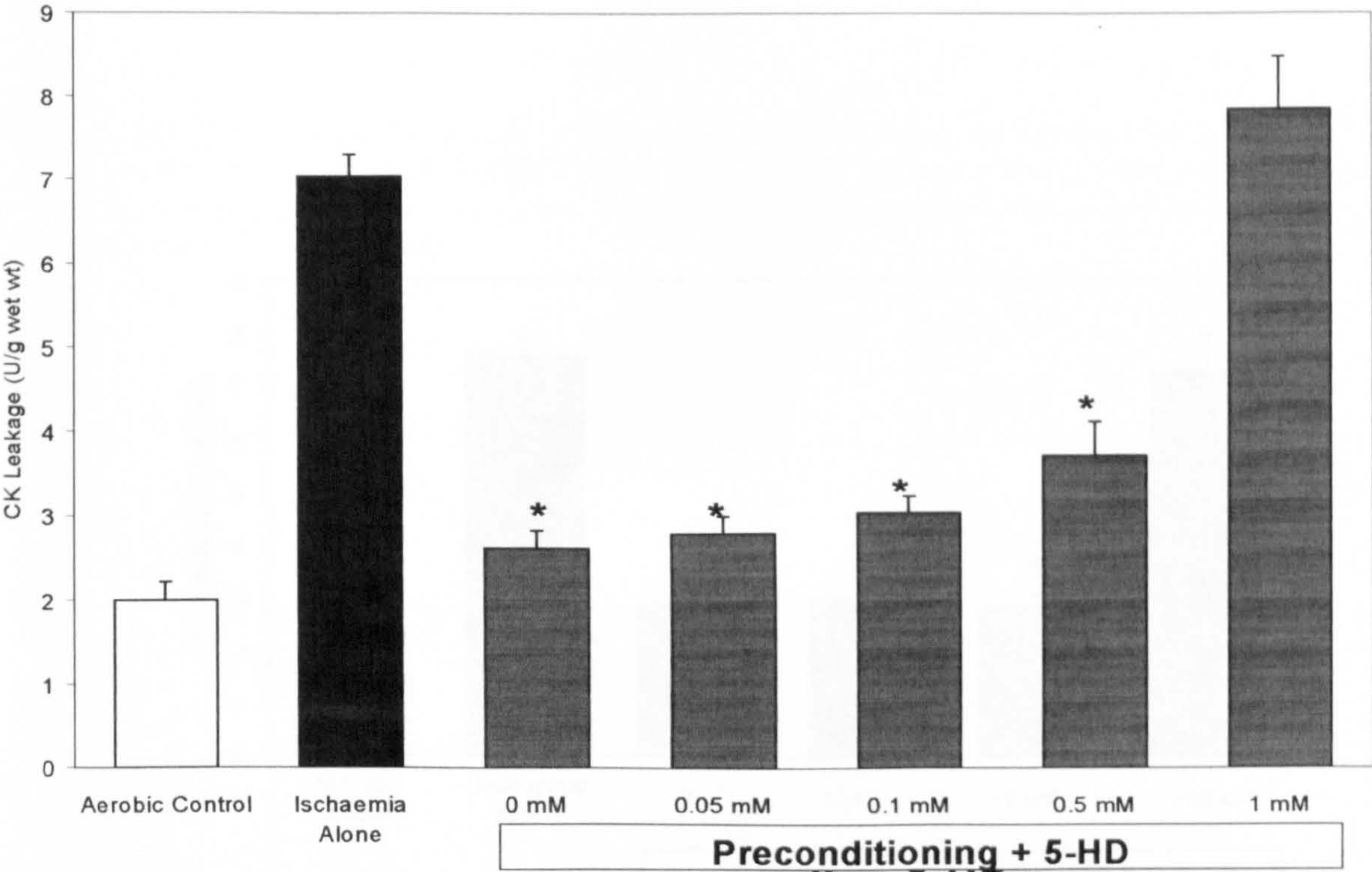


Figure 4.6 Dose-response experiments of HMR-1883 on leakage of creatinine kinase (CK) into the media during the 120min reoxygenation period (last 120min in the aerobic control period) and MTT reduction. Data are expressed as mean \pm standard error of mean of 6 experiments. *p <0.05 vs Ischaemia Alone group.

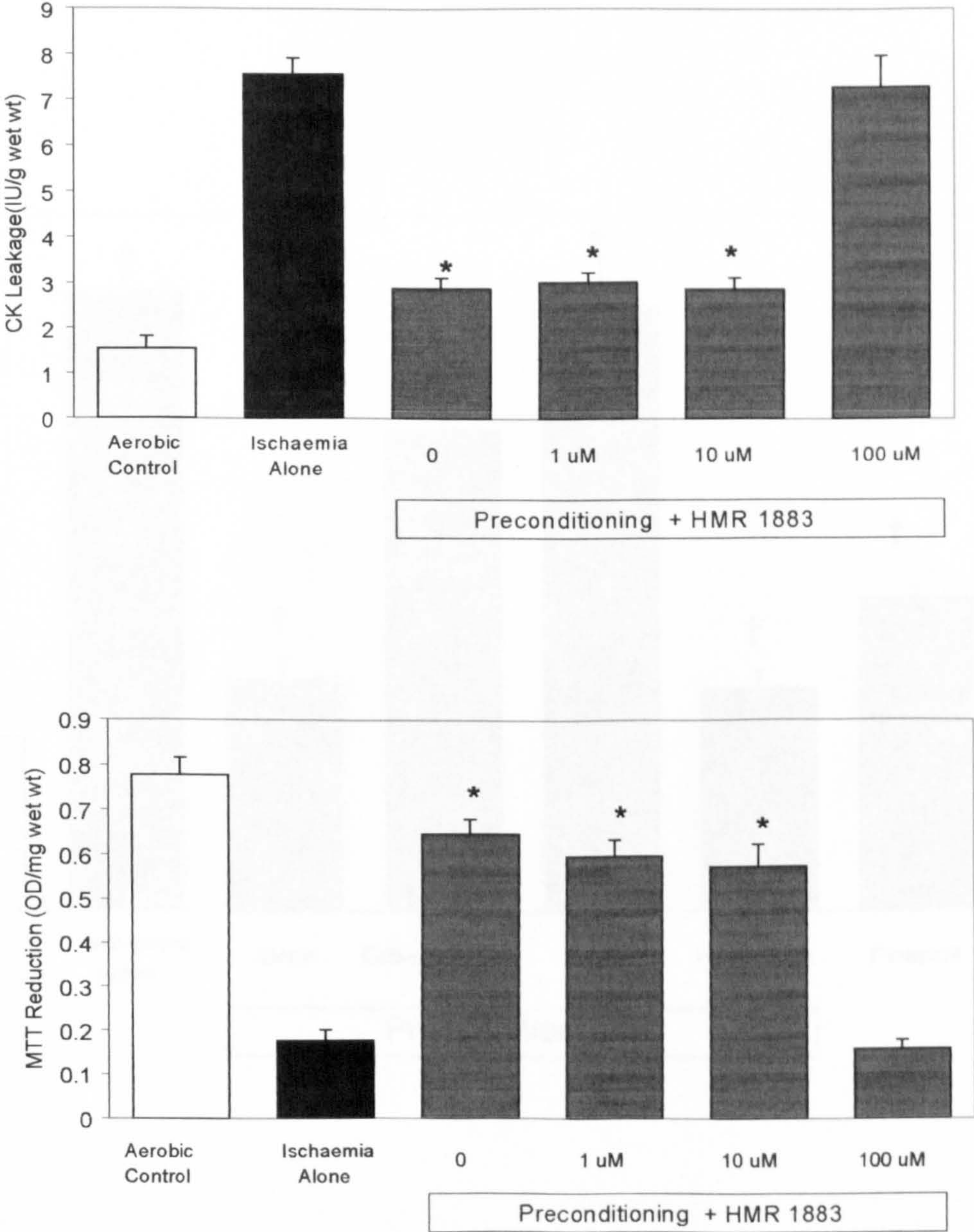


Figure 4.7: Leakage of creatinine kinase (CK) into the media during the 120min reoxygenation period (last 120min in the aerobic control period). Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs Aerobic control group, † $p < 0.05$ vs Ischaemia Alone group and ¶ $p < 0.05$ vs PC group.

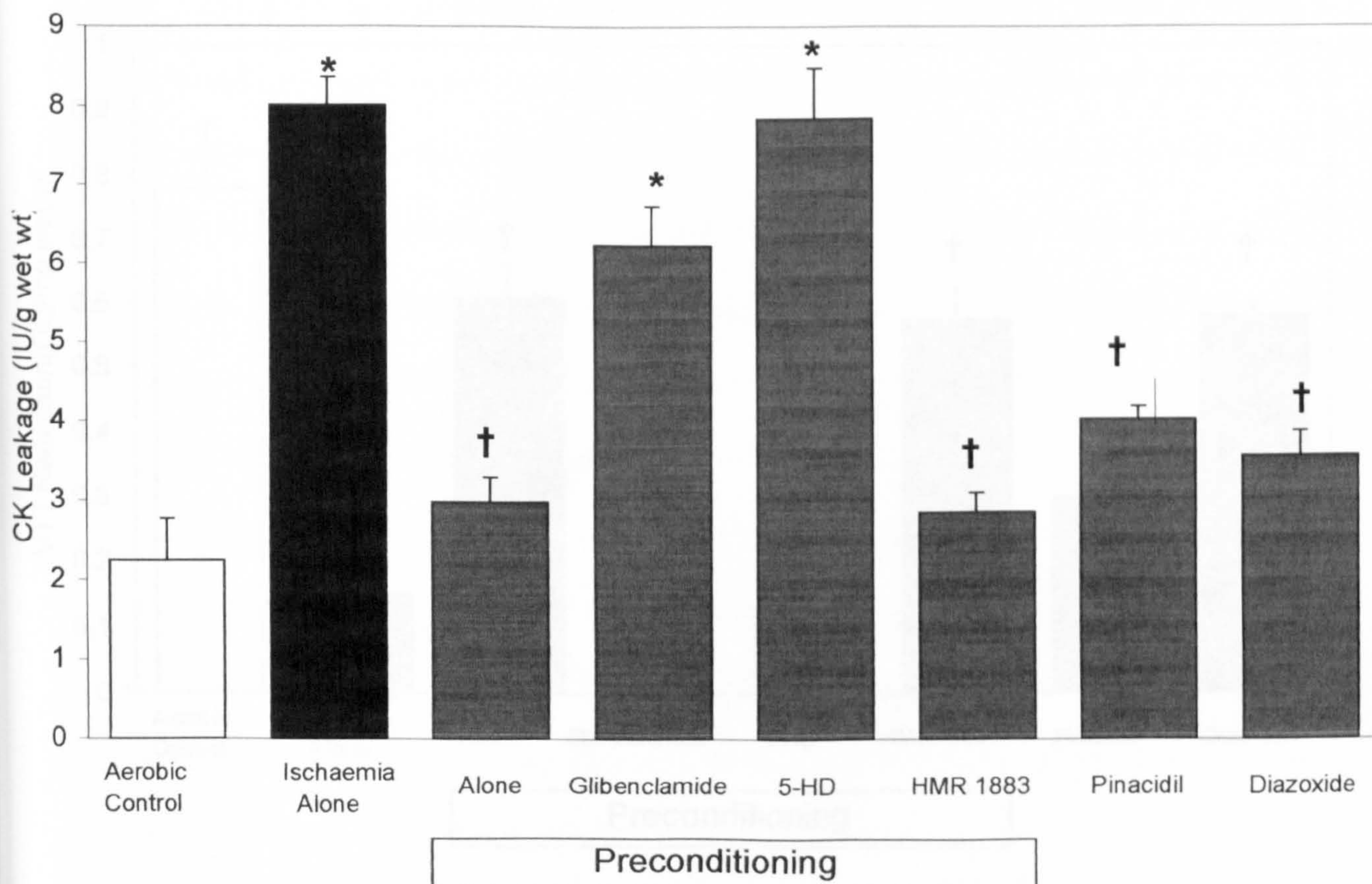
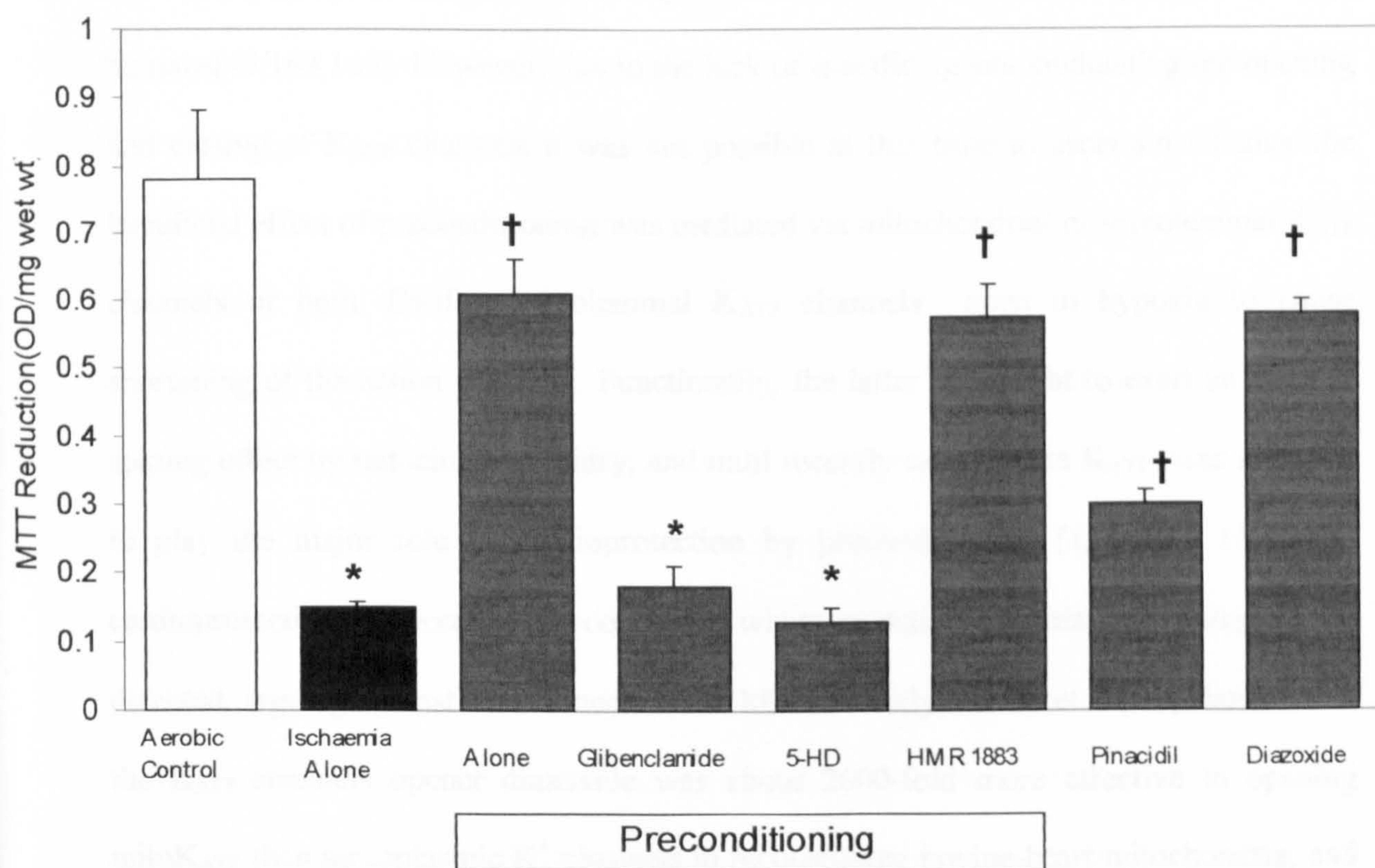


Figure 4.8: Measurement of MTT reduction by the slices at the end of the reoxygenation period. Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs Aerobic control group, † $p < 0.05$ vs Ischaemia Alone group and ¶ $p < 0.05$ vs PC group.



4.4 DISCUSSION:

The involvement of K_{ATP} channels in preconditioning was first suggested by Gross and Auchampach [107] and Auchampach et al [17] in the canine heart. These authors showed that K_{ATP} channels blockers, glibenclamide and 5-HD, blocked the protection induced by preconditioning and also revealed that aprikalim, a K_{ATP} channel opener, mimicked the cytoprotective effect of preconditioning in reducing infarct size. Subsequently, a plethora of pharmacological studies have shown that the opening of K_{ATP} channels contributes to the cardioprotection of preconditioning in number of models and species including humans[45,164,165]. However, due to the lack of specific agents modulating the opening and closing of K_{ATP} channels it was not possible at that time to ascertain whether the beneficial effect of preconditioning was mediated via mitochondrial or sarcolemmal K_{ATP} channels or both. Cardiac sarcolemmal K_{ATP} channels open in hypoxia to cause shortening of the action potential. Functionally, the latter is thought to exert an energy-sparing effect by reducing Ca^{2+} entry, and until recently sarcolemma K_{ATP} was assumed to play the major role in cardioprotection by preconditioning [134,302]. However, cardioprotection can occur under conditions where no action potential shortening can be detected, arguing against such a mechanism. More recently, Garlid et al [99] showed that the K_{ATP} channels opener diazoxide was about 2000-fold more effective in opening mito K_{ATP} than sarcoplasmic K^+ channels in reconstituted bovine heart mitochondria, and that its cardioprotective potency in rat hearts correlated with its effectiveness on mitochondrial rather than sarcolemmal channels. Liu et al [196] using isolated rabbit ventricular myocytes used flavoprotein oxidation as an index of mito K_{ATP} channel

activity, and showed that diazoxide induced oxidation at concentrations that correlated well with its cardioprotective effects, but which did not activate sarcolemmal K_{ATP} channels. The present study provides evidence for the first time that opening of mito K_{ATP} channels may be responsible for the protection induced by ischaemic preconditioning in the human myocardium.

K_{ATP} channels are composed of 2 proteins, an inwardly rectifying potassium channel (Kir 6.x) and a sulphonylurea receptor (SUR) subunit. It has been suggested that SUR2A and Kir 6.2 are found in cardiac sarcolemma. Unfortunately, to date the mito K_{ATP} channel has not been cloned as yet but there are suggestions that Kir 6.1 subunit [293] is involved in the mitochondrial membrane of the rat skeletal muscle and liver. Certainly, mitochondrial and sarcolemmal K_{ATP} channels appear to exhibit minor differences in structure but the function of the mitochondrial K_{ATP} channels appear to be intimately involved in matrix volume control as opposed to electrical activity for sarcolemmal K_{ATP} channels. In this instance, opening of the mito K_{ATP} channel leads to membrane depolarization, matrix swelling, slowing of ATP synthesis, and accelerated respiration [108]. There is good evidence that diazoxide and 5-HD show good selectivity for mitochondrial over cardiac sarcolemmal K_{ATP} channels [99,266] and this present study confirms that preconditioning can be mimicked with diazoxide and abolished with both 5-HD and glibenclamide. These results are in close agreement with those of Garlid et al [99] and further suggest that the mito K_{ATP} channels are the possible effectors of cardioprotection produced by ischaemic preconditioning. To more clearly address this issue, I used a specific sarcolemmal K_{ATP} channel blocker, HMR 1883. The novel blocker HMR 1883 shows good selectivity for the cardiac sarcolemmal K_{ATP} channel over those

of pancreatic beta cells and the vasculature[266]. Further, a brief report suggests that HMR 1883 did not block the protection induced by ischaemic preconditioning in the rabbit [102]. My results are in agreement with this, since I found that the protective effect of PC persisted in the presence of HMR 1883. Taken together with my findings with 5-HD and diazoxide, this suggests that HMR 1883 does not block mito K_{ATP} channels of human myocardium at the concentration used (10 μ M), and that blockade of the sarcolemmal channel does not abolish protection.

The mechanisms by which the opening of mito K_{ATP} channels exert the cardioprotective effect of preconditioning are not fully understood. Consequences of opening of the mito K_{ATP} channel include depolarization of the intramitochondrial membrane as K^+ enters leading to decreased calcium in-port and matrix swelling. In this regard, Halestrap [120] suggested that if cell swelling, such as occurs during ischaemia, were to activate both the sarcolemmal and mitochondrial K_{ATP} channels simultaneously by stretch-induced protein phosphorylation, a loss of K^+ would occur from the cytosol and an increase in K^+ into the mitochondria would be expected to occur that might produce intramitochondrial swelling and a subsequent increase in ATP production. Membrane depolarization produced by the K^+ entry would also be expected to reduce mitochondrial calcium entry through the calcium uniport, thus reducing calcium overload. In addition, Holmuhamedov et al [135] have shown that preloaded mitochondria release calcium in response to activation by a K_{ATP} channel opener, which suggests that a cell in which calcium overload is already present may also be protected by a K_{ATP} opener or enhanced activation of the channel after PC.

Furthermore, Van den Hoek et al [316] have suggested that reactive oxygen species released by mitochondria during a brief period of hypoxia can precondition isolated myocytes, and Becker et al [27] have shown that PC reduces superoxide production and prevents the impairment of state 3 mitochondrial respiration induced by ischaemia and reperfusion. The interaction between the mito K_{ATP} channel and free radicals is not fully elucidated; however, several studies have demonstrated that free radicals open sarc K_{ATP} channels[20,303] and thus, it is possible that such an interaction may also occur within mitochondria [173,316]. The mechanism of how this ultimately stimulates mitochondrial respiration and consequently the cytoprotective effect is much less clear and warrants further research.

A potential limitation of this study was the use of atrial tissue as opposed to ventricular myocardium and therefore any extrapolation must be conducted with caution; however, Yellon and colleagues have suggested that preconditioning exerts identical protection in both tissues [52,282]. Undoubtedly, as mentioned previously, K_{ATP} channels are present in both atrium and ventricle [132], although their density in both tissues is unknown. As mentioned previously also, another possible limitation might be that right atrial appendages were obtained from patients subjected to various medical treatments (e.g. nitrates, β -blockers, calcium antagonists) and that in principle may have influenced ischaemia/reperfusion injury and the protection induced by preconditioning. However, it should be emphasized, that all medication was stopped the day before surgery when specimens were taken for the study and that significant effect of the medication was unlikely since all preparations responded to ischemia/reperfusion with a similar degree of injury and preconditioning was protective in all instances when applied.

It should be mentioned that the preparation used in this study was not electrically stimulated (i.e. non-beating) and therefore one should be cautious when extrapolating to the *in vivo* situation.

It should also be emphasized, that in common with previous studies in non-human hearts [99,196,266], my evidence for the involvement of mitoK_{ATP} rather than sarcolemmal K_{ATP} channels depends strongly on the selectivity of the K_{ATP} channel openers and blockers used, in particular diazoxide, 5-HD, and HMR1883. In this context, the concentration of diazoxide that I used (100 μ M) has been reported to activate mitoK_{ATP} but not sarcolemmal K_{ATP} in rabbit ventricular myocytes [196]. HMR 1883 should be an effective blocker of sarcolemmal K_{ATP} channels at 10 μ M, since its reported K_i for these channels is 0.8 μ M [102], though its effects on mitoK_{ATP} have not been tested directly. 5-HD is an effective blocker of mitoK_{ATP} provided that the channel is opened by a physiological or pharmacological opener [145,196], but its selectivity for mitoK_{ATP} over sarcolemmal K_{ATP} merits further study. Notsu et al [232] reported block of sarcolemmal K_{ATP} channels in guinea pig myocytes by 5-HD, though more recently Hu et al [137] have argued that 5-HD at a concentration of 0.5 mM selectively blocks mitoK_{ATP} without affecting sarcolemmal K_{ATP} channels of rabbit ventricular cells. Certainly, 5-HD at a concentration of 1 mM was an effective blocker of cardioprotection in this human model.

In conclusion, the present study provides strong evidence that mitochondrial rather than sarcolemmal K_{ATP} channels are effectors of ischaemic preconditioning in the human myocardium. The finding has obvious important clinical implications, however, the

mechanism by which the opening of mito K_{ATP} channels is protective is not fully understood and merits further investigation. With the establishment of the role of K_{ATP} channels in human myocardial preconditioning, I turned my attention to exploring the responses of these channels in human myocardium effected by diabetes mellitus and chronic ischaemia causing heart failure and this is the subject of the experiments discussed in the next chapter.

CHAPTER V

**PRECONDITIONING – A HEALTHY HEART
PHENOMENON?**

5.1 INTRODUCTION:

Within the enormous amount of research describing the cellular basis of the preconditioning response, relatively few studies have focussed on the effect of preconditioning in hearts with concurrent abnormalities relevant to coronary artery disease in humans. More importantly, even amongst those studies, the conclusions have been conflicting. Clinical studies clearly identify a number of conditions that increase mortality due to myocardial infarction; these include heart failure, diabetes, hypertension, aging and hypercholesterolaemia [159,255]. It is plausible that these conditions interfere with the biochemical pathways underlying the preconditioning response.

Cardiovascular disease associated with diabetes mellitus is a major cause of death in diabetic patients [160]. In the vast majority of animal studies, diabetic hearts demonstrate a reduced tolerance to anoxia, hypoxia or ischaemia [86,127,299] but studies that have investigated the effect of preconditioning on diabetic hearts have yielded confusing data. Tosaki et al [312] have shown in the streptozotocin-induced diabetic rat heart that preconditioning does not confer cardiac protection. Their results were opposed to those by Liu et al [195] who had earlier shown, also in the rat heart, that myocardial infarction is reduced in diabetes and that preconditioning further increases the protection of these hearts. There are very few studies in human diabetic tissue. Cleveland et al [53] used a functional isolated atrial trabeculae model and showed that preconditioning did not confer any protection of the myocardium from patients taking long term oral hypoglycemic agents and hypothesized that long term inhibition of K_{ATP} channels with these agents may be responsible for the excess cardiovascular mortality associated with diabetes.

Heart failure is common in all forms of heart diseases. Mechanical dysfunction of the failing heart is due to many factors, including neurohormonal disturbance, accumulation of extracellular matrix, alteration of excitation-contraction coupling and a maladaptation of myocardial energetics [268]. There are very few studies that have investigated the effects of the preconditioning response in the failing myocardium in light of alterations in the cellular metabolic and biochemical pathways associated with heart failure. Cleveland et al [53] showed in isolated ventricular trabeculae from patients requiring heart transplantation that preconditioning conferred protection. However, more recently, Dekker et al [74] have studied perfused papillary muscles from rabbits in which cardiac failure has been induced by a combination of pressure and volume overloading. The end-points used to assess responses to ischaemia, namely the time to onset of a rise in the intracellular concentration of Ca^{2+} (cellular uncoupling) and of ischaemic contracture, were delayed by preconditioning in normal myocardium but were exaggerated by preconditioning in the failing myocardium.

The aims of the studies in this chapter were to investigate: (i) the effects of preconditioning on the diabetic and failing human myocardium; and (ii) the role of mitochondrial K_{ATP} channels in the responses of these pathological conditions. These studies were carried out in an in vitro model of human right atrial myocardium of simulated ischaemia and reoxygenation.

5.2 MATERIALS AND METHODS:

5.2.1 Experimental Preparation

Experiments were performed on myocardium obtained from the right atrial appendage of patients undergoing open heart surgery as described in previous chapters. Atrium from patients were excluded if they had enlarged right atriums, atrial arrhythmias, right ventricular failure or were taking opioid analgesia. Patient characteristics are detailed in Table 5.1. Experiments were performed as described in section 2.3 (Chapter 2).

5.2.2 Solutions

The incubation medium was prepared daily as described in section 2.3 (Chapter 2). The K_{ATP} channel blocker glibenclamide was made up to a concentration of 10 μmol in 100 mL K-H solution and the K_{ATP} opener diazoxide was dissolved in DMSO immediately before being added into the experimental solutions. All reagents were obtained from Sigma Chemical Co.

5.2.3 Experimental Protocols

After sectioning the atrium, the preparations were allowed to stabilize for 30min and then randomly allocated to various protocols. In all studies simulated ischaemia was induced for a period of 90min followed by 120min of reoxygenation. The drugs were applied to the sections for 10min after the initial 30min of stabilisation and then removed before ischaemia. The following two studies were performed:

Study 1: To investigate whether diabetes influences the protective effect of PC, atrial specimens were collected from 4 groups of patients: (I) non-diabetics, (II) diet controlled diabetics (DCD), (III) non-insulin dependent diabetics (NIDD) on long term oral

suplhonylureas and (IV) insulin dependent diabetics (IDD). Preparations from each group of patients were then randomly allocated to various protocols (n=6/group) shown in Figure 5.1.

Study 2: To investigate the effect of cardiac function on the protection induced by PC, atrial specimens were collected from 3 groups of patients: (I) with normal left ventricular function (LVEF >50%); (II) with moderately impaired function (LVEF=30-50%) and (III) with severely impaired function (LVEF <30%). Preparations from each group were then randomly allocated to various protocols (n=6/group) shown in Figure 5.2.

In the above two studies, PC was induced by a single cycle of 5min ischaemia/5min reoxygenation, a protocol that I have demonstrated provides maximal protection in this model. Preliminary studies (data not shown) had demonstrated that increasing the number of cycles of 5min ischaemia/5min reoxygenation from 1 to 3 does not elicit protection beyond that obtained with a single cycle. In the groups receiving diazoxide and glibenclamide, the drugs were used at a concentration of 0.1 mM and 10 μ M respectively. I have already shown that these drug concentrations are the minimal effective concentration required to elicit a response and to block PC.

5.3 Assessment of tissue injury and viability:

At the end of each experimental protocol, tissue injury was determined by measuring the leakage of creatinine kinase (CK) into the incubation medium and tissue viability by the reduction of 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) to blue formazan product.

CK Leakage: The activity of CK leakage into the media during the reoxygenation period (U/g wet wt) was assayed as described in section 3.2.6B (Chapter 3).

MTT reduction: At the end of the experimental time, MTT analysis was performed as described in section 3.2.6B (Chapter 3) and the results expressed as mmol/g wet

5.4 Statistical Analysis

All data are presented as mean \pm SEM. All values were compared by two-way ANOVA with application of a post hoc Bonferroni's test. Statistical significance was assumed at the $p < 0.05$ level.

Table 5.1. Patient characteristics

	Non-Diabetics	DCD	NIDD	IDD	LVEF>50%	LVEF=30-50%	LVEF<30%
Number	6	6	6	6	6	6	6
Age (yrs±SEM)	68.1±3.2	65.1±1.9	59.4±5.4	70.2±2.8	58.7±9.8	66.1±2.8	69.4±5.9
Sex (male/female)	4/2	3/3	5/1	4/2	3/3	2/4	1/5
Hypertension (%)	50	33	50	67	33	33	50
Previous MI (%)	0	33	33	33	0	0	83
Medication							
Glibenclamide			3				
Gliclazide			2				
Tolbutamide			1				
Nitrates	6	6	5	5	6	6	5
Ca2+ antagonists	6	6	6	6	5	5	6
β-blockers	5	6	3	2	6	6	3
K _{ATP} opener	0	0	0	0	0	0	0
LVEF (%±SEM)	58.1±12.4	60.2±5.4	55.8±3.9	52.1±6.1	63.2±1.6	35.2±3.1	23.1±5.1

LVEF, left ventricular ejection fraction; MI, myocardial infarction.

Figure 5.1: Experimental protocols for Study 1. All groups were equilibrated for 30min in aerobic conditions (37°C). Then, the right atrial slices were randomly assigned to one of the study groups (n=6/group): **Aerobic control**, aerobically incubated for the entire experimental time. **Ischaemia alone**, subjected to 90min simulated ischaemia followed by 120min reoxygenation. **PC**, preconditioning with 5min ischaemia followed by 5min reoxygenation before undergoing 90min ischaemia. **Diazoxide**, incubation in 0.1mM of the drug for 10min before exposure to 90min ischaemia. **Glibenclamide**, pre-treatment in 10µM of the drug for 10min before PC was applied.

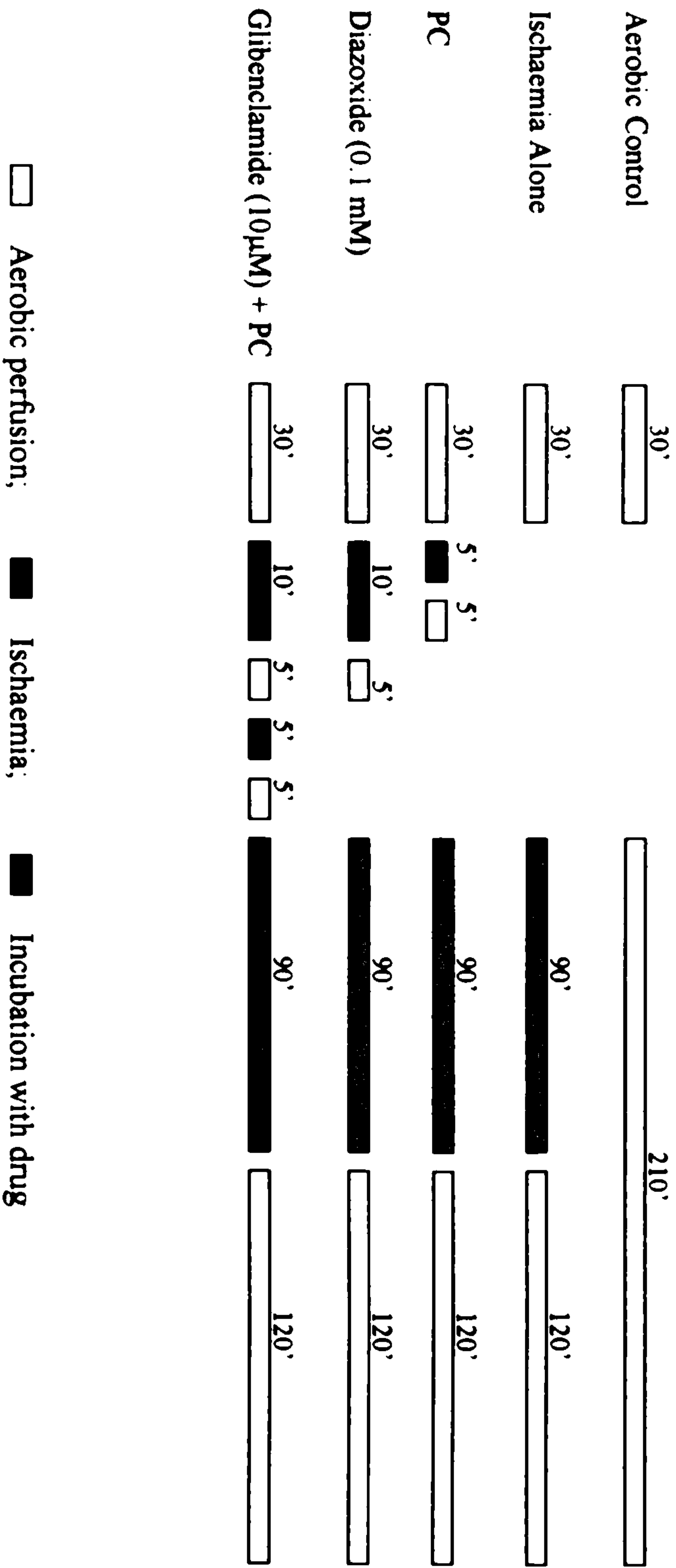
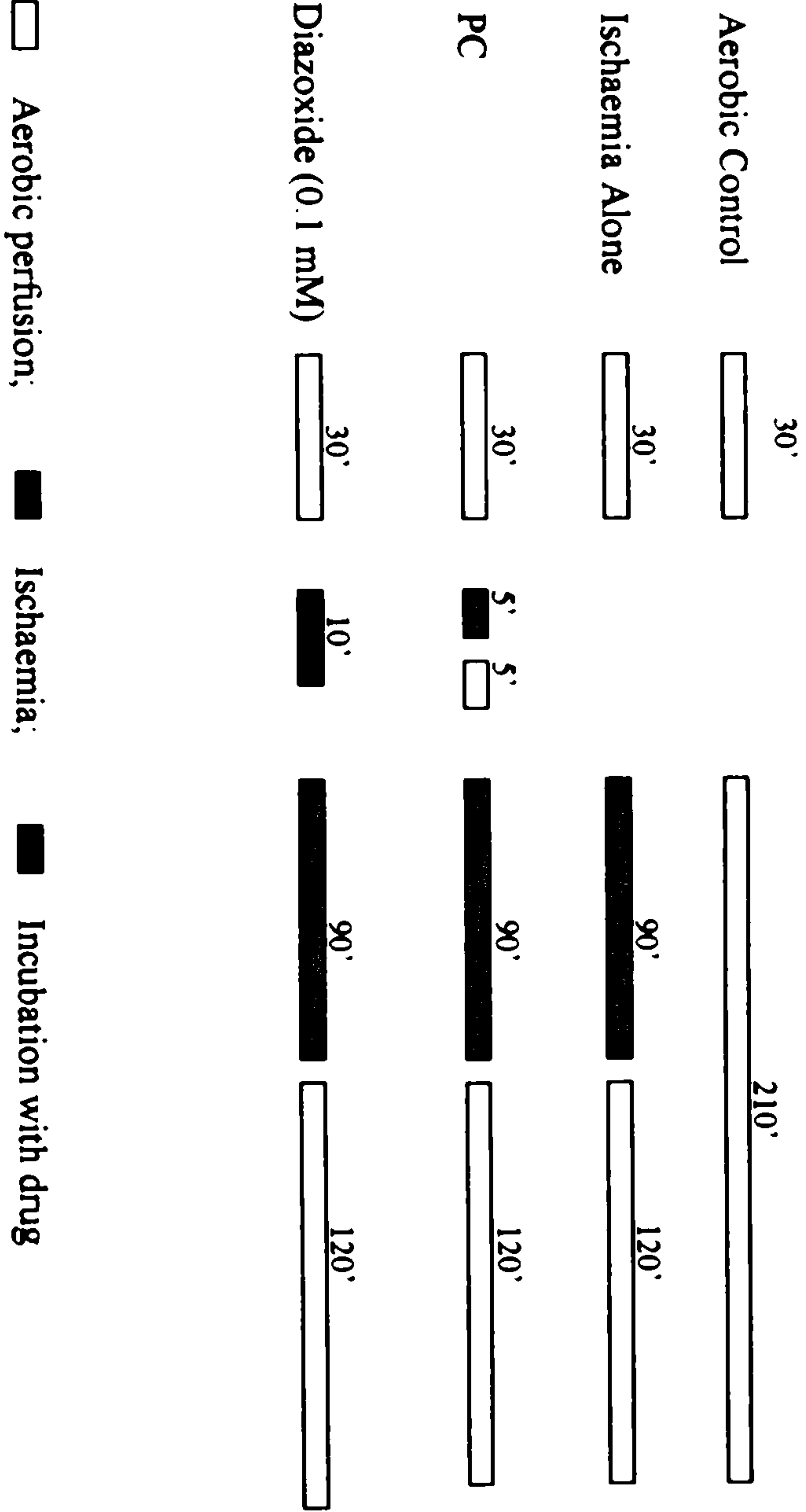


Figure 5.2: Experimental protocols for Study 2. All groups were equilibrated for 30min in aerobic conditions (37°C). Following this, the right atrial slices were randomly assigned to one of the following study groups (n=6/group). **Aerobic control**, aerobically incubated for the entire experimental time course. **Ischaemia alone**, subjected to 90min simulated ischaemia followed by 120min reoxygenation. **PC**, preconditioning with 5min ischaemia followed by 5min reoxygenation before undergoing 90min ischaemia **Diazoxide**, incubation in 0.1mM of the drug for 10min before exposure to 90min ischemia.



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5.5 RESULTS:

All samples entering the studies completed the applied experimental protocol and were included in the analysis.

Effect of Preconditioning in Diabetes (Study 1): Figure 5.3A shows that CK leakage was increased to a similar degree by ischaemia alone in all study groups. It also shows that PC completely reversed the effect of ischaemia in the non –diabetics and DCD groups, so that mean CK leakage values were similar to that seen in the aerobic control group, but did not have a protective effect in NIDD and IDDM groups. Interestingly, diazoxide, a specific mito K_{ATP} channel opener, mimicked the protection afforded by PC in non –diabetics and DCD groups but it failed to protect the NIDD and IDD groups. As expected, glibenclamide, a non-specific K_{ATP} channel blocker, abolished the protection of PC in non –diabetics and in DCD and had no effect in the NIDD and IDD groups. The MTT results shown in Figure 5.3B are a mirror-image of the CK leakage results with PC and diazoxide exhibiting a similar protection in the non –diabetics and DCD groups and no protection in the NIDD and IDD groups. Overall, the results suggest that changes in mito K_{ATP} channels in patients with NIDD and IDD are the most likely cause for the failure to precondition the myocardium, however the possibility that other elements of the signal transduction pathways of preconditioning could also contribute cannot be completely excluded.

Effect of Preconditioning in Patients with Contractile Cardiac Dysfunction (Study 2):

Figures 5.4A also shows that ischaemia alone resulted in a significant increase in CK leakage similar in all study groups regardless of the severity of cardiac dysfunction. PC

resulted in a decrease of CK leakage in the groups with LVEF $\geq 30\%$ but not in the group with LVEF $<30\%$. As observed in Study 1, diazoxide mimicked the effect of PC on CK leakage in the groups with LVEF $\geq 30\%$ and, interestingly, also was protective in the group with LVEF $<30\%$. The results on MTT reduction shown in Figure 5.4B were again a mirror-image of the CK leakage suggesting that the cause for the absence of protection by PC in the LVEF $<30\%$ group is probably due to alterations in some element(s) of the signal transduction pathway that exclude the mito K_{ATP} channels.

It should be noted that in the above two studies the number of patients involved in each study group was too small to be able to analyze the possible influence of the different clinical characteristics on the effects of ischaemia and preconditioning.

5.6 DISCUSSION:

The major findings of the present study are that myocardium from patients with diabetes and poor cardiac function are not protected from ischaemic preconditioning although the injury induced by ischaemia/reperfusion is not exacerbated in these conditions. Furthermore, the demonstration that activation of mitoK_{ATP} channels in myocardium from hearts with poor cardiac function mimicks the protection induced by preconditioning in the myocardium from hearts with good contractility but not from patients with diabetes suggests that the failure to precondition in these two conditions is due to alterations of different elements of the signal transduction pathway. Cardiovascular mortality is increased in patients with heart failure [92,170] and diabetes [160] and therefore any myocardial adaptation to ischaemia would be beneficial to

decrease mortality associated with these diseases. The results of my studies may have important clinical implications and a number of points warrant further discussion.

Preconditioning and diabetes

Insulin regulates the balance of energy substrates available to the heart and also regulates metabolism and myocardial perfusion via actions on various intracellular regulatory proteins and messenger systems [284], it is therefore conceivable that diabetes may affect ischaemic injury. However, my results show that the diabetic human myocardium is not more sensitive to lethal ischemic injury than the non-diabetic myocardium under the conditions of the present study. Since epidemiological studies have clearly shown that patients with either IDD or NIDD are more prone to develop myocardial infarction and post infarction complications [160,289], then it may be argued that the cause of cardiac complications in diabetics is not the tolerance of the heart to ischaemia. The literature is inconsistent with respect to how susceptible the hearts of diabetic animals are to injury from ischaemia/reperfusion and whereas some studies have observed a greater susceptibility to ischaemia/reperfusion injury [195], others have reported no significant effect [87,240]. The divergent results may find a possible explanation in the experimental differences but if our results in the human atrial myocardium can be confirmed in ventricular myocardium then our attention to reduce cardiac complications in diabetes should be centred in the context of blood components and the vasculature rather than in the own myocardium.

My studies have demonstrated that preconditioning affords protection of the myocardium from patients with diet controlled diabetes but that this is lost when patients

are on long-term hypoglycaemics or become insulin dependent. These results are in agreement with those of reported by Cleveland et al [53]. These investigators showed that myocardium from diabetic patients on long term K_{ATP} blockers also do not precondition in a model of myocardial stunning. However, the same authors also suggested that the myocardium from IDD subjects can be preconditioned although the protective effect obtained was not to the same extent as that in non-diabetics. Again these results in the human tissue contrast with the reported experimental results. Liu and co-workers [195] were the first to examine preconditioning in experimental streptozotocin-induced diabetic rats and found that diabetic hearts were more resistant to infarction than normal control hearts and that preconditioning conferred additional protection in *in vivo* experimental conditions. However, a subsequent study examined the effect of the streptozotocin-induced diabetes on the response to ischaemia/reperfusion and preconditioning in the isolated rat heart at different stages following its induction [312] and showed that the diabetic heart is more resistant to ischemia/reperfusion in the early phase of the diabetes (two weeks after onset); but that this protection is lost by 4-6 weeks. In addition, they observed that the diabetic heart can be preconditioned after 6-8 weeks. It must be mentioned that the disparity in the results observed between the two studies could be explained, at least in part, by the differences in the models used. Thus for example, in the study by Liu and colleagues [195] the model of diabetes is unique in that diabetes was induced by streptozotocin in the neonate and then animals were allowed to grow into adulthood before any intervention was carried out.

The present studies do not provide an explanation for the above disparity of results, however, they suggest that abnormalities of mito K_{ATP} channels may be

responsible for the failure to precondition the myocardium of diabetics. Several investigators [99,196] including my work described in the chapter previously have demonstrated that mito K_{ATP} channels are involved in the protection of ischaemic preconditioning. The findings by Smith et al [279] that K_{ATP} channels are altered possessing a greater outward single-channel current in the ventricular myocardium of diabetic rats further support the above thesis. Clearly, further research is needed to fully elucidate the contribution of the alteration of this channel to the failure to precondition the myocardium from diabetics.

Preconditioning and the failing heart

LV hypertrophy and LV chamber dilation are among others compensatory mechanisms of the failing heart. There is experimental evidence that the hypertrophied myocardium is at greater risk from ischaemia/reperfusion injury and it is generally believed that the failing heart is less tolerant to ischaemia/reperfusion injury. My results, however, have shown for the first time that the effects of ischaemia/reoxygenation are similar in the failing and non-failing myocardium. In addition, I have demonstrated also for the first time, that the myocardium from hearts exhibiting a LVEF <30% cannot be preconditioned. My results contrast with those of Cleveland et al [53] showing that the isolated ventricular trabeculae obtained from patients undergoing cardiac transplantation can be preconditioned. Again, the explanation for this difference cannot be found in the reported experimental studies and whereas some investigators have shown protection of the failing heart by preconditioning [52], others have observed no effect [223] or even further tissue damage [74]. The diversity of results is not entirely surprising due to the

absence of uniformity of experimental design and the degree of heart failure as I have shown in the present studies.

The protection observed with diazoxide in the myocardium from hearts with LVEF <30% was commensurate with the protection induced by preconditioning in the myocardium from non-failing hearts supports the thesis that the failure to precondition the failing human heart is not due to an alteration in the response of the mitochondrial K_{ATP} channel but caused by abnormalities in other elements of the preconditioning signalling pathway. Considerable evidence indicates that PKC is intimately involved in ischaemic preconditioning [58] and a potential candidate to explain the failure to precondition the failing heart may be the chronic activation of PKC observed in this condition [35]. There are several PKC isoforms, some of which have been involved with preconditioning, and in future studies the type of isoforms and their expression in the myocardium of the failing heart should be investigated. Indeed, if specific PKC isoforms are proved to be responsible for the failure to precondition then their manipulation could represent a potential therapeutic intervention to reduce myocardial injury in ischaemia/reperfusion of the failing heart.

Possible limitations of the study and clinical implications

A potential limitation of our study was the use of atrial tissue as opposed to ventricular myocardium and therefore any extrapolation must be conducted with caution; however, Yellon and colleagues have suggested that preconditioning exerts identical protection in atrial and ventricular myocardium [52]. The present study also used atrial tissue to characterize the effects of ischaemia and reperfusion in the failing and diabetic

human myocardium. However, atrial and ventricular myocardium possess characteristics of their own that may influence the susceptibility to ischaemia/reperfusion injury and as a consequence results from one may not be applicable to the other. Thus, for example, the reported differences in the distribution of potassium channels [5,132], which contribute to the characteristic differences between atrial and ventricular action potentials, may determine a different response to ischaemia/reperfusion. Undoubtedly, K_{ATP} channels are present in both atrium and ventricle [132], although their density in both tissues is unknown. It must also be mentioned that the preparation is superfused ("simulated ischaemia") as opposed to being arterially perfused and simulated ischaemia is achieved by removal of oxygen and blocking glycolytic ATP production with 2-deoxyglucose. This results in metabolic conditions within the myocardium that may be different than that which occurs in the myocardium during clinical ischaemia.

Another possible limitation might be that right atrial appendages were obtained from patients subjected to various medical treatments (e.g. nitrates, β -blockers, calcium antagonists) and that in principle may have influenced ischaemia/reperfusion injury and the protection induced by preconditioning. However, it should be emphasized, that all medication was stopped the day before surgery when specimens were taken for the study and that significant effect of the medication was unlikely since all preparations responded to ischaemia/reperfusion with a similar degree of injury. It should be mentioned that the preparation used in this study was not electrically stimulated (i.e. non-beating) and therefore one should be cautious when extrapolating to the in vivo situation.

5.7 Conclusion

Preconditioning is a potent protective intervention which use has been advocated in clinical situations such as angioplasty and cardiac surgery. The results of my studies have obvious clinical implications in that preconditioning cannot be beneficial to patients with NIDD and IDD and those with cardiac failure. They also show that in the failing heart a similar degree of protection to that seen with preconditioning can be obtained by the administration of a selective opener of mito K_{ATP} channels, an intervention that is not effective in diabetics.

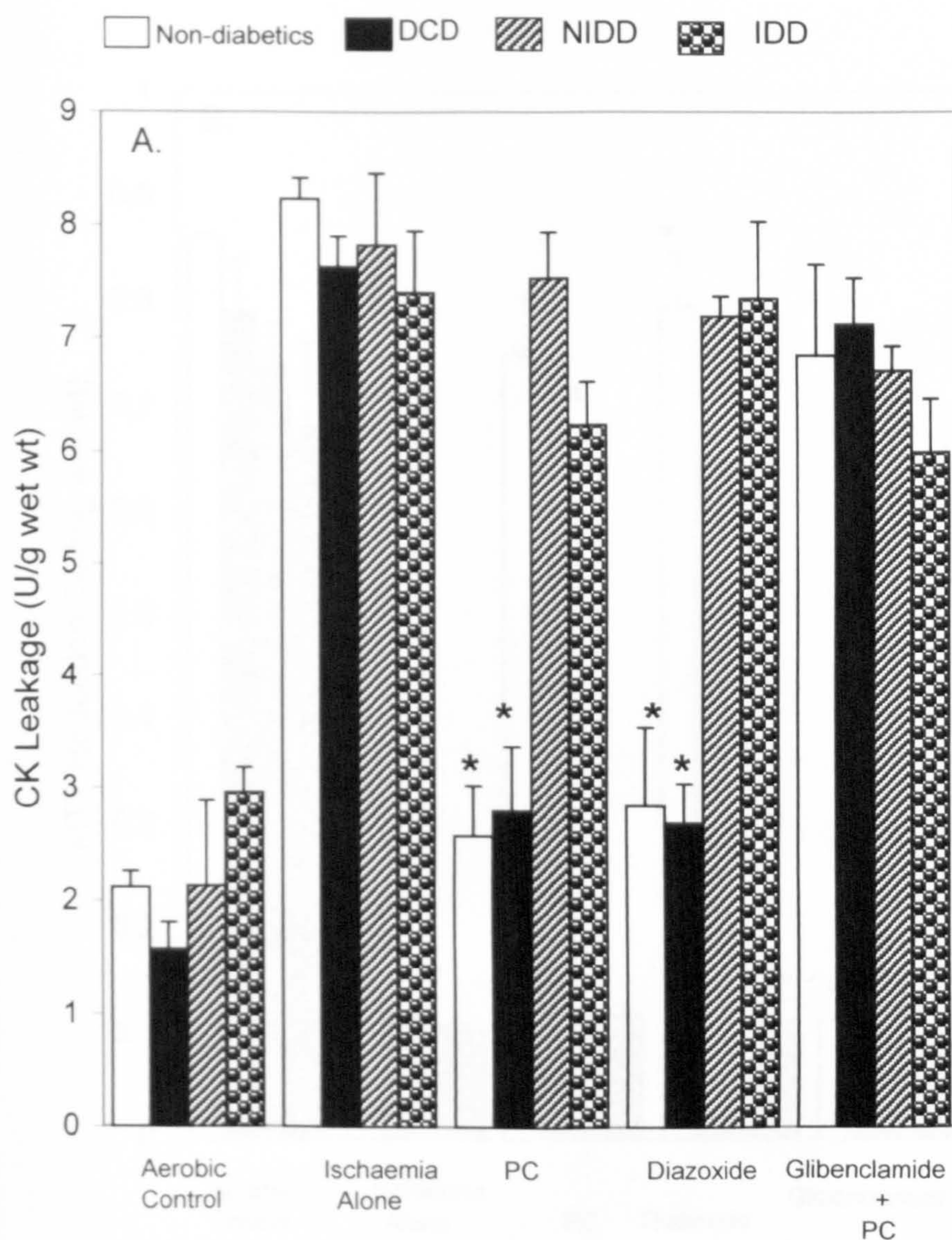


Figure 5.3A: Creatine kinase (CK) leakage at the end of the 120min reoxygenation period (last 120min in the aerobic control group). Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs corresponding group subjected to Ischaemia Alone.

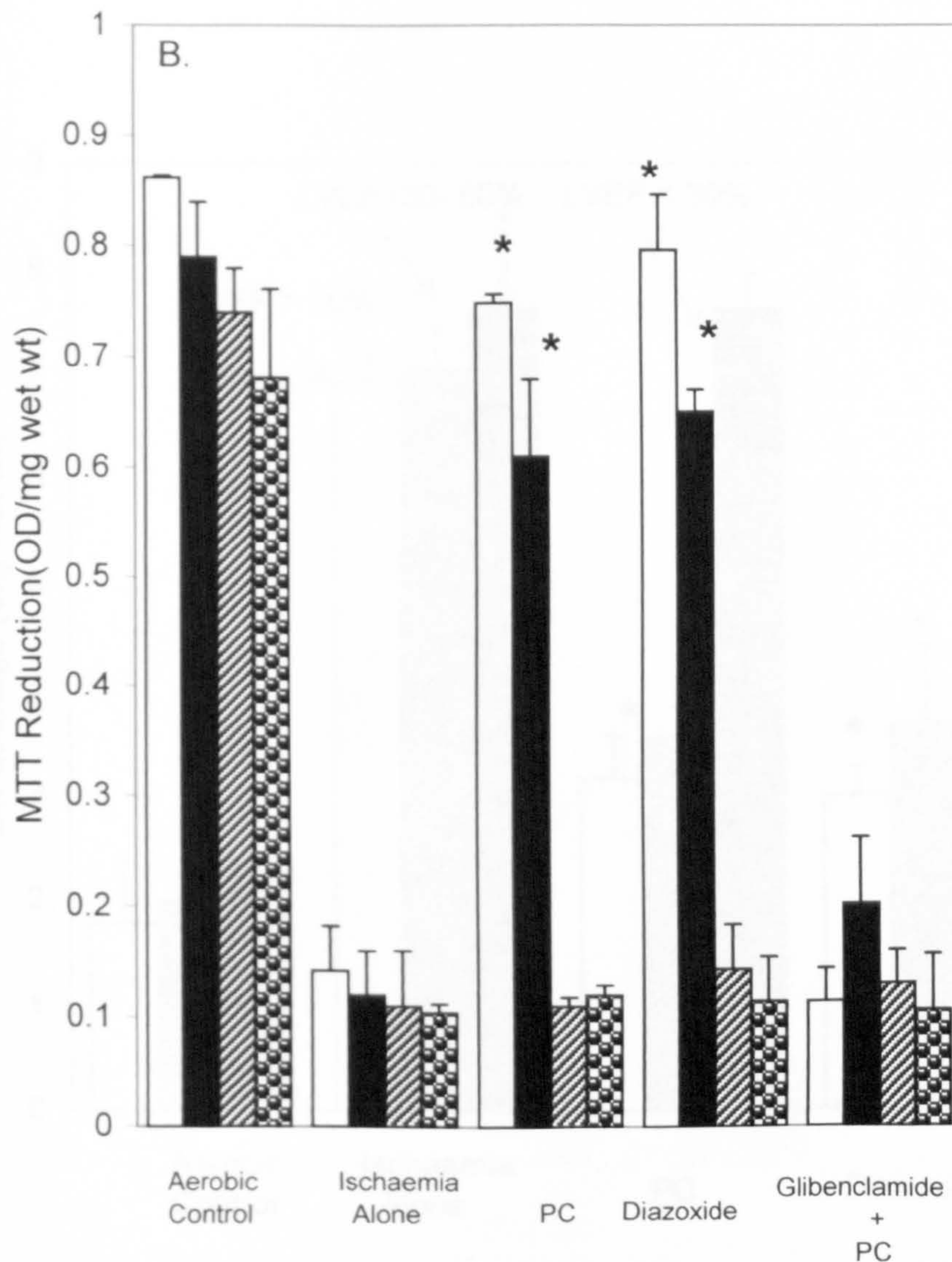


Figure 5.3B: MTT reduction at the end of the 120min reoxygenation period (last 120min in the aerobic control group). Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs corresponding group subjected to Ischaemia Alone.

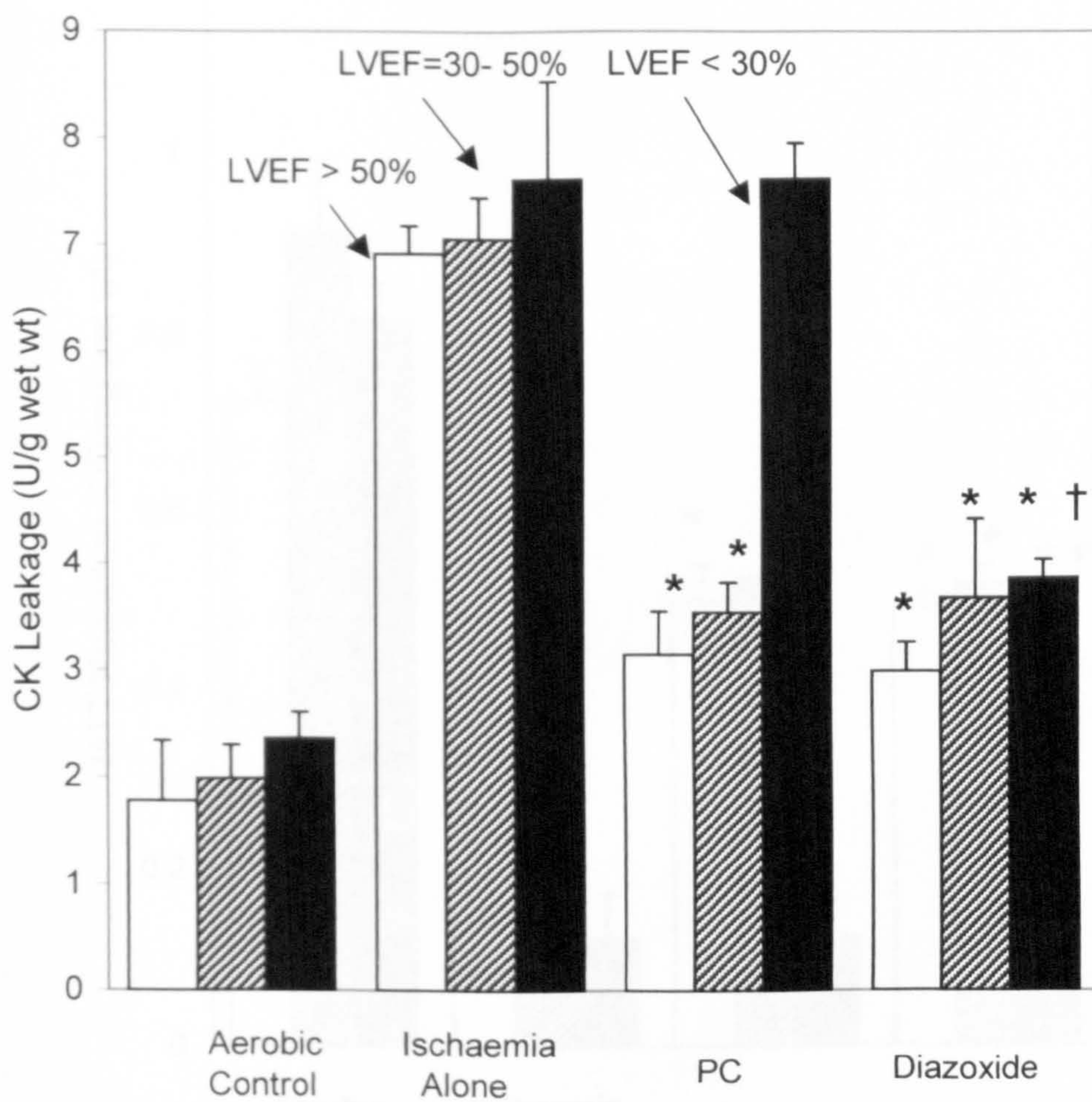


Figure 5.4A. Creatine kinase (CK) leakage during the 120min reoxygenation period (last 120min in the aerobic control group). Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs corresponding group subjected to Ischaemia Alone; † $p < 0.05$ vs corresponding group subjected to PC.

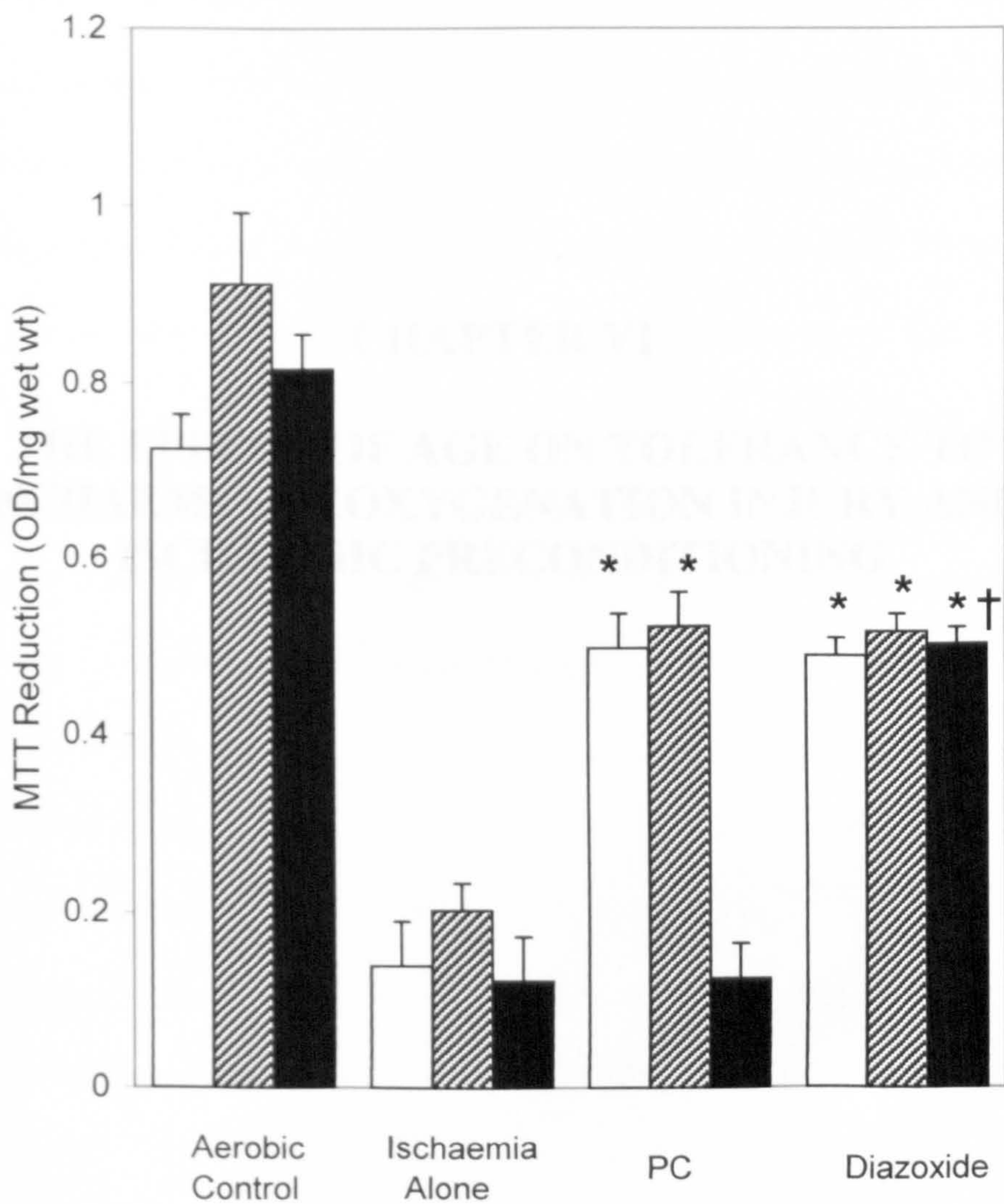


Figure 5.4B. MTT reduction at the end of the 120min reoxygenation period (last 120min in the aerobic control group). Data are expressed as mean \pm standard error of mean of 6 experiments. * $p < 0.05$ vs corresponding group subjected to Ischaemia Alone; † $p < 0.05$ vs corresponding group subjected to PC.

CHAPTER VI

THE EFFECT OF AGE ON TOLERANCE TO ISCHAEMIA/REOXYGENATION INJURY AND ISCHAEMIC PRECONDITIONING

6.1 INTRODUCTION

Life expectancy continues to grow and the increased expenditure in the health care of an ageing population is a reality in the developed world. Increasing age has been recognized as a cause for adverse prognosis with myocardial infarction [123,181], coronary angioplasty [331] and also following cardiac surgery [239]. The hearts of aged animals are less tolerant than those of young adult animals to ischaemia/reperfusion injury [144,314]. Anatomical, mechanical, ultrastructural and biochemical alterations may comprise the adaptive responses of the senescent hearts. This thesis is supported by animal [300] and human *in-vitro* studies [202] suggesting that the aged myocardium is more susceptible to ischaemic injury than the young myocardium. The possibility of death due to coronary artery disease increases progressively with age [241], but this is largely attributed to a reduction in the use of thrombolytic therapy. However, recently Ivanov et al [144] have reported an improvement in operative mortality during heart surgery over the last two decades despite an increase in the prevalence of elderly patients and greater severity of their risk factors, which suggests that the associated co-morbid conditions but not age *per se* are the main detrimental factors. The undefined role of age as a risk factor during cardiac surgery is also reflected by the different risk scoring between the Parsonnet [239] and Euroscore (European System for Cardiac Operative Risk Evaluation) systems [256].

It is also unclear whether the protection obtained with interventions such as ischaemic preconditioning is altered in the ageing heart. Ischaemic preconditioning with brief periods of ischaemia has been demonstrated to protect both animal [216,224] and human [52,282] myocardium from a subsequent ischaemic insult. This has been

demonstrated in the aged sheep hearts [208] but it has been questioned in the aged myocardium of the rat [248,301] and rabbit [41] heart.

The aims of this study were (i) to determine the effect of age on the tolerance of the human myocardium to ischaemia and (ii) to investigate whether age affects the protection afforded by ischaemic preconditioning.

6.2 METHODS

6.2.1 Experimental preparation

Experiments were performed on myocardium obtained from the right atrial appendage of 90 patients undergoing open heart surgery as described in previous chapters. Experiments were performed as described in section 2.3 (Chapter 2).

6.2.2 Solutions

The incubation medium was prepared daily as described in section 2.3 (Chapter 2).

6.2.3 Experimental protocol

After sectioning the atrium, the preparations were allowed to stabilise for 30min and then randomly allocated to one of the following groups: (i) aerobically incubated for 210min to serve as aerobic time matched controls, (ii) simulated ischaemia for a period of 90min followed by 120min of reoxygenation, and (iii) ischaemic preconditioning induced by 5min ischaemia/5min reoxygenation immediately before the 90min ischaemia. Patients were divided by age into three different groups: between 30-49 (n=12), 50-69 (n=48) and 70-90 (n=30) years old.

6.2.4 Assessment of tissue injury and viability:

At the end of each experimental protocol, tissue injury was determined by measuring the leakage of creatinine kinase (CK) into the incubation medium and tissue viability by the reduction of 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide

(MTT) to blue formazan product

CK Leakage The activity of CK leakage into the media during the reoxygenation period ($\mu\text{g wet wt}$) was assayed as described in section 3.6B (Chapter 3).

MTT reduction At the end of the experimental time, MTT analysis was performed as described in section 2.6B (Chapter 2) and the results expressed as mmol/g wet wt .

6.2.5 Statistical analysis

All data are presented as mean \pm SD. ANOVA was used for multiple comparisons with application of a post hoc Tukey's test. Linear regression analysis was used where appropriate. Statistical significance was taken as $p < 0.05$.

6.3 RESULTS

Table 6.1 shows that the demographic data and the risk factors were similar in the three age groups. Figures 6.1A and 6.1B show that simulated ischaemia/reoxygenation resulted in significant increases in CK leakage and decreases in MTT reduction that were similar in all three age groups. They also show that ischaemic preconditioning afforded similar protection in all age groups. The linear regression analysis shown in Figures 6.2A and 6.2B clearly demonstrates that the degree of ischaemic injury and the protection obtained with ischaemic preconditioning does not change with increasing age.

6.4 DISCUSSION

The present study is the first to report that ischaemic injury of the human myocardium is not exacerbated by increased age and that ischaemic preconditioning equally protects the myocardium of all ages. These findings have important clinical implications regarding the selection and treatment of patients presenting with ischaemic syndromes and they warrant further discussion.

Ischaemia occurs in a number of clinical settings that include myocardial infarction, coronary angioplasty and cardiac surgery. Ischaemic heart syndromes are the commonest cause of death in the elderly [123,181] and it has been suggested that a greater vulnerability to ischaemic injury of this age group is a major contributor [123,181,331]. This thesis finds support in experimental studies showing that a greater calcium overload [12] and increased oxygen free radical generation [64] occur in the senescent rat heart and that hearts become less tolerant to ischaemia with age [300]. In contrast, my results have clearly demonstrated that in the human myocardium the degree of ischaemic injury is unrelated to age. The controversy is further fuelled by the results recently reported by Mariani et al [202] showing that the aged human atrial myocardium, also obtained from patients undergoing cardiac surgery, has a reduced capacity to recover developed force after hypoxia or simulated ischaemia than younger myocardium although contraction duration, time to peak tension and time to 50% relaxation were unaffected by age. Therefore, the results of the study by Mariani et al [202] may be subject to different interpretations depending on the parameter examined.

The demonstration that ischaemic injury is not exacerbated by age questions the validity of the use of age as a risk factor for mortality and morbidity following myocardial infarction [123,181] and in patients undergoing coronary angioplasty [331] or cardiac surgery [144,314]. Therefore, in the light of the present results, therapeutical interventions such as cardiac surgery should not be denied on the basis of age alone and they prompt us to re-evaluate the impact and inclusion of age in risk scoring systems like Parsonnet and Euroscore.

The second major finding of the present study is that the protection of the human myocardium by ischaemic preconditioning is not influenced by age. This contrasts with the findings of Abete et al [1] showing that the senescent rat heart cannot be preconditioned with ischaemia. However, in spite of these results they also showed that both adult and senescent hearts can be equally pharmacologically preconditioned with exogenous norepinephrine suggesting that the preconditioning signal transduction pathway is preserved in both age groups. This is further supported by reports from Przyklenk et al [248] and Burns et al [41] demonstrating their ability to precondition the senescent hearts of rabbits and sheep.

The mechanism of ischaemic and pharmacological preconditioning is at present the object of intense investigation and as yet is not fully understood; however, Tani et al [300] observed differences in the translocation of PKC between young and middle-aged rats that demonstrates the possibility of distinct characteristics in the signalling pathway of preconditioning in different age groups.

The elucidation of this pathway will be invaluable to exploit the protective action of preconditioning and to combat ischaemic injury. The use and refinement of preconditioning will help to improve the prognosis and outcome of the sufferers of

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ischaemic heart disease in the elderly population and will make safer procedures such as coronary angioplasty and cardiac surgery.

Having established the preconditioning phenomenon in the human myocardium *in vitro* and its role in pathological myocardium together with the role of K_{ATP} channels in these states, I turned my focus of attention to exploring the role of preconditioning in the clinical arena and this is the subject of the next chapter.

Table 6.1 Patients' demographic and preoperative risk factors.

	Group 1 (30-49 years)	Group 2 (50-69 years)	Group 3 (70-90 years)
Number of patients	12	48	30
Sex (Male : Female)	8 : 4	29 : 19	18 : 12
Hypertension	7(58%)	31(65%)	12(40%)
Hypercholesterolemia	10(83%)	33(69%)	14(47%)
Cerebrovascular accident	1(8%)	3(6%)	2(7%)
Peripheral vascular disease	1(8%)	6(13%)	5(17%)
Medications			
Nitrates	6(50%)	41(85%)	24(80%)
Diuretics	6(50%)	17(35%)	12(40%)
Ca channel antagonists	9(75%)	37(77%)	21(70%)
β -blockers	10(83%)	29(60%)	14 (47%)
ACE inhibitors	5(42%)	19(39%)	12(40%)

TEXT BOUND INTO THE SPINE

Figure 6.1: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in the human atrial myocardium from the different age groups. Data are expressed as mean \pm SD of the patients in each group. n=12 in the 30-49 years of age group; n=48 in the 50-69 years of age group; and n=30 in the 70-90 years of age group. *p<0.05 vs. aerobic control group; †p<0.05 vs. simulated ischaemia/reoxygenation (SI/R) alone group.

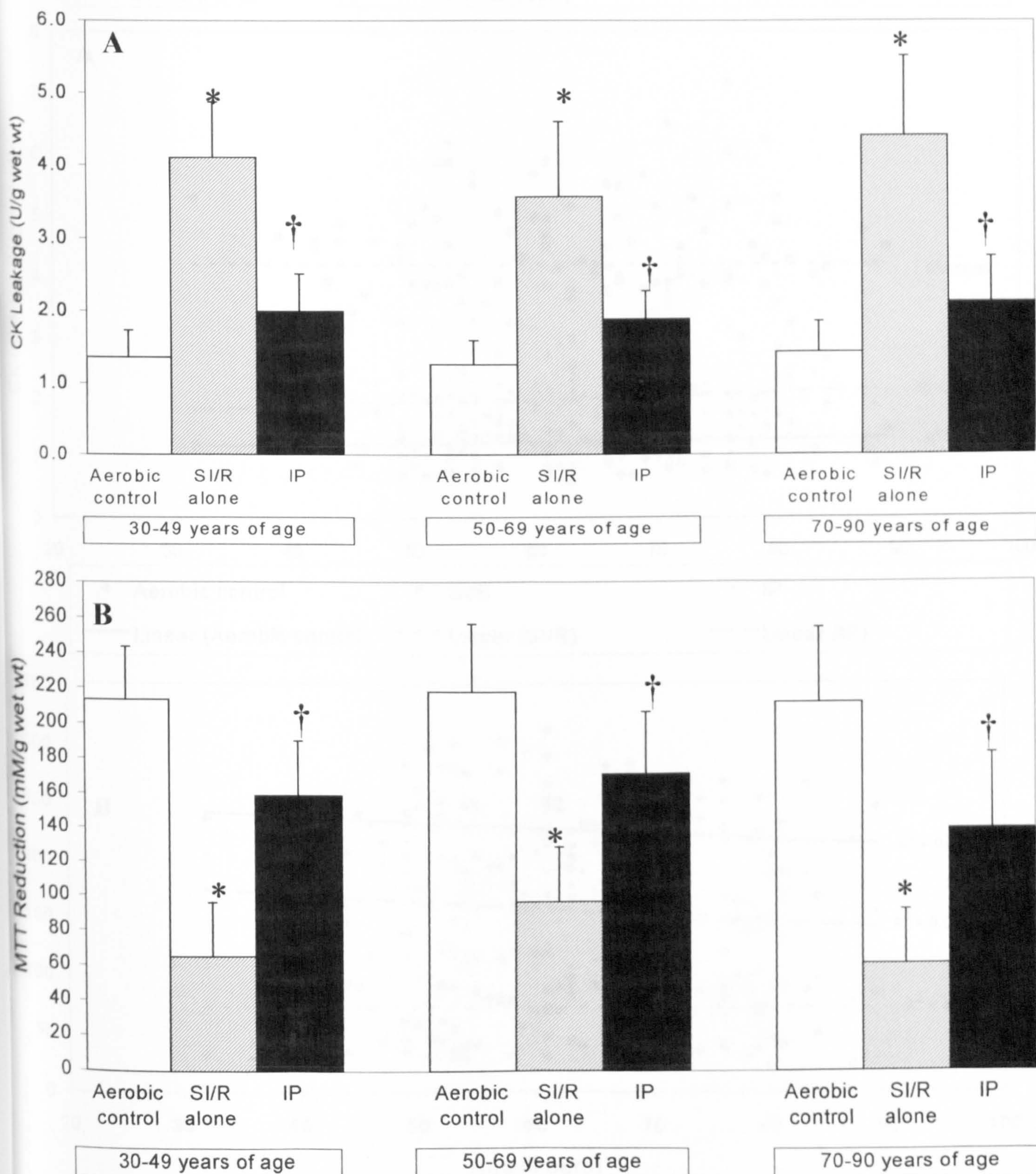
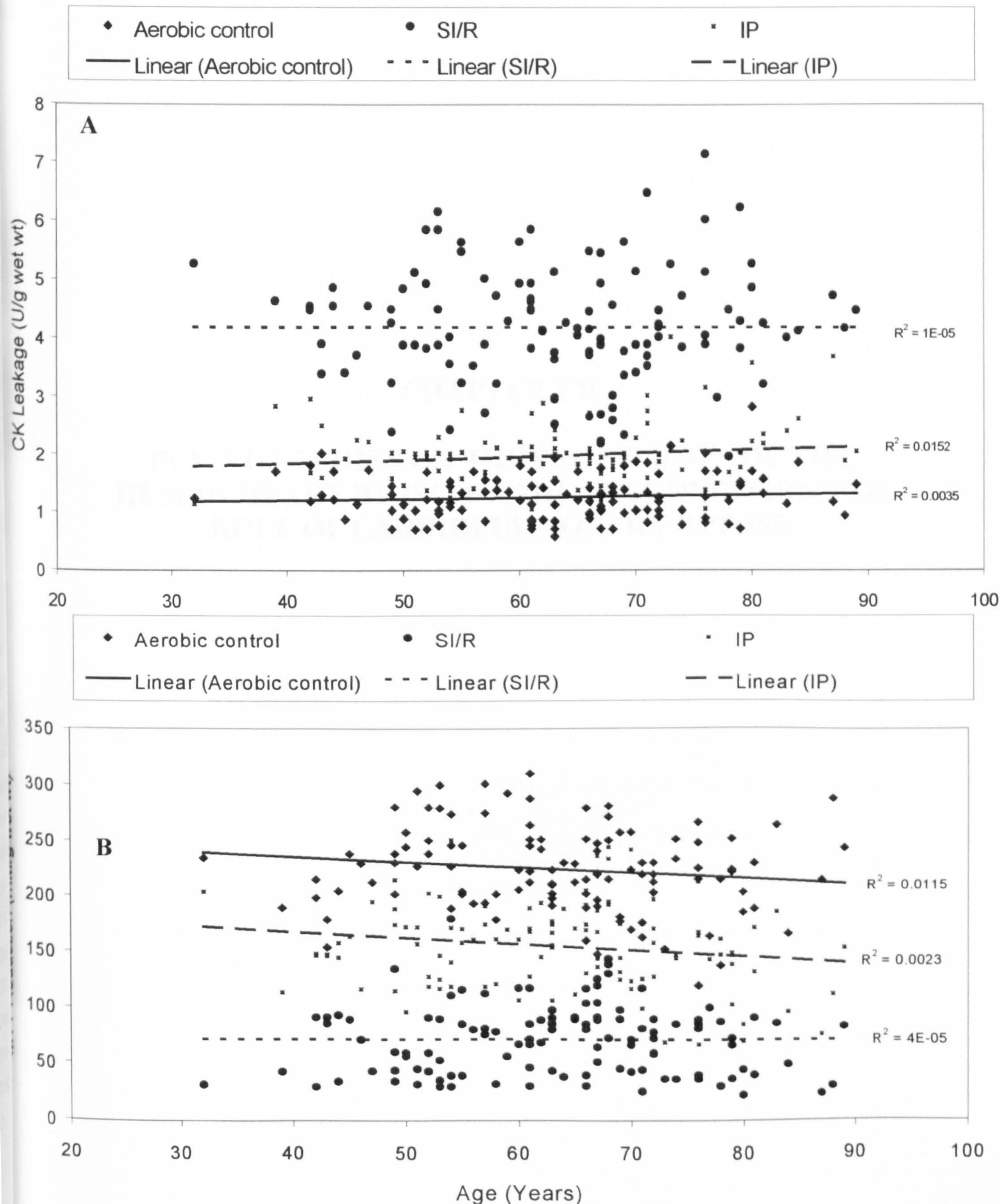


Figure 6.2: Linear regression analysis on Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in the human atrial myocardium from all ages subjected to 90 min of simulated ischaemia and 120 min reoxygenation preceded or not by ischemic preconditioning.



CHAPTER VII

IN VIVO STUDIES ON THE PROTECTION OF THE HUMAN HEART BY ISCHAEMIC PRECONDITIONING: ROLE OF CARDIOPULMONARY BYPASS

7.1 INTRODUCTION

Myocardial injury, manifested as transient cardiac contractile dysfunction ('stunning') and myocardial necrosis, is the most frequent complication during heart surgery [38]. Ischaemic preconditioning has been demonstrated in a variety of animal species [104,214,269] and in *in vitro* experiments involving isolated human cardiomyocytes [140] and human atrial trabeculae [282] to be protective against stunning and infarction. For logistic and ethical reasons, no clinical study can meet the strict conditions of experimental studies on preconditioning with infarct size as the end-point and because of this human *in vivo* studies have produced conflicting results and the role of preconditioning in man remains controversial.

Yellon and coworkers [346] were the first to report that preconditioning protects the human heart in the setting of cardiac surgery using the conservation of myocardial adenosine triphosphate content as the major endpoint. However, Perrault and colleagues [242] have reported that in the presence of cardioplegic arrest, there was no difference in the release of biochemical markers (CK-MB) between the preconditioned and control groups. The lack of additional protection conferred by ischaemic preconditioning was further confirmed by the absence of difference in the post-arrest myocardial levels of ribonucleic messengers coding for cardioprotective heat shock proteins between the two groups. Similar negative results have been reported by Kaukoranta et al [161] and interestingly by Yellon and colleagues [81], who went on to report that in the presence of hypothermia, no beneficial effect of preconditioning was observed in similar patients undergoing surgery using intermittent fibrillation techniques.

The apparent discrepancy between results obtained with non cardioplegic and cardioplegic techniques could be reconciled if one takes into account one possible hypothesis - that preconditioning and its salutary effects are only observed in situations of unprotected ischaemia. With this in mind, the aims of this study were (i) to investigate whether ischaemic preconditioning with 5 min ischaemia followed by 5 min reperfusion is protective in patients undergoing coronary artery bypass graft (CABG) surgery with cardiopulmonary bypass using cardioplegia and ventricular fibrillation techniques and in patients undergoing CABG on the beating heart without cardiopulmonary bypass, and (ii) to elucidate the underlying cause of any protection

7.2. METHODS

7.2.1 In Vivo Studies

Patient selection and Study groups. 120 patients with stable angina operated on by a single surgeon (MG) accepted to enter the study. They were divided in those to be operated on using cardiopulmonary bypass (CPB) (ie on-pump) and exhibiting three vessel coronary disease (n=80) and those to be operated on the beating heart without CPB (ie off-pump) and having single or double vessel coronary artery disease (n=40). Patients in the CPB group were randomised using computer generated random table to be operated on (i) using cardiopulmonary bypass (CPB) and intermittent cross clamp fibrillation + ischaemic preconditioning (n 20 in each subgroup) or (ii) with cold blood cardioplegia + ischaemic preconditioning (n 20 in each subgroup). Similarly, patients in the beating heart without CPB group were randomised to have or not have ischaemic preconditioning (n 20 in each subgroup). Ischaemic preconditioning was induced by clamping the ascending aorta for 5 minutes at 37 °C when CPB was used or by occlusion of the coronary artery to be

grafted for the same period and then reperfused for another 5-minutes before proceeding with bypass grafting. Patients with low ejection fractions ($EF < 30\%$), unstable angina, recent myocardial infarctions (< 1 month), additional cardiac diseases, severe non-cardiac diseases, diabetes, and on medication that included ATP-dependent potassium channel (K_{ATP}) openers were excluded. The study was approved by the local ethics committee and all patients gave written informed consent to the study. Pre-operative characteristics of the patients are highlighted in Table 7-1

7.2.2 Operative Procedure

Anaesthetic technique was standardized for all patients. Anaesthesia was induced by fentanyl and maintained with enflurane in all patients. All operations were performed through a median sternotomy and full heparinisation (3 mg/kg iv). CPB was conducted with non-pulsatile perfusion flow (2.2-2.4 l/min/m²) with ascending aortic cannulation and 2-staged venous cannulation and moderate systemic hypothermia (32°C). When cardiac arrest was achieved with cold blood (6-10°C) cardioplegia, 1000 mL of the solution (composition in mmol/l: 16 $MgCl_2 \cdot 6H_2O$, 2 $CaCl_2$, 20 KCl, 147 NaCl, 1.0 procaine HCl, pH 7.40) was mixed with blood from the pump in a ratio of 1:1 and injected into the aortic root immediately after aortic cross-clamping to obtain a myocardial temperature of 12°C to 15°C. An additional dose of 500 mL of cardioplegia was injected after 30min of ischaemia. In the group in which intermittent aortic cross-clamp fibrillation was used, reperfusion was carried out for 3 minutes between each period. One single distal coronary artery anastomosis was performed during each ischaemic period. Proximal anastomosis were completed on the beating heart with aortic partial occlusion clamp in all groups.

In the off-pump group, coronary bypass grafting was performed on the beating heart using the Octopus myocardial stabilisation device (Medtronic Inc). The suction cups were placed on the epicardial surface on either side of the artery to be grafted, with a suction pressure of no more than 600 mm Hg. Small coronary clamps were applied to proximal and distal to the site of the anastomosis with just enough pressure to occlude coronary flow and therefore allow grafting in a bloodless field. They were released when the anastomosis was complete. The proximal anastomosis was performed using a partial occlusion aortic clamp.

Ischaemic preconditioning was applied prior to the first dose of cardioplegia or immediately before the aortic cross application or the coronary artery occlusion. The use of a Khuri Tissue pH Analyser (Vascular Tech., Mass. USA) showed that the pH of the myocardium decreased from >7.3 to < 7.0 at the end of the 5 min ischaemic preconditioning period in all cases.

7.2.3 Assessment of myocardial injury

Serial venous blood samples were collected prior to induction of anaesthesia and at 1, 4, 8, 24 and 48 hr after termination of ischaemia for the assessment of Troponin T (TnT). This was measured using a commercially available enzyme linked immunoabsorbent assay kit (ELISA Troponin T, Boehringer Mannheim, Mannheim, Germany). The lower detection limit of the assay was 0.05 ng/ml, and concentrations above the discriminator value of 0.1 ng/ml were considered elevated.

7.2.4 *Haemodynamic measurements*

Heart rate (HR), mean pulmonary artery pressure (MPAP), pulmonary capillary wedge pressure (PCWP), and cardiac output (CO) were monitored using a pulmonary artery floatation catheter. Derived cardiovascular variables, including cardiac index, systemic and pulmonary vascular resistance were calculated using standard formulas. Haemodynamic data were collected at 4 time points: baseline, just after induction of anaesthesia, and 1, 3 and 24 hours after termination of ischaemia. Changes in derived variables were calculated and compared.

7.2.5 *In Vitro Studies*

Specimens of human right atrium appendage were obtained from patients undergoing elective coronary artery surgery using CPB. Samples were obtained prior to the institution of CPB and again 10 min after the initiation of CPB. Two 6/0 Prolene encircling sutures were placed in the epicardial layer of the appendage once the pericardium was opened. During the entire period of harvesting, the atrium was exposed to the systemic circulation. The pre-bypass sample was then harvested and a 2-stage venous cannula was then inserted in the free-wall of the right atrium 3 cm away from the appendage. Cardiopulmonary bypass was then instituted and after 10 min, a further sample of appendage was harvested. The specimens were prepared as described in section 2.3 (Chapter 2). The specimens were equilibrated for 30min before being randomly allocated to one of the following two groups (n = 6 each from different patients/group): (1) ischaemia alone - 90min ischaemia followed by 120min reoxygenation, (2) ischaemic preconditioning with 5min ischaemia /5min reoxygenation before 90min ischaemia followed by 120min reoxygenation. Tissue injury was determined by measuring the leakage of creatine kinase (CK) and MTT

reduction as described in section 3.2.4 (Chapter 3) and the results expressed as U/g wet wt and mM/mg wet wt respectively.

7.3 Statistical Analysis

Statistical analyses were carried out using the SPSS 9.0 statistical package program. A non-parametric test (Mann-Whitney U) was carried out for non-Gaussian distribution of data. Unpaired Student's t test was used for continuous data (two-tailed) and χ^2 test for categorical data was used to compare variables between the groups. Repeated-measures analysis of variance (ANOVA) was used to test the repeated observation variables post-operatively. The area under curve was calculated using the method of Matthews and Altman [204]. Data was presented as mean \pm standard deviation (SD). The level of significance was set at a p value < 0.05 .

7.4. Results

7.4.1 In Vivo Studies

There were no operative deaths (first 30 postoperative days) or perioperative myocardial infarctions in any of the study groups. Table 7.1 shows that the patient characteristics and perioperative data were similar within each study group and that there was no difference between patients who were treated with or without ischaemic preconditioning.

1. Plasma Troponin T

Figures 7.1A & B show that the profile of TnT release in plasma was identical in patients undergoing coronary surgery using CPB whether they were protected using

intermittent fibrillation or cold blood cardioplegia. Thus, there was a significant increase in plasma TnT by 1 hr after termination of ischaemia that peaked at 4hr with mean values still remaining elevated at 48 hr. Interestingly, preconditioning did not alter this profile in both groups, suggesting that preconditioning conveys no benefit to patients undergoing coronary surgery using cardiopulmonary bypass.

By contrast, as shown in Figure 7.1C, the profiles of plasma TnT were different in patients with or without preconditioning and operated off-pump. Peak release occurred by 8 hr after termination of ischaemia in the control group but by 1 hr in the preconditioned group with a sharp decrease over the next 3 hr with mean plasma TnT values significantly lower than in the control group. Only by the end of 48 hr TnT levels were similar in both groups.

Figure 7.2 shows that the cumulative plasma release of TnT (i.e area under the curve) was similar in the groups operated under CPB with no significant effect of preconditioning. It also shows that TnT release was lower in patients operated without CPB and, importantly that preconditioning in this group significantly reduced the total TnT release by 33% when compared to the control group (2.1 ± 0.1 vs 3.1 ± 0.2 ng.hr/ml, $p < 0.05$).

2. *Haemodynamic data*

Table 7.2 shows that the mean systemic arterial pressures, heart rate, mean pulmonary artery pressures, systemic and pulmonary vascular resistance and cardiac indices fluctuated within normal ranges after the operation in both the control and preconditioned groups and in the groups operated with and without CPB

7.4.2 *In vitro studies*

Figures 7.3A and 7.3B show the results of the CK leakage and MTT reduction of the atrial slices obtained before CPB and 10min after initiation of CPB. They demonstrate that the increase in CK leakage and the decrease in MTT reduction caused by ischaemia/reoxygenation in the atrial muscles obtained prior to the institution of bypass were significantly improved in the slices obtained 10 min after the initiation of bypass and that in fact this level of protection was identical to that of preconditioning. Thus, muscles that were obtained 10 min after the initiation of bypass were already preconditioned and the application of ischaemic preconditioning did not result in additional benefit to that seen with ischaemic preconditioning alone.

7.5 DISCUSSION

The present studies have shown that the human heart is preconditioned by the institution of cardiopulmonary bypass and that the use of ischaemic preconditioning in combination with other protective interventions such as cardioplegia do not result in additional protection. They have also clearly demonstrated that the human heart can be protected by ischaemic preconditioning when patients are operated on without the use of cardiopulmonary bypass. These findings have obvious important clinical implications and they warrant further discussion.

Preconditioning of the Human Heart

Experimental findings on ischaemic preconditioning cannot be directly extrapolated to humans because its mechanisms may be different from other animal species. As a result, for both logistic and ethical reasons, no clinical study can meet the strict conditions of experimental studies on preconditioning in which infarct size is

the primary end-point and instead surrogate end-points have to be used. Because of this the demonstration of this phenomenon in the setting of cardiac surgery has been controversial. Yellon et al [346] were the first to examine the effect of two 3-minute ischaemic episodes, where each was followed by 2-minute reperfusion on myocardial high energy phosphate content in patients undergoing coronary artery bypass graft surgery using cardiopulmonary bypass. They claimed that the human myocardium showed the typical biochemical features of preconditioning observed by Murry and colleagues [224] in their classic canine model of ischaemic preconditioning and thus could be preconditioned. Following that, there have been some recent studies [294,334] which also highlight the potential benefits of preconditioning in the cardiac surgery setting. However, Perrault et al [242] failed to show a beneficial effect of ischaemic preconditioning when this was induced with 3-minute aortic cross-clamping followed by 2-minute reperfusion before the administration of warm blood cardioplegia. Similar findings have been reported by other investigators [65,161] questioning the ability of preconditioning to protect the human heart. The dispute on the occurrence or not of cardioprotection by preconditioning during cardiac surgery is further fuelled by a more recent study by Yellon and colleagues [3] using a protocol identical to the one used in their first study, they showed a reduction of TnT release at 72 hrs in patients exposed to preconditioning but not at 24 or 48 hrs. Interestingly, in contrast with their first study, the same authors reported an absence of protection on myocardial high energy phosphates. These opposed results are puzzling and contrast with the overwhelming evidence that preconditioning is cardioprotective during coronary angioplasty [78] and in *in vitro* experimental conditions using atrial trabeculae [282] or isolated myocytes [52]. My finding that CPB can act as a

preconditioning stimulus in man is supported by another study in sheep [40] and sheds light on the above controversy.

During cardiac surgery, there may be pre- and intraoperative factors such as opioid agonist [275] and anaesthetic agents [63] that may mimic the protection of preconditioning. These include the use of opioid agonists, aprotinin and importantly cardiopulmonary bypass itself. Anaesthetic agents did not play a significant role in this study because ischaemic preconditioning exerted protection in all the patients operated on without CPB (ie off-pump) and the atrial myocardium harvested prior to the institution of CPB was protected by ischaemic preconditioning in all instances. Hypothermia is another cardioprotective factor [120] that may influence the cardioprotection of preconditioning. Recently, Takeshima et al [296] have also demonstrated that preconditioning is not protective with deep hypothermia, however moderate hypothermia alone, as used in the present studies, does not inhibit the preconditioning response.

Mechanism of preconditioning by cardiopulmonary bypass

Although the precise mechanism of ischaemic preconditioning still remains unclear, recent investigations have clearly identified a number of factors that are essential to achieve protection. CPB induces a systemic inflammatory reaction and it is possible that some elements of this reaction may be responsible for the observed protection. In this connection, Yamashita and co workers [338] have recently reported that IL-1 and TNF- α , production of which is increased by CPB, causes an elevation in tissue Mn-SOD, as has been shown to occur when brief sub-lethal ischaemia or anoxic insults are induced [337]. However, this thesis is unlikely since the production of cytokines is a late event in response to CPB that requires more than 10 min.

Recently, our laboratory has shown that the generation of free radical species occurs soon after the institution of CPB [203]. Therefore, it is possible to speculate that free radicals are the primary cause of the cardioprotection by CPB. The relationship between free radicals and preconditioning was first suggested by Richard and colleagues [253], who showed that administration of oxygen free radical scavengers during the first reperfusion period could block the beneficial effect of preconditioning on infarct size in dogs. They therefore proposed that the generation of low amounts of free radicals during the short ischaemic episode is not sufficient to cause cell necrosis, but enough to modify cellular activity and induce preconditioning. More recently, Pain et al [236] have demonstrated that opening of the mito K_{ATP} channels triggers protection through the generation of free radicals which activate PKC, an obligatory step in the signal transduction mechanism of ischaemic preconditioning.

It must be mentioned that one of the potential limitations with my *in vitro* studies is the use of atrial myocardium as opposed to ventricular myocardium and therefore any extrapolation must be conducted with caution; however, Yellon and coworkers [52] have suggested that preconditioning exerts identical protection in both tissues. Undoubtedly, K_{ATP} channels are present in both atrium and ventricle [132] although as mentioned before in a previous chapter their density in both tissues is unknown.

The induction of CPB affects the body haemodynamics that may provoke a number of tissue responses. Thus, the loss of atrial and ventricular filling may stimulate a sympathetic-receptor mediated release of local catecholamines, whereas the interruption of pulsatile systolic and diastolic blood flow to the adrenal glands may stimulate a systemic catecholamine release. Therefore an altered adrenergic state may also be partially responsible for CPB-associated preconditioning in human.

myocardium. Several investigators [22,59] including work from this laboratory [197] have observed that norepinephrine or phenylephrine triggered PC and that the protection was prevented by adrenergic blockade. Similarly, Thornton et al [304] have demonstrated that tyramine, an agent that causes the release of endogenous catecholamines, reduced infarct size in rabbits when given prior to a sustained period of ischaemia. Certainly, more studies are required to elucidate the mechanism of cardioprotection effected by CPB.

Clinical Implications

Cardiac surgical practice is rapidly evolving and an increasing number of surgeons are adopting surgery on the beating heart, without the use of CPB, in their practice. Cardioplegic solutions cannot be used in this situation and the demonstration that interventions such as ischaemic preconditioning are protective can have important clinical implications. It should be however, recognised that the clinical application of ischaemic preconditioning may still be difficult and cumbersome, particularly if minimally invasive approaches are used. Because of this, the pharmacological manipulation of the signal transduction cascade of preconditioning may appear a more attractive. In this regard, several investigators including ourselves, are endeavouring to fully elucidate the mechanism of preconditioning in man to make this intervention a clinical reality.

CPB is known to induce a systemic inflammatory reaction that is believed to be responsible for increased morbidity. The present studies have demonstrated that CPB can also trigger preconditioning and be cardioprotective

Table 7.1 – Patients’ characteristics and perioperative data

Variable	<u>With Cardiopulmonary Bypass</u>				<u>Without Cardiopulmonary Bypass</u>			
	Intermittent X-clamp		Cold Blood Cardioplegia					
	Control	PC	Control	PC	Control	PC	Control	PC
Number	20	20	20	20	20	20	20	20
Age (years ± SD)	66.9±5.4	63.7±4.2	61.4±9.2	63.8±5.9	61.2±2.7	59.2±6.4	61.2±2.7	59.2±6.4
LVEF (% ± SD)	60.2 ± 5.5	61.8±4.1	62.1±5.7	63.7±4.9	59.7±9.2	65.1±6.4	59.7±9.2	65.1±6.4
No. of Grafts/patient	3.8 ± 1.5	3.6 ± 0.8	3.7 ± 1.2	4.1±0.7	1.15 ± 0.6	1.2 ± 0.5	1.15 ± 0.6	1.2 ± 0.5
Ischaemic time/graft (min ± SD)	16.1±2.5	15.4±3.1	17.2±4.9	14.7±5.7	17.8±7.9	16.9±4.7	17.8±7.9	16.9±4.7
CPB time (min ± SD)	66.4±12.7	70.4±11.4	71.4±13.4	69.8±6.7				

PC=preconditioning; LVEF= left ventricular ejection fraction (expressed as a percentage ± standard deviation); CPB=cardiopulmonary bypass.

Table 7.2 - Patients haemodynamic data.

Groups	Variable	HR	Mean MAP (mmHg)	CVP (mmHg)	Mean PAP (mmHg)	PCWP (mmHg)	CI (L/m2 per min)	PVRI (dyn/sec/cm5/m2)	SVRI
Pre-operative									
X-clamp	-PC	68.4±2.7	71.8±5.7	10.3±0.8	20.4±3.1	5.8±2.1	2.8±0.2	195±11	1568±102
	+PC	75.8±2.3	69.4±3.5	9.8±2.1	19.4±5.2	6.4±3.9	2.4±0.4	182±20	1602±53
BCP	-PC	59.4±9.4	65.7±6.9	7.8±5.7	22.6±6.4	5.1±2.6	2.9±0.4	201±13	1497±33
	+PC	65.1±6.8	70.2±2.1	9.1±2.4	25.1±9.3	7.4±1.3	3.05±0.19	215±25	1629±98
Off-pump	-PC	52.4±2.9	59.8±8.7	5.6±2.2	15.9±11.4	9.3±1.5	2.52±0.54	156±12	1307±109
	+PC	49.5±9.7	61.4±11.2	6.7±4.1	21.7±4.8	5.7±2.8	2.74±0.29	168±25	1567±63
1 hr post-termination of Ischaemia									
X-clamp	-PC	101.3±1.9	65.4±4.7	9.4±1.2	18.1±2.7	10.4±1.3	2.56±0.31	217±20	2001±52
	+PC	95.6±2.4	59.4±7.2	11.2±2.7	22.4±5.1	8.5±2.1	2.06±0.28	271±16	1959±257
BCP	-PC	99.8±10.4	71.2±5.9	8.6±3.5	16.4±6.9	11.3±1.4	2.76±0.22	301±27	2106±554
	+PC	92.7±4.9	64.8±6.8	10.2±2.9	21.9±2.4	9.4±3.8	2.98±0.15	357±12	1903±261
Off-pump	-PC	105.5±6.5	75.4±8.7	11.6±3.9	24.7±9.4	12.1±2.6	2.02±0.26	298±21	1506±23
	+PC	112.2±9.2	77.6±6.2	13.5±5.7	19.7±3.1	10.5±1.1	2.43±0.18	312±12	1709±143
3 hours post-termination of Ischaemia									
X-clamp	-PC	92.4±11.5	67.5±5.9	7.7±3.2	20.9±5.2	11.9±6.4	2.52±0.38	256±17	1954±106
	+PC	84.2±12.7	71.8±11.8	9.2±2.7	19.8±3.4	9.7±2.9	2.21±0.27	291±67	2304±328
BCP	-PC	95.3±15.1	67.8±11.9	9.8±2.4	23.7±6.8	10.2±1.9	2.64±0.12	333±29	1796±447
	+PC	105.3±24.9	77.4±4.9	10.7±3.1	22.7±3.6	11.6±2.7	2.57±0.25	401±46	1667±169
Off-pump	-PC	79.5±12.9	71.5±12.7	9.4±2.5	21.9±2.7	8.4±2.5	2.11±0.15	412±29	1509±225
	+PC	86.7±14.7	75.1±8.3	7.9±4.2	26.4±5.9	9.6±1.6	2.92±0.26	368±36	1882±203
24 hours post-termination of Ischaemia									
X-clamp	-PC	61.5±6.4	62.7±2.9	6.4±2.9	19.2±2.7	5.8±2.3	2.96±0.26	208±22	1804±234
	+PC	72.9±5.8	58.4±9.1	7.6±6.1	16.7±4.9	10.4±3.7	2.35±0.36	214±50	2004±102
BCP	-PC	77.9±9.6	61.9±4.6	5.4±3.8	21.6±5.8	9.2±1.5	2.55±0.46	289±16	1620±98
	+PC	75.4±11.3	71.3±6.1	7.8±1.6	24.9±1.1	4.6±2.8	3.19±0.53	295±26	1492±207
Off-pump	-PC	91.6±8.7	88.1±5.3	4.4±0.8	19.6±4.6	7.8±3.8	2.46±0.34	312±28	1623±115
	+PC	84.3±10.4	90.1±2.8	6.8±1.7	23.3±3.3	10.2±1.4	2.89±0.41	256±33	2006±204

BCP blood cardioplegia; CI cardiac index; CVP central venous pressure; HR heart rate; MAP mean arterial pressure; PAP pulmonary artery pressure; PCWP pulmonary capillary wedge pressure; PVRI pulmonary vascular resistance index; SVRI systemic vascular resistance index. Data are expressed as mean ± standard deviation.

Figure 7.1A: Time course of release of plasma cardiac troponin T concentrations in patients undergoing coronary artery bypass grafting with CPB and intermittent aortic cross-clamping. In each group, patients were randomly subdivided into control and preconditioning (n=20/group). Data are expressed as mean \pm standard deviation. *p<0.05 vs control group

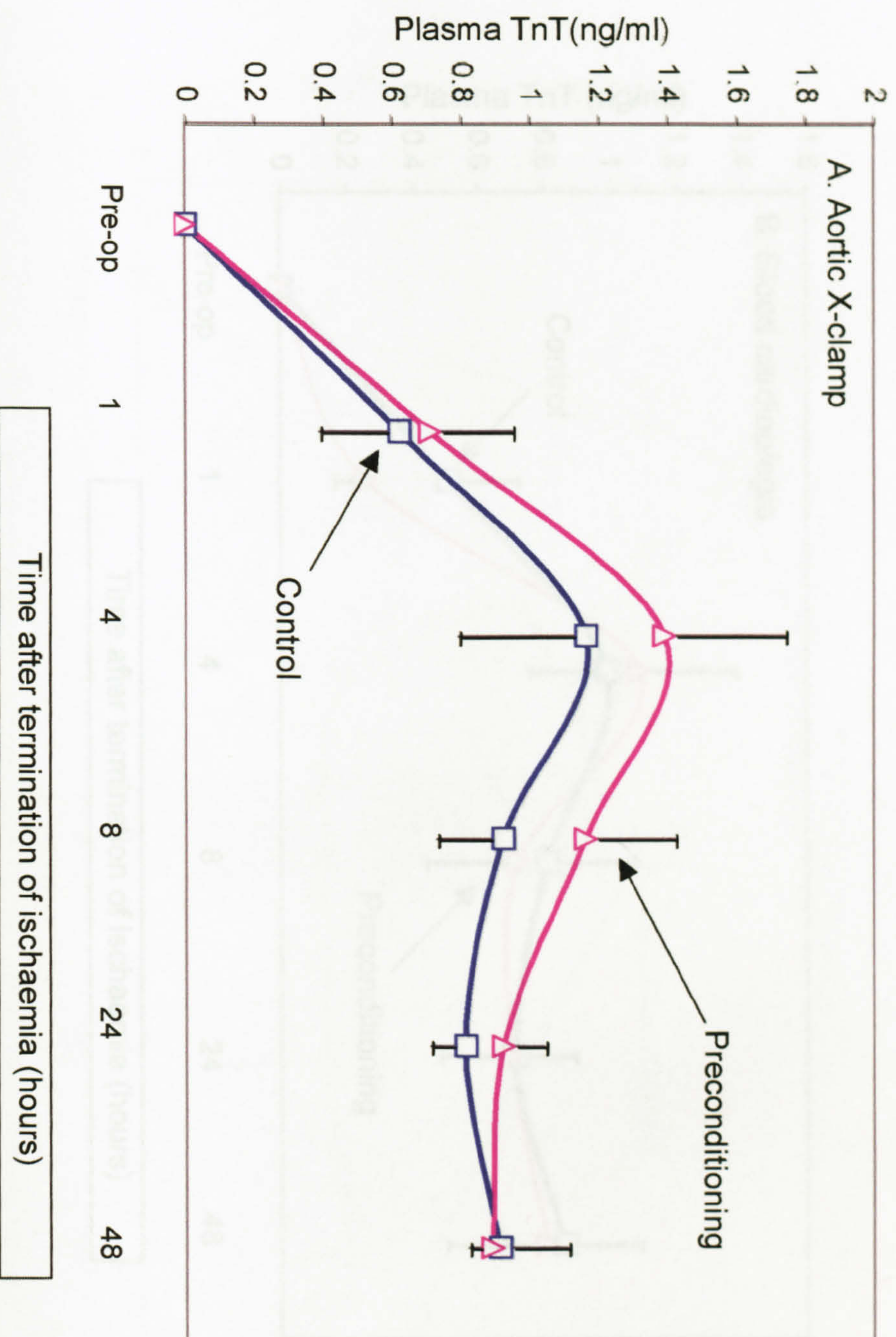


Figure 7.1B: Time course of release of plasma cardiac troponin T concentrations in patients undergoing coronary artery bypass grafting with CPB and cold blood cardioplegia. In each group, patients were randomly subdivided into control and preconditioning (n=20/group). Data are expressed as mean \pm standard deviation. *p<0.05 vs control group

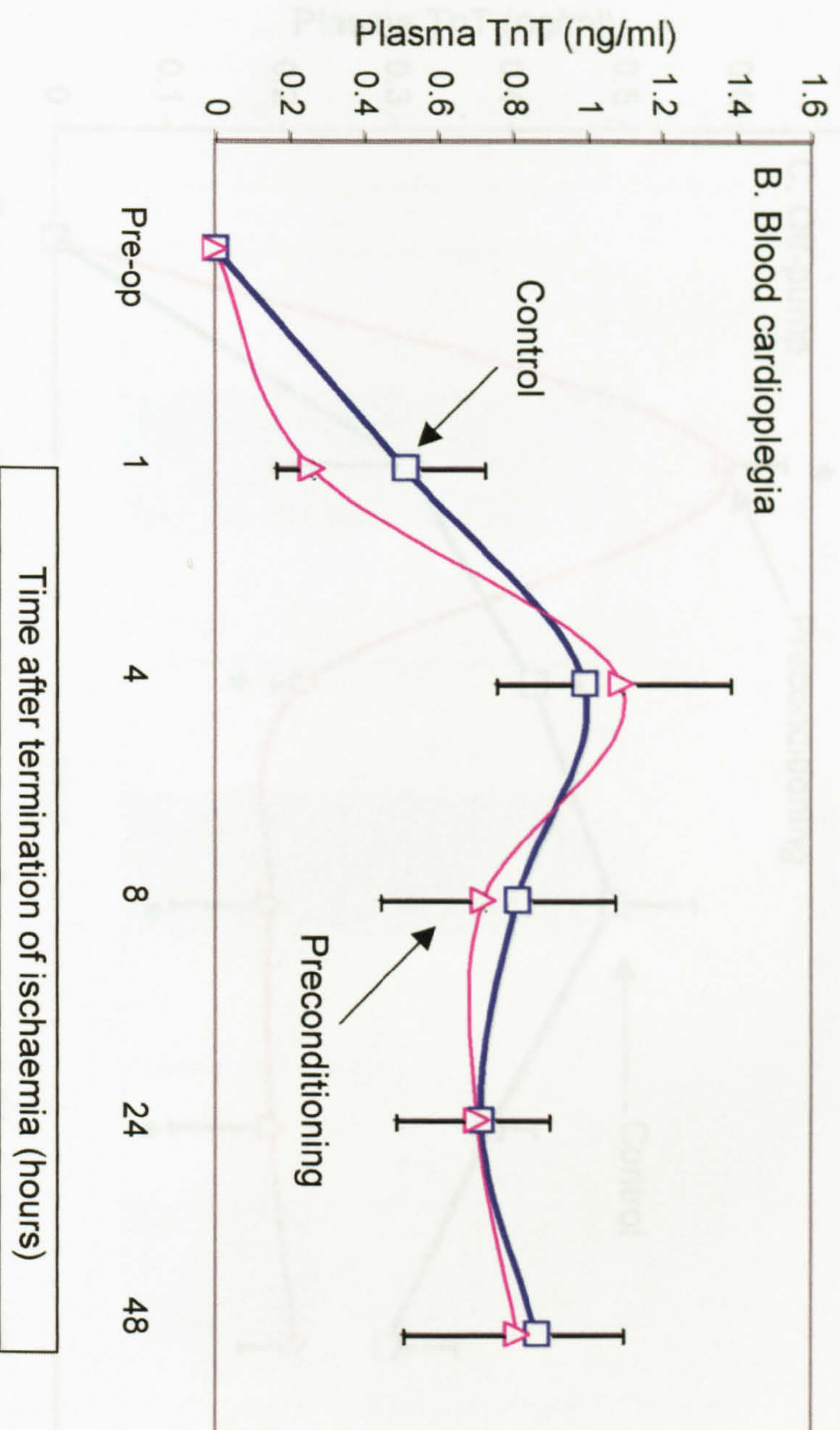
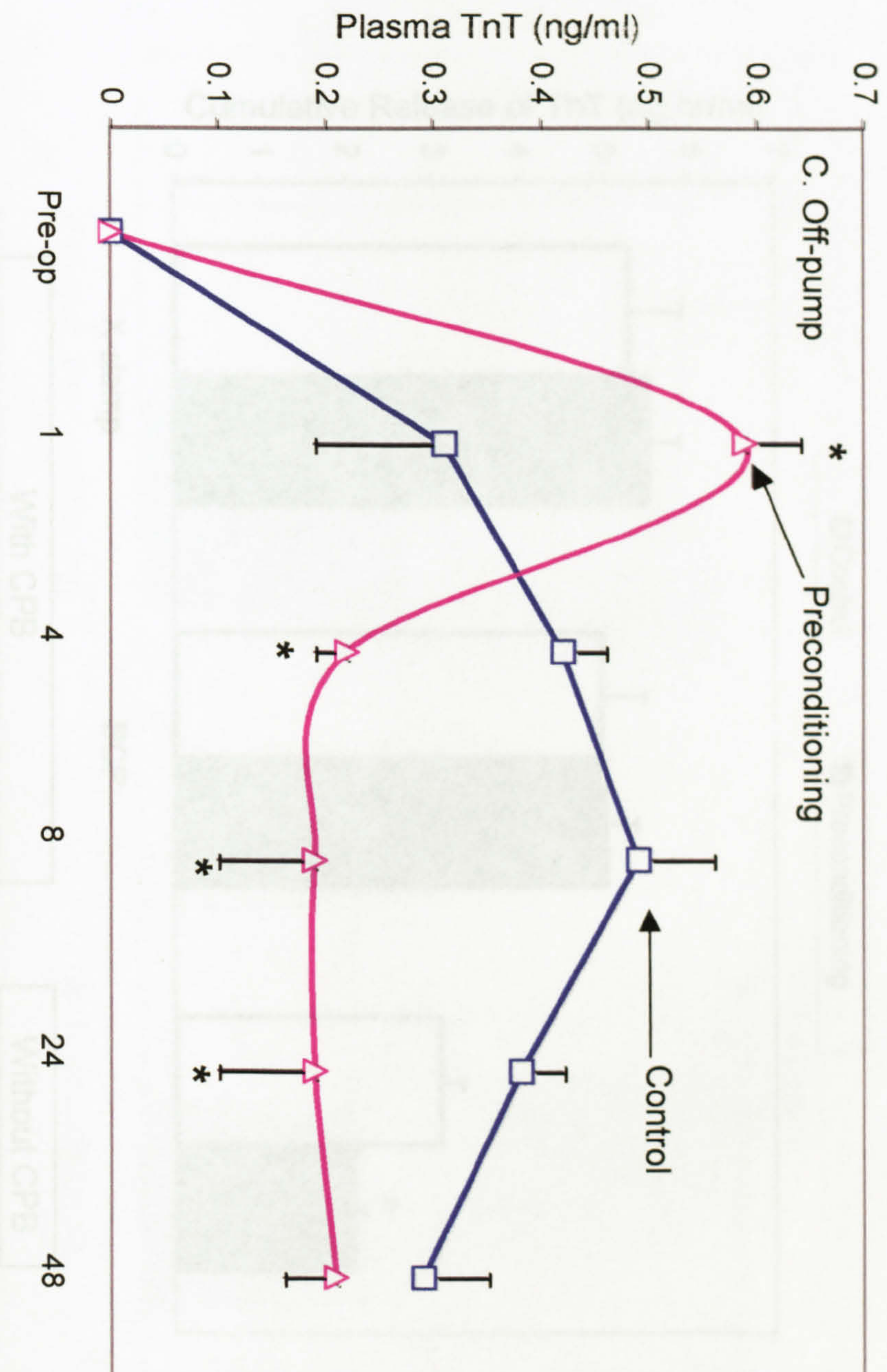


Figure 7.1C: Time course of release of plasma cardiac troponin T concentrations in patients undergoing coronary artery bypass grafting on the beating heart without CPB. In each group, patients were randomly subdivided into control and preconditioning (n=20/group). Data are expressed as mean \pm standard deviation. *p<0.05 vs control



Time after termination of ischaemia (hours)

Figure 7.2: Area under the curve of the plasma release cardiac troponin T in patients undergoing coronary artery bypass grafting with CPB and intermittent aortic X-clamp, CPB and cold blood cardioplegia and on the beating heart without CPB. *p<0.05 vs corresponding control group

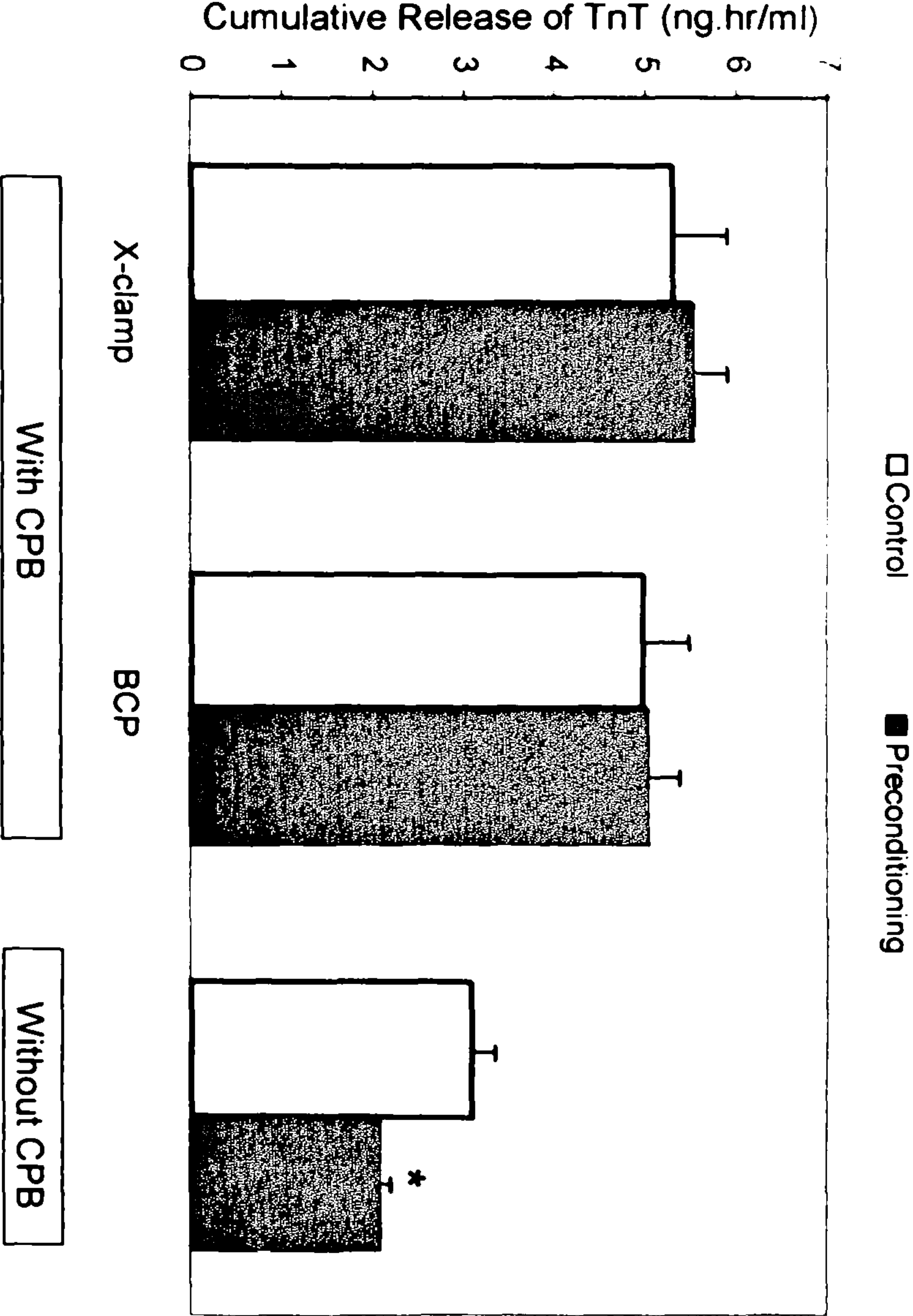


Figure 7.3A: Creatine kinase (CK) leakage during the 120min reoxygenation period following 90min of normothermic global ischaemia with or without ischaemic preconditioning. Data are expressed as mean \pm standard deviation of mean of 6 experiments. * $p < 0.05$ vs corresponding group subjected to without preconditioning.

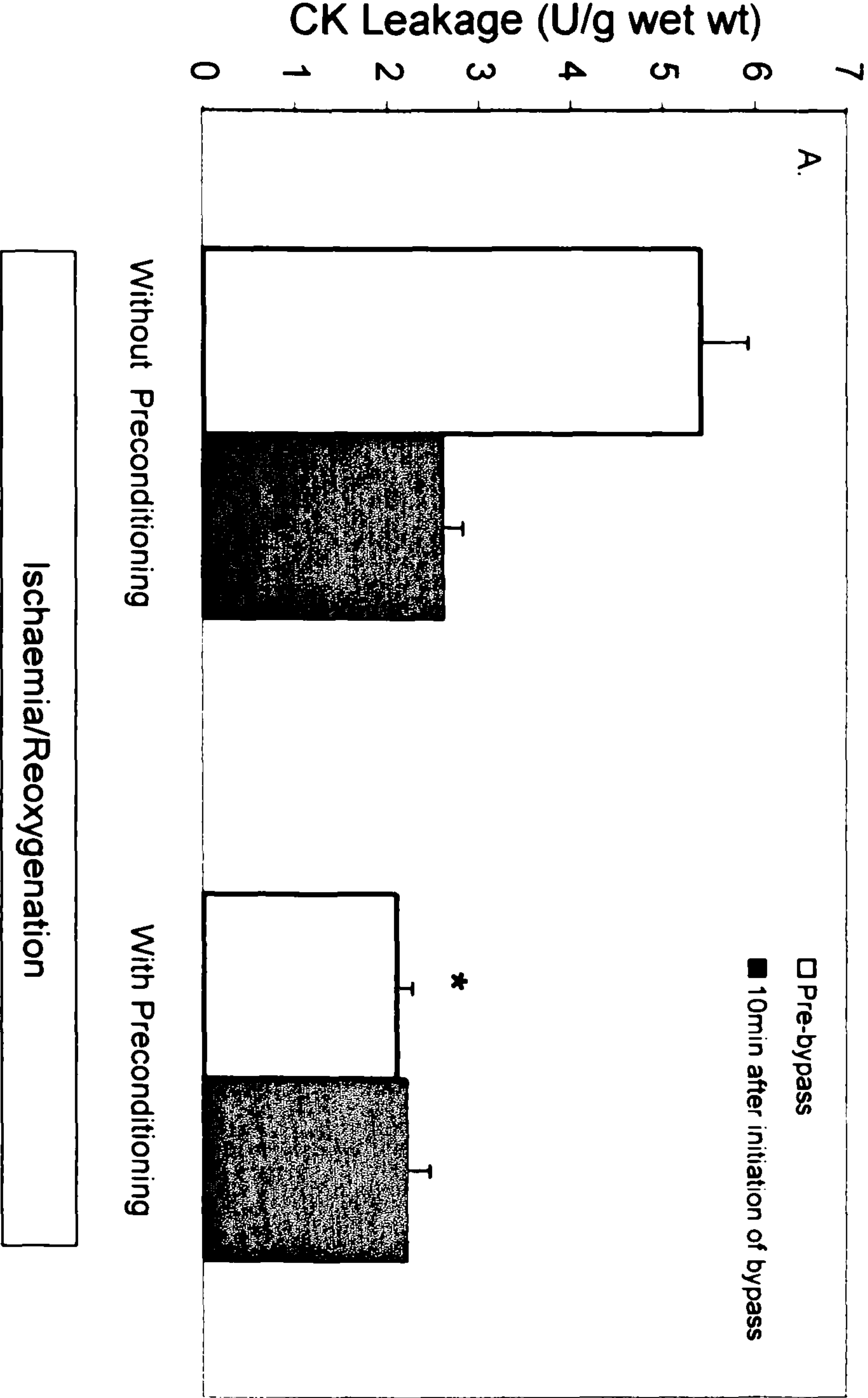
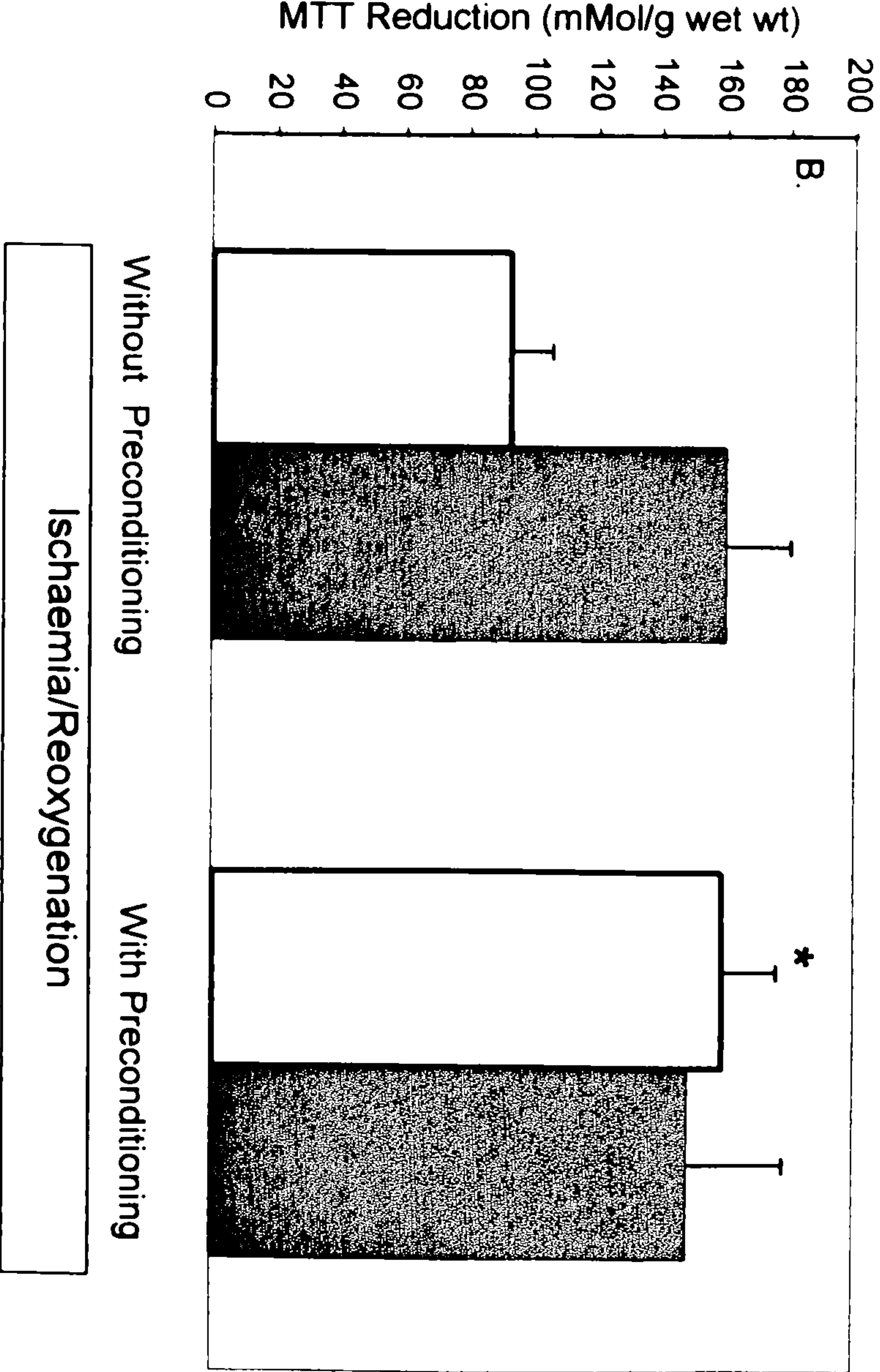


Figure 7.3B: MTT reduction at the end of the reoxygenation period following 90min of normothermic global ischaemia with or without ischaemic preconditioning. Data are expressed as mean \pm standard deviation of mean of 6 experiments. *p<0.05 vs corresponding group subjected to without preconditioning.



CHAPTER VIII
CONCLUSION

When ischaemic preconditioning was first described it became evident to the scientific world that a unique phenomenon of endogenous myocardial protection had been identified. Although initially there were many sceptics as to the originality and uniqueness of the phenomenon, further studies proved without doubt that ischaemic preconditioning is a strong form of myocardial protection manifesting itself in infarct size limitation, as well as other benefits.

It used to be thought that recruitment of collateral blood flow could account for this ischaemic protection, but this thesis has been disproved. Following this several theories have been tested and essentially they fall into two categories. Firstly, there are the advocates of the so-called metabolic theory of protection stating that specific metabolic changes/reactions take place in the cytosol to bring about protection. These metabolic changes may include the preservation of high energy phosphates, limitation of acidosis and lactate accumulation, as well as Ca^{2+} accumulation. Secondly, individuals have concentrated on the triggers of protection which may act on the cell membrane via adenosine receptors, adrenoreceptors, acetylcholine and other receptor groups. Stimulation of these receptors or the opening of a specific channel may result in a cascade of events inside the cell including opening of K_{ATP} channels, that may lead to protection against an ischaemic insult. These two lines of investigation are not mutually exclusive, and in fact they may be fundamentally linked, since membrane (sarcolemmal or mitochondrial) receptors/channels can trigger a set of biochemical reactions, and that it is possible that these reactions in the cytosol may ultimately be responsible for protection.

The adaptive response to ischaemia occurs quickly, but remains relatively short lived: the sustained ischaemic insult must follow soon after the brief period of

ischaemia. I have shown that this is valid in human myocardium. Protection lasts for only two hours but there is a second window of protection seen at 24 hr that in my studies appears to be much less potent than the protection of the first or early window. It is also important that the heart becomes unresponsive to preconditioning stimuli beyond 4-6 min of ischaemia and that the number of cycles applied is unimportant.

Substantial progress has been made towards the determination of the mechanisms that underlie classical ischaemic preconditioning. The two major trigger theories of protection include adenosine receptor stimulation and the opening of the K_{ATP} channel. Human evidence for the involvement of the K_{ATP} channel comes from angioplasty model [78] and from the functional atrial trabeculae model [282]. It has been suggested that mitochondrial channel rather than the sarcolemmal site may be responsible for the protection induced by ischaemic preconditioning in animal studies. In this thesis, I have shown that the protection brought about by various specific mitochondrial K_{ATP} channel openers is comparable to that brought about by prior treatment with an ischaemic preconditioning stimulus and that the protection can be abolished by the application of specific mitochondrial K_{ATP} blockers, thereby suggesting that it is the mitochondrial K_{ATP} channel and not the sarcolemmal channel that is responsible for the powerful protection induced by preconditioning. More recently, Pain et al [236] have demonstrated that opening of the mito K_{ATP} channels triggers protection through the generation of free radicals which activate PKC and MAPK, an obligatory step in the signal transduction mechanism of ischaemic preconditioning suggesting that these channels are probably triggers rather than end-effectors. Evidence as to the possible interplay between PKC activation and the opening of the K_{ATP} channels has been provided by Hu et al [136] who used a whole-cell voltage clamp technique applied to rabbit ventricular myocytes. Experimental

evidence for the involvement of PKC activation following opening of mitochondrial K_{ATP} channels is scant and clearly warrants further research.

Within the enormous amount of research describing the cellular basis of the preconditioning response, relatively few studies have focussed on the effect of preconditioning in hearts with concurrent abnormalities relevant to coronary artery disease in humans. More importantly, even amongst those studies, the conclusions have been conflicting. Clinical studies clearly identify a number of conditions that increase mortality due to myocardial infarction; these include heart failure, diabetes, hypertension and aging. It is plausible that these conditions interfere with the biochemical pathways underlying the preconditioning response. The data on the involvement of these factors in human ischaemic preconditioning is lacking and it was my aim to proceed down this line of investigation and attempt to establish a link between these factors and protection induced by preconditioning in human myocardium. The major findings of the studies involving heart failure and diabetes were that myocardium from patients with diabetes and poor cardiac function are not protected from ischaemic preconditioning although the injury induced by ischaemia/reoxygenation is not exacerbated in these conditions. Furthermore, the demonstration that activation of $mitoK_{ATP}$ channels mimicks the protection induced by preconditioning in the myocardium from hearts with poor contractility but not from patients with diabetes suggests that the failure to precondition the former is due to alterations in the signal transduction pathway upstream of K_{ATP} channels and to alterations in the response of the K_{ATP} channels in the latter. This area warrants further research and is the subject of future investigations in our laboratory. Increasing age has been recognized as a cause for adverse prognosis in patients presenting with a myocardial infarction and is also an independent risk factor for patients undergoing

coronary angioplasty and cardiac surgery that is believed to be attributed to a greater susceptibility of the senescent heart to ischaemic injury and to a lower response to protective interventions. Interestingly, the experimental evidence from my studies suggested that age did not influence the tolerance of the human myocardium to ischaemia or the protective effect of ischaemic preconditioning. These results question the validity of regarding age as a risk factor in heart disease and in cardiac surgery on the basis of tolerance to ischaemia and suggest a re-evaluation of its importance in risk scoring.

Having established *in vitro* experimental evidence for the powerful protection of preconditioning in human myocardium, it was my aim to extrapolate the findings of laboratory work to the clinical *in vivo* setting. For logistic and ethical reasons, no clinical study can meet the strict conditions of experimental studies on preconditioning with infarct size as the end-point and because of this human *in vivo* studies have produced conflicting results and the role of preconditioning in man remains controversial. The conflicting results obtained in studies designed within the surgical setting may be explained by different study designs, hence reconciled if one takes into account one possible hypothesis - that preconditioning and its salutary effects are only observed in situations of unprotected ischaemia. For this reason, my aims were to investigate the role of preconditioning in both off and on-pump surgery. Interestingly, the studies showed that the human heart was preconditioned by the institution of cardiopulmonary bypass and that the use of ischaemic preconditioning in combination with other protective interventions such as cardioplegia and hypothermia does not result in additional protection. They also clearly demonstrated that the human heart can be protected by ischaemic preconditioning when patients are operated on without the use of cardiopulmonary bypass. The mechanism by which

institution of cardiopulmonary bypass alone results in protection that mimicks that of ischaemic preconditioning is still elusive and clearly needs further research.

Future Directions

The mechanism of protection induced by ischaemic preconditioning is yet to be completely understood. It would appear that insult to the myocyte activates a receptor-based trigger system which leads to activation of PKC and MAPK and mitochondrial K_{ATP} channels which ultimately leads to protection. What remains unclear is whether the activation of mitochondrial K_{ATP} channels is up or downstream of PKC and MAPK phosphorylation. Cellular stresses in many mammalian cell types activate a distinct subset of the MAPK family of enzymes, termed the Stress-Activated Protein Kinases (SAPKs). The SAPKs are analogous to the Extracellularly Regulated Kinase or ERK family of MAPKs which regulate predominantly the growth and proliferation of cells in response to factors acting on tyrosine kinase receptors and G protein-coupled receptors. To date, two distinct groups of SAPKs have been identified. The c-Jun N-terminal Kinases or JNKs and the p38 family. The mitogen activated protein kinases-p38 have been demonstrated to be activated by ischaemia/reperfusion injury in the heart [245,303,337,348]. The role of the p38 MAPK signaling pathway in the early phase of preconditioning seems to be controversial. The two lines of studies performed to date have sought to determine (1) whether preconditioning induces activation of p38 MAPKs and (2) whether inhibition of p38 MAPK abrogates the cardioprotective effect. Unfortunately, both have yielded conflicting results. Moreover, in man, the role of p38 MAPK signaling in preconditioning and the final activation of the end-effector(s) responsible for cell protection has yet to be elucidated.

p38 MAPKs are likely to be critically involved in both the short and long-term responses of cardiac myocytes to ischaemia and reperfusion injury and possibly ischaemic preconditioning, by bringing about changes in the phosphorylation state of regulatory proteins in the signaling pathways involved. It is in this area that I think future research in preconditioning should be directed and in our laboratory, work has begun to address these issues which are a subject of a future programme grant.

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Characterization of an *in vitro* model for the study of the short and prolonged effects of myocardial ischaemia and reperfusion in man

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A B S T R A C T

The mechanisms underlying myocardial ischaemia and reperfusion-induced injury have been investigated, mainly by using animal experimental preparations *in vitro* and *in vivo*, but little is known of the process in human myocardium. The present studies characterize an *in vitro* model using human myocardium for the study of early and delayed effects of ischaemia and reperfusion. The right atrial appendage was manually sliced and incubated in buffer through which was bubbled O₂/CO₂ (19:1, v/v) for various time periods. Lactate dehydrogenase (LDH) leakage, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction, oxygen consumption, nucleotide levels and tissue morphology were all investigated as markers of myocardial injury. The specimens remained stable and viable up to 24 h, but had significantly deteriorated by 48 h. The preparation responded to ischaemia in a time-related manner. Tissue viability was reduced by 25% after 30 min ischaemia, declined to 60% after 60 min ischaemia and to 75% after 120 min ischaemia. Interestingly, the tissue was more susceptible when ischaemia was induced after 24 h of aerobic incubation. The effects of the duration of reperfusion were investigated after a fixed 60 min ischaemic insult. The results of LDH leakage suggest that reperfusion injury is mainly sustained within the first 2 h of reperfusion. However, the results of MTT reduction show that there is a progressive decrease in tissue viability over the 24 h reperfusion period, possibly reflecting the occurrence of tissue necrosis and apoptosis at different reperfusion times. In conclusion, the data provide evidence that the incubation of human atrial tissue *in vitro* is stable, and slices are viable for at least 24 h, which permits the study of early and delayed consequences of ischaemia and reperfusion in the human myocardium.

INTRODUCTION

Ischaemic heart disease is the single most common cause of mortality in the Western world. Over the last two decades, a great deal has been learned about the pathophysiology of myocardial ischaemia, the consequences of reperfusion and how the adverse effects may be com-

bated. Most of our knowledge has been gained by using *in vivo* and *in vitro* experimental animal models, and the extrapolation of this information to the human heart has resulted in the implementation of novel therapeutic approaches and in a progressive decrease in the death rate attributed to cardiac ischaemic events.

Studies on cardiac ischaemia and reperfusion in man

Key words: human myocardium, ischaemia, reperfusion, right atrium.

Abbreviations: ANOVA, analysis of variance; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide; PMNs, polymorphonuclear cells.

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are difficult because of the presence and potential influence of a whole host of clinical factors. The utilization of human isolated myocytes [1,2], papillary muscle [3,4] and atrial myocardium [5–8] has provided a means to investigate directly the effects and mechanisms of ischaemia and reperfusion in man without the need to resort to assumptions made from animal studies, and to safely test interventions intended to be used clinically. Thus the use of human cultured myocytes [1,9] and right atrial tissue [5,10], for example, has served to identify some of the mechanisms involved in ischaemic preconditioning in the human myocardium, which, compared with those found in other animal species [11–15], has opened the door for its clinical application [16].

The right atrial preparation is of particular interest, because the tissue is easily obtainable from patients undergoing open-heart surgery, it is simple to prepare and the procedure is inexpensive. Yet the preparation has not been fully characterized. The aim of the present studies was therefore to investigate the stability of the human right atrium when incubated in a buffered medium, its response to various degrees of ischaemic insult and the short and prolonged effects of reperfusion.

METHODS

Preparation of atrial slices

Specimens of human right atrium appendage were obtained from patients undergoing elective heart surgery. During surgery, the right atrial tissue is routinely removed for venous cannulation and establishment of cardiopulmonary bypass. Samples were quickly immersed in cold (4 °C) Krebs/Henseleit/Hepes medium (118 mM NaCl, 4.8 mM KCl, 27.2 mM NaHCO₃, 1 mM KH₂PO₄, 1.2 mM MgCl₂, 1.25 mM CaCl₂, 10 mM glucose, 20 mM Hepes). The medium had been pre-bubbled with O₂/CO₂ (19:1, v/v) to attain a *P*O₂ of 25–30 kPa and pH 7.4. The atrial appendage was immediately sliced manually with skin-graft blades (Swann-Morton Ltd, Sheffield, U.K.), each slice to a thickness of 0.5 mm and a weight of 5–10 mg, as originally described for the preparation of rat renal slices [17]. Briefly, the tissue was placed with the epicardial surface face down on filter paper fixed to a rectangular glass base (5 × 25 cm). A ground-glass slide (2.5 × 7.5 cm) was then pressed against the tissue and the blade was drawn between the slide and the tissue. The slicing apparatus and the tissue was kept wet at all times with ice-cold medium (4–10 °C).

Experimental time course

After preparation, the slices (3–5 slices per specimen) were blotted with wet filter paper, loaded into glass 25 ml Erlenmeyer flasks and 5 ml of medium, which was

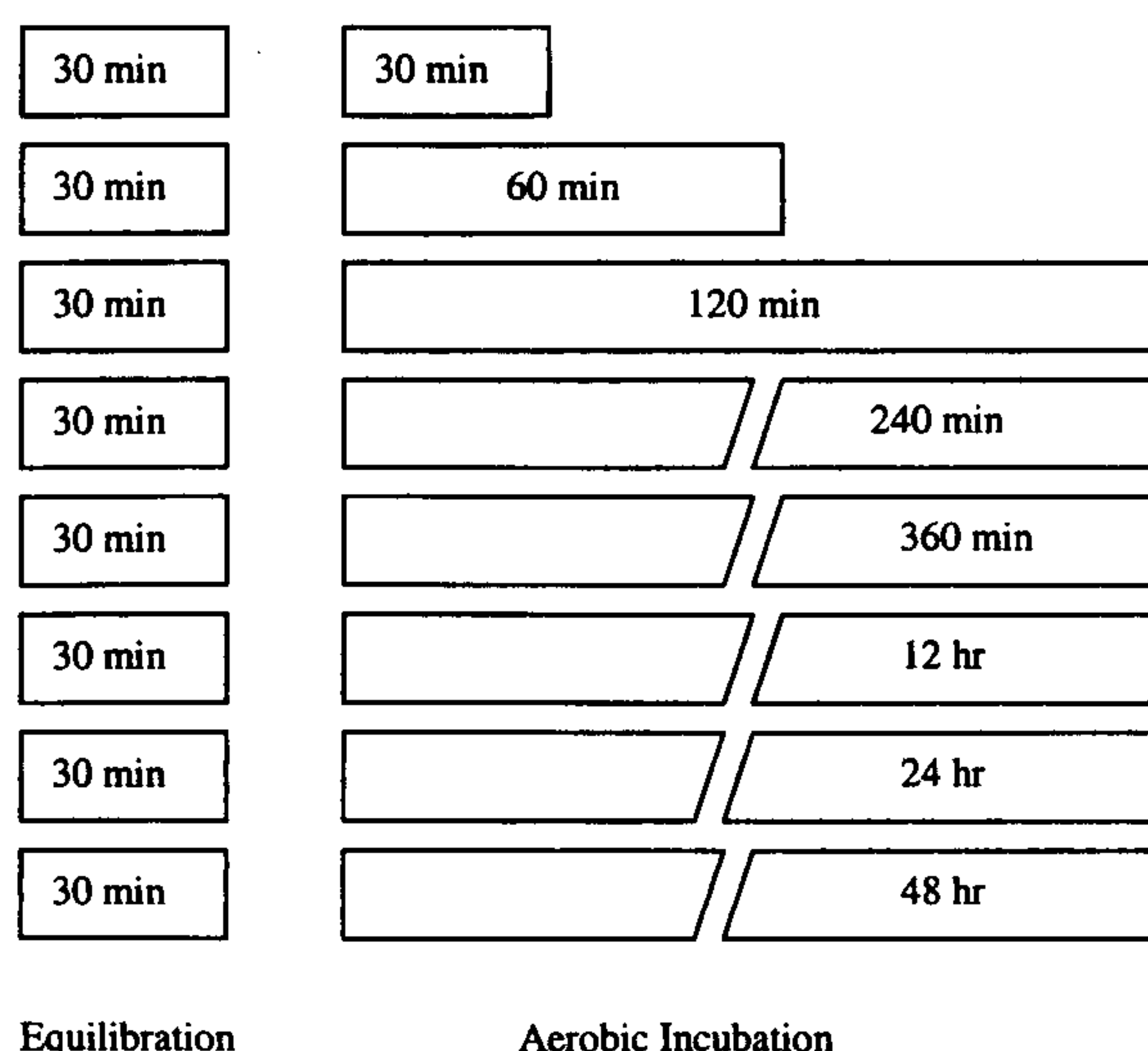


Figure 1 Experimental protocol for Study 1

Tissues from Groups 1–4 were equilibrated for 30 min in aerobic conditions at 37 °C. The right atrial slices (*n* = 6/group) were further incubated aerobically for the time periods shown.

continuously bubbled with O₂/CO₂ (19:1) to maintain a *P*O₂ of 25 kPa and a pH of 7.4, was added. The flasks were then placed in a shaking water bath (100 cycles/min) at 37 °C for 30 min to allow equilibration. The slices were then rinsed with the medium, blotted and placed in clean flasks containing 5 ml of oxygenated medium for various time periods to serve as time-matched aerobic controls.

For the induction of simulated ischaemia, the slices were washed with one rinse of medium bubbled with N₂/CO₂ (19:1) at pH 6.8. In this case, glucose in the medium was replaced with 10 mM 2-deoxy-D-glucose (grade II). The slices were transferred to clean flasks containing 5 ml of the same medium which was continuously bubbled with N₂/CO₂ (19:1) and maintained at 37 °C during the entire ischaemic period. Monitoring of *P*O₂ with an oxygen detector electrode (Oxylite[®]; Optronix Ltd, Oxford, U.K.) revealed that the *P*O₂ in the medium was 0 kPa. At the end of each ischaemic period, the non-oxygenated medium was removed, the slices were rinsed with oxygenated medium (O₂/CO₂, 19:1) and incubated in 5 ml of oxygenated medium containing 10 mM glucose at 37 °C for a further 120 min.

Study groups

Three different studies were performed to investigate: the stability of the preparation (Study 1), the effect of the severity of ischaemia (Study 2), and the effect of the duration of reperfusion (Study 3).

In Study 1 (Figure 1), atrial slices (*n* = 6/group) were subjected to various periods of aerobic incubation after an initial 30 min equilibration period. At the end of the experimental time, samples of the incubation medium

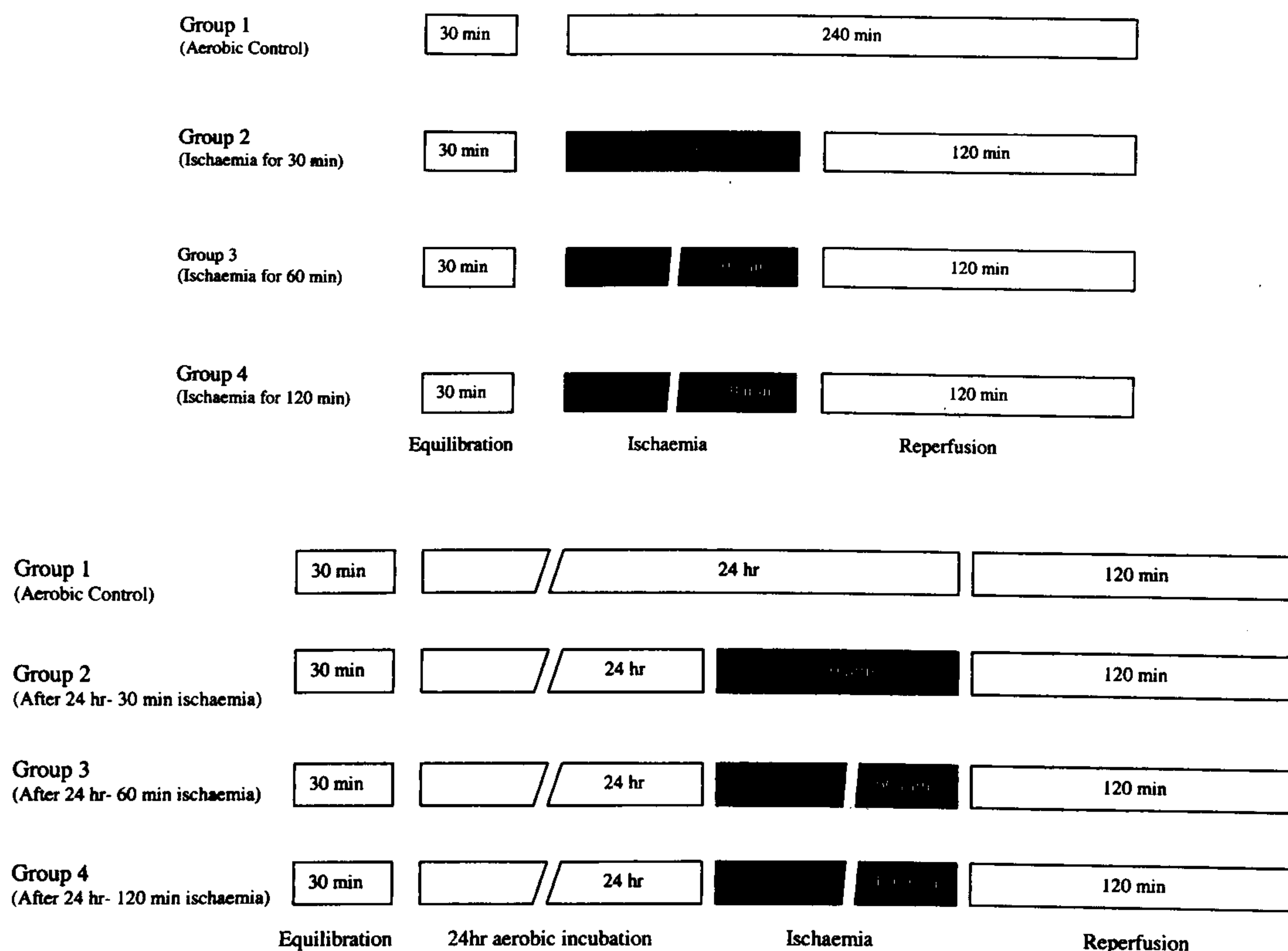


Figure 2 Experimental protocols for Studies 2A and 2B

Study 2A (upper panel): right atrial slices from Groups 1–4 ($n = 6/\text{group}$) were equilibrated for 30 min. In Group 1, the slices were then incubated aerobically for 240 min. After 120 min, the incubation medium was changed and the slices were incubated for a further 120 min. In Groups 2, 3 and 4, after equilibration, the slices were subjected to 30, 60 or 120 min of ischaemia respectively, and then subjected to 120 min of reperfusion. Study 2B (lower panel): right atrial slices from Groups 1–4 ($n = 6/\text{group}$) were equilibrated for 30 min. In Group 1, the slices were then aerobically incubated for 26 h to act as time-matched controls. In groups 2, 3 and 4, the slices were aerobically incubated for 24 h before being subjected to 30, 60 or 120 min of ischaemia respectively, followed by 120 min of reperfusion.

were taken for the assessment of lactate dehydrogenase (LDH) leakage and the slices were removed for the determination of oxygen consumption, tissue viability, nucleotide metabolite analysis and morphological examination.

Study 2 was divided into two parts. In study 2A (Figure 2, upper panel), slices ($n = 6/\text{group}$) were initially equilibrated for 30 min and then randomly subjected to various periods of ischaemia (30, 60 or 120 min) followed by 120 min of reperfusion. In Study 2B (Figure 2, lower panel), the slices ($n = 6/\text{group}$) were randomly subjected to an identical protocol of ischaemia and reperfusion as in Study 2A, except that they were incubated aerobically for 24 h before they were subjected to ischaemia and reperfusion. At the end of the experimental time, samples of the incubation medium were taken for the measurement of LDH leakage and the slices were used for determination of tissue viability (Studies 2A and 2B) and oxygen consumption (Study 2A only).

In Study 3 (Figure 3), slices ($n = 6/\text{group}$) were subjected to ischaemia for 60 min and then randomly allocated to various reperfusion times (2, 4, 12 or 24 h). An additional study ($n = 4/\text{group}$) was performed using

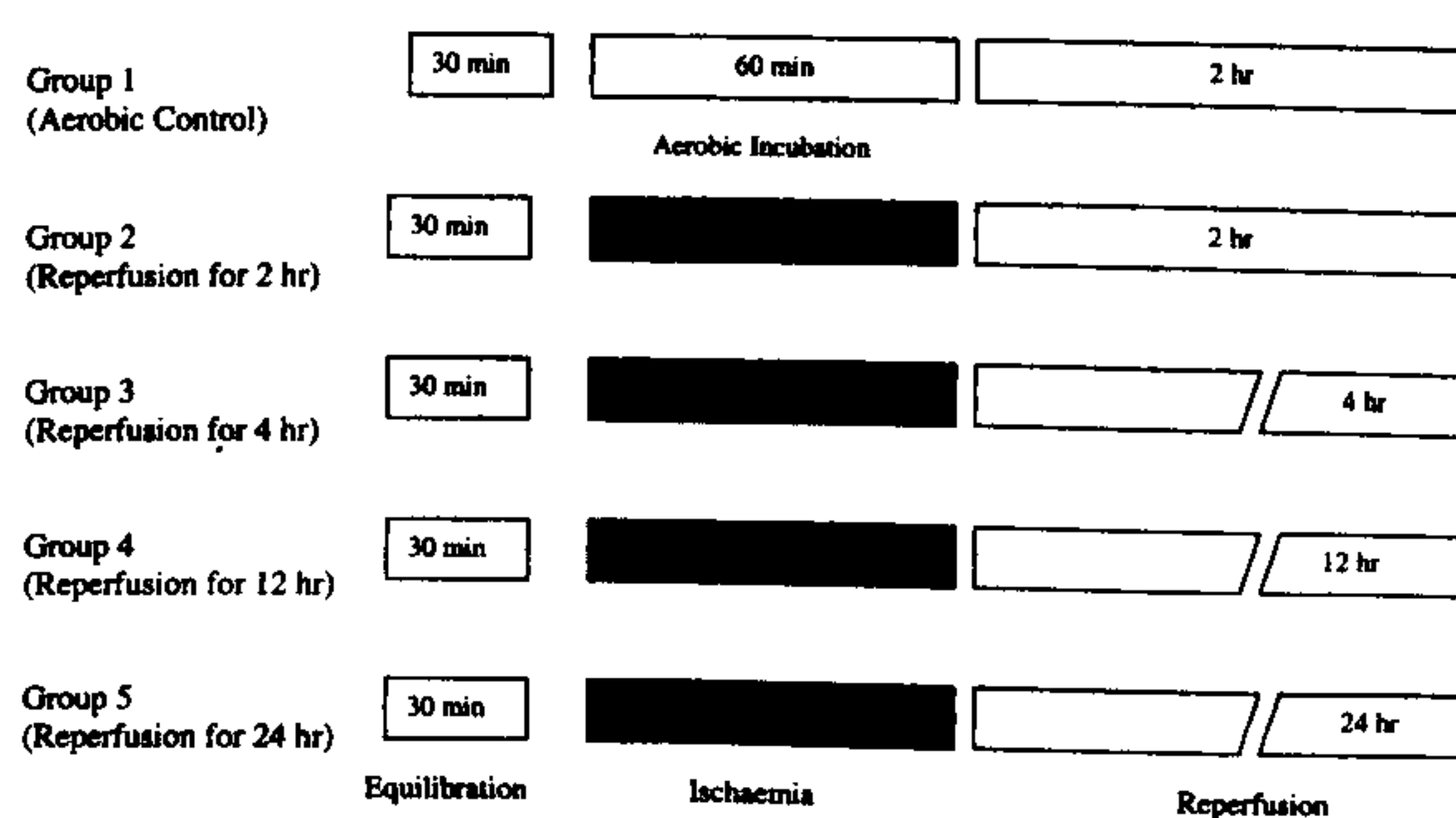


Figure 3 Experimental protocol for Study 3

Right atrial slices from Groups 1–5 ($n = 6/\text{group}$) were equilibrated for 30 min. In Group 1 the slices were then incubated aerobically for 180 min. In Groups 2–5 the slices were subjected to 60 min of ischaemia followed by 2, 4, 12 or 24 h, respectively, of reperfusion.

the same experimental protocol in the presence of neutrophils obtained from the same patients from whom the right atrial appendage was removed. As in previous studies, LDH leakage and tissue viability were determined at the end of the experimental period.

Assessment of LDH leakage

The activity of LDH in the media (units/g wet weight) was assayed spectrophotometrically by monitoring the oxidation of NADH at A_{340} (Sigma Catalogue No. 1340-K).

Tissue viability

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl-2*H*-tetrazolium bromide (MTT) assay was used to quantify tissue viability. In this assay, the yellow MTT is reduced to a blue formazan product by the mitochondria of viable tissue. Briefly, at the end of each experiment, the slices were placed in 15 ml Falcon conical tubes (Beckton-Dickinson, Franklin Lakes, NJ, U.S.A.), 2 ml of PBS (0.05 M) containing 3 mM MTT (final concentration) was added and the slices were incubated for 30 min at 37 °C. The slices were then homogenized (Ultra-Turrax T25, dispersing tool G8; IKA Laboratories, Staufen, Germany) in 2 ml of DMSO at 9500 rev./min for 1 min and centrifuged at 1000 *g* for 10 min. Portions of the supernatant (0.2 ml) were dispensed into 98-well flat-bottom microtitre plate (Nunc Brand Products, Denmark) and the A_{570} was measured on a plate reader (Benchmark; Bio-Rad, Hercules, CA, U.S.A.) and results were expressed as A/mg wet weight.

Oxygen consumption

Oxygen consumption by the slices was measured using a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.). The electrode contained 1 ml of air-saturated incubation medium (pH 7.4) and was maintained at 37 °C. The slices were carefully loaded into the chamber to avoid the formation of bubbles, and oxygen consumption was recorded for 5–8 min. Tissue respiration was calculated as the decrease in oxygen concentration in the medium after the addition of the slices and the results were expressed as $\text{nmol O}_2/\text{g}$ wet wt.

Metabolite analysis

Atrial slices were frozen in liquid nitrogen at the end of 0.5, 4, 24 and 48 h of aerobic perfusion. For tissue metabolite analysis, the dried slices were extracted into 0.6 M perchloric acid (25 $\mu\text{l}/\text{mg}$ of dry tissue) and the extract was centrifuged at 11 000 *g* for 5 min at 4 °C. The supernatant was removed and neutralized with an appropriate volume of 2 M KOH. Aliquots (20 μl) were taken for analysis by HPLC [18]. The values obtained ($\mu\text{mol}/\text{g}$ dry weight) were used to calculate the myocardial energy status (ATP + ADP + AMP).

Morphological examination

Samples of atrial slices were taken at the end of 0.5, 4, 24 and 48 h of aerobic perfusion and fixed in 3% (w/v)

glutaraldehyde, post-fixed in 2% aqueous osmium tetroxide, dehydrated in ethanol and embedded in Araldite resin. A semiquantitative estimate of cell damage was performed on 60-nm sections. Blocks from each tissue sample were randomly chosen and cell damage was quantified without prior knowledge of the group to which the tissue belonged. Cell morphology was assessed according to the following classification [19,20]: grade 1 (normal), compact myofibrils with uniform staining of nucleoplasm, well defined rows of mitochondria between myofibrils and the non-separation of opposing intercalated disks; grade 2 (mild damage), similar to grade 1, except for the presence of some vacuoles adjacent to the mitochondria; grade 3 (severe damage), reduced staining of cytoplasmic organelles, clumped chromatin, wavy myofibrils and granular cytoplasm or cells with contraction-band necrosis.

Preparation of neutrophils

Heparinized, venous blood (4 ml) was obtained from patients the day before surgery, in accordance with a protocol approved by the local Ethics Committee. Polymorphoneutrophils (PMNs) were isolated as described previously using Hypaque-Fico density-gradient dextran sedimentation [21]. Purified PMNs were resuspended in PBS and kept on ice until use. PMNs (1×10^6 to 10×10^6 cells/ml) were added to the atrial slices during the experimental protocol.

Statistical analysis

Data were expressed as means \pm S.E.M. One-way analysis of variance (ANOVA) was used for comparisons of more than two means. ANOVA for repeated measurements was used to test the significance of mean values over time. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Stability of the preparation (Study I)

LDH leakage

The leakage of LDH into the medium during the incubation period is shown in Figure 4. LDH leakage steadily increased during the first 12 h of incubation, from 2.8 ± 0.2 units/g wet wt at 0.5 h to 10.4 ± 0.7 units/g wet wt at 12 h (Figure 4, upper panel). However, if the leakage was calculated as the net LDH leakage (the difference in the enzyme leakage between two adjacent time points divided by the period of incubation in hours), the greatest leakage occurred during the first 0.5 h (2.96 ± 0.31 units/g wet wt/h) (Figure 4, lower panel), with a continuous decrease in the leakage during the

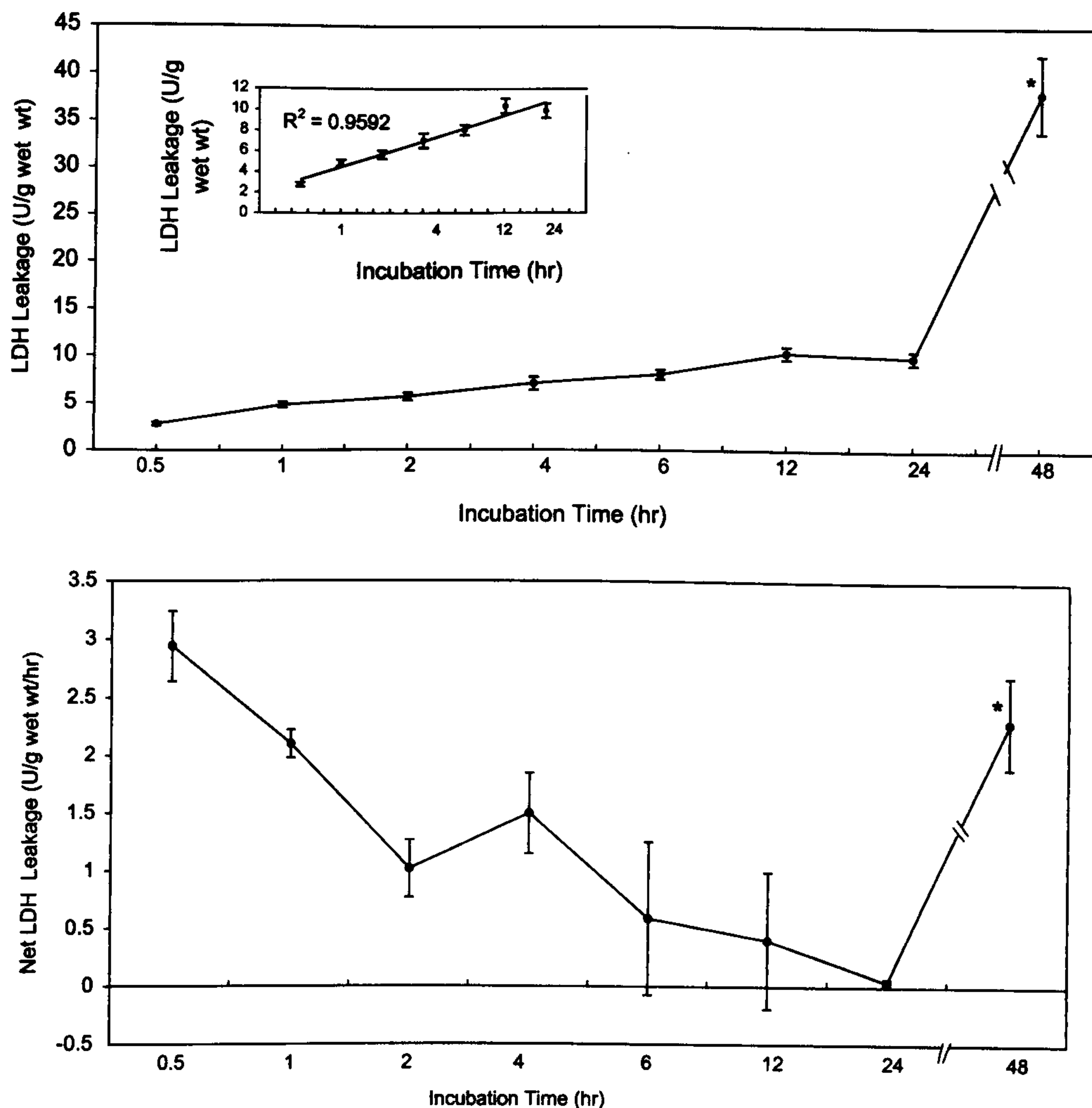


Figure 4 LDH leakage from right atrial slices incubated in aerobic conditions at 37 °C for different time periods

Upper panel: data are expressed as means \pm S.E.M. of six experiments. * $P < 0.05$ versus 24 h value. Inset: linear regression analysis of the data over the 24 h period.

Lower panel: net LDH leakage from right atrial slices. Data are expressed as means \pm S.E.M. of six experiments. * $P < 0.05$ versus 24 h value.

ensuing 12 h. Interestingly, irrespective of the way the results are expressed, there was little LDH leakage between 12 and 24 h of incubation, but leakage had dramatically increased at 48 h of incubation. Regression analysis (inset; Figure 4, upper panel) revealed that the profile of LDH leakage for the first 24 h of incubation was linear, suggesting continuous enzyme leakage into the media. These results suggest that the preparation does not sustain significant tissue injury for the first 24 h of incubation but that it has deteriorated by 48 h.

Tissue viability

The reduction of MTT by the atrial slices (Figure 5), an index of tissue viability, was 0.89 ± 0.05 absorbance units/mg wet wt after 30 min of incubation. These values were not significantly decreased after either 4 h (0.84 ± 0.02 absorbance units/mg wet wt) or 24 h (0.84 ± 0.03 absorbance units/mg wet wt). However, by 48 h these values were significantly decreased to only

10% (0.09 ± 0.01 absorbance units/mg wet wt; $P < 0.05$) of the values at 30 min incubation. These results also indicate that the preparation was stable for at least the first 24 h of incubation but that tissue viability was not maintained for 48 h.

Myocardial oxygen consumption

As shown in Figure 6, oxygen consumption by the slices was approx. 907 ± 39 nmol O_2 /g wet wt per min after 30 min of incubation. Similar values were obtained after 4 h (827 ± 21 nmol O_2 /g wet wt per min; not statistically different) and 24 h (876 ± 40 nmol O_2 /g wet wt/min) of incubation, but again by 48 h oxygen consumption dramatically decreased to 20% of the initial values (184 ± 12 nmol O_2 /g wet wt per min; $P < 0.05$). These findings further support that the human atrial preparations were viable and stable for at least 24 h of incubation but not for 48 h.

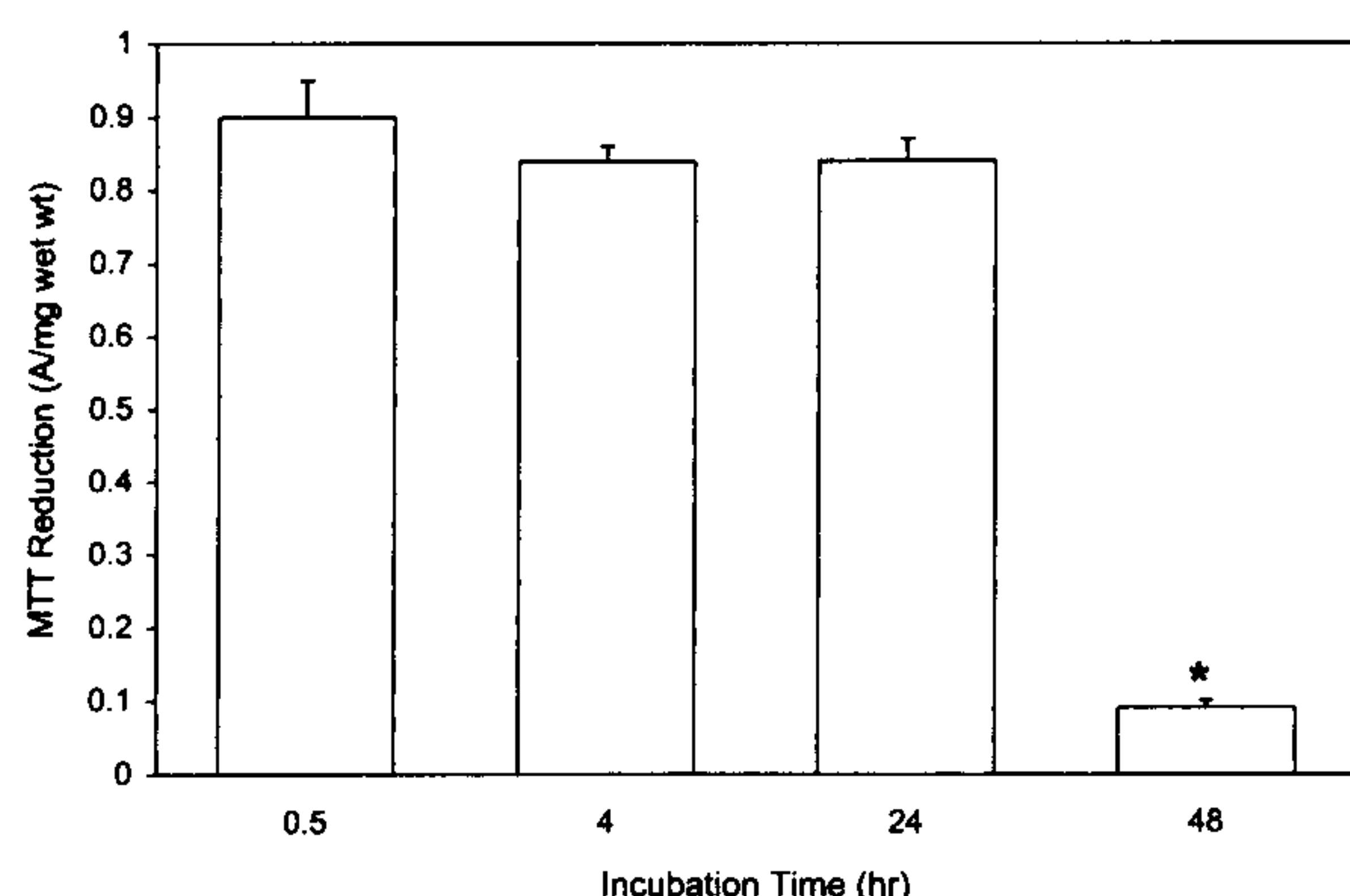


Figure 5 MTT reduction in right atrial slices incubated in aerobic conditions at 37 °C for different time periods

Data are expressed as means \pm S.E.M. of six experiments. * $P < 0.05$ versus the other time periods.

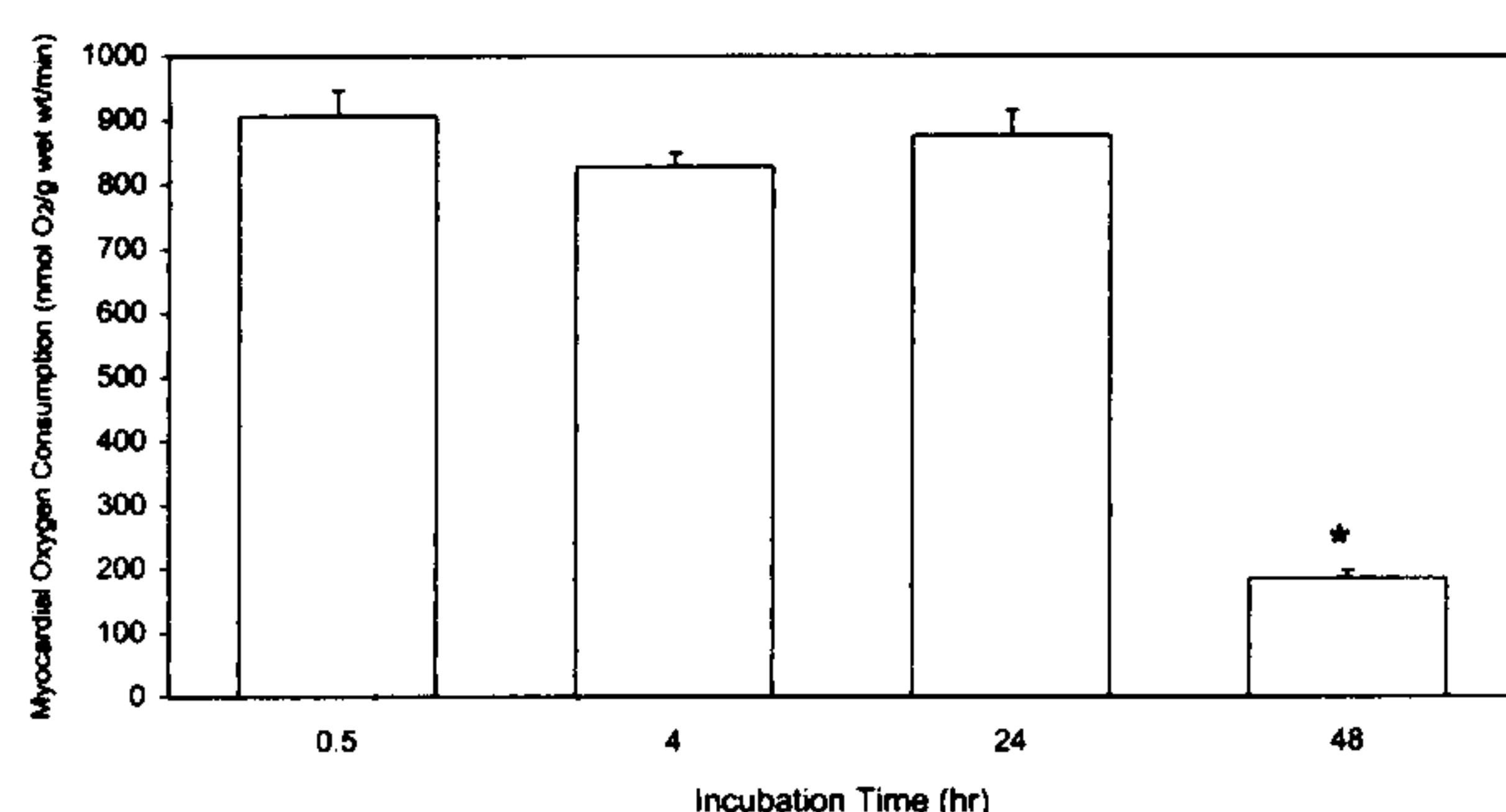


Figure 6 Oxygen consumption of right atrial slices in aerobic conditions at 37 °C for different time periods

Data are expressed as means \pm S.E.M. of six experiments. * $P < 0.05$ versus the other time periods.

Tissue adenosine nucleotide content

Table 1 shows the adenosine nucleotide content of the atrial slices at various time periods of aerobic incubation. The ATP, ADP and AMP content, ADP/ATP ratio and the total adenylate pool were similar in the slices aerobically incubated for 0.5, 4 and 24 h. However, in line with the previous results, there was a significant

decrease in all metabolites after 48 h of aerobic incubation.

Morphological assessment

Figure 7 (upper panels) shows representative high resolution transmission electron micrographs of atrial tissue: grade 1, normal (Figure 7a); grade 2, mild damage (Figure 7b); and grade 3, severe damage (Figure 7c). The morphological appearance of specimens aerobically perfused for 0.5, 4 and 24 h was similar; however, significant damage was observed in specimens aerobically perfused for 48 h. A semi-quantitative grading of the morphological examination is shown in Figure 7 (bottom panel).

Effect of the severity of ischaemia (Study 2)

LDH leakage

As shown in Figure 8 (top panel), ischaemia caused a time-related increase in LDH enzyme leakage from the atrial slices, which was exacerbated when the preparation was incubated for 24 h before being subjected to ischaemia.

Tissue viability

The level of tissue damage suggested by LDH leakage was mirrored by the MTT reduction results (Figure 8, middle panel). Ischaemia for 30 min significantly decreased the ability of the slices to reduce MTT to 75 % of the aerobic control mean values, with a further decrease when the period of ischaemia was extended to 60 or 120 min. Again, viability was even less when the tissue was incubated for 24 h prior to ischaemia.

Oxygen consumption

As shown in Figure 8 (bottom panel), oxygen consumption by the atrial slices was significantly decreased by 30 min of ischaemia, to almost 50 % of the aerobic control values. However, in contrast with the pattern observed for LDH leakage and MTT reduction, the increase in ischaemic time was not accompanied by a

Table 1 Adenosine nucleotide content (μ mol/g dry wt) of right atrial slices subjected to aerobic perfusion at 37 °C for various time periods

* $P < 0.05$ versus 0.5 h aerobic incubation group.

Period of aerobic incubation	n/group	Nucleotide				
		ATP	ADP	AMP	ADP/ATP ratio	Total adenylate pool
0.5 h	6	5.56 \pm 0.80	3.52 \pm 0.35	0.73 \pm 0.19	0.45 \pm 0.15	9.80 \pm 1.97
4 h	6	5.50 \pm 0.55	4.54 \pm 0.27	1.03 \pm 0.33	0.52 \pm 0.14	10.65 \pm 1.05
24 h	6	4.30 \pm 0.42	3.38 \pm 0.27	0.61 \pm 0.12	0.46 \pm 0.11	8.17 \pm 0.89
48 h	6	2.16 \pm 0.73*	1.07 \pm 0.15*	0.16 \pm 0.06*	0.23 \pm 0.04*	3.40 \pm 1.36*

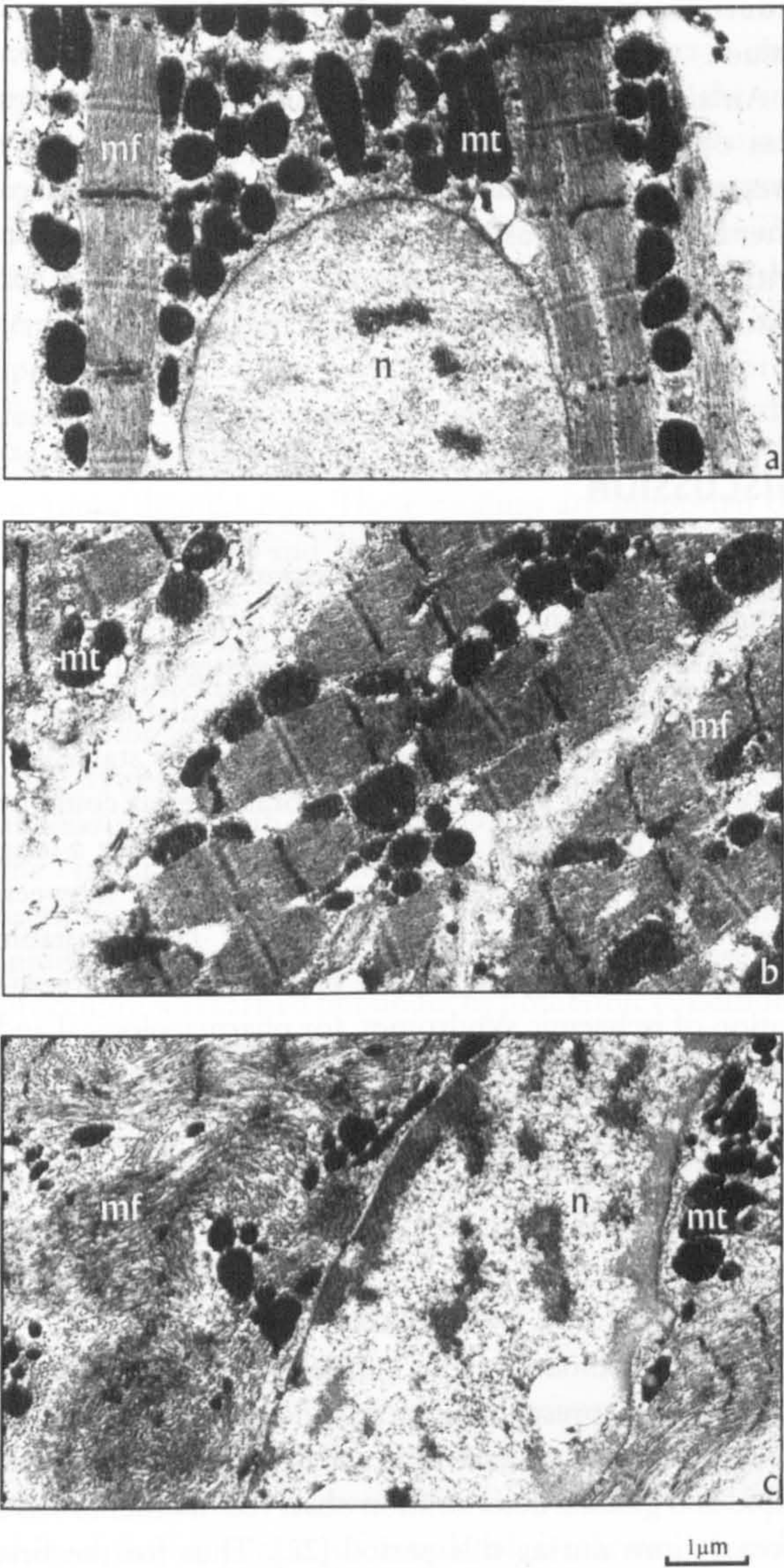


Figure 7 Representative transmission electron micrographs showing morphological changes in right atrial slices incubated in aerobic conditions for different time periods

Upper panel: (a) normal tissue, grade 1; (b) mild damage, grade 2; (c) severe damage, grade 3 (see text for details). n, nucleus; mf, myofibril; mt, mitochondria. Scale bar = 1 μm (applies to each panel). Lower panel; semiquantitative assessment of tissue damage after aerobic incubation for various time periods. Data are expressed as means ± S.E.M. of six experiments. **P* < 0.05 versus rest of the groups.

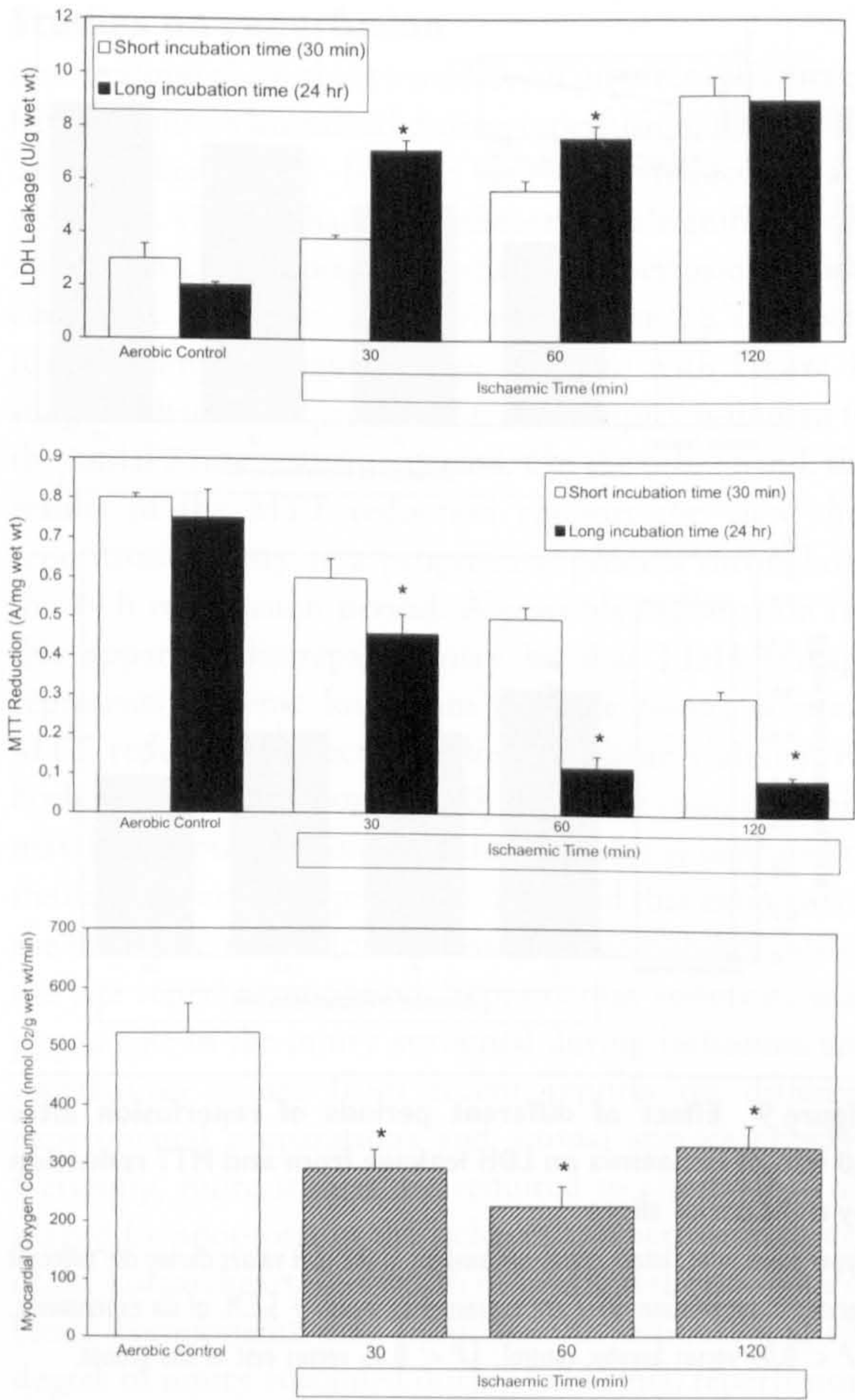


Figure 8 Effect of ischaemia/reperfusion of right atrial slices on LDH leakage, MTT reduction and oxygen consumption

Top panel: LDH leakage (units/g wet wt) from right atrial slices is shown after 30, 60 or 120 min of ischaemia followed by 120 min reperfusion for short or long incubation times. Data are expressed as means ± S.E.M. of six experiments. **P* < 0.05 versus the corresponding short incubation time group. Middle panel: MTT reduction by right atrial slices after 30, 60 or 120 min of ischaemia followed by 120 min reperfusion for long or short incubation times. Data are expressed as means ± S.E.M. of six experiments. **P* < 0.05 versus the corresponding short incubation time group. Bottom panel: oxygen consumption by right atrial slices after 30, 60 or 120 min of ischaemia followed by 120 min reperfusion for 30 min (short) incubation time. Data are expressed as means ± S.E.M. of six experiments. **P* < 0.05 versus aerobic control.

commensurate decrease in oxygen consumption and the mean values after 60 or 120 min of ischaemia were not significantly different from those observed after 30 min ischaemia.

Effect of the duration of reperfusion (Study 3)

In this study, the period of ischaemia was 60 min in all groups but the atrial muscle slices were reperfused for 2, 4, 12 or 24 h.

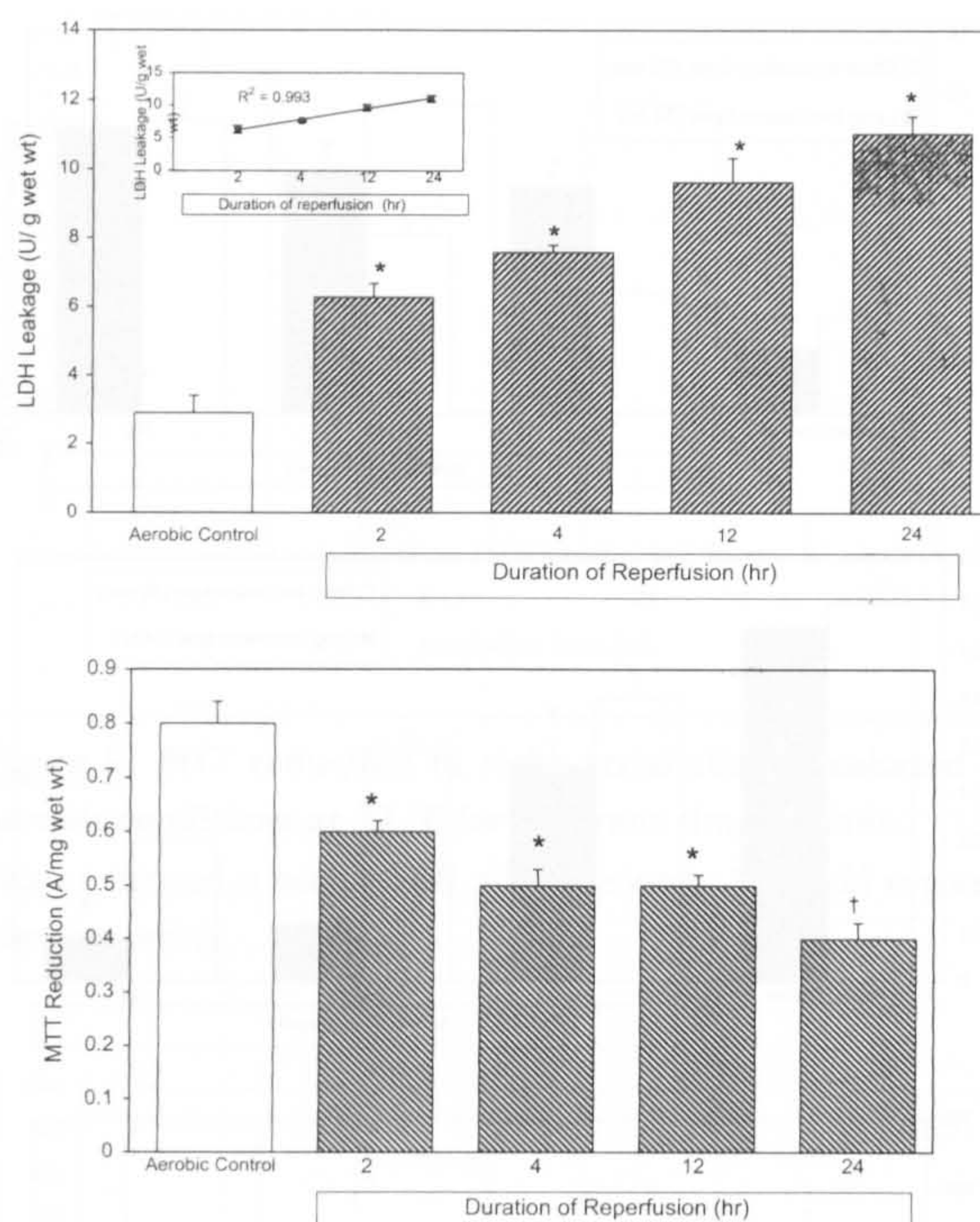


Figure 9 Effect of different periods of reperfusion after 60 min of ischaemia on LDH leakage from and MTT reduction by right atrial slices

Upper panel inset: linear regression analysis of the LDH values during the different periods of reperfusion. Data are expressed as means \pm S.E.M. of six experiments. * $P < 0.05$ versus aerobic control; † $P < 0.05$ versus rest of the groups.

LDH leakage

Figure 9 (upper panel) shows that LDH leakage gradually increased with the duration of reperfusion; however, as shown in Study 1, this increase may represent the overall accumulation of the enzyme rather than net release of LDH. Thus regression analysis (Figure 9, inset) revealed an LDH leakage profile similar to that seen in Study 1.

The LDH leakage profile was similar to controls when atrial slices were incubated in the presence of neutrophils for the first 12 h of reperfusion; however, after 24 h of reperfusion, LDH leakage was substantially increased when compared with the group without neutrophils (20.32 ± 0.64 versus 11.02 ± 0.5 units/g wet wt respectively; $P < 0.05$).

Tissue viability

As shown in Figure 9 (lower panel) and in Study 2, ischaemia for 60 min followed by 120 min reperfusion significantly decreased MTT reduction by atrial slices. Interestingly, and in contrast with the LDH leakage results, extension of the reperfusion period beyond 2 h revealed a delayed reperfusion injury with three possible phases: the first corresponding to the initial 2 h, the second observed between 4 and 12 h of reperfusion and the third manifesting after 24 h, where the mean MTT

reduction values decreased to 50 % of the aerobic control values.

An identical profile of MTT reduction by atrial slices was observed for the first 12 h of reperfusion in the presence of neutrophils, but a greater decrease was seen after 24 h of reperfusion than was observed in the group without neutrophils (0.10 ± 0.04 versus 0.40 ± 0.02 absorbance units/mg wet wt respectively; $P < 0.05$).

DISCUSSION

The present studies have demonstrated that the incubation of human atrial slices in a buffered medium is a useful preparation for the investigation of the mechanisms of injury underlying myocardial ischaemia and reperfusion in man. The preparation was stable and viable for at least 24 h, the severity of ischaemia could be readily evaluated, and the possibility of attaining a long reperfusion period allowed us to distinguish between early and delayed reperfusion injury. These notable features of the preparation are of value for the investigation of ischaemic syndromes, for pharmacological and toxicological studies and, potentially, for the evaluation of the effects of genetic manipulation of the human myocardium. A number of aspects of our studies warrant further discussion.

Stability of the preparation

The *in vitro* model of the human right atrium characterized in the present studies has the major advantage of being stable for at least 24 h and it did not exhibit the rapid and gradual deterioration observed in other *in vitro* preparations during this period [22]. Thus for the first 24 h of incubation, tissue viability was not decreased and myocardial oxygen consumption and adenosine nucleotides were maintained at baseline values. However, by 48 h of incubation the preparation had deteriorated significantly, so that viability of the tissue was decreased to only 10 %, oxygen consumption by the atrial slices had decreased to less than 20 % of the starting values, and the total adenosine nucleotides had decreased to 30 % of the starting values. Morphological examination of the tissue also supported this thesis.

The stability of the preparation is also reflected by the profile of LDH leakage, an index widely accepted as a marker of tissue damage [23]. In both instances, there was a small but gradual leakage of LDH into the incubation media that, at first sight, could be interpreted as an indication of ongoing tissue injury; however, although this cannot be ruled out completely, it is most probably due to a physiological transmembrane movement of proteins and enzymes [24,25]. The massive LDH leakage observed at 48 h of incubation supports the argument that significant tissue damage has occurred by this time.

A number of factors, including the temperature of the medium into which the atrial tissue is collected and processed, the slice thickness and the PO_2 of the incubation media may affect the viability of the preparation. In preliminary studies (data not shown), we observed that all these factors play an important role in maintaining the stability of the preparation. To ensure stability, the temperature of the medium for the collection of the specimen should be 4–10 °C; for recovery and processing, the media temperature should be 37 °C and the PO_2 25–30 kPa, and the thickness of the slices should be no more than 0.5 mm. These findings are supported by Paradise et al. [4] and Prasad and Callaghan [26], who reported that the thickness of the muscle preparation and the PO_2 of the media are the determinants of the oxygen diffusion rate and the viability of the preparation. The small, not statistically significant decrease in tissue viability seen after the initial 30 min equilibration period, and associated with increased LDH leakage, is possibly the result of mechanical injury sustained during sectioning of the tissue. It is unlikely that ischaemic injury is a contributor to this phenomenon, because the procedure was carried out under hypothermic conditions and the time spent in processing the tissue samples was less than 2 min, which is clearly insufficient time to induce myocardial damage. Furthermore, during this short period of sample processing, the muscles are not preconditioned when subjected to a long period of ischaemia [27].

Studies on ischaemia

To the best of our knowledge, the present study is the first to characterize the response of the human myocardium to various degrees of ischaemic insult. We have shown that a period of simulated ischaemia of only 30 min induced significant tissue injury, as measured by MTT reduction and LDH leakage, and that lengthening the ischaemic time to 2 h resulted in a loss of more than 75% viable tissue and substantial LDH leakage. It is worth noting that the decrease in oxygen consumption observed after 30 min of ischaemia was not further affected by 60 or 120 min of ischaemia. This suggests that, in fact, the remaining viable tissue augments oxygen consumption when compared with aerobically perfused tissue or with tissue subjected to shorter periods of ischaemia. This phenomenon of an oxygen-sparing effect after ischaemia has been described for several animal preparations [28–31], suggesting that it may not be a reliable index of tissue damage.

It is evident from these studies, not unexpectedly, that, although the atrial myocardial slices aerobically incubated for 24 h are still viable, they become more susceptible to ischaemic injury. This observation is of particular relevance when investigating the delayed effects of ischaemic syndromes, such as the second window of ischaemic preconditioning.

Studies on reperfusion

Our findings show that two different pictures can emerge from the injury sustained during reperfusion, depending on whether LDH leakage or MTT reduction are examined. On one hand, the absence of a significant net increase in LDH leakage over the 24 h reperfusion period compared with that seen during the first 2 h of reperfusion [compare Figure 9 (upper panel) with Figure 4] suggests that in our preparation tissue injury is limited to the initial 2 h reperfusion period. On the other hand, the results of the MTT reduction support the view that reperfusion injury is a progressive process throughout the 24 h reperfusion period. A possible explanation for this apparent discrepancy may be that LDH leakage represents enzyme loss from necrotic tissue, whereas MTT reduction reflects the loss of tissue viability via both necrosis and apoptosis. If this is the case, then one may be tempted to conclude that necrosis is confined to the early reperfusion period (≤ 2 h), and that apoptosis is the main mechanism for the loss of tissue viability during the late reperfusion period. Support that apoptosis may play a role in the injury sustained during ischaemia and reperfusion comes from recent reports on different experimental preparations and animal species [32–34]. Certainly, more studies are required to clarify the role played by apoptosis in the ischaemia/reperfusion injury of the human heart. It is worth noting that the presence of blood components may influence the response and the degree of injury sustained during ischaemia/reperfusion. Our studies have shown that neutrophils may exacerbate late reperfusion injury and therefore the presence or absence of blood components should be taken into account when designing a study and interpreting the results.

The present model may facilitate the investigation of the underlying mechanisms of injury during early and delayed reperfusion in the human myocardium. This model will also allow us to elucidate the true effects of therapeutical interventions. By looking exclusively at the early reperfusion period, it is possible that many interventions, thought to be beneficial in the past, in fact may have limited therapeutic value if their action is delaying rather than reducing myocardial injury.

Comparison with other preparations

The use of *in vivo* or *in vitro* experimental models each has advantages and disadvantages but both are regarded as necessary and complementary. Thus, for example, *in vivo* models are useful to study the physiological relevance and long-term effects of the processes under investigation; however, they are complex and the influence of factors such as blood elements, the neuro-endocrine system and even animal welfare and seasonal variations cannot be ruled out. By contrast, *in vitro* models are not exposed to the internal and external

effects of living animals, but they are limited by their short duration (usually a few hours) and stability.

Studies on myocardial ischaemia in the clinical setting are difficult to carry out and to interpret and frequently they are ethically unacceptable. To overcome these difficulties, an alternative is the use of the right atrial preparations, such as the one described in the present study. Certainly, our right atrial preparation is relatively easy to use, the tissue is readily available and inexpensive, since it is regarded as 'surgical waste' in open-heart procedures, and, more importantly, it provides meaningful information on the human myocardium. The use of atrial tissue for studies of ischaemia may also offer advantages over isolated and cultured myocytes, because these are more difficult to obtain, they do not retain the normal cell-to-cell contact, ischaemia is more difficult to accomplish and they are viable only for short periods of time [35,36]. A notable benefit derived from the prolonged stability of our preparation is that the effects of reperfusion can be studied for a period that extends beyond the first few hours, which is usually not possible with *in vitro* preparations.

Limitations of the preparation

The present study has several limitations which need to be mentioned. First, the preparation is superfused ('simulated ischaemia') as opposed to being arterially perfused. However, the preclusion of the vasculature as the natural pathway for the provision of substrate may also be advantageous in that the confounding effects of the vascular changes and collateral flow induced by ischaemia and reperfusion are separated.

In the present study, atrial tissue was used to characterize the effects of ischaemia and reperfusion in the human myocardium. However, atrial and ventricular myocardium possess characteristics of their own that may influence the susceptibility to ischaemia/reperfusion injury and consequently the results from one may not be applicable to the other. Thus, for example, the reported differences in the distribution of potassium channels [37,38], which contribute to the characteristic differences between atrial and ventricular action potentials, may determine a different response to ischaemia/reperfusion.

Although atrial tissue is generally stable and disease free, individual biological variations between patients may result in different susceptibility to ischaemia/reperfusion injury and this must be accounted for when interpreting results from any human study.

Conclusion

We have characterized a model of ischaemia and reperfusion in the human myocardium using right atrial tissue obtained from patients undergoing cardiac surgery. This is readily available, the preparation is inexpensive and stable for at least 24 h, which permits the study of the

early and delayed consequences of ischaemia and reperfusion. In addition, the extended stability of the model may be potentially useful for genetic manipulation to investigate the pathophysiological mechanisms underlying injury sustained during ischaemia and reperfusion, and to develop new therapeutic strategies to combat the undesirable effects.

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Evidence for mitochondrial K_{ATP} channels as effectors of human myocardial preconditioning

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Abstract

Background: Sublethal periods of ischemia preceding a prolonged interval of ischaemia protect the myocardium. This myocardial preconditioning (PC) appears to be effected by K_{ATP} channels. These channels occur both in the sarcolemma and the mitochondrial membrane. We investigated whether mitochondrial K_{ATP} channels are the end-effector of PC in the human myocardium. **Methods:** Right atrium specimens obtained from patients undergoing cardiac surgery were prepared and incubated in buffer solution at 37°C. After 30-min stabilisation, the muscles were made ischemic for 90 min and then reperfused for 120 min. The preparations were randomised into eight experimental groups ($n=6$ /group): (1) Aerobic control — incubated in oxygenated buffer for 210 min, (2) ischemia alone — 90 min ischemia followed by 120 min reperfusion, (3) PC — preconditioned with 5 min ischemia/5 min reperfusion, (4) Glibenclamide (10 μ M) in the incubation media for 10 min before PC, (5) 5-hydroxydecanoate (5-HD, Mito K_{ATP} blocker, 1 mM) in the incubation media for 10 min before PC, (6) HMR 1883 (Sarc K_{ATP} blocker, 10 μ M) in the incubation media for 10 min before PC, (7) Pinacidil (0.5 mM) in the incubation media for 10 min before ischemia, and (8) Diazoxide (Mito K_{ATP} opener, 0.1 mM) in the incubation media for 10 min before ischemia. Creatinine kinase leakage into the medium (CK, IU/g wet wt) and MTT reduction (OD/mg wet wt.), an index of cell viability, were assessed at the end of the experiment. **Results:** Ischemia alone resulted in a significant increase in CK leakage (8.01 ± 0.35) and decrease in MTT (0.15 ± 0.01) from the values seen in the aerobic control (2.24 ± 0.52 and 0.78 ± 0.10 respectively, $P < 0.05$ in both instances). PC fully reversed the effect of ischemia (CK = 2.97 ± 0.31 and MTT = 0.61 ± 0.05 ; $P < 0.05$ vs. ischemia alone group but $P = NS$ vs. aerobic control group). Both Glibenclamide and 5-HD abolished the protection induced by PC (CK = 6.23 ± 0.5 and 7.84 ± 0.64 ; MTT = 0.18 ± 0.03 and 0.13 ± 0.02 , respectively, $P < 0.05$ vs. PC), but interestingly, the protective effect of PC was not abolished by HMR 1883 (CK = 2.85 ± 0.24 and MTT = 0.58 ± 0.05 , $P = NS$ vs. PC). Diazoxide mimicked the protective effect of PC (CK = 3.56 ± 0.32 and MTT = 0.58 ± 0.02 , $P = NS$ vs. PC), however pinacidil exhibited less protection than PC (CK = 4.02 ± 0.16 and MTT = 0.30 ± 0.02 , $P < 0.05$ vs. PC). **Conclusions:** These studies demonstrate that K_{ATP} channels are the end-effectors of ischemic preconditioning and that protection is mediated by mitochondrial K_{ATP} channels in human right atrial myocardium. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Ion channels; K-ATP channel; Mitochondria; Preconditioning

1. Introduction

Brief interruptions of coronary blood flow paradoxically protect the heart from a subsequent prolonged ischemic insult. This inherent form of cardioprotection, known as 'ischemic preconditioning' was first described by Murry et al. [1] in 1986 in dogs. Subsequently, this phenomenon has

been shown to exist in all mammalian species studied to date [2–4] and there is also evidence that it exists in man [5]. The basis of such endogenous cardioprotection is not fully elucidated despite intensive investigation. The most favoured current hypothesis for preconditioning suggests that a variety of endogenous ligands such as adenosine, bradykinin, catecholamines and opioids activate receptors linked to protein kinase C to initiate an intracellular signal transduction pathway. PKC may activate a tyrosine kinase,

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which in turn activates MAP or JUN kinases leading finally to phosphorylation of an effector protein, which ultimately leads to protection [6].

The ATP-sensitive K^+ channel (K_{ATP}) has been suggested as a possible end-effector in the mechanism of ischemic preconditioning [7]. This evidence arises primarily from pharmacological studies [8–10]. K_{ATP} channels exist in the sarcolemma and in mitochondria; however, since the effects of sarcolemmal K_{ATP} channels [11,12] on excitability cannot alone account for the protection of preconditioning, it has been suggested that mitochondrial K_{ATP} channels may be the true effectors mediating the beneficial action of preconditioning. This hypothesis is supported by recent reports by Garlid et al. [13] showing that diazoxide opens reconstituted mitochondrial K_{ATP} channels at cardioprotective concentrations but is much less potent on sarcolemmal channels and by Liu et al. [14], who showed that diazoxide caused oxidation of mitochondrial flavoproteins in isolated cardiac myocytes, consistent with activation of mitochondrial K_{ATP} .

It has been reported that K_{ATP} channels also participate in the protection of preconditioning in man [15,16], however, to the best of our knowledge, there is no evidence as yet that mitochondrial K_{ATP} channels are involved in human preconditioning. The aim of the present study was to investigate whether the protection induced by preconditioning in the human myocardium is mediated by mitochondrial K_{ATP} channels. To achieve this, we used a model of simulated ischemia with right atrial trabeculae obtained from patients undergoing elective cardiac surgery and investigated the effects of the mito K_{ATP} channel opener diazoxide, the blockers 5-hydroxydecanoate (5-HD) and glibenclamide, and the sarcolemmal K_{ATP} blocker HMR 1883.

2. Materials and methods

2.1. Experimental preparation

Experiments were performed on trabeculae obtained from the right atrial appendage of patients undergoing elective open heart surgery. Patients were excluded if they had enlarged right and left atria, atrial arrhythmias, poor left ventricular function (ejection fractions <30%), right ventricular failure or were taking oral hypoglycaemic agents or opioid analgesia. Local ethical committee approval was obtained for the harvesting technique. The specimens were collected in oxygenated HEPES buffered solution at 4–5°C and immediately sectioned and prepared for study as described previously [17]. Briefly, the appendage was mounted onto a ground glass plate with the epicardial surface faced down and then sliced using surgical skin graft blades (Swann–Morton, UK) to a thickness of between 300 and 500 μ m. The specimen and the slide

were always kept moist throughout the procedure. The slices (weight 30–50 mg) were then transferred to conical flasks (25 ml Erlenmeyer flasks, Duran, Astell Scientific, Kent, UK) containing 10 ml of oxygenated buffered solution. Following this, the flasks were placed in a shaking water bath maintained at 37°C. The oxygenation of the incubation medium was maintained by a continuous flow of 95% O_2 /5% CO_2 gas mixture to obtain a pO_2 between 25 and 30 kPa and a pCO_2 between 6 and 6.5 kPa. The pO_2 , pCO_2 and pH in the incubation medium were monitored by intermittent analyses of the effluent by using an automated blood gas analyser (model 855 Blood Gas System, Chiron Diagnostics) and the pH was kept between 7.36 and 7.45. For the induction of simulated ischemia, the medium was bubbled with 95% N_2 /5% CO_2 (pH 6.80 to 7.00) and D-glucose removed and substituted with 2-D-deoxyglucose.

2.2. Solutions

The incubation medium was prepared daily with de-ionized distilled water and contained (in mmol/l): NaCl (118), KCl (4.8), $NaHCO_3$ (27.2), KH_2PO_4 (1), $MgCl_2$ (1.2), $CaCl_2$ (1.25), D-glucose (10) and HEPES (20). As mentioned above, during simulated ischemia, D-glucose was removed and substituted with 2-deoxy glucose (10 mmol/l) and this kept the osmolarity of the incubation medium constant. All reagents were obtained from Sigma Chemical. The K_{ATP} channel blockers, glibenclamide and HMR 1883 were made up to a concentration of 10 μ mol in 100 ml KH solution and sodium 5-hydroxy decanoic acid (5-HD), was made up to a concentration of 1 mmol in 500 ml KH solution. The K_{ATP} openers pinacidil and diazoxide, were dissolved in DMSO immediately before being added into experimental solutions. The final concentration of DMSO was <0.1%. Pinacidil and 5-HD were purchased from Research Biochemical Int. Glibenclamide and diazoxide were purchased from Sigma Chemicals and HMR 1883 was a gift from Hoechst Marion Roussel, Frankfurt.

2.3. Experimental protocols

Initially, a dose–response analysis was undertaken with various doses for each of the five different drugs used in the experimental protocols. The drugs were applied to the sections for 10 min after 30 min of stabilisation, followed by prolonged ischemia and reperfusion. Once the optimal dose for each drug was determined, the preparations were randomly allocated to various protocols ($n=6$ /group). In the groups subjected to simulated ischemia, this was induced for a period of 90 min and then it was followed by 120 min of reperfusion. The time course and the incubation times with the various agents are shown in Fig. 1.

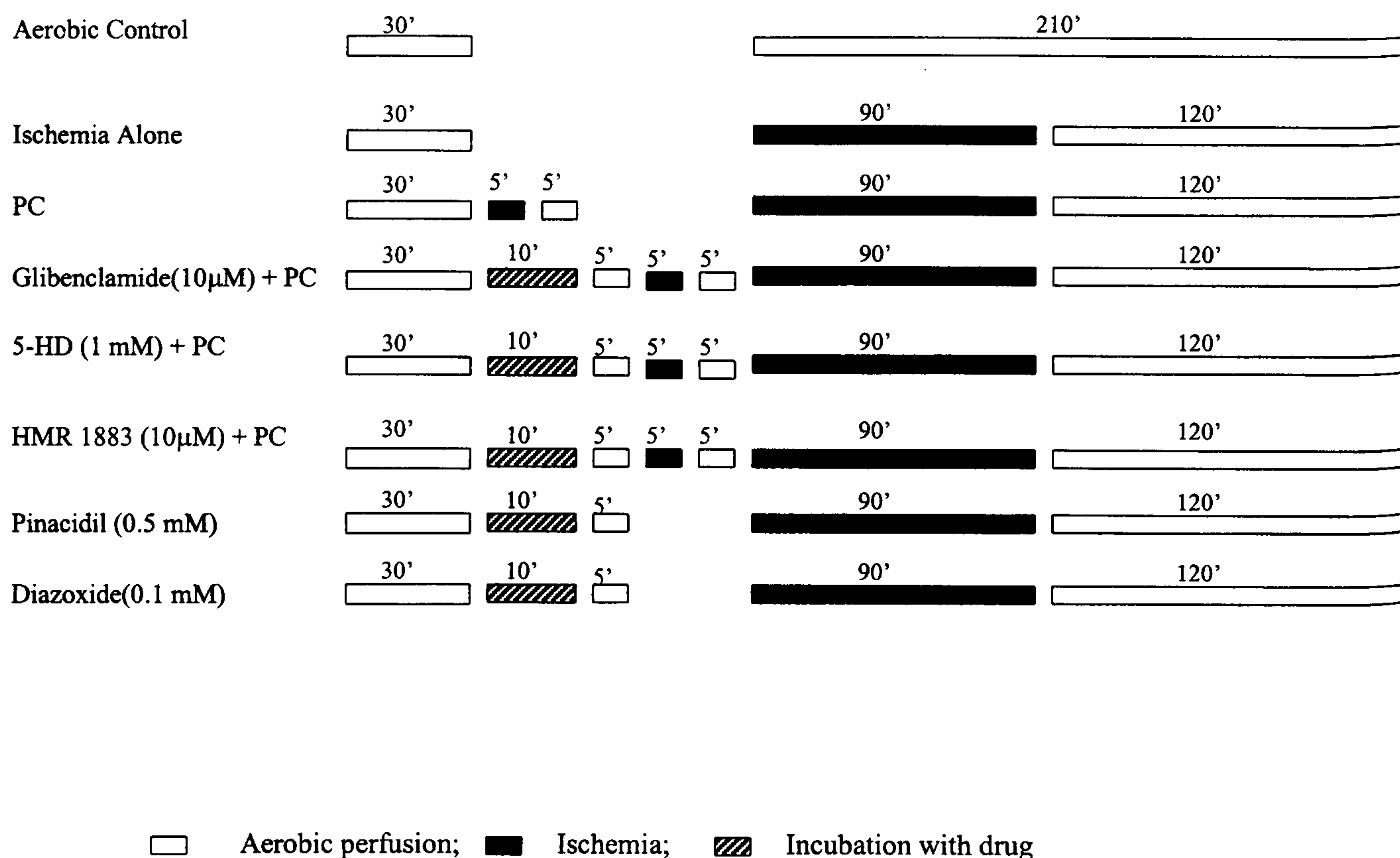


Fig. 1. Experimental protocols for Study 1. All groups were equilibrated for 30 min in aerobic conditions (37°C). Following this, the right atrial slices ($n=6/\text{group}$) were randomly assigned to the study groups. Aerobic control: slices were aerobically perfused for the entire experimental time course. Ischemia alone: the slices underwent 90-min simulated ischemia followed by 120-min reperfusion. PC: the slices were preconditioned with 5-min ischemia followed by 5-min reperfusion before undergoing 90-min ischemia. Glibenclamide: the slices were pretreated in 10 μM of the drug for 10 min before preconditioning. 5-HD: the slices were pretreated in 1 mM of the drug for 10 min before preconditioning. HMR 1883: the slices were pretreated in 10 μM of the drug for 10 min before preconditioning. Pinacidil: the slices were incubated in 0.5 mM of the drug for 10 min before exposure to 90-min ischemia. Diazoxide: the slices were incubated in 0.1 mM of the drug for 10 min before exposure to 90-min ischemia.

2.4. Assessment of tissue injury and viability

Tissue injury was determined by measuring the leakage of creatinine kinase (CK) into the incubation medium during the 120-min reperfusion period. This was assayed by a kinetic ultraviolet method based on the formation of NAD (Sigma Catalogue No. 1340-K) and the results expressed as IU/g wet wt.

Tissue viability was assessed by the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) to a blue formazan product at the end of the experimental time. The tissue was loaded into a Falcon conical tube (15 ml, Becton Dickinson Labware, New Jersey, USA) to which 2 ml of phosphate buffer solution (0.05 M) containing MTT (1.25 mg/ml, 3 mM at final concentration) was added and then incubated for 30 min at 37°C. Following this, the tissue was homogenized in 2 ml of dimethyl sulfoxide (Homogenizer Ultra-Turrax T25, dispersing tool G8, IKA-Labortechnik, Staufen, Germany) at 9500 rpm for 1 min. The homogenate was then centrifuged at 1000 g for 10 min and 0.2 ml of the supernatant was dispensed into a 98-well flat-bottom microtiter plate (Nunc Brand Products, Denmark). After

this, the absorbance was measured on a plate reader (Benchmark, Bio-Rad Laboratories, California, USA) at 550 nm and the results expressed as Optical Density (OD)/mg wet wt.

2.5. Statistical analysis

All data are presented as mean \pm S.E.M.. All values were compared by ANOVA with application of a post hoc Tukey's test. A P value of <0.05 was considered statistically significant.

3. Results

Samples were obtained from patients with stable ischemic heart disease or aortic valve disease undergoing elective heart surgery. All samples entering the studies completed the applied experimental protocol and were included in the analysis. A dose-response analysis (0 to 5 mM) based on both CK leakage and MTT reduction, revealed that diazoxide and pinacidil were found to be most protective at a dose of 100 μM and 0.5 mM

respectively. Diazoxide lost its protective effect at doses $\geq 500 \mu\text{M}$ and pinacidil at doses $\geq 1 \text{ mM}$. At the above optimal doses, the greatest degree of protection was afforded when the K_{ATP} openers was applied for 10 min before ischemia (pretreatment) followed by wash-out.

Glibenclamide abolished the protective effects of preconditioning at doses $\geq 10 \mu\text{M}$. A dose response analysis for 5-HD was performed between concentrations of 0 and 10 mM. The minimal effective concentration for 5-HD which abolished protection afforded by preconditioning was 1 mM. The dose–response analysis for HMR 1883 revealed that concentrations between the range 0 to 100 μM had no effect on the protection afforded by preconditioning. Again pretreatment with these drugs for 10 min before ischemia was the most effective protocol.

Fig. 2 shows that ischemia alone resulted in a significant increase in CK leakage and that preconditioning completely reversed the effect of ischemia so that CK leakage was similar to that seen in the aerobic control group. It also shows that glibenclamide (10 μM), which blocks K_{ATP} channels both in the sarcolemma and the mitochondrial inner membrane, partially blocked the beneficial effect of preconditioning on CK leakage. 5-HD is a K_{ATP} channel blocker which appears to show selectivity for $\text{mitoK}_{\text{ATP}}$ channels over sarcolemmal K_{ATP} .

Thus 5-HD has been shown to block K_{ATP} channels in isolated mitochondria [18] but did not affect sarcolemmal K_{ATP} currents activated by cromakalim [19]. In isolated

rabbit cardiac myocytes, Sato et al. [20] have recently shown that 5-HD inhibited oxidation in response to the opener pinacidil, but did not block the sarcolemmal K_{ATP} current activated by the same opener, consistent with 5-HD showing selectivity for blocking of $\text{mitoK}_{\text{ATP}}$ over sarcolemmal K_{ATP} . Fig. 2 also shows that 5-HD (1 mM) abolished the protective effect of PC on CK leakage in human myocardium. We also used the novel sulphonylthiourea HMR 1883, which is thought to have the reciprocal selectivity to 5-HD, preferentially blocking the sarcolemmal K_{ATP} channel [21,22]. HMR 1883 did not block the protective effect of preconditioning.

The results in Fig. 2 also show the effects of pretreatment with K_{ATP} channel openers in the absence of an ischemic preconditioning stimulus. Both the non-selective K_{ATP} channel opener, pinacidil and the selective opener of $\text{mitoK}_{\text{ATP}}$ channels, diazoxide were protective; with diazoxide reducing CK leakage to levels not significantly different from those obtained with PC itself and pinacidil exhibiting a less potent effect than PC. In this connection, it is worth noting that Garlid et al. [13] found that diazoxide was around 2000-fold more potent at opening $\text{mitoK}_{\text{ATP}}$ than cardiac sarcolemmal K_{ATP} channels.

Fig. 3 shows the results of MTT reduction. In essence, it reveals a mirror image of the results with CK leakage in the aerobic control, ischemia alone and preconditioning groups. Thus, ischemia alone caused a five-fold decrease in MTT reduction values to those seen in the aerobic control

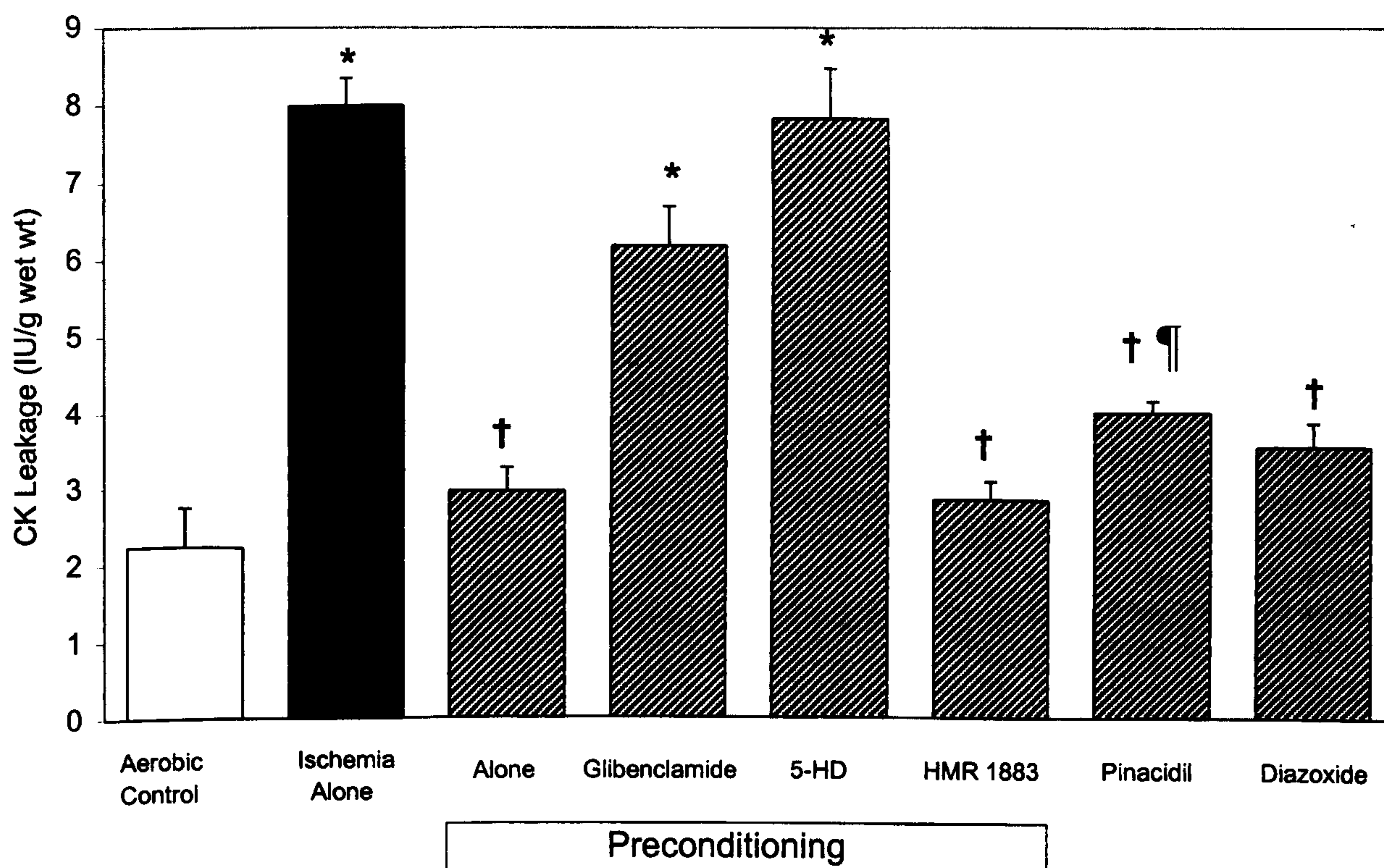


Fig. 2. Leakage of creatinine kinase (CK) into the media during the 120-min reperfusion period (last 120 min in the aerobic control period). Data are expressed as mean \pm S.E.M. of six experiments. * $P < 0.05$ vs. aerobic control group, † $P < 0.05$ vs. ischemia alone group and ‡ $P < 0.05$ vs. PC group.

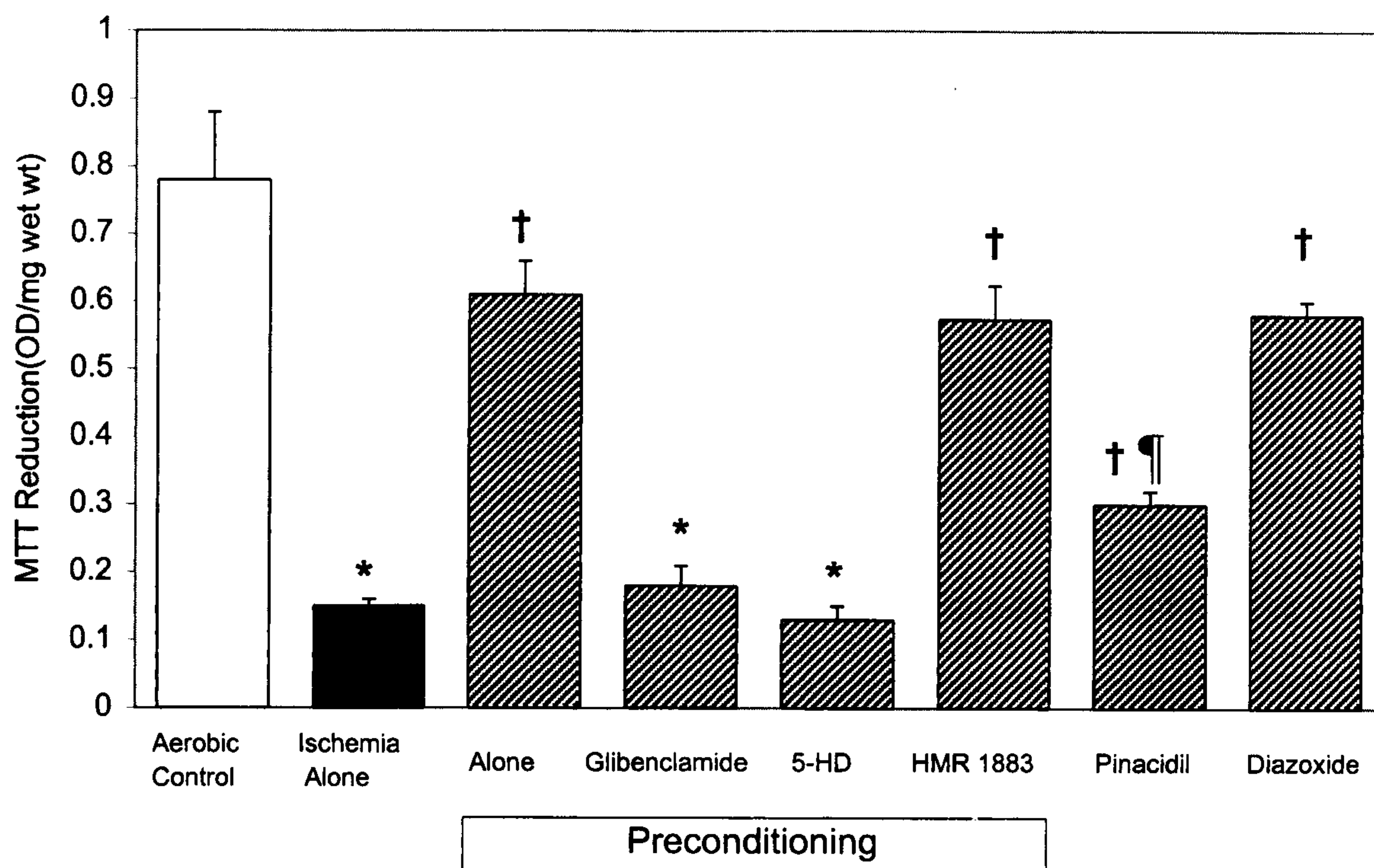


Fig. 3. Measurement of MTT reduction by the slices at the end of the reperfusion period. Data are expressed as mean \pm S.E.M. of six experiments. * $P < 0.05$ vs. aerobic control group, † $P < 0.05$ vs. ischemia alone group and ‡ $P < 0.05$ vs. PC group.

group, and this effect was significantly prevented by preconditioning. Both glibenclamide and 5-HD similarly abolished the protective effect of preconditioning. However, HMR 1883 decreased MTT reduction values to a similar degree as that seen in the PC group. Interestingly, diazoxide was as effective as preconditioning in that the MTT reduction values were similar in the two groups; however, pinacidil was less effective and the MTT reduction values in this group were only one half of those seen in the preconditioning and diazoxide groups.

4. Discussion

The involvement of K_{ATP} channels in preconditioning was first suggested by Gross and Auchampach [8] and Auchampach et al. [23] in the canine heart. These authors showed that K_{ATP} channels blockers, glibenclamide and 5-HD, blocked the protection induced by preconditioning and also revealed that aprikalim, a K_{ATP} channel opener, mimicked the cytoprotective effect of preconditioning in reducing infarct size. Subsequently, a plethora of pharmacological studies have shown that the opening of K_{ATP} channels contributes to the cardioprotection of preconditioning in a number of models and species including humans [24,25]. However, due to the lack of specific agents modulating the opening and closing of K_{ATP} channels, it was not possible at that time to ascertain whether the beneficial effect of preconditioning was mediated via mitochondrial or sarcolemmal K_{ATP} channels or

both. Cardiac sarcolemmal K_{ATP} channels open in hypoxia to cause shortening of the action potential. Functionally, the latter is thought to exert an energy sparing effect by reducing Ca^{2+} entry, and until recently sarcolemma K_{ATP} was assumed to play the major role in cardioprotection by preconditioning [26,27]. However, cardioprotection can occur under conditions where no action potential shortening can be detected, arguing against such a mechanism. More recently, Garlid et al. [13] showed that the K_{ATP} channel opener diazoxide was about 2000-fold more effective in opening mito K_{ATP} than sarcoplasmic K^+ channels in reconstituted bovine heart mitochondria, and that its cardioprotective potency in rat hearts correlated with its effectiveness on mitochondrial rather than sarcolemmal channels. Liu et al. [14] using isolated rabbit ventricular myocytes used flavoprotein oxidation as an index of mito K_{ATP} channel activity, and showed that diazoxide induced oxidation at concentrations that correlated well with its cardioprotective effects, but which did not activate sarcolemmal K_{ATP} channels. The present study provides evidence for the first time that mito K_{ATP} channels may be the effectors of ischemic preconditioning in the human myocardium.

K_{ATP} channels are composed of two proteins, an inwardly rectifying potassium channel (Kir 6.x) and a sulphonyl urea receptor (SUR) subunit. It has been suggested that SUR2A and Kir 6.2 are found in cardiac sarcolemma. Unfortunately, to date the mito K_{ATP} channel has not been cloned as yet but there are suggestions that the Kir 6.1 subunit [28] is involved in the mitochondrial membrane of

the rat skeletal muscle and liver. Certainly, mitochondrial and sarcolemmal K_{ATP} channels appear to exhibit minor differences in structure but the function of the mitochondrial K_{ATP} channels appear to be intimately involved in matrix volume control as opposed to electrical activity for sarcolemmal K_{ATP} channels. In this instance, opening of the mito K_{ATP} channel leads to membrane depolarization, matrix swelling, slowing of ATP synthesis, and accelerated respiration [29]. There is good evidence that diazoxide and 5-HD show good selectivity for mitochondrial over cardiac sarcolemmal K_{ATP} channels [13,20] and our present study confirms that preconditioning can be mimicked with diazoxide and abolished with both 5-HD and glibenclamide. These results are in close agreement with those of Garlid et al. [30] and further suggest that the mito K_{ATP} channels are the end-effectors of cardioprotection produced by ischemic preconditioning. To more clearly address this issue, we used a specific sarcolemmal K_{ATP} channel blocker, HMR 1883. The novel blocker HMR 1883 shows good selectivity for the cardiac sarcolemmal K_{ATP} channel over those of pancreatic beta cells and the vasculature [20]. Further, a brief report suggests that HMR 1883 did not block the protection induced by ischemic preconditioning in the rabbit [21]. Our results are in agreement with this, since we found that the protective effect of PC persisted in the presence of HMR 1883. Taken together with our findings with 5-HD and diazoxide, this suggests that HMR 1883 does not block mito K_{ATP} channels of human myocardium at the concentration used (10 μ M), and that blockade of the sarcolemmal channel does not abolish protection.

The mechanisms by which the opening of mito K_{ATP} channels exert the cardioprotective effect of preconditioning are not fully understood. Consequences of opening of the mito K_{ATP} channel include depolarization of the intramitochondrial membrane as K^+ enters leading to decreased calcium in-port and matrix swelling. The mechanism of how this ultimately stimulates mitochondrial respiration and consequently the cytoprotective effect is much less clear and warrants further research.

A potential limitation of our study was the use of atrial tissue as opposed to ventricular myocardium and therefore any extrapolation must be conducted with caution; however, Yellon and colleagues have suggested that preconditioning exerts identical protection in both tissues [16,31]. Undoubtedly, K_{ATP} channels are present in both atrium and ventricle [32], although their density in both tissues is unknown. Another possible limitation might be that right atrial appendages were obtained from patients subjected to various medical treatments (e.g. nitrates, β -blockers, calcium antagonists) and that in principle may have influenced ischemia/reperfusion injury and the protection induced by preconditioning. However; it should be emphasized, that all medication was stopped the day before surgery when specimens were taken for the study and that a significant effect of the medication was unlikely since all

preparations responded to ischemia/reperfusion with a similar degree of injury and preconditioning was protective in all instances when applied. It should be mentioned that the preparation used in this study was not electrically stimulated (i.e. non-beating) and therefore one should be cautious when extrapolating to the in vivo situation.

It should also be emphasized, that in common with previous studies in non-human hearts [13,14,20], our evidence for the involvement of mito K_{ATP} rather than sarcolemmal K_{ATP} channels depends strongly on the selectivity of the K_{ATP} channel openers and blockers used, in particular diazoxide, 5-HD, and HMR1883. In this context, the concentration of diazoxide that we used (100 μ M) has been reported to activate mito K_{ATP} but not sarcolemmal K_{ATP} in rabbit ventricular myocytes [14]. HMR 1883 should be an effective blocker of sarcolemmal K_{ATP} channels at 10 μ M, since its reported K_i for these channels is 0.8 μ M [21], though its effects on mito K_{ATP} have not been tested directly. 5-HD is an effective blocker of mito K_{ATP} provided that the channel is opened by a physiological or pharmacological opener [14,18], but its selectivity for mito K_{ATP} over sarcolemmal K_{ATP} merits further study. Notsu et al. [33] reported block of sarcolemmal K_{ATP} channels in guinea pig myocytes by 5-HD, though more recently Hu et al. [34] have argued that 5-HD at a concentration of 0.5 mM selectively blocks mito K_{ATP} without affecting sarcolemmal K_{ATP} channels of rabbit ventricular cells. Certainly, 5-HD at a concentration of 1 mM was an effective blocker of cardioprotection in our human model.

In conclusion, the present study provides strong evidence that mitochondrial rather than sarcolemmal K_{ATP} channels are effectors of ischemic preconditioning in the human myocardium. The finding has obvious important clinical implications, however, the mechanism by which the opening of mito K_{ATP} channels is protective is not fully understood and merits further investigation.

Acknowledgements

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Failure to Precondition Pathological Human Myocardium

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- OBJECTIVES** We investigated the effects of ischemic preconditioning (PC) on diabetic and failing human myocardium and the role of mitochondrial K_{ATP} channels on the response in these diseased tissues.
- BACKGROUND** There is conflicting evidence to suggest that PC is a healthy heart phenomenon.
- METHODS** Right atrial appendages were obtained from seven different groups of patients: nondiabetics, diet-controlled diabetics, noninsulin-dependent diabetics (NIDD) receiving K_{ATP} channel blockers, insulin-dependent diabetics (IDD), and patients with left ventricular ejection fraction (LVEF) >50%, LVEF between 30% and 50% and LVEF <30%. After stabilization, the muscle slices were randomized into five experimental groups (n = 6/group): 1) aerobic control—incubated in oxygenated buffer for 210 min, 2) ischemia alone—90 min ischemia followed by 120 min reoxygenation, 3) preconditioning by 5 min ischemia/5 min reoxygenation before 90 min ischemia/120 min reoxygenation, 4) diazoxide (Mito K_{ATP} opener, 0.1 mm)—for 10 min before the 90 min ischemia/120 min reoxygenation and 5) glibenclamide (10 μ m)—10 min exposure prior to PC (only in the diabetic patient groups). Creatine kinase leakage into the medium (CK, U/g wet wt) and MTT reduction (OD/mg wet wt), an index of cell viability, were assessed at the end of the experiment.
- RESULTS** Ischemia caused similar injury in both normal and diseased tissue. Preconditioning prevented the effects of ischemia in all groups except NIDD, IDD and poor cardiac function (<30%). In the diazoxide-treated groups, protection was mimicked in all groups except the NIDD and IDD groups. Interestingly, glibenclamide abolished protection in nondiabetic and diet-controlled NIDD groups and did not affect NIDD groups receiving K_{ATP} channel blockers or IDD groups.
- CONCLUSIONS** These results show that failure to precondition the diabetic heart is due to dysfunction of the mitochondrial K_{ATP} channels and that the mechanism of failure in the diabetic heart lies in elements of the signal transduction pathway different from the mitochondrial K_{ATP} channels. (J Am Coll Cardiol 2001;37:711–8) © 2001 by the American College of Cardiology

Ischemic preconditioning (PC) falls within a spectrum of adaptive responses to ischemia and represents the ability of the myocardium to adapt to sublethal ischemic stress in the short term so that it is more resistant to a subsequent, potentially injurious period of ischemia. Preconditioning consists of two phases of protection: an early or first window of protection (≤ 2 h) and a delayed or second window of protection (≥ 24 h). The underlying mechanism of PC has been extensively investigated; however, the basis of such cardioprotection is not fully elucidated. The most favored hypothesis for the first window of PC suggests that a variety of endogenous ligands such as adenosine, bradykinin, catecholamines and opioids activate receptors linked to protein kinase C (PKC) to initiate an intracellular signal transduction pathway. Protein kinase C may activate a tyrosine kinase, which in turn activates mitogen-activated protein or c-Jun-N-terminal kinases (JNK) kinases leading to phosphorylation of K_{ATP} channels (1). There is convincing evidence in the literature that K_{ATP} channels are involved in

the protection of ischemic PC in the human myocardium (2,3), although the exact place of these channels in the signal transduction pathway is still unclear (4–6). Recently we have shown that the mitochondrial, not the sarcolemmal, K_{ATP} channel is responsible for this powerful protective mechanism in the human myocardium (7).

Within the enormous amount of research describing the cellular basis of the PC response, relatively few studies relevant to coronary artery disease in humans have focused on the effect of PC in hearts with concurrent abnormalities. More importantly, even amongst those studies, the conclusions have been conflicting. Clinical studies identify a number of conditions that increase mortality from myocardial infarction; these include heart failure, diabetes, hypertension, aging and hypercholesterolemia (8,9). It is plausible that these conditions interfere with the biochemical pathways underlying the PC response.

Cardiovascular disease associated with diabetes mellitus is a major cause of death in patients with diabetes (10). In the vast majority of animal studies, diabetic hearts demonstrate a reduced tolerance to anoxia, hypoxia or ischemia (11–13), but studies that have investigated the effect of preconditioning on diabetic hearts have yielded confusing data. Tosaki et al. (14) have shown in the streptozotocin-induced diabetic rat heart that PC does not confer cardiac protection. Their results were opposed to those of Liu et al. (15), who had

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Abbreviations and Acronyms

CK	= creatine kinase
DCD	= diet-controlled diabetes
IDD	= insulin-dependent diabetes
K _{ATP}	= ATP-dependent potassium channels
LVEF	= left ventricular ejection fraction
MTT	= 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
NIDD	= noninsulin-dependent diabetes
PC	= preconditioning
PKC	= protein kinase C

earlier shown, also in the rat heart, that myocardial infarction is reduced in diabetes and that PC further increases the protection of these hearts. There are very few studies in human diabetic tissue. Cleveland et al. (16) used a functional isolated atrial trabeculae model and showed that PC did not confer any protection of the myocardium on patients taking long-term oral hypoglycemic agents. They hypothesized that long-term inhibition of K_{ATP} channels with these agents may be responsible for the excess cardiovascular mortality associated with diabetes.

Heart failure is common in all forms of heart disease. Mechanical dysfunction of the failing heart is due to many factors, including neurohormonal disturbance, accumulation of extracellular matrix, alteration of excitation-contraction coupling and maladaptation of myocardial energetics (17). Very few studies have investigated the effects of the PC response in the failing myocardium in light of alterations in the cellular metabolic and biochemical pathways associated with heart failure. Cleveland et al. (3) showed in isolated ventricular trabeculae from patients requiring heart transplantation that PC conferred protection. However, more recently Dekker et al. (18) have studied perfused papillary muscles from rabbits in which cardiac failure has been induced by a combination of pressure and volume overload-

ing. The end points used to assess responses to ischemia, namely the time to onset of a rise in the intracellular concentration of Ca²⁺ (cellular uncoupling) and of ischemic contracture, were delayed by PC in normal myocardium but were exaggerated by PC in the failing myocardium.

The aims of the present study were 1) to investigate the effects of PC on the diabetic and failing human myocardium, and 2) to investigate the role of mitochondrial K_{ATP} channels in the responses of these pathological conditions. These studies were carried out in an in vitro model of human right atrial myocardium of simulated ischemia and reoxygenation.

MATERIALS AND METHODS

Experimental preparation. The right atrial appendage of patients undergoing elective coronary artery surgery or aortic valve replacement was obtained. Patients were excluded if they had enlarged right atriums, atrial arrhythmias or right ventricular failure, or were taking opioid analgesia. Patient characteristics are detailed in Table 1. Local ethical committee approval was obtained for the harvesting technique, and the investigation conforms to the principles outlined in the Declaration of Helsinki. The specimens were collected in oxygenated HEPES buffered solution at 4° to 5°C and immediately sectioned and prepared for study. Briefly, the appendage was mounted onto a ground glass plate with the epicardial surface face down and was then sliced with surgical skin graft blades (Shwann-Morton, Sheffield, United Kingdom) to a thickness of between 300 and 500 μm. The specimen and the slide were kept moist throughout the procedure. Then 30 to 50 mg of muscle were transferred to conical flasks (25 ml Erlenmeyer flasks, Duran, Astell Scientific, Kent, United Kingdom) containing 10 ml of oxygenated buffered solution. Following this, the flasks were placed in a shaking water bath maintained at

Table 1. Patient Characteristics

	Nondiabetics	DCD	NIDD	IDD	LVEF >50%	LVEF = 30%-50%	LVEF <30%
Number	6	6	6	6	6	6	6
Age (yrs ± SEM)	68.1 ± 3.2	65.1 ± 1.9	59.4 ± 5.4	70.2 ± 2.8	58.7 ± 9.8	66.1 ± 2.8	69.4 ± 5.9
Gender (male/female)	4/2	3/3	5/1	4/2	3/3	2/4	1/5
Hypertension (%)	50	33	50	67	33	33	50
Previous MI (%)	0	33	33	33	0	0	83
Medication							
Glibenclamide			3				
Gliclazide			2				
Tolbutamide			1				
Nitrates	6	6	5	5	6	6	5
Ca ²⁺ antagonists	6	6	6	6	5	5	6
Beta-blockers	5	6	3	2	6	6	3
K _{ATP} opener	0	0	0	0	0	0	0
Mean LA size (cm ± SEM)	<4	<4	<4	<4	2.8 ± 0.4	3.2 ± 0.36	4.2 ± 0.19
Mean RA size (cm ± SEM)	<4	<4	<4	<4	<4	3.4 ± 0.14	3.7 ± 0.24
Mean PA pressures (mm Hg ± SEM)	ND	ND	ND	ND	ND	24.1 ± 5.4	36.8 ± 11.1
LVEF (% ± SEM)	58.1 ± 12.4	60.2 ± 5.4	55.8 ± 3.9	52.1 ± 6.1	63.2 ± 1.6	35.2 ± 3.1	23.1 ± 5.1

DCD = diet-controlled diabetics; IDD = insulin-dependent diabetics; LA = left atrial; LVEF = left ventricular ejection fraction; MI = myocardial infarction; ND = not determined; NIDD = noninsulin-dependent diabetics; PA = pulmonary artery; RA = right atrial.

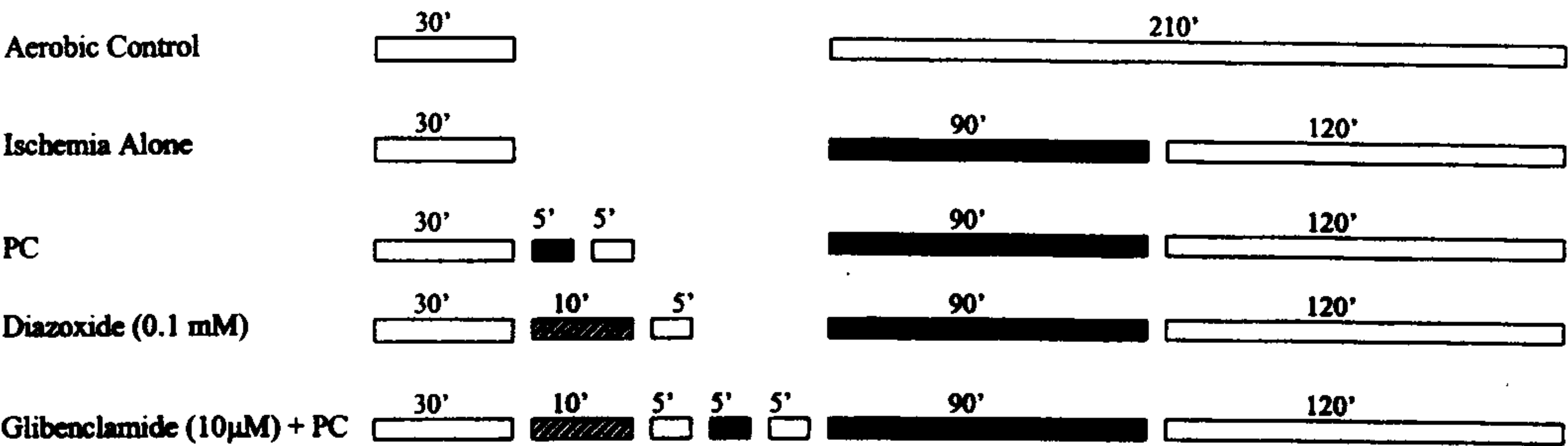


Figure 1. Experimental protocols for study 1. All groups were equilibrated for 30 min in aerobic conditions (37°C). Then the right atrial slices were randomly assigned to one of the study groups (n = 6/group): Aerobic Control = aerobically incubated for the entire experimental time. Ischemia Alone = subjected to 90 min simulated ischemia followed by 120 min reoxygenation. PC = preconditioning with 5 min ischemia followed by 5 min reoxygenation before undergoing 90 min ischemia. Diazoxide = incubation in 0.1 mm of the drug for 10 min before exposure to 90 min ischemia. Glibenclamide = pretreatment in 10 µm of the drug for 10 min before preconditioning was applied.

37°C. The oxygenation of the incubation medium was maintained by a continuous flow of 95% O₂/5% CO₂ gas mixture to obtain a pO₂ between 25 and 30 kPa and a pCO₂ between 6.0 and 6.5 kPa. The pO₂, pCO₂ and pH in the incubation medium were monitored by intermittent analyses of the effluent by using an automated blood gas analyzer (Model 855 Blood Gas System, Chiron Diagnostics, Halstead, Essex, United Kingdom) and the pH was kept between 7.36 and 7.45. For the induction of simulated ischemia, the medium was bubbled with 95% N₂/5% CO₂ (pH 6.8 to 7.0) and D-glucose was removed and the pO₂ was maintained at 0 kPa. In this preparation, tissue injury and viability were assessed but the atrium was not paced and force development was not measured.

Solutions. The incubation medium was prepared daily with deionized distilled water and contained: NaCl (118 mmol/l), KCl (4.8 mmol/l), NaHCO₃ (27.2 mmol/l), KH₂PO₄ (1 mmol/l), MgCl₂ (1.2 mmol/l), CaCl₂ (1.25 mmol/l), D-glucose (10 mmol/l) and HEPES (20 mmol/l). During simulated ischemia, the substrate D-glucose was removed and replaced with 2-deoxy glucose (10 mmol/l) to maintain a constant osmolarity. The K_{ATP} channel blocker glibenclamide was made up to a concentration of 10 µmol in 100 ml K-H solution and the K_{ATP} opener diazoxide was dissolved in dimethylsulfoxide immediately before being added into the experimental solutions. All reagents were obtained from Sigma Chemical Co. (Poole, Dorset, United Kingdom).

Experimental protocols. After the atrium was sectioned, the preparations were allowed to stabilize for 30 min and then randomly allocated to various protocols. In all studies simulated ischemia was induced for 90 min followed by 120 min of reoxygenation. The drugs were applied to the sections for 10 min after the initial 30 min of stabilization and then removed before ischemia. Two studies were performed:

STUDY 1. To investigate whether diabetes influences the protective effect of PC, atrial specimens were collected from four groups of patients: nondiabetics, diet-controlled diabetics (DCD), noninsulin-dependent diabetics (NIDD) on long-term oral sulphonylureas and insulin-dependent diabetics (IDD). Preparations from each group were then randomly allocated to various protocols (n = 6/group) shown in Figure 1.

STUDY 2. To investigate the effect of cardiac function on the protection induced by PC, atrial specimens were collected from three groups of patients: with normal left ventricular ejection fraction (LVEF >50%); with moderately impaired function (LVEF 30% to 50%) and with severely impaired function (LVEF <30%). Preparations from each group were then randomly allocated to various protocols (n = 6/group) shown in Figure 2.

In both these studies, PC was induced by a single cycle of 5 min ischemia/5 min reoxygenation, a protocol that we have demonstrated provides maximal protection in this

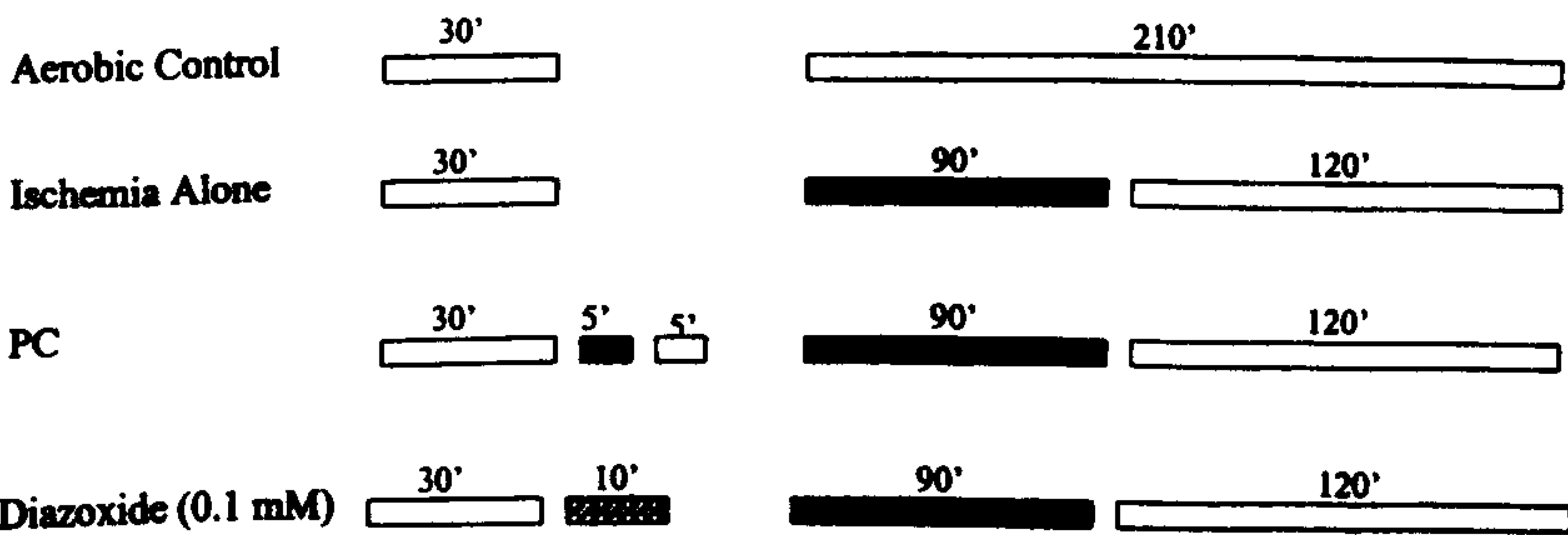


Figure 2. Experimental protocols for study 2. All groups were equilibrated for 30 min in aerobic conditions (37°C). Following this, the right atrial slices were randomly assigned to one of the following study groups (n = 6/group): Aerobic Control = aerobically incubated for the entire experimental time course. Ischemia Alone = subjected to 90 min simulated ischemia followed by 120 min reoxygenation. PC = preconditioning with 5 min ischemia followed by 5 min reoxygenation before undergoing 90 min ischemia. Diazoxide = incubation in 0.1 mM of the drug for 10 min before exposure to 90 min ischemia.

model (19). Preliminary studies (data not shown) had demonstrated that increasing the number of cycles of 5 min ischemia/5 min reoxygenation from one to three does not elicit protection beyond that obtained with a single cycle. In the groups receiving diazoxide and glibenclamide, the drugs were used at a concentration of 0.1 mM and 10 μ M, respectively. We have shown that these drug concentrations are the minimum effective concentration required to elicit a response and to block PC (7).

Assessment of tissue injury and viability. At the end of each experimental protocol, tissue injury was determined by measuring the leakage of creatine kinase (CK) into the incubation medium and tissue viability by the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) to blue formazan product.

CK LEAKAGE. The activity of CK leakage into the medium during the reperfusion period was assayed by a kinetic ultraviolet method based on the formation of NAD (Sigma Catalogue No. 1340-K) at 37°C and the results expressed as U/g wet wt.

MTT REDUCTION. At the end of the experimental time, the tissue was loaded into a Falcon conical tube (15 ml, Becton Dickinson Labware, Cowley, United Kingdom) and 2 ml of phosphate buffer solution (0.05 M) containing MTT (1.25 mg/ml, 3 mM at final concentration) was added, incubated for 30 min at 37°C and then homogenized in 2 ml dimethyl sulfoxide (Homogenizer Ultra-Turrax T25, dispersing tool G8, IKA-Labortechnik, Staufen, Germany) at 9,500 rpm for 1 min. The homogenate was then centrifuged at 1,000 g for 10 min and 0.2 ml of the supernatant was dispensed into a 98-well flat-bottom microtiter plate (Nunc Brand Products, Roskilde, Denmark). After this, the absorbance was measured on a plate reader (Benchmark, Bio-Rad Laboratories, Hercules, California) at 550 nm and the results expressed as OD/mg wet wt.

Statistical analysis. All data are presented as mean \pm SEM. All values were compared by two-way analysis of variance with application of a post hoc Bonferroni's test. Statistical significance was assumed at the $p < 0.05$ level.

RESULTS

All samples entering the studies completed the applied experimental protocol and were included in the analysis.

Effect of PC in diabetes (Study 1). Figure 3A shows that CK leakage was increased to a similar degree by ischemia alone in all study groups. It also shows that PC completely reversed the effect of ischemia in the nondiabetics and DCD groups, so that mean CK leakage values were similar to those in the aerobic control group, but did not have a protective effect in NIDD and IDDM groups. Interestingly, diazoxide, a specific mito K_{ATP} channel opener, mimicked the protection afforded by PC in nondiabetics and DCD groups but failed to protect the NIDD and IDDM groups. As expected, glibenclamide, a nonspecific K_{ATP} channel

blocker, abolished the protection of PC in nondiabetics and in DCD and had no effect in the NIDD and IDDM groups. The MTT results shown in Figure 3B are a mirror image of the CK leakage results with PC and diazoxide exhibiting a similar protection in the nondiabetic and DCD groups and no protection in the NIDD and IDDM groups. Overall, the results suggest that changes in mito K_{ATP} channels in patients with NIDD and IDDM are the most likely cause for the failure to precondition the myocardium. However, the possibility that alterations in signal transduction pathways could also contribute cannot be completely excluded.

Effect of PC in patients with contractile cardiac dysfunction (Study 2). Figure 4A also shows that ischemia alone resulted in a significant increase in CK leakage similar in all study groups regardless of the severity of cardiac dysfunction. Importantly, PC was significantly protective; decrease of CK leakage was observed in the groups with LVEF $\geq 30\%$ but not in the group with LVEF $< 30\%$. As observed in study 1, diazoxide mimicked the effect of PC on CK leakage in the groups with LVEF $\geq 30\%$. However, in contrast with the absence of protection by PC in the group with LVEF $< 30\%$, diazoxide reduced CK leakage in the LVEF $< 30\%$ group to an extent similar to that seen in LVEF $\geq 30\%$ groups. The results on MTT reduction shown in Figure 4B were again a mirror image of the CK leakage, suggesting that the cause for the absence of protection by PC in the LVEF $< 30\%$ group is probably due to alterations in some element(s) of the signal transduction pathway that exclude the mito K_{ATP} channels.

It should be noted that in these two studies the number of patients in each study group was too small for analysis of the possible influence of different clinical characteristics on the effects of ischemia and preconditioning.

DISCUSSION

The major findings of this study are that myocardium from patients with diabetes and poor cardiac function is not protected from ischemic PC although the injury induced by ischemia/reperfusion is not exacerbated in these conditions. Furthermore, the demonstration that activation of mito K_{ATP} channels in myocardium from hearts with poor cardiac function mimics the protection induced by preconditioning in the myocardium from hearts with good contractility, but not from patients with diabetes, suggests that the failure to precondition in these conditions is due to alterations of different elements of the signal transduction pathway. Cardiovascular mortality is increased in patients with heart failure (20,21) and diabetes (10) and therefore any myocardial adaptation to ischemia would decrease mortality associated with these diseases. The results of our studies may have important clinical implications and a number of points warrant further discussion.

Preconditioning and diabetes. Insulin regulates the balance of energy substrates available to the heart and also regulates metabolism and myocardial perfusion via actions

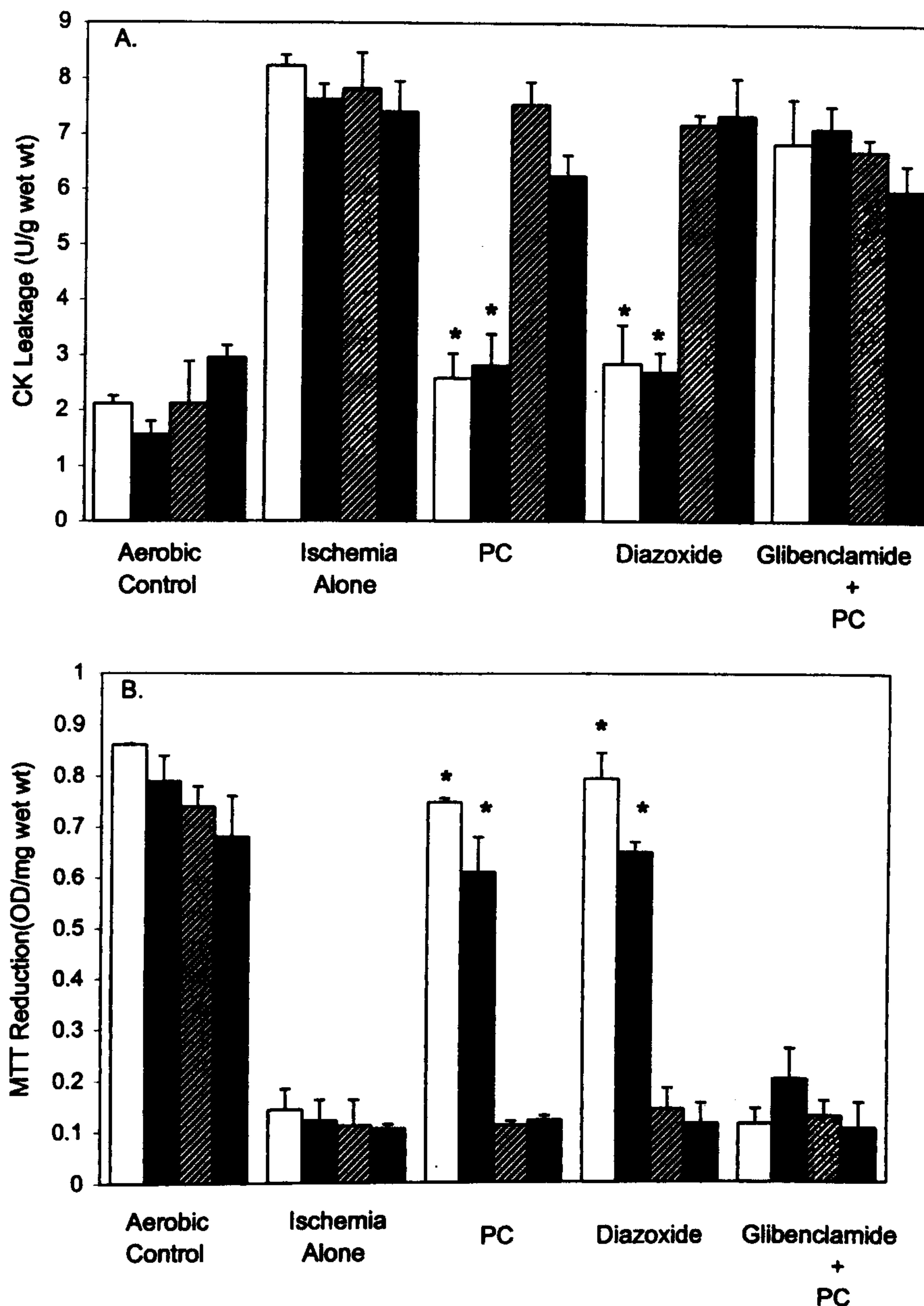


Figure 3. Creatine kinase (CK) leakage (A) during the 120 min reoxygenation period (last 120 min in the aerobic control group) and MTT reduction (B) at the end of the reoxygenation period in study 1 (for protocol see Fig. 1). Data are expressed as mean \pm standard error of mean of six experiments. * $p < 0.05$ versus corresponding group subjected to ischemia alone. White bar = nondiabetics; black bar = diet-controlled diabetes; striped bar = noninsulin-dependent diabetes; hatched bar = insulin-dependent diabetes; PC = preconditioning.

on various intracellular regulatory proteins and messenger systems (22). It is therefore conceivable that diabetes may affect ischemic injury. However, our results show that the diabetic human myocardium is not more sensitive to lethal ischemic injury than the nondiabetic myocardium under the conditions of the present study. Because epidemiological studies have clearly shown that patients with either IDD or NIDD are more prone to develop myocardial infarction and postinfarction complications (10,23), it may be argued that the cause of cardiac complications in diabetics is not the tolerance of the heart to ischemia. The literature is inconsistent with respect to how susceptible the hearts of diabetic animals are to injury from ischemia/reperfusion, and whereas some studies have observed a greater susceptibility

to ischemia/reperfusion injury (15), others have reported no significant effect (24,25). The divergent results may be explained by experimental differences, but if our results in the human atrial myocardium can be confirmed in ventricular myocardium then our attention to reduce cardiac complications in diabetes should be centered in the context of blood components and the vasculature rather than in the own myocardium.

Our studies have demonstrated that PC affords protection of the myocardium from patients with DCD but that this is lost when patients are on long-term hypoglycemics or become insulin dependent. These results are in agreement with those reported by Cleveland et al. (16). These investigators showed that myocardium from patients with dia-

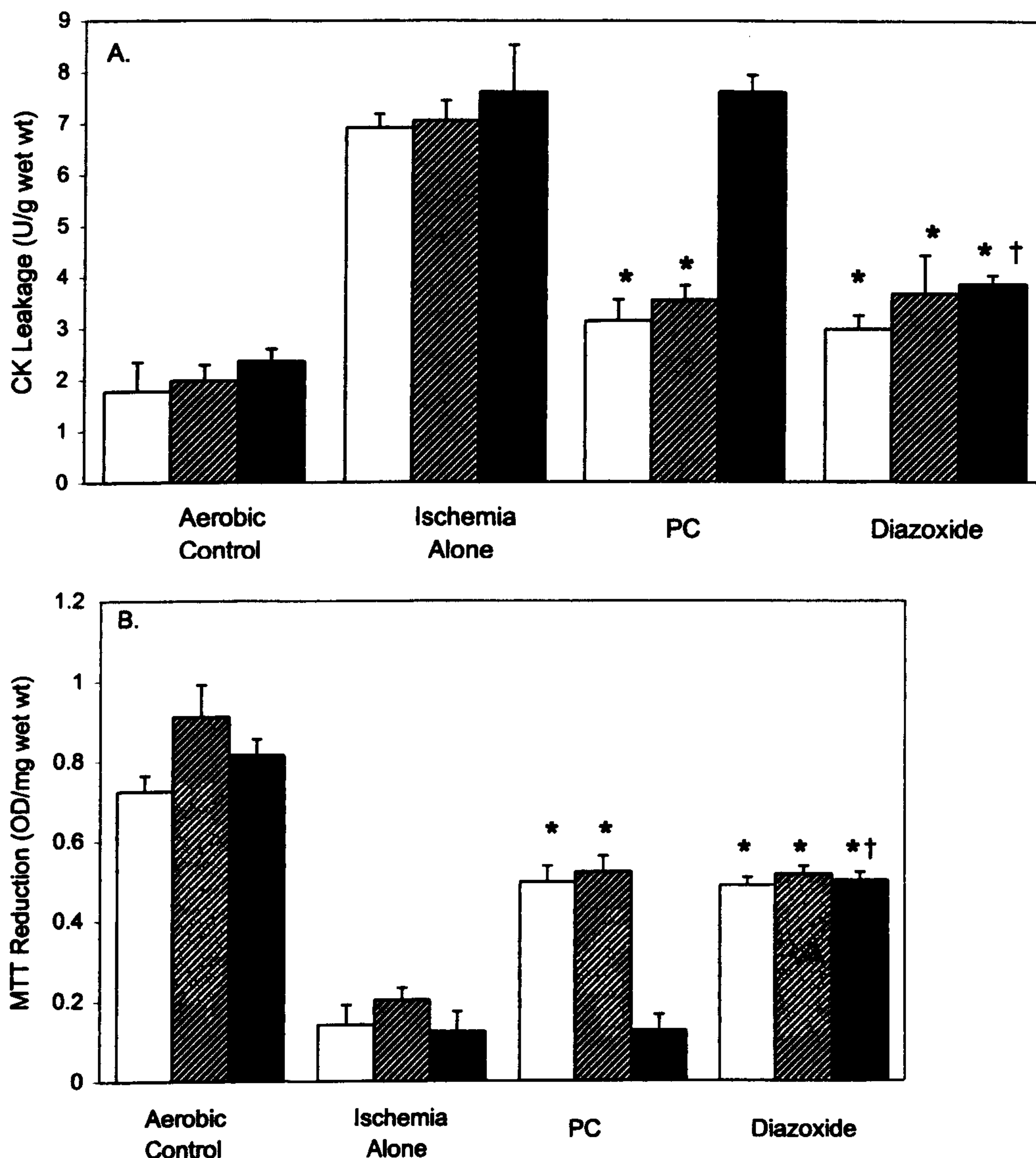


Figure 4. Creatine kinase (CK) leakage (A) during the 120 min reoxygenation period (last 120 min in the aerobic control group) and MTT reduction (B) at the end of the reoxygenation period in study 2 (for protocol see Fig. 2). Data are expressed as mean \pm standard error of mean of six experiments. * $p < 0.05$ versus corresponding group subjected to ischemia alone; † $p < 0.05$ versus corresponding group subjected to preconditioning (PC). White bar: left ventricular ejection fraction (LVEF) $>50\%$; striped bar: LVEF = 30% to 50%; black bar: LVEF $<30\%$.

betes on long-term K_{ATP} blockers also does not precondition in a model of myocardial stunning. However, the same authors also suggested that the myocardium from IDD subjects can be preconditioned, although the protective effect obtained was not to the same extent as that in nondiabetics. Again, these results in the human tissue contrast with the reported experimental results. Liu and co-workers (15) were the first to examine preconditioning in experimental streptozotocin-induced diabetic rats and found that diabetic hearts were more resistant to infarction than normal control hearts and that preconditioning conferred additional protection under in vivo experimental conditions. However, a subsequent study examined the effect of streptozotocin-induced diabetes on the response to ischemia/reperfusion and preconditioning in the isolated rat heart at different stages following its induction (26) and showed that the diabetic heart is more resistant to ischemia/

reperfusion in the early phase of the diabetes (two weeks after onset); but that this protection is lost by four to six weeks. In addition, they observed that the diabetic heart can be preconditioned after six to eight weeks. It must be mentioned that the disparity in the results observed between the two studies could be explained, at least in part, by the differences in the models used. Thus, for example, in the study by Liu and colleagues the model of diabetes is unique in that diabetes was induced by streptozotocin in the neonate and then animals were allowed to grow into adulthood before any intervention was carried out.

These studies do not provide an explanation for such a disparity of results; however, they suggest that abnormalities of mito K_{ATP} channels may be responsible for the failure to precondition the myocardium of diabetics. Several investigators (27,28), including ourselves (7), have previously demonstrated that mito K_{ATP} channels are involved in the

protection of ischemic PC. The findings by Smith et al. (29) that K_{ATP} channels are altered, with a greater outward single-channel current in the ventricular myocardium of diabetic rats, further support this thesis. Clearly, further research is needed to fully elucidate how the alteration of this channel contributes to the failure to precondition the myocardium of diabetics.

Preconditioning and the failing heart. Left ventricular hypertrophy and LV chamber dilation are among the compensatory mechanisms of the failing heart. There is experimental evidence that the hypertrophied myocardium is at greater risk from ischemia/reperfusion injury, and it is generally believed that the failing heart is less tolerant to such injury. Our results, however, have shown for the first time that the effects of ischemia/reoxygenation are similar in the failing and nonfailing myocardium. In addition, we have also demonstrated for the first time that the myocardium from hearts exhibiting a LVEF <30% cannot be preconditioned. Our results contrast with those of Cleveland et al. (3) showing that the isolated ventricular trabeculae obtained from patients undergoing cardiac transplantation can be preconditioned. Again, the explanation for this difference cannot be found in the reported experimental studies, and whereas some investigators have shown protection of the failing heart by PC (3), others have observed no effect (30) or even further tissue damage (18). The diversity of results is not entirely surprising because of the lack of uniformity of experimental design and the degree of heart failure we have shown in this study.

The protection observed with diazoxide in the myocardium from hearts with LVEF <30% was commensurate with the protection induced by PC in the myocardium from nonfailing hearts. This supports the thesis that the failure to precondition the failing human heart is not due to an alteration in the response of the mitochondrial K_{ATP} channel but is caused by abnormalities in other elements of the preconditioning signaling pathway. Considerable evidence indicates that PKC is intimately involved in ischemic PC (31), and the failure to precondition the failing heart may be due to the chronic activation of PKC observed in this condition (32). There are several PKC isoforms, some of which have been involved with PC, and in future studies the type of isoforms and their expression in the myocardium of the failing heart should be investigated. Indeed, if specific PKC isoforms are proved to be responsible for the failure to precondition, then their manipulation could become a therapeutic intervention to reduce myocardial injury in ischemia/reperfusion of the failing heart.

Possible limitations of the study and clinical implications. A potential limitation of our study was the use of atrial tissue as opposed to ventricular myocardium, and therefore any extrapolation must be conducted with caution; however, Yellon and colleagues have suggested that PC exerts identical protection in atrial and ventricular myocardium (2). The present study also used atrial tissue to characterize the effects of ischemia and reperfusion in the

failing and diabetic human myocardium. However, atrial and ventricular myocardium possess characteristics of their own that may influence susceptibility to ischemia/reperfusion injury, and as a consequence results from one may not be applicable to the other. Thus the reported differences in the distribution of potassium channels (33,34), which contribute to the characteristic differences between atrial and ventricular action potentials, may determine a different response to ischemia/reperfusion. Undoubtedly, K_{ATP} channels are present in both atrium and ventricle (33), although their density in both tissues is unknown. It must also be mentioned that the preparation is superfused ("simulated ischemia") as opposed to being arterially perfused, and simulated ischemia is achieved by removal of oxygen and blocking glycolytic ATP production with 2-deoxyglucose. This results in metabolic conditions within the myocardium that may be different from those that occur in the myocardium during clinical ischemia.

Another limitation might be that right atrial appendages were obtained from patients subjected to various medical treatments (e.g., nitrates, beta-blockers, calcium antagonists), which may have influenced ischemia/reperfusion injury and the protection induced by PC. However, it should be emphasized that all medication was stopped the day before surgery when specimens were taken for the study, and that significant effect of the medication was unlikely because all preparations responded to ischemia/reperfusion with a similar degree of injury. The preparation used in this study was not electrically stimulated (i.e., nonbeating) and therefore one should be cautious when extrapolating to the *in vivo* situation.

Conclusions. Preconditioning is a potent protective intervention whose use has been advocated in clinical situations such as angioplasty and cardiac surgery. The results of our studies have obvious clinical implications in that PC cannot be beneficial to patients with NIDD or IDD and those with cardiac failure. The results also show that in the failing heart a degree of protection similar to that seen with PC can be obtained by the administration of a selective opener of mito K_{ATP} channels, an intervention that is not effective in diabetics.

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