The Role of TNF α in Cardiac Protection

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Declaration

I, Naushaad Suleman, hereby declare that the work on which this theses is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole work nor any part of it has been, or is being, or is to be submitted for another degree in this or any other university.

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To steal from one is plagiarism, to steal from many research

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Abstract

Introduction - Brief periods of sub-lethal ischemia that precede sustained ischemia results in a marked limitation of infarct size and is described as ischemic preconditioning. Understanding this innate biologic phenomenon could enable us to develop newer strategies to enhance myocardial tolerance against ischemic injury. Until recently, the role of the innate immune system in preconditioning has not been extensively explored. Interestingly, the pleiotropic cytokine TNF α which is known to be an apical regulator of innate immunity has been shown to be modestly elevated in the serum of rabbits following ischemic preconditioning. Moreover, pharmacologic administration and endogenous production of TNF α has induced preconditioning-like cardioprotection in both rats and rabbits. Preconditioning which in essence unmasks an innate cytoprotective program is classically thought to be activated predominantly via G_i-coupled receptor mediated signalling. The ischemic preconditioning induced ligands, which activate G_i-receptor coupled signalling and are proposed to activate the preconditioning cytoprotective programs, include adenosine, bradykinin and opioids. In contrast TNF α acts via type II membrane receptors and directs a multitude of diverse downstream signalling events, which are thought to be both distinct from and potentially overlapping with Gi-receptor, associated signalling. Whether TNF α signalling is essential to or whether a parallel TNF α-independent signalling pathway exists needs to be studied. To evaluate the role of TNF α in ischemic preconditioning we determined infarct-sparing effect of ischemic preconditioning comparing TNF α null (TNF α -/-) and wild-type mice. In conjunction to this we determine whether the TNF α and G-protein coupled receptor pathways, converge at the mitochondrial K_{ATP} channel.

Methods and Results – We utilised an isolated mouse heart model of ischemia and ischemic preconditioning. The ischemic preconditioning protocol included 4 x 5 minutes ischemia/reperfusion prior to the index 35 minutes of global ischemia followed by 45 minutes of reperfusion in isolated perfused murine hearts. Infarct size was measured as a percentage of cardiac volume. Following ischemic preconditioning the infarct was reduced to 19.7±3.9%* in the wild-type mice (from 34.5±3.2%) but was unchanged at 36.4±2.8% in the TNF α-/- group, * $p < 0.05$ vs. baseline wild-type ischemia/reperfusion control. Measurement of TNF α levels, using immunoprecipatation and western blot analysis, after the ischemic preconditioning stimulus resulted in a two-fold increase in TNF α levels in the wild-type mouse when compared to constitutive baseline levels. Induction of preconditioning-like protection using the pharmacological agents adenosine (100μM) and diazoxide (30μM) mimicked ischemic preconditioning in the wild-type (Infarct size – 11.0±3.7%* and 18.4±1.8%*) and in the TNF α -/- mice (Infarct size – 14.5±4.0%* and 23.1±2.8%*) * $p< 0.05$ vs. respective ischemia/reperfusion controls. Recombinant TNF α (0.5 ng/ml) administered for 7 minutes followed by a 10 minutes washout mimicked ischemic preconditioning in wild-type mice (Infarct size – 10.5±1.5%, $p<0.01$ vs ischemia/reperfusion controls). The cardioprotective effects of ischemic preconditioning, adenosine and TNF α were abolished by the co-administration of the mitochondrial K_{ATP} blocker 5-hydroxydecanoate.

Conclusion -In conclusion we demonstrate that cardiac TNF α production is required for ischemic preconditioning induced cardioprotection but not necessary in pharmacologic preconditioning with adenosine or diazoxide in TNF α -/- mice. Moreover, TNF α administration is sufficient to activate preconditioning in wild-type

mice. Finally, as 5-hydroxydecanoate abrogates ischemic, adenosine and TNF α induced preconditioning, this suggests that there distinct signalling pathways converge at the level of mitochondrial K_{ATP} channel activation to mediate cardioprotection in this model.

Abbreviations

5-HD	5-Hydroxy Decanoate
ATP	Adenosine Triphosphate
DAG	Daicel Glycerol
DD	Death Domain
EDTA	Ethylene Diamine Tetrahacetic Acid
HSP 72	Heat Shock Protein 72
GDP	Guanosine Diphosphate
Gi-protein	Pertussis toxin sensitive inhibitory GTP-binding protein
GTP	Guanosine Triphosphate
IP3	Inositol 1,4,5–Triphosphate
K_{ATP}	$Potassium_{ATP}$
kDa	Kilo Dalton
Kn	Knockout
MAPK	Mitogen Activated Protein Kinase
MnSOD	Manganese Superoxide Dismutase
NFκB	Nuclear Factor Kappa B
PBS	Phosphate Buffered Saline
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLAD	Pre-ligand Binding Domain
PLC	Phospholipase C
RIPA	Radioimmunoprecipitation assay

RPM	Revolutions per Minute
SDS PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SWOP	Second Window of Protection
TACE	TNF α Converting Enzyme
TNF α	Tumour Necrosis Factor alpha
TNF α -/-	TNF α knockout
TNF α R1	TNF α Receptor 1
TNF α R2	TNF α Receptor 2
TTC	2,3,5 – Triphenyl Tetrazlium Chloride

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1.)Introduction

a) What is preconditioning?

A major cause of death world-wide is from heart disease, of which ischemic heart disease is a substantial factor (Feuerstein *et al.*, 1998). Ischemic heart disease occurs as a result of myocardial ischemia, which occurs when the supply of blood to the myocardium is inadequate (Opie, 1991). This results in cardiocyte cellular dysfunction (stunning, hibernation) and/or heart muscle cell death (myocardial infarction) which can lead to heart failure (ischemic cardiomyopathy)(Narula *et al.*, 1996). Understanding the biochemical mechanisms by which the heart instinctively protects itself from damage will lead to new and novel therapies with which to treat heart disease.

In 1986, while performing experiments on ATP levels in the failing canine heart, Murry *et al* came across such a mechanism (Murry *et al.*, 1986). They observed that when the animal was subjected to four 5 minute circumflex occlusions, each separated by 5 minutes of reperfusion, before a sustained 40 minute occlusion, the heart had an increased tolerance against ischemia resulting in a reduction in infarct size. Here, brief episodes of ischemia and reperfusion preconditioned heart (myocardium) against sub-lethal ischemia (Murry *et al.*, 1986). This type of preconditioning, known as classical preconditioning, is the first of two windows of protection that can be observed following ischemic preconditioning. The Second Window of Protection (SWOP) or delayed protection (Baxter *et al.*, 1997; Yellon,Baxter, 1995), occurs 24-72 hours following the original ischemic preconditioning stimulus. In this study we concentrate on classical preconditioning.

The protective effect of preconditioning the myocardium is observed, post ischemia, as a reduction in infarct size (the extent of irreversible damage) (Alkhulaifi *et al.*, 1993) and/or an improvement in contractile function (Amsterdam *et al.*, 1993).

Preconditioning-like protection can be induced by many chemical and physical stimuli. These include hypoxia (Bruer *et al.*, 1997), thermal stress (Cumming *et al.*, 1996), stretch (Gysembergh *et al.*, 1998) and a wide variety of pharmacological agents (Nakano *et al.*, 2000). Although first observed in the heart, this phenomenon has been shown to occur in multiple tissue types, including skeletal muscle (Kohin *et al.*, 2001), brain (Cui *et al.*, 2001), liver (Matsumoto *et al.*, 2001) and kidneys (Chien *et al.*, 2000) of numerous mammalian species. These widespread observations of induced protection due to preconditioning suggests that it may be an evolutionary conserved cell survival programme, enabling many tissue types to have heightened tolerance to an ischemic insult.

b) Classical Mechanism of Cardiac Preconditioning

i) Activating the pathway

As with all biological systems, specific signal transduction pathways are followed to achieve a desired state or result. Similarly, preconditioning of the heart requires a signal, which will result in the activation of or the translation of proteins that are required to protect the myocardium from sustained ischemia. Although a large amount of work has gone into studying this signalling pathway, the mystery surrounding its mechanism of protection has not yet been fully uncovered.

Activation of the preconditioning pathway has been shown to be induced by many paracrine and neuroendocrine triggers. These include adenosine (Cain *et al.*, 1998), acetylcholine (Mullane,Bullough, 1995), angiotensin II (Liu *et al.*, 1995), bradykinin (Goto *et al.*, 1995),endothelin (Erikson,Velasco, 1996) and opioids (Nakano *et al.*, 2000). Interestingly the concentration and the type of preconditioning trigger used to confer protection varies between species. With regards to the concentration, a threshold must be reached before the heart can be preconditioned against further damage (Horton *et al.*, 2000). The required concentration of a ligand to induce preconditioning pathway is very specific and can be easily overshot which may affect the heart adversely.

As previously mentioned, the stimulus chosen to induce preconditioning-like protection is important as some, like adenosine which preconditions the rabbit, dog, pig and human myocardium and has no effect in rats (Yellon *et al.*, 1998), do not induce protection in all species. However, this inability of adenosine to precondition is questionable as recent evidence, in the isolated rat heart, has shown that it can induce preconditioning-like protection in the rat heart (Awan *et al.*, 2000).

ii) G_i-protein coupled receptors

Once a trigger, such as adenosine, has been administered to the heart, the ligands activated receptor is thought to couple to pertussis toxin sensitive inhibitory GTP-binding proteins (Gi proteins) (Schultz *et al.*, 1998; Thornton *et al.*, 1993) to transduce their signal. These G-proteins are found within the lipid bilayer of the cell where they move about freely (Voet, 1990). In the inactive state GDP is bound to the

Gi-protein which, when stimulated, releases the bound GDP and binds GTP, activating this protein. The activated G_i-protein moves within the cell membrane activating a multitude of downstream signalling molecules amplifying the original signal. Adenosine, which has been shown to accumulate rapidly in ischemic myocardium (Belardinelli *et al.*, 1989), is coupled to these Gi-proteins (Sumeray,Yellon, 1997).

The role of these Gi-proteins in preconditioning is controversial. In the rat evidence exists both for (Piacentini *et al.*, 1993) and against (Lawson *et al.*, 1993; Liu,Downey, 1993) the involvement of the Gi-protein in infarct size limitation. In other animals, studies involving the blockade of these Gi-proteins using pertussis toxin, attenuated protection is induced by ischemic preconditioning (Sumeray,Yellon, 1997).

iii) Protein Kinase C

Once these Gi-proteins have been activated by a preconditioning stimulus, they in turn activate phospholipase C (PLC) (Guillon *et al.*, 1992). This results in the formation of Inositol 1,4,5–Triphosphate (IP_3) and 1,2–Diacylglycerol (DAG) from the membrane lipid Phosphatidylinositol 4,5–Bisphosphate (Yellon *et al.*, 1998). Formation of DAG activates protein kinase C (PKC) (Oishi *et al.*, 1988), an enzyme which is thought to play a key role in the intracellular signalling pathway of ischemic preconditioning (Puceat,Vassort, 1996).

PKC is thought to play an important role in transduction of the preconditioning signal (Brooks,Hearse, 1996; Li *et al.*, 2000). Direct activation of this kinase, either by a DAG analogues or a PKC agonist, phorbol myristate acetate (Speechly-Dick *et al.*,

1994) induces preconditioning-like protection. In tandem to this, by blocking PKC with the inhibitors polymoxin B and staurosporine (Yellon *et al.*, 1998) the protection afforded by these agents could be abolished. Controversially these inhibitors failed to block precondition in pig and dog hearts (Przyklenk *et al.*, 1995; Vahlhaus *et al.*, 1996). In these species, preconditioning may occur via an alternate signalling pathway utilising activation of a protein Tyrosine kinase instead of PKC which may just preconditioning some species and not in others (Yellon *et al.*, 1998). This data hints at the existence of an alternative preconditioning signal transduction pathway, one which is independent to the G-protein coupled receptor mediated pathway and independent of PKC activation.

iv) Mitochondrial K_{ATP} Channel and Preconditioning

Although controversy surrounds the role PKC is thought to play in the preconditioning signal transduction pathway, the next important step thought to occur is K_{ATP} channel activation (Schultz R, 2001). Two distinct populations of K_{ATP} channels exist within the cardiomyocytes (Kim *et al.*, 1994; Kukreja *et al.*, 1990), the sarcolemmal and the mitochondrial K_{ATP} pools. Each displays distinctive properties and can be activated or inhibited using specific pharmacological compounds. Even though such pharmacological tools exist, a large amount of controversy exists as to which channel is important for cardioprotection due to ischemic preconditioning (Schultz R, 2001).

Specific activation of the mitochondrial K_{ATP} channel, by diazoxide can confer preconditioning-like protection against lethal ischemia in the hearts of both rabbits and rats (Garlid *et al.*, 1997; Pain *et al.*, 2000). Also, 5-Hydroxy-decanoate (5HD), a

mitochondrial K_{ATP} blocker, abrogates cardioprotection offered by ischemic preconditioning (Garlid *et al.*, 1997) in both the above species. In contrast the sarcolemmal K_{ATP} inhibitor, HMR 1883, could not duplicate these results. Therefore the activation of the mitochondrial K_{ATP} channel is likely to be involved in preconditioning signal transduction.

c) Alternative Mechanism for Preconditioning

Nature has demonstrated a tendency for redundancy in many signal transduction pathways. In these systems, inhibiting only one protein within the pathway does not prevent the original signal from inducing the desired result. The ischemic preconditioning signal transduction pathway is filled with such debatable roles of signalling molecules, especially where inhibition of important intermediates within the pathway does not inhibit preconditioning-like protection. This leads us to believe that an alternate to the classical signal transduction pathway exists, one that is independent of the Gi-protein coupled receptor pathway.

As an example, the pleiotropic cytokine, Tumour Necrosis Factor α (TNF α) has been identified as a ligand in the activation of this Gi-protein independent pathway that may also precondition the myocardium against ischemic damage. First discovered and described in tumour masses, this cytokine is expressed as a 26kDa transmembrane protein that is cleaved by TNF α Converting Enzyme (TACE). This releases the 17kDa soluble form allowing for more widespread effects of this cytokine. TNF α has been implicated as mediator of diverse physiological and pathophysiological events including inflammation, cellular survival, growth, differentiation and apoptosis (for review see (Idriss,Naismith, 2000). It achieves these

varied effects by binding to either one of its cognate receptors (Meldrum, 1998), TNF α Receptor 1 and TNF α Receptor 2 (TNF α R1 and TNF α R2). Both of these receptors are expressed in the myocardium (Torre-Amione *et al.*, 1995).

These receptors occur as single transmembrane glycoproteins sharing 28% homology, mostly in their extracellular regions. Also found in this extracellular region is a pre-ligand binding domain (PLAD) that precomplexes the receptors and encourages them to homotrimerise particularly upon activation by TNF α (Chan, 2000). Intracellularly they are, in contrast, very distinct (Grell, 1995). This allows the receptors to activate distinct pathways in response to TNF α activation.

Of the two receptors, TNF α R1 has been more extensively studied. The signalling pathways coupled to TNF α R1 include activation of PLC (Schutze *et al.*, 1992), activation of Phosphlolipase 2 (PL2) and activation of acidic and neutral sphingomyelinases (Wiegmann *et al.*, 1994). The intracellular region has been shown to contain a death domain (DD) motif that is critical in the death inducing, via apoptosis, activity of the receptor (Tabei *et al.*, 1995). TNF α R1 also has two phosphorylation sites in this cytoplasmic domain, a consensus Mitogen Activating Protein Kinase (MAPK) site as well as a candidate Tyrosine phosphorylation site (Aggarwal,Natarajan, 1996; Cottin *et al.*, 1999). Although a specific role for phosphorylation in the receptor has not been established, it is important for the activation of downstream signalling pathways. In contrast the signalling pathways that are coupled to TNF α R2 are largely unknown. Information is limited but it has been shown to lack a DD, and have greater affinity for the membrane bound type of TNF α than the soluble form.

The role of TNF α in the myocardium can be described as a double-edged sword. On the one hand, TNF α is thought to be required for the adaptive response of preconditioning (Mann, 1996), on the other hand TNF α is thought to induce apoptosis. TNF α is largely produced by macrophages but recent studies have shown it to be expressed by the myocardium (Benigni *et al.*, 1996).

Recent studies have highlighted the pathogenic role of TNF α in the development of myocardial disease, such as the direct correlation between serum TNF α levels and the severity and progression of heart failure (Sack *et al.*, 2000). Also transgenic mice, designed to over-express TNF α in a cardiac restricted pattern, were shown to develop myocarditis while the wild type animals remained healthy (Kubota *et al.*, 1997). Although TNF α is associated with maladaptive end points there is considerable support for the hypothesis that TNF α could be protective in the short term and detrimental to the myocardium if the signal is sustained (Mann, 1996).

Pretreatment of rabbits with TNF α intravenously 24 hours prior to a sustained ischemic insult improved contractile function and decrease lactate dehydrogenase secretion (Nelson *et al.*, 1995). It was also noted that this pre-treatment of the myocardium with TNF α was linked to increases in the free radical scavenger manganese superoxide dismutase (MnSOD). The upregulation of this free radical scavenger, possibly via transactivation of the transcription factor nuclear factor κB (NFκB), may be a link to the pathway through which TNF α is thought to protect the myocardium from ischemia. NFκB is thought to upregulate a large number of

cytoprotective genes. To establish as to whether TNF α was indeed required in protection from ischemia in the heart, TNF α R1 and TNF α R2 were knocked out both separately and together in mice (Kurrelmeyer *et al.*, 2000). Here left ventricular infarct size was assed at 24h after acute coronary occlusion in wild type mice (both receptors present), mice lacking either the TNF α R1 or TNF α R2 receptor and in mice lacking both receptors. Upon comparison, the infarct size in the combined TNF α R1 and TNF α R2 knockout mouse was much larger than the infarct size of wild type and TNF α R1 or TNF α R2 knockout mice indicating that TNF α signalling gives rise to a cytoprotective signal. Interestingly the infarct sizes of the TNF α R1 or TNF α R2 knockout mouse were similar to that of the wild type mouse (Kurrelmeyer *et al.*, 2000). This elegant experiment illustrated that the loss of TNF α signalling resulted in a diminished ability to resist ischemic injury.

d) Objectives

Evidence as to the requirement of TNF α for delayed preconditioning (SWOP) leads us to believe that this cytokine, although shown to be maladaptive, may also be able to precondition the heart against an ischemic insult in classical preconditioning (Kurrelmeyer *et al.*, 2000). What may be the important variable as to which end result is achieved, adaptive or maladaptive, may depend on the relative concentration of TNF α within the heart (Smith *et al.*, 2002). This leads us to question as to whether TNF α mediated signalling is indeed required for classical ischemic preconditioning, whether this form of preconditioning is distinct form the Gi-protein coupled receptor mechanism, whether TNF α on its own can confer preconditioning like protection and

whether it occurs via mitochondrial K_{ATP} channel activation. To tackle this issue, of whether TNF α is able to confer classical protection in the heart, we took the reductionist approach, utilising an isolated perfused heart model (Bell,Yellon, 2001). It has been shown previously that the operational procedures involved in inducing preconditioning and ischemia, including the sham procedures, have resulted in the sustained elevation of numerous cytokines including TNF α (Nossuli *et al.*, 2000). To most effectively achieve our objectives it was decided that comparing infarct sizes of TNF α null mice versus wild type mice would ideally suite our needs.

We hypothesise that a signal transduction mechanism exists, independent to the Gi-protein coupled receptor pathway, which can be activated by the cytokine TNF α, and confers protection from ischemic injury via the activation of the mitochondrial K_{ATP} channel.

To test the hypothesis we aimed:

1. To establish whether TNF α is indeed required to confer the protective response in ischemic preconditioning.
2. To determine whether TNF α is required in Gi protein coupled preconditioning via adenosine administration.
3. To confirm the role of the mitochondrial K_{ATP} channel in classical preconditioning and establish whether it has a role in TNF α mediated preconditioning.
4. To initiate the evaluation of downstream candidate signalling molecules.

2. Materials and Methods

This study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academic Press, Washington DC, 1996), and all procedures were approved by the University of Cape Town Medical School Research Ethics Committee.

To test our hypotheses we utilised several techniques described below: -

a) Mouse isolated heart perfusion model

Male mice (18-25g) were anaesthetised (pentabarbitone sodium, 60 mg kg^{-1} i.p.) and heparinized, to prevent the blood from clotting, (25 IU IP). Once an adequate level of anaesthesia had been achieved the chest was opened excising the sternum and attached costal cartilage to give adequate access to the mediastinum. The heart of the mouse was rapidly removed and placed in ice cold (4°C) Krebs-Henseleit buffer and the aorta cannulated. Hearts were then perfused with a modified Krebs-Henseleit buffer (NaCl 118.0mM; $NaHCO_3$ 24.0mM; KCl 4.0mM; NaH_2PO_4 1.0mM; $CaCL_2$ 2.5mM; $MgCl_2$ 1.2mM; di-sodium EDTA 0.5mM; glucose 10mM; gassed with 95% O_2 / 5%CO_2 at 37 °C) in a retrograde fashion with a constant pressure of 110cm H_2O. Temperature was measured by the placement of a fine thermocouple wire (Physitemp, NJ, USA) and monitored on a Digitron 2600T (Torquay, UK). Hearts were fastened, *via* a rigid lightweight lexan coupling rod, to a force displacement transducer (Grass FT03C, Mass. USA) by means of a 4-0 silk (on a 20mm curved atraumatic needle) placed through the apex of the heart. Diastolic tension was adjusted to 2g and hearts paced at 600 beats per minute (by placing an electrode through the left atrium) and the developed tension was recorded on a chart recorder

(Lectromed Multitrace-2, Letchworth, UK). The coronary flow rate was measured by timed collection.

Hearts were first stabilised before being subjected to the protocols shown in figure 1. At the end of each experimental protocol the infarct size was assessed by 2,3,5-Triphenyl tetrazolium chloride (TTC) staining as described previously (Sumeray *et al.*, 2000). Hearts were then frozen and sectioned into 1.5 mm slices. These slices were then laid out and compressed between thin glass plates 0.5mm apart. The sections were then scanned, enlarged, and infarct size assessed using computerised planimetry (Planimetry+, Boreal Software, Norway) by a researcher blinded to the groups.

b) Immunoprecipitation and western blot analysis

To measure the levels of TNF α after ischemic preconditioning hearts were perfused and freeze clamped immediately after the final reperfusion step (fig.1) using Wollenberger tongs precooled in liquid nitrogen. This instantly froze the tissue sample to -196^0C preventing protein degradation. This tissue was then stored at -80°C until protein extraction was performed. Cell lysates were prepared in ice-cold radioimmunoprecipitation assay (RIPA) buffer (1 X Phosphate Buffered Saline (PBS); 1% Igepal; 0.5% sodium deoxycholate; 0.1% SDS) supplemented with protease inhibitors (100µg/ml phenyl methyl sulphonyl flouride (PMSF); 40 µg/ml aprotinin (Sigma) and 1mM sodium orthovanadate (Sigma), pH 10.0). Cellular debris was removed by centrifugation at 5000 X g for 5 minutes, and protein concentrations were determined in duplicate by the Lowry method (Lowry R, 1951).

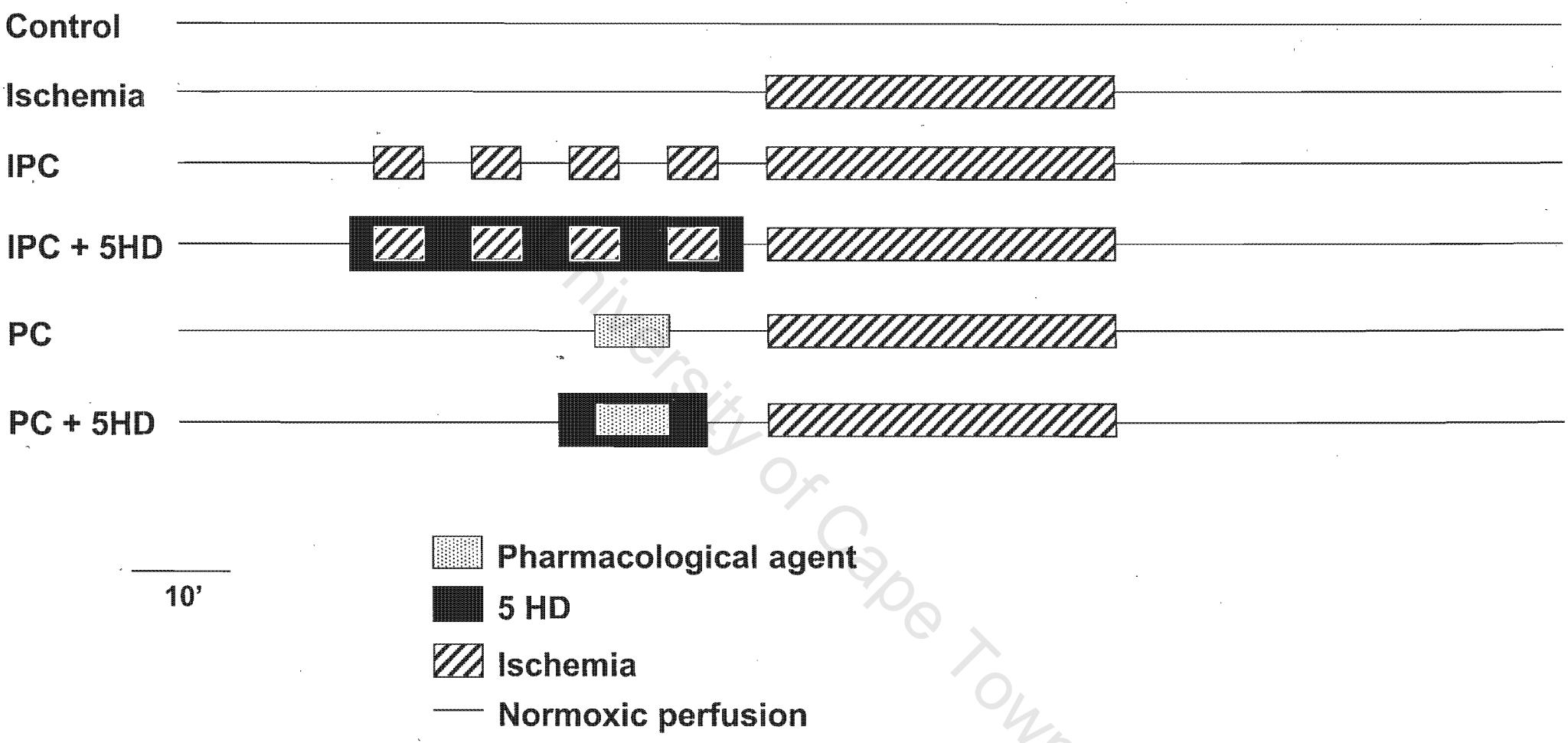

Figure 1: Schematic Representation of preconditioning protocols in the isolated perfused murine hearts. In all experiments, the index ischemia lasted for 35 minutes and the final reperfusion time for 45 minutes. Ischemic preconditioning was conferred by four times 5 minutes of ischemia and reperfusion prior to the index ischemia insult.The pharmocological mimetic agents were administered for 7 minutes followed by a 10 minute washout prior to the index ischemia. 5-HD bracketed both ischemic and pharmacological preconditioning by administration for 5 minutes after the preconditioning triggers.

For all samples 500 μg of the total protein was immunoprecipitated using a rabbit polyclonal TNF α antibody, 1 hour of shaking at 4°C, (Santa Cruz Biotech, CA, USA) and agarose conjugated Protein A/G (Santa Cruz Biotech, CA, USA), 1 hour of shaking at 4°C. Washed proteins (4 x in PBS, pH 7.4) were resolved by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and were transferred to PVDF membranes by electrophoretic transfer. Non-specific binding sites were blocked by incubation in Tris-buffered saline containing 0.1% Tween 20 (Sigma) and 5% (wt/volume) skimmed milk powder, 2 hours at room temperature. Immunoblotting analyses was performed with and immunocomplexes were visualised with the appropriate horseradish peroxidase-conjugated immunoglobulin G (IgG; Santa Cruz Biotech, CA, USA) and chemiluminescent reagents (Amersham Pharmacia Biotech, Amersham, UK). Relative peptide levels were measured using densitometric analysis with UVI band (UVI Tech, Cambridge UK) software on a PC.

c) Genomic DNA extraction from mouse tails

Initially homozygous TNF α deficient (TNF -/-) mice and wild type control mice, a generous gift from Bernard Ryffel, Department of Immunology, University of Cape Town Medical School, had to be genotyped to confirm their genetic backgrounds. The genomic DNA required to test the mice, was extracted from there respective tails (Disch *et al.*, 1996). Approximately 1 cm of a candidate mouse tail was removed from the mouse being tested and digested in 400μl tail digestion buffer (1M Tris pH 8.0; 5M NaCl; 0.5M EDTA pH 8.0; autoclaved then 10% SDS added) with Proteinase K (1mg/ml) added fresh to the digestion buffer upon usage. Tails were then digested overnight, at 55°C, in a waterbath. The following day 400μl of phenol: Chloroform:

Isoamyl Alcohol, 25:24:1, (Sigma) mixture was added to each digested tail, which was then gently mixed and cellular debris removed via centrifugation (13 000 rpm at 4^0C for 10 minutes). This resulted in two separate layers, the lower layer containing cellular debris and the upper layer containing the required DNA. To separate the two layers, without contaminating the samples, the bottom layer was removed and discarded using barrier tips (Quality Scientific Plastics) and 200µl of chloroform (Sigma) added to the remaining upper phase and the tube inverted. This was then spun in a centrifuge at 13 000rpm for 5 minutes. Once again two separate phases occur and the bottom phase is removed and discarded while the upper phase is kept. Samples were then spun at 13 000rpm for 10 minutes to force the remaining debris into a pellet at the bottom of the tube. The aqueous phase was then transferred to a new Eppendorff and 45µl of 7.5M ammonium acetate (Merck) and 1 ml 100% ethanol (BDH) added and the tube inverted to precipitate the DNA. This white stringy DNA precipitate was immediately removed, using sterile yellow tips, to a new tube containing 70% ethanol to hydrate the DNA. The tube with the DNA precipitate is now spun at 13 000rpm for 10 minutes, so that the DNA may form a pellet and the bottom, and the alcohol decanted carefully so as not to loose the DNA pellet. Tubes are then left to lyophilise for 1 hour and sterile water added to dissolve the DNA.

d) Genotyping of TNF α deficient and wild type mice

Targeted deletion of the TNF α gene has been previously described (Marino *et al.*, 1997). Genotyping involved testing as to whether the respective mouse's TNF α gene was intact or had been disrupted by the insertion of a phosphoglycerate kinase-

neomycin expression cassette within the gene. Random genotyping was performed to check the status of the breeding colonies.

The following primers were used: TNF α forward 5'-AGA TAG CAA ATC GGC AGA CGG-3' and TNF α reverse: 5'-ATC AGT TCT ATG GCC CAG ACC-3' (fig.2a) to genotype mice for the presence of an intact TNF α gene. Reactions were performed in 25 μl volumes containing magnesium (Mg) free buffer (Promega), 4mM Mg (Promega), 100μM dNTP's, 0.2μM primer mixture, 0.625U Taq polymerase (Promega) and 1.0μl of DNA. The reaction mixtures were incubated at 94°C for 4 minutes then run for 35 cycles at 94°C for 1 minute (melting), 55°C for 1 minute (annealing) and 72°C for 2 minute (elongation) finishing with a 10 minute incubation at 72°C in a Perkin Elmer GeneAmp PCR System 2700.

To test as to whether the mice were TNF α -/-, PCR for the inserted phosphoglycerate kinase-neomycin expression cassette using the forward primer 5'-CTA AAT GAA CTG CAG GAC GA-3' and the reverse primer, 5'-ATA CTT TCT CGG CAG GAG CA-3' was performed (fig.2b). Reactions were performed in 25 μl volumes containing Mg free buffer, 4mM Mg, 100μM dNTP's, 0.2μM primer mixture, 0.625U Taq polymerase and 1.0μl of DNA. These reaction mixtures were incubated at 94°C for 1 minute 30 seconds then run for 35 cycles at 94°C for 30s, 60°C for 30s and 72°C for 30 seconds finishing with a 2 minute incubation at 72°C.

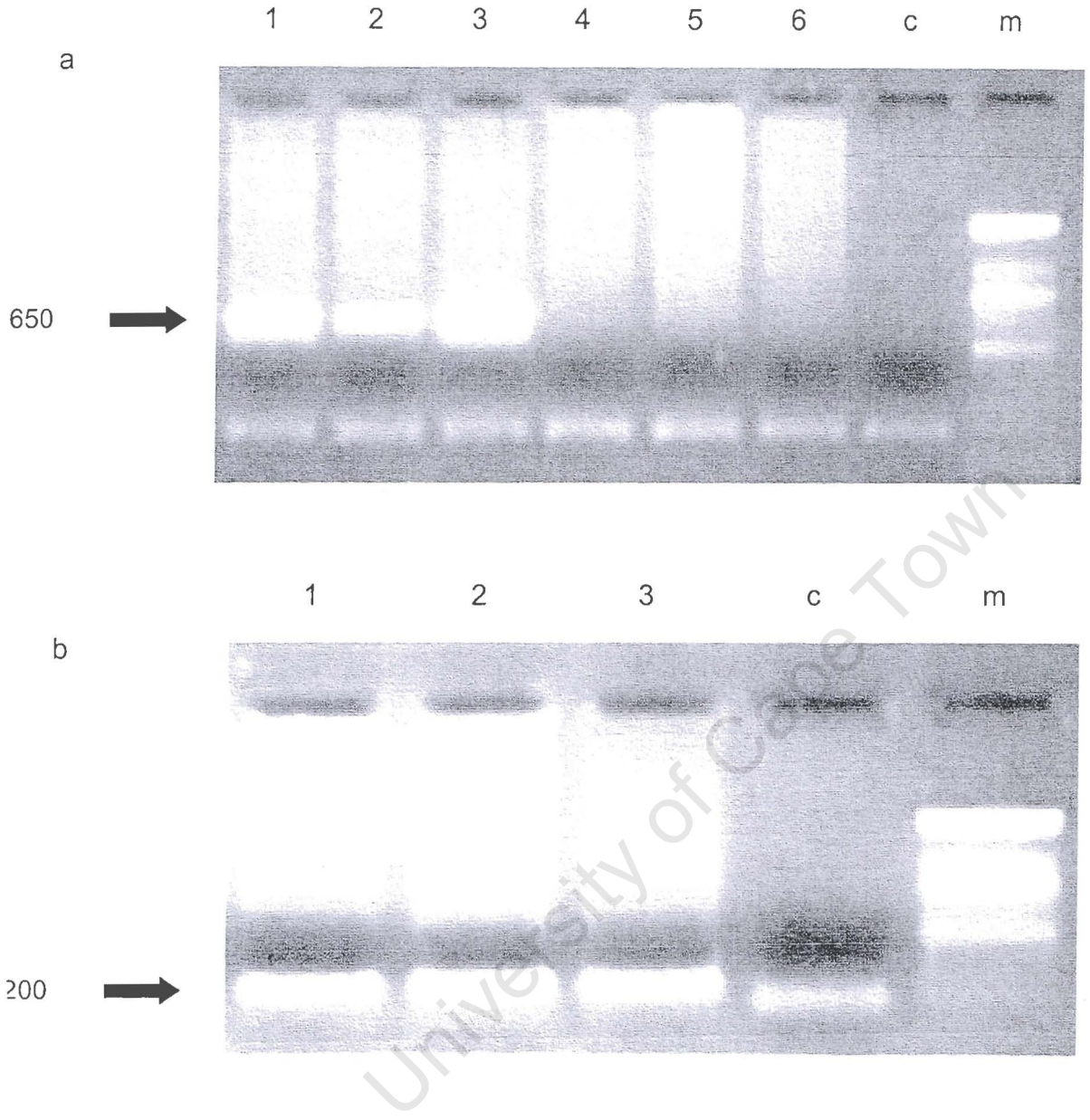

Figure 2 : Analysis of extracted mouse genomic DNA for the determination of targeted disruption of the TNF α gene. a) TNF α specific primers were utilised to distinguish between wild type (lanes 1-3) and knockout (lanes 4-6) mice. b) Neomycin cassette specific primers (lanes 1-3) were utilised as a secondary indication of TNF α disruption. c - Water control, m - marker.

e) Ischemic Preconditioning and Pharmacological Studies

Mice were subjected to four cycles of five minutes of ischemia and reperfusion to precondition them (fig.1). During ischemia temperatures were maintained at 37.0 $\pm 0.2^0$C by immersing the hearts in Krebs Henseleit buffer that was maintained at 37.0^0C in a waterbath.

During pharmacological analysis of preconditioning, TNF α was administered for 7 minutes followed by a 10 minute washout period (fig.1). We utilised a washout period as the adaptive effect of TNF α is thought to be as a result of short-term expression of TNF α (Mann, 1996) whereas long term expression may be maladaptive by producing cardiac decompensation. Administration of adenosine as a preconditioning mimetic utilised the same protocol.

The mitochondrial K_{ATP} channel inhibitor 5-HD was utilised in studying the role of this mitochondrial channel in preconditioning. This inhibitor was administered during the reperfusion periods of ischemic preconditioning in an attempt to abolish the protective effects of preconditioning the myocardium. In the pharmacological studies when attempting to precondition using either TNF α or adenosine, we administered 5-HD from 3 minutes before the addition of either of the mimetics, up until 5 minutes after we stopped perfusing with the either of the mimetics. This was followed by 5 minutes of standard perfusion to wash out all the pharmacological agents we had added (fig.1).

f) Identification of downstream TNF α linked signalling agents

Recent evidence has revealed a possible role for Signal Transducer and Activator of Transcription 3 (STAT3) in ischemic preconditioning (Bolli *et al.*, 2001; Hattori *et al.*, 2001). In addition, STAT 3 is an integral signalling peptide in the innate immune system (Smith *et al.*, 2002) and could be activated by TNF α preconditioning. We hereby examined this transcription factors role in ischemic preconditioning by measuring STAT 3 activation in response to an ischemic preconditioning trigger. Immunoprecipatation and western blot analysis was carried out, as before, utilising a rabbit polyclonal STAT 3 antibody or a mouse monoclonal tyrosine-p antibody (Santa Cruz Biotech, CA, USA). Tyrosine phosphorylation of STAT 3 was an indication of STAT 3 activation.

g) Statistical analysis

Results are expressed as mean values ± standard error of the mean and were analyzed by one-way ANOVA with Dunn's post test, using GraphPad InStat version 3.01 (GraphPad Software, San Diego California USA). Differences were considered statistically significant at values of $P<0.05$.

3. Results

a) Establishing a isolated mouse heart perfusion model

The isolated perfused heart model has been shown to be effective in studying ischemic preconditioning (Yellon,Dana, 2000). An *ex vivo* model of preconditioning suited our needs research as we wished to use a reductionist approach and the cytokine we were interested in, TNF α has been shown to be elevated in *in vivo* cardiac perfusion models (Nossuli *et al.*, 2000). As the option to utilise a TNF α knockout mouse was made available to us (Marino *et al.*, 1997), we decided to perform our study in this mouse.

Before we could utilise the knockout mouse, an isolated heart perfusion model of the mouse had to be set up in our lab. A mouse model of ischemia and ischemic preconditioning had as yet not been established in South Africa.

To set up the model we had to show that removal of the myocardium and the subsequent perfusion did not cause large-scale infarct damage and that the heart could be preconditioned against sustained ischemic damage. Excision and normoxic of the heart was shown to cause negligible myocardial damage, less than 5% of the myocardium was infarcted (fig.3). Sustained ischemia of 35 minutes was shown to increase the infarct size (fig.3) while preconditioning the myocardium with 4 times 5 cycles of ischemia and reperfusion (fig.1), reduced the infarct size by 42.8± 9.0% (fig.3 and fig.4) when compared to the ischemic controls.

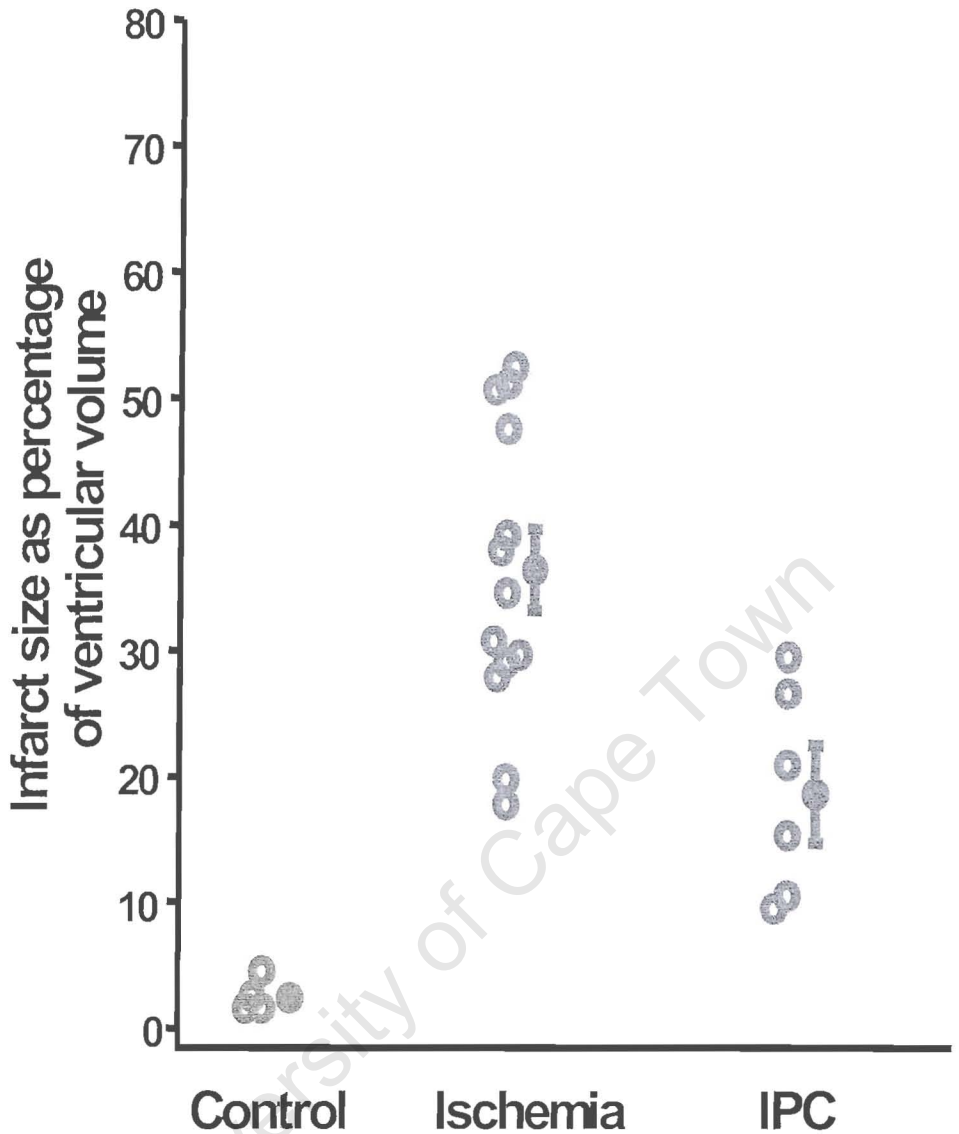

Figure 3: Infarct size assessment of wild type mice subjected to, control, ischemia and IPC (protocols as in figure1). Preconditioning the myocardium resulted in a decrease in infarct size when compared to ischemic controls ($P<0.05$). Group S.E.M.

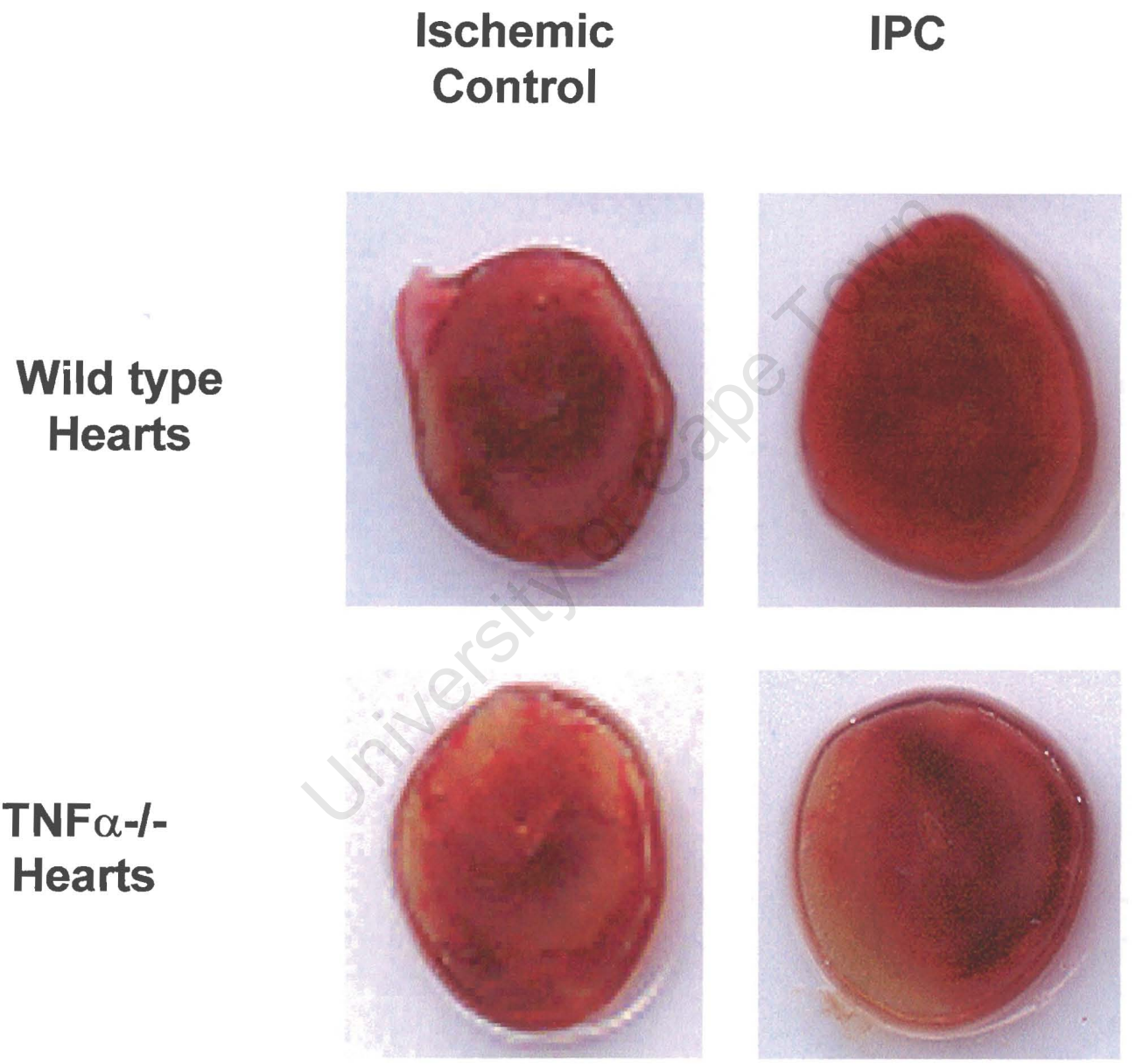

Figure 4: Representative sections of hearts stained with TTC following the ischemic control and the ischemic preconditioning protocols (see figure 1). Coloured regions represent viable tissue and colourless regions the area of infarction.

b) TNF α regulation in response to ischemic preconditioning

With our mouse heart perfusion model operational, we could now look at other parameters of ischemic preconditioning. Previously, in rabbits, pre-treatment with TNF α was shown to provide the heart with delayed protection (Nelson *et al.*, 1995) against sustained ischemia. With our model of ischemic damage and preconditioning set up, we decided to look at the response when TNF α is the ischemic preconditioning stimulus.

Wild type mice were subjected to control and ischemic preconditioning perfusion protocols (fig.5) at the end of which hearts were snap frozen using Wollenberger tongs and immersed in liquid nitrogen. Whole heart protein extract was subjected to immunoprecipatation for TNF α and their respective levels compared. Preconditioning the myocardium, with ischemia, resulted in a two-fold increase in TNF α levels (fig.5) when compared to control levels of TNF α. We therefore show that in response to the ischemic preconditioning trigger, TNF α levels were upregulated in the heart.

a) Preconditioning TNF α knockout mouse

As we wished to study the role of the cytokine TNF α, we utilised a TNF α knockout mouse to assist in elucidating the role of TNF α in ischemic preconditioning. These mice had had a neomycin cassette inserted into a TNF α exon, nullifying the protein (Marino *et al.*, 1997). Mice were genotyped for this neomycin cassette as well

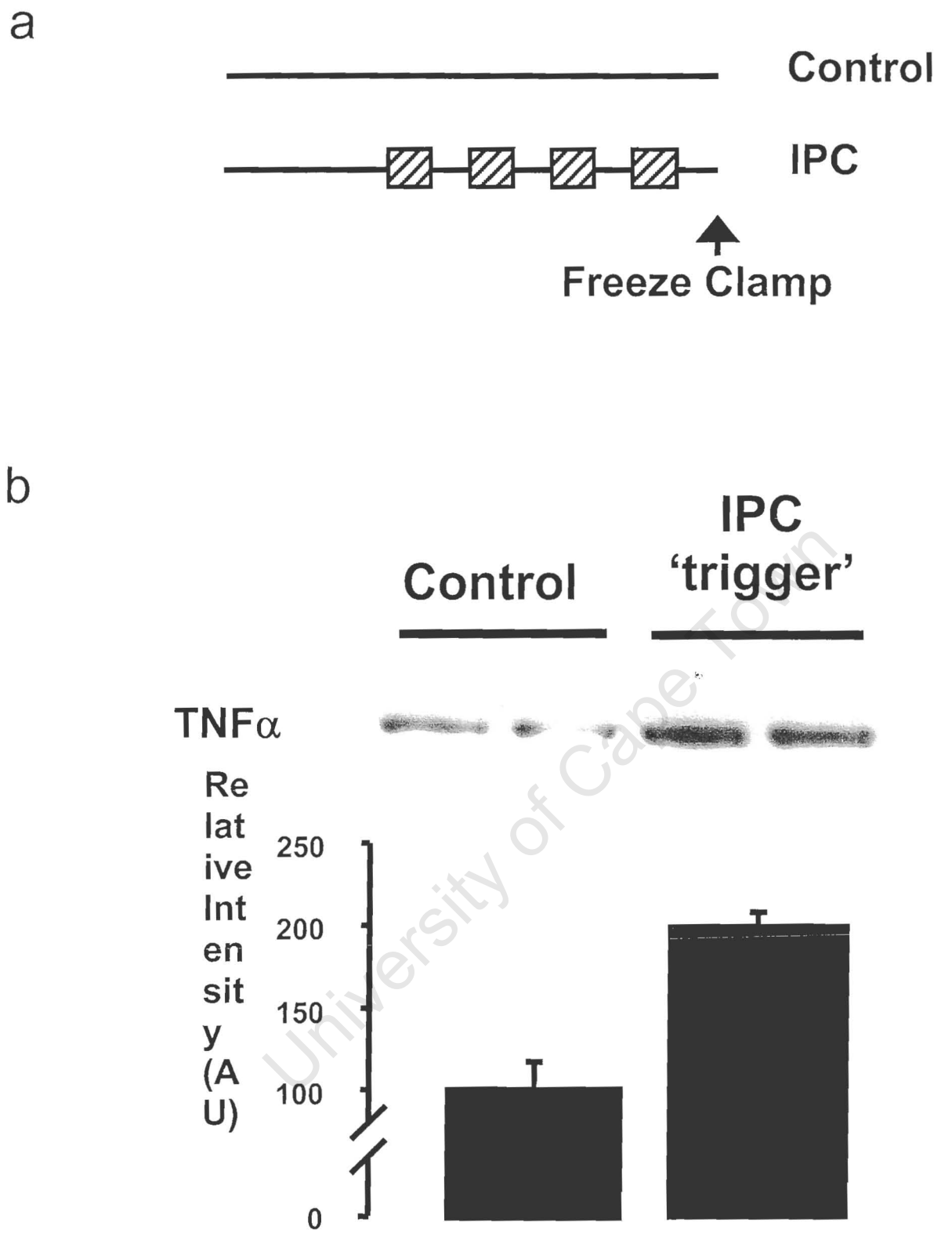

Fig.5: Endogenous TNF α levels as a result of ischemic preconditioning. a) Perfusion protocol of IPC trigger utilised. b) Western blot of TNF α levels as a result of the IPC trigger in comparison to baseline TNF α levels. ▨ Ischemic episode — perfusion.

as for TNF α to establish their genetic background, with regards to TNF α (fig.2).

Initially we showed that in the TNF α -/- mice, normoxic as well as 35 minutes of global ischemia, had relatively similar infarct sizes as in the wild type mice (fig.6). Our next step involved preconditioning the myocardium against a sustained ischemic insult with ischemic preconditioning. We were unable to precondition the TNF α null mouse by using ischemia as a trigger (fig.6). We believed that this inability to precondition the myocardium via ischemia was a direct result of TNF α being knocked out in this mouse.

As we could not precondition these knockout mice, we decided to rescue their inability to produce TNF α by giving them recombinant TNF α (o.5ng/ml) during perfusion experiments (fig.7a and 7c). TNF α administration, in wild type mice, was shown to decrease infarct size by 69.4± 4.3% when compared to ischemic controls (fig.c). When the same protocol was applied to the TNF α -/- mouse we could not protect the myocardium form sustained ischemic damage.

This data, although not conclusive, supported the hypothesis that in these TNF α null mice, the TNF α signalling pathways may be down regulated. Therefore we attempted to prime the system by administering 1μg of recombinant TNF α i.v. 24 hours prior to the ischemic insult (fig.7b). By combining this TNF α injection to our TNF α administering perfusion protocol (fig.7c), we were able to induce

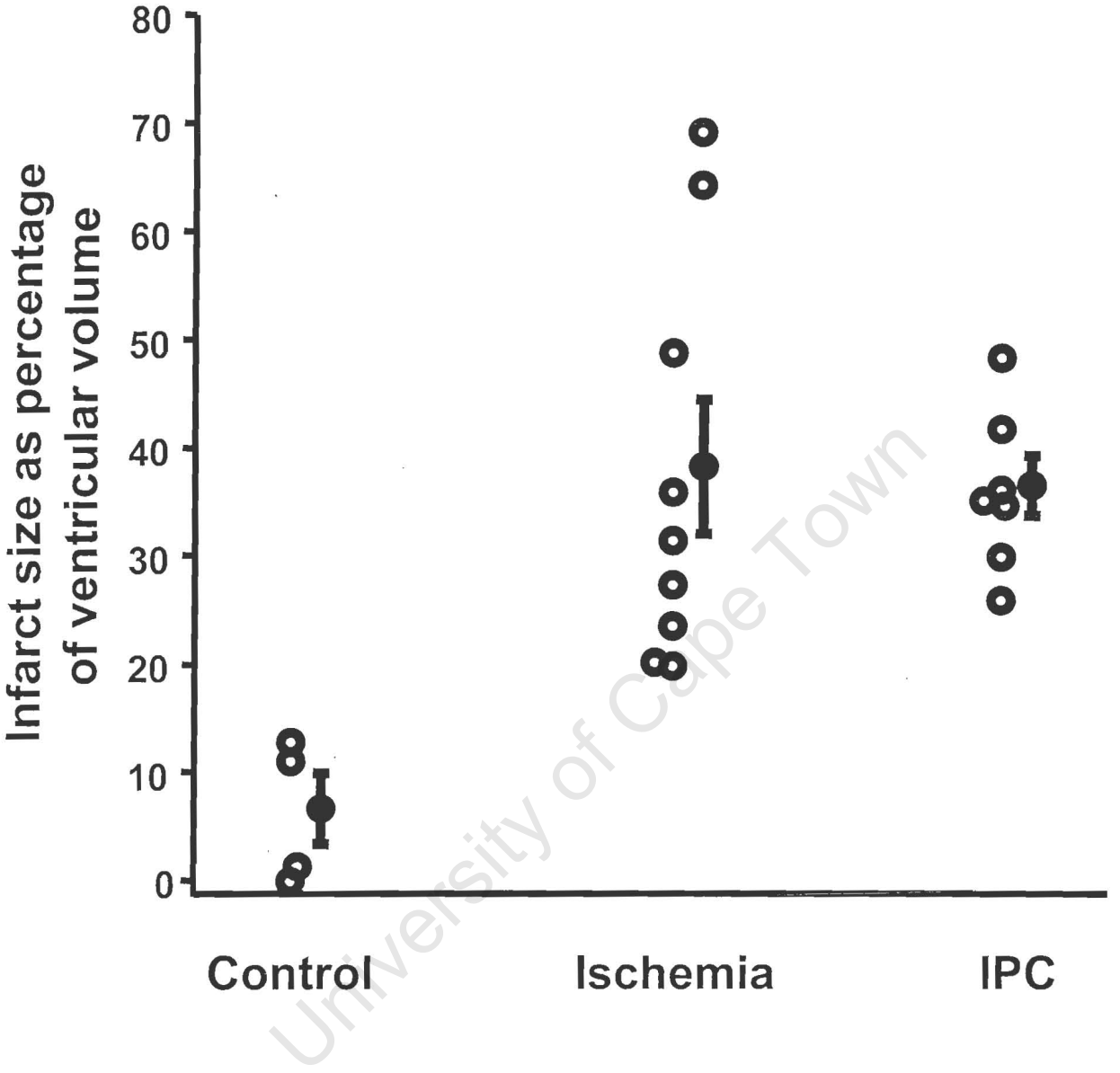

Figure 6: Infarct size assessment of TNF α knockout mice subjected to control (8%), ischemia (39%) and IPC (38%) protocols (protocols as in figure 1). IPC was not effective as a preconditioning stimulus in these animals ($P<0.05$).● S.E.M.

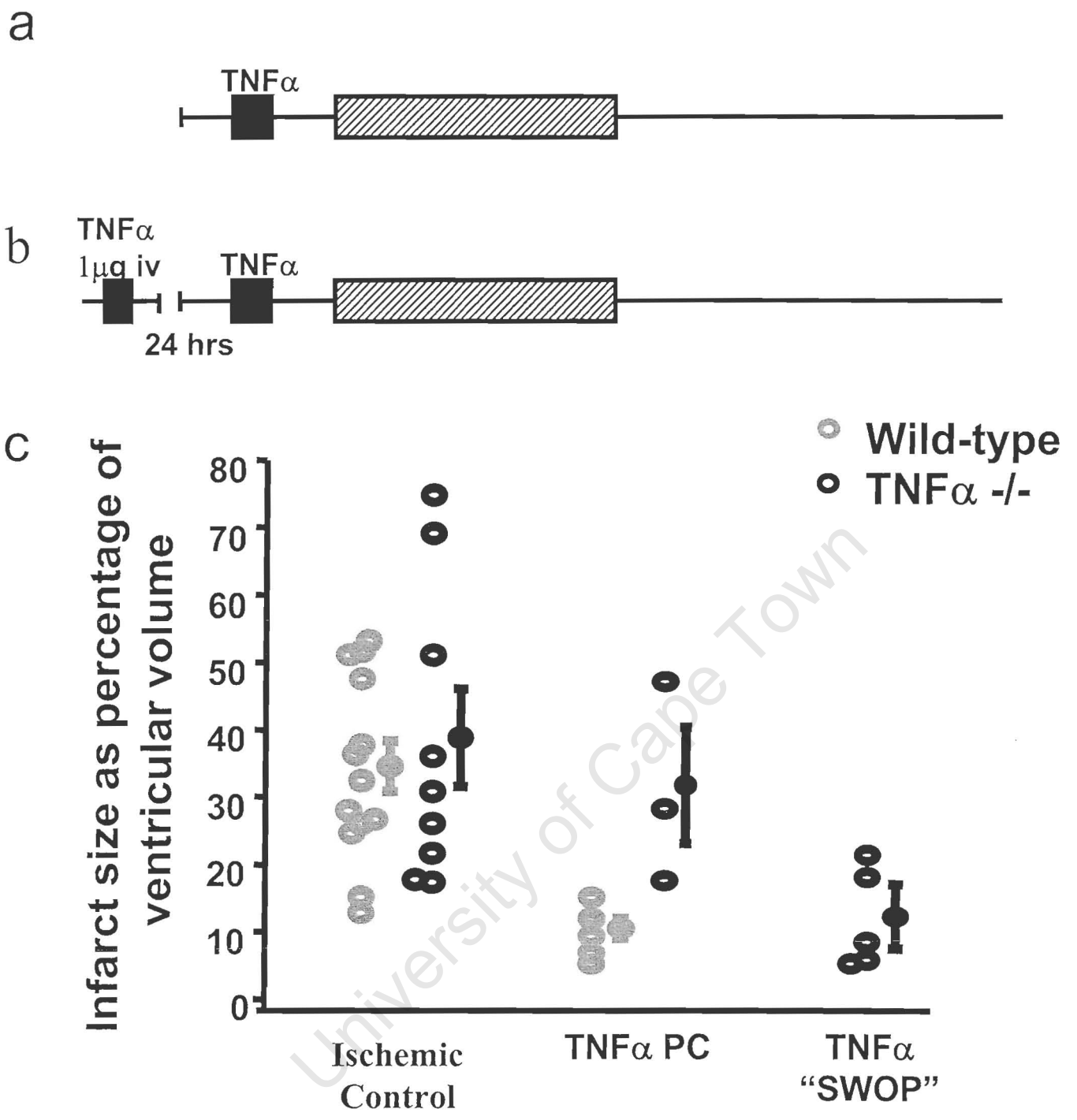

Fig.7: Preconditioning-like protection of the myocardium via TNF α administration. a)Schematic representation of the protocol used in artificially inducing preconditioning-like protection in the myocardium wild type mice.b) Protocol utilised in inducing protection via TNF α administration in knockout mice.1μg of TNF α was administered IV as a primer for protection.c) TNF α administration prior to global ischemia reduced the infarct sizes significantly in both wild type and knockout mice. -($P<0.05$). ● Wild type and ● knockout group S.E.M.

preconditioning-like protection in the knockout mouse as well, showing a 59.4± 12.3% decrease in the size of the infarct when compared to the ischemic control (fig.7c). This pointed to the existence of an alternate pathway via which preconditioning could occur.

b) Does preconditioning involve the Mitochondrial K_{ATP} channel?

Activation of the mitochondrial K_{ATP} channel is thought to be an end effector of a preconditioning stimulus in both the rat and the rabbit heart (Baines *et al.*, 1999; Schultz R, 2001). Whether this was the case in the mouse has yet to be established.

To determine whether the mitochondrial K_{ATP} channel played an active role in preconditioning we attempted to induce preconditioning-like protection using diazoxide, the mitochondrial K_{ATP} channel activator. In addition to this we attempted to abolish protection, due to ischemic preconditioning, by administering 5-HD during the reperfusion periods of the ischemic preconditioning protocol (fig.1).

Administration of diazoxide (30 μM) prior to index ischemia was shown to decrease the infarct size by 46.7±5.2% when compared to ischemic controls while 5-HD (100 μM) abolished the infarct reducing ability of ischemic preconditioning (fig.8).

This dual approach at determining the role of the mitochondrial K_{ATP} channel allowed us to relate mitochondrial K_{ATP} channel activation and preconditioning.

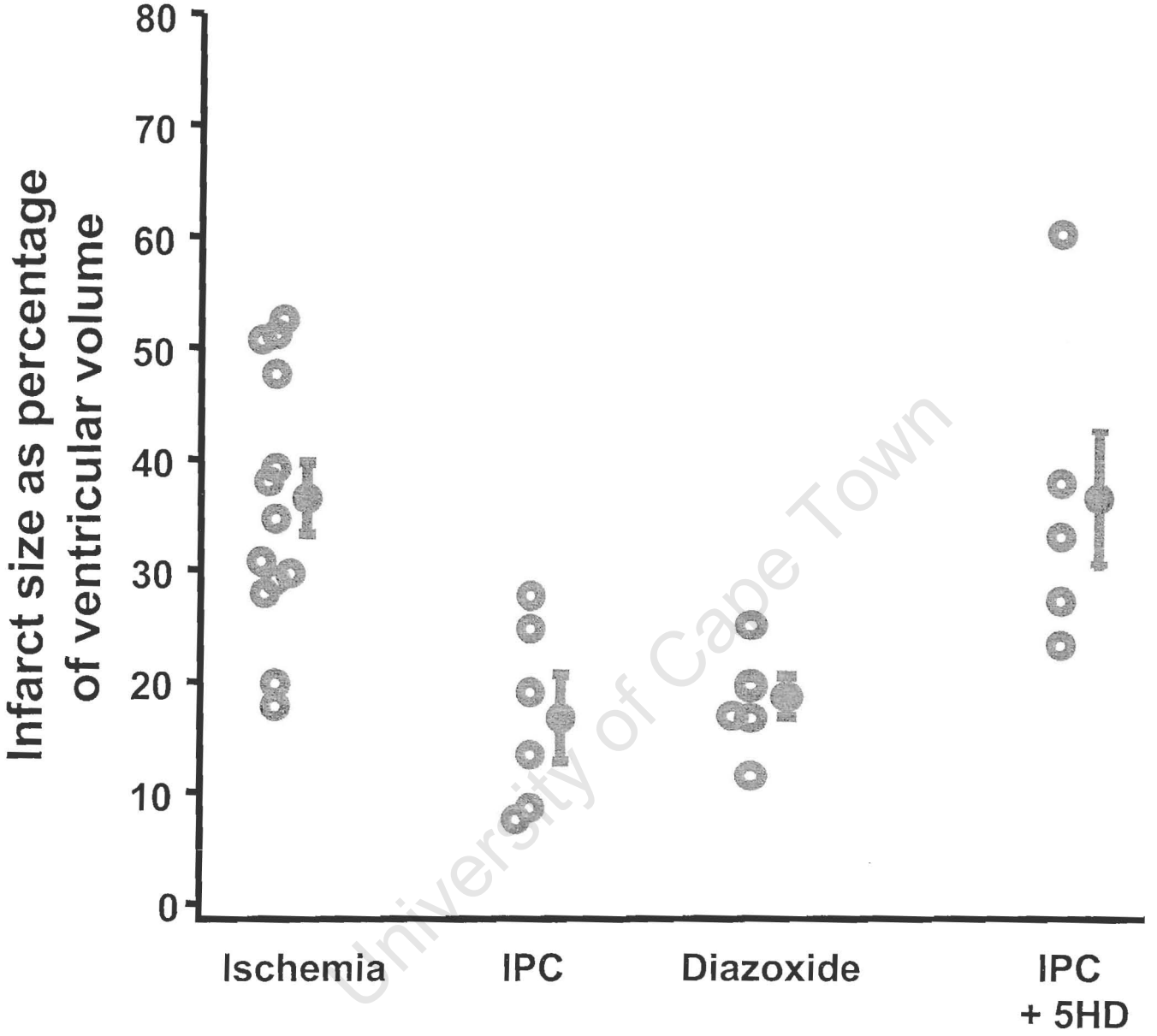

Figure 8: Pharmacological preconditioning of wild type mice. Infarct size is presented as a percentage of the ventricular volume in an isolated mouse heart. Perfusion protocols utilised are illustrated in figure 1. Diazoxide (30μM) , the mitochondrial K_{ATP} channel activator, administration is shown to provide the myocardium with protection equal to that of IPC while 5 HD (100μM) blocked protection due to IPC. ● Group S.E.M.

e) Pharmacological Preconditioning

As TNF α administration and not ischemia could precondition the myocardium of TNF α kn mice against sustained ischemia, we wondered if these knockout mice could also be preconditioned via the classical G_i-protein coupled receptor pathway (Cohen *et al.*, 2000).

We made use of the established preconditioning mimetic adenosine (Ely,Berne, 1992; Kitakaze *et al.*, 1995; Meldrum, 1998) to induce preconditioning-like protection in the hearts. By administering adenosine (100μM) prior to the sustained ischemia, we were able to confer preconditioning like protection in both the wild type and the TNF α -/- mouse. Infarct sizes were reduced by 68.0±10.8% and 62.0±10.5% respectively (fig.9). As we had previously established a putative role for the mitochondrial K_{ATP} channel in preconditioning, we extended the diazoxide (30 μM) perfusion protocol (fig.1) to the knockout mouse. Pharmacological preconditioning with diazoxide restored the cardioprotective phenotype in the TNF α null mice reducing the infarct size by 39.4±7.3% when compared to ischemic controls (fig.10).

f) Convergence of the Respective preconditioning pathways

Recent evidence, including our results, has implicated the mitochondrial K_{ATP} channel as a possible end point of preconditioning. With evidence from the knockout mouse

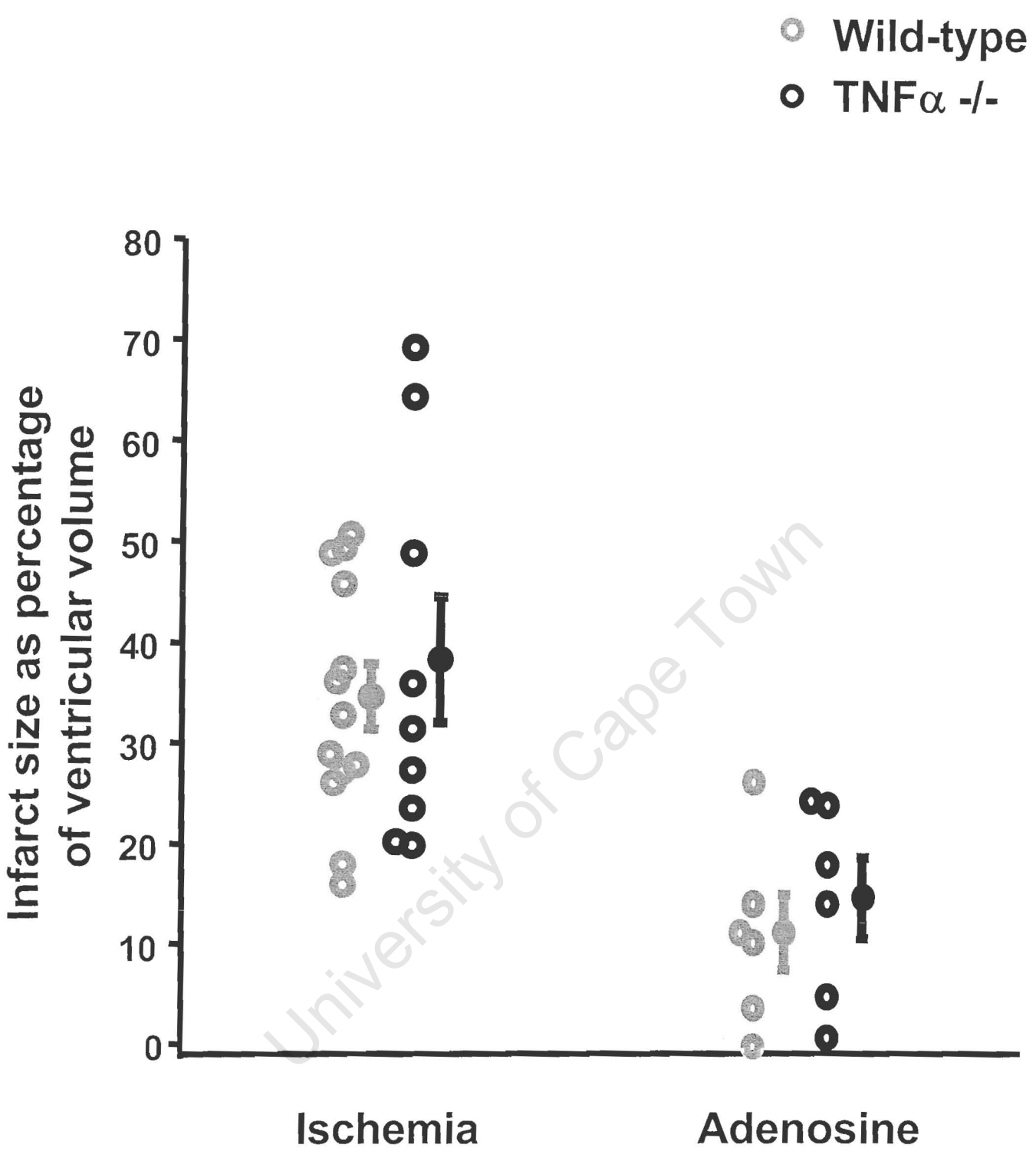

Figure 9: Pharmacological preconditioning of both wild type and knockout mice with adenosine (100μM). Adenosine administration reduced the infarct sizes in both mice significantly.($P<0.05$) Wild type and knockout group S.E.M.

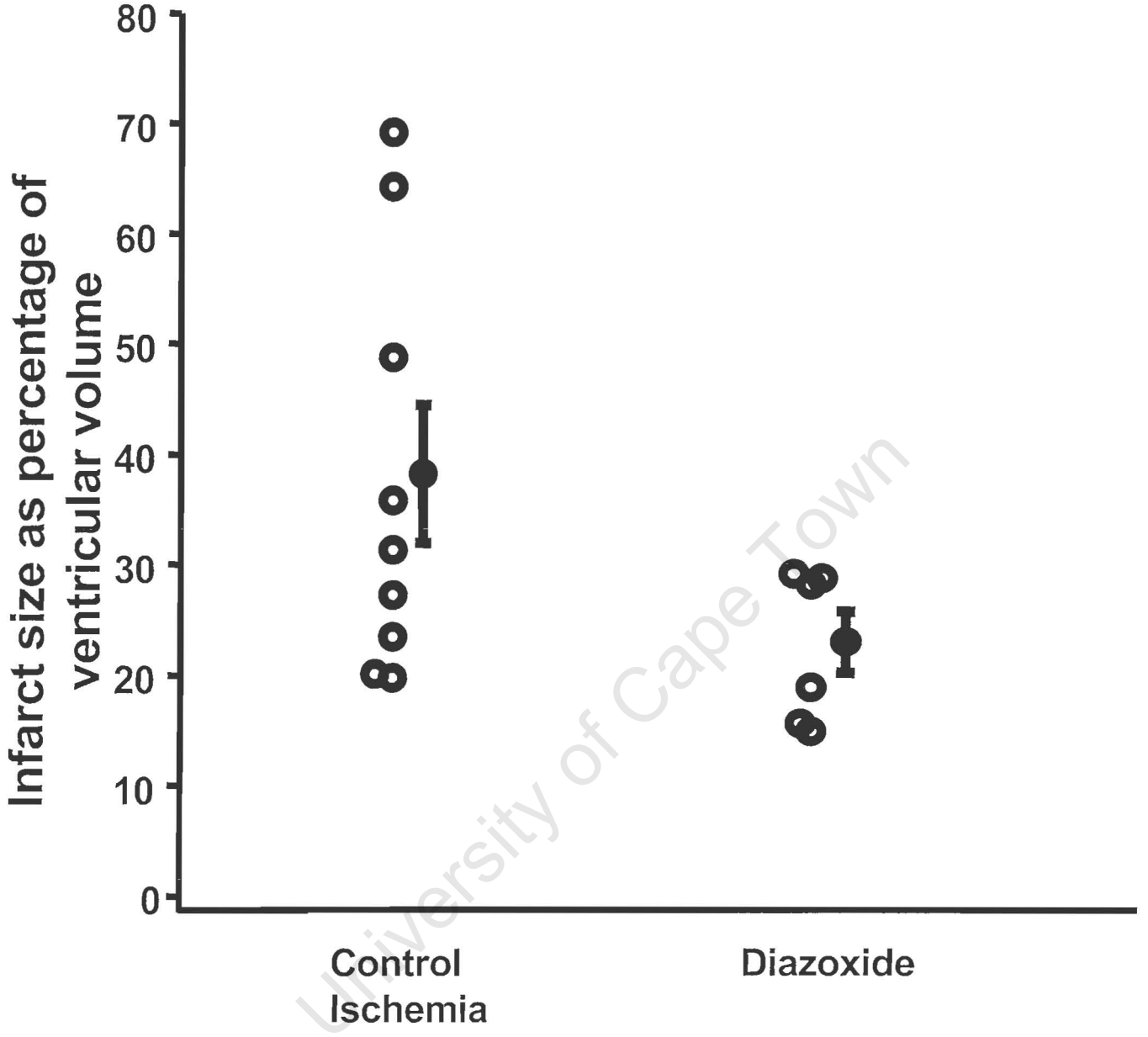

Figure 10: TNF α -/- hearts were given diazoxide (30 μm), the mitochondrial K_{ATP} channel activator.This resulted in a decrease in infarct size when compared to ischemic controls. Symbols as in figure 7. ($P<0.05$). ● Group S.E.M.

supporting the existence of two independent preconditioning signalling pathways, we aimed to determine whether these pathways converged at the mitochondrial K_{ATP} channel.

As the preconditioning-like protection afforded by adenosine, which acts via G_i-protein coupled receptors could be abolished by 5-HD (fig.11), we hypothesised that if this could be repeated using TNF α as the preconditioning mimetic, then we would have established a link between the two pathways. Similar to all the other cases of 5-HD administration, protection due to recombinant TNF α was abrogated when administered with 5-HD.These results indicate that both the TNF α and a Gi-protein coupled receptor pathways converged and resulted in activation of the mitochondrial K_{ATP} channel.

g) Signal Transducer and Activator of Transcription 3 (STAT3)

As we could establish that TNF α plays an important role in the ischemic preconditioning pathway, and that it activated a separate pathway to the classical G_i-protein coupled receptor pathway, we decided to look at possible signalling intermediates within this TNF α signalling pathway.

Recent evidence has pointed to the transcription factor Signal Transducer and Activator of Transcription 3 (STAT 3) as to having a role in preconditioning the myocardium (Hattori *et al.*, 2001; Imada,Leonard, 2000; Negoro *et al.*, 2000). As STAT 3 is an integral-signalling molecule in the innate immune

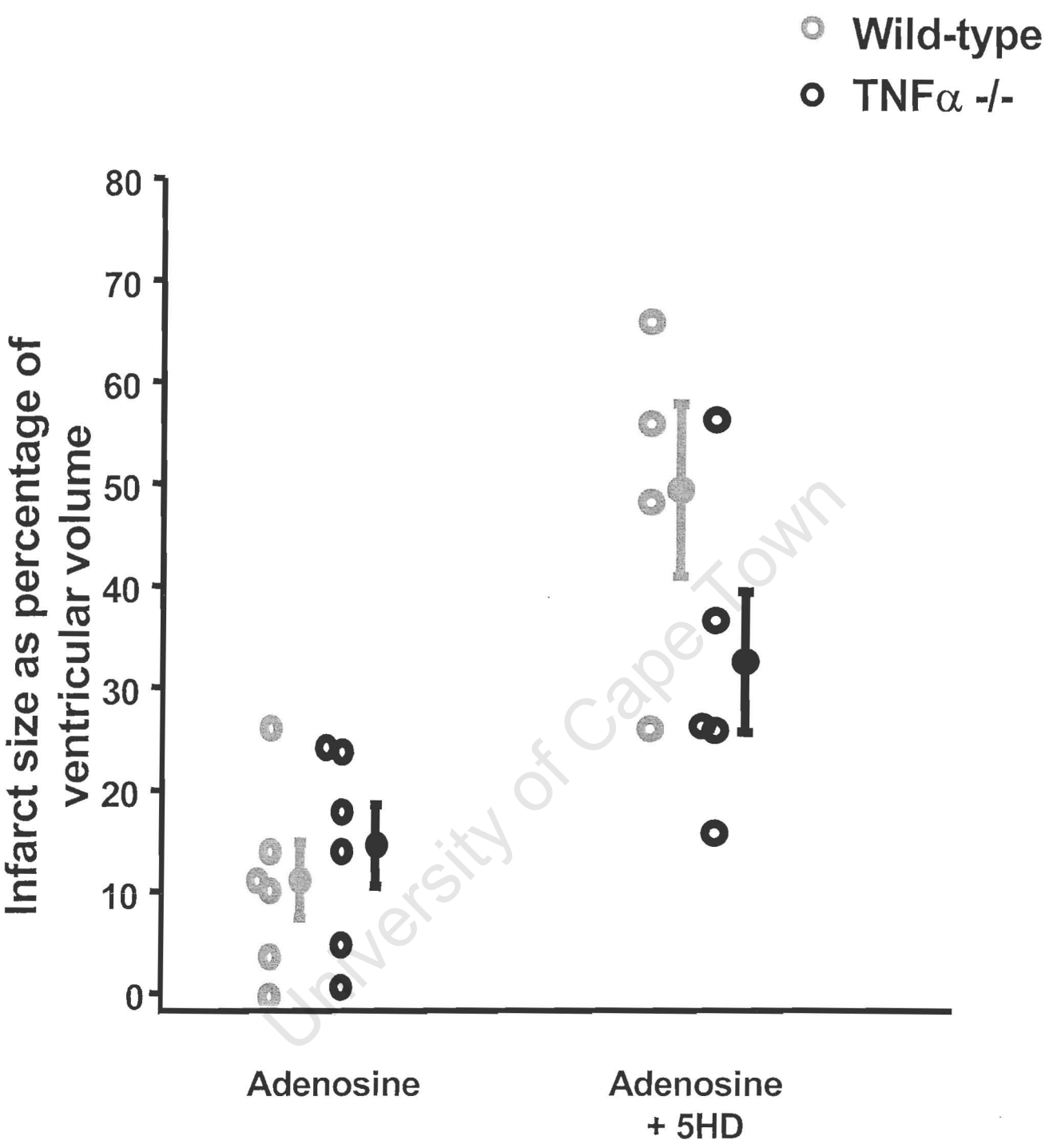

Figure 11: Wt and TNF α -/- mice were given adenosine (100 μM) as a preconditioning mimetic. Protection was blocked using the mitochondrial K_{ATP} channel blocker 5-HD ($P<0.05$). Symbols as in figure 7. ◍ Wild type and ● knockout group S.E.M.

system (in which TNF α is an apical signalling molecule), this led us to question whether this transcription factor was involved in the preconditioning pathway we were examining. To test this, we aimed to determine as to whether STAT 3 was activated in response to an ischemic preconditioning stimulus. STAT 3 is thought to be activated via phosphorylation by one of the receptor associated tyrosine kinases of the Janus Kinases (JAK) family (Hattori *et al.*, 2001).

By looking at the amount of STAT 3 tyrosine phosphorylation, we were able to show that in response to an ischemic preconditioning stimulus (hearts were removed from the rig at the end of the final reperfusion step) STAT 3 was activated (fig.12).

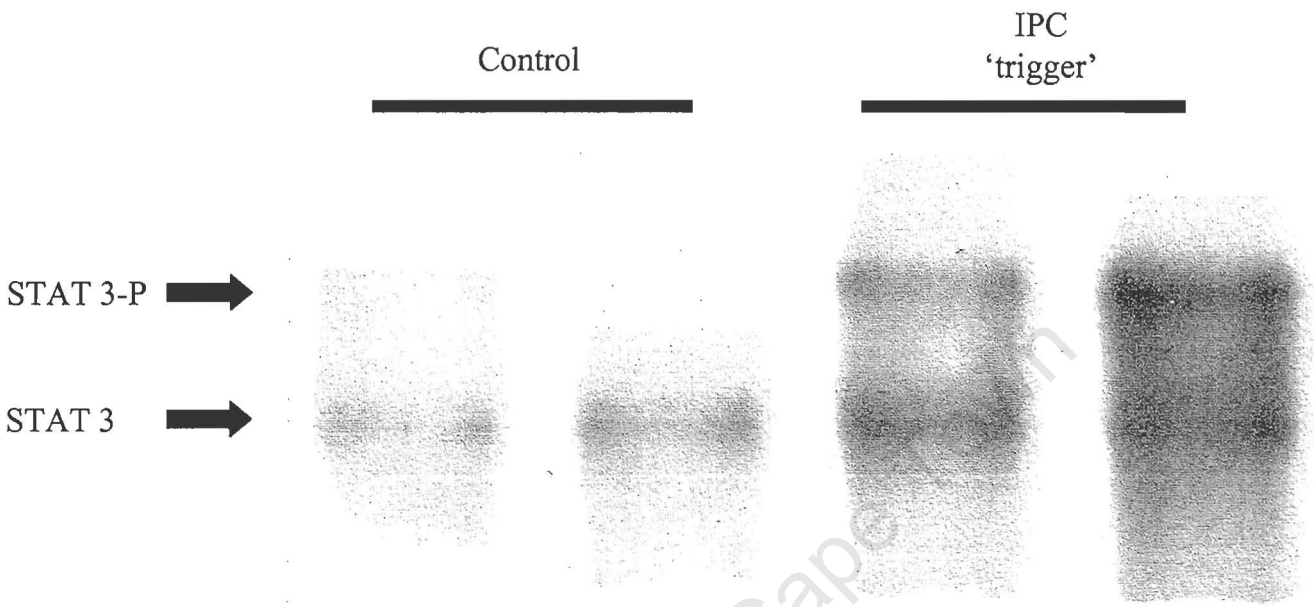

Figure 12: Representative western blot showing an increase in STAT 3 as well as phosphorylated, activated STAT 3 (STAT 3-P) levels in hearts subjected to the control and ischemic preconditioning protocols of fig.5a.

4. Discussion

As early as 1896, the isolated perfused heart has been used as a model of the hearts functions. Nearly one hundred years later, perfusion studies in the dog (Murry *et al.*, 1986) unmasked an innate cardioprotective programme which came to be known as preconditioning. Preconditioning the myocardium was shown to initiate an unknown mechanism within the heart, which protected the myocardium against sustained ischemia and subsequent reperfusion. With the mechanism and mechanics of preconditioning unknown and controversial in nature, I decided to look at the less studied preconditioning pathway that involves the pleiotropic cytokine TNF α.

In this study I demonstrate that ischemic preconditioning stimulates endogenous cardiac TNF α production, in mice and that this ligand is required in ischemic preconditioning induced cardioprotection against myocardial infarction in mice. Conversely we demonstrate that endogenous TNF α is not necessary for pharmacologic preconditioning when either adenosine or diazoxide are used to 'trigger' this cardioprotective program. Moreover, TNF α administration is sufficient to activate preconditioning in wild-type mice. Finally, the fact that 5-hydroxydecanoate abrogates ischemic, adenosine and TNF α induced preconditioning in mice suggest that distinct signalling pathways converge at the level of mitochondrial K_{ATP} channel activation to mediate this cardioprotective regulatory program.

a) The Isolated mouse heart perfusion model and TNF α mediated cardiac protection

To date, data surrounding the role of TNF α in the heart has been not only of a conflicting nature, but also predominantly maladaptive as to its function in the heart. Not only has TNF α been shown to be expressed in virtually all forms of cardiac injury (Mann, 1996), but the serum levels of TNF α have been correlated with the progression of heart failure (Sack *et al.*, 2000), and over expression of the cytokine in the hearts of transgenic mice was shown to result in myocarditis (Kubota *et al.*, 1997). However, recently there seems to be the emergence of data revealing a new role for TNF α in the heart, that of protection (Mann, 1996). This cytokine may have adaptive effects directing innate cardiac protection against ischemia (Kurrelmeyer *et al.*, 2000), against viral cardiac pathogens (Wada *et al.*, 2001) and in promoting adaptive cardiac hypertrophic growth (Smith *et al.*, 2001).

The cell surface ligands that have been shown to induce preconditioning have classically acted via G_i-protein coupled receptors and were postulated to act predominantly via similar intracellular signalling events (Nakano *et al.*, 2000). Interestingly, recent data is beginning to question the commonality of cell surface receptor mediated signalling in preconditioning (Cohen *et al.*, 2001). Our data adds to this knowledge base and identifies a possible G_i-protein-independent signalling cascade that may also induce the preconditioning mediated cytoprotective program. Mann and colleagues (Kurrelmeyer *et al.*, 2000) have provided the strongest evidence in support of a role for TNF α in cardiac protection utilising TNF α R1and TNF α R2 knockout mice. Their data suggested that TNF α signalling gave rise to

one or more cytoprotective signals that prevented or possibly delayed the development of cardiac myocyte apoptosis after ischemic injury.

By setting up a model of ischemic preconditioning in the mouse heart we were able to establish an intrinsic adaptive role for TNF α. The cytokine levels were shown to be elevated in response to an ischemic preconditioning stimulus and we were able to use recombinant TNF α as a preconditioning mimetic in both the wild type and the TNF α knockout mouse. In conjunction to this ischemic preconditioning could not protect the myocardium in the TNF α knockout mouse.

The fact that pre-administration of TNF α, 24 hours prior to TNF α preconditioning, was required to induce preconditioning-like protection suggests that in the knockout mouse, the signalling pathways downstream to TNF α may be down regulated. Although the downstream mechanisms of the protective effects of TNF α are unknown, stimulation of cultured cardiomyocytes with TNF α has been shown to provoke Heat Shock Protein 72 (HSP 72) expression. HSP 72 is the major HSP which has been shown to be induced in response to certain forms of environmental stress (Marber *et al.*, 1995). TNF α has also been linked to increased expression of the anti-oxidant MnSOD (Nelson *et al.*, 1995) as well as upregulation of NFκB dependent cytoprotective pathways within minutes of TNF α exposure (Natoli *et al.*, 1998). Interestingly, adenosine was able to confer preconditioning-like protection in the murine heart.

b) The Mitochondrial K_{ATP} channel and ischemic protection in the murine heart

In this study the mitochondrial K_{ATP} pharmacologic antagonist data does implicate a common role for mitochondrial biology as a pivotal intracellular regulator of ischemic preconditioning. However, it has been postulated that activation of the sarcolemmal K_{ATP} channel was required for ischemic preconditioning (Gross,Fryer, 1999).

Two distinct populations of K_{ATP} channels have been shown to exist in cardiomyocytes (Kim *et al.*, 1994; Kukreja *et al.*, 1990). It is a matter of debate as to which channel is important for the cardioprotection due ischemic preconditioning (Gross,Fryer, 1999). Evidence for the importance of both as well as for one or the other exists. In studies in the rat as well as the rabbit heart, pre-treatment of the myocardium with diazoxide could protect the heart from ischemic damage (Garlid *et al.*, 1997; Pain *et al.*, 2000). While in a separate study in the rabbit, diazoxide failed to protect the myocardium from ischemic damage. In addition to this evidence 5-HD attenuated protection in both the rat and rabbit. Interestingly, in the dog, both channels had to be blocked in order to attenuate myocardial protection due to ischemic preconditioning.

The question that still needs to be answered is how activation of the mitochondrial K_{ATP} channel results in cardioprotection? Two main theories have been proposed. Some believe that activation of the mitochondrial K_{ATP} channel decreases mitochondrial calcium overload via a reduction in calcium uptake and increased calcium release. This would in turn preserve the integrity of the mitochondria (Schultz R, 2001). A second hypothesis relates to change in mitochondrial volume which is regulated by opening the K_{ATP} channels. These volume changes have been shown to

be important as they influence efficient energy transfer between mitochondria and cellular ATPases (Schultz R, 2001). However, there is still a possibility that the mitochondrial K_{ATP} channel is not the end-effector but rather a signal transduction element. Only time will tell.

Conclusion

We have shown that the pleiotropic cytokine TNF α is required to induce ischemic preconditioning in the mouse heart and that in parallel to Gi-coupled receptor signalling seems to confer this cardioprotection via activation of the mitochondrial K_{ATP} channel. Additionally, this study in conjunction with other findings in studies described above identifies TNF α signalling as a novel system to study in order to facilitate our understanding of the molecular and cellular mechanisms underlying the cardio-protected phenotype induced by preconditioning. Moreover, the data presented support the emerging concept that the innate immune system may play an important role in adaptive cyto-protective effects in the heart.

Future Directions

The TNF α signal transduction pathway still requires a large amount of work to delineate the pathway. In recent studies of the TNF α signalling pathway, the transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) has been implicated to play an important role in TNF α mediated cardioprotection. This transcription factor is part of a signalling pathway known as the Janus Kinase (JAK) – STAT pathway.

STAT proteins, which were discovered while investigating gene regulation of type 1 interferons, are cytoplasmic proteins that are actively involved in the activation of target genes. Seven STAT proteins are known to occur, STAT 1 – STAT 4 as well as STAT 5a, STAT 5b and STAT6 (Copeland *et al.*, 1995). They are about 700 – 850 amino acids in length and possess several conserved domains, which have been found to be critical to STAT functional abilities. They are activated by tyrosine phosphorylation by JAK, which is associated with cell surface receptors. Recruitment of STAT transcription factors to the cellular membrane occurs via a conserved Src Homology 2 (SH2) domain, which binds to an activated phosphorylated receptor. The JAK associated with the activated receptor, phosphorylates the STAT molecule, which then dimerises and translocates to the nucleus (Darnell, 1997) where it can increase transcription of numerous genes. Various Receptor Tyrosine Kinases (RTK) can activate STAT 1, STAT3 and STAT 5 but not much is known about the specificity of this mechanism of STAT activation.

The JAK – STAT pathway has been implicated in cardiac hypertrophy (Kunisada *et al.*, 1996), apoptosis (Sheng *et al.*, 1997) and inflammation (McWhinney *et al.*, 1997). In recent studies, evidence for a role of the JAK – STAT pathway in cardiac

protection has come to the surface. Activation of STAT3 (Omura *et al.*, 2001) has been reported in rat models of myocardial infarction produced by permanent coronary occlusion, where it has been suggested to limit apoptosis. STAT3 has also been found to exert antiapoptotic effects in cultured neonatal rat cardiac myocytes subjected to anoxia, metabolic inhibition and acidosis (Stephanou *et al.*, 2000). In a recent study in rats (Hattori *et al.*, 2001) a potential role for STAT3 was elucidated. Here they showed that JAK 2 and STAT3 were both rapidly activated in response to ischemic preconditioning and that inhibition of JAK 2 blocked the cardioprotective abilities of preconditioning suggesting the importance of STAT3 activation. Also, TNF α activation has been linked to STAT3 upregulation in cardiac myocytes. Interestingly, in the SWOP, Xuan *et al* were able to show a protective role for the JAK-STAT pathway in preconditioning (Bolli *et al.*, 2001).

With strong evidence for a protective role for STAT3 activation, we looked at the levels of STAT3 in response to the preconditioning stimulus in our model of early protection. Our preliminary results showed a marked increase in the level of STAT3 phosphorylysation in response to the ischemic preconditioning stimulus.

As we had successfully set up the first model of ischemic preconditioning in the isolated mouse heart in Africa, and as we were interested in TNF α mediated cardioprotection, we decided to generate a heart specific STAT3 knockout mouse to further study this innate mechanism of cardioprotection (Craig *et al.*, 2000).

a) Generation of a myocardial specific STAT 3 knockout mouse

Earlier attempts to knock STAT3 out in a mouse were unsuccessful, as this such a knockout was found to be embryo lethal (Takeda,Akira, 2000). Due to this we obtained a STAT3 floxed mouse from Prof. Shizuo Akira of Osaka University in Japan and the Cre recombinase expressing mouse was a gift from Prof. K Chien of the UCSD. To generate this mouse, a Cre-Lox P system was used, employing STAT3 floxed mice (Takeda *et al.*, 1998).

In these mice Lox P sites were introduced into introns 21 and 22 surrounding the exon coding for the activation site of STAT3 (fig.13). Expression of the Cre recombinase protein, which cleaves DNA at these floxed sites within the organ, would result in the excision of this exon. The mouse would then produce a non-functional truncated form of STAT3 (fig.13). To ensure a heart specific knockout mouse was produced, the inserted Cre gene was placed under the control of the Myosin Light Chain 2V (MCL 2V) gene which is activated primarily in the adult mouse heart ensuring a heart specific knockout (Chen *et al.*, 1998; Hirota *et al.*, 1999).

To achieve this, female homozygous floxed STAT3 mice (STAT3 $^{F/F}$) were crossed with male heterozygous Cre mice (Cre $^{T/N}$)(fig.14). The resultant offspring were screened via PCR and the male Cre positive heterozygous STAT3 floxed (Cre $^{T/N}$ STAT3 $^{F/N}$) mice were back-crossed with female homozygous STAT3 floxed mice (fig.14). This gave us heart specific STAT3 knockout mice (Cre $^{T/N}$ STAT3 $^{F/F}$). These

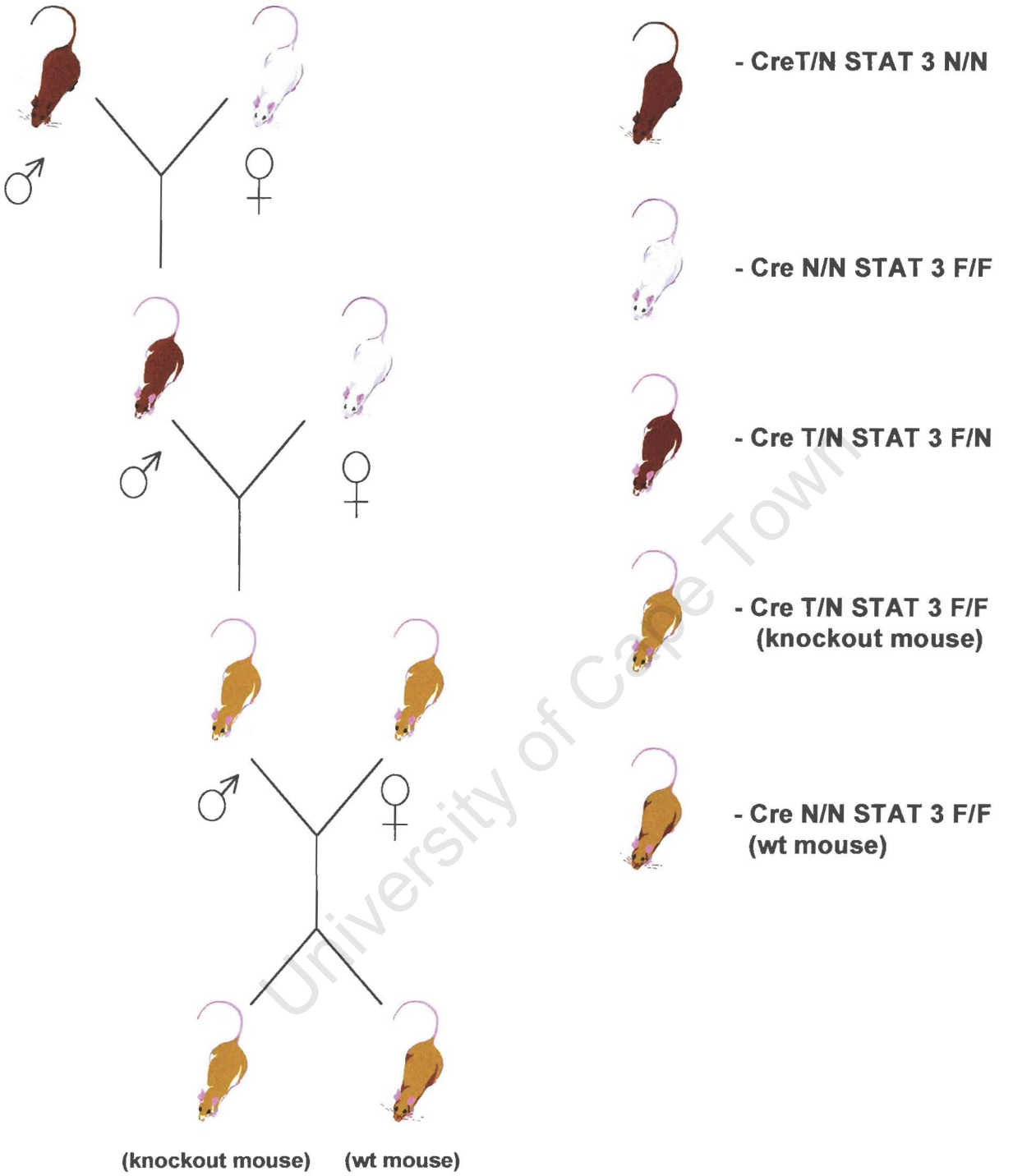

Figure 14: Generation of ventricle specific knockout mice.N - normal, T - Transgenic Cre insert, F-Floxed STAT 3. Of note is that Cre $^{T/T}$ is embryo lethal due to the absence of MLC 2V. Here no offspring can be Cre $^{T/T}$

transgenic knockout mice were then bred to give us both the knockout mice as well as a littermate control strain (Cre $^{N/N}$ STAT3 $^{F/F}$) (fig.14).

b) Genotyping of STAT3 knockout mice

At each step of the breeding process mice had to be genotyped as the breeding of STAT3 knockout mice gave rise to both the wild type and the knockout mice. Therefore mice had to be tested to see whether they were harbouring the Cre recombinase insertion as well as whether they were floxed or not.

For this, the forward Cre primer, 5' - GTT CGC AAG AAC CTG ATG GAC A - 3' and Cre reverse primer, 5' - CTA GAG CCT GTT TTG CAC GTT C - 3',was used to determine whether the mice were Cre positive (fig.15). Besides genotyping for Cre recombinase, mice were genotyped for the MLC 2V gene as a positive control for the presence of DNA. For this the MLC 2V forward primer, 5' - GGC AAC CCT CAG ACA CCA T - 3' and the MLC 2V reverse primer, 5' - TGT GGA GGC TCT GGA TCA GGA C - 3', was used (fig.15). For both, reactions were performed in 50μl volumes containing Mg free buffer, 4mM Mg, 100μM dNTP's, 0.2μM primer mixture, 1.25U Taq polymerase and 1.5μl DNA. The reaction mixtures were incubated at 94°C for 4 minutes then run for 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds and at 72°C for 1 minute finishing with a 10 minute incubation at 72°C.

To determine whether STAT3 was floxed or not, one set of primers were used yielding PCR fragments of different lengths (fig.16). If harbouring a floxed STAT3, then a 250 bp fragment is seen on the gel, if STAT3 is not floxed, then a 200 bp

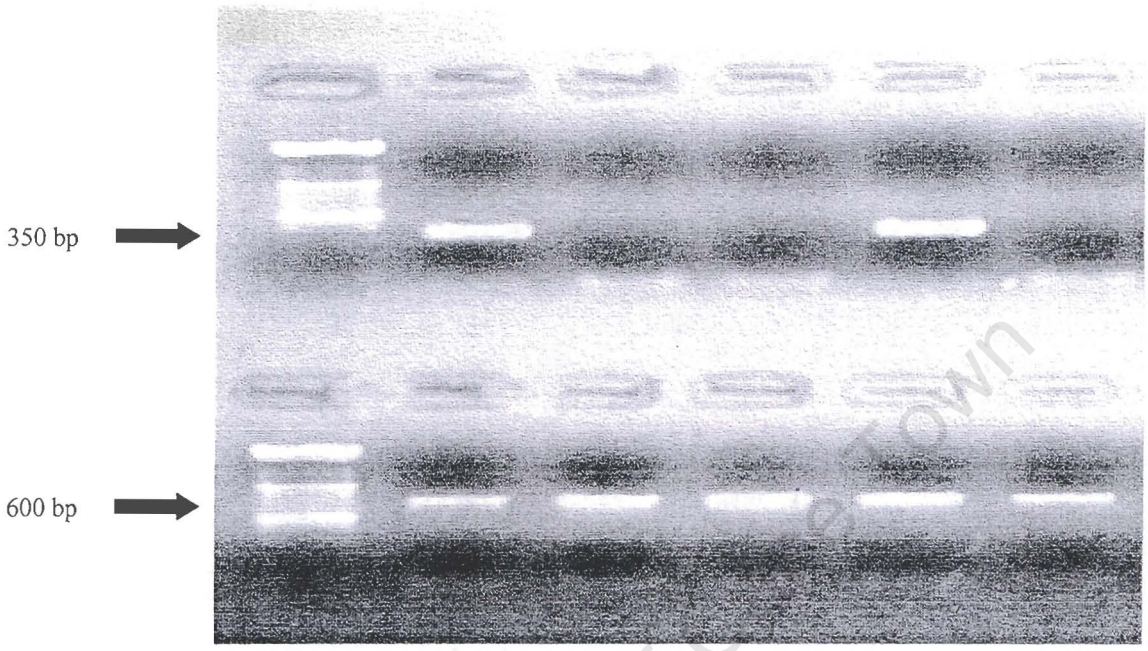

Figure 15 : Agarose gel showing Cre positive band (350 bp) as well as the control Myosin Light chain 2V (MLC 2V) band at 600bp. Presence of the larger MLC 2V band was used as a positive control for the presence of DNA. All Cre positive mice were heterozygous for Cre as loss of both MLC 2V genes resulted in embryo lethality. M - marker

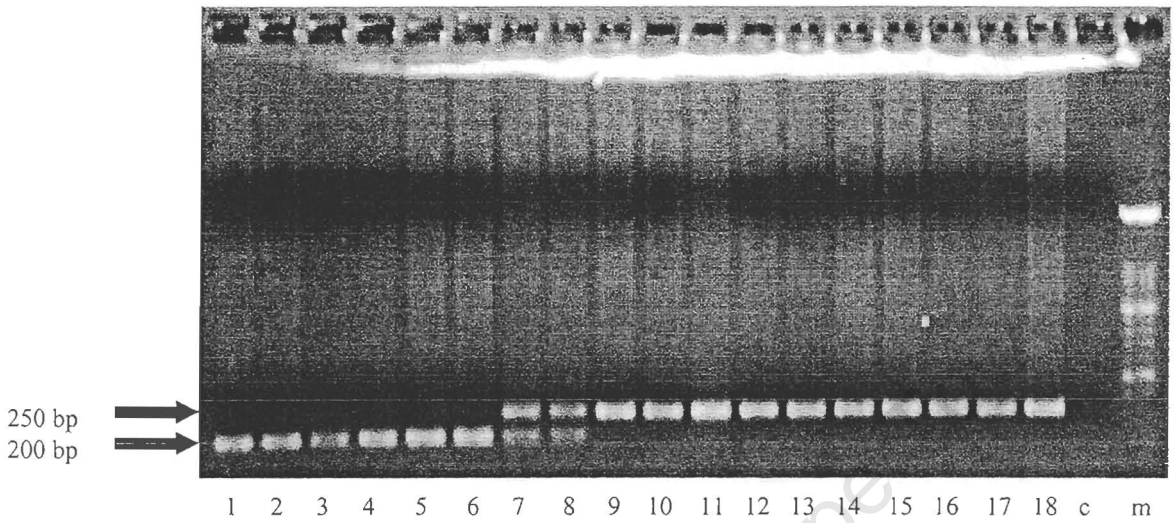

Figure 16 : Agarose gel showing STAT 3 $^{N/N}$ mice (1-6), no flox sites inserted, heterozygous STAT 3 floxed mice, STAT 3 $^{F/N}$ (7-8) and homozygous STAT 3 floxed mice, STAT 3 $^{F/F}$ (9-18).C is the water control and M is the marker

fragment is visualised on the gel. If the mouse being tested is heterozygous (STAT3 $^{F/N}$) then a double band is seen (fig.16). For the amplification of the STAT3 floxed or non-floxed DNA fragment, STAT3 primer a, 5"- CCT GAA CAA GTT CAT CTG TGT GAC – 3' and STAT3 primer b, 5' – CAC ACA AGC CAT ACT CTG GTC TCC – 3' was used (fig.16). Reactions were performed in 25μl volumes containing Mg free buffer, 4mM Mg, 100μM dNTP's, 0.2μM primer mixture, 0.625U Taq polymerase and 1.0μl DNA. The reaction mixtures were incubated at 94°C for 7 minutes then run for 35 cycles at 94°C for 30s, 60°C for 1 minute and 74°C for 1 minute finishing with a 10 minute incubation at 74°C. Sterile water controls were included with each batch of reactions (fig.16).

12μl of each PCR mixture was mixed with 2μl gel loading buffer (0.25% Bromophenol Blue–Xylene Cyanole Dye Solution, Sigma) and electrophoresed on a 1.2% agarose (Whitehead Scientific) gel. Gels were stained with ethidium bromide (0.1μg/ml) and photographed.

We have now generated the required knockout mouse for further studies regarding cardiac protection via TNF α signalling. Our future studies will delineate and characterise the requirement/role of this regulatory peptide in both TNF α and ischemic preconditioning.

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