EFFECTS OF *VISCUM ALBUM* ON CARDIAC TISSUE IN RATS

by

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ABSTRACT

EFFECTS OF *VISCUM ALBUM* ON CARDIAC TISSUE IN RATS

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Viscum album L. (European mistletoe) comprises a semi parasitic plant which grows on various host trees in Turkey and Europe. It has been widely used as a herbal treatment since ancient times for hypertension and other cardiovascular disorders. Its cardioprotective action has been attributed to the vasodilatation increased by nitric oxide synthase activity and antioxidant activity. However, little is known about its effects on atrial tissue and myocardial ischaemia-reperfusion injury. We hypothesized that *V. album* is able to limit infarct size under ischeamia-reperfusion injury. This study was designed to evaluate the cardiac & cardioprotective effects of *V. album* crude extracts on isolated heart preparation, using the Langendorff technique in an ischaemia-reperfusion infarct model and isolated rat atrial muscle.

Both administration of aqueous *V. album* (AVa) and methanolic *V. album* (MVa) extracts during ischaemia - reperfusion significantly limited infarct size compared to controls. Inhibition of nitric oxide synthesis completely abrogated this protection in both extracts. K_{ATP} channel blockage by glibenclamide also abrogated

the protection afforded by MVa but perfusion of glibenclamide along with AVa showed a synergistic action. Nevertheless, aqueous *V. album* extract induced negative inotropic and chronotropic effects in the rat atrial muscles, in vitro. None of the pharmacological agents used in this study abrogated the negative inotropic effects of *V. album* extract.

These data suggest that *V. album* extracts can afford cardioprotection by limiting infarct size during ischaemia reperfusion. Furthermore, our study is of great significance in terms of being the first research in the experimental field of infarct model with V. *album*. Further studies need to be performed to investigate the interaction of *V. album* extracts and more specific agents. These need to be focused on the isolation, purification, structural dilucidation, pharmacological reevaluation in order to find the chemical compound responsible for such a pharmacological effect.

Keywords: *Viscum album* L., cardioprotective, ischaemia - reperfusion injury, nitric oxide, vasodilation, Langendorff, infarct, K_{ATP} channel, inotropy, antioxidant.

ÖZET

SIÇAN KALP DOKUSUNDA ÖKSE OTU' NUN (*VİSCUM ALBUM* L.) ETKİLERİ

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Viscum album L. *(*Avrupa Ökse Otu*),* Türkiye ve Avrupa'da pek çok konakçı ağaç üzerinde yaşayan yarı parazitik bir bitki türüdür. Antik çağlardan beri yaygın olarak yüksek tansiyon ve diğer kalp rahatsızlıkları için bitkisel tedavide kullanılmaktadır. Bitkinin kalp koruyucu etkisi nitrik oksit sentezi ve anti- oksidant aktivitesi aracılığı ile arttırılan vazodilatasyona dayandırılmaktadır. Ancak, bitkinin myokardial iskemi - reperfüzyon hasarı ve izole atrium kası üzerine olan etkileri hakkında çok az şey bilinmektedir. Bu noktada, *V. album'*un iskemi reperfüzyon hasarı ile oluşan enfarkt alanı azaltan bir etki gösterebileceğini öne sürmekteyiz. Bu çalışma, *V. album* özütlerinin izole atrium kasında ve iskemi - reperfüzyon enfarkt modelini, Langendorff tekniği kullanılarak izole kalp preparatlarında kalp dokusuna ve koruyucu etkilerini değerlendirmek amacı ile tasarlanmıştır.

İskemi – reperfüzyon süresince uygulanan her iki su ve methanolik *V. album* özütleri enfarkt alanı kontrole göre anlamlı bir şekilde azaltmıştır. Her iki özütün koruyucu etkisi NO sentezinin inhibisyonu ile tamamen ortadan kalkmıştır. Potasyum kanallarının glibenclamide ile kapatılması MVa'nın koruyucu etkisini ortadan

kaldırmıştır ancak AVa'ın glibenclamide ile perfüzyonu sinerjik etki göstermiştir. Ayrıca, *V. album* su özütü izole sıçan atrium kasında negatif inotropik ve kronotropik etki göstermiştir. Çalışmamızda kullanılan farmakolojik ajanların hiçbiri AVa'nın negatif inotropik etkisini ortadan kaldırmamıştır.

Bulgular, *V. album* özütlerinin iskemi reperfüzyon hasarında enfarkt alanı azaltarak korucu etkisi olduğunu desteklemektedir. Dahası çalışmamız deneysel enfarkt model araştırmalarında ilk olması bakımından önemlidir. İleride yapılması gereken araştırmalarda, *V. album* özütlerinin ve farmakolojik ajanların etkileşimlerinin incelenmesi gerekmektedir. Bu tür bir farmakolojik etkiden kaynaklanan kimyasal bileşiği bulmak için izolasyon, saflaştırma, yapısal tayin belirleme ve yeniden farmakolojik değerlendirmelere odaklanmak gerekmektedir.

Anahtar kelimeler: *Viscum album* L., kalp koruyucu, iskemi – reperfüzyon hasarı, nitrik oksit, vazodilatasyon, Langendorff, enfarkt, ATP bağımlı potasyum kanalları, inotropi, antioksidan

To my lovely family and my fiancé Dr. C. Andrew Ford

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ABBREVIATIONS

5-HD	5-hydroxy decanoate
Ach	Acetylcholine
AMI	Acute myocardial infarction
ANF	Atrial natriuretic factor
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AR	Area at risk
ATP	Adenosine triphosphate
AUC	Area under the curve
Ava	Aqueous *Viscum album*
BNP	Brain natriuretic peptide
BPM	Beats per minute
cAMP	Adenosine-3', 5'-cyclic monophosphate
CAO	Coronary artery occlusion
CFR	Coronary flow rate
cGMP	Guanosine-3', 5'-cyclic monophosphate
CHD	Coronary heart disease
CNP	C-type natriuretic peptide
C-PTIO	2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-Imidazolyl-1-oxy-3-oxide, monopotassium salt

CTEPH	Chronic thromboembolic pulmonary hypertension
CVD	Cardiovascular disease
DETA-NO	Diethylenetriamine NONOate
DMSO	Dimethyl sulphoxide
ECG	Electrocardiogram
EDRF	Endothelium derived relaxing factore
NOS	Endothelial nitric oxide synthase
EPR	Electro paramagnetic resonance
GC	Guanylyl cyclase
GSK3β	Glycogen synthase kinase 3 beta
GSNO	S-Nitrosoglutathione
GTP	Guanosine triphosphate
HPLC	High performance liquid chromatography
HR	Heart rate
HSP	Heat shock protein
I	Infarct
iNOS	Inducible nitric oxide synthase
IPC	Ischaemic preconditioning
JAK	Janus kinase
K_{ATP}	Adenosine triphosphate-sensitive potassium channel
KO	Knockout
LDCA	Left descending coronary artery
L-NAME	Nω-Nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
LV	Left ventricle

LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end diastolic pressure
MAPK	Mitogen activated protein kinase
MVa	Methanolic *Viscum album*
mPTP	Mitochondrial permeability transition pore
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOA	Nitric oxide analyser
NOC-9	6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-Hexanamine
NOS	Nitric oxide synthase
NP	Natriuretic peptide
NPR	Natriuretic peptide receptor
NSB	Nonspecific binding
OBC	Ozone based chemiluminescence
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PAH	Pulmonary arterial hypertension
PDE	Phosphodiesterase
pGC	Particulate guanylyl cyclase
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKG	Protein kinase G
PLB	Phospholamban
PPCI	Primary percutaneous coronary intervention

PVDF	Polyvinylidene fluoride
RIA	Radioimmunosorbent assay
RISK	Reperfusion injury salvage kinase
ROS	Reactive oxygen species
RPP	Rate pressure product
RV	Right ventricle
SAFE	Survivor activating factor enhancement
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	Sarcoplasmic endoplasmic reticulum calcium ATPase
sGC	Soluble guanylyl cyclase
SNAP	*S*-Nitroso-*N*-acetylpenicillamine
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
STAT	Signal transduction activator of transcription
STEMI	ST elevation myocardial infarction
TCA	Trichloroacetic acid
VF	Ventricular fibrillation
VPB	Ventricular premature beat
WHO	World health organization

CHAPTER I

1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Viscum album L. (mistletoe) is an evergreen, perennial, epiphytic, dioecious small shrub that lives semi parasitically on a wide range of tree host plant species. It is native to Europe that is known as European mistletoe (Chevallier, 1996) and also known a pathogen, a pharmaceutical plant and a symbol in mythology (Efraim et al. 2011). *Viscum* includes approximately 100 species most of them in Africa and Madagascar and a smaller number in southern Asia. Only a few species are known from Europe and temperate Asia, Malaysia and eastern Australia (Barlow, 1983 & Barlow, 1987). The European white – berry mistletoe (Figure 1.1) belongs to the Loranthaceae family within the order Santales (Bremer, 1998). Originally the plant belonged to Viscaceae which has now been included in Loranthaceae (Nickrent, 2002). In Turkey, it is found in North West of Turkey and North, South and East parts of Anatolia (Davis, 1972). Other names for this species include European mistletoe, golden bough, bird lime, devil's fuge, herb de la croix (Fr), lignum crucis, visci albi fructus (berries), visci albi herba (leaves) and viscum. It is called as ökse otu, gökçe, purç and pür in Turkish.

Three subspecies are recognised one covering the whole range of host species (Ball, 1993; Böhling et al, 2002). Two of them grow on conifer hosts: *V. album* L. subsp. *abietis* (Wiesb.) Abromeit on fir (*Abies* Mill.)*, V. album* L. subsp. *austriacum* (Wiesb.) Vollmann on Scots pine *(Pinus sylvestris* L.). The best known Eurpean mistletoe is *V. album* L. subsp. *album*, growing on decidous trees, mostly on apple, ash, poplar, hawthorn (Figure 1.2&1.4), willow and many other species growing in range but very rarely on oak and never on beech. The species has linear lanceolate leathery leaves which last for several seasons and whitish, transculucent berries that develop in the late fall and early winter from the yellow to green flowers which grow in the sprout axil (Büssing, 2000).

1.2. Literature Review

1.2.1. Botany

V. album is an evergreen hemiparasitic shoot parasite and maximum age of a shrub is about 27-30 years. *V. album* reproduces predominantly sexually. Vegetative propagation is rare. Birds are the main vectors of mistletoes. The seeds survive the passage through the digestive tract unharmed and are released with feces. *V. album* grows up to 1m high, with numerous, regularly forked branches (Grieve, 1982).

The stem grows without branching for the first 3 or 4 years. The leaves are borne in pairs, each leaf 5–7cm long, widest above the middle, narrowly oval and slightly curved in outline (Figure 1.3). They are matt yellowish - green in colour and have a distinctive leathery texture. The flowers are fly pollinated and are very small and inconspicuous, appearing from February to April in clusters of 3 to 5. Male and

female flowers are borne on different plants. Both sexes have 4 small, yellowish green petals - the sepals of female flowers are extremely small and are completely absent in male flowers.The waxy berries are 1 cm in diameter, white and sticky and are only produced by female plants. Birds eat the berries and disperse the seeds, but the berries are poisonous to humans.

Figure 1.1 Picture of the white berry mistletoe taken by Eylem Suveren, 2009.

Figure 1.2. *V. album* hosted by *Crataegus tanacetifolia* (Lam.) Pers (Tansy-leaved Thorn) in AIBU campus area by Eylem Suveren, 2013.

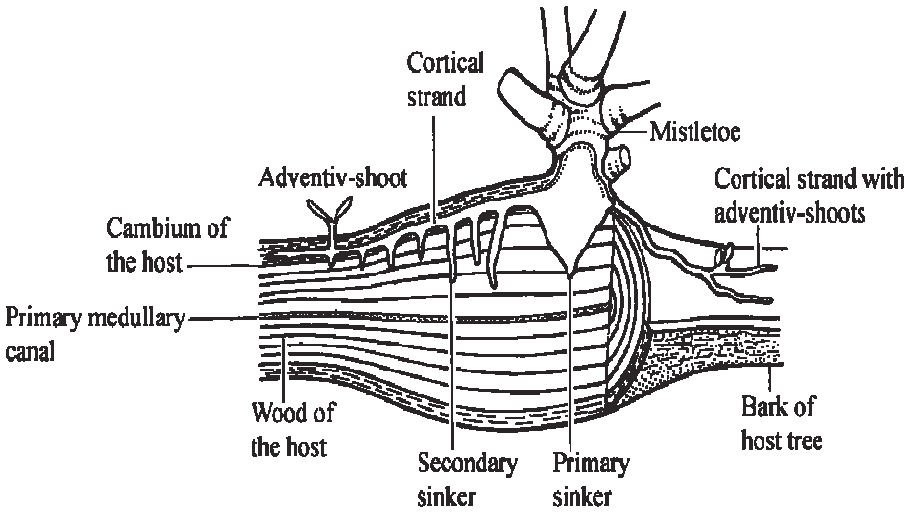


Figure 1.3. Perennial mistletoe on a host shoot. Left part shows longitudinal section, right part topview with partly removed bark (Nierhaus & Lawrenz, 1997).

1.2.2. Folkloric Usages of *V. album*

V. album is widely used in the folk medicine and traditionally used for the prevention and treatment of several diseases. In Classical times Dioscorides (1st century CE) described the Ixos, which was identified with white mistletoe and listed its medical uses: to treat swellings, to soothe, to cure stomach ulcers, to treat problems of the spleen, and to remove stones from the urinary tract (Gunther, 1959). Pliny describes mistletoe and the methods of making birdlime out of it, as well as the superstitions about it of the Gauls, including its worship on the fifth day of the moon (Pliny, 2000). Paracelsus and Matthiolus (15 and 16th century) mention mistletoe for the treatment of epilepsy (Dymocck, 1972). The tradition of "kissing under the mistletoe" originated in Scandinavian legend. Bakder, the god of peace, was killed by an arrow made of mistletoe and was resurrected by the other deities. Common mistletoe was then entrusted to the goddess of love, who established it as a symbol of

love; it was customary that anyone passing beneath it should receive a kiss (Bown, 1995). A festival in honour of the mistletoe, called Guilanlen, was celebrated in France as late as the 16th century; in England the plant is still hung in the living rooms at Christmas (Dymocck, 1972).

In German folklore mistletoe bestows the power to see ghosts and make them speak. In traditional European medicine it was believed that the medicinal uses of the common mistletoe varied according to the host tree; the Druids held mistletoe on oak supreme, probably compounds that effect protein synthesis, and benefit the immune and circulation systems and the heart (Bown, 1995). Common mistletoe has a pungent, bitter-sweet taste, and is a warming herb that lowers blood pressure, stimulates the immune system, slows heart beat, relaxes spasms, and has sedative, diuretic, and anti-cancer effects. The plant is used internally, for mild hypertension, hardening of the arteries, nervous tachycardia, nervous tension, and cancer (mainly of lung and ovaries). Externally the common mistletoe is used for arthritis, rheumatism, chilblains, leg ulcers, and varicose veins. It is also used for the treatment of mild hypertension with *Cartaegus laevigata* and *Mellisa officinalis* and hardening of the arteries with *Ginkgo biloba* and *Vinca major* (Bown, 1995).

Many authors of Arabic medical books mention mistletoe identified in various dictionaries as *V. album* (IssaBey, 1930 & Levey, 1966). But this plant does not grow in the Middle East. It suggest that the plant used by the medieval Middle Eastern physicians, herbalists and pharmacologists was the local *V. cruciatum*; in those times they often could not differentiate species of the same genus, so many names were collective. These sources also describe mistletoe's nature and various uses, including medicinal. It was used as laxative and a solvent of corrupt humours, to cleanse the system of adust bile and phlegm (with walnuts and castor oil) and

remove obstructions, and as a remedy for lumbago and piles. Applied externally, it was used to promote suppuration, or cause dispersion of tumours or enlargements. According to al - Kindi (9^{th} century Iraqi physician), the mistletoe resin was a medication for mouth cancer, tooth decay, and stomach ulcers (Levey, 1966).

Ibn al - Baytar claimed that the plant grew mainly on olive, almond, and pear trees. He cited various physicians who listed the medical properties and uses of the plant; they included knitting broken bones and treating cracks in the skin, treating torn muscles and helping the cure coughs (when boiled with figs), and skin diseases. In his view the plant was cold, constricting, and dry (Leclerc, 1983).

According to Daud al-Antaki (16^{th} century Turkish physician) and known physicians that he mentioned, mistletoe is a cold, dry and astringent drug, and rather bitter. It healed damaged muscles, treated blood spitting, opened obstructions, cleansed the mind and the stomach, knitted fractures, cured cough and skin diseases, and dried haemorrhoids .

By the 18th century the medical use of *V. album* had become widespread in Europe and was associated with superstitious and magical practices (Kanner, 1939). In the 19th century several physicians in Europe and the USA started to study the plant and its medicinal uses with modern tools, and thereafter used it in various forms to treat, among other things, menorrhagia and post-partum haemorrhage, and as a substitute for digitalis. The active materials of the plant's berries and leaves were studied from the 19th century as well (Dymocck, 1972). *V. album* is used extensively in Turkish traditional medicine, and is sold in shops specializing in traditional *materia medica*. Its main medicinal uses are for pains, allergy, bedwetting children, hypotension, tuberculosis, stomach and duodenal ulcers; as a prophylactic in arteriosclerosis, and for stomachache and wounds (Baser, 1986). In traditional

medicine in Egypt the plant is used for the treatment of epilepsy, arteriosclerosis, and diseases of cardiac arteries, and as a hypotensive (Kamal, 1975).

V. album has been used for therapeutic purposes in human cultures for centuries and it has been taken as tea or decoction as a traditional therapy in treatment of many diseases.

Figure 1.4. Picture of *V. album* on host tree-*Crataegus tanacetifolia* (Lam.) Pers (Tansy-leaved Thorn) in AIBU campus area by Eylem Suveren, 2013.

1.2.3. Biologically active components of *V. album*

The first investigation into the chemical content of mistletoe was carried out by Reinsch in 1860 (Franz, 1985). He analysed the sticky substance contained in berries, which he named viscin. Now, we know that *V. album* is a rich source of various pharmacologically active compounds. The main group of secondary metabolites found in mistletoe are; glycoproteins, polypeptides, alkaloids, amines, flavanoids, acids and terpenoids. Franz et al. (1981), isolated three lectins from *V. album* which are the main constituents of mistletoe, they also known as viscumin or agglutinin are cytotoxic glycoproteins (Mistletoe Lectin I, II, III). Lectins cause cells to agglutinate and inhibit protein synthesis on the ribosomal level (Stirpe et al. 1992). ML I is widely used in immunomodulation and anticancer studies since it was shown to pronounced immunomodulatory (Hajto et al. 1989). Bussing et al. (1996) reported MLIII to be most active (followed by ML II and ML I) in the induction of apoptic cell death. The other important active group of compounds synthesised by the mistletoe are the viscotoxins. In 1942, first isolation of viscotoxin was made by Winterfeld and its toxicity was reported. The most important viscotoxins are A2, A3 and B (Lamont, 1983). Viscotoxin is a 46 aminoacid peptide that damages cell membrane. Viscotoxin is found only in *V. album* Various polysaccharides are thought to be involved in mistletoe's antineoplastic effects. The leaves and stem contain esterified galacturonan, while the berries contain primarily arabinogalactan. Alkaloids are nitrogenous compounds thay may contribute to mistletoe's cytotoxicity (Jordan & Wagner, 1986).

The amounts and biologically active *V. album* compounds may show great variability depending upon the host tree and harvesting time. Concerning the

quantitative composition, only a few studies were conducted on the particular content of the subspecies; viscotoxins (Schaller et al. 1998), phenylpropanaoids (Deliorman, 1999) and flavonoids (Lorch, 1993) were comparatively investigated and shown to be exist in different concentrations depending up on the subspecies. Moreover, it was reported that leaves and twigs of this plant contain ursolic acid, kaempferol, quercetin, peçtin, β – sitosterol, syringin, and lectins as main components (Ergun & Deliorman, 1995).

1.2.4. Potential clinical benefits of *V. album*

Mistletoe is reputed to possess hypotensive, cardiac depressant and sedative properties, used particulary in the aged for the treatment of symptoms of cardiovascular diseases including dizziness, fatigue, and irritability (Calvin, 2006). A number of in vivo and in vitro studies were conducted on the plant material have revealed that mistletoe exert various biological activities including, antioxidant (Deliorman et al. 2005 & Ucay et al. 2006), antidiabetic (Gray & Flatt, 1999 & Yeşilada et al. 1998 & Simsek et al. 2004 & Deliorman et al. 2004), antibacterial (Ertürk et al. 2003), antimycobacterial (Deliorman et al. 2001), antiviral (Karagöz et al. 2003), anti-inflammatory (Newall et al. 1996), immunostimulant (Fischer et al. 1997), antihypertensive (Eno et al. 2004 & Ofem et al. 2006 & Duke, 2001), anticancer (Maier et al. 2002), and antihypercholesterolaemic (Avcı et al. 2006).

1.2.4.1. Cardiovascular features of *V. album*

Viscotoxin is decreased contractile force and exerted its negative inotrophic effect on the heart muscle which has been reported by Rosell & Samuelsson in 1966. Wagner et al. (1986), investigated that flavanoids, phenol carboxlic acids, phenyl propanes and lignans are postulated as the possible derivatives of cardiovascular activity in *V. album* and they also suggested that butanolic extracts of *V. album* play a role in the inhibition of $_{C}$AMP - phosphodiesterase (PDE) and a correlation was proposed between the invitro inhibition of PDE and in vivo pharmacological activity. Ergun et al. (1995) has shown that some ethanol extracts caused significant relaxations on rat aortic ring whereas aqueous & n- butanolic extracts did not exhibit any relaxant activity. Deliorman et al. (2000) reported that only one isolated phenolic compound (VA-15) displayed very slight relaxant response and there was no clear correlation between the weak relaxant effects of subfractions and the inhibitory effect on $_{C}$AMP –PDE. In an experimental study, it has been reported that GABA isolated from ground leaves of *V. album* produced sharp reduction in blood pressure of anesthesized rats (Samulsen, 1959). Similarly, a few investigations suggested that hypertension could be managed successfully using mistletoe therapy (Yucecan, 1988; Duke, 2001; Khosh and Khosh, 2001; Thompson, 2003). Ofem et al. (2006) have shown that the effects of crude aqueous leaf extract produced anti-hypertansive effect without any alteration in heart rate in rats. Another study has shown that crude extract from *V. album* cause deppression of blood pressure in normotensive and hypertensive rats in a dose-dependent manner (Eno et al. 2004).

Tenorio et al. (2005) has shown a significant coronary vasodilator activity of the aqueous extract of *V. album* by using the langendoorff‘s isolated and perfused

heart model. They reported that vasodilatory activity elicited by extract is mediated by the NO/sGC pathway (Tenorio et al. 2006). The effects of butanolic extracts of *V. album* samples were studied on platelet activating factor, collagen, arachidonic acid and thromboxane induced platelet aggregation (Sener et al. 1996).

1.2.4.2. Hypoglycemic effects of *V. album*

Mistletoe leaves was reported to exert a beneficial effect to alleviate the symptoms of diabetes in local medicines worlwide (Gray & Flatt, 1999). Hypoglycemic properties of *V. album* have been reported by Ohiri et al. (2003), in alloxan induced diabetic animals. Obatomi et al. (1994) have shown the anti-diabetic activity of aquoues African mistletoe extracts in STZ- induced rats. In Anatolia, the extracts of *V. album* species have been used for its antidiabetic activities as a folk remedy (Yeşilada et al. 1998; Simsek et al. 2004). Deliorman et al. (2004) demonstrated that *V. album* possess potent antihyperglycaemic activity. Another study has been shown that methanolic extacts of African mistletoe exhibited marked hypoglcemia and improved serum lipid profile of STZ –diabetic rats (Oluwatosin et al. 2012).

1.2.4.3. Antioxidant profile of *V. album*

In recent years, antioxidants derived from natural sources, mainly from plants, have been intensively used to prevent oxidative damage. Natural antioxidants have some advantages over synthetic ones. They can be obtained easily, economically and have slight or neglible side effects. Onay & Ucar et al. (2006)

investigated the free radical scavenging activity (DPPH), ferric reducing antioxidant capacity (FRAP) of methanolic *V. album* ssp. *album* extract and also they found that antioxidant capacity of plant differed according to the harvesting time as well as the host tree. Deliorman et al. (2005) studied the antioxidant activity of aqueous and ethanolic extracts of three *V. album* ssp. *album,* ssp. *abietis,* spp. *austriacum* was reported in liver, kidney, heart tissues of streptozotocin induced diabetic rats and determine antioxidant activity of tha extracts, tissue MDA and GSH levels were measured by using spectrophotometric methods. Avcı et al. (2006) showed that antihypercholesterolaemic and antioxidant activity of *V. album* were used as a remedy in Turkish folk medicine.

1.2.4.4. *V. album* as a potential anticancer drug

In 1916, Rudolf Steiner suggested for the first time the use of mistletoe extracts as possible for cancer therapy (Selg et al. 2007). *V. album* has gained notable attention due to its central role as leading remedy in cancer care in Anthroposophic medicine. Ita Wegman (1876–1943), one of the first Anthroposophic physicians, took up his proposal and with a pharmacist in Zürich developed the first mistletoe medication, Iscar, which was renamed as Iscador in 1926 (Annette, 1999). Mistletoe therapy has become the best-known Anthroposophic therapy, and in German-speaking central Europe it is now one of the most commonly prescribed complementary therapies for cancer (Kienle, 2006). In 2007, 9.2 million daily doses were sold in Germany, amounting to 22.5% of all chemotherapy agents that year (Schwabe et al. 2008). Other Anthroposophic *Viscum* preparations besides Iscador are Abnoba viscum, Iscusin, and Helixor. In modern times *V. album* has won notable

attention due to its central role as leading remedy in cancer care in Anthroposophic medicine. Subsequent in vitro and clinical studies followed *V. album's* use in Anthroposophic clinics in central and Western Europe. Laboratory studies indicate that *V. album* extract induces cancer cell apoptosis (Khil et al. 2007), cytotoxic activity (Valentiner et al. 2002), DNA repair in peripheral blood mononuclear cells (Kovacs, 2002), angiogenesis inhibition (Elluru et al. 2009), antiproliferative effects in cancer cell lines (Urech et al. 2006), maturation and activation of human dendritic cells (Elluru et al. 2008), and stimulation of GM-CSF, IL-5 and IFNgamma production (Huber et al. 2005). Clinical trials indicate that *V. album* may improve the quality of life of patients with breast cancer (Hornebar et al. 2008 & Semiglazov et al. 2006) and reduce the side effects of chemotherapy and radiotherapy (Bock et al. 2004). Preliminary studies suggest that *V. album* may also improve survival rates of patients with malignancies of breast (Ziegler et al. 2008), cervix and ovary (Grossarth & Ziegler, 2007), uterus (Grossarth & Ziegler, 2008) and colon (Cazacu et al. 2003), and of those with melanoma (Augustin et al. 2005). It may also lower recurrence of superficial bladder cancer (Elsasser et al. 2005). A systematic review of 37 clinical studies on mistletoe therapy showed an improvement of quality of life; significantly fewer adverse effects from chemotherapy, radiation treatment or surgery, tumor remission, and survival benefit, were shown in eight of 37 studies (Kienla & Kiene, 2007). Ostermann et al. (2009) reported on a pooled analysis of clinical studies and suggested that adjuvant *Viscum* treatment is associated with a better survival rate of cancer patients. Nevertheless, a Cochrane Database systematic review published in 2008 by Horneber et al. concluded that the evidence from randomized controlled trials to support the impact of *V. album* on survival is weak and that more high

quality, independent clinical research is needed to assess *Viscum*'s safety and effectiveness (Horneber et al. 2008).

1.2.4.5 Other studies

The bacteriostatic effect of *V. album* was investigated by Fulder in 1998. Antiviral (Karagöz et al. 2003) and antimicrobial (Ertürk et al. 2003) activities of *V. album* have been reported. Deliorman et al. (2001) reported the antimycobacterial activity of *V. album* extract. Gupta et al. (2012) revealed that the aqueous extract of *V. album* exibited sedative, anti-epileptic and anti-psychotic properties via facilitation of GABA transmission as well as blockade of D_2 receptors. Karakas et al. (2008) reported that *V. album* extracts decreased intestinal contractions in all intestinal segments in male Syrian hamsters.

1.3. PLANT MATERIAL PREPARATION

European mistletoe (*Viscum album* L. subsp. *album*) hosted by Tansy-leaved Thorn trees [*Crataegus tanacetifolia* (Lam.) Pers] was collected in Abant Izzet Baysal University (AIBU) Campus, Bolu, Turkey in May of 2009 and identification of the species was made by using "Flora of Turkey and the East Aegean Islands" (Davis, 1982). Voucher specimens (AUT-1604) were deposited at the AIBU Herbarium, Bolu, Turkey. Collected mistletoe leaves were dried in an oven at 35 °C and then ground into a powder. Two different extraction solvents (water and methanol) were used.

1.3.1 Aqueous extract preparation (AVa)

200 grams of mistletoe leaves were extracted with 600 ml of water at 35 °C in water bath for 12 h. Extract was filtered and then centrifuged. Pellet portion was discarded and supernatant was lyophilized using a freeze dryer at –65 °C. The final yield of crude extract was 12.4 gr.

1.3.2. Methanolic extract preparation (MVa)

200 grams of mistletoe leaves were extracted with 600 ml of 80 % methanol at 35 °C in water bath for 12 h. Extract was filtered and concentrated in vacuo. Residue was dissolved with 10 ml distilled water and lyophilized using a freeze dryer at –65 °C. The final yield of crude extract was 6.8 gr.

1.4. Objectives of the study

A. Preparation of aqueous (AVa) and methanol extracts (MVa) of *V. album* hosted by *Crataegus tanacetifolia* (Lam.) Pers (Tansy-leaved Thorn).

B. Determination of the effective concentration of aqueous *V. album* extract in isolated atrial preparation and administration of K_{ATP} channel blocker (glibenclamide), NOs inhibitor (L-NAME), muscarinic receptor antagonist (atropine) and β adrenergic blocker (propranolol).

C. Characterization of a model of coronary artery occlusion with infarct size.

D. Confirmation of efficacy of Ischaemic Preconditioning (IPC).

E. Determination of the effective concentration of aqueous and methanolic extracts of *V. album* in Ishaemia/Reperfusion injury (I/R).

F. Administration of K_{ATP} channel blocker (glibenclamide) and NOs inhibitor (L-NAME).

CHAPTER II

2. ISOLATED HEART PREPARATION WITH LANGENDORFF APPARATUS

2. 1. Cardiovascular disease

Ishaemic heart disease (IHD) or heart attack is still largest cause of death and disability worldwide, claiming more victims than any other disease. Acute myocardial infarction is fundamental clinical manifestation of heart attack following coronary thrombosis. The primary manifestations of myocardial I/R injury are myocyte death, arrhythmias and contractile dysfunctions. The World Health Organization (WHO) reports that there were over 17 million deaths in 2008 attributable to cardiovascular disease (CVD) (Mendis, 2011). Nearly 8 million of these deaths were as a direct result of coronary artery failure (CAF), a disease that results in insufficient blood supply to the myocardium. CAF deaths are most often caused by sudden rupture of an atherosclerotic plaque that results in insufficient blood supply to the myocardium. The major reason cause of death is the lethal arrhythmias that appear after the coronary occlusion or reperfusion.

2.1.1. What is the acute myocardial infarction?

Acute myocardial infarction (AMI) as a consequence of coronary artery thrombosis is a major cause of morbidity and mortality worldwide. Coronary thrombosis of a major blood vessel supplying the myocardium leads to myocardial ischaemia, which is described as the sudden and sustained lack of blood flow and associated hypoxia to part of the heart, resulting in permanent damage to the heart tissue. This is clinically diagnosed as acute myocardial infarction (AMI) (Baker et al., 2011; Buja & Weerasinghe, 2008; Fuster et al., 1988). WHO guidelines for clinical diagnosis of AMI suggest that three criteria are satisfied. These are: clinical history of ischaemic like pain of duration 20 minutes, changes in repeated electrocardiograms (ECG), and rise and/or fall of cardiac biomarkers such as troponin and creatine kinases (Lippincott et al., 1979). As a result of the increasing sensitivity of biomarker assays, WHO guidelines were revised in 2000 to give more weighting towards increases in these "soluble markers"(Antman et al., 2000).

Atherosclerotic lesions form when leukocytes adhere to the endothelial monolayer of an artery, followed by maturation of monocytes into macrophages and their uptake of lipid resulting in the production of foam cells. Smooth muscle cells migrate towards the intima adjacent to the endothelial monolayer, followed by collagen, elastin and proteoglycans. A lipid or necrotic core forms in the center of the lesion resulting from dead or dying smooth muscle cells, macrophages and an accumulation of cholesterol. A fissure in the fibrous cap of the plaque allows blood coagulation components to interact with tissue factors in the lesion, triggering platelet aggregation, thrombus formation and occlusion of the vessel lumen. If the occluded

vessel is a major artery supplying the myocardium then this can lead to the rapid development of an ischaemic risk zone or zone of jeopardised tissue. The propensity to arrhythmias and decreased left ventricular contractility, which can be fatal, is directly related to the size of the risk zone. The portion of the area at risk that has undergone irreversible cell damage is called the infarct.

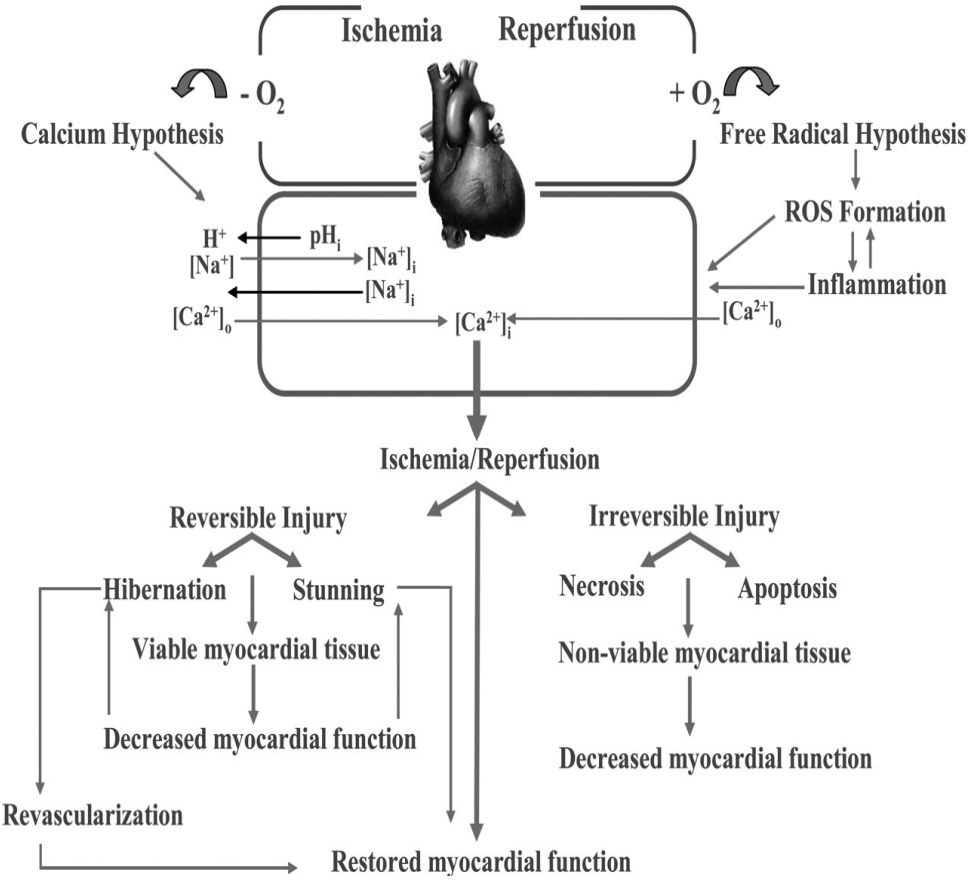


Figure 2.1. Mechanism of Ischaemia-Reperfusion (I/R) injury. Putative mechanisms of calcium and free radical hypothesis and inflammation in generation of I/R (Levitsky, 2006).

2.1.2. Cellular changes during myocardial ischaemia

Occlusion of the coronary artery restricts the coronary blood flow through the myocardium. Myocardial ischemia occurs when the oxygen and substrate supply to the myocardium is less than its demand. In the ischemic zone, deprived of oxygenated blood flow, hypoxia swiftly leads to cessation of mitochondrial oxidative phosphorylation and ultimately the loss of the major source of adenosine trisphosphate (ATP). Concomitant with the ceasing of oxidative reactions, cytosolic glycogen becomes the major substrate for anaerobic glycolysis, which becomes accelerated (Reimer & Ideker, 1987). This compensatory mechanism is a vain attempt of the myocardium to survive. Unfortunately anaerobic glycolysis can only produce up to 7 % of ATP required for the normal functioning of the myocardium (Wollenberger & Krause, 1968). Although contractile function of the heart, the major energy demanding process of the myocardium is suppressed rapidly, ATP demand quickly outstrips production and stores are depleted by half within the first 10 minutes of a severe ischaemic episode (Jennings et al., 1978).

Accumulation of H^+ and lactate, as well as mitochondrial fatty acid metabolism, results in the production and release of reactive oxygen species (ROS) and reactive nitrogen species, both of which contribute to impaired contraction with persistent electrical activity and culminating in ventricular arrhythmias (Buja & Vela, 2008; Thandroyen et al., 1992). In the first minutes of ischaemic insult, there is a net loss of cytosolic K^+ by efflux, initially not altering the Na^+/K^+ adenosine triphosphatase (ATPase). This is followed by an increase in free Mg^{2+} and a decrease in total Mg. Eventually the ATP demand to maintain electrochemical gradients

cannot be met and this result in the inhibition of the Na^+/K^+-ATPase. As a consequence of this, there is net Na^+ influx and further K^+ efflux as well as cell swelling due to increased uptake of water and Cl^-. The rise in intracellular Na^+ activates the Na^+/Ca^{2+} exchanger, which addresses the ion imbalance by extruding three Na^+ in exchange for one Ca^{2+}. Changes in the sarcolemma and sarcoplasmic reticulum (SR) come about as result of an increase in cytosolic free Ca^{2+}, also activating proteases causing alterations in contractile proteins. There is a decrease in Ca^{2+} sensitivity due to phosphate and hydrogen ions, which leads to sustained impairment of contractility despite elevated cytosolic Ca^{2+}(Allen & Kurihara, 1982). Ca^{2+} overload leads to cell damage by activating membrane phospholipases, depressing mitochondrial respiration and increasing mitochondrial permeability (Figure 2.3). During prolonged ischaemic episodes, contracture occurs. The interaction between myosin heads and actin is maintained because of the lack of ATP production (Stapleton & Allshire, 1998).

2.1.3. Why myocytes die during myocardial ischaemic injury?

There are three types of cell death that occur during myocardial ischaemia and reperfusion. Apoptosis, oncosis and autophagy are the three terms used to describe the demise of cells. Necrosis is seen as the final manifestation, at the tissue level, of both oncotic and apoptotic cell death. (Majno & Joris, 1995; Buja & Weerasinghe, 2008). Oncosis is characterized by its development through exogenous stimuli, as a consequence of energy depletion and/or membrane damage and it accounts for most necrotic damage. Environmental insults that initiate oncotic

pathways include hypoxia, inflammatory processes and ischaemia. In contrast to apoptosis and autophagy, oncosis is described as accidental and passive cell death (Buja & Vela, 2008). Cellular homeostasis is lost due to progressive membrane damage caused by products of activated leukocytes, the complement attack complex (C5b-9) and osmotic fluctuations caused in part by fluctuations in Ca^{2+}. Cell swelling occurs due to uncontrolled influx of water as well as Na^{+} and Ca^{2+}. Ultimately, cell swelling leads to membrane blebbing and cell rupture (Majno & Joris, 1995). Leakage of intracellular components upon membrane rupture in cells undergoing oncosis results in exudative inflammation.

Apoptosis has been described as "programmed cell death", it requires energy and results in cell and nuclear shrinkage and fragmentation without exudative inflammation (Kerr et al., 1972). There are two molecular pathways that lead to apoptotic death, both of which occur in cardiac myocytes. The external pathway utilizes cell surface receptors and the intrinsic pathway involves the calcium-dependent organelles, mitochondria and SR. Both pathways involve the activation of caspases (cytosolic aspartate residue-specific cysteine proteases) such as caspase-8, but differ in that the extrinsic pathway is activated by binding of death receptors e.g., FasL, and the intrinsic pathway involves the formation of the mitochondrial permeability transition pore (mPTP) and cytochrome c (Kumar et al., 2007). The most recent studies, although in agreement that apoptotic cell death occurs during coronary occlusion, do not agree concerning the extent or time period in which apoptosis occurs. Kajstura et al. (1996) reported that apoptotic cell death was the major form of cell death during up to 6 hours coronary occlusion in the rat. Conversely, Fliss et al. (1996) and Gottlieb et al. (1994) reported that apoptosis

occurred only during reperfusion. Scarabelli et al. (1999) employed multiple staining techniques to report a time course of apoptosis in specific cell types during ischaemia/reperfusion. They reported that endothelial cells are the predominant cell type affected by apoptosis, and that the cells undergo apoptosis before cardiomyocytes. The percentage of cells undergoing apoptosis, contributing towards ischaemic injury, has been reported between 5 and 33 % (Garg et al., 2003; Takashi & Ashraf, 2000) whilst Bialik et al. (1997) report a range between 3 and 12 %. Reported data for apoptotic contribution to ischaemic cell death has, in the main, been confirmed by terminal uridine deoxynucleotidyl transferase staining. It is important to mention that there are numerous reports that suggest that current sensitivity and specificity of this method in determining apoptotic cell death is questionable, which may in part explain the inconsistency in reported results (Fliss & Gattinger, 1996; Bialik et al., 1997; Buja & Vela, 2008).

Autophagy or macro autophagy is a physiological process that results in cells digesting internal organelles or dysfunctional cytoplasmic components via the lysosomal degradative pathway. This process occurs both under physiological and pathophysiological conditions, including myocardial ischaemia (Yan et al., 2005; Kanamori et al. 2011). During prolonged ischaemia autophagy is substantially increased and is said to be pro survival as degraded membrane lipids within autophagosomes are recruited to maintain needed levels of ATP production and protein synthesis (Levine & Klionsky, 2004). Hickson-Bick et al. (2008) reported that in neonatal cardiomyocytes, autophagy can protect cells against programmed cell death. They showed that apoptotic markers were increased when lipopolysaccharide (LPS) treated cardiomyocytes were treated with inhibitors of autophagy. This work

supports data from an acute hypoxia-reoxygenation model of cardiomyocytes that suggests that autophagy protects against ischaemia-reperfusion injury by selective sequestration of damaged mitochondria, resulting in the limitation of pro-apoptotic factors (Hamacher et al., 2006).

2.1.4. Progression of infarct

During ischaemia, myocytes can be classified into three categories: cells that are viable and so far unaffected by ischaemic insult; cells that have been irreversibly damaged and undergone cell death; and those that have been reversibly damaged, which may recover function with timely reperfusion (Reimer et al., 1993). The speed with which myocytes progress through these states, and the ultimate size of the infarct depends on a number of factors. Collateral blood flow and the severity of index ischaemia as well as duration of ischaemia will affect this end point (Reimer et al., 1977). Many non-human primates and pigs have little or no preformed collateral anastomoses between coronary arterial regions, and so occlusion of a coronary artery will result in total or near total ischaemia of the affected region (Fujiwara et al., 1982; Lavallee & Vatner, 1984). This is also the case for most human patients who have very few physiologically occurring anastomoses. The exception to this would be if physical stimuli such as brief ischaemic episodes (angina pectoris) have occurred historically, resulting in collateral development (Fulton, 1963). In contrast, canine and feline models have been shown to have considerable collateral anastomoses, meaning that occlusion of one coronary artery rarely leads to total ischaemia due to perfusion of the ischaemic risk zone by the collateral circulation (Jennings et al.,

1978). Similarly, the guinea pig heart is well documented as having an extensive collateral coronary circulation (Maxwell et al., 1987), so much so that Winkler et al. (1984) were unable to detect infarct in guinea pig hearts following 6 hours of coronary artery ligation.

Seminal work published in 1977 by Keith Reimer and colleagues described the first reliable infarct model, reporting the importance of controlling the above mentioned parameters (Reimer et al., 1977). They were the first group to characterize robustly the progression of infarct in a canine model of ischaemia-reperfusion, known as the "wave front phenomenon" (see Figure 2.2). Cell death occurs in a wave like fashion from the subendocardial myocardium progressing toward the subepicardial myocardium over time, with many cells in the subepicardial myocardium surviving up to six hours after coronary artery occlusion. Following 24 hours of coronary artery occlusion, they showed that infarct had become almost transmural. Any tissue that was still viable at this point was associated with adjacent blood vessels perfusing the anterior myocardium. They concluded that the wave front progression of the infarct was a consequence of the collateral blood flow, which is greatest at the epicardial myocardium (Reimer et al., 1977; Reimer et al., 1993).

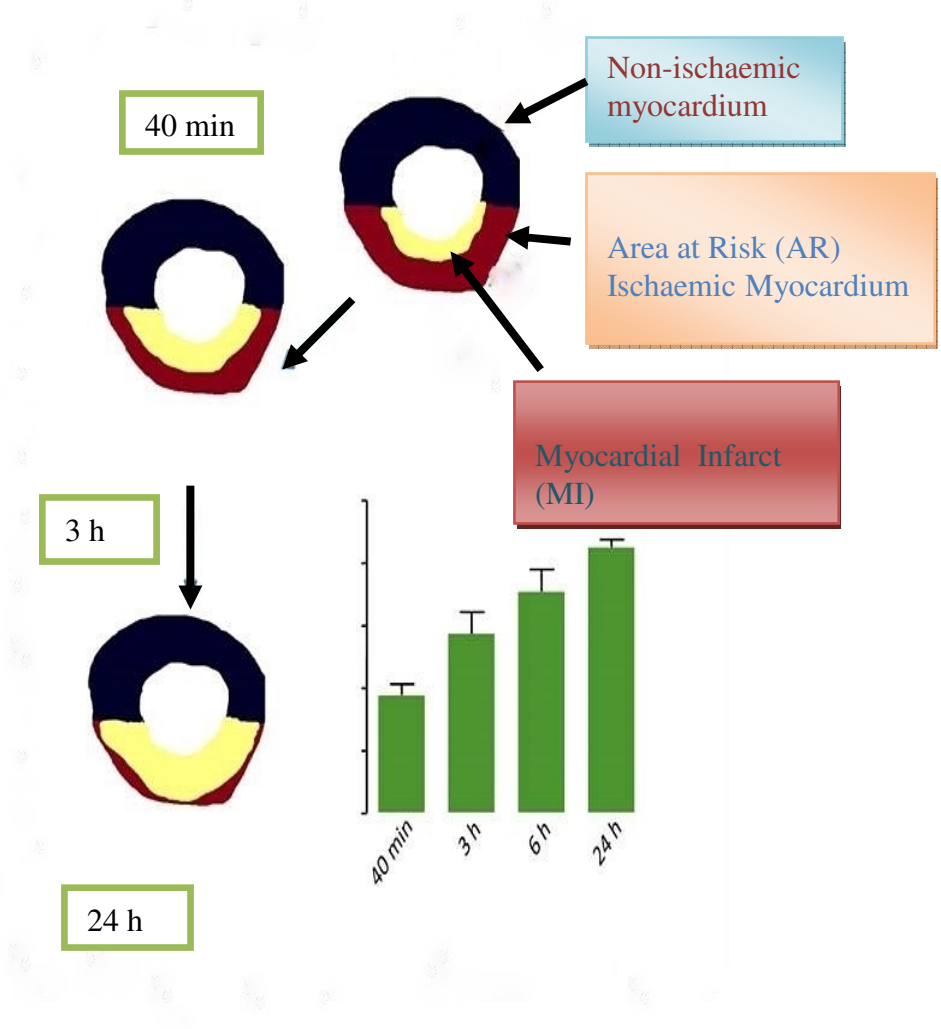


Figure 2.2. Schematic of the wavefront phenomenon of myocardial necrosis proposed by Reimer and Jennings (1977). Infarct develops in a wave like fashion from the subendocardial myocardium to the subepicardial myocardium with time. Histogram adapted from Reimer et al. (1977), showing increasing infarct (I) size as a percentage of the area at risk (AR) over time.

2.1.5. Collateral circulation

Coronary collateral circulation varies depending on animal model and even between human patients (Kloner et al., 1976; Reimer & Jennings, 1979;

Maxwell et al., 1987; Leshnower et al., 2007). The conclusions drawn by the seminal work of Reimer et al. (1977) were based partly on the presence of collateral anastomoses. Leshnower et al. (2007) have recently investigated the progression of infarct upon coronary artery occlusion in a model that is more akin to species that have limited collateral circulation, or human patients whose collateral circulation is poorly developed. They chose an ovine model because of its consistent paucity of preformed collaterals. In contrast to the work of Reimer and Jennings 30 years earlier, Leshnower and colleagues reported a more evenly distributed infarct throughout the ventricular wall, following 45 minutes coronary artery occlusion. When occlusion time was increased to 1 hour, distribution of the infarct was altered again, the majority of infarct now focused in the mid myocardium, with the least damage in the endocardial myocardium. Although these more recent data highlight differences between the progression of infarction in well-collateralized hearts and those with limited collateral circulation, there is certainly agreement in the relationship between size of infarct and duration of index ischaemia. Interestingly, the most recent studies investigating this phenomenon continue to adopt the view of Reimer and colleagues that the endocardial myocardium is most susceptible to ischaemia and ultimately infarct.

2.1.6. Cellular changes in reperfusion

In the first few minutes of reperfusion, further metabolic and biochemical insult occurs. The oxygen paradox reported by Hearse et al. (1978) describes the requirement for restoration of oxygen to the ischaemic area to allow aerobic

respiration to restart. Paradoxically, the sudden return of oxygen allows the re-energisation of the mitochondria, which, in turn, generates detrimental ROS in the first few minutes of reperfusion. In re-addressing the ion homeostasis during early reperfusion, the Na^+/Ca^{2+} exchanger is activated in reverse mode, resulting in an overload of intracellular Ca^{2+} uptake and subsequent release by the SR. Intracellular pH is brought back to physiological levels rapidly with wash out of lactic acid and the activation of the Na^+/H^+ exchanger and the Na^+/HCO_3^- symporter. Elevated intracellular Ca^{2+} cause several detrimental actions, which result in cell death. Through protease activation, sarcolemmal rupture occurs and through uptake of Ca^{2+} into the mitochondria, mPTP is formed (Halestrap et al., 2004). Release of inflammatory mediators results in recruitment of neutrophils, which cause microvascular plugging, further generation of ROS and release of deleterious enzymes. Cellular swelling and microvascular plugging and associated interstitial oedema contribute to the no-reflow phenomenon even with an open coronary artery (Schwartz & Kloner, 2011).

Reperfusion injury can manifest in several ways; it can be described as either reversible or irreversible (lethal) reperfusion injury. Vascular injury that results as a consequence of inflammation and endothelial dysfunction around the site of reperfusion are examples of lethal injury. Myocardial stunning, the prolonged contractile dysfunction that occurs after a short period of ischaemia, is reversible over time; however it generally manifests for much longer than the ischaemic episode itself, often days (Hearse & Bolli, 1992). Reperfusion-induced arrhythmias can contribute to either reversible or irreversible reperfusion injury, depending on their severity, displaying as ventricular premature beats (VPB) or in

the most severe cases ventricular fibrillation (VF). The most widely held concept of lethal reperfusion-induced injury is the killing of cells that, prior to reperfusion, were still viable (Basso & Thiene, 2006; Burke & Virmani, 2007). It is generally accepted that the majority of reperfusion induced cell death occurs in the first few minutes of reperfusion and is a result of sarcolemmal rupture (Piper et al., 1998; Yoshida et al., 1995).

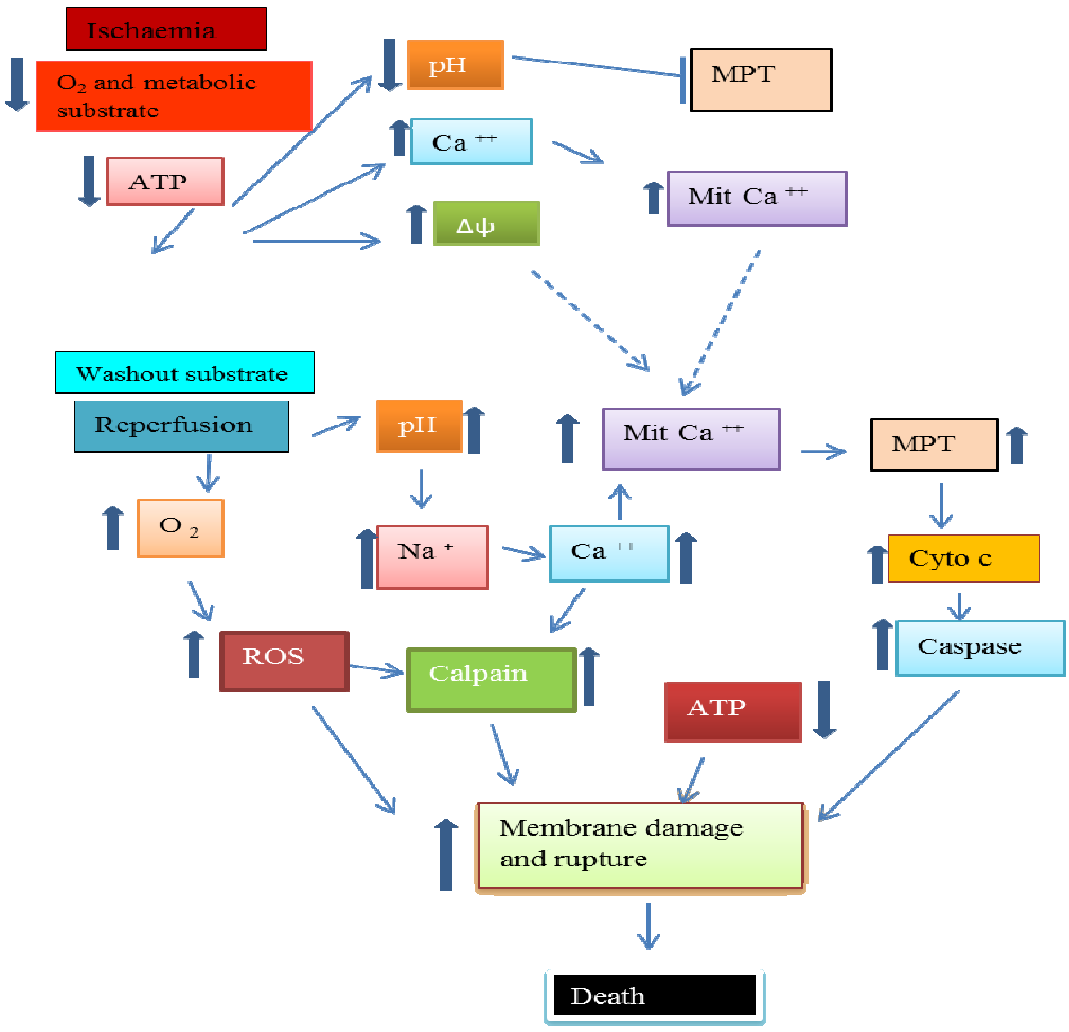


Figure 2.3. Pathways leading to cell death in ischaemia- reperfusion.

2.1.7. Ischemic preconditioning

Ischaemic pre and post-conditioning are powerful cardioprotective phenomena, in which infarct size is reduced by one or several short periods of myocardial ischaemia with reperfusion preceding or following, respectively, the infarct – inducing sustained ischaemia. Both phenomena have been identified in all species studied so far, including man; they are relevant in the context of interventional and surgical coronary revascularization, but also in preinfarction angina. Early experiments that investigated the possibility of salvaging myocardial tissue after an ischaemic episode came to no avail. The models being used were inconsistent and no standardized approach was taken to allow data to be reliably compared. Any success in reducing infarct size was modest, in the order of 10 %, which was not sufficient to proceed to the clinic. It was Reimer and Jennings' group who led the way in tackling the cardioprotection paradigm. Charles Murry, working in their laboratory, undertook a seminal study which showed that performing 4 cycles of 5 minute coronary artery occlusion/5 minute reperfusion prior to 40 minutes of index ischaemia would substantially protect the heart (Murry et al., 1986). They showed infarct limitation of up to 75%, far greater protection than anyone had reported before and reproducible by all whom subsequently applied it. The protection afforded was termed ischaemic preconditioning (IPC), later known as classic or early IPC, and was not dependent on collateral blood flow. As well as reduction in infarct size, it has been reported that IPC can afford cardioprotection in other ways. In dogs (Vegh et al., 1990) and rats (Shiki & Hearse, 1987) IPC has been shown to reduce the incidence of

ischaemia-reperfusion arrhythmias. Interestingly, the rat is the only species where improved recovery of mechanical function can be shown (Steenbergen et al., 1993), and is reported to be as a result of altered adenosine metabolism in the rat heart (Gelpi et al., 2002).

Murry et al. (1986) also reported data for a longer ischaemic model where hearts were not reperfused for three hours following IPC. The data from these experiments was in stark contrast to the acute ischaemic model. IPC was unable to protect hearts subjected to three hours ischaemia, with infarct sizes comparable to controls, suggesting that prompt reperfusion after the ischaemic insult is a necessity. Later studies showed that the preconditioned state is very transient, lasting only 60-120 minutes and is completely lost between 2 and 4 hours in conscious rabbits (Burckhartt et al., 1995). This means that if index ischaemia is not ensued within this time period, the IPC mediated reduction in infarct size will be lost.

2.1.7.1. Mechanisms of classical IPC

Many autacoids have been shown to trigger classical IPC, the first to be described being adenosine (Liu et al., 1991). Downey's laboratory showed that the adenosine A_1 receptor activation triggered IPC and thus showed that IPC was receptor mediated. They showed that blocking of the adenosine A_1 receptor abolished the characteristic protection seen, suggesting the importance of endogenous G_i-protein-coupled adenosine A_1 receptor activation to afford IPC

protection (Liu et al., 1991). Further studies have characterized other receptors that can trigger IPC when activated by their ligands. Noradrenaline was shown to afford similar protection through activation of α-receptors in the rat heart (Banerjee et al., 1993). Bradykinin B_2 and opioid δ1 receptor activation have also been shown to trigger IPC mediated protection (Baxter & Ebrahim, 2002; Gross et al., 2005). To date, it is suggested that any G_i-coupled receptor can trigger the preconditioned state through activation of G_i protein, although a limited number of endogenous autacoids participate naturally in the phenomenon. Downey's group identified that pharmacological inhibition of one autacoid receptor could only block the protection afforded by one cycle of IPC, but not from multiple cycles (Goto et al., 1995). They introduced the idea of an IPC threshold, suggesting that any one receptor stimulus only contributed towards IPC and that increasing the stimulus via other receptors (pharmacologically) or increasing the number of IPC cycles (mechanically) could compensate and meet the threshold to initiate IPC. Similarly, other G_i receptors such as endothelin ET_1 (Wang et al., 1996) and muscarinic M_2 receptors (Yao & Gross, 1993) have been shown to afford IPC when activated pharmacologically (meeting the IPC threshold), however their antagonism does not impair IPC as agonists to these receptors are not produced during IPC (Yellon & Downey, 2003).

The protection afforded by the stimulation of the above mentioned receptors has been reported to be mediated by protein kinase C (PKC). Inhibition of PKC will eliminate the protection from a preconditioned heart but has no effect on a nonprenconditioned heart (Ytrehus et al., 1994). ATP sensitive potassium channels

(K_{ATP}) have also been reported to play a crucial role in IPC. The K_{ATP} inhibitor glibenclamide abrogated the protective effects of IPC (Ferdinandy et al., 1995), whereas the pharmacological activators, pinacidil and chromakalin afford protection quantitatively similar to that of IPC (Grover et al., 1989). In a rabbit model of IPC, Pain et al. (2000) suggested that the end effector of IPC was not opening of K_{ATP} channels. They demonstrated that this in fact triggered small bursts of ROS that trigger entrance into a preconditioned state that then activates downstream kinases, including PKC, and ultimately inhibition of mPTP formation. Other kinases that are involved in IPC signaling include activation of phosphatidylinositol 3-kinase (PI3K), and its substrate kinase Akt (Hausenloy et al., 2005), p38 mitogen activated protein kinase (MAPK), p42/p44, the Janus kinase/ signal transduction activator of transcription (JAK/SAT) pathway (Dawn et al., 2004) and more recently inhibition of glycogen synthase kinase 3β (GSK3β) (Gao et al., 2009). Evidence that suggests these kinases play an important role in IPC relies on abolishing or abrogation of protection when their pharmacological inhibitors have been used in models of IPC (Hausenloy & Yellon, 2007).

2.1.7.2 Ischemic preconditioning affords protection at reperfusion

Although many studies had been published documenting the kinases involved in IPC, it remained unclear how IPC afforded protection through K_{ATP} channel activation. It was well accepted that these channels played an important role in both pharmacological (Garlid et al., 1997) and mechanical IPC (Hide & Thiemermann, 1996), yet whether they were triggers or mediators was not agreed.

In 2002, Hausenloy and colleagues examined the role of the mPTP in the context of IPC. Using an isolated rat heart model, they reported that the infarct limitation afforded by both mechanical and pharmacological IPC was abolished if the mPTP was opened pharmacologically with atractyloside. They also showed that inhibiting the opening of the mPTP at reperfusion with cyclosporine A, resulted in similarly protected hearts as those treated with IPC (Hausenloy et al., 2002). Work by Halestrap's group the following year further investigated the role of the mPTP in IPC, using a more direct approach to assessing mPTP activity. They used the same technique that allowed them to show some years earlier that mPTP opening occurs during early reperfusion and not during index ischaemia (Griffiths & Halestrap, 1995). The method involved the mitochondrial entrapment of 2-deoxy[^{3}H]glucose ([^{3}H]DOG) which allowed them to measure mPTP opening. Their findings supported those of Hausenloy et al. (2002), in that protection afforded by IPC is associated with indirect inhibition of the mPTP. They further concluded that in their hands, direct targeting of the mPTP was less effective than removing the conditions that are responsible for pore opening in the first place (Javadov et al., 2003). As momentum gathered during the early 2000's, Yellon's group investigated the link between IPC and protection afforded in early reperfusion further. Two studies suggested that the kinases previously thought to be activated during ischaemia in response to IPC were in fact phosphorylated during early reperfusion in response to this stimulus (Hausenloy et al., 2004). Furthermore, IPC increased phosphorylation of PI3K-Akt and MEK-1/2-ERK-1/2 pathways, but during reperfusion, after index ischaemia. They also suggested that these kinases are critical for IPC induced protection (Hausenloy et al., 2005). Work

published by both Yellon's and Halestrap's laboratories shifted the focus of IPC mediated protection towards the first few minutes of reperfusion. The so-called survival kinase pathways had now been shown to be implicated in IPC mediated protection as well as targeting reperfusion induced injury (Yang et al., 2006; Burley et al., 2007). Clinically these observations were appealing as it has long been a criticism of IPC mediated protection that it had limited clinical applicability. Clinical studies had been employed where high risk patients received K_{ATP} channel agonists or nitric oxide (NO) donors. However, the protective effects were limited, and dosing was a challenge due to the generally unpredictable nature of AMI (Lee et al., 2002; Rezkalla & Kloner, 2007). The focus had now shifted towards targeting ischaemia-reperfusion injury in the first few minutes of reperfusion, a strategy which is both clinically desirable and practical (Hausenloy et al., 2005).

2.1.8. Nitric Oxide

Over the last 30 years, interest in NO and our understanding of it as a biological mediator has grown at increasing pace. Originally described as a noxious gas, it was the seminal work of Furchgott and Zawadzki (1980) and Ignarro et al. (1982) who discovered an endothelium derived relaxing factor (EDRF) and later Palmer and colleagues (1987) who identified it as NO that created the foundation for this large body of research. In 1867, it was documented in the Lancet that organic nitro vasodilators (amyl nitrite) were efficacious in patients presenting with angina pectoris (Brunton, 1867). It was not until the latter part of the

20th century that pharmacology of these compounds was understood. Furchgott et al. (1980) noticed that there were inconsistencies in data reporting the vasoactive properties of acetylcholine (ACh) on blood vessels *in vitro*. They observed in thoracic aortic rings from rabbits that if the endothelium was damaged on dissection then the responses to ACh were different. This led them to conclude that the endothelium must be present for ACh mediated relaxation. Furthermore, they proposed that ACh acting on muscarinic receptors in the endothelial cells stimulates the release of a substance that causes relaxation of vascular smooth muscle. It was then Palmer et al. (1987) who documented that relaxation induced by both EDRF and NO was inhibited by hemoglobin and enhanced by superoxide dismutase (SOD) to a similar degree. They deduced that NO released from endothelial cells was indistinguishable from EDRF in terms of biological activity, stability and susceptibility to an inhibitor. They concluded that EDRF and NO are identical.

NO is synthesized by a group of enzymes called nitric oxide synthases (NOS). To date there are three distinct NOS isoforms located in different tissues. They are distinguished by their dependence on calcium and calmodulin (Bredt & Snyder, 1990). Neuronal NOS (nNOS, NOS I), isolated from brain tissue, and inducible NOS (iNOS, NOS II) are mostly found in soluble portions (Stuehr et al., 1991). Endothelial NOS (eNOS, NOS III) is mainly found in the particulate fraction in endothelial cells (Forstermann et al., 1991). nNOS and eNOS are constitutive enzymes, constantly producing nanomolar amounts of NO. In contrast, iNOS is not present constitutively; it is induced by pro-inflammatory cells of the immune system (Griffith & Stuehr, 1995). NOS produce NO by catalysis of a 5-electron oxidation of one nitrogen atom of the guanidine group of L-arginine to

form NO and L-citrulline. It is important to consider the distribution and expression of NOS isoforms when thinking about the actions NO has in different tissues. eNOS and nNOS are relatively low output enzymes and associated with basal physiological function. On the other hand, iNOS is a high output enzyme, generating 1000-fold more NO than eNOS (Singh & Evans, 1997). The sheer amount of NO that iNOS can produce can result in detrimental effects, not directly by NO, but by the ROS produced in an NO and super oxide rich environment (Ferdinandy & Schulz, 2003). It is important to note that studies have also shown that eNOS can be regulated directly by PI3K/Akt (Fulton et al., 1999), and more recently it has been reported that oestrogen receptor binding can lead to phosphorylation and activation of eNOS (Haynes et al., 2000; Russell et al., 2000).

NO's charge neutrality enables it to diffuse freely in aqueous solutions across cell membranes (Figure 2.4). The rate of diffusion is dependent on its half-life, which in turn is dependent on the rate of formation. Typically, in aqueous solution, the intracellular half-life of NO is in the millisecond range (Bolli, 2001; Hakim et al., 1996). Biological breakdown of NO can occur in numerous ways. The most common intermediate breakdown product is nitrite (NO_2^-) (Kelm, 1999). Nitrite can then be taken up by red blood cells, where further oxidation by a hemoglobin dependent mechanism leads to production of nitrate (NO_3^-), which can be redistributed in plasma.

2.1.8.1. Nitric oxide and cardioprotection

For almost as long as research has been carried out exploring cardioprotective paradigms, particularly ischaemia-reperfusion injury, there has been controversy over the role that NO plays (Bolli, 2001; Ferdinandy & Schulz, 2003; Schulz et al., 2004). It would appear that in recent years this controversy is dwindling and it is generally accepted that NO is cardioprotective in the ischaemia-reperfusion setting (Figure 2.5). It is however important to note when drawing conclusions from the literature that various end points and animal models have been used and there is certainly species variation in NO production and distribution (Jones & Bolli, 2006). The source of NO and the amount produced or the NO donor being used, will affect whether NO affords protective or deleterious actions. NO was initially documented as the only endogenous ligand of soluble guanylyl cyclase (sGC), activating it in all tissues tested (Waldman & Murad, 1987). However, it was later demonstrated that carbon monoxide (CO), produced by haem oxygenase could mediate smooth muscle relaxation and platelet aggregation via sGC (Brüne & Ullrich, 1987). Most recently it has been demonstrated that nitroxyl could activate sGC, however it was noted that the nitroxyl donors used could not activate the oxidised form of sGC. The authors further reported that the activation observed was independent of NO (Miller et al., 2009). When acting as a signaling molecule, NO binds to the haem iron moiety, resulting in its activation and generation of guanosine-3', 5'-cyclic monophosphate (cGMP) from guanosine triphosphate (GTP), and cGMP acts at distal targets. It is worthy to note that NO can act independently of sGC and cGMP to initiate other

actions such as protein S-nitrosylation (Ziolo, 2008). It has also been demonstrated that NO can directly activate adenylate cyclase, thus increasing adenosine-3', 5'-adenosine monophosphate (cAMP) levels and myocardial contractility (Burgoyne & Eaton, 2009). The role NO plays differs depending on whether it is involved in the classical or late phase preconditioning phenomenon. In classical IPC it has been shown that inhibition of NOS by pharmacological intervention has no effect on IPC's ability to limit infarct size. However, exogenous delivery of NO, by NO donors such as SNAP, NOC-9 and diethylenetriamine NONOate (DETA-NO) prior to index ischaemia protect the heart comparably to IPC itself (Nakano et al., 2000; Post et al., 2000). More recently, du Toit et al. (2007) reported that mice over expressing eNOS demonstrated a maximally protective state, comparable to wild type littermates who had undergone IPC. In contrast to reports of protection, others have reported deleterious actions of NOS inhibition during preconditioning. Vegh et al. (1993) showed in some of the earliest NO IPC studies, that inhibition of NO by the NOS inhibitor L-NAME, both pre and post IPC abolished the antiarrhythmic effects of IPC in an open chest dog model of coronary artery occlusion. Inhibition of NOS was also shown to increase post ischaemic contractile dysfunction (Lochner et al., 2002).

Evidence suggests that NO plays an important part in both classical and delayed IPC. Although the exact mechanism that affords protection under the delayed IPC is unclear, pharmacological mimetics and triggers such as bradykinin and adenosine, NO and ROS have been identified. Several of these triggers initiate signaling that converges on NOS activation, demonstrating the importance of NO production in initiating delayed IPC. Bolli and colleagues, who have led the way

in investigating NO's role in delayed IPC, propose that NO has a functional role both in initiating, but also mediating delayed IPC (Bolli et al., 1998). It is suggested that stimulation of production of NO by eNOS triggers downstream signaling that results in upregulation of cardioprotective mediators, including iNOS. Bolli et al. (2001) propose that phosphorylation of PKC, recruitment of tyrosine kinases and activation of NF-κB, as well as ROS production play crucial roles. More recently, studies have shown that NO is a potent stimulator of mitochondrial biogenesis which is consistent with the NO hypothesis of IPC (McLeod et al., 2005; Nisoli et al., 2003). Although there is increasing evidence to support the notion that both eNOS and iNOS are important in IPC, the role of nNOS remains unclear (Murillo et al., 2011). As with the other isoforms of NOS, the literature remains divided about nNOS. Recent studies have shown that while inhibition of nNOS is protective in ischaemia-reperfusion, its function is required to appreciate the protective effects of delayed IPC (Barua et al., 2010; Lu et al., 2009).

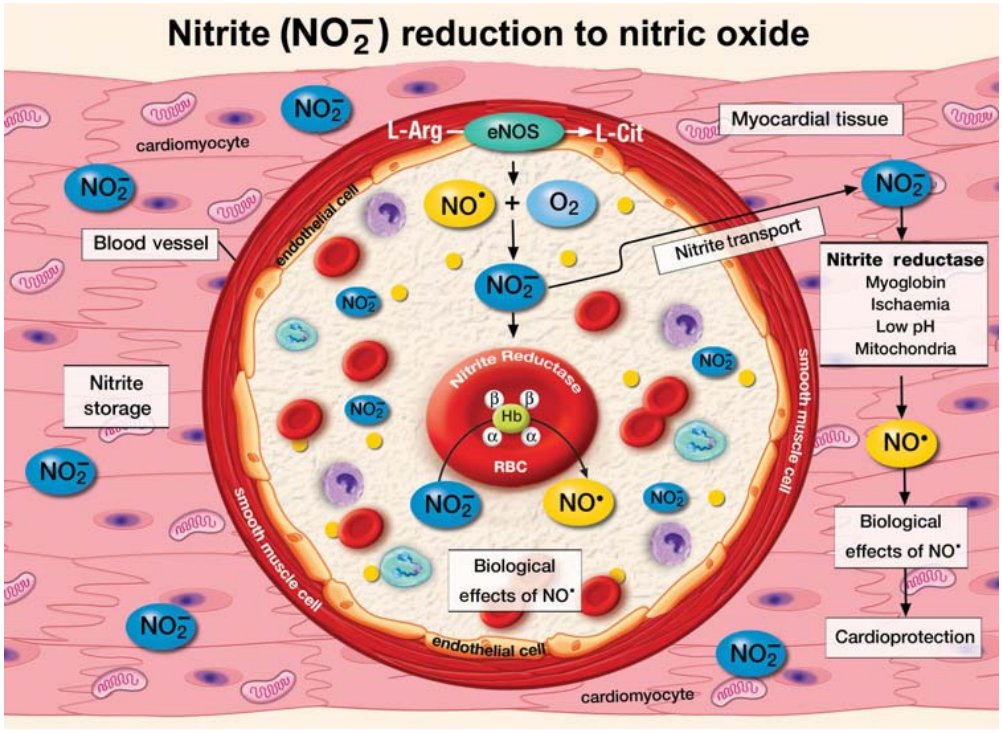


Figure 2.4. Predominant pathways for the generation of nitric oxide (NO) from nitrite. During conditions of hypoxia and/or ischaemia nitrite stored in the blood and heart is converted to NO via the action of a number of nitrite reductases. In the myocardium, nitrite is thought to be reduced to NO by myoglobin, low pH, hypoxia, and mitochondria. Conversion of nitrite to NO increases myocardial blood flow and directly protects the myocardium against ischaemic injury (Calvert & Lefer, 2009).

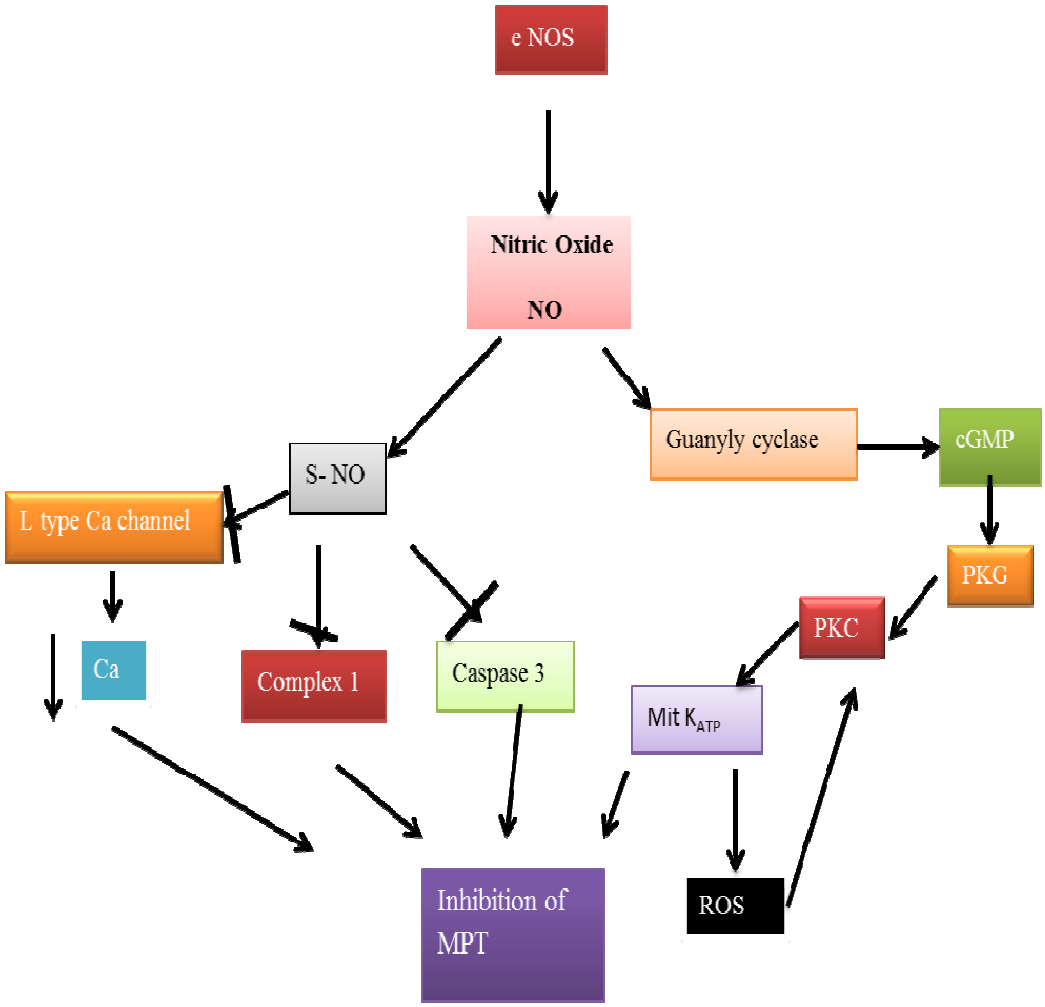


Figure 2. 5. Nitric oxide (NO) signaling in cardioprotection. NO is generated by nitric oxide synthases (NOS). NO can activate guanylyl cyclase resulting in activation of protein kinase G. Alternatively, NO can result in S-nitrosation of a number of proteins.

2.2. Materials and Methods

2.2.1. The isolated perfused heart according to Oscar Langendorff

Isolated heart perfusion has been a highly valued technique for studying numerous physiological and pathophysiological aspects of the myocardium for more than a century. Oscar Langendorff first reported his method of studying the mechanics of the completely isolated mammalian heart in 1895 (Langendorff, 1895). The basic principles of the method were to create a preparation whereby the heart could be studied in an ex vivo setting, whilst perfusing the organ and maintaining basic cardiodynamic function. Perfusate, whether it be whole blood or other physiological fluid such as Krebs' buffer is forced towards the heart through a cannula inserted in the ascending aorta. The pressure of the perfusate flowing through the ascending aorta in a retrograde direction (reverse to that in vivo) causes the aortic valve to close, diverting the perfusate through the coronary arteries. The direction of flow then follows that of blood in vivo, through the arterioles, capillaries, the venous system and finally through the coronary sinus into the right atrium. Unlike the in vivo heart the cardiac cavities remain basically empty throughout the experiment (Figure 2.6).

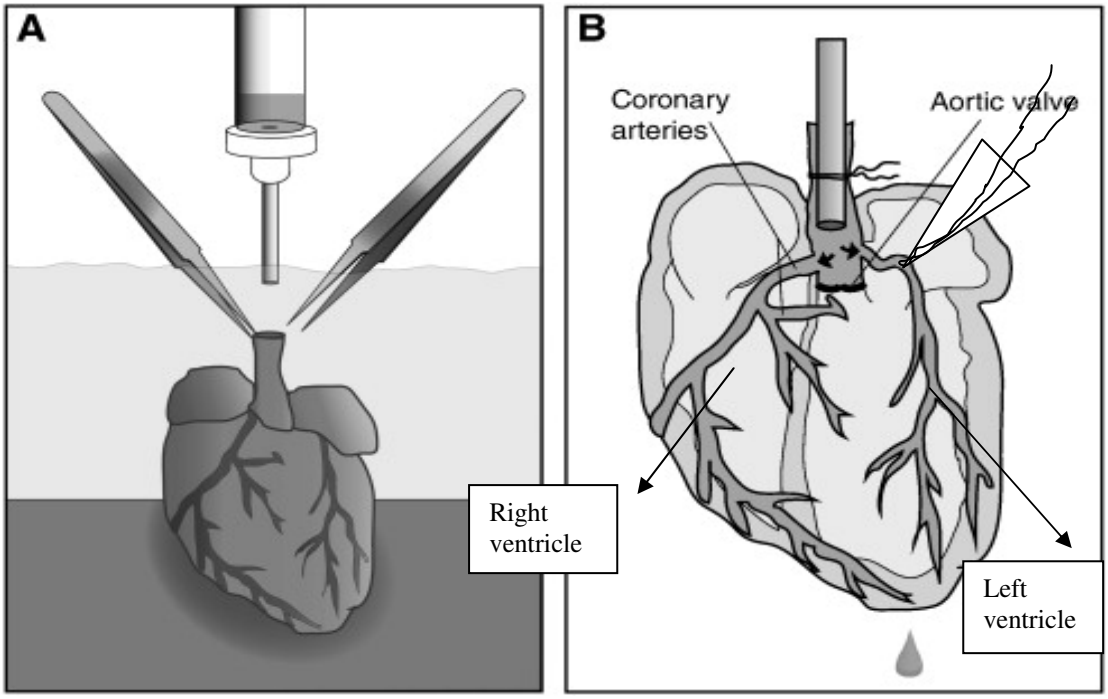


Figure 2.6. Diagram of the myocardium highlighting the retrograde perfusion of Krebs Henseleit buffer through the coronary ostia. Also shown is the placement of a ligature to occlude the LDCA.

The fact that the Langendorff perfused heart preparation has been employed as the method of choice when studying many diverse myocardial parameters is a testament to its simplicity and utility. With the increasing number of genetic knock-out and knock-in animals available, the Langendorff method allows for the characterisation of phenotype, investigation of complex signalling processes, pharmacological intervention and isolation of myocytes for primary culture. It does, however, have to be acknowledged that the isolated perfused heart is a deteriorating preparation; work by Sutherland and Hearse (2000) using a mouse model and others since have reported a decrease in contractile and chronotropic function of between 5 and 10 % per hour under control conditions. Whilst important to note, suitable experimental protocols can be designed within these parameters.

There are two methods widely described to perfuse the isolated heart in the Langendorff model. Both constant pressure and constant flow systems have been used; each offers advantages and disadvantages. Constant pressure perfusion allows the accommodation of the heart's natural regulation of coronary tone and can be achieved by maintaining a constant hydrostatic pressure between the meniscus of the buffered perfusate and the tip of the cannula attached to the ascending aorta (Doring & Dehnert, 1988). Constant pressure can be achieved reasonably inexpensively using standard laboratory glassware and tubing or using a pump system with pressure feedback control. The latter is typically the set up of the commercially available perfusion systems which have the advantage over the hydrostatic systems of reducing dead space which, when perfusing pharmacological agents, can be an important cost consideration. The constant flow system allows the perfusion apparatus to be much more compact, again removing dead space and is the method of choice if vasoactive parameters and resistance are of interest (Bell et al., 2011). However, a constant pressure system overrides the auto-regulatory functions of the heart and therefore there is no feedback on the flow demands of the heart when vascular interventions are performed, such as occlusion of a major vessel in an ischaemia-reperfusion protocol. This can cause shear stress and damage to the vasculature in this setting (Sutherland & Hearse, 2000).

The longevity of the Langendorff perfused heart method may also be attributed to the wide variety of species that can be used. For the most part, the rat is the species of choice. The isolated rat heart has been experimentally characterised, especially with respect to ischaemia-reperfusion studies. However for experiments where genetically modified animals are needed, then the mouse heart has been extensively used. In early work, the canine heart was reported as well as the rabbit.

Practical considerations need to be reviewed when selecting animal models, particularly the economics of perfusing larger animal hearts, especially when pharmacological agents are to be used. The anatomy of the heart must also be considered. The guinea-pig heart has been Langendorff perfused but for regional ischaemia models is not suitable because of its extensive native collateral circulation; it is more suited for global ischaemia studies. The pig isolated heart is still used in some laboratories, for transplant and stem cell studies due to the continuing exploration of the similarities between the swine and human hearts.

2.2.2 Animals

Male Sprague Dawley rats sourced from B&K Universal Ltd. (Bristol, UK), Harlan UK Ltd (Oxfordshire, UK) and Charles River Laboratories Inc. (Maidenhead, UK) were selected for use in this thesis. Rats were housed in the institutional animal house and allowed to acclimatise for 7 days. The care and use of animals was in accordance with the Animals (Scientific Procedures) Act 1986 (The Stationery Office, London, UK). Both water and food were available ad libitum. Food pellets contained 4% fat and 18% protein. Animals were exposed to 12 h on, 12 h off light cycles.

2.2.3 Langendorff heart perfusion

Group sizes were determined based on historical data from our laboratory and others that suggests that n=5-6 is sufficient to resolve statistical differences between groups. Animals were used at 300-400g body weight. The animals were placed under

surgical anaesthesia using pentobarbital sodium (175mg/kg) with heparin (200 units) given concomitantly by intraperitoneal injection. Once animals were unconscious and surgical plane anaesthesia had been reached, (characterised in part by a lack of pedal reflex), the animal was placed in the supine position, a sagittal incision was made ventrally exposing the xiphoid process and diaphragm. The thoracic cavity was then entered by bilateral caudal-cranial dissection of the rib-cage. The ribcage was then reflected cranially to expose the thoracic cavity containing the heart. Excision of the heart was then performed by cradling the heart gently between the thumb and fore-finger and cutting above the heart ensuring a sufficient length of ascending aorta was also removed. The heart was immediately placed in ice cold modified Krebs Henseleit (KH) buffer, NaCl 118.5 mM, NaHCO3 24.8 mM, d-Glucose 11 mM, KCl 4.7 mM, MgSO4.7H2O 1.2 mM, KH2PO4 1.2 mM, CaCl2.2H2O 1.3 mM, aerated with 95 % O2/5 % CO2 (pH 7.3-7.5 at 37.0 ˚C).

The ascending aorta of the rat heart was cannulated with a metal cannula (Fisher Scientific) using mini surgical forceps, and initially held with a small metal clip. The heart was rotated so that the anterior surface of the heart was facing forward. At this point the heart was perfused only partially (half opened tap) with modified KH buffer to ensure that there was no leaking of perfusate whilst positioning the heart. Once the heart was positioned correctly it was secured with two braded silk ligatures, the metal clip was removed and the heart was perfused fully at a constant hydrostatic pressure of 74 mmHg (100 cm H_2O). Warming of KH buffer was achieved by warming the jacketed pre-warmers and Baker coil by a thermo-regulated circulator (see Figure 2.7). Actual heart temperature was monitored by a thermocouple probe positioned under the right atrium and maintained between 36.8-37.4 °C. The left atrial

appendage was removed to expose the atrial chamber. An 80 mm length of 3-0 suture material (Ethicon, UK) was placed through the myocardium to sit posterior to the LDCA at its origin, ensuring a margin of approximately 2 mm either side of the anterior LDCA was achieved on entrance and exit of the heart tissue.

A latex balloon (Harvard Apparatus, UK) attached to a polypropylene cannula was then inserted through the bicuspid valve into the left ventricle (LV) and held just above the apex (see Figure 2.8&2.9). The balloon was then filled with distilled water and the cannula was attached to a pressure transducer and Powerlab data acquisition system apparatus (AD instruments, Abington, UK). The left ventricular end diastolic pressure (LVEDP) was set between 5 and 10 mmHg. This allowed isovolumetric pressure measurements to be recorded continuously. Coronary flow rate (CFR) was measured by collecting the coronary effluent from the apex of the heart for 30 seconds and expressed in mL/min.

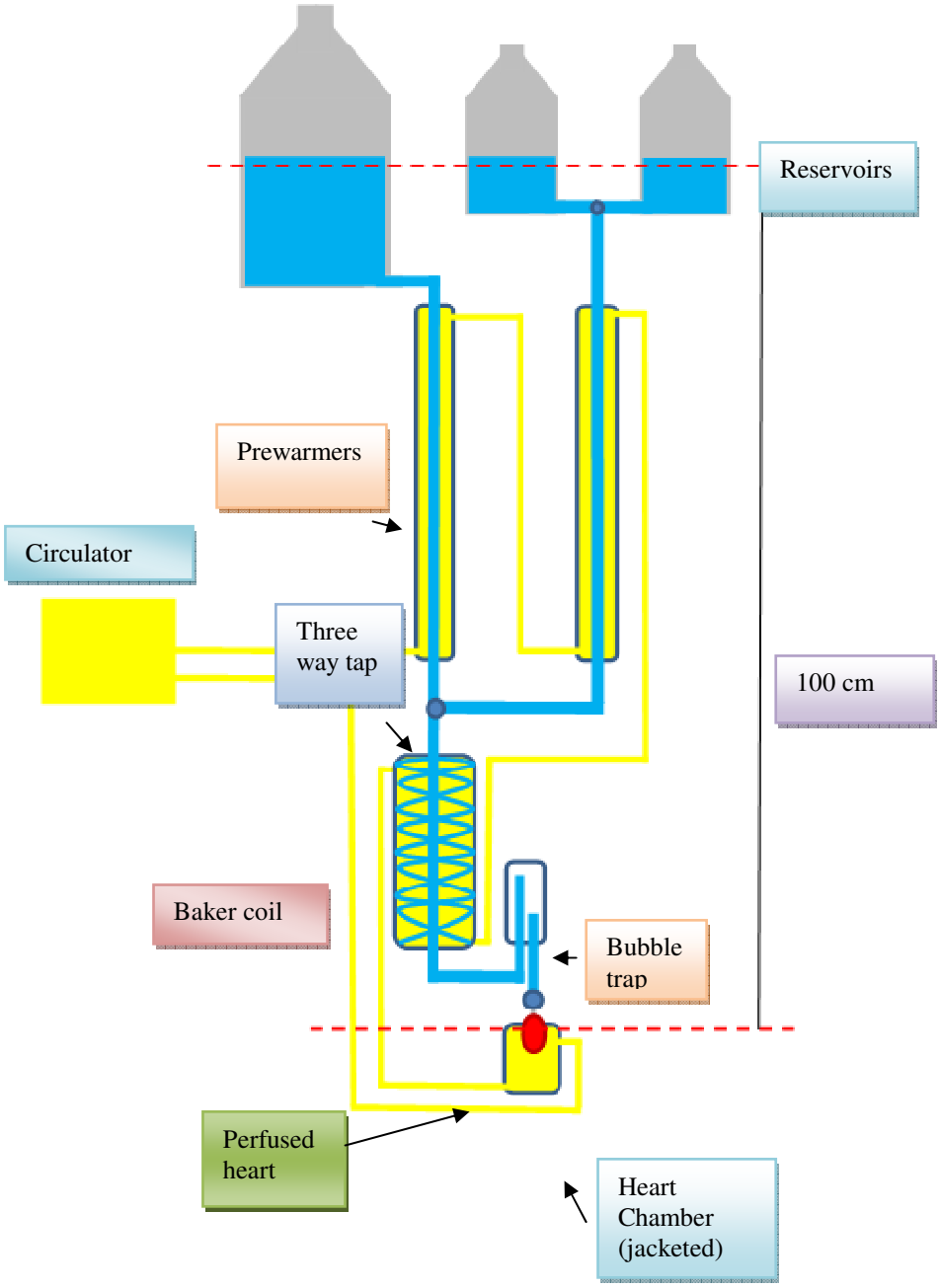


Figure 2.7. Diagram representing the apparatus used for Isolated Heart perfusion experiments. Of importance is the difference in height between the perfused heart and the KH level in the reservoir. Yellow indicates glass wear that is jacketed and warmed to 37 ˚C by the heated circulator. The bubble trap ensures that any air in the system is captured and does not pass into the myocardium through the aorta.

2.2.4. Stabilisation

A period of stabilisation was initiated to ensure that the haemodynamic functions of the heart met the inclusion criteria. These included a LVEDP of between 5 and 10 mmHg, a left ventricular developed pressure (LVDP) of at least 50 mmHg, a CFR between 10 and 24 mL/min, heart rate (HR) of 200-350 beats per minute (BPM) and a maintained temperature of 36.8-37.4˚C. During stabilisation these haemodynamic measurements were monitored.

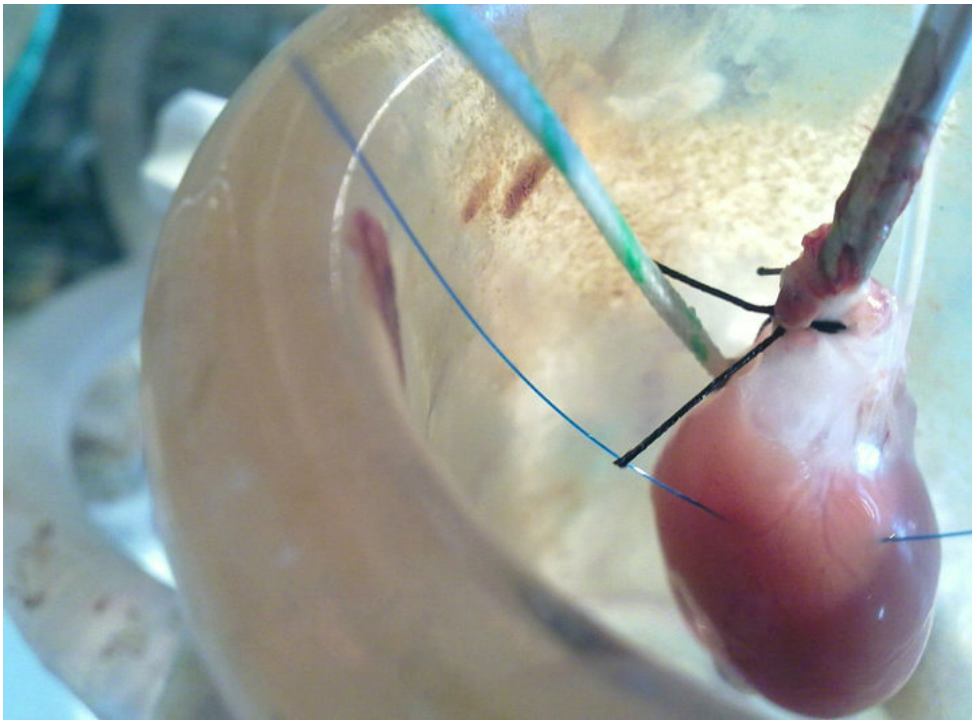


Figure 2.8. Location of threading on anterior LDCA, Isolated rat heart with balloon inserted into the left ventricle and connected to a pressure transducer via a sealed water filled cannula.

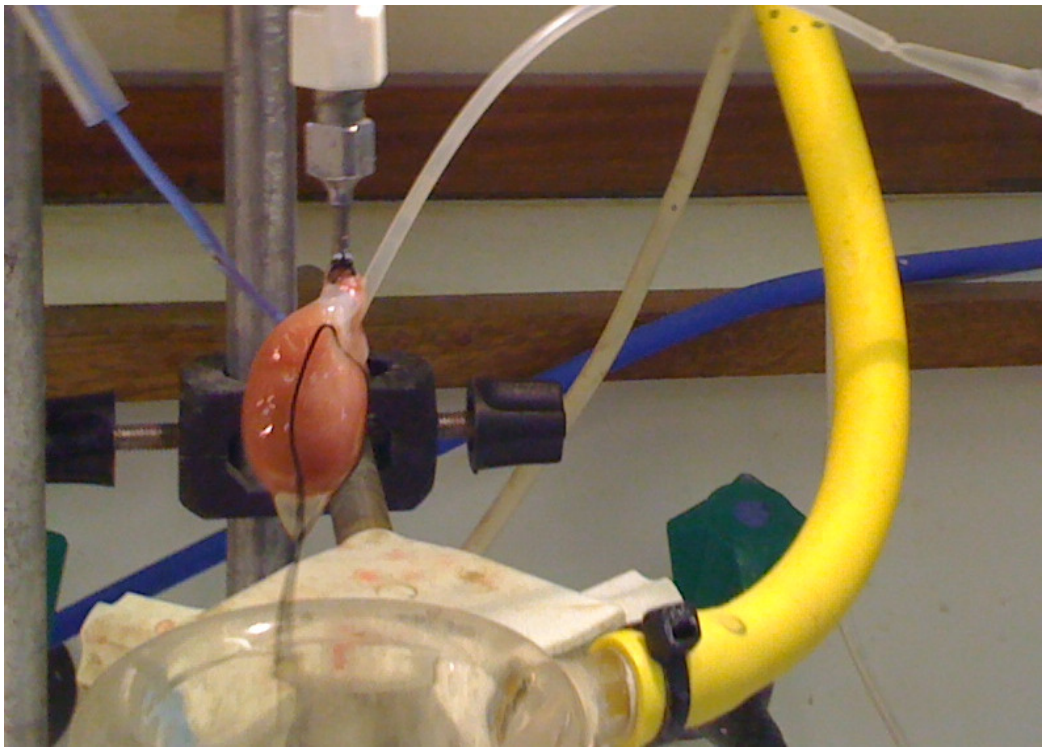


Figure 2.9. Isolated rat heart with balloon inserted into the left ventricle and connected to a pressure transducer via a sealed water filled cannula.

2.2.5. Induction of ischaemia

Regional ischaemia was induced following the stabilisation period by occluding the anterior LDCA by threading both ends of the silk suture though a shortened 200 μL pipette tip (Figure 2.8-B). A second 200 μL pipette tip was placed inside the first creating a snare that could be tightened, occluding the LDCA with the surrounding tissue. Confirmation that the artery was sufficiently occluded was gained by a >30 % decrease in CFR. The heart was subjected to 35 min regional ischaemia and then reperfused for 120 min. Reperfusion was achieved by removing the pipette tip snare, ensuring that the silk suture was still in place, but not occluding the artery, confirmed by an increase in CFR towards baseline. 120 min of reperfusion allowed washout of reducing enzymes and co-factors from irreversibly damaged cells that

would otherwise have interfered with staining the heart in the next part of the protocol.

2.2.6. Re-occluding and staining

Following 120 min of reperfusion the LDCA was ligated with a surgeon's knot using the silk ligature that was already in place. Between 0.5 and 1.0 mL of Evans Blue (0.4 %), dye was then perfused through a side arm in the cannula tap into the heart, staining the non-risk zone (see Figure 2.8- C). The heart was then removed from the cannula and frozen at -20˚C for 3-24 hours.

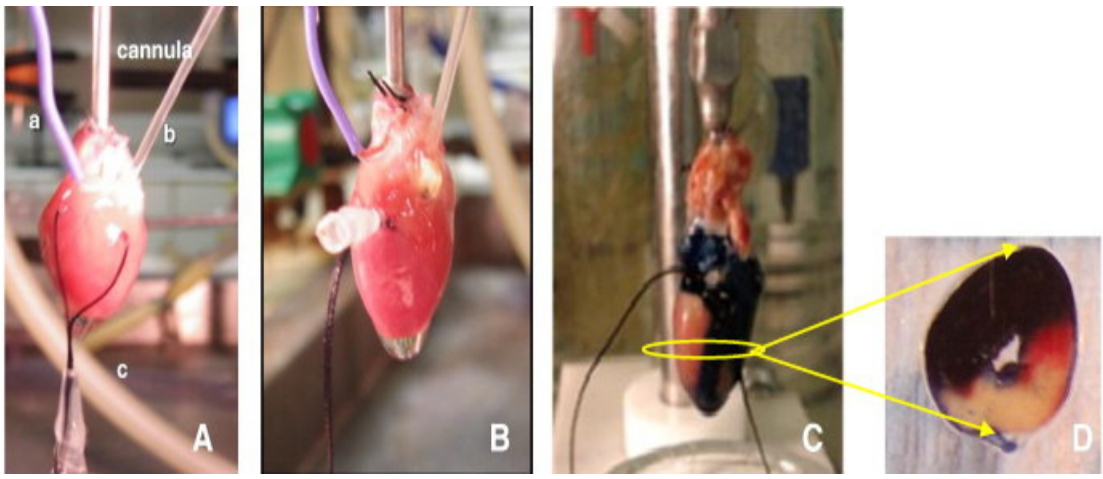


Figure 2.10. Rat heart mounted in the Langendorff system. A- In stabilisation, B- During ischaemia, C-temporary ligation is released during reperfusion but in the end of this period, a secure knot is tied and Evans blue is perfused via the aortic cannula, staining the non risk zone dark blue. D- TTC stained rat heart slice (Bell & Yellon, 2011).

The frozen heart was allowed to partially thaw for 2-3 min and then sectioned transversely from the apex into 2 mm thick sections. These sections were then thawed completely before being incubated in 1 % triphenyltetrazolium chloride (Sigma-Aldrich, UK) at 37.0 ˚C for 20 min, with frequent agitation. The red

formazan pigment observed in the non-blue stained regions was produced by the reduction of the triphenyltetrazolium chloride by enzymes and the nicotinamide adenine dinucleotide phosphate (NADPH) cofactors in viable tissue. The stained sections were then fixed in 10 % formalin for 48 hours before being imaged from both sides of the cross section using computer imagery software ImageJ version 1.45q (National Institute of Health, USA) (Figure 2.9).

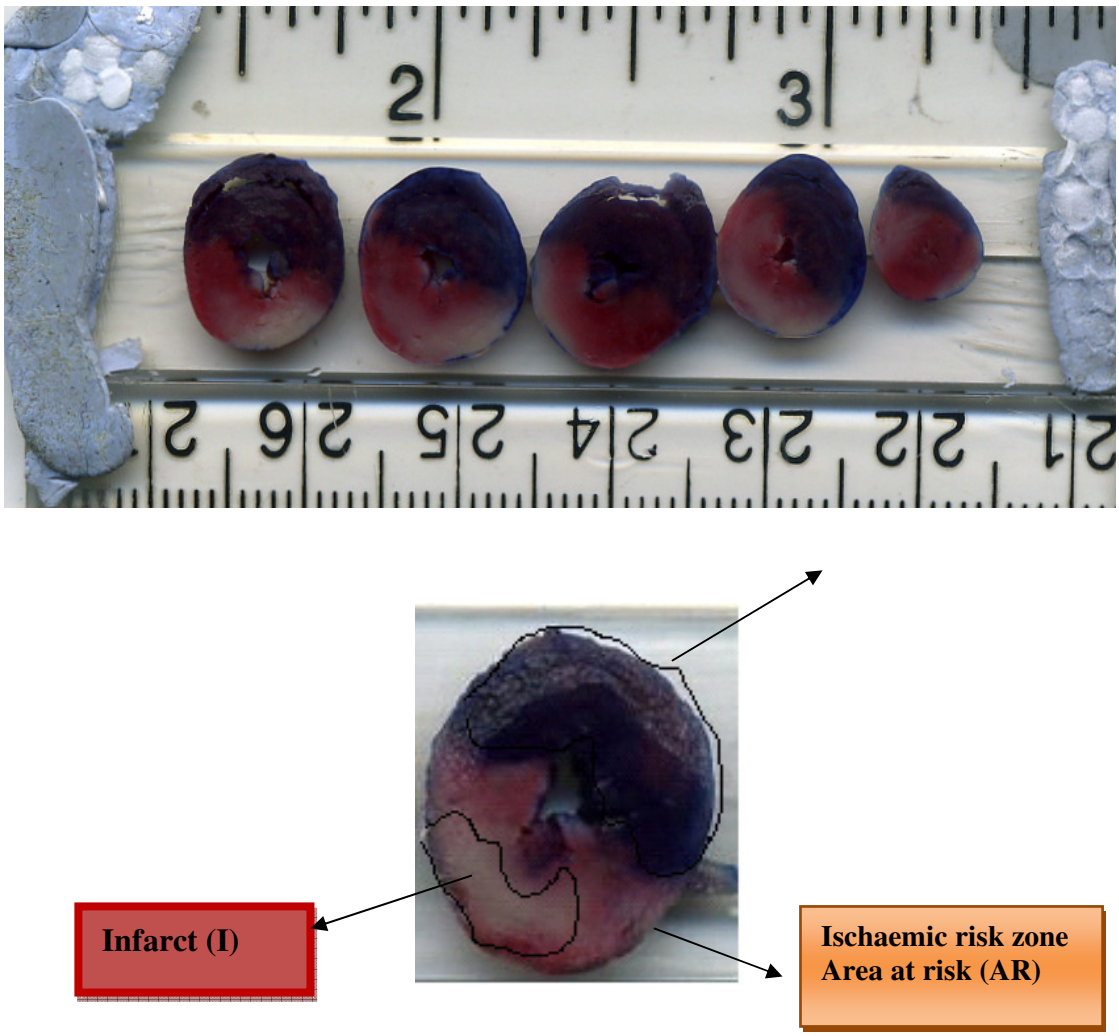


Figure 2.11. Scanned image of heart sections following tetrazolium staining and 48 hours fixation in formalin. Blue colouring delineates tissue not subjected to ischaemia. Red coloured tissue delineates tissue subjected to ischaemia but not infarcted. White colouring highlights infarcted tissue. The sum of red and white tissue equals the risk zone.

Planimetry was then performed on each image using a graphics tablet (Trust, USA), measuring the total area, risk zone and infarct size of each section. Planimetry was performed blindly and the recorded values were converted into volumes and combined to give total heart area at risk and infarct size as a percentage of the risk zone.

2.2.7. Statistical analysis

Experimental data were analysed using Prism 5.0. Data are expressed as mean ± standard error of the mean (SEM). Normality testing was performed prior to subsequent analysis. All data sets were found to be normally distributed, confirmed by the Kolmogorov-Smirnov test. ONE-way ANOVA was used when stated to analyse the arithmetic means followed by Newman-Keuls post-hoc test when significance was reported. This was used to compare arithmetic means for raw data corresponding to specific treatment groups. Cardiodynamic data including HR, LVDP and rate pressure product (RPP, heart rate multiplied by LVDP) were analysed using repeated measures ANOVA followed by Newman-Keuls post-hoc test. Values were considered statistically significant if $p<0.05$.

2.2.8. Pharmacological Compounds

All salts used to make modified KH solution were sourced from Fisher Scientific LTD (UK). Aquaeous *V. album* (AVa) extract was dissolved in the KH. Methanolic *V. album* (MVa) extract was dissolved in methanol. Glibenclamide (K_{ATP}

channel blocker) was dissolved in dimethyl sulphoxide (DMSO), the final concentrations of DMSO in the KH was 0.04 % v/v.

2.2.9. Concentration response to AVa and MVa : Study-I

This study was undertaken to assess the infarct limiting properties of the aqueous and methanolic *V. album* extracts. A concentration response to AVa and MVa were carried out by perfusing hearts with AVa 1 mg, 10 mg, 50 mg and MVa 5 mg, 10 mg, 20 mg at reperfusion. The control hearts were perfused for 20 min with KH for AVa group and were subjected to 35 min ischaemia then reperfused for 120 min. In MVa groups, hearts were perfused with the same volume of methanol.

Group 1, Control for AVa: This group includes some hearts that were perfused with the KH from 10 min before ischaemia until 10 min reperfusion. There was no statistical significant difference between control hearts perfused with or without methanol vehicle and so all hearts were pooled for statistical analysis.

Group 2, AVa-1mg : The hearts were perfused with KH buffer containing 1 mg/L AVa lyophlized extract from 10 min before ischaemia until 10 min reperfusion.

Group 3, AVa-10mg: The hearts were perfused with KH buffer containing 10 mg/L AVa lyophlized extract from 10 min before ischaemia until 10 min reperfusion.

Group 4, AVa-50mg: The hearts were perfused with KH buffer containing 50 mg/L AVa lyophlized extract from 10 min before ischaemia until 10 min reperfusion.

Group 5, Control for MVa: The hearts were perfused with KH buffer containing same volume of methanol (50, 100, 200 µl) from 10 min before ischaemia until 10 min reperfusion.

Group 6, MVa-5 mg/L: The hearts were perfused with KH buffer containing 5 mg/L MVa lyophlized extract from 10 min before ischaemia until 10 min reperfusion.

Group 7, MVa-10 mg/L: The hearts were perfused with KH buffer containing 10 mg/L MVa lyophlized extract from 10 min before ischaemia until 10 min reperfusion.

Group 8, MVa-20 mg/L: The hearts were perfused with KH buffer containing 20 mg/L MVa lyophlized extract from 10 min before ischaemia until 10 min reperfusion.

Group 9, IPC, 3X3 cycle: The hearts were subjected to ischaemic preconditioning regimes consisting of 3 cycles of 3 min global ischaemia followed by 3 min reperfusion.

2.2.10. Effect of AVa&MVa Study –II

Group 1, Control for AVa & MVa: This group includes some hearts that were perfused with the KH and methanol, respectively, from 10 min before ischaemia until 10 min reperfusion. There was no statistical significant difference between control hearts perfused with or without methanol vehicle and so all hearts were pooled for statistical analysis.

Group 2, AVa-10mg/L: The hearts were perfused with KH buffer containing alone 10 mg / L AVa lyophlized extract from 10 min before ischaemia until 10 min reperfusion.

Group 3, Glibenclamide+AVa: The hearts were perfused with KH buffer containing combination of 10 mg/L AVa lyophlized extract and 10µM Glibenclamide from 10

min before ischaemia until 10 min reperfusion.

Group 4, Glibenlamide: The hearts were perfused with KH buffer containing alone 10µM Glibenclamide from 10 min before ischaemia until 10 min reperfusion.

Group 5, L-Name + AVa: The hearts were perfused with KH buffer containing combination of 10 mg /L AVa lyophlized extract and 100µM L-Name from 10 min before ischaemia until 10 min reperfusion.

Group 6, L-Name: The hearts were perfused with KH buffer containing alone 100µM L-Name from 10 min before ischaemia until 10 min reperfusion.

Group 7, MVa-5mg/L: The hearts were perfused with KH buffer containing alone 5 mg /L MVa lyophlized extract from 10 min before ischaemia until 10 min reperfusion.

Group 8, Glibenclamide+MVa: The hearts were perfused with KH buffer containing concomitant with 5mg/L MVa lyophlized extract and 10µM Glibenclamide from 10 min before ischaemia until 10 min reperfusion.

Group 9, Glibenclamide: The hearts were perfused with KH buffer containing alone 10µM Glibenclamide from 10 min before ischaemia until 10 min reperfusion.

Group 10, L-Name+MVa: The hearts were perfused with KH buffer containing combination of 5 mg/L MVa lyophlised extract and 100µM L-Name from 10 min before ischaemia until 10 min reperfusion.

Group 11, L-Name: The hearts were perfused with KH buffer containing alone 100µM L-Name from 10 min before ischaemia until 10 min reperfusion.

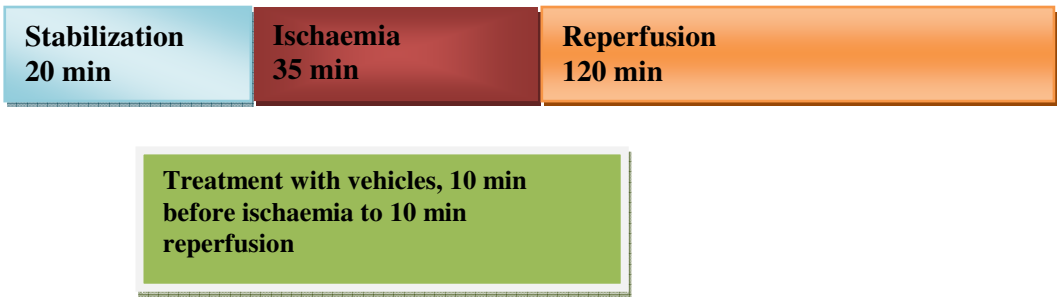


Figure 2.12. Experimental protocol for groups in 1-8 in study I-II. All hearts were stabilised for 20 min followed by 35 min regional ischaemia and then reperfused for 120 min.

Figure 2.13. Experimental protocol for group in 9 in study I. All hearts were stabilised for 20 min followed by 3 X 3 cycle IPC then 35 min regional ischaemia and reperfused for 120 min.

2.3. RESULTS

2.3.1. Summary of experiments

In this Study, 121 rats were used. Three hearts died after 50 mg/L AVa administration. Three animals died before their hearts were excised and seven hearts were excluded from the study due to technical error, thus 108 completed experiments were reported. The period of stabilisation before the onset of ischaemia was carried out to allow the hearts to stabilise and reach pre determined criteria

(see below). For a heart to be included and subjected to ischaemia it had to achieve the following baseline cardiodynamic criteria.

CFR between 10 and 24 mL/min, LVEDP 5-10 mmHg, HR 200-350 BPM, LVDP greater than 50 mmHg and a steady sinus rhythm. Hearts were also excluded during analysis if there was inadequate delineation of the risk zone and infarcted tissue (poor staining).

2.3.2. Infarct size data: Study -1

This study contains Langendorff perfusion experiments for the initial dose response to AVa and MVa. The area at risk for all hearts in all groups was 45 to 55 % of the combined left and right ventricular tissue. There were no statistical differences between groups for risk zone sizes. Infarct size was expressed as a percentage of the risk zone, calculated as described in Chapter 2 and reported in Figures 2. 8 & 2 .9. Under control conditions (35 min ischaemia followed by 120 min reperfusion) hearts had infarct sizes of 38.06 ± 1.38 % (n=56) compared to IPC 21.49 ± 2.43 % (n=7) ($p<0.05$)

Treatment with MVa 5mg/L and 10mg/L showed a concentration dependent reduction in infarct size compared to control (20.25 % ± 2.46 (n=6), 27.27 % ± 4.1 (n=7), respectively. But treatment with MVa, higher concentration, 20 mg/L had no statistically significant effect on infarct size (34.7 % ± 2.64 (n=5). The lowest concentration of MVa showed a 53.20 % relative reduction in mean infarct size compared to control hearts. (Figure 2.12).

Treatment with 1mg, 10mg AVa showed a concentration dependent

reduction in infarct size compared to control (39.05 % ± 1.69 (n=5), 17.46 % ± 1.5 (n=7) and highest concentration of AVa (50 mg) was stopped the all hearts (n=3). 10 mg concentration of AVa showed a 45.87 % relative reduction in mean infarct size compared to control hearts (Figure 2.13).

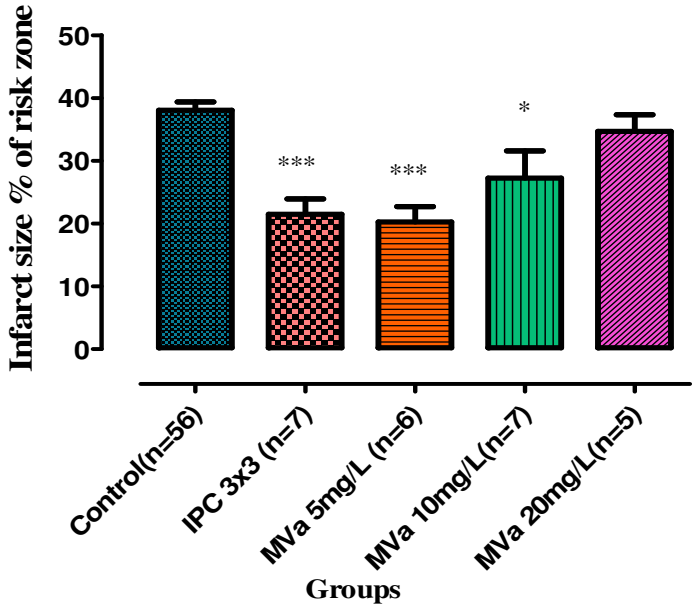


Figure 2. 14. Infarct size expressed as percentage of ischaemic risk zone for MVa (*** p<0.01 vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-17).

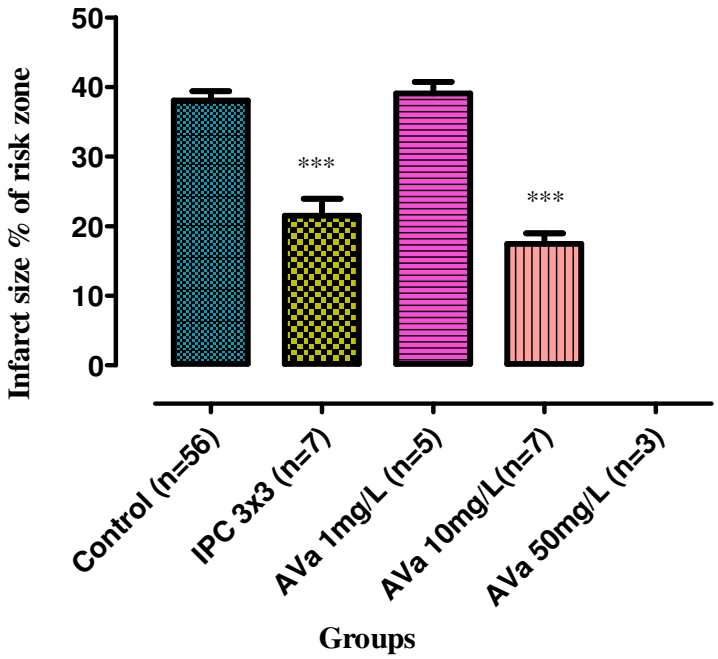


Figure 2. 15. Infarct size expressed as percentage of ischaemic risk zone for AVa (*** p<0.01 vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-17).

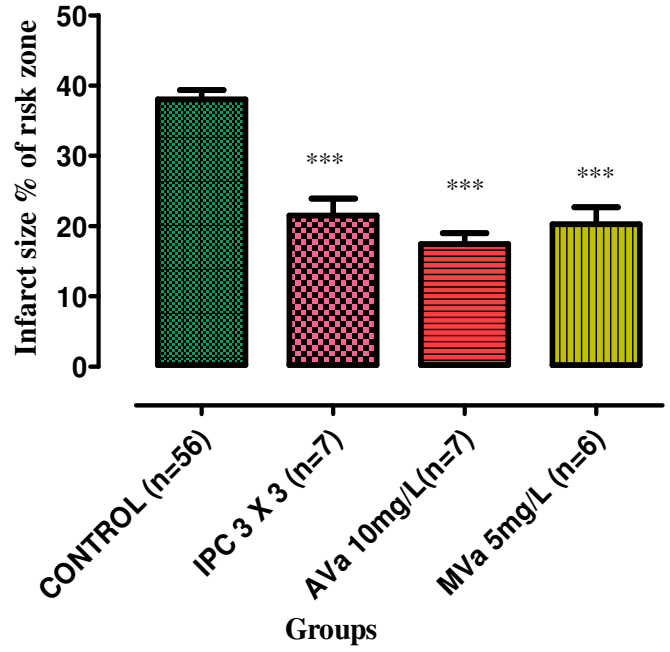


Figure 2.16. Infarct size expressed as percentage of ischaemic risk zone (*** p<0.01 vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-17).

2.3.3. Cardiodynamic Data: Study –I

Baseline cardiodynamic data are presented in Table 2.1 & 2.2 There was no statistical difference in any of the parameters between groups. RPP dropped by as much as 95 % upon induction of ischaemia in all treatment groups, recovering partially throughout ischaemia and then dropping again upon reperfusion due to the tendency for the hearts to fibrillate. This recovered before a gradual decline throughout reperfusion (Figures 2 .15). There is no differences in RPP and CFR (Figures 2 .15 A & B) parameters between Control, AVa (1 mg/L, 10mg/L) and MVa (5 mg/L) groups. Higher concentratons of MVa (10 mg/L and 20 mg/L) significantly reduced recovery throughout the reperfusion than the others, Control = AVa = MVa (5mg/L) > MVa (10mg/L) > MVa (20mg/L). Upon induction of ischaemia, CFR dropped in all experiments by at least 45 %. CFR increased towards baseline once the ligature had been removed from the LDCA, confirming successful reperfusion and then decreased gradually during reperfusion.

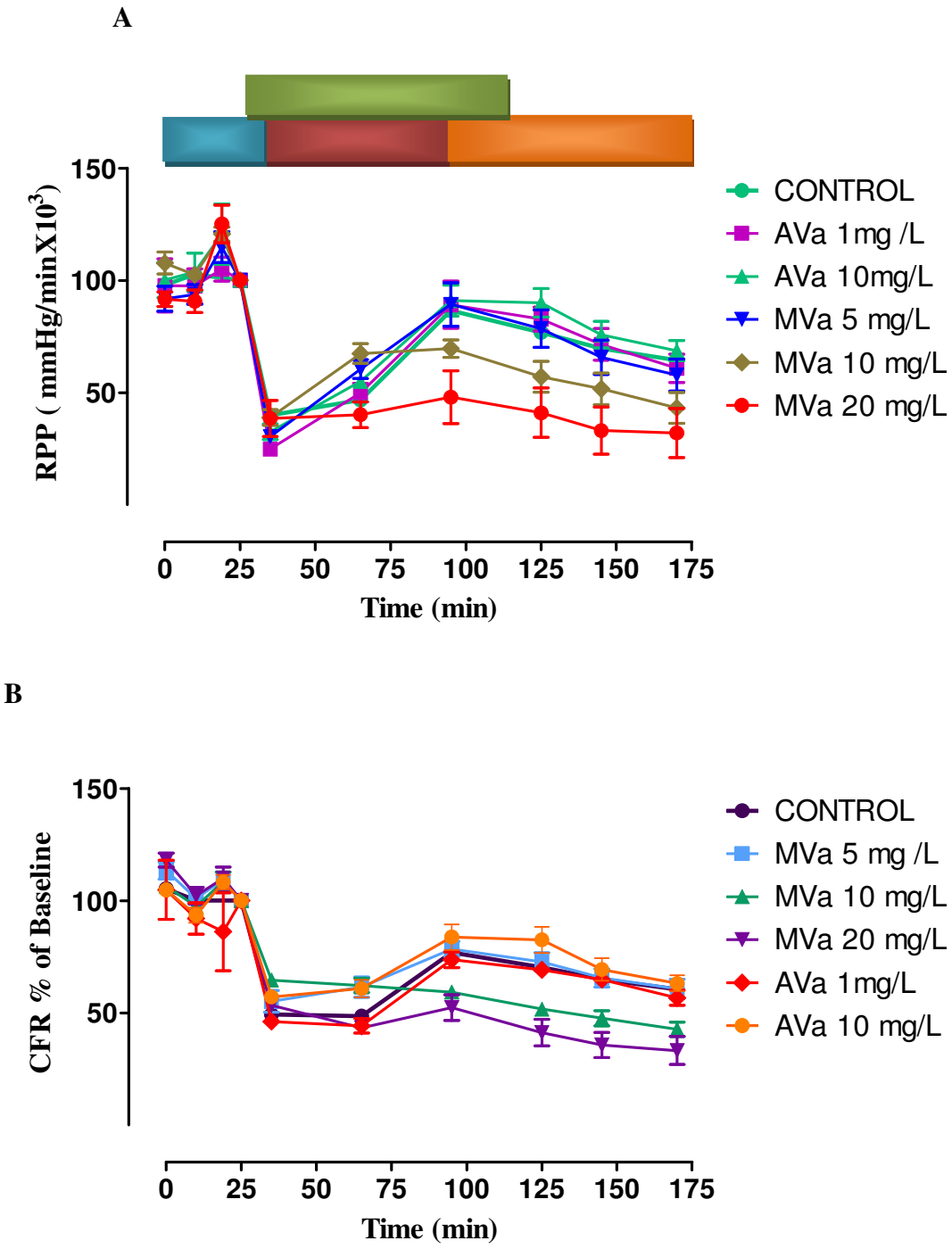


Figure 2. 17. A. Percentage change from baseline, rate pressure product and coronary flow rate B. from -1 min stabilisation (19 min) through 120 min reperfusion (175 min). Green box indicates time at which drugs were perfused. Repeated measures ANOVA.

2.3.4. Infarct size data: Study - II

Concomitant perfusion of 10 μM glibenclamide (K_{ATP} channel blocker) with aquoeus extract (AVa) was carried out to explore the role of K_{ATP} channel. It was decided that co-treatment would be perfused for 10 min prior to ischaemia till 10 min of reperfusion. Treatment with glibenclamide alone had no statistically significant effect on infarct size (%35.45 ± 4.0 (n=7)) ($p<0.05$) compared to controls. Administration of glibenclamide with AVa showed a reduction infarct size (%24.08 ± 3.5 (n=7)) ($p<0.05$) compared to controls.

Concomitant perfusion of 100 μM L-NAME (NOS inhibitor) with aquoeus extract (AVa) was carried out to explore the role of NO releasing activity. It was decided that co-treatment would be perfused for 10 min prior to ischaemia till 10 min of reperfusion. Treatment with L-NAME alone had no statistically significant effect on infarct size (%28.4 ± 3.79 (n=6)) ($p<0.05$) compared to controls. However, L-NAME abrogated the effect of AVa (30.70± 3.64 (n=6) ($p<0.05$).

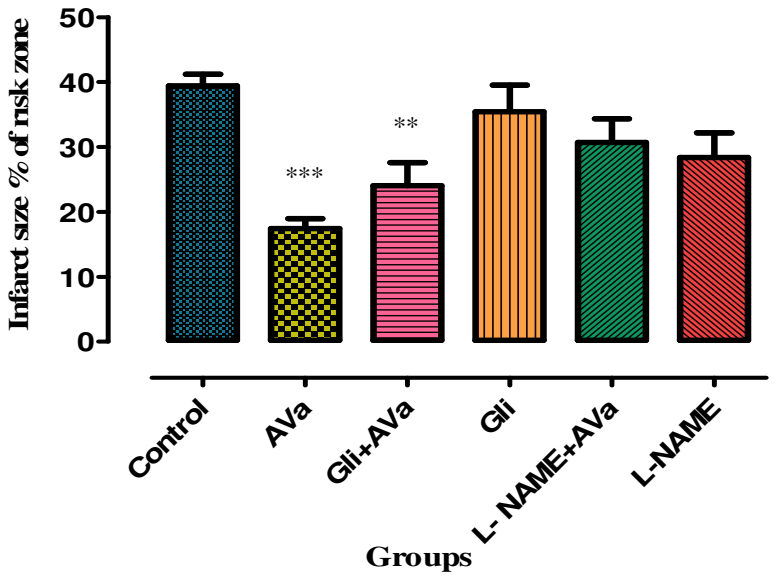


Figure 2. 18. Infarct size expressed as percentage of ischaemic risk zone (** $p<0.01$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-17).

Concomitant perfusion of 10 μM glibenclamide (K_{ATP} channel blocker) with methanolic extract was carried out to explore the role of K_{ATP} channel. It was decided that co-treatment would be perfused for 10 min prior to ischaemia till 10 min of reperfusion. Treatment with glibenclamide alone had no statistically significant effect on infarct size (%35.45 ± 4.0 (n=7)) ($p<0.05$) compared to controls. However, glibenclamide abrogated the protective effect of MVa (%33.74 ± 2.23 (n=7)) ($p<0.05$) compared to controls.

Concomitant perfusion of 100 μM L-NAME (NOS inhibitor) with methanolic extract was carried out to explore the role of NO releasing activity. It was decided that co-treatment would be perfused for 10 min prior to ischaemia till 10 min of reperfusion. Treatment with L-NAME alone had no statistically significant effect on infarct size (28.4 ± 3.79 % (n=6)) ($p<0.05$) compared to controls. However, L-NAME abrogated the effect of MVa (29.10± 2.17 % (n=7) ($p<0.05$).

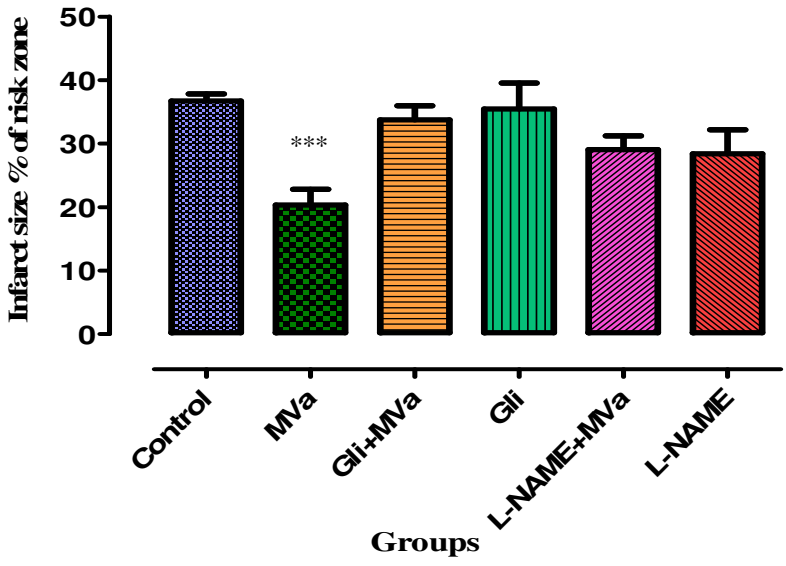
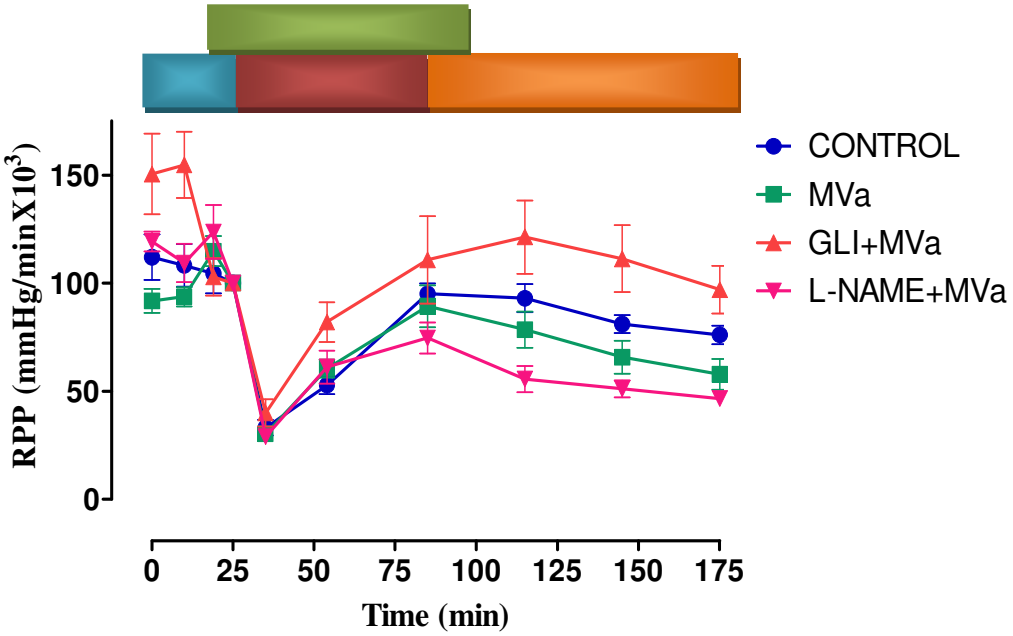


Figure 2.19. Infarct size expressed as percentage of ischaemic risk zone (*** $p<0.01$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-17).

2.3.5. Cardiodynamic Data: Study – II

Baseline cardiodynamic data are presented in Table 3.1. There was no statistical difference in any of the parameters between groups. RPP dropped by as much as 95 % upon induction of ischaemia in all treatment groups, recovering partially throughout ischaemia and then dropping again upon reperfusion due to the tendency for the hearts to fibrillate. This recovered before a gradual decline throughout reperfusion (Figures 2. 18 & 2. 19). Upon induction of ischaemia, CFR dropped in all experiments by at least 45 % (Figures 2.18 B & 2. 19 B). CFR increased towards baseline once the ligature had been removed from the LDCA, confirming successful reperfusion and then decreased gradually during reperfusion.

A.



B.

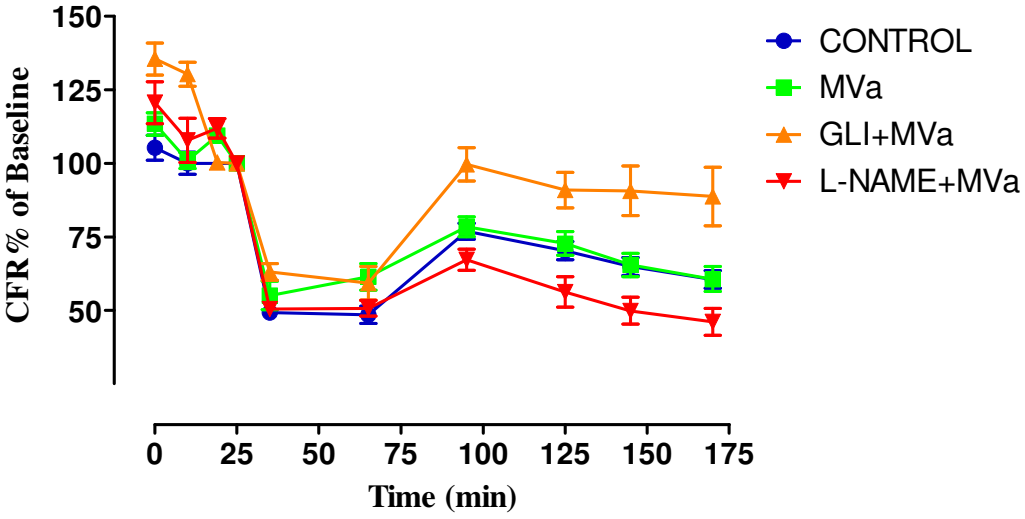
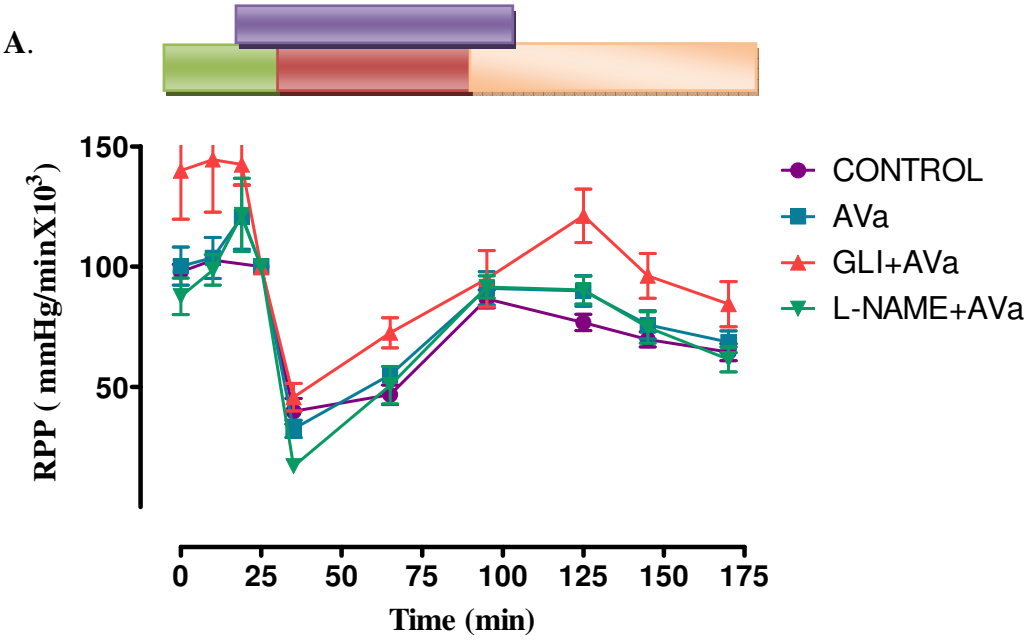


Figure 2.20. A. Percentage change from baseline, rate pressure product and coronary flow rate B. from -1 min stabilisation (19 min) through 120 min reperfusion (175 min). Green box indicates time at which drugs were perfused. Repeated measures ANOVA.

A.



B.

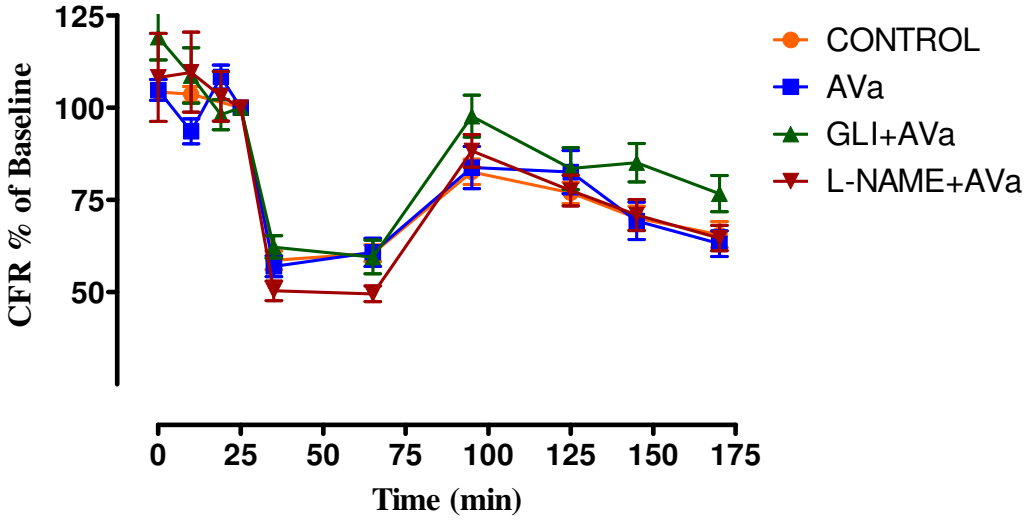


Figure 2.21. A. Percentage change from baseline, rate pressure product and coronary flow rate B. from -1 min stabilisation (19 min) through 120 min reperfusion (175 min). Repeated measures ANOVA. Purple box indicates time at which drugs were perfused.

Table 2.1 Baseline cardiodynamic data. No statistical differences between groups (ONE-way ANOVA + Newman-Keuls post-hoc)

Treatment Group	n	CFR (mL/min)	HR (BPM)	LVDP (mmHg)	RPP (mmHg/minx10^3)	Vol LV and RV (cm^3)	Risk Zone % Vol LV and RV	Risk ZoneVol. (cm^3)
Control	17	15.2±0.7	289±10.4	64±2.7	18.4±1.1	0.81±0.03	44.0±0.9	0.37±0.02
IPC	7	13.4±0.8	302±11.8	61±2.4	19.4±1.7	0.74±0.04	38.7±1.3	0.29±0.02
AVa 1 mg/L	7	13.0±1.4	266±17.1	60±6.0	15.8±2.0	0.80±0.06	45.2±0.9	0.34±0.03
AVa 10 mg/L	7	14.1±0.6	306±13.1	63±4.6	19.1±1.6	0.72±0.05	43.5±0.9	0.31±0.02

Table 2.2. Baseline cardiodynamic data. No statistical differences between groups (ONE-way ANOVA + Newman-Keuls post-hoc)

Treatment Group	**n**	**CFR (mL/min)**	**HR (BPM)**	**LVDP (mmHg)**	**RPP (mmHg/minx10^3)**	**Vol LV and RV (cm^3)**	**Risk Zone % Vol LV and RV**	**Risk ZoneVol. (cm^3)**
Control	12	13.5±0.5	285±15.2	67.0±2.9	19.0±1.2	0.79±0.02	41.3±0.8	0.32±0.007
IPC	7	13.4±0.8	302±11.8	61±2.4	19.4±1.7	0.74±0.04	38.7±1.3	0.29±0.02
MVa 5mg/L	6	14.2±1.0	302±8.5	64.7±6.3	19.4±1.7	0.79±0.03	42.3±1.0	0.33±0.01
MVa 10mg/L	7	15.2±1.1	296±10.1	67.7±4.3	20.0±1.5	0.82±0.03	41.6±1.1	0.35±0.01
MVa 20 mg/L	5	13.7±1.0	285±4.3	68.5±2.2	19.5±0.7	0.74±0.05	41.0±1.3	0.34±0.02

Table 2.3. Baseline cardiodynamic data. No statistical differences between groups (ONE-way ANOVA + Newman-Keuls post-hoc)

Treatment Group	n	CFR (mL/min)	HR (BPM)	LVDP (mmHg)	RPP (mmHg/minx10^3)	Vol LV and RV (cm^3)	Risk Zone % Vol LV and RV	Risk ZoneVol. (cm^3)
Control	17	15.2±0.7	289±10.4	64±2.7	18.4±1.1	0.81±0.03	44.0±0.9	0.37±0.02
IPC	7	13.4±0.8	302±11.8	61±2.4	19.4±1.7	0.74±0.04	38.7±1.3	0.29±0.02
AVa	7	14.1±0.6	306±13.1	63±4.6	19.1±1.6	0.72±0.05	43.5±0.9	0.31±0.02
AVa +Gli	7	12.7±0.7	300±15.3	61±3.4	18.4±1.4	0.81±0.03	39.6±1.2	0.32±0.01
Gli	7	14.0±0.6	315±16.1	69±5.7	21.1±2.5	0.76±0.03	41.6±1.5	0.31±0.01
AVa + L-Name	6	12.1±1.3	279±10.2	64±2.4	17.9±0.9	0.87±0.04	47.1±1.4	0.41±0.02
L-Name	6	13.5±1.4	296±16.9	60±4.5	17.7 ±2.1	0.80±0.04	45.2±1.1	0.36±0.02

Table 2.4. Baseline cardiodynamic data. No statistical differences between groups (ONE-way ANOVA + Newman-Keuls post-hoc)

Treatment Group	n	CFR (mL/min)	HR (BPM)	LVDP (mmHg)	RPP (mmHg/minx10^3)	Vol LV and RV (cm^3)	Risk Zone % Vol LV and RV	Risk ZoneVol. (cm^3)
Control	12	13.5±0.5	285±15.2	67.0±2.9	19.0±1.2	0.79±0.02	41.3±0.8	0.32±0.007
IPC	7	13.4±0.8	302±11.8	61±2.4	19.4±1.7	0.74±0.04	38.7±1.3	0.29±0.02
MVa	6	14.2±1.0	302±8.5	64.7±6.3	19.4±1.7	0.79±0.03	42.3±1.0	0.33±0.01
MVa +Gli	7	15.7±0.8	318±13.3	69.5±4.6	22.1±1.7	0.74±0.02	43.4±0.9	0.32±0.01
Gli	7	14.0±0.6	315±16.1	69.0±6.9	21.8±2.5	0.77±0.03	41.6±1.5	0.32±0.02
MVa + L-Name	7	13.1±0.7	288±13.1	59.0±2.7	15.5±1.0	0.82±0.03	41.5±1.0	0.34±0.01
L-Name	5	13.5±1.4	296±17.1	60.0±4.7	17.7±2.1	0.80±0.04	45.2±1.1	0.36±0.02

2.4. Discussion

This study was designed to evaluate the cardioprotective effects of *V. album* crude extracts on isolated heart preparation, using the langendorff technique in ischaemia-reperfusion infarct model. We hypothesised that *V. album* is able to limit infarct size under ischeamia-reperfusion injury. Its action has been attributed to vasodilator properties associated with increased nitric oxide synthase activity. Little is known about its effects on myocardial ischaemia-reperfusion injury. In this study lyophilised aqueous and methanolic *V. album* extracts have been shown to significantly reduced infarct size in isolated rat perfused heart when given 10 minute before ischaemia till 10 minutes of reperfusion. Since the agents possesses a known vasodilator effect, we further hypothesized that NO generation and K_{ATP} channel opening are involved in the cardioprotective action of *V. album.* Pharmacological observations of the mechanisms by which the K_{ATP} channel blocker and NOS inhibitor are illustrated in the ischaemia-reperfusion infarct limitation model. These two vasodilator mechanisms are known to have mediated infarct limitation in many earlier studies of ischaemic preconditioning, a proven endogenous protective mechanism.

The NOS inhibitor L-NAME was given with or without AVa 10 mg/L during ischaemia reperfusion to investigate the dependency of endogenous NO on AVa extract induced infarct limitation. Concomitant perfusion of L-NAME abrogated the protection afforded by the AVa extract suggesting that this protection is dependent on the function of NOS. Administration of the NOS inhibitor alone resulted in an infarct size similar to that of the control experiments; this

observation is consistently seen in many laboratories (Burley & Baxter, 2007; Ren et al., 2007; Krieg et al., 2009). This suggests that inhibition of NO production by NOS at reperfusion does not increase infarct size beyond control levels. It remains to be elucidated whether compensatory mechanisms are activated or increased to protect the heart from further damage. Pre-ischaemic treatment of the myocardium with L-NAME has however been shown to improve contractile recovery (Andelova et al., 2005). It has been suggested that this may be of benefit as less NO produced during early reperfusion will limit the amount of deleterious ROS species produced (Zweier & Talukder, 2006). There is no evidence to suggest that this translates to infarct limiting protection in a model of reperfusion. Tenorio et al. (2005) reported that coronary vasodilator action elicited by aqueous *V. album* extract is mediated by the NO/ sGC pathway and furthermore that *V. album* increased in NOS-2 and NOS-3 expression. Our findings are support these reports, up to point. Another study reported that *V. album* reduced mean arterial pressure of both normotensive and hypertensive rats. In addition, it was shown to be very unlikely that extract employs the autonomic pathways in depressing heart rate (Eno et al., 2004). Conversely, Ofem et al. (2007) suggested that *V. album* extracts produced an antihypertensive effect without any alteration in heart rate due to vasopressive effect. In our study, we have not found any differences in heart rate between all groups after treated V. *album* extracts. Interestingly, the percentage of coronary flow in effective concentration of *V. album* extracts has been increased by the same amount.

The K_{ATP} channel blocker Glibenclamide was given with or without AVa during ischaemia - reperfusion to investigate the role of K_{ATP} channels on AVa

induced infarct limitation. Concomitant perfusion of glibenclamide did not abrogate the protection afforded by the AVa suggesting that this protection is independent of K_{ATP} function. Administration of the glibenclamide alone resulted in an infarct size similar to that of the control experiments; this observation is consistently seen in many laboratories (Burley & Baxter, 2007; Ren et al., 2007; Krieg et al., 2009). This suggests that blockage of K_{ATP} channels by glibenclamide during ischaemis - reperfusion does not increase infarct size beyond control levels. It remains to be elucidated whether compensatory mechanisms are activated or increased to protect the heart from further damage. Like many other studies demonstrates that targeting a specific component of the so – called RISK pathway can afford infarct limitation. The fact that inhibition of some components of the pathway does not exacerbate infarct size beyond control levels demonstrates the dynamic non – linear structure of the signalling. This suggests that other components of the pathway can be activated or that components can be bypassed by non – linear signaling.

On the other hand some research reported antioxidant activity of *V. album* extracts and these have also shown that this activity depends on the host tree and harvesting time (Deliorman et al., 2005; Ucar et al., 2006). Cardioprotective effect of *V. album* extracts may be due to its high antioxidant capacity. It can act as primary and / or secondary antioxidants, being free radical scavengers in prevention of lipid oxidation. In a previous study, the chemotactic and anti-apoptosis activity of extract was found by Basaran et al. (1995). Many studies also reported anti-apoptic (Khil et al. 2007), cytotoxic (Valentiner et al. 2002), angiogenesis (Elluru et al. 2009), anti-cancer (Kovacs, 2002), immunomodulatory and antiprolierative (Urech et al. 2006) properties of *V. album* extracts.

Experiment in Chapter 2 showed that concomitant perfusion of the methanolic extract with L- NAME abrogated the protection afforded by the MVa alone, suggesting NO releasing plays role in its action. Similarly, concomitant perfusion of the MVa with Glibenclamide abrogated the protection afforded by the MVa alone, suggesting blockage of the K_{ATP} channels also play role this protection. Concomitant perfusion of the aqueous extract with L-NAME abolished the protection afforded by the AVa alone, suggesting NO dependence in AVa action like MVa protection, however this protection not abrogated by blockage of the K_{ATP} channels, suggesting that it is independent of K_{ATP} channels.

Adjunct data in the form of RPP and CFR recorded during the reperfusion experiments highlight no haemodynamic or functional differences between in effective concentrations of MVa and AVa groups when compared the control group. Although this may appear surprising, the percentage of the total heart damaged by CAO in our model varies only between approximately 8-17 %, insufficient to observe differences in LVDP. These observations are consistent with previous studies that report comparable limitation in infarct size following pharmacological interventions; yet document no difference in either LVDP or RPP (Hausenloy et al., 2002; D'Souza et al., 2003; Burley & Baxter, 2007). These data in the form of CFR suggest that any infarct limitation documented for a given treatment group is independent of vasodilatation. However, specific investigation of any vasoactive properties of the interventions used would require an adapted protocol. Hearts would be perfused at constant flow and not constant pressure and any changes in vascular tone would be recorded as a change in pressure. This model is better suited to investigating vasoactive parameters, but is not optimal for

infarct studies as constant pressure represents physiological conditions more robustly.

RPP is a reliable indicator of heart function, represented as a function of both HR and LVDP. Heart rate fluctuates very little throughout the described Langendorff experiments and so any large changes in RPP are because of changes in LVDP. Differences were reported for % change of RPP and CFR between different treatment groups irrespective of their ability to limit infarct size. Highest recovery in RPP was seen when concomitant perfusion of Glibenclamide with MVa, Gli+MVa> Control> MVa> LNAME+ MVa after reperfusion period, respectively. % change of coronary flow were found that Gli+MVa> Control = MVa> LNAME+ MVa. However, data in the form of RPP and CFR recorded during the reperfusion, no haemodynamic or functional differences between in effective concentrations of AVa, LNAME+AVa and Control but only concomitant perfusion of glibenclamide with AVa shown highest recovery. Our study shown that the methanolic and aqueous extracts do not similar action on the cardioprotection; their chemical components probably differed from eachother. Cardioprotective action of *V. album* extracts in folk remedy has not been proven, either in controlled clinical studies or on experimental animal models. Further studies need to be focused on the isolation, purification, structural dilucidation, pharmacological reevaluation in order to find the chemical compound responsible for such a pharmacological effect.

2.5. Conclusions

Results from these studies demonstrate that both lyophilized aqueous and methanolic *V. album* crude extracts are cardioprotective when given at 10 min before ischaemia till 10 min of reperfusion. The extracts given at concentrations of 10 mg/L for AVa and 5 mg/L for MVa limited infarct size in regionally ischaemic isolated rat heart. This cardioprotective effect was abrogated with L-NAME in both extracts, AVa & MVa administration. The protection afforded by the *Viscum* extracts alone, suggesting NO releasing dependence in its action. While the cardioprotective activity of AVa was attenuated by the blockage of the K_{ATP} channels with Glibencliamide, this protection was abolished with glibenclamide in MVa administration. Further studies need to be performed to investigate the interaction of *V. album* extracts and more specific agents. These need to be focused on the isolation, purification, structural dilucidation, pharmacological reevaluation in order to find the chemical compound responsible for such a pharmacological effect.

CHAPTER 3

3. ISOLATED ATRIUM MUSCLE IN ORGAN BATH

3.1. Introduction

3.1.1. Anatomy of the atrium

Atriums possess the same basic components. Thus, each atrium is made up of a venous component, an appendage, and a vestibule, with the chambers separated one from the other by the septum (Anderson et al. 2007). These various components themselves are supported by the bodies of the atriums. The body is much more obvious in the left than in the right atrium, and is derived from medisatinal myocardium. The venous components of the two chambers are then derived from different embryonic sources, with the entirety of the embryonic systemic venous sinus being incorporated into the morphologically right atrium (Anderson et al. 2007). The right atrium is dominated by an extensive array of pectinate muscles within the extensive appendage, whereas the left atrium is relatively smooth-walled, with a much smaller tubular appendage. The atrial walls vary markedly in their thickness between the atriums, and also in their structure. For the most part, the

walls of the left atrium are thicker than their right atrial counterparts, but parts of the right atrium are also thickened, such as the terminal crest and the pectinate muscles (Wang et al. 1995). Within these thickened parts of the right atrium, the myocytes are aligned with their own long axis parallel to the long axis of the muscular bundles. This arrangement potentiates to more rapid conduction along the long axis than parallel to it. In similar fashion, it is the parallel arrangement of the myocytes within Bachman's bundle that potentiates to somewhat more rapid interatrial conduction across the anterior interatrial groove, a mechanism emphasized by Bachman himself in his original description of this muscular bridge (Bachmann, 1916). Within the left atrium itself, there is a much more irregular arrangement of the myocytes, the alignment often changing at different depths within the atrial walls. Inhomogeneities both in gross structure and myoarchitecture are common in the normal heart. The histologically specialized cells in the atrial walls are found only within the right atrium. They make up the sinus node, positioned laterally and sub-epicardially within the terminal groove, and the atrioventricular node, located at the apex of the triangle of Koch. Limited areas of transitional myocardium form the entrances to the atrioventricular node, but such transitional zones are very short at the margins of the sinus node (Ho et al. 2002).

3.1.2. Neuronal Regulate of Atrium

Atriums are innervated by vagal and sympathetic fibers. The right vagus nerve primarily innervates the SA node, whereas the left vagus innervates the AV node; however, there can be significant overlap in the anatomical distribution (Opie,

1998). Cardiac function is altered by neural activation. Sympathetic stimulation increases heart rate (positive chronotropy), inotropy and conduction velocity (positive dromotropy), whereas parasympathetic stimulation of the heart has opposite effects. Sympathetic and parasympathetic effects on heart function are mediated by beta-adrenoceptors and muscarinic receptors, respectively. Cholinergic parasympathetic simulation from the vagus nerve causes a decrease in the heart rate via muscarinic receptors, which hyperpolarize pacemaker cells and slow phase 4 depolarization. There are three types of parasympathetic reflex receptors found in the atria that function to regulate changes in heart rate or volume status. Type A receptors are innervated by myelinated vagal afferent fibers and are located throughout the atria as well as at the junction between the vena cava and the RA and the pulmonary vein and the LA. These receptors seem to be more responsive to heart rate rather than atrial chamber volume, and they discharge impulses continuously throughout the normal cardiac cycle that correspond to the timing of the av wave. Type B receptors are also innervated by myelinated vagal afferent fibers and occur in a similar distribution to that of type A receptors. These receptors seem to be more responsive to atrial stretch and changes in intravascular volume than to heart rate, and they discharge impulses late in systole that correspond to the timing of the v wave. These receptors are normally inactive in atrial contraction, but they increase their activity in conditions such as tachyarrhythmias, in which the rate of rise of intra-atrial pressure increases. Finally, a group of receptors innervated by group C parasympathetic fibers and located throughout the atria respond to changes in atrial pressure with a threshold of 2 to 3 mm Hg, but in general they are much less responsive and have a lower rate of activity than type B receptors. Most sympathetic afferent nerves are unmyelinated,

although the significance of myelinated versus unmyelinated nerve fibers remains unclear. Both myelinated and unmyelinated spinal afferents have been detected in the atria, fire throughout the cardiac cycle, and appear to be sensitive to both mechanical and chemical stimulation including K^+ and bradykinin (Berne & Levy 2001).

3.1.3. Ion Channels

Ion channels are selective for ions so there are Na^+, K^+, $Ca^{2+,}$ Cl^- channels, among others. And each ion can have some different channels which are used in different situations. Most of them are controlled by the membrane potential and are the so-called voltage-gated ion channels. Others are ligand-gated channels what mean they need the presence of a chemical ligand to open its gate. Voltage-gated ion channels have transmembrane voltage sensors. Ligand-gated channels have receptors where the ligand will be bound to unleash an action. Everything is regulated by genes. Most of these mechanisms are yet under research and belong to molecular biology. **Funny channels**; Excitatory cells have the so-called pacemaker channels of the HCN family channels, Hyperpolarization-activated, Cyclic Nucleotide-gated channels. These poorly selective cation channels conduct more current as the membrane potential becomes more negative, or hyperpolarized. They conduct both potassium and sodium ions. The activity of these channels in the SA cells causes the membrane potential to slowly become more positive (depolarize). They are the so-called "funny" channels and are responsible for the phase 4 diastolic depolarization. **The fast Na^+ channel;** The fast sodium channels are voltage-dependent and have a very important role in cardiac action potential, these channels have three main

functions: they permit Na^+ to go in, keep K^+ from going out and prevent Ca^{2+} from getting stuck in the channel and interfering with Na^+permeability. **Potassium channels**; there are two main types of K^+ channels but all have a basic common function: the creation of a transmembrane "leak" of potassium ions. This leak is the cause of a hyperpolarization. The voltage gated (Kv) channels are activated by a specific the depolarizing voltage change. They are located mainly inside the cellular membrane. The inward-rectifier channels (Kir) are gated by nucleotides and G proteins among others and most of them are located outside the cellular membrane. **Calcium channels**; two voltage-dependent calcium channels play critical roles in the electro-physiology of cardiac muscle: L-type calcium channel ('L' for Long-lasting) and T-type calcium channels ('T' for Transient) voltage-gated calcium channels. These channels respond differently to voltage changes across the membrane: L-type channels respond to higher membrane potentials, open more slowly, and remain open longer than T-type channels. Because of these properties, L-type channels are important in sustaining an action potential, while T-type channels are important in initiating them. Because of their rapid kinetics, T-type channels are commonly found in cells undergoing rhythmic electrical behavior. For example, T-type channels are commonly found in some neuron cell bodies involved in rhythmic activity such as walking and breathing. These T-type calcium channels are also found in pacemaker cells, the sinoatrial node and the atrioventricular node (Opie 1998; Berne & Levy 2001).

Most of the signal transduction pathways that regulate inotropy involve Ca^{++}. Briefly, inotropic state can be enhanced by 1) increasing Ca influx across the sarcolemma during action potential via L- type Ca channels, 2) increasing the release

of Ca by the sarcoplasmic reticulum or 3) sensitizing troponin C to Ca. For example, β_1 adrenoreceptor activation acting through Gs protein increase cAMP, which activates protein kinase A.This enzyme, can phosphorylate different intracellular sites to influence Ca entry, Ca release and Ca affinity. Cardiac glycosides such as digoxin inhibit the Na/K ATP ase, leading to an increase in intracellular Ca and increase in inotropy (Opie, 1998; Berne & Levy 2001).

3.2. Method

3.2.1. Dissection and preparation of isolated atrium

The Guiding Principles in the Care and Use of Laboratory Animals together with The Recommendations from the Declaration of Helsinki were strictly adhered to during the execution of all the procedures described within this manuscript. This project was approved by the institutional Experimental Animal Care and Use Ethics Committee of Abant Izzet Baysal University before the commencement of any intervention. Sprague – Dawley rats weighing 230 – 300 g were anesthetized by ether inhalation. An incision was made on the skin of the thorax and the underlying muscle layer. The ribs were cut open to expose the heart. The entire heart was excised rapidly and transferred into an ice-cold Feigen solution (in mM; NaCl 153.8, KCl 5.6, NaHCO3 23.8, glucose 11.1, CaCl2 5.5) which was bubbled with 95% O_2 plus 5% CO2 (pH 7.4). Two atria were isolated from the heart and tied by silk threads and suspended inside a tissue chamber containing 20 ml of feigen solution aerated continuously at 34 ^{0}C.

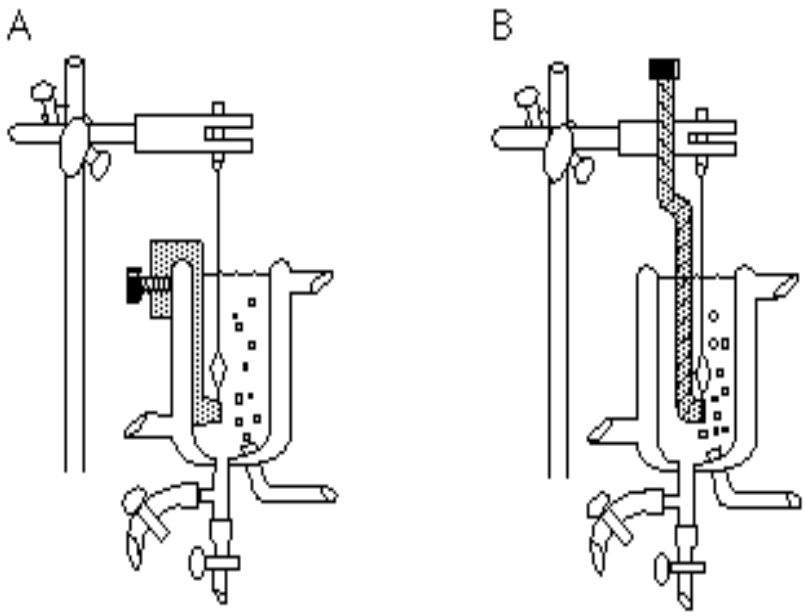


Figure 3.1. Isolated tissues mounted in organ baths. (A) For tissue fixed to a holder mounted on the organ bath, response is measured from a free-floating holder. (B) An apparatus in which the tissue and recording device (isometric transducer) are fixed together, not touching the walls of the organ bath.

3.2.2. Measurement of cardiac muscle contractility

The contractile force was measured isometrically, using force displacement (Biopac, MP30) and recorded on a computer. Beating rate of the spontaneous contractions was monitored simultaneously. Atrial tissue was equilibrated for 30 min under resting tension of 0.5 g, which gave maximum contractile force in the rat atrium. During this period, the solution in the bath was changed every 15 min. Plant extract were added directly into the tissue chamber and cumulative concentration responses were obtained.

Figure 3. 2. Isolation of atrium by Eylem Suveren in Hacettepe Univ., Pharmacology Dept. 2008.

3.2.3. Statistical Analysis

Experimental data were analysed using Prism 5.0. Data are expressed as mean ± standard error of the mean (SEM). Normality testing was performed prior to subsequent analysis. All data sets were found to be normally distributed, confirmed by the Kolmogorov-Smirnov test. ONE-way ANOVA was used when stated to analyse the arithmetic means followed by Newman-Keuls post-hoc test when significance was reported. This was used to compare arithmetic means for raw data corresponding to specific treatment groups. Heart rate and

contraction force were analysed using repeated measures ANOVA followed by Newman-Keuls post-hoc test. Values were considered statistically significant if $p<0.05$.

3.2.4. Pharmacological Compounds

All salts used to make modified Feigen solution and drugs Atropin (muscarinic cholinergic receptor antagonist), Propanolol (beta adrenergic receptor antagonist), L-NAME (nitric oxide inhibitor), Glibenclamide (K_{ATP} channel blocker) were sourced from Sigma Scientific LTD. Aquaeous *V. album* (AVa) extract was dissolved in the ultrapure distilled water. Glibenclamide was dissolved in dimethyl sulphoxide (DMSO). All other compounds were dissolved in fresh physiological solution.

3.2.5. Effect of individual plant extract on isolated atria and the role of different drugs on this effect:

This study was undertaken to assess the atrial functions of the aqueous extract of *V. album*. A concentration response to AVa was carried out with 5 µg, 10 µg, 12.5 µg, 15 µg and 25 µg. When the atrial beating and tension were steady after first concentration, next higher concentration was applied. Choronotropic and inotropic responses were then obtained. Effective concentration of extract was also examined in the presence of Glibanclamide 10^{-6} M, L-NAME 10^{-5} M, Atropine 10^{-6} M, Propranolol 10^{-6} M. To exclude the effect of the solvent DMSO used in glibenclamide, control experiments were also performed in the presence of DMSO

at equivalent concentration alone. In control group atrium muscles received no treatment.

3.3. Results

3.3.1. Summary of experiments:

In this study, sixty nine rats were used. Nine animals died before their hearts were excised and six hearts were excluded from the study due to technical errors, thus fifty two completed experiments were reported. The period of stabilisation was carried out to allow the hearts to stabilise and reach pre determined criteria. This study contains isolated atrial muscle experiments for initial concentration response to for aqueous *V. album* extracts (AVa). In control group, the amplitudes of spontenous beating atrium muscles and the frequency of beat, the basal tension of atrium were found respectively; 238. 8 ± 55.8 mg, 340.7 ± 36.46 beat/min and 1203 ± 50.04 mg (n=25). 5 µg/ml, 10 µg/ml, 12.5 µg/ml, 15 µg/ml, 25 µg/ml concentrations of AVa was given to spontaneously beating atria.

Effective concentration was found 15 µg/ml for aquoeus *V. album* extract and this concentration significantly increased % change of basal tension 18.4 ± 4.2 in atrium compared to control. AVa signifacantly decreased % change of beat per minute, 22.4 ± 6.5. AVa significantly reduced % change of contraction force, 25.0 ± 5.7. AVa exhibited negative inotrophic and chronotropic effect consentration dependently. It was not recorded more than fifteen minutes for each experiment. Spontanously atrium beats were completely inhibited within the few min (2-3) at a

higher concentration than 15 µg/ml. Lower concentrations than 15 µg/ml were slightly not effected.

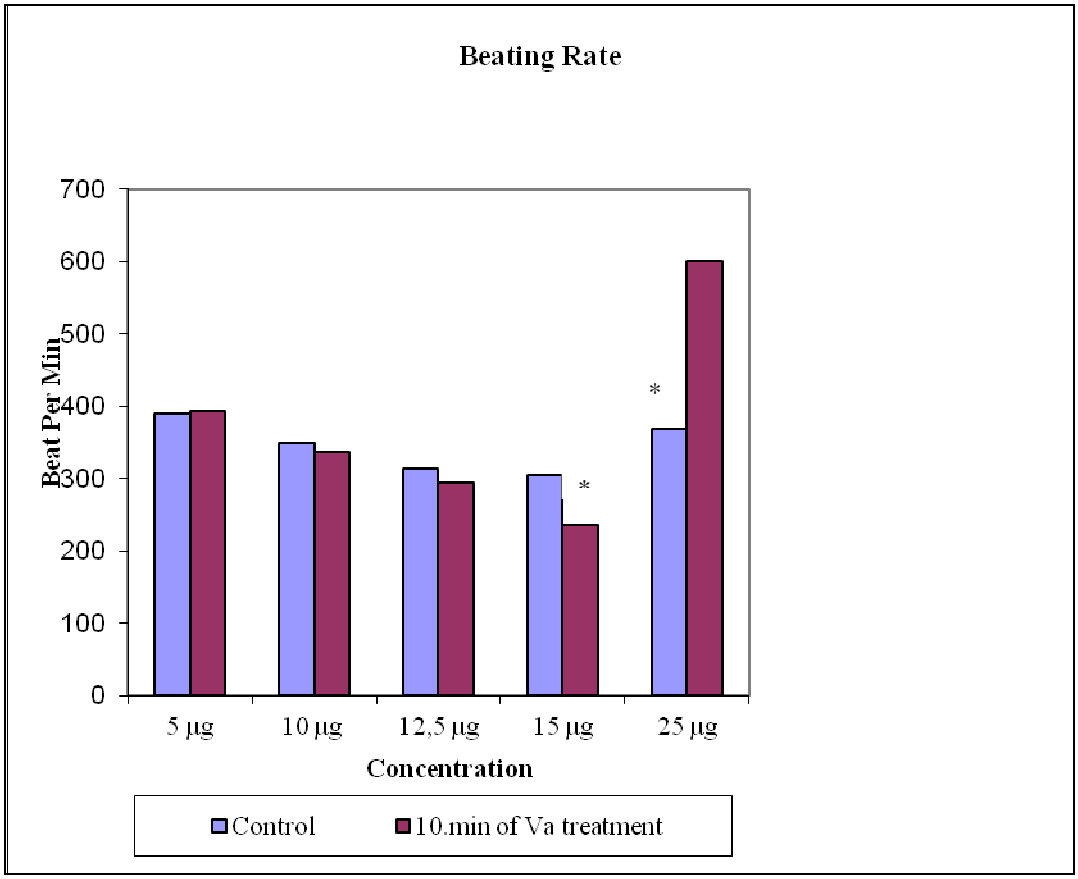


Figure 3. 3. The effect of different concentrations of AVa extracts on the heart rate in isolated atrial muscle preperations (** $p<0.05$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=5-7).

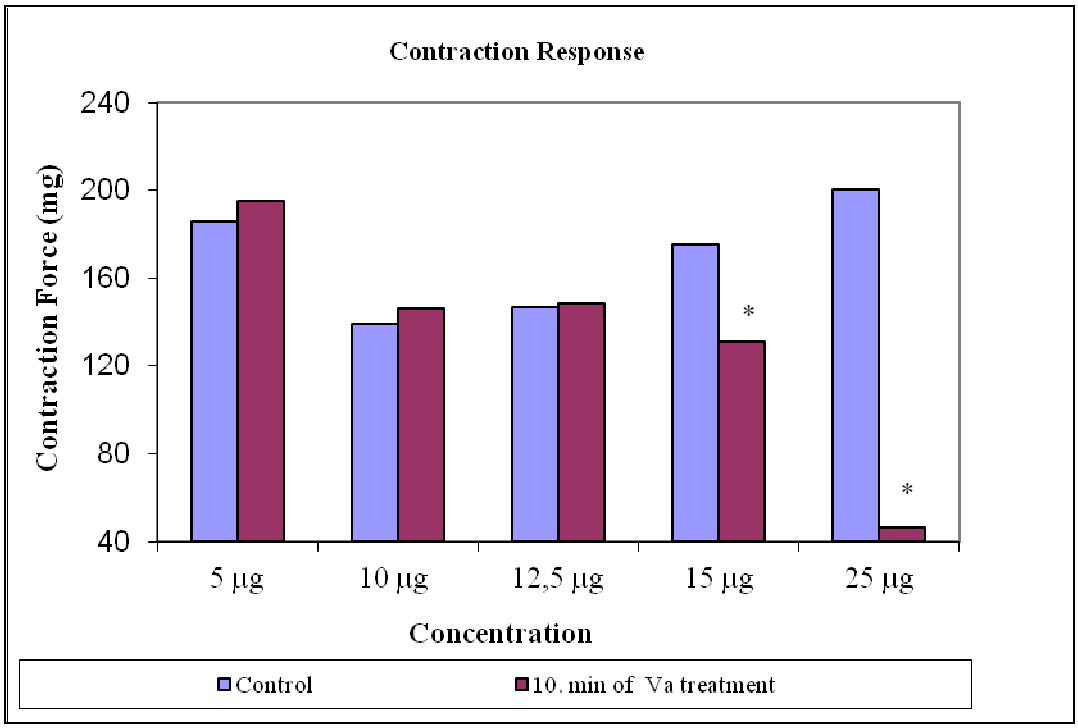


Figure 3. 4. The effect of different concentrations of AVa extracts on the contraction force (mg) in isolated atrial muscle preperations (** $p<0.05$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=5-7).

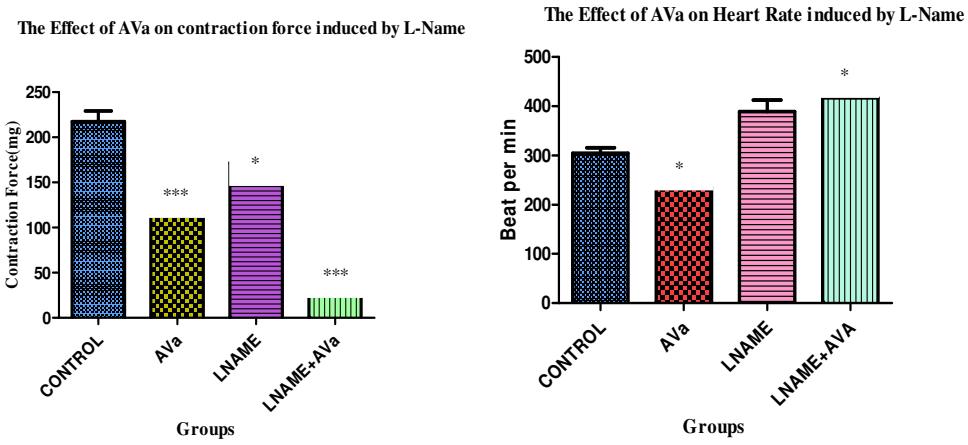


Figure 3. 5. The effect of AVa on contraction force and heart rate induced by L-NAME (*** p<0.01 vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-8).

In control groups, heart rate and contraction force are found 305 ± 11 per minute and 217 ± 12 mg, respectively. Administration of extract significantly decreased heart rate, 236.1 ± 28 and contraction force 131.4 ± 5.1 (p< 0.01). Treatment with L-NAME alone significantly deracreased contraction force 172.1± 13 and increased heart rate 388.7 ± 21 compared to control. Concomitant administration of LNAME with *V. album* extract significantly increased heart rate 416.3± 31 but decreased contraction force 49.8 ± 4.1 (p< 0.01).

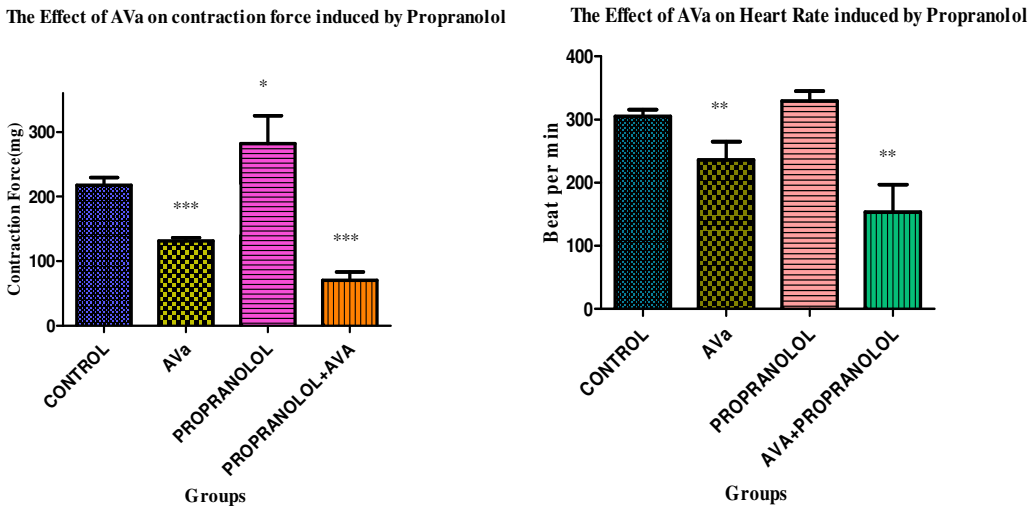


Figure 3. 6. The effect of AVa on contraction force and heart rate induced by Propranolol (** * p<0.01 vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=5-7).

Treatment with propranolol alone significantly increased contraction force 283.1 ± 27 compared to control and heart rate 329 ± 15 was found. Concomitant administration of propranolol with *V. album* extract significantly decreased heart rate 156.3± 37 and decreased contraction force 71.0 ± 13 (p< 0.01).

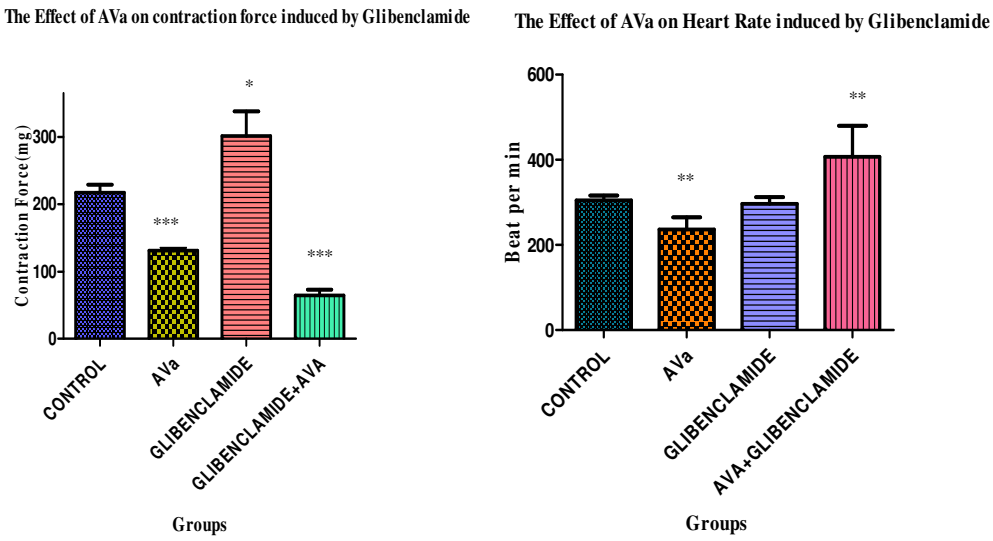


Figure 3. 7. The effect of AVa on contraction force and heart rate induced by Glibenclamide (*** $p<0.01$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=6-8).

Treatment with glibenclamide alone significantly increased contraction force 296.7 ± 16 ($p< 0.01$) compared to control and heart rate 302 ± 15 was found. Concomitant administration of glibenclamide with *V. album* extract significantly increased heart rate 406 ± 35 and decreased contraction force 64.6 ± 9 ($p< 0.01$).

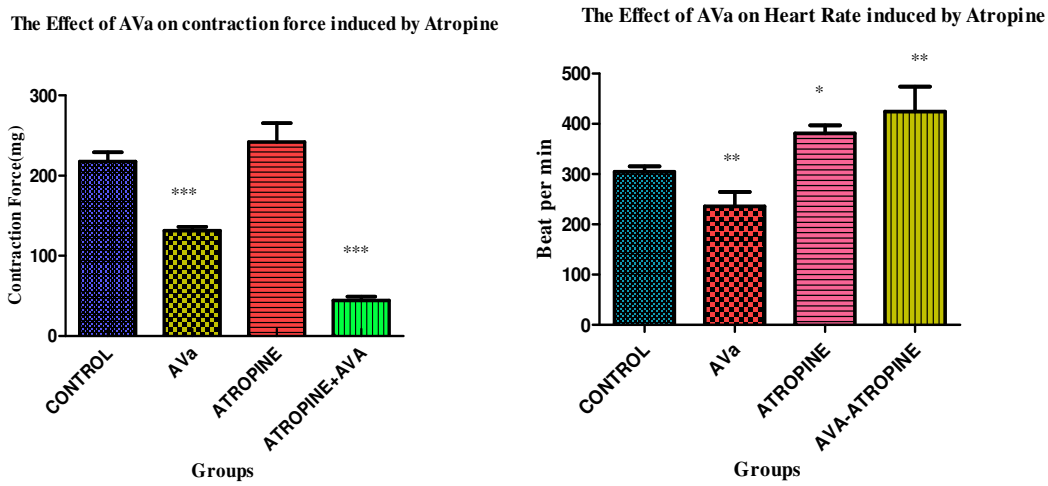


Figure 3. 8. The effect of AVa on contraction force and heart rate induced by Atropine (*** $p<0.01$ vs. control) ONE-way ANOVA + Newman-Keuls post-hoc (n=5-7).

Treatment with atropine alone significantly increased heart rate 382 ± 15 ($p< 0.01$) compared to control and contraction force 242.6 ± 23 was found. Concomitant administration of atropine with *V. album* extract significantly increased heart rate 424 ± 33 and decreased contraction force 46.4 ± 9 ($p< 0.01$).

3.4. Discussion

The results of this study demonstrate that *V. album* extract induce negative inotropic and chronotropic effects in the rat atrial muscles in vitro. Our results are in agreement with the findings of some of the earlier investigators who have reported negative inotropic and chronotropic effect of the *V. album* extract in experimental animal model (Rosell & Samuelsson 1966; Anderson et al., 1973). Negative inotropic effect of extract, reversible and reproducible effects seem to not be mediated by well known pathways that enhance contraction such as Glibenclamide and Atropine. None of the pharmacological agents used in this study abrogated the cardiac effects of *V. album* extract. However, negative chronotropic effect of extract inhibited by Glibenclamide and Atropine. Anderson et al. (1973) and Rosell et al. (1966) reported that viscotoxins and phoratoxin produced reflex bradycardia, negative inotropic effects and vasoconstriction in cardiac muscle of cats, progressive depolarization in rabbit heart preparations, a process reversed by calcium.

Some investigators have shown that an important correlation between the pharmacological activity of various therapeutic agents including natural products and inhibition of PDE activity (Weinbry et al., 1972; Nikaido et al., 1981). Wagner et al. (1986) reported that n- butanolic extract of *V. album* displayed an inhibitor activity on $_{C}$AMP-PDE activity. Mainly phenylpropanoids and lignan derivatives were suggested to contribute in this effect. Ergun et al. (1995) demonstrated that alcoholic extract of *V. album* produced marked relaxation in aorta. However, n- butanolic fraction of *V. album* did not produced relaxation in aorta (Deliorman et al., 2000), furthermore there was no clear correlation between the weak relaxant effect and an

inhibitory effect on $_{C}$AMP-PDE. In East and South regions of Anatolia, the 1-2 % infusion of the leaves of *V. album* is reported to be used against hypertension (Yucecan et al., 1988). In an experimental study, it has been reported that GABA (γ – amino butyric acid) isolated from ground leaved of *V. album* produced sharp reduction in blood pressure anesthesized rats (Samuelsson, 1959).

Endothelial nitric oxide plays a vital role in the control of vasomotor tone and structure. The role of NO in modulating cardiac contractility has been examined in different cardiac preparations and in humans. Administration of inhibitors of NO synthase in experimental animals and in humans has also been associated with cardiac depression. In this study, L-NAME did not affect the negative inotropic contractile responses to extract in the rat atria. The negative chronotropic effect of *V. album* was inhibited by glibenclamide, L-NAME and atropine. This result suggests that the effect was partially related to the K_{ATP} channel, NO releasing and muscarinic receptors.

In conclusion, we have shown that *V. album* extract produces a negative inotropic and chronotropic effect in a concentration-dependent manner. The mechanism of the negative chronotropic effect induced by extract on the hearts might be related to the K_{ATP} channels, activation of the NO–cGMP pathway and muscarinic receptor. These results suggest that extract at physiological concentrations might play a modulatory role in heart function, but at high concentrations has a damaging effect on isolated rat atrium.

3.5. Conclusion

Results from these studies demonstrate that lyophilized aqueous *V. album* crude extracts are produced concentration dependent negative inotropic and chronotropic effect on rat isolated spontaneously beating atrial muscle preperations. Moreover, negative inotropic effect of AVa did not abrogated by pharmacological agents used in this study, but negative chronotropic effect of AVa abrogated by L-NAME, Glibencliamide and Atropine. Further studies need to be performed to investigate the interaction of *V. album* extracts and more specific agents. These need to be focused on the isolation, purification, structural dilucidation, pharmacological reevaluation in order to find the chemical compound responsible for such a pharmacological effect.

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SKILLS

Teaching Skills

General Biology Laboratory I-II, undergraduate students class-I

Cell Biology Laboratory, undergraduate students class-II

Biochemistry Laboratory I-II, undergraduate students class-II

Animal Diversity Laboratory, undergraduate students class-II

Animal Physiology Laboratory, undergraduate students class-III

Haematology Laboratory, undergraduate students (Election Lesson III-IV)

Technical Skills

In - vitro organ bath (papillary muscle and atrium)

In – vivo ischemia - reperfusion arrhythmia model (Anesthetized and Conscious rats)

Isolated perfused heart (langendorff)

Extraction methods from plants (soxhlet, rotary evaporator, lyophilisator)
Experimental models of septic shock
Stereology Certificate

SCIENTIFIC PUBLICATIONS

1.Effects of adrenaline pretreatment on the arrhythmias observed following ischemia and reperfusion in conscious and anesthetized rats. Omer Bozdogan, Nuran Ekerbicer, **Eylem Suveren**, Sevda P. Bikmaz. **Exp.Clin.Cardiol.Vol.7, 20-24. 2002.**

2. Mechanisms of the glibenclamide-mediated anti-arrhythmia and ischemic conditionining in a rat model of myocardial infarction: Role of yohimbine treatment. Ömer Bozdoğan, Ersöz Gonca, **Eylem Suveren**, Fatih Gökçe .**Turk. J. Med. Sci. Vol. 34. 21- 27. 2004.**

4**.** The effect of sex on ischemia – reperfusion induced arrhythmias and the role of ATP-dependent potassium channel blockage. Ersöz Gonca, **Eylem Suveren**, Ömer Bozdoğan **Turk. J. Biol. Vol. 28. 39-46.2004.**

5. Effect of Thimerosal on Arrhythmia Induced by Coronary Ligation. The Involvement of ATP- dependent Potassium Channels. Ömer Bozdogan, Ersöz Gonca, Melih Nebigil, **Eylem Suveren**. **International Heart Journal Vol.46, No.4 pp.711-721, 2005.**

6. The Effect of Thimerosal on the Arrhythmias Observed During the Reperfusion and the Role of ATP Dependent Potassium Channels in Anesthetized Rats" Ömer Bozdoğan, **Eylem Suveren**, Ersöz Gonca **Journal of Düzce Medicine Faculty, 2002; 4 (3): 5-9.**

PRESANTATIONS

1. The Role Of GYY 4137, A novel H2S Donor, In Ischaemia – Reperfusion Injury. **ESC-Frontiers in Cardiovascular Biology. Imperial Collage, 2012, London – UK.**

2. Effects of adrenaline pretreatment on the arrhythmias observed following ischemia and reperfusion in anesthetized rats. Ömer Bozdoğan, **Eylem Suveren (26. National Physiology Congress, 2000 Eskişehir).**

3. Effects of adrenaline pretreatment on the arrhythmias observed following ischemia and reperfusion in conscious rats. Ersoz Gonca, Ömer Bozdoğan, **Eylem Suveren (27. National Physiology Congress, 2001 Istanbul).**

4. The combination effect of glibenclamide (K_{ATP} blocker) and Yohimbine (α_2 adrenergic blocker) on the arrhythmias observed following ischemia and reperfusion in conscious rats . Ersoz Gonca, **Eylem Suveren**, Ömer Bozdoğan **(28. National Physiology Congress, 2002 Izmir).**

5. Effect of Thimerosal on Arrhythmia Induced by Coronary Ligation. The Involvement of ATP- dependent Potassium Channels. Ömer Bozdoğan, **Eylem Suveren**, Ersoz Gonca **(29. National Physiology Congress, 2003 Ankara).**

6. The effect of sex on ischemia – reperfusion induced arrhythmias and the role of ATP-dependent potassium channels. Eylem Suveren **(30. National Physiology Congress, 2004 Konya).**

7. The effects of gender differences and L-Name (Inhibitor of NO synthase) on ischemia – reperfusion induced arrhythmias in rats. İclal Karalar, **Eylem Suveren**, Ersoz Gonca **(31. National Physiology Congress, 2005 Gaziantep).**

SYMPOSIUM & COURSE

1. The symposium about Ischemia-reperfusion arrhythmias in Gaziantep University Faculty of Medicine, Department of Pharmacology, Gaziantep. 21, April, 2004.

2**.** Turkish Pharmacological Society, Prof. Dr. M. Oğuz Güç Summer School, 22- 28 June, 2009.

REFERENCES

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