The effects of ageing on the myocardium

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THE EFFECTS OF AGEING ON THE MYOCARDIUM

By

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Award of

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ABSTRACT

Investigating the causes of ageing on the myocardium is especially important for the future health of Australia's ageing population, which is increasing due to increased life expectancy (AIHW, 1999).

Cardiovascular disease is currently Australia's greatest health problem (AIHW, 1999), and since the aged population is most commonly affected by this disease (HSVD, 1999), investigating the causes of ageing of the myocardium and the predisposing factors of cardiovascular disease, is important for the future health of Australia's ageing population.

In this study, male and female Wistar rats [young (n = 8, aged 12-30 weeks old), middle aged (n = 6, 1-1.5 years old) and senescent (n = 7, 2-2.5 years old)] had cardiac tissue removed for contractile, histological and morphometric analysis.

The data derived from the contractile investigations comparing young, middle-aged and senescent rats, do not demonstrate any major differences across the age spectrum. For the contractile analysis, mitochondria were inactivated and an exogenous energy supply was used. The results showed that the cardiomyocytes are able to develop the same levels of force regardless of age, suggesting that it is the mitochondria that exert an influence on ageing cardiomyocyte contractile function.

Statistical analysis of one contractile parameter (pSr10, the pSr that produces 10% of maximum activated force) yielded a significant (P < 0.05) difference between young and middle-aged rats, and young and senescent rats. It was speculated that these results might reflect a developmental change in the Troponin C (TnC) protein that may only occur early in life ie. between young and middle-age in the rat.

During the histological and morphometric analyses, significant (P < 0.05) changes were observed in the volume of mitochondria, cytoplasm and 'other' structures, in both sides of the heart, when comparing young, middle-aged and senescent rats. In the left side of the heart the volume of mitochondria significantly (P < 0.05) decreased whilst the
volume of cytoplasm significantly (P < 0.05) increased with age. This provides further evidence of the influence of mitochondria on the functioning of the ageing heart.

In the right side of the heart, the volume of mitochondria significantly decreased between young and middle-aged rats, then significantly (P < 0.05) increased between middle-aged and senescent rats. The volume of cytoplasm significantly (P < 0.05) increased between young and middle-aged rats whilst the volume of ‘other’ structures ie. connective tissue, vascular and interstitial space, significantly (P < 0.05) decreased between middle-aged and senescent rats.

The knowledge gained from the current investigation will further the information already available on the effects of ageing on the myocardium. The results will be discussed in terms of human myocardial ageing.
DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

(i) incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;

(ii) contain any material previously published or written by another person except where due reference is made in the text; or

(iii) contain any defamatory material.

Signature

Date 20/1/01
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ABBREVIATIONS

AIF-  ‘apoptosis inducing factor’.
Apaf-1-  apoptotic-protease-activating-factor 1
ATP-  adenosine triphosphate; is energy synthesised from glucose within mitochondria during oxidative phosphorylation, needed for cellular functioning.
ATPase-  adenosine triphosphatase; hydrolysis of the high energy phosphate bonds of ATP is catalysed by ATPase activity in the myosin heads when in contact with the actin molecules.
bcl-2-  the gene involved in the regulation of apoptosis, acting to keep cells alive and functional.
CPD-  cell population doubling
CR-  calorie restriction
EDL-  extensor digitorum longus, a fast-twitch skeletal muscle.
EM-  electron microscopy
ETC-  electron transport chain, consists of a sequence of proteins called ‘cytochromes’. The site of oxidative phosphorylation (OXPHOS).
FDL-  flexor digitorum longus, a fast-twitch skeletal muscle.
FOV-  field of view
H$_2$O$_2$-  hydrogen peroxide free radical
IMM-  inner mitochondrial membrane
LS-  lifespan
MHC-  myosin heavy chains (ie. α- & β-MHC).
MLC-  myosin light chains
MLS-  maximum lifespan
mRNA-  messenger RNA
MtDNA-  mitochondrial deoxyribonucleic acid
MtPTP-  the mitochondrial permeability transition pore.
nDNA-  nuclear deoxyribonucleic acid
O$_2^-$-  superoxide free radical
OH$^-$-  hydroxyl free radical
OMM-  outer mitochondrial membrane
OXPHOS- oxidative phosphorylation; the process by which ATP is synthesised. Takes place on the ETC.

PCR- polymerase chain reaction

RNA- ribonucleic acid

rRNA- ribosomal RNA

SOL- soleus, a predominantly slow-twitch skeletal muscle.

SR- sarcoplasmic reticulum; a tubular membrane network surrounding individual myofibrils; stores Ca^{2+} which when released into the sarcoplasm, initiates muscle contraction.

STZ- streptozotocin (alloxan); an agent that induces diabetes.

TEM- transmission electron microscope/micrograph.

TnC- troponin C, the troponin subunit that binds to Ca^{2+}.

TnI- the troponin subunit that binds to G actin, holding the TnT-Tm complex in place.

TnT- the troponin subunit that binds to tropomyosin forming the troponin-T-tropomyosin complex (TnT-Tm).

tRNA- transfer RNA

TR3- a nuclear transcription factor

TR3- a nuclear transcription factor

V1- isoenzyme of myosin-ATPase (fast)

V3- isoenzyme of myosin-ATPase (slow)
DEFINITION OF TERMS

Actin- the ‘thin’ myofilament protein.
Active site- where a G actin molecule binds to a thick (myosin) filament; not unlike the binding of a substrate molecule to the active site of an enzyme.
‘ad libitum’- free access to unlimited food and water whenever desired.
Aerobic- requiring the presence of oxygen.
Antioxidant- protects against free radical damage; is a substance when present at low concentrations, delays or inhibits oxidation of an oxidisable substrate.
Apoptosis- a genetically controlled form of cell death.
Apoptosome- a complex containing cytochrome c, Apaf-1 & procaspase 9.
Atherosclerosis- the formation of fatty plaques in the walls of arteries, leading to circulatory impairment.
Cardiomyocytes- cardiac muscle cells.
Cardiolipin- a diphosphatidyl glycerol derivative found principally in mitochondria; plays an important role in mitochondrial membrane structure and function.
Catabolism- chemical reactions that break down complex organic compounds into simple ones with the release of energy.
Chromatin- a histological term referring to the grainy material visible in cell nuclei during interphase; the appearance of the DNA content of the nucleus when the chromosomes are uncoiled.
Contractile proteins- actin and myosin
Coronary blood flow- the blood supply to the myocardium.
Cristae- the numerous folds of the inner mitochondrial membrane.
Cross-bridge- a myosin head that projects from the surface of a thick filament; binds to an active site of a thin (actin) filament in the presence of Ca^{2+}.
Cytochrome- has two components: a protein and a pigment containing a metal ion (ie. Fe^{3+} or Cu^{2+}). Cytochrome a, a_3, b & c make up the ETC.
<table>
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<th>Term</th>
<th>Definition</th>
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<td>Cytochrome c-</td>
<td>is an electron-transfer protein in the ETC, and also acts as a signalling molecule to induce apoptosis.</td>
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<tr>
<td>Cytokines-</td>
<td>are released by cellular defenders, acting as hormones, affecting cells and tissues throughout the body.</td>
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<tr>
<td>Delta psi-</td>
<td>the 'transmembrane potential'.</td>
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<tr>
<td>Dementia-</td>
<td>a loss of mental abilities.</td>
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<tr>
<td>Diastole-</td>
<td>the period of cardiac relaxation.</td>
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<tr>
<td>Diploid-</td>
<td>having a complete somatic complement of chromosomes (23 pairs in human cells).</td>
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<td>Electron-</td>
<td>a fundamental subatomic particle bearing a negative charge, and normally orbits the protons of the nucleus.</td>
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<td>Excitation-</td>
<td>contraction coupling- the link between the generation of an action potential in the sarcolemma and the start of a muscle contraction.</td>
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<td>Fibroblasts-</td>
<td>cells of connective tissue proper responsible for the production of extracellular fibres and the secretion of the organic compounds of the extracellular matrix.</td>
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<td>Free radicals-</td>
<td>derived from oxygen; free radicals are highly reactive and unstable molecular subgroups with and unpaired electron in their outer orbital.</td>
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<tr>
<td>G actin-</td>
<td>individual globular molecules of actin.</td>
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<td>Gene-</td>
<td>a portion of DNA that functions as a hereditary unit; is located on a particular site on a specific chromosome, and codes for a specific protein.</td>
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<td>Growth factors-</td>
<td>stimulate the division of specific cell types.</td>
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<td>Hepatocytes-</td>
<td>liver cells</td>
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<td>Histones-</td>
<td>proteins associated with the nDNA; the DNA strands are wound around them.</td>
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<tr>
<td>Hydrolyse-</td>
<td>the breakage of a chemical bond through the addition of a water molecule.</td>
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<td>Hypertrophy-</td>
<td>an increase in size without cell division.</td>
</tr>
<tr>
<td>Ischaemia-</td>
<td>inadequate blood supply to a region of the body.</td>
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<tr>
<td>Isoenzymes-</td>
<td>'iso' = 'same'; enzymes that differ in structure but catalyse the same reaction.</td>
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</table>
Matrix- the fluid contents of the mitochondria containing metabolic enzymes.
Macrophage- a phagocytic cell on the monocyte-macrophage system.
Mitochondrion- an intracellular organelle responsible for producing most of the ATP required for cellular operations.
Myocardium- the cardiac muscle tissue of the heart.
Myofilament- fine muscle protein filaments, composed of actin and myosin.
Myosin- the ‘thick’ myofilament protein.
Myocyte- muscle cells
Necrosis- a form of cell/tissue death due to disease or injury.
Oedema (cytoplasmic)- the cytoplasmic accumulation of water, as a result of cell injury
Oxidants- ‘free radicals’
“Oxidative stress”- when an imbalance between antioxidant and free radical production occurs, favouring free radicals.
Peptide- a chain of amino acids linked by peptide bonds.
Phagocytosis- the engulfing of extracellular materials or pathogens.
Postmitotic- ‘after’; post-replicating cells ie. neurones & muscle cells.
Polypeptides- a chain of amino acids strung together by peptide bonds; those containing more than 100 peptides are called proteins.
Progeria- premature ageing
Proteases- are enzymes that breaks down proteins into peptides and amino acids.
Proton- a fundamental particle bearing a positive charge.
Regulatory proteins- troponin (TnC, TnI & TnT), tropomyosin (Tm).
Replication- duplication
Sarcolemma- the membrane of a muscle cell.
Sarcomere- the smallest contractile unit of a striated muscle cell.
Sarcoplasm- the cytoplasm of a muscle cell.
Senescence- ‘old age’, and/or ‘aged/ageing’.
Somatic- pertaining to the body.
Systole- the period of cardiac contraction.
Transcription- the encoding of genetic instructions on a strand of mRNA.
Translation- the process of peptide formation from the instructions carried by a mRNA strand.

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CHAPTER 1

INTRODUCTION
1.1 BACKGROUND TO THE STUDY

The present study investigates the effects of ageing on rat myocardium at the cellular level. Two different techniques were used. Firstly, contractile investigations and secondly histological and morphometric analyses. In combination, these techniques will be used to study functional and structural changes with age in myocardial tissue. The results of both techniques were correlated and analysed in comparison to reports in scientific and medical literature.

The literature review details the current knowledge available regarding the effects of ageing on the myocardium; incorporating theories of ageing, free radical damage, mitochondrial changes with age, apoptosis and changes in protein structure and other cellular components within individual muscle cells of the myocardium.

1.2 SIGNIFICANCE OF THE STUDY

Investigating the effects of ageing on myocardium is important, due to cardiovascular disease being Australia's greatest health problem (AIHW, 1999). In addition, the aged population is increasing due to increased life expectancy (AIHW, 1999).

With the above information in mind, this study will contribute to the knowledge of the effects of ageing on the myocardium as well as help in the identification of the causes of ageing. The information yielded will also help in the treatment of diseases and conditions that occur in the myocardium as a result of ageing.
1.3 RESEARCH QUESTIONS

- What are the effects of ageing on the function of the contractile and regulatory proteins of myocardium?
- What are the effects of ageing on the volume of mitochondria in myocardium?
- Is there a relationship between the function of the myocardium and the volume of mitochondria?

1.4 HYPOTHESES

- That there will be a difference in the kinetics of calcium and strontium activated force in senescent cardiomyocytes in comparison to young cardiomyocytes.
- That the volume of mitochondria in senescent cardiomyocytes will be greater than the volume of mitochondria in young cardiomyocytes.
CHAPTER 2

LITERATURE REVIEW
2.1 Ageing

Understanding the effects of ageing on the myocardium has taken on special significance in recent years, as cardiovascular disease is now Australia's greatest health problem (AIHW, 1999). Next to cancer, cardiovascular disease is the second greatest cause of death among Australians, accounting for 52,641 deaths (41% of all deaths) in 1997 (ABS, 1999).

The prevalence of cardiovascular conditions (ie. coronary heart disease, stroke and hypertension) increases quite dramatically with advancing age. For example, in 1995 over 60% of Australians aged 75 years and over had a cardiovascular condition, compared to less than 9% of those aged 35 years and under (AIHW, 1999).

Coronary heart disease is the greatest single cause of death in Australia, claiming 29,051 lives in 1997. It is estimated that every day approximately 80 Australians die from coronary heart disease (HSVD, 1999). Stroke is the second greatest single cause of death in Australia, after coronary heart disease, claiming 12,133 lives in 1997. Stroke is also the leading cause of long-term disability in adults (HSVD, 1999).

Additionally, since the older population in Australia is expanding due to increased life expectancy (AIHW, 1999), investigating the causes and effects of ageing on the myocardium is especially important for the aged, among whom cardiovascular disease is most common (HSVD, 1999).

Ageing in general is characterised by a decline in physiological function that leads to morbidity and eventual death (Paradies, Ruggiero, Petrosillo, Gadaleta, & Quagliariello, 1994; Pollack & Leewenburgh, 1999; Sohal & Weindruch, 1996; Svanborg, 1997). The specific causes of this decline are poorly understood (Shigenaga, Hagen, & Ames, 1994), and many hypotheses have been proposed to try and explain the cause of ageing, the classic example being Harman's 1956 'free radical theory' (Goldstein, Gallo, & Reichel, 1989; Miquel, 1998).
The study of ageing is also complicated by the pathologic processes that become more prevalent with advancing age (Goldstein et al., 1989; Lakatta, 1993; Svanborg, 1997). Examples are diseases such as osteoporosis, atherosclerosis and primary degenerative dementia which often present almost identical morphological and functional changes to those characteristic of normal ageing (Goldstein et al., 1989; Safar, 1990; Svanborg, 1997).

2.2 THEORIES OF AGEING

To believe there is a single "magic bullet" (as referred to by Bittles, 2001) cause of ageing is indeed naive as clearly many different tissues and systems of the body are altered during the ageing process. A single theory of ageing is highly unlikely to be able to scientifically support and explain a 'single' cause of ageing that affects the whole body. It would appear less naive to consider the possibility that many different theories could collectively explain the process of ageing.

Currently, genetic-based theories of ageing are the most popular, stimulating research worldwide. Theories of ageing involving mitochondria also currently receive much attention. Some of the relevant theories of ageing are reviewed below.

Genetic based theories are just that. Theories based on the concept that ageing is associated with changes in the genetic constitution of an individual (Bittles, 2001). The notion of 'programmed ageing' arose from lifespan studies of both mammal and non-mammal species, with the discovery of remarkable constancy in the life expectancy of various species (Bittles, 1997, p. 8). Genetic based theories of ageing are also strengthened by the well-known finite lifespan of human diploid cells when cultured in a laboratory (Bittles, 2001).

In relation to evolution, it has been suggested that ageing may be a form of natural selection to limit the lifespan of an individual, thereby ensuring the turnover of future generations. Genes of ageing were said to be 'pleiotropic' in their action, exerting beneficial effects when an individual was young or reproducing, followed by unfavourable ageing effects during an individual's later, post-reproductive years. This
The first ‘evolutionary theory’ of ageing was proposed by Rubner (1908; cited in Miquel, 1998) who “observed an inverse relationship between longevity and ‘rate of living’, expressed as the amount of energy necessary for body growth” (Miquel, 1998, p. 123). Along similar lines, Kirkwood (1977) suggested that ageing was the end result of an ‘energy conservation strategy’ operating in somatic cells. During an individual’s lifespan, their total available energy is differentially allocated to a variety of functions, i.e. macromolecular synthesis and degradation, cell and organ maintenance, and reproduction. Since this energy supply is finite, a compromise is reached in that the maximum available energy is allocated to ensure the successful transmission of genes to future generations (Bittles, 2001). However, after reproductive success, total energy available is reduced and the effects of ageing set in.

The concept of energy and ageing is ultimately related to the source of energy production within the body, i.e. the mitochondria. Since mitochondria supply the energy needed for cellular functioning, a decline in mitochondrial function with age would ultimately lead to an overall decline in bodily functioning within an individual. Miquel’s (1998) oxygen-stress mitochondrial mutation theory of ageing relates senescence to mutations of the mitochondrial genome (mtDNA) leading to the physiological decline seen with age. This theory is particularly related to postmitotic cells, such as those found in the myocardium (Miquel, 1998).

Miquel’s (1998) theory is also related to the ‘free radical theory’ postulated by Harman in 1956, which implicates free radicals as the cause of nonspecific damage to macromolecules (DNA, lipids and proteins), which ultimately leads to cellular ageing and overall bodily senescence (Goldstein et al., 1989; Miquel, 1998). Miquel’s (1998) ‘oxygen stress mitochondrial mutation theory’ agrees with and expands on the 1956 ‘free radical theory’ of Harman, suggesting free radicals as the ‘central senescence-causing factor’. Of interest to note at this point is the main location of free radical production within the body; the mitochondria. Hence, mitochondria are akin to a ‘double edged sword’ in that they generate the crucial energy needed for cellular functioning and survival, as well as the free radicals that potentially destroy somatic cells. Within the
following literature review, a strong case is presented implicating mitochondria as possessing a central role in cellular and overall somatic ageing.

2.3 AGEING MYOCARDIUM

Cardiac function deteriorates with age (Andreu, Arbos, Perez-Martos, Lopez-Perez, Asin, Lopez, Montoya, & Schwartz, 1998; Hattori, Tanaka, Sugiyama, Obayashi, Ito, Satake, Hanaki, Asai, Nagano, & Ozawa, 1991; Paradies et al., 1994), with some authors postulating that ageing of the myocardium begins in the third decade of life (Lakatta, 1987; Müller-Höcker, 1990; Safar, 1990). However, it could be said that ageing of the myocardium begins in childhood with the development of a ‘fatty streak’ in the arteries that could lead to atherosclerosis and eventual cardiovascular disease (Cotran, Kumar & Robbins 1998, p. 503).

With advancing age, there is an increased ‘stiffening’ of the cardiomyocytes, heart valves, mural connective tissue and the blood vessels (particularly the aorta), which reduces compliance in the cardiovascular system (Lakatta, 1993; Macdonald & O'Rourke, 1998; Safar, 1990; Svanborg, 1997). In fact it is the age related changes in arterial pressure, due to increased arterial ‘stiffening’, that physicians have used to assess cardiovascular ageing (Macdonald & O'Rourke, 1998).

Perhaps the most prominent physiological characteristic associated with the ageing myocardium is a prolonged contraction and relaxation time (Andreu et al., 1998; Bhatnagar, Walford, Beard, Humphreys, & Lakatta, 1984; Fraticelli, Josephson, Danziger, Lakatta, & Spurgeon, 1989; Lakatta, 1987 & 1993; Lakatta & Yin, 1982; Müller-Höcker, 1990; Muscari, Caldarera, Rapezzi, Branzi, & Caldarera, 1992; Safar, 1990; Svanborg, 1997; Xu & Narayanan, 1998), leading to a decreased maximal heart rate and ejection volume, increased end diastolic volume and decreased resting cardiac output (Lakatta, 1987; Müller-Höcker, 1990).
2.4 **Contractile and Regulatory Proteins**

Muscari et al. (1992) and other authors (Fraticelli et al., 1989; Lakatta, 1987; Müller-Höcker, 1990) have partly explained the prolonged contraction and relaxation time in aged myocardium by attributing it to an alteration of excitation-contraction coupling, due to an increased action potential duration, reduced biosynthesis of the Ca\(^{2+}\)-stimulated ATPase pump of the sarcoplasmic reticulum (SR), and the prevalence of the V3 isoform of myosin with a slow ATPase activity (Fraticelli et al., 1989; Lakatta, 1987; Müller-Höcker, 1990; Muscari et al., 1992).

Xu and Narayanan (1998) reported that the ATP-dependent Ca\(^{2+}\)-uptake activity of the SR was significantly lower in aged myocardium and concluded that this finding was most likely the major factor contributing to the slow rate of myocardial relaxation seen with ageing.

In addition, Fitzsimons, Patel and Moss (1999) proposed that the prolonged contraction and relaxation time in aged myocardium was due to the increased expression of the β-myosin heavy chain (MHC). In rodent heart, the normal ageing process is associated with a significant age-induced alteration in ventricular MHC expression, from ~80% α-MHC/20% β-MHC at 3 months to ~30% α-MHC/70% β-MHC by 24 months (Fitzsimons et al., 1999).

Fitzsimons et al. (1999) hypothesised that the increased expression of β-MHC contributes to the prolonged contraction and relaxation by depressing the kinetics of cross-bridge interaction. Indeed, the results demonstrate that the age-related increase in β-MHC expression was associated with significant slowing of myocardial cross-bridge interaction kinetics, particularly a decline in the rates of submaximal and maximal tension development (Fitzsimons et al., 1999). In addition, the authors summarised that the ageing-dependent relationship of kinetics and β-MHC content is consistent with the approximately threefold greater ATPase activity of α-MHC versus β-MHC (Fitzsimons et al., 1999).

With senescence the composition of the two principle isoenzymes of myosin-ATPase, V1 and V3, shift from predominantly V1, a rapid ATPase, to predominantly V3, a slower
ATPase, indicating a slower rate of ATP hydrolysis in ageing myocardium (Muscari et al., 1992). The level of mRNA coding for the V1 isoform decreases during the lifespan of the rat, whilst the coding for the V3 isoform increases, indicating that contractile changes during ageing are, at least partially, regulated at the level of gene expression (Muscari et al., 1992).

In 1996, Hill, Stewart and Verrier conducted a study investigating the ‘age and sensitivity of rat skeletal muscle fibres to calcium and strontium’. Since the skeletal muscle fibres of older rats displayed increased sensitivity to calcium, but not strontium, compared to younger rats, it was speculated that modifications to the contractile proteins, particularly the myosin regulatory light chain, may occur with age (Hill et al., 1996).

A study by Stephenson, O'Callaghan & Stephenson (1994) using a ‘skinned fibre technique’, investigated the effects of Streptozotocin (STZ)-induced diabetes on the contractile and regulatory proteins of single fast (EDL-extensor digitorum longus; predominantly fast)- and slow (SOL-soleus; predominantly slow)-twitch skeletal muscle fibres from the rat. Approximately 28 days after STZ administration, 37.5% of the fast-twitch EDL fibres developed maximum activated tensions (77.1 ± 10.4 kN/m²), which was significantly lower than maximum activated tensions developed by the controls (244.0 ± 14.3 kN/m²). In addition, the slow-twitch SOL fibres demonstrated a slightly higher sensitivity to Ca²⁺ (and Sr²⁺) than the controls. Hence, 28 days after STZ treatment, the ability of many, but not all, of the fast-twitch EDL fibres to develop maximum tension decreases dramatically, and the sensitivity of the contractile/regulatory proteins to Ca²⁺ (and Sr²⁺) increased in most of the slow-twitch SOL fibres (Stephenson et al., 1994).

Stephenson et al. (1994) adopted a novel approach by determining the electrophoretic profiles of the myofibrillar proteins in the muscle fibres that were used in their experiments. This validates their skinned fibre method as being able to identify and correlate functional changes in contractile and regulatory proteins with their chemical isoforms.
All of the fast-twitch EDL and slow-twitch SOL muscle fibres displayed the myofibrillar protein isoforms characteristic of these two major fibre types: MHC2 (myosin heavy chain 2), fast MLCs (myosin light chains) (MLC1-F, MLC2-F, and MLC3-F), and fast troponin T (TnT)-tropomyosin (Tm) complex for fast EDL fibres; and MHC1, slow MLCs (MLC1-S and MLC2-S), and slow TnT-Tm complex for slow-twitch SOL fibres. Of the fast-twitch muscle fibres found in the SOL muscles of control rats, combinations of both fast- and slow-twitch myofibrillar isoforms with prominent MHC2 versus MHC1 were seen (Stephenson et al., 1994).

2.5 **MYOCARDIAL MORPHOLOGY**

Age-related morphological changes associated with the myocardium are dominated by left ventricular wall and septal hypertrophy, and left atrial and ventricular widening (Macdonald & O'Rourke, 1998; Safar, 1990; Svanborg, 1997). According to Safar (1990), cardiac hypertrophy is an adaptive response to the increase in cardiac after load caused by age-related arterial dilation and loss of ventricular compliance.

The hypertrophy is caused by increases in individual cardiomyocyte size (Fraticelli et al., 1989; Macdonald & O'Rourke, 1998; Svanborg, 1997), even though the actual number of cardiomyocytes declines (Anversa, Palackal, Sonnenblick, Olivetti, Meggs & Capasso, 1990; Macdonald & O'Rourke, 1998; Svanborg, 1997) by approximately 30% as the heart ages (Pollack & Leewenburgh, 2000). In the human heart, some 38 million nuclei disappear from the left ventricle each year due to either necrosis (Pollack & Leewenburgh, 2000) or continuous apoptosis (Macdonald & O'Rourke, 1998; Pollack & Leewenburgh, 2000).

The myocardium has the ability to compensate for the loss of cardiomyocytes by increasing their average size (hypertrophy), hence, the ventricular fibre mass is regenerated during ageing (Anversa et al., 1990; Svanborg, 1997). Fraticelli et al. (1989) report that the average cardiomyocyte volume, measured via Coulter counter techniques, almost doubled between 2-24 months in rats. The authors attribute the structural basis for the increased volume of cardiomyocytes with age to the addition of morphologically similar sarcomeres, which they also implicate in the age-related prolonged contraction seen in aged cardiomyocytes (Fraticelli et al., 1989).
Fraticelli et al. (1989) also reported an increase in average cardiomyocyte length with age. Measured under high power light microscopy, average cardiomyocyte length in unattached cells increased from 133µm at 2 months, to 146µm at 6-9 months to 162µm at 24-25 months, in individual cardiomyocytes, isolated from rat hearts (Fraticelli et al., 1989).

In relation to morphological changes with ageing, a common observation in aged cardiomyocytes is the progressive decline in mitochondrial numbers, increases in mitochondrial structural abnormalities, losses in mitochondrial enzyme activities and decreased respiratory control (Bandy and Davison, 1990). Furthermore, mitochondria from older rats also demonstrate higher levels of lipid peroxides, losses of polyunsaturated fatty acids, and correspondingly increased oxygen free radical production (Bandy & Davison, 1990; Sawada & Carlson, 1987).

Supporting these age-related changes to mitochondria, Tauchi and Sato (1968) report a decrease in hepatic mitochondrial numbers, however the size of individual mitochondrial increased with age in senescent human and rat liver (Tauchi & Sato, 1968).

According to Tauchi & Sato (1968) the increase in size (hypertrophy) of hepatic mitochondria, seen with age, was considered a 'compensatory hyperfunction' for the decline in mitochondrial numbers. In support of this statement, Goldstein, Moerman and Porter (1984) also suggested that the age-related hypertrophy of mitochondria was a compensatory attempt to produce adequate capacity for oxidative phosphorylation (OXPHOS).

In addition, Goldstein et al. (1984), using electron microscopy, also report an age-related increase in mitochondrial ‘blebs’ seen in aged (76 year old female) and progeria (5 year old female and 9 year old male) human fibroblasts (progeria being premature ageing). The authors described ‘Blebs’ as ‘mitochondrial cystic dilations’ caused by weakening or destruction of the inner mitochondrial membrane (IMM) which allowed outward dilation of the outer mitochondrial membrane (OMM) (see Figure 1). A decrease in width was also seen in aged and progeria human fibroblast mitochondria compared to young (11 year old male) fibroblasts, as well as the observation that aged and progeria fibroblast
mitochondria contain less transverse cristae (Goldstein et al., 1984). With these results in mind, it was speculated by Goldstein et al. (1984) that since mitochondrial cristae are formed by invagination of the inner IMM, and blebs may result from IMM weakening; the decreased mitochondrial width in aged and progeria human fibroblasts may reflect a generalised age-dependent loss of IMM integrity which leads simultaneously to blebs and reduced numbers of transverse cristae.

2.6 MITOCHONDRIA

Mitochondria are said to be the 'powerhouse' of a cell because their metabolic activities provide the energy required to support the life of mammals and other aerobic organisms (Tortora, 1995, p. 41; Rhoades & Pflanzer, 1996, p. 87). Mitochondria (mitos, thread + chondrion, granule) are small organelles present in the cytoplasm of aerobic cells, which may take on a variety of shapes and sizes, from long and slender with branches to short and fat (Martini, 2001, p. 87), spherical or highly irregular in shape (Bozzola & Russell, 1991, p. 441). Brown (2000b) describes mitochondria as best resembling bacteria. Both are approximately the same size, with roughly 1 billion mitochondria fitting into a grain of sand. In addition, Brown (2000b) estimates that human bodies contain roughly 10 million billion mitochondria.

Cardiomyocytes are characterised by very large mitochondria that are densely packed between the myofibrils (Ross, Romrell & Kaye, 1995, p. 228), often appearing cylindrical, although usually flattened by the tight packing (Bers, 1991, p. 15). These large mitochondria often extend for the full length of a sarcomere and have abundant, closely packed cristae. In addition, glycogen granules are also concentrated between the myofibrils, hence the structures that store energy (glycogen granules) and the structures that release and recapture energy (mitochondria), are located adjacent to the structures that use the energy to drive contraction, ie. the myofibrils (Ross et al., 1995, p. 228).

Mitochondria are unusual in that they have a double membrane: an outer mitochondrial membrane (OMM) surrounding the whole organelle, and an inner mitochondrial membrane (IMM) containing numerous folds called 'cristae' (Tortora, 1995; p. 41), which may be lamellar (sheet-like) or tubular in appearance (Bozzola & Russell, 1991, p.441). The folding cristae provide a large surface area for a group of reactions,
collectively known as ‘cellular respiration’, to take place (Tortora, 1995, p. 41, 1995). The area enclosed by the IMM is fluid filled, otherwise known as the mitochondrial ‘matrix’ (Martini, 2001, p. 87-88; Tortora, 1995, p. 41). The structure of a mitochondrion is shown in Figure 1.

Mitochondrial numbers within cells vary depending upon the type of cell and its energy demands (Martini, 2001, p. 87). Bers (1991) estimates that approximately 35% of the volume of mammalian and avian ventricular cardiomyocytes is occupied by mitochondria (p. 14). Further, Martini (2001, p. 87) estimates that approximately 20% of an active liver cell is occupied by mitochondria. However, these authors do not specify the age of the samples, and therefore it is assumed that they are from adult specimens.
Figure 1. A 3-dimensional diagram (a) and a transmission electron micrograph (TEM) (b) of a typical mitochondrion (TEM x 46,332). All of the relevant structures are labelled. Taken from Martini (2001, p. 88).
The enzymes that catalyse the reactions involved in cellular respiration, are located on the cristae, with the reactions taking place within the matrix (Martini, 2001, p. 87; Tortora, 1995, p. 41). The cristae within cardiac mitochondria are said to be more tightly packed than the mitochondria of hepatocytes, which possibly reflects the more intense energy demands of the myocardium (Bers, 1991, p. 15).

Cellular respiration only occurs in the presence of oxygen (O\textsubscript{2}), and results in the catabolism of nutrient molecules ie. glucose, to generate energy in the form of adenosine triphosphate (ATP). Active cells eg. muscle, liver and kidney tubule cells, have large numbers of mitochondria, and use ATP at a high rate (Tortora, 1995, p. 41).

The mitochondrion provides this energy by generating a proton motive force, used to fuel ATP synthesis, via the process of oxidative phosphorylation (OXPHOS) (Ozawa, 1997). OXPHOS is the principle source of energy for several organs and tissue systems, including the brain, muscle, heart, kidney, liver and pancreatic islets. Therefore, if the OXPHOS process is modified, via damage to mitochondrial DNA (mtDNA), mitochondrial ATP production declines until it falls below the minimum energy levels necessary for oxidative tissues and organs to function, resulting in tissue-specific diseases and senescence (Wallace, 1992 & 1999).

The OXPHOS system declines with age (Wallace, 1992). According to Ozawa (1997), the loss of OXPHOS capacity is related to mtDNA damage, and the accumulation of mtDNA mutations could be one of the main causes of disease associated with ageing. This interpretation, together with the known increase in the level of mtDNA mutations in the heart (Hayakawa, Sugiyama, Hattori, Takasawa, & Ozawa, 1993), suggests that cardiomyocyte mitochondrial senescence could be, at least in part, a consequence of accumulated mitochondrial damage (Wallace, 1992), resulting in decreased mtDNA transcription (Andreu et al., 1998).
2.7 **Mitochondrial Genetics**

OXPHOS genetics is complicated because the genes for component peptides are distributed throughout both the nuclear DNA (nDNA) and mtDNA. Human mtDNA is a 16,569-base pair (bp) closed circular molecule located within the mitochondrial matrix (Wallace, 1992).

The expression of the mtDNA OXPHOS genes requires functional mitochondrial replication, transcription, and translation systems, and all of the polypeptides for these processes are encoded by the nDNA. Therefore, the biogenesis of OXPHOS requires hundreds of nuclear and cytoplasmic genes (Wallace, 1992, p. 629).

In addition, the genetics of OXPHOS is further complicated by the unique characteristics of mtDNA inheritance. Mitochondrial DNA is almost exclusively maternally inherited (Brown, 2000b; Wallace, 1992 & 1999; Yowe & Ames, 1998) by offspring through the oocyte cytoplasm (Ozawa, 1997). Once a sperm has penetrated, it delivers a full load of nDNA but only a few, if any, mitochondria. However, the larger oocyte contains tens of thousands of mitochondria (Brown, 2000b).

As mentioned previously, myocardial function deteriorates with age, and progressive age-dependent damage to mtDNA and mitochondrial functions is believed to be an important contributor to human ageing (Hattori et al., 1991; Ozawa, 1997).

In recent years an abundance of mutations and deletions in mtDNA of aged myocardium, which could affect mtDNA transcription and result in deficient synthesis of the mtDNA encoded OXPHOS polypeptides, have been described (Andreu et al., 1998; Cortopassi & Arnheim, 1990; Hattori et al., 1991; Ozawa, 1997; Takasawa et al., 1993; Wallace, 1999).

Using PCR (polymerase chain reaction) techniques, Hattori et al. (1991) detected a 7.4kb deletion of mtDNA occurring in elderly subjects, and the proportion of deleted mtDNA to normal mtDNA increased with age. The deleted mtDNA fragments prevent the biosynthesis of some subunits of the mitochondrial energy transducing system, leading to inhibition of energy transduction. This effect induces a chronic ischaemia-like
state in the myocardium, which may contribute to the genesis of ageing heart (presbycardia) (Hattori et al., 1991).

A 7.4kb deletion in mtDNA has also been identified in cardiomyopathy patients as well as in the myocardium of normal aged people (Miquel, 1998). As pointed out by Ozawa (1995), the ratio of mtDNA with a 7.4kb deletion to total DNA increases exponentially with age up to 7% at age 97, with an estimated 100% mutation rate of the mtDNA at about 130 years of age. This might set an upper limit to longevity, since in Ozawa’s (1995) words, “it could be speculated that 129 years may be the maximum lifespan of humans with regards to myocardial bioenergetic function” (p. 281).

Andreu et al. (1998) studied the effect of ageing on mtDNA transcription with an in organello system using intact isolated rat heart mitochondria. A comparison of the electrophoretic patterns of mtDNA transcription products in young (4 weeks), adult (12 months) and senescent (24 months) rats demonstrated an age-related decrease in newly synthesised mitochondrial RNAs that reflects a decrease in the synthesis rate. In fact, the mitochondria from senescent (24 months) rat hearts synthesised RNA about 2.4 times less efficiently than the mitochondria of young (4 weeks) animals, and about 1.5 times less efficiently than adult (12 months) rats.

Mitochondrial DNA has approximately a 10-fold higher rate of mutation, is much more susceptible to free radical damage, and has higher turnover rates compared to nDNA (Yowe & Ames, 1998). This higher rate of damage and mutation to mtDNA is due to its location inside the inner mitochondrial membrane (IMM) near the electron transport chain (ETC) (Ozawa, 1997; Yowe & Ames, 1998). The ETC is a major source of ‘free radical’ production, hence mtDNA is directly susceptible to attack by free radicals (Ozawa, 1997).

Furthermore, compared to nDNA, mtDNA lacks protective histones and has inefficient DNA repair mechanisms, therefore the mutated mitochondrial genomes tend to accumulate in cells, especially in stable tissues such as muscle and nerve, which consist of postmitotic cells (Linnane, Marzuki, Ozawa, & Tanaka, 1989; Ozawa, 1997; Yowe & Ames, 1998).
The cause of mtDNA damage leading to mutations and deletions has been postulated to be due to 'free radicals'. In 1956, Harman proposed a 'free radical theory' of ageing, implicating free radicals as the cause of nonspecific damage to macromolecules, such as DNA, lipids and proteins (Miquel, 1998; Ozawa, 1997). Miquel (1998) agreed with Harman in that 'free radicals' were the central 'senescence-causing factor'. However, the current information available on the mitochondrial genome led Miquel (1998) to propose an 'oxygen stress-mitochondrial mutation theory' of ageing in which cellular ageing derives from mitochondrial membrane and genetic damage caused by free radicals. Miquel (1998) has been able to explain better the reasons for the lack of repair and regeneration mechanisms, to counteract the harmful effects of the free radicals; explanations that were lacking in Harman's 1956 'free radical theory' of ageing.

Free radical production in brain and cardiac mitochondria increases significantly with age (Sawada & Carlson, 1987). Sohal and Weindruch (1996) reported that the rate of free radical production increases generally about 15-50% during the ageing process in mammals. In addition, the damage caused by free radicals also increases with age (Muscari et al., 1992; Sohal & Weindruch, 1996).

2.8 FREE RADICALS

Free radicals are derived from oxygen \((O_2)\) and are molecular subgroups with an unpaired electron in their outer orbital. This unpaired electron renders them highly unstable and reactive, with the potential to become destructive oxidising agents (Goldstein et al., 1989; Halliwell, 1994; Sawada & Carlson, 1987; Bittles, 1997, p. 11). Free radicals are a natural by-product of OXPHOS because electrons can be transferred directly from reduced flavin dehydrogenase, Coenzyme Q, and cytochrome b to oxygen (Wallace, 1992). As a result, about 1-4 % of the oxygen consumed by the cells is converted to oxygen free radicals (Finkel, 1997; Halliwell, 1994; Linnane, Baumer, Maxwell, Preston, Zhang & Marzuki, 1990; Pollack & Leewenburgh, 1999; Sohal & Weindruch, 1996, Wallace, 1992), and of that 1-4%, 98% of the oxygen metabolised is handled by a single enzyme, cytochrome oxidase, located in mitochondria. The cytochrome oxidase transfers four electrons of oxygen in an intensive reaction to produce two molecules of water as the end product (McCord, 2000).
Superoxide ($O_2^-$) is the major free radical produced from the utilisation of oxygen by mitochondria (Goldstein et al., 1989; Halliwell, 1994; Shigenaga et al., 1994; Sohal & Weindruch, 1996; Pollack & Leewenburgh, 1999). Superoxide ($O_2^-$) is produced at two sites in the electron transport chain (ETC), namely complex I and ubiquinone (coenzyme Q) (Murphy, 2000), and since it is a charged molecule it tends to remain within the inner mitochondrial compartment where it is converted into hydrogen peroxide ($H_2O_2$). $H_2O_2$ can easily diffuse through the mitochondrial membrane and other cellular membranes to be distributed outside the cell. However, if it is not eliminated, $H_2O_2$ may undergo transition-metal-catalysed homolytic scission to produce highly reactive hydroxyl free radical (OH) (Sohal & Weindruch, 1996).

Free radicals react with other molecules to cause cellular damage and DNA mutation (Halliwell, 1994). Free radicals attack cell and organelle membranes, causing the lipid to undergo a chain reaction known as ‘lipid peroxidation’ (Shigenaga et al., 1994). This may result in severe damage and the eventual death of the cell via apoptosis (Bittles, 2001).

The fluidity of cellular membranes decreases with age, and this is supported by Lewin & Timiras’s (1984) research into lipid changes with ageing in cardiac mitochondrial membranes. They found reduced fatty acid unsaturation of phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine in older rats. Membrane fluidity is dependent upon the proportional content of saturated and unsaturated fatty acids. Young rat mitochondrial membranes are rich in unsaturated fatty acids and are more fluid than the membranes of older rats with a high proportion of saturated fatty acids. The decrease in membrane fluidity was also supported by the marked (approximately 260%) increase in cholesterol content between 4 month old and 33 month old rats used in the study. Such an increase in cholesterol de-fluidises the membrane by reducing thermal motion of the hydrocarbon (Lewin & Timiras, 1984). The decreased fluidity of cellular membranes is a change that may be attributed in part to oxidation of plasma and mitochondrial membrane lipid components by free radicals (Lewin & Timiras, 1984; Shigenaga et al., 1994).

Castelluccio, Baracca, Fato, Pallotti, Maranesi, Barzanti, Gorini, Villa, Castelli, Marchetti & Lenaz (1994) investigated the mitochondrial activities of rat heart during
In support of the above-mentioned study by Lewin and Timiras (1984) the authors also found that the fatty acid composition of the mitochondrial membranes revealed a percentage increase of polyunsaturated fatty acids (20:4 n-6, 22:6 n-3) up to 12 months. However, no changes in membrane fluidity were noticed. The authors proposed that the fluorescence polarisation methods used to detect fluidity may not have been sensitive enough to reveal small fluidity changes due to variations in lipid unsaturation (Castelluccio et al., 1994).

Cardiolipin, a diphosphatidyl glycerol derivative found principally within mitochondria, plays an important role in mitochondrial membrane structure and function. Cardiolipin interacts with various proteins of the inner mitochondrial membrane (IMM) and plays a central role in maintaining their activities (Shigenaga et al., 1994). In addition, cardiolipin appears to be important in controlling the permeability of the IMM to small molecules as well as establishing mitochondrial proton gradients (Shigenaga et al., 1994).

Lewin and Timiras (1984) have reported that the content of cardiac mitochondrial cardiolipin decreases with age, and Paradies et al. (1994) found that the phospholipid decreased by more than 30%. Cardiolipin is required for optimal activity of cytochrome-c-oxidase and adenine nucleotide translocase in mitochondria. Paradies et al. (1994) found that the activity of both cytochrome-c-oxidase and adenine nucleotide translocase is also reduced in the heart mitochondria of aged (28 months) rats. However, treatment of older rats with acetyl-L-carnitine restores the level of cardiolipin in the IMM to the level of young (5 months) rats (Paradies et al., 1994).

In a study by Hudson et al. (1998) cytochrome-c-oxidase demonstrated an age-related decrease in activity. In support of this finding, Hayakawa et al. (1993) observed that the age-related decrease of cytochrome-c-oxidase activity in the rat heart correlates with a decrease in mtDNA content and an increase in 8-hydroxy-deoxyguanosine (8-OH-dG), an oxidative product of deoxyguanosine (dG). The authors suggest that the decrease in cytochrome-c-oxidase activity is the manifestation of an overall deterioration of the mitochondrial genome.
2.9 Oxidative Stress

Overall, it seems that oxidative stress and damage increases with age because of an increased rate of free radical production and an increased susceptibility of tissues to free radical damage (Sohal & Weindruch, 1996).

Aerobic organisms are well protected against oxidative challenges by sophisticated antioxidant defence systems (Pollack & Leewenburgh, 1999). The term ‘antioxidant’ usually refers to chain-breaking compounds, such as tocopherol (vitamin E). Generally speaking, an antioxidant is any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate (Harman, 1998).

However, it appears that during the ageing process an imbalance between oxidant and antioxidant production may occur, favouring oxidants and rendering the antioxidants useless. This is referred to as ‘oxidative stress’ (Pollack & Leewenburgh, 1999; Halliwell, 1994).

If mild oxidative stress occurs, tissues often respond by making extra antioxidants. However, severe oxidative stress can cause cell injury and death. As mentioned earlier, free radical induced cell death can progress as necrosis or apoptosis (Halliwell, 1994).

2.10 Apoptosis

Recently, apoptosis has been implicated as a contributing factor to cellular ageing, and mitochondria play a critical role (Halestrap, 2000; Brown, 2000a; Pollack & Leewenburgh, 2000). It was discovered that mitochondria release factors that activate cytoplasmic proteases and induce apoptosis. The most surprising of these factors is cytochrome c, which appears to play two roles: firstly as an electron-transfer protein in the ETC, and secondly as a signalling molecule to induce apoptosis (Cooper & Halestrap, 2000).
Mitochondrial ‘damage’ of some kind is required to trigger apoptosis. However, too much damage and the cell will be depleted of ATP, preventing apoptosis and promoting necrosis (Brown, 2000a).

Cells can die in two different ways: ‘chaotic explosion’ or ‘controlled suicide’. The former is known as necrosis and the latter apoptosis (Brown, 2000b). Necrosis involves a swelling of the cell and its intracellular organelles, culminating in plasma-membrane rupture and the release of intracellular proteins. Therefore, unlike apoptosis, necrosis induces a localised inflammatory response that can cause further tissue injury (Halestrap, 2000). Apoptosis, is a carefully controlled, non-inflammatory form of cell death, orchestrated by growth factors and cytokines, resulting in phagocytosis by macrophages (Brown, 2000a; Halestrap, 2000).

Apoptosis plays a vital role in protecting the body. However, when apoptosis is overactivated (eg. in neurodegenerative conditions such as Alzheimer’s and Parkinson’s diseases), an excessive number of brain neurons die. On the other hand, the inactivation of apoptotic processes is one of the key changes that allow cancerous cells to survive (Brown, 2000b).

Mitochondria also provide a major switch for the initiation of apoptosis. This switch is thought to involve the opening of a nonspecific inner mitochondrial membrane (IMM) channel, the ‘mitochondrial permeability transition pore’ (mtPTP) (Wallace 1999). The mtPTP is a large pore in the IMM that is normally fully closed. Opening of the pore allows protons to leak through, causing the mitochondria to lose their proton-motive force and rapidly hydrolyse cellular ATP (Brown, 2000a & b).

Opening of the mtPTP, followed by the death of the cell, can be initiated by the mitochondrion’s excessive uptake of Ca\(^{2+}\), increased exposure to free radicals, increased presence of phosphate (conditions present during ischaemia or reperfusion) (Brown, 2000a & b), or a decline in energetic capacity. Thus, a noticeable reduction in mitochondrial energy production and a chronic increase in free radical production could theoretically activate the mtPTP and initiate apoptosis (Wallace, 1999).
The IMM space contains a number of cell death-promoting factors, including cytochrome c, apoptosis-inducing factor (AIF, a flavoprotein), and latent forms of specialised proteases called caspases. The opening of the mtPTP causes the collapse of Delta Psi (the transmembrane potential), swelling of IMM, and release of the above mentioned death-promoting factors (Wallace, 1999). Cytochrome c activates the cytosolic caspase protein degradation pathway, which leads to the destruction of the cytoplasm. AIF translocates to the nucleus, inducing chromatin destruction (Wallace, 1999).

Cytochrome c serves as the key regulator of apoptosis because once it is released from the IMM space, the cell is irreversibly committed to death. Either apoptosis occurs through the caspase-mediated process or the cell undergoes a necrosis-like death due to the collapse of the ETC. Release of cytochrome c interrupts the transfer of electrons between electron transport chain complexes III and IV, resulting in the generation of detrimental free radicals and the end of ATP synthesis (Pollack & Leewenburgh, 2000).

Once cytochrome c has been released into the cytosol, it binds to Apaf-1 (apoptotic protease-activating factor 1) and, in the presence of ATP, recruits and activates procaspase 9 to form a complex known as the ‘apoptosome’. In turn, activated caspase 9 activates other caspases that eventually dismantle the cell (Martinou & Green, 2001).

Apoptosis depends on ATP, and if mitochondria are damaged and fail to produce ATP in the early stages of apoptosis, the apoptosome cannot form and caspase 9 is not activated, therefore, cells die by necrosis (Martinou & Green, 2001; Brown, 2000a)

The gene involved in the regulation of apoptosis has been identified. This gene, called bcl-2, is a 26 kDa protein located on the OMM (Halestrap, 2000). The gene acts to prevent apoptosis, allowing a cell to remain alive and functional. If something interferes with the function of bcl-2, the cell will self-destruct (Martini, 2001, p. 96).

A loss of mitochondrial function is observed during apoptosis, and since the over-expression of bcl-2 has previously been found to block apoptosis induced by a wide range of stimuli (Halestrap, 2000), it has been suggested that mitochondria may play a significant role in the regulation of bcl-2 (Bittles, 2001). In support of this suggestion,
experimental findings have recently been produced, demonstrating that apoptosis is
initiated following the translocation of a nuclear transcription factor, TR3, to the
mitochondria (Li, Kolluri, Gu, Dawson, Cao, Hobbs, Lin, Chen, Lu, Lin, Xie, Fontana,
Reed, & Zhang, 2000). The translocation of TR3, initiated by apoptotic stimuli, triggers
the mitochondria to release cytochrome c (Li et al., 2000), the known initiator of
apoptosis (Halestrap, 2000).

2.11 Cardiac Ischaemia and Apoptosis

The heart is known to have one of the highest oxygen consumption rates in the body
(Roffe, 1998), and since the heart works continuously, a reliable source of oxygen and
other respiratory substrates is required to provide energy for the exclusive aerobic
metabolism of the myocardium. The coronary circulation supplies blood containing these
essentials needed by the myocardium (Martini, 2001, p. 664). Hence, even the slightest
alteration in the blood supply to the myocardium ultimately influences cardiac efficiency.

Energy depletion is one of the most common causes of cell death in our bodies. Cells die
due to cessation of the blood supply (ischaemia) and therefore cessation of the
continuous supply of respiratory substrates and oxygen. As mentioned previously, the
main causes of death in the developed world are cardiovascular disease (including
atherosclerosis and stroke) and cancer, which normally cause death by ischaemia of
surrounding tissue (Brown, 2000a).

Complete ischaemia or energy depletion, where both oxygen and glucose are absent,
ultimately causes necrosis, blocking apoptosis, because a certain level of ATP is required
to carry out apoptosis. However, either transient ischaemia or mitochondrial inhibitors
can trigger apoptosis, and allow sufficient ATP in the cell to execute it (Brown, 2000a).

Next to the brain, which is most sensitive to energy depletion, being irreversibly damaged
within 15-30 minutes of ischaemia (eg. during a stroke), the heart is the next most
sensitive tissue to ischaemia (Brown, 2000a). During coronary heart disease, the heart is
often subject to ischaemia. However, reperfusion of the heart with oxygen results in a
burst of free radical production, which contributes to the activation of the mtPTP, and
subsequent necrosis or apoptosis of cardiomyocytes (Brown, 2000a; Jeremias, Kupatt, Martin-Villalba, Habazettl, Schenkel, Boekstegers & Debatin, 2000).

Of interest is the brief ischaemia that the myocardium, and hence the cardiomyocytes, would be subject to during each cardiac contraction. During a single cardiac contraction, the coronary blood vessels supplying the myocardium are compressed. And since left ventricular pressure is slightly higher than aortic pressure during systole, but less than aortic pressure during diastole, the blood flow through the coronary arteries that supply the subendocardial area of the left ventricle, will only occur during diastole. Hence, the left ventricular subendocardium is vulnerable to transient ischaemia, as well as being the most common site of myocardial infarction (Tadlock, 1996, p. 61).

Since ischaemia followed by reperfusion results in a burst of free radical production (Brown, 2000a), it can be hypothesised that the myocardium is much more susceptible to free radical damage and subsequent cardiomyocyte apoptosis via opening of the mtPTP, (than perhaps the brain). This is because a burst of free radicals is produced following each cardiac cycle and transient ischaemia imposed on the myocardium.

Pharmaceutical companies are currently developing drugs that block apoptosis or prevent the opening of the mtPTP. However, blocking apoptosis may not be very beneficial as ‘rescued’ cells may end up dying via necrosis, causing further damage. Also, even if necrosis was blocked, the damaged cell may function abnormally or produce free radicals (Brown, 2000b).

### 2.12 Calorie Restriction (CR)

It has been known for approximately 60 years that calorie restriction (CR) of approximately 60% of the caloric intake of an ad libitum diet, performed without malnutrition, extends the lifespan (LS) and maximum lifespan (MLS) of rodents, by approximately 40% (Harman, 1998; Pollack & Leewenburgh, 1999; Sohal & Weindruch, 1996).

The mechanism by which CR increases MLS is postulated to be due to a reduction in oxygen consumption, with a simultaneous reduction in metabolic rate and body
temperature in CR animals, which lowers the chronic oxidative stress with age. In addition, CR also retards a variety of age-related detrimental biochemical and physiological changes, such as the development of cancer and diabetes (Pollack & Leewenburgh, 1999).

Rodents subject to CR demonstrate an attenuation of age-associated increases in rates of mitochondrial O$_2^-$ and H$_2$O$_2$ generation, slower accumulation of free radical damage, and delayed loss of membrane fluidity (Sohal & Weindruch, 1996).

Harman (1998), who proposed the 'free radical theory' of ageing in 1956, believes that CR increases LS by decreasing free radical reaction initiation rates. Since less free radicals are generated, there will be less injury to the mitochondria and their DNA, which overall should reduce ageing and age-associated diseases. Alternatively, CR may cause more efficient electron transport chain coupling, resulting in less leakage of electrons and therefore a decreased generation of free radicals (Harman, 1998).

2.13 Conclusion

As mentioned previously, investigating the effects of ageing on the myocardium is important for the future health of Australia's increasing aged population. The information from such investigations will contribute to the knowledge already gained from similar experiments by previous authors. Information yielded relating to the causes of ageing in general, as well as in the myocardium, is especially important from a health perspective in that various medical treatments can be devised to treat, and/or prolong the effects of ageing, to ensure maximum quality of life for aged Australians.

Although many theories exist to explain the cause of ageing, it is highly plausible that no single theory is entirely correct. A combination of theories may indeed best explain the cause of ageing as it appears to be a very complicated process affecting almost every aspect of an individual's body. Hence a single theory explaining the cause of ageing couldn't possibly explain the far-reaching effects of this currently inevitable biological process.
Also mentioned previously was the difficulty such investigations into the cause of ageing have when trying to distinguish between ‘normal’ ageing effects as well as various pathological processes that present almost identical morphological and functional changes (Goldstein et al., 1989; Safar, 1990; Svanborg, 1997). Such pathological conditions include osteoporosis, atherosclerosis and primary degenerative dementia, with onset becoming more prevalent with advancing age (Goldstein et al., 1989; Lakatta, 1993; Svanborg, 1997).

The literature reviewed above appears to relate the possible cause of ageing, in general and in the myocardium, to alterations in the function of mitochondria, the cellular organelles responsible for the production of energy (ATP) required for efficient cellular functioning (Martini, 2001, p. 87-88).

With age, mitochondrial numbers decrease, yet their size increases, in senescent human and rat hepatocytes (Tauchi & Sato, 1986). In addition, the OXPHOS system, responsible for the production of ATP, decreases with age (Wallace, 1992).

It has been suggested by Ozawa (1997), that the loss of OXPHOS capacity could be partly related to the accumulation of mtDNA mutations, already reported to increase with age by Hayakawa et al. (1993). A 7.4kb deletion of mtDNA has also been reported (Miquel, 1998), and since OXPHOS components are encoded partly within mtDNA, as well as in nDNA (Wallace, 1992), mutations and deletions of mtDNA ultimately affect the production of ATP in body cells.

Within the current literature review, ‘free radicals’ have been widely postulated as the cause of damage to mtDNA, resulting in the mutations and deletions mentioned above (Lewin & Timiras, 1984; Miquel, 1998; Ozawa, 1997; Shigenaga et al., 1994).

Apoptosis has also been related to cellular ageing, and mitochondria implicated as the ‘trigger’ of apoptosis (Brown, 2000a; Halestrap, 2000; Li et al., 2000; Pollack & Leewenburgh, 2000; Wallace, 1992). However, some kind of mitochondrial ‘damage’ is required to ‘trigger’ apoptosis (Brown, 2000a), hence ‘free radicals’ are once again implicated (Wallace, 1992).
It has been hypothesised that the progressive loss of transcription from mutated or deleted DNA, leading to deficient electron transfer and ATP synthesis, and resulting in defective energy conservation could be the basis of ageing. This view is rapidly gaining support worldwide (Castelluccio et al., 1994).
CHAPTER 3

MATERIALS AND METHODS
3.1 Subjects

Wistar rats of both sexes were used. Age groups ranged from young \((n = 8, \, 12-30 \text{ weeks old})\), middle-aged \((n = 6, \, 1-1.5 \text{ years old})\) and senescent \((n = 7, \, 2-2.5 \text{ years old})\). Data were not obtained for all animals, as 3 rats (2 senescent, 1 young) were sacrificed for dissection practice, as well as trial storage of cardiac tissue in various solutions.

Animals of the same sex were kept 2-3 per cage, and were supplied with food and water ad libitum, at room temperature (approximately 19-23°C). All animals used were specific pathogen free and obtained from the Animal Resource Centre, Murdoch University.

Rats were an appropriate animal model to study the physiological aspects of ageing because, like humans, rats are omnivorous and thus have much in common concerning intermediary metabolism and gastrointestinal function. Their normal lifespan is comparatively short, up to 2.5-3 years, making it relatively easy to study them from birth through to death. Environment, nutrition and water-salt intakes can be easily controlled and rats rarely become atherosclerotic, a disorder which often complicates investigations into human cardiovascular ageing (Folkow & Svanborg, 1993).

Rats in captivity are commonly referred to as ‘senescent’ at that age at which approximately 50% mortality occurs, which in many strains is approximately 2 years (Lakatta & Yin, 1982).

3.2 Ethical Considerations

Clearance for this project has been obtained from the Edith Cowan University Animal Experimentation Ethics Committee, approval code: 00-A8.
3.3 INSTRUMENTS AND EQUIPMENT

- Microdissecting surgical instruments, including jewellers forceps and vanna scissors
- Leica dissecting binocular microscope
- Lynx Tissue Processor
- LKB Nova Ultramicrotome
- 3-Dimensional micromanipulator
- Macintosh Maclab 2e
- Macintosh LC 575 computer
- Maclab GP Amp
- Micro-force transducer, using Piezoelectric resistive strain gauges, type AME 801
- Light microscope (with 42 point Weibel graticule)

3.4 PROCEDURE

Rats were anaesthetised using "Fluothal" (Halothane), then sacrificed by cervical dislocation. A review of the literature found no evidence of Halothane affecting mitochondria. However, Halothane sensitises the heart to adrenalin (Fenton, 1995).

The rat hearts were rapidly excised and placed into carbogenated Krebs-Henseleit solution (see appendix I). The hearts were dissected while in solution, and tissue samples removed and placed in two media. Some tissue was set aside in chilled paraffin oil, at approximately 4°C, for immediate contractile investigations. Other tissue dissected at the same time was placed in Karnovsky's fixative (see appendix II) for later histological processing.

The tissue samples taken from the myocardium include left and right papillary muscles and ventricle. After adequate tissue samples were taken for experimental procedures, the remaining heart tissue was stored in Karnovsky's fixative.
3.4.1 Contractile Investigations

The contractile investigations were carried out immediately after dissection of the myocardial tissue, to minimise deterioration. Only tissue from the left side of the heart i.e. left ventricle and left papillary muscles, was used for the contractile investigations. This was decided as i) the left side of the heart operates at a higher energy output and should manifest any age-related changes in function before the right side of the heart, and ii) it was not possible to use papillary muscles from the right side of the heart as they were too small for contractile investigations.

Using methods similar to those described by Stephenson & Williams (1981) and Fink, Stephenson & Williams (1986), functional changes in the contractile and regulatory proteins that may occur as a result of ageing can be detected. This was achieved by immersing semi-permeable muscle preparations in solutions containing Ca$^{2+}$ or Sr$^{2+}$. Chemical treatment of muscle fibres with Triton X-100 partially disintegrates the sarcolemma, allowing the composition of the fluid surrounding the myofibrils to be directly controlled. When immersed in these solutions, muscle fibres contract, producing force (West, Barclay, Luff & Walker, 1999). This technique bypasses the need for nervous stimulation or ATP production, as ATP is provided in the activating and relaxing solutions. This permits analysis of the contractile apparatus and regulatory proteins in isolation (Hill, Stewart & Verrier, 1997).

Strontium was used in addition to calcium, as Sr$^{2+}$ has several properties characteristic of the other divalent cation, Ca$^{2+}$ (West, et al., 1999; Spencer & Berlin, 1997). Strontium ions are able to permeate through sarcolemmal Ca$^{2+}$ channels and be transported by the Na$^+$/Ca$^{2+}$ exchanger, as well as bind to troponin C (Tn-C) to initiate muscle contraction (Spencer & Berlin, 1997). Comparing Ca$^{2+}$ and Sr$^{2+}$ activated contractions permits a greater level of comparison of functionality.

Slow-twitch skeletal muscle fibres eg. soleus (SOL) (West et al., 1999), as well as cardiac muscle fibres (Kerrick, Malencik, Hoar, Potter, Coby, Pocinwong & Fischer, 1980), have a similar sensitivity to Ca$^{2+}$ and Sr$^{2+}$. However, cardiac muscle fibres have been found to be more sensitive to Sr$^{2+}$ compared to fast-twitch skeletal muscle fibres
(Kerrick et al., 1980), eg. flexor digitorum longus (FDL), which have a greater sensitivity to Ca\(^{2+}\) than Sr\(^{2+}\) (West et al., 1999).

Hence, the use of both Ca\(^{2+}\) and Sr\(^{2+}\) solutions provides a means of greater differentiation between muscle fibre phenotypes (Kerrick et al., 1980; Stephenson & Williams, 1981; West et al., 1999).

The contractile investigations involved dissecting small segments of myocardial tissue (approximately 2-3 mm); tying silk sutures at either end; mounting the tissue between a calibrated force transducer and clamp assembly (see Figure 2, i & ii), and then placing the mounted tissue into a series of solutions, starting with solution I (high relaxing solution) (see appendix III) containing a detergent (‘2% v/v Triton X-100’), for approximately 15 minutes.

Triton X-100 chemically disintegrates the sarcolemma of cardiomyocytes, rendering them semi-permeable, as well as destroying the integrity of the sarcoplasmic reticulum (SR) and mitochondria (Harrison & Bers; 1989), therefore allowing the experimenter to control the intracellular environment (Bers, 1991, p. 29).

After pre-treatment with Triton X-100, the myocardial segments were placed in a series of titrated, buffered activating Ca\(^{2+}\) (termed the 190 & 200 series) and Sr\(^{2+}\) (termed the MA & NA series) solutions, while measuring the resultant force.
Figure 2. (i) (Top) A photograph showing the equipment used for measuring force output from the individual myocardial segments. On the right, labelled (a) is the micromanipulator, which allows 3 dimensional movement of the transducer (b) and the anchoring forceps (c). The preparation was mounted between the transducer pin and anchoring forceps by attachment with silk suture. Passive stretch of the preparation was adjusted by means of a micrometer screw that varied the separation of the two stainless steel arms. The calcium or strontium solutions were held in the multiple wells in a rotary carousel (d), which was mounted beneath the transducer assembly.

Figure 2. (ii) (Bottom) An enlargement of Figure 2, (i) (above) showing the carousel beneath the transducer pin and anchoring forceps. At the bottom left (arrow) is the plasticine weight used for calibrating the transducer. The whole assembly was rotated 90 degrees and the weight suspended from the transducer pin such that the force exerted was in the same alignment as for the tissue preparation.
3.4.1.1 Calibration

Absolute force was calculated by first calibrating the transducer by suspending an object (usually a round ball of plasticine, see Figure 3, i) of known mass (see Figure 2, ii) and measuring the amplitude of the signal (see Figure 3, ii). Force was calculated by Equation 1 below.

Equation 1.

\[
\text{Force (Newton)} = \text{Mass of plasticine ball (Kg)} \times g
\]

Where:

\( g = \text{the gravitational acceleration constant} = 9.8 \text{ m/s}^{-2} \)
Figure 3. (i) (Top) A photograph showing the transducer after rotation through 90 degrees with the ball of plasticine (arrow) suspended from the pin (a). The anchoring forceps (b) have been moved sideways such that they do not make contact with the suture loop supporting the ball of plasticine.

Figure 3. (ii) at bottom shows a photograph taken of the MacLab screen showing the electrical output of the transducer versus time during calibration. Several signals like this were recorded to ensure the output was reproducible.
3.4.1.2 Titrations

The activating $\text{Ca}^{2+}$ and $\text{Sr}^{2+}$ solutions, of varying pCa or pSr were made by mixing fractions of Solution I (High Relaxing) and Solution II (activating $\text{Ca}^{2+}$ or $\text{Sr}^{2+}$) (see appendix III) in proportions predicted by 1st order rate-reaction chemistry. The amount of free EGTA buffer was assayed by an in-house titration method based on the methods of Stephenson and Williams (1981).

An aliquot of solution was placed in a stable ionic environment adjusted to a pH of between 8 and 9 (see Figure 4). This was back titrated using 100mM Calcium Chloride and a curve of pH versus µmol of Calcium added was plotted. (See Equation 2 below).

Equation 2.

$$\text{EGTA.H}_2 + \text{CaCl}_2 = 2\text{HCl} + \text{CaEGTA}$$

The precise amount of Calcium added was evaluated by recording the end point of the titration curve. If the total amount of the EGTA is known, the amount of free (unbound) calcium or strontium (and therefore the pCa or pSr) can be calculated by Equation 3.

Equation 3.

$$\text{End point of titration (µmols)} \times \text{Concentration of CaCl}_2 \ (\text{mM}) \quad \frac{\text{Volume of Aliquot (mls)}}{}$$

Titrations were usually repeated twice until the pCa or pSr calculated varied by less than 1%.
Figure 4. A close up photograph taken of an aliquot of calcium solution being titrated. A micro pH probe (arrow) is inserted to the left of the beaker. Calcium chloride solution is added in 10-20μL amounts by means of a micropipettor (a) on the right. The end-point of the pH change is measured, and the amount of free calcium (or strontium) ions subsequently measured.
A third solution, Solution III (low relaxing), which did not contain Ca\(^{2+}\) or Sr\(^{2+}\), was also used in the series to relax the tissue before another series was carried out (see appendix III). This prevented any cross-contamination between solutions I and II.

Force was measured using Piezoelectric resistive strain gauges (type AME 801) coupled directly to the myocardial segments via silk suture, and a 15mm stainless steel pin (see Figure 2, i & ii).

The magnitude of force development provides an index of the degree of activation of the myofilaments by Ca\(^{2+}\) or Sr\(^{2+}\). Increasing the concentration of Ca\(^{2+}\) and Sr\(^{2+}\) in the activating solutions, above the threshold for contraction, results in a progressive increase in the production of force until the concentration is reached where all the calcium binding sites are occupied by either Ca\(^{2+}\) or Sr\(^{2+}\) (West et al., 1999).

Force displacement from the transducer pin generated a resistance disparity in a wheatstone bridge circuit connected to the general purpose amplifier of the MacLab 2e system. This was set in a single ended operational amplifier mode and produced an output in the range of 1-20mV, which was displayed on the Macintosh LC 575 computer using MacLab chart v3.4.3 software.

Examination of the calcium and strontium activated force was by the mathematical analysis of the force-pCa or force-pSr relationship in the manner of Stephenson and Williams (1981). Statistical analysis was performed using the commercial software packages Excel 97 and Deltagraph 3.5.

All contractile investigations were conducted at room temperature (21-25\(^\circ\)C).
3.4.2 Histology and Morphometry

Histological techniques were used to preserve, section and stain the tissues, whilst morphometric techniques were used to measure the volume differences between young, middle-aged and senescent cardiomyocytes.

Using histological and morphometric techniques, similar to those described in Roberts and McGeachie (1996) as well as Roberts, McGeachie and Grounds (1997), the volume of mitochondria, cytoplasm, nuclei and 'other' structures (ie. connective tissue, vascular space, interstitial space) was determined in the myocardial tissue samples from across the age range.

To obtain these data, the tissue samples were stained with p-phenylenediamine, which stains mitochondria to make them visible at the light microscope level, as well as enabling the visualisation of nuclei and cytoplasm. Mitochondria were particularly targeted due to their apparent crucial involvement in cellular and overall ageing, as reviewed in the above literature.

Tissue samples were taken from both the left and right side of the heart to compare age related changes in the volume of various structures ie. mitochondria, and relate the possible changes to the function of each side of the heart.

3.4.2.1 Tissue Processing

The tissue samples taken at the time of dissection ie. left and right papillary muscles and ventricle, and kept until needed in Karnovsky’s fixative, were processed in a Lynx automatic tissue processor at 24°C with gentle agitation. Initially, the tissue samples were washed in cold 0.1 Molar phosphate buffer (pH 7.2) for 15 minutes before post-fixation in 1% Osmium Tetroxide (in distilled water) for one hour. The tissue samples were then washed in distilled water for 15 minutes following post-fixation before placement in a block-stain of 1% p-phenylenediamine solution for one hour (prepared in 70% ethanol). Principally for staining of mitochondria, and secondly of nuclei and cytoplasm.
Another 15 minute wash in distilled water followed staining, before the tissue samples were dehydrated in a series of graded ethanols, for 30 minutes/per ethanol. The tissue samples were then placed in pure acetone for two 30 minute sessions, followed by immersion in Epon Araldite (2:1) for 12 hours and Pure Epon Araldite for 9 hours.

Whilst still immersed in Pure Epon Araldite, the tissue samples were removed from the tissue processor and placed in a vacuum chamber (30 Torr, 25°C) for 30 minutes to remove any air/acetone bubbles from within the tissue samples.

3.4.2.2 Embedding and Sectioning

The tissue samples were then placed into rubber moulds and covered with the original Epon Araldite used during tissue processing, before placement in a 60°C oven for 24 hours to polymerise. Sections (1μm) were cut using a LKB Nova Ultramicrotome. Sections were placed on microscope slides and cover-slipped for later morphometric analysis.

3.4.2.3 Morphometric Analysis

All analyses were performed on a light microscope with a 10x ocular and 100x oil immersion objective (1000x total magnification). All slides were coded and analysed ‘blindly’ to avoid experimenter bias.

Stereology is a mathematical methodology, which enables 3D values to be derived from 2D histological sections (Weibel, 1979). Stereological techniques were used for the morphometric analyses as they take into account the volume of the cardiomyocytes occupied by mitochondria, as well as nuclei and cytoplasm.

Unlike skeletal muscle, the morphometric examination of cardiac muscle does not pose the problem of oriented structures. That is, because cardiac muscle is randomly oriented it is an ideal candidate tissue for morphometry (Aherne & Dunnill, 1982, p.103).

To determine the percentage volume of cardiomyocytes occupied by mitochondria, cytoplasm, nuclei and ‘other’ structures, the ‘point counting’ method of Weibel (1979)
was used. Briefly, an eyepiece graticule containing the multipurpose test system (M42) was placed in the microscope eyepiece. Areas were quantitated in a clockwise direction in the shape of a square, beginning in the top left hand corner of a well stained area of tissue. Four fields of view (FOV) were counted from each section. Within each FOV, points falling on mitochondria, cytoplasm, nuclei were counted, with all other tissue components counted as ‘other’ eg. connective tissue, vascular space and interstitial space. Figure 10 is an example of a FOV at 1000x magnification, with immersion oil.

To determine the fraction (volume) that each of these components (ie. mitochondria, cytoplasm, nuclei and ‘other’ structures) occupies in a particular tissue, a method based on the Delesse principle was used (Aherne & Dunnill, 1982, p. 33). Briefly, the principle states that the fraction of the total volume occupied by a tissue component (the volume density) can be directly estimated from the relative areas of the outline of that component viewed on random histological sections. This relationship was expressed by Weibel and Gomez (1962) as:

Equation 4.

\[ A_{Ai} = V_{Vi} \]

Where:

- \( A \) = the area of the histological section under investigation.
- \( A_{i} \) = the section area occupied by profiles of a structure \( i \).
- \( V \) = the unit volume of the cell or tissue.
- \( V_{i} \) = represents the volume of the structure \( i \) as part of the unit volume (Roberts, 1991).

Points were counted by projecting the point lattice (graticule) onto the histological sections. The ratio of the number of points lying on the component \( i \) (\( P_{i} \)) and the total number of points (\( P_{T} \)) within the graticule lattice is the area fraction of \( i \).

Equation 5.

\[ A_{Ai} = P_{i}/P_{T} = V_{Vi} \]
When a point fell between two structures, each structure was allocated half of one point (Roberts, 1991).
3.5 DATA ANALYSIS

3.5.1 Contractile Investigations

The raw experimental data took the form of force outputs displayed oscillographically (see Figure 5). From these, maximal force peaks were measured, and relative force was calculated and matched with the corresponding calcium and strontium concentrations. A mathematical analysis of each force-calcium/strontium relationship was undertaken following the methods of Stephenson and Williams (1981). Data were fitted to the Hill Equation (see Equation 6 below) and the following parameters recorded:

\[ p_{Ca10}, p_{Ca50}, n_{Ca}, P_o(Ca), p_{Sr10}, p_{Sr50}, n_{Sr}, P_o(Sr), p_{Ca50} - p_{Sr50} \]

Where:
\( p_{Ca10} \) & \( p_{Ca50} \) = the pCa that produces 10% and 50%, respectively, of maximum activated force.
\( n_{Ca} \) = steepness of the force-pCa curves
\( P_o(Ca) \) = normalised tension (N/cm\(^2\))
\( p_{Sr10} \) & \( p_{Sr50} \) = the pSr that produces 10% and 50%, respectively, of maximum activated force.
\( n_{Sr} \) = steepness of the force-pSr curves
\( P_o(Sr) \) = normalised tension (N/cm\(^2\))
\( p_{Ca50} - p_{Sr50} \) = reflects the differential sensitivity of a muscle fibre to the two activating ions (Fink et al., 1986; Fink, Stephenson & Williams, 1990; Hill, Stewart & Verrier, 1996; Stephenson et al., 1994).

\[ P_r = K[X^{2+}]^n/(1 + K[X^{2+}]^n) \]

Where:
- \( P_r \) = the relative force
- \([X^{2+}]\) = the concentration of the divalent cations Ca\(^{2+}\) and Sr\(^{2+}\)
- \( n \) = (the Hill coefficient) is a constant determined by the slope of the force-pCa or force-pSr curves
- \( K \) = a constant related to the lateral shift of the curves (Hill et al., 1996).

Normalised tension (\( P_o \)) was calculated by first measuring the cross-sectional area (CSA) of the preparation in cm\(^2\) (see Equation 7 below) and then multiplying the measured force per preparation by its reciprocal.

Equation 7.

\[ \text{CSA} = \pi \times r^2 \]

Where:
- \( \text{CSA} \) = cross-sectional area
- \( r \) = the radius of the preparation in cm
- \( \pi = 3.141572 \)

A detailed quantitative analysis of the kinetics of Ca\(^{2+}\) and Sr\(^{2+}\) activation not only accurately describes force generation, but also provides a precise basis for demonstrating any age-related changes.
3.5.2 Histology and Morphometry

Statistical analysis begins with an estimation of the minimum number of points that must fall on the structure of interest ie. mitochondria, to obtain an acceptably small standard of error. The following formula is employed to determine the minimum number of points needed to fall on a particular structure at a particular level of confidence ie. minimum significance level of < 0.05.

Equation 8.

\[ RSE = \frac{1 - Vv}{\sqrt{n}} \]

Where:
RSE = the relative standard error
Vv = the volume fraction
n = the square root of the number of points falling on the structure of interest (Roberts, 1991).

3.5.2.1 Arcsine Transformations

Morphometric analysis involved performing an Arcsine Transformation to normalise the data yielded from the stereological analyses followed by an ANOVA to compare within group and between group results. A Student’s t-test was used if the results were significant.

As described earlier, the point counting technique was used to calculate volume densities using Formula 2. Since these values were proportions, a statistical analysis was performed on the arcsine transformed values to adjust for non-normality.

The data derived from both the contractile investigations and the morphology were compared to determine (if indeed), decreased contractility was related to increased mitochondrial volume within cardiomyocytes.
3.6 LIMITATIONS

- Age of animals
- Number of animals
- Number of preparations per animal
- Number of samples
- Deterioration of the cardiac tissue with time
CHAPTER 4

RESULTS
4.1 Contractile Investigations

A total of 8 Young (12-30 weeks old), 6 middle-aged (1-1.5 years) and 7 senescent (2-2.5 years) Wistar rats, of both sexes, were used in the present study. Of those animals, 33 young, 44 middle-aged and 26 senescent segments of myocardial tissue from the left side of the heart were sampled for the contractile investigations.

Examples of the graphical analyses are shown in Figures 6-8. Force was detected using an amplified microtransducer system (see methods p. 46-52). The millivolt (mV) output was amplifier and displayed oscillographically using a Maclab 2e system coupled to a Macintosh 575 computer. This allowed for continuous adjustment of both time base and voltage sensitivity, thus ensuring a large, clear signal was always displayed.

Overall, the data derived from the force-pCa and force-pSr curves comparing the 3 age groups ie. young, middle-aged and senescent, showed no major differences. The mean and standard deviation data of the parameters analysed ie. pCa10, pCa50, nCa, P0(Ca), pSr10, pSr50, nSr, P0(Sr), pCa50- pSr50, are shown in Table 1. The data derived from Student’s t-test (P values), also using the above parameters, are shown in Table 2, at a significance level of < 0.05.
Figure 5. (i) (Top) and (ii) (Bottom) are photographs taken of the MacLab screen showing examples of the electrical outputs derived during the contractile investigations when myocardial segments were placed in either a calcium (a) or strontium (b) series of solutions. The vertical lines are event markers indicating solution identity just before the preparation was raised from one carousel well and dipped into the next. The maximum voltage was tabulated with respect to baseline for each solution. Note the greater scale in mV required during the strontium series compared to the lower mV of the calcium series, indicating higher force outputs when myocardial segments were placed in the strontium solutions. Also observe the immediate decline in force output after the myocardial segments were placed in relaxing solution I (arrows) in the calcium series, whereas in strontium, force appears to increase or remain high for some time after placement in solution I (arrows).
Figure 6. Shows a representative sample of 4 charts representing the force-pCa (pSr) relationships derived from tissue from young animals. All curves display the classic sigmoidal shape typically seen with cardiac and skeletal muscle. There was very little variation amongst the slopes of the individual curves. Each of the 4 charts represents a different test animal.
Figure 7. Shows a representative sample of 4 charts representing the force-pCa (pSr) relationships derived from tissue from middle-aged animals. All curves display the classic sigmoidal shape typically seen with cardiac and skeletal muscle. There was very little variation amongst the slopes of the individual curves. Each of the 4 charts represents a different test animal.
Figure 8. Shows a representative sample of 4 charts representing the force-pCa (pSr) relationships derived from tissue from senescent animals. All curves display the classical sigmoidal shape typically seen with cardiac and skeletal muscle. It is apparent that variation amongst the slopes of the individual curves exists, as evidenced by the nCa value of the senescent group. Each of the 4 charts represents a different test animal.
Figure 9. A photograph taken from the screen of the MacLab system showing maximal force output during Ca$^{2+}$ activation at two different temperatures. The peak on the left (a) was achieved at 30°C, while the peak on the right (b) was a repeat at room temperature ie. 21-25°C. The difference in electrical output (hence force output) was approximately 2 times.
Table 1. Contractile characteristics for myocardial segments from young, middle-aged and senescent rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Young (n = 8, 12-30 weeks)</th>
<th>Middle-aged (n = 6, 1-1.5 years)</th>
<th>Senescent (n = 7, 2-2.5 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>2.35 ± 1.17</td>
<td>1.84 ± 0.59</td>
<td>2.81 ± 2.28 *</td>
</tr>
<tr>
<td>p&lt;sub&gt;Ca&lt;sub&gt;10&lt;/sub&gt;</td>
<td>6.18 ± 0.22</td>
<td>6.22 ± 0.05</td>
<td>6.10 ± 0.24</td>
</tr>
<tr>
<td>p&lt;sub&gt;Ca&lt;sub&gt;50&lt;/sub&gt;</td>
<td>5.72 ± 0.18</td>
<td>5.67 ± 0.14</td>
<td>5.64 ± 0.17</td>
</tr>
<tr>
<td>P&lt;sub&gt;o&lt;/sub&gt;(Ca)</td>
<td>0.05 ± 0.04</td>
<td>0.05 ± 0.03</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>n&lt;sub&gt;Sr&lt;/sub&gt;</td>
<td>1.59 ± 0.66</td>
<td>2.41 ± 0.51</td>
<td>2.71 ± 1.37</td>
</tr>
<tr>
<td>p&lt;sub&gt;Sr&lt;sub&gt;10&lt;/sub&gt;</td>
<td>5.60 ± 0.39</td>
<td>5.28 ± 0.11</td>
<td>5.27 ± 0.17</td>
</tr>
<tr>
<td>p&lt;sub&gt;Sr&lt;sub&gt;50&lt;/sub&gt;</td>
<td>4.90 ± 0.29</td>
<td>4.87 ± 0.14</td>
<td>4.84 ± 0.10</td>
</tr>
<tr>
<td>P&lt;sub&gt;o&lt;/sub&gt;(Sr)</td>
<td>0.19 ± 0.20</td>
<td>0.26 ± 0.19</td>
<td>0.10 ± 0.11</td>
</tr>
<tr>
<td>pCa&lt;sub&gt;50&lt;/sub&gt;-pSr&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.63 ± 0.34</td>
<td>0.80 ± 0.08</td>
<td>0.77 ± 0.18</td>
</tr>
</tbody>
</table>

* Indicates variations within the senescent age group of animals.

n<sub>Ca</sub> & n<sub>Sr</sub> = the steepness of the force-pCa and force-pSr curves
p<sub>Ca<sub>10</sub> & p<sub>Sr<sub>10</sub> = the pCa & pSr that produces 10% of maximum activated force; a parameter reflecting the threshold for activation of a muscle fibre by each activating ion.
pCa<sub>50</sub> & pSr<sub>50</sub> = the pCa & pSr that produces 50% of maximum activated force; a parameter indicating the relative sensitivity of a muscle fibre to each activating ion.
P<sub>o</sub>(Ca) & P<sub>o</sub>(Sr) = normalised tension (N/cm<sup>2</sup>)
pCa<sub>50</sub>-pSr<sub>50</sub> = reflects the differential sensitivity of a muscle fibre to the two activating ions (Fink et al., 1986; Fink et al., 1990; Hill, Stewart & Verrier, 1996; Stephenson et al., 1994).

Mean ± Standard Deviation

The slope of the force-pCa curve of senescent rats, indicated by the nCa value, displayed a large variation (2.81 ± 2.28) within the myocardial segments of the senescent age group.
Table 2. P values derived from Student’s t-test performed on contractile data

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Young vs Middle-aged</th>
<th>Young vs Senescent</th>
<th>Middle-aged vs Senescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCa_{10}</td>
<td>P = 0.258</td>
<td>P = 0.180</td>
<td>P = 0.057</td>
</tr>
<tr>
<td>pCa_{50}</td>
<td>P = 0.247</td>
<td>P = 0.107</td>
<td>P = 0.356</td>
</tr>
<tr>
<td>pSr_{10}</td>
<td>P = 7.68x10^{-3} *</td>
<td>P = 7.66x10^{-5} **</td>
<td>P = 0.337</td>
</tr>
<tr>
<td>pSr_{50}</td>
<td>P = 0.286</td>
<td>P = 0.152</td>
<td>P = 0.384</td>
</tr>
</tbody>
</table>

* Indicates a significant difference between the young versus middle-aged age group.
** Indicates a significant difference between the young versus senescent age group.

Significant differences were seen at pSr_{10} between the young versus middle-aged (P < 0.05) and the young versus senescent (P < 0.05) age groups.
4.2 **Histology and Morphometry**

Of the 21 animals used in the present study, a total of 3,696 points were counted from myocardial tissue sections cut from the 8 Young (12-30 weeks old) animals; 4,536 points from the 6 middle-aged (1-1.5 years) animals; and 4,032 points from the 7 senescent (2-2.5 years) animals used. The points were counted at a significance level of 0.05.

The percentage volume of myocardial tissue occupied by structures of interest ie. mitochondria, cytoplasm, nuclei and 'other' (connective tissue, interstitial space, vascular space), for both the left and right sides of the heart, are shown in Table 3 and Table 4 respectively, with graphical representations in Figures 10-15.

Figure 16 demonstrates a typical field of view (FOV), at 1000x magnification with oil immersion, counted during the present study. The structures of interest are labelled, as well as some additional structures characteristic of myocardial tissue ie. intercalated discs. Figure 17 is an interesting example of an arteriole (coronary artery/arteriole) within the right papillary muscles of a senescent rat. Once again various structures are labelled.

The data derived from Student's t-test (P values), using the arcsine transformed values and the percentage volume values, from both the left and right sides of the heart, are shown in Table 5 and Table 6 respectively, at a significance level of < 0.05.

In each side of the heart, data were separately derived and analysed from the papillary muscles and ventricle alone. The data were also combined to yield overall figures on the activity of the left and right sides of the heart as a whole. This was considered valid as papillary muscles are part of the ventricle on both sides of the heart, with both acting in unison when the heart is contracting.
4.2.1 Left Side of the Heart

In relation to the papillary muscles on left side of the heart, statistical analyses of the arcsine transformed data demonstrated a significant decrease in mitochondrial volume between young and middle-aged rats (P < 0.05), and young and senescent rats (P < 0.05). No significant changes in mitochondrial volume were observed between middle-aged and senescent rats. The percentage volume of mitochondria decreased from 54.5% in young to 43.1% in middle-aged and 34.1% in senescent rats.

The volume of cytoplasm in left papillary muscles increased significantly between young and senescent rats (P < 0.05). The percentage volume of cytoplasm increased from 31.2% in young to 37.7% in middle-aged rats; further increasing to 47.7% in senescent rats. No significant volume changes were observed in relation to nuclei or ‘other’ structures, across the age range in left papillary muscles.

Statistically significant changes in the volume of mitochondria, cytoplasm, nuclei and ‘other’ structures were not observed in the left ventricle of rats when compared across the age range. Although the percentage volume of mitochondria decreased between young and middle-aged rats (49.9% to 45.7% respectively), and then increased between middle-aged and senescent rats (45.7% to 48.7% respectively), whilst the volume of cytoplasm increased between young and middle-aged rats (30.2% to 34.2% respectively) and then decreased slightly between middle-aged and senescent rats (34.2% to 33.8%). Volume changes in nuclei or ‘other’ structures were unremarkable.

When the data from the left side of the heart were combined, significant differences in mitochondrial volume were observed between young and middle-aged rats (P < 0.05) and young and senescent rats (P < 0.05). The percentage volume of mitochondria decreased from 52.2% in young to 44.4% in middle-aged rats, also decreasing from 52.2% in young rats to 41.4% in senescent rats.

A significant change in cytoplasmic volume was observed between young and senescent rats (P < 0.05), with the percentage volume of cytoplasm increasing from 30.7% in young to 40.8% in senescent rats. No other significant volume differences were observed from the combined data of the left side of the heart.
4.2.2 Right Side of the Heart

On the right side of the heart, statistically significant changes in ventricular mitochondrial volume were observed between young and middle-aged rats (P < 0.05) and between middle-aged and senescent rats (P < 0.05). Between young and middle-age rats the percentage volume of mitochondria decreased from 48.6% to 40.0% respectively. Between middle-aged and senescent rats the percentage volume of mitochondria increased from 40.0% to 49.7% respectively. No significant changes in mitochondrial volume were observed between young and senescent rats.

Between middle-aged and senescent rats, a significant (P < 0.05) decrease in ‘other’ structures ie. connective tissue, vascular space or interstitial space, was observed in the right ventricle. The percentage volume of ‘other’ structures decreased from 21.3% in middle-aged to 12.4% in senescent rats. No other significant volume differences were observed from across the age range in the right ventricle.

In the right papillary muscles, no significant volume changes were observed across the age range. The percentage volume of all structures of interest demonstrated little change, although the volume of mitochondria increased from 43.9% in young rats to 46.0% in senescence, which was statistically non-significant.

The combined data from the right ventricle and right papillary muscles demonstrated significant changes in mitochondrial volume between young and middle-aged rats (P < 0.05) and middle-aged and senescent rats (P < 0.05). The percentage volume of mitochondria decreased from 46.3% in young to 41.8% in middle-aged rats, and then increased from 41.8% in middle-aged to 47.9% in senescent rats. A significant difference in cytoplasmic volume was also observed between young and middle-aged rats (P < 0.05), with the percentage volume increasing from 33.0% in young to 36.9% in middle-aged rats.

Interestingly a significant difference in the volume of ‘other’ structures was observed between middle-aged and senescent rats (P < 0.05), with the percentage volume decreasing from 19.4% in middle-aged to 15.0% in senescent rats. No other significant volume differences were observed from the combined data of the right side of the heart.
Table 3. Percentage volume of myocardial tissue from the left side of the heart occupied by structures of interest.

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>Mitochondria</th>
<th>Cytoplasm</th>
<th>Nuclei</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>LV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>49.9</td>
<td>30.2</td>
<td>1.3</td>
<td>18.6</td>
<td>100</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>45.7</td>
<td>34.2</td>
<td>0.8</td>
<td>19.3</td>
<td>100</td>
</tr>
<tr>
<td>Senescent</td>
<td>48.7</td>
<td>33.8</td>
<td>0.5</td>
<td>17.0</td>
<td>100</td>
</tr>
<tr>
<td>LP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>54.5</td>
<td>31.2</td>
<td>1.5</td>
<td>12.8</td>
<td>100</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>43.1</td>
<td>37.7</td>
<td>1.7</td>
<td>17.5</td>
<td>100</td>
</tr>
<tr>
<td>Senescent</td>
<td>34.1</td>
<td>47.7</td>
<td>1.3</td>
<td>16.9</td>
<td>100</td>
</tr>
<tr>
<td>LV &amp; LP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>52.2</td>
<td>30.7</td>
<td>1.4</td>
<td>15.7</td>
<td>100</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>44.4</td>
<td>35.9</td>
<td>1.3</td>
<td>18.4</td>
<td>100</td>
</tr>
<tr>
<td>Senescent</td>
<td>41.4</td>
<td>40.8</td>
<td>0.9</td>
<td>16.9</td>
<td>100</td>
</tr>
</tbody>
</table>

LV = Left Ventricle
LP = Left Papillary Muscles
LV & LP = the combined data from both the left ventricle and left papillary muscles.
Figure 10. Percentage volume occupied by structures of interest in left ventricular muscle.

Y = Young
MA = Middle-aged
S = Senescent

Mito = Mitochondria
Cyto = Cytoplasm
Nuclei = Nucleus/Nuclei
Other = Connective tissue, vascular space, interstitial space
Figure 11. Percentage volume occupied by structures of interest in the left papillary muscles.

Y = Young
MA = Middle-aged
S = Senescent

Mito = Mitochondria
Cyto = Cytoplasm
Nuclei = Nucleus/Nuclei
Other = Connective tissue, vascular space, interstitial space
% Volume Occupied by Structures of Interest in the Left Side of the Heart

Figure 12. Combined percentage volume occupied by structures of interest in left papillary and ventricular muscle.

Y = Young
MA = Middle-aged
S = Senescent

Mito = Mitochondria
Cyto = Cytoplasm
Nuclei = Nucleus/Nuclei
Other = Connective tissue, vascular space, interstitial space
Table 4. Percentage volume of myocardial tissue from the right side of the heart, occupied by structures of interest.

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>Mitochondria</th>
<th>Cytoplasm</th>
<th>Nuclei</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>48.6</td>
<td>32.4</td>
<td>2.5</td>
<td>16.5</td>
<td>100</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>40.0</td>
<td>36.9</td>
<td>1.8</td>
<td>21.3</td>
<td>100</td>
</tr>
<tr>
<td>Senescent</td>
<td>49.7</td>
<td>36.1</td>
<td>1.8</td>
<td>12.4</td>
<td>100</td>
</tr>
<tr>
<td>RP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>43.9</td>
<td>33.8</td>
<td>2.3</td>
<td>20.0</td>
<td>100</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>43.6</td>
<td>37.0</td>
<td>1.9</td>
<td>17.5</td>
<td>100</td>
</tr>
<tr>
<td>Senescent</td>
<td>46.0</td>
<td>34.9</td>
<td>1.4</td>
<td>17.7</td>
<td>100</td>
</tr>
<tr>
<td>RV &amp; RP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>46.3</td>
<td>33.0</td>
<td>2.4</td>
<td>18.3</td>
<td>100</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>41.8</td>
<td>36.9</td>
<td>1.9</td>
<td>19.4</td>
<td>100</td>
</tr>
<tr>
<td>Senescent</td>
<td>47.9</td>
<td>35.5</td>
<td>1.6</td>
<td>15.0</td>
<td>100</td>
</tr>
</tbody>
</table>

RV = Right Ventricle
RP = Right Papillary Muscles
RV & RP = the combined data from both the right ventricle and right papillary muscles.
Figure 13. Percentage volume occupied by structures of interest in right ventricular muscle.

Y = Young
MA = Middle-aged
S = Senescent

Mito = Mitochondria
Cyto = Cytoplasm
Nuclei = Nucleus/Nuclei
Other = Connective tissue, vascular space, interstitial space
Figure 14. Percentage volume occupied by structures of interest in the right papillary muscles.

Y = Young  
MA = Middle-aged  
S = Senescent  

Mito = Mitochondria  
Cyto = Cytoplasm  
Nuclei = Nucleus/Nuclei  
Other = Connective tissue, vascular space, interstitial space
Figure 15. Combined percentage volume occupied by structures of interest in right papillary and ventricular muscle.

Y = Young
MA = Middle-aged
S = Senescent

Mito = Mitochondria
Cyto = Cytoplasm
Nuclei = Nucleus/Nuclei
Other = Connective tissue, vascular space, interstitial space
Table 5. P values derived from Student’s t-test performed on the arcsine transformed data from the left side of the heart and the percentage volume occupied by structures of interest.

* Indicates a significant difference between young and middle-aged animals.
** Indicates a significant difference between young and senescent animals.

Structures of Interest = mitochondria, cytoplasm, nuclei and ‘other’ (interstitial space, vascular space, connective tissue).
LV = Left Ventricle
LP = Left Papillary Muscles
LV & LP = the combined data from both the left ventricle and left papillary muscles.
Table 5. P values derived from Student’s t-test performed on the arcsine transformed data from the left side of the heart and the percentage volume occupied by structures of interest.

<table>
<thead>
<tr>
<th>Structures of Interest</th>
<th>Young vs Middle-aged</th>
<th>% Volume of Young vs Middle-aged</th>
<th>Young vs Senescent</th>
<th>% Volume of Young vs Senescent</th>
<th>Middle-aged vs Senescent</th>
<th>% Volume of Middle-aged vs Senescent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>P = 0.147</td>
<td>49.8% vs 45.7%</td>
<td>P = 0.405</td>
<td>49.9% vs 48.7%</td>
<td>P = 0.217</td>
<td>45.7% vs 48.7%</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>P = 0.049</td>
<td>30.2% vs 34.2%</td>
<td>P = 0.060</td>
<td>30.2% vs 33.8%</td>
<td>P = 0.434</td>
<td>34.2% vs 33.8%</td>
</tr>
<tr>
<td>Nuclei</td>
<td>P = 0.209</td>
<td>1.3% vs 0.8%</td>
<td>P = 0.157</td>
<td>1.3% vs 0.5%</td>
<td>P = 0.126</td>
<td>0.8% vs 0.5%</td>
</tr>
<tr>
<td>Other</td>
<td>P = 0.432</td>
<td>18.6% vs 19.3%</td>
<td>P = 0.378</td>
<td>18.6% vs 17.0%</td>
<td>P = 0.280</td>
<td>19.3% vs 17.0%</td>
</tr>
<tr>
<td><strong>LP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>P = 0.036 *</td>
<td>54.5% vs 43.1%</td>
<td>P = 0.001 **</td>
<td>54.5% vs 34.1%</td>
<td>P = 0.103</td>
<td>43.1% vs 34.1%</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>P = 0.156</td>
<td>31.2% vs 37.7%</td>
<td>P = 0.008 **</td>
<td>31.2% vs 47.7%</td>
<td>P = 0.098</td>
<td>37.7% vs 47.7%</td>
</tr>
<tr>
<td>Nuclei</td>
<td>P = 0.329</td>
<td>1.5% vs 1.7%</td>
<td>P = 0.280</td>
<td>1.5% vs 1.3%</td>
<td>P = 0.176</td>
<td>1.7% vs 1.3%</td>
</tr>
<tr>
<td>Other</td>
<td>P = 0.055</td>
<td>12.8% vs 17.5%</td>
<td>P = 0.115</td>
<td>12.8% vs 16.9%</td>
<td>P = 0.409</td>
<td>17.5% vs 16.9%</td>
</tr>
<tr>
<td><strong>LV &amp; LP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>P = 0.010 *</td>
<td>52.2% vs 44.4%</td>
<td>P = 0.005 **</td>
<td>52.2% vs 41.4%</td>
<td>P = 0.219</td>
<td>44.4% vs 41.4%</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>P = 0.059</td>
<td>30.7% vs 35.9%</td>
<td>P = 0.005 **</td>
<td>30.7% vs 40.8%</td>
<td>P = 0.110</td>
<td>35.9% vs 40.8%</td>
</tr>
<tr>
<td>Nuclei</td>
<td>P = 0.329</td>
<td>1.4% vs 1.3%</td>
<td>P = 0.179</td>
<td>1.4% vs 0.9%</td>
<td>P = 0.356</td>
<td>1.3% vs 0.9%</td>
</tr>
<tr>
<td>Other</td>
<td>P = 0.120</td>
<td>15.7% vs 18.4%</td>
<td>P = 0.307</td>
<td>15.7% vs 16.9%</td>
<td>P = 0.254</td>
<td>18.4% vs 16.9%</td>
</tr>
</tbody>
</table>
Table 6. P values derived from Student’s t-test performed on the arcsine transformed data from the right side of the heart and the percentage volume occupied by structures of interest.

* Indicates a significant difference between young and middle-aged animals.

** Indicates a significant difference between middle-aged and senescent animals.

Structures of Interest = mitochondria, cytoplasm, nuclei and ‘other’ (interstitial space, vascular space, connective tissue).

RV = Right Ventricle
RP = Right Papillary Muscles
RV & RP = the combined data from both the right ventricle and right papillary muscles.
Table 6. P values derived from Student’s t-test performed on the arcsine transformed data from the right side of the heart and the percentage volume occupied by structures of interest.

<table>
<thead>
<tr>
<th>Structures of Interest</th>
<th>Young vs Middle-aged</th>
<th>% Volume of Young vs Middle-aged</th>
<th>Young vs Senescent</th>
<th>% Volume of Young vs Senescent</th>
<th>Middle-aged vs Senescent</th>
<th>% Volume of Middle-aged vs Senescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>P = 0.018 *</td>
<td>48.6% vs 40.0%</td>
<td>P = 0.372</td>
<td>48.6% vs 49.7%</td>
<td>P = 0.017 ***</td>
<td>40.0% vs 49.7%</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>P = 0.121</td>
<td>32.4% vs 36.9%</td>
<td>P = 0.182</td>
<td>32.4% vs 36.1%</td>
<td>P = 0.368</td>
<td>36.9% vs 36.1%</td>
</tr>
<tr>
<td>Nuclei</td>
<td>P = 0.277</td>
<td>2.5% vs 1.8%</td>
<td>P = 0.206</td>
<td>2.5% vs 1.8%</td>
<td>P = 0.331</td>
<td>1.8% vs 1.8%</td>
</tr>
<tr>
<td>Other</td>
<td>P = 0.120</td>
<td>16.5% vs 21.3%</td>
<td>P = 0.114</td>
<td>16.5% vs 12.4%</td>
<td>P = 0.004 ***</td>
<td>21.3% vs 12.4%</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>RP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>P = 0.447</td>
<td>43.9% vs 43.6%</td>
<td>P = 0.331</td>
<td>43.9% vs 46.0%</td>
<td>P = 0.308</td>
<td>43.6% vs 46.0%</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>P = 0.144</td>
<td>33.8% vs 37.0%</td>
<td>P = 0.338</td>
<td>33.8% vs 34.9%</td>
<td>P = 0.221</td>
<td>37.0% vs 34.9%</td>
</tr>
<tr>
<td>Nuclei</td>
<td>P = 0.265</td>
<td>2.3% vs 1.9%</td>
<td>P = 0.063</td>
<td>2.3% vs 1.4%</td>
<td>P = 0.183</td>
<td>1.9% vs 1.4%</td>
</tr>
<tr>
<td>Other</td>
<td>P = 0.145</td>
<td>20.0% vs 17.5%</td>
<td>P = 0.300</td>
<td>20.0% vs 17.7%</td>
<td>P = 0.482</td>
<td>17.5% vs 17.7%</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>RV &amp; RP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>P = 0.033 *</td>
<td>46.3% vs 41.8%</td>
<td>P = 0.239</td>
<td>46.3% vs 47.9%</td>
<td>P = 0.026 ***</td>
<td>41.8% vs 47.9%</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>P = 0.044 *</td>
<td>33.0% vs 36.9%</td>
<td>P = 0.141</td>
<td>33.0% vs 35.5%</td>
<td>P = 0.217</td>
<td>36.9% vs 35.5%</td>
</tr>
<tr>
<td>Nuclei</td>
<td>P = 0.178</td>
<td>2.4% vs 1.9%</td>
<td>P = 0.050</td>
<td>2.4% vs 1.6%</td>
<td>P = 0.186</td>
<td>1.9% vs 1.6%</td>
</tr>
<tr>
<td>Other</td>
<td>P = 0.346</td>
<td>18.3% vs 19.4%</td>
<td>P = 0.075</td>
<td>18.3% vs 15.0%</td>
<td>P = 0.043 ***</td>
<td>19.4% vs 15.0%</td>
</tr>
</tbody>
</table>
Figure 16. A photomicrograph of a typical field of view (FOV) counted during the present study, at 1000x magnification. This tissue sample is from the left papillary muscle of a young rat. The characteristic structures of myocardial tissue are clearly seen, such as the striated appearance of the myofibrils (↑) and the intercalated discs (↑). Nuclei are clearly seen with a central dark stained nucleolus (→). Mitochondria are also visible, often between myofibrils (←). Blood vessels (coronary venules) containing red blood cells (a) are visible between cardiomyocytes.
Figure 17. A photomicrograph of a coronary arteriole within the right papillary muscle of a senescent rat, at 1000x magnification. The lumen of the arteriole contains numerous red blood cells (a). Smooth muscle cells can be seen within the wall of the arteriole (←) as well as the connective tissue layer (b) of the tunica externa, surrounding the arteriole. The cardiomyocytes are to the right of the photomicrograph, and various structures such as mitochondria (↑) and nuclei (→) can be seen.
CHAPTER 5

DISCUSSION AND CONCLUSIONS
5.1 CONTRACTILE INVESTIGATIONS

The present study used two activating solutions, one containing Calcium ions (Ca$^{2+}$), the other, Strontium ions (Sr$^{2+}$). As previously mentioned, Sr$^{2+}$ has several properties characteristic of the other divalent cation calcium (Ca$^{2+}$) (West et al., 1999; Spencer & Berlin, 1997). Strontium ions are able to permeate through sarcolemmal Ca$^{2+}$ channels, be transported by the Na$^+-$Ca$^{2+}$ exchanger, and bind to troponin C (Tn-C) to initiate muscle contraction (Spencer & Berlin, 1997).

5.1.1 Contractile Parameters

A major finding in the contractile investigations was that the force generated in the cardiomyocytes was essentially the same across the age range. This suggests that young cardiomyocytes contract more efficiently than older cardiomyocytes as it has been shown by Fraticelli et al. (1989) that there is a statistically significant increase in sarcomere numbers in senescent (24-25 months) rat cardiomyocytes ie. young cardiomyocytes generate the same force as senescent cardiomyocytes, despite comparatively fewer sarcomeres. This will be discussed further in combination with the results of the histological and morphometric analyses.

Several quantitative characteristics can be derived from a set of force-pCa and force-pSr curves. Of the parameters recorded during the present study, the pCa$_{50}$ and pSr$_{50}$ values represent the pCa and pSr corresponding to 50% maximum Ca$^{2+}$- and Sr$^{2+}$-activated force responses respectively. These two characteristics are able to describe the sensitivity of the contractile apparatus to Ca$^{2+}$ and Sr$^{2+}$ respectively (Fink et al., 1986; Fink et al., 1990; Stephenson et al., 1994). During the present study, no significant differences were observed when the age groups were compared at pCa$_{50}$ and pSr$_{50}$.

The difference between the two, pCa$_{50}$-pSr$_{50}$, refer to the relative sensitivity of a particular fibre to Ca$^{2+}$ and Sr$^{2+}$ (Fink et al., 1986; Fink et al., 1990; Stephenson et al., 1994), and have been used to describe the functional differences between slow- and fast-twitch skeletal muscle fibres (Stephenson & Williams, 1981; West et al., 1999) as well as cardiac muscle fibres (Kerrick et al., 1980). No significant changes were observed during
the present study, when the difference between pCa_{50} and pSr_{50} \( (pCa_{50} - pSr_{50}) \) was calculated across the age ranges.

The pCa_{10} and pSr_{10} parameters gives an indication of the contraction threshold for the activating ion and represents the pCa and pSr corresponding to a relative force value of 0.1 (Fink et al., 1986; Fink et al., 1990; Stephenson et al., 1994). During the present study, statistical analysis of the pSr_{10} parameter using Student's t-test demonstrated a significant difference between the young and middle-aged \( (P < 0.05) \) animals, and the young and senescent \( (P < 0.05) \) animals, with no significant difference demonstrated between the middle-aged and senescent \( (P < 0.05) \) animals. It is speculated that these results might be a reflection of developmental changes in the TnC protein that may only occur early in life ie. between young and middle-age in the rat.

It is interesting that this difference was not seen at ionic concentrations that generated 50% relative force (ie. at pCa_{50} and pSr_{50}). This could be the result of other mechanisms upregulating force output such as myosin light chain (MLC) phosphorylation (Stephenson, 1988). It might be interesting to repeat these experiments whilst regulating the concentration of phosphate \([P_i]\).

The n_{Ca} and n_{Sr} parameters represent numbers 'n' in the Hill equation (see Equation 6 in the methods section, p. 58), which provide the closest fit to the experimental points, and give an indication of the maximum steepness of the force-pCa and force-pSr curves (Fink et al., 1986; Fink et al., 1990; Stephenson et al., 1994). The slope of the force-pCa curve demonstrated a large variation \( (2.81 \pm 2.28) \) within the myocardial segments of only the senescent age group during the present study. This result indicates a wide range of functional phenotypes of the contractile protein (myosin) in the cardiomyocytes of the senescent rat population itself. It is hypothesised that senescent rats are at various stages of physiological deterioration, impacting on the function of the myosin heavy chains (MHC). Hence accounting for the large standard deviation of within group force-pCa curve results.

The P_{0}(Ca) and P_{0}(Sr) parameters relate to the maximum Ca^{2+}- and Sr^{2+}-activated tension (in N/cm^{2}) developed by each myocardial segment, determined from the initial force response in the maximally activating Ca^{2+} and Sr^{2+} solution and from the cross-
sectional area of the myocardial segments (Stephenson et al., 1994). The cross sectional area (CSA) was measured whilst the myocardial segments were mounted on the force measuring apparatus (see methods section, p. 58), using a pre-calibrated graticule within a low power binocular microscope. No significant changes were observed across the age range during the present study, when the P_0(Ca) and P_0(Sr) parameters were calculated.

5.1.2 Contractile and Regulatory Proteins

During the present study, across three defined age ranges ie. young, middle-aged and senescent, the myocardial segments appeared to be more sensitive to solutions containing Sr^{2+} rather than Ca^{2+} ie. greater absolute force was produced when myocardial segments were maximally activated in solutions containing Sr^{2+} rather than solutions containing Ca^{2+}. Also, while not statistically significant, on average, maximum tension was reached at a lower pSr when compared to pCa. It was generally observed that the force output took 2-3 times longer to return to baseline if activated in Sr^{2+} rather than Ca^{2+} (see Figure 5, i & ii). It is speculated that this might be a reflection of a greater affinity of Sr^{2+} to TnC. Additionally, the diffusion co-efficient (which is a function of the size and shape of the molecule) of Sr^{2+} would be smaller than Ca^{2+} as the atomic weight of strontium (87.6) is more than double that of calcium (40.1) (Lehninger, 1975), hence slowing the rate of Sr^{2+} diffusion and therefore slowing the rate of return to baseline.

The observations from the present study are in contrast with those of Kerrick et al. (1980) who found rabbit and bovine cardiac muscle fibres to be equally well activated by Sr^{2+} and Ca^{2+}, and hence, have similar sensitivities to both divalent cations. Also in contrast, Spencer and Berlin (1997) found that the binding of cardiac troponin C (TnC) to Sr^{2+} is 2- to 4-fold weaker than its binding to Ca^{2+}. However, these comparisons should be made cautiously, as the observed differences could be species specific.

The prolonged contraction and relaxation time seen in aged myocardium, has often been attributed to age-related changes in the contractile apparatus involved in muscle contraction ie. the contractile and regulatory proteins. Indeed, age-related changes in the sensitivity of muscle fibres to Ca^{2+} and or Sr^{2+} are hypothesised to be due to changes in the contractile and regulatory proteins with age (Hill et al., 1996; Stephenson & Williams, 1981; Fink et al., 1986).
A previously mentioned study by Hill et al. (1996) found that the skeletal muscle fibres of older (18-19 months) rats displayed increased sensitivity to Ca\(^{2+}\), but not Sr\(^{2+}\), compared to younger (5-6 months) rats. It was suggested that age-related modifications of the contractile proteins, particularly the regulatory myosin light chain (MLC), was the cause of the increased sensitivity to Ca\(^{2+}\), not Sr\(^{2+}\).

Myocardial fibres have a similar myofibrillar protein composition to skeletal muscle fibres ie. \(\alpha\text{-MHC} & \beta\text{-MHCs, MLCs, TnT-Tm complex, TnC and TnI}\); with slightly different isoforms (Walsh, 1987). As previously mentioned, Fitzsimons et al. (1999) attributed the age-related decline in cardiac function, ie. the prolongation of myocardial contraction and relaxation time seen with age, to changes in the expression of the \(\beta\)-myosin heavy chain (\(\beta\text{-MHC}\)). The authors found that the age-related increase in the expression of \(\beta\text{-MHC}\) contributes to the prolonged contraction and relaxation by depressing the kinetics of cross-bridge interaction, summarising that the ageing-dependent relationship of kinetics and \(\beta\text{-MHC}\) content is consistent with the approximately threefold greater ATPase activity of \(\alpha\text{-MHC}\) versus \(\beta\text{-MHC}\) (Fitzsimons et al., 1999).

Using electrophoretic techniques, Fitzsimons et al. (1999) were also able to determine the expression of other myofibrillar proteins, in addition to MHC. Interestingly, ageing was found not to alter the relative distribution of the ventricular myosin light chain (MLC) isoforms as well as the thin (actin) filament-associated regulatory proteins (TnT-Tm complex, TnC & TnI). Thus, because the ventricular MHC isoforms were the only myocardial contractile proteins that demonstrated an age-related change in phenotypic expression, Fitzsimons et al. (1999) concluded that the age-related changes in the kinetics of myocardial cross-bridge interaction are due to age-related alterations in MHC expression.

In the present study, similar age-related changes in the contractile protein myosin, as described above by Fitzsimons et al. (1999), would be expected. As previously mentioned, during the present study the nCa value derived from the senescent age group displayed a large variation \((2.81 \pm 2.28)\) within the myocardial segments of the age group. A variation postulated to be due to changes in the myosin heavy chains (MHC) with age, causing the large standard deviation.
5.1.3 Calcium Sensitivity

Unexpected low force outputs were yielded from the Ca\textsuperscript{2+} activating solutions used during the present study, whilst the Sr\textsuperscript{2+} solutions activated the myocardial segments adequately. Two batches of Ca\textsuperscript{2+} solutions were used (termed the 190 and 200 series). The '190' calcium series was used for only 8 (4 young, 1 middle-age and 3 senescent rats) out of a total of 21 animals used during the present study, yielding consistent calcium activated force responses from the myocardial segments. On the other hand, the '200' calcium series activated the myocardial segments poorly, yielding depressed, if any, force responses.

The '200' calcium series solutions were 're-titrated' (see Appendix IV) and were found slightly weaker than the '190' series. However, allowing for this, force responses should have been unaffected, as the difference in Ca\textsuperscript{2+} strength between the '190' and '200' series was minimal ie. the pCa of the '190' solution II (maximum activating) series was 5.17 compared to the pCa of the '200' series 5.21.

Alternatively, it was speculated that perhaps other components, other than Ca\textsuperscript{2+}, could have been mistakenly omitted or added to the solution blend in an incorrect amount eg. ATP or MgCl\textsubscript{2} (see appendix III for correct ingredients list), or that the pH of the calcium solutions was too low. Mg\textsuperscript{2+} and low pH decreases the Ca\textsuperscript{2+} sensitivity of the cardiac myofilaments by decreasing the affinity of troponin C (TnC) to Ca\textsuperscript{2+} (Gao, Backx, Azan-Backx & Marban, 1994). However, by a 'trial-and-error' process, each component of this solution was further added to see if force output improved. No changes were observed. The pH was checked and was within range.

The protocol used by this laboratory to conduct contractile investigations using skeletal muscle, was employed during the present study investigating the contractile properties of rat myocardium. Given the poor results yielded from the contractile investigations during the present study, it is proposed that perhaps the protocol used should be reviewed.

Since the time available to complete laboratory work was limited by the time constraints imposed by the Honours system, the formation and generation of new solutions
standardised for mammalian myocardial tissue activation was beyond the scope of the present study.

5.1.4 Triton X-100

Triton X-100 chemically disintegrates the sarcolemma of cardiomyocytes, rendering them semi-permeable, as well as destroying the integrity of the sarcoplasmic reticulum (SR) and mitochondria (Harrison & Bers; 1989). The results of a study by Gao et al. (1994) found that 'chemical skinning' using Triton X-100 decreases the calcium sensitivity of cardiac myofilaments. The authors postulated that the decrease in calcium sensitivity was due to decreased Ca\textsuperscript{2+} binding to TnC (either by loss of natural calcium sensitisers or inadvertent proteolysis), alterations in MLC phosphorylation, and changes in cross-bridge kinetics as a result of chemical skinning (Gao et al., 1994).

The possibility that the use of 2% Triton X-100 during the present study, was the cause of altered calcium sensitivity in the myocardial segments has not been overlooked. Further investigation into the effects of chemical skinning on cardiomyocytes is warranted, as clearly chemical skinning affects calcium sensitivity of skinned versus intact cardiomyocytes, as demonstrated by Gao et al. (1994).

Another possibility to consider is that the branching nature of cardiomyocytes may impair the ability of various detergent agents eg. Triton X-100, to disintegrate the sarcolemma sufficiently to allow the penetration of the activating solutions to generate force.

5.1.5 Temperature and Calcium Sensitivity

In a study by Harrison & Bers (1989), it was discovered that over the temperature range of 37°C to 1°C there was a progressive decrease in the Ca\textsuperscript{2+} sensitivity of the myofilaments with cooling in chemically skinned rabbit ventricular muscle. The authors attributed much of the difference between the influence of temperature on cardiac and skeletal muscle to the TnC type in the muscle. These conclusions were based on experiments where rat ventricular TnC was extracted and replaced by rabbit skeletal TnC or bovine cardiac TnC (Bers, 1991, p. 26). The results demonstrate an interesting thermodynamic difference between cardiac and skeletal muscle regulation at the level of
TnC, ie. cooling decreases the calcium sensitivity of cardiac myofilaments (Harrison & Bers, 1989), but increases the sensitivity of skeletal myofilaments (Stephenson & Williams, 1981); largely due to the TnC type (Bers, 1991, p. 26).

During the present study, all contractile investigations were conducted at room temperature (21-25°C) using activating Ca\(^{2+}\) and Sr\(^{2+}\) solutions also kept at room temperature. This was done for experimental standardisation and consistency, as any changes in the kinetics of activation with age should manifest at this temperature.

The cardiac tissue was kept in chilled paraffin oil at approximately 4°C until needed. It was essential that the cardiac tissue be kept cool whilst not in use as it deteriorates rapidly when kept at room temperature. The possibility that cardiac tissue deterioration was occurring whilst in use ie. during a series of experimentation at room temperature (21-25°C), using activating Ca\(^{2+}\) and Sr\(^{2+}\) solutions, has not been overruled. Even though it appears that increased temperature increases the calcium sensitivity of cardiac myofilaments (Harrison & Bers, 1989), the likelihood that the room temperature and time taken to perform each experiment, led to cardiac tissue deterioration and hence poor force responses, is still possible.

A short experiment was conducted during the present study to test the possibility that by increasing the temperature of the solutions used, calcium sensitivity may also increase. After warming a few vials of activating calcium solutions in hot tap water to 30°C, a segment of myocardial tissue which was unresponsive in room temperature activating calcium solutions, was passed through the warmed solutions. A larger force output was observed, ie. 2 times greater (see Figure 9), indicating an increased response to calcium. However, it must be borne in mind that the transducer could be responding to the increase in temperature by generating a higher voltage output. A separate series of experiments would need to be devised to test for this and compensate for any electrothermal effects.
5.1.6 Problems Encountered

In addition to problems involving calcium solutions and tissue deterioration, the time taken to complete the contractile investigations took longer than estimated, hence limiting the time available to conduct histological and morphometric analyses.

Approximately 9 hours was needed to conduct contractile experiments on one animal alone. This was due to the complexity of the procedures involved, as well as the length of each protocol. The fact that myocardial tissue could not be successfully stored, either refrigerated or frozen, for short periods of time (i.e. a day or two), made it difficult to derive a large amount of data from the myocardial tissue taken from one animal.

During the present study, myocardial tissue was tested for refrigerated or frozen short-term storage (12-24 hours) in solutions containing the following combinations: solution I (see appendix III) + 50% glycerol, solution I + Triton X-100, solution I + 50% glycerol + Leupeptin, Krebs-Henseleit solution (see appendix I) + 50% glycerol or Krebs-Henseleit solution; as well as refrigerated paraffin oil. However, these tests demonstrated that no force could be generated from myocardial tissue after storage. Deterioration of the myocardial tissue was believed to be the cause.

The laboratory in which the present study was conducted, routinely stores skeletal muscle successfully in solution I + 50% glycerol, either refrigerated or frozen, for up to a year. Force outputs from skeletal muscle stored this way are reported to be just as good as if the tissue were freshly harvested (A. Stewart personal communication).

This laboratory also works with single skeletal muscle fibres. The difficulty in dissecting single cardiomyocytes, given the size and branching nature of cardiomyocytes compared to skeletal muscle fibres, led to the dissection of small myocardial segments containing up to approximately 20 cardiomyocytes.

The protocol for contractile investigations used in the present study was taken from an existing one for single skeletal muscle fibres. It appears that refinement of the skeletal muscle fibre protocol, which better suits the labile nature of myocardial tissue (as
described above), is required. Such refinement was beyond the scope of the present study, but future investigations are warranted.

5.2 HISTOLOGY AND MORPHOMETRY

Interesting results were yielded from the morphometric analysis of the myocardial tissue samples taken from both the left and right sides of the rat heart. Age-related changes in the left side of the heart were of most interest during the present study as the left ventricle is responsible for pushing blood through the large systemic circuit, whereas the right ventricle only needs to push blood a short distance through the pulmonary circuit (Martini, 2001, p. 662). Although the left and right ventricle handle equal volumes of blood, the myocardium of the left ventricular wall is much thicker to enable the development of sufficient pressure to pump blood to the head and body. The myocardium of the right ventricle wall is thinner as it pumps blood at low pressure, approximately 15cm to and from the lungs (Martini, 2001, p. 662).

With the functional differences between the left and right side of the heart in mind, the most intriguing finding during the present study was the fact that in the left papillary muscles, mitochondrial volume significantly decreases with age (P < 0.05), whereas in right papillary muscles, mitochondrial volume increases, although not significantly.

Another interesting observation on the right side of the heart was that the mitochondrial volume of the right ventricle significantly decreased (P < 0.05) between young and middle-age rats with the percentage volume decreasing from 48.6% in young to 40.0% in middle-aged rats. However, between middle-age and senescence, the volume of mitochondria significantly (P < 0.05) increased from 40.0% in middle-age to 49.7% in senescence.

In the left ventricle, no significant changes in mitochondrial volume were observed from across the age range, although mitochondrial volume decreased from 49.8% in young to 45.7% in middle-aged rats, and then increased from 45.7% in middle-aged to 48.7% in senescent rats.
It is speculated that these volume changes are due to age-related changes in mitochondrial numbers and hypertrophy of individual mitochondria. Generally speaking, within the literature, an age-related decrease in mitochondrial numbers is observed concurrently with an age-related increase in mitochondrial size (hypertrophy) (Goldstein et al., 1984; Tauchi & Sato, 1968). These observations have been reported from studies using rat and human hepatocytes (Sastre, Pallardó, Plá, Pellin, Juan, O'Connor, Estrela, Miquel and Vina, 1996; Tauchi and Sato, 1968) and human fibroblasts (Goldstein et al., 1984), although Sastre et al. (1996) did not observe any age related changes in mitochondrial numbers.

Using rat myocardial tissue, Frenzel and Feimann (1984) compared the structural changes in the myocardium of three 6 week old and three 2 year old, female Wistar rats, using samples taken from the side wall of both the left and right ventricle.

5.2.1 Left Side of the Heart

The study by Frenzel and Feimann (1984) observed that in the cardiomyocytes of 2 year old rats, the actual number of mitochondria had significantly increased by 42%, yet the mean size of a mitochondrion had significantly decreased by 36%, leading to a 9% reduction in the volume of mitochondria in the left ventricular wall, compared to 6 week old rats. The volume of myofibrils had increased by 10%, with the actual number of myofibrils significantly increasing by 36% in the cardiomyocytes of the left ventricular wall compared to 6 week old rats (Frenzel & Feimann, 1984).

In support of Frenzel and Feimann's (1984) observations, during the present study, the volume of mitochondria significantly decreased (P < 0.05) by 11.4% between young and middle-aged rats, and by 20.4% between young and senescent rats, in the left papillary muscles. In addition, the volume of cytoplasm significantly increased (P < 0.05) by 6.5% between young and senescent rats, in the left papillary muscles. It is speculated that the observed age-related increase in cytoplasmic volume is related to an increased volume of myofibrils, as myofibrils were counted as ‘cytoplasm’ during the present study.

No significant volume changes were observed in the left ventricle during the present study, unless combined with the data obtained from the left papillary muscles. When
combined, the data from both the left ventricle and left papillary muscles indicate a significant decrease (P < 0.05) in the volume of mitochondria between young and middle-aged rats, with the percentage volume decreasing from 52.2% to 44.4% respectively; and between young and senescent rats, with the percentage volume decreasing from 52.2% to 41.4% respectively. In addition, the volume of cytoplasm significantly increased (P < 0.05) between young and senescent rats, with percentage volume increasing from 30.7% to 40.8%.

5.2.2 Right Side of the Heart

The study by Frenzel and Feimann (1984) observed that in the cardiomyocytes of 2 year old rats, the number of mitochondria had non-significantly increased by 9%, whilst the mean size of a mitochondrion decreased by 11% in the right ventricular wall, compared to 6 week old rats. Mitochondrial volume changes were not recorded for the right ventricle, however it was stated that no significant difference was observed between the mitochondrial volume in the left or right ventricle of young (6 weeks old) compared to aged (2 year old) rats (Frenzel & Feimann, 1984).

During the present study, the volume of mitochondria in the right ventricle significantly decreased (P < 0.05) between young and middle-aged rats, the percentage volume decreasing from 48.6% to 40.0% respectively. However, the volume of mitochondria significantly increased (P < 0.05) between middle-aged and senescent rats, the percentage volume increasing from 40.0% to 49.7% respectively. It is speculated that the decrease in mitochondrial volume seen between young and middle-aged rats is a result of a decrease in actual mitochondrial numbers, and between middle-aged and senescent rats, the remaining mitochondria are speculated to undergo hypertrophy to compensate for the loss of mitochondrial numbers, as previously shown by Goldstein et al. (1984) and Tauchi and Sato (1968).

No significant volume changes were observed during the present study in regard to the right papillary muscles alone, although a non-significant increase in mitochondrial volume was observed between young and senescent rats, the percentage volume increasing from 43.9% to 46.0% respectively. However, when combined, the data from both the right ventricle and right papillary muscles indicated a significant decrease (P < 0.05) in
mitochondrial volume between young and middle-aged rats, the percentage volume decreasing from 46.3% to 41.8% respectively. Also a significant increase (P < 0.05) in mitochondrial volume between middle-aged and senescent rats, the percentage volume increasing from 41.8% to 47.9% respectively. Once again it is speculated that between young and middle-aged animals, the decrease in mitochondrial volume reflects a decrease in mitochondrial numbers; and between middle-aged and senescent animals, the increase in mitochondrial volume is due to hypertrophy of the remaining mitochondria to compensate for the loss of mitochondrial numbers.

Frenzel and Feimann (1984) observed that the volume of myofibrils in 2 year old rats had increased by 4%, with the actual number of myofibrils increasing by 12% in the right ventricular wall compared to 6 week old rats. In support of these results, during the present study, when combined, the data from both the right ventricle and the right papillary muscles indicated a significant increase (P < 0.05) in the volume of cytoplasm between young and middle-aged rats, the percentage volume increasing from 33.0% to 36.9% respectively. Once again, since myofibrils were counted as ‘cytoplasm’ during the present study, it is speculated that the age-related increased volume of cytoplasm is due to an increased volume of myofibrils with age.

Additionally, in the right ventricle alone and in the combined right ventricle and right papillary data, a significant decrease (P < 0.05) in ‘other’ structures was observed between middle-aged and senescent rats. The percentage volume in the right ventricle alone decreased from 21.3% to 12.4% between the middle-aged and senescent rats respectively; and in the combined right ventricle and right papillary muscles, decreased from 19.4% to 15.0% between the middle-aged and senescent rats respectively. The ‘other’ structures included interstitial space, vascular space and connective tissue. In the study by Frenzel and Feimann (1984) the interstitial space alone increased significantly by 52% in the right ventricle of 2 year old rats. The authors report that the widening of the interstitial space in 2 year old rats was due an age-related increase in collagen fibres, as well as oedema (cytoplasmic) and the presence of blood capillaries. Since the ‘other’ structures were found to decrease with age in the present study, it could be speculated that cardiomyocyte hypertrophy is occurring in the right ventricle.
alone and in the combined right ventricle and papillary muscles, resulting in an age-related decrease in interstitial space, vascular space and connective tissue.

Frenzel and Feimann (1984) summarise that in the cardiomyocytes of aged (2 year old) rats, the quantitative alterations are more obvious in the more stressed left ventricular wall than in the right ventricular wall. Also that their findings signify a close relationship between the number of mitochondria versus myofibrils in cardiomyocytes, postulating that a reduced mitochondria/myofibril ratio may contribute to the diminished functional adaptability seen in the ageing heart (Frenzel & Feimann, 1984).

As previously mentioned in the literature review, age-related morphological changes associated with the myocardium are dominated by left ventricular wall hypertrophy, caused by increased individual cardiomyocyte size (Macdonald & O’Rourke, 1998; Safar, 1990; Svanborg, 1997). Cardiomyocyte hypertrophy is an adaptive response to the age-related increased loss of cardiomyocytes (Anversa et al. 1990; Macdonald & O’Rourke, 1998; Svanborg, 1997) through necrosis (Pollack & Leewenburgh, 2000) or continuous apoptosis (Macdonald & O’Rourke, 1998; Pollack & Leewenburgh, 2000).

It could be speculated that the age-related increase in myofibrillar numbers observed by Frenzel and Feimann (1984) in the left ventricular myocytes, is the cause of cardiomyocyte hypertrophy. Indeed Fraticelli et al. (1989) attribute the structural basis for the age-related hypertrophy of cardiomyocytes to the addition of morphologically similar sarcomeres. The age-related diminution of the mitochondria/myofibril ratio is implicated in the cause of the diminished functional adaptability seen in the ageing heart (Frenzel & Feimann, 1984).

During the present study, determining age-related changes in cardiomyocyte mitochondrial numbers and structure, as well as changes in the myofibrillar composition, using electron microscopic (EM) techniques, would have provided invaluable information and support for the present study’s findings. Unfortunately the use of EM techniques was beyond the scope of the present study, given the time constraints.

However, if age-related changes in mitochondrial numbers could have been determined during the present study, direct correlations could have been made between the number
and volume of mitochondria, and the number and volume of myofibrils, in relation to age-related changes in the efficiency of myocardial contractility. Indeed if more significant data were yielded from the contractile investigations, a direct correlation between age-related changes in cardiomyocyte function and structure could have been made.

As previously mentioned, a significant ($P < 0.05$) age-related decrease in mitochondrial volume was observed between young and middle-aged, and between young and senescent rats, in the left papillary muscles. This finding is speculated to be related to an age-related decrease in mitochondrial numbers and an increase in myofibril numbers and/or volume, as a significant increase ($P < 0.05$) in cytoplasm was also observed between young and senescent rats in the left papillary muscles. If the ratio of mitochondria to myofibrils decreases, it is speculated that a decrease in the ability of the cardiomyocytes to contract would be observed.

An adequate supply of ATP to the myofibrils is required for adequate contraction of the cardiomyocytes. Since approximately 95% of the energy required for contraction, is produced by the mitochondria (Frenzel & Feimann, 1984), age-related changes in mitochondrial volume, numbers, functional ability and ratio to myofibrils, directly affect the ability of the myofibrils to contract.

In the aged myocardium an abundance of mtDNA mutations and deletions have been described, which could affect mtDNA transcription, resulting in deficient synthesis of the mtDNA encoded OXPHOS polypeptides (Andreu et al., 1998; Hattori et al., 1991; Ozawa, 1997; Takasawa et al., 1993; Wallace, 1999). These mutations and deletions of mtDNA are postulated to be an important contributor to human ageing (Hattori et al., 1991; Ozawa, 1997). It is speculated that the damaged mitochondria may accumulate in cardiomyocytes, appearing at light microscope level to be intact structurally, however functionally inefficient. Hence, whilst it appears that mitochondrial numbers and/or volume may increase with age, perhaps not all of these mitochondria are functioning efficiently, leading to decreased ATP production and compromised contractile ability of the myocardium.
5.3 Combined Conclusions

The purpose of this project was to gather, analyse and compare data attained from both techniques i.e. the contractile investigations and the morphometric analyses, to ascertain whether a relationship can be established between contractility and volume of mitochondria in cardiomyocytes with ageing. A causal relationship can be inferred, as it is known that cardiac function decreases with age. With mitochondria removed as the energy source, it was found that cardiomyocytes could generate the same contractile force, regardless of age, while mitochondrial volume decreased with age in the functionally critical left side of the heart. That cardiomyocytes can generate this force across the age spectrum (when mitochondrial energy is not used) may be as a result of the increased cytoplasm observed in this present study, which probably indicates and increase in the levels of contractile tissue, as observed by Frenzel and Feimann (1984).

During the contractile investigations, minimal differences were observed in the kinetics of activation using exogenous Ca\(^{2+}\) and Sr\(^{2+}\) across the age spectrum. Significant (P < 0.05) changes in the threshold of activation to Sr\(^{2+}\) were observed between young and middle-aged rats and between young and senescent rats, however not between middle-aged and senescent rats. Since changes in strontium sensitivity denote changes in the regulatory proteins of striated muscle (Stephenson et al., 1994), the age-related changes in threshold of activation to Sr\(^{2+}\), are speculated to be due to functional changes in myocardial troponin C (TnC) that may be occurring early in life, remaining unchanged throughout normal ageing.

In comparison, morphometric analyses on the myocardial tissue samples from across the age range indicated significant (P < 0.05) changes in the volume of various structures, particularly mitochondria, across all the age groups. Hence, in terms of the energy needed to drive myocardial contraction, changes in mitochondrial volumes were of great importance.

The contractile and regulatory proteins of striated muscle do not work in isolation, but are controlled and influenced by the electrical, mechanical and biochemical events that are part of muscle contraction (Stephenson, 1988). During the present study, it was hoped that age-related changes in the volume of mitochondria would correlate with a
significant difference in the kinetics of activation of the contractile apparatus driving muscle contraction. The experimental outcomes of the present study did not support this supposition, although it was clear that there was both mitochondrial and some functional changes occurring with age.

The contractile investigations provided a means of determining age-related changes to the contractile machinery in isolation, without the influence of the mitochondria and sarcoplasmic reticulum (SR), which are destroyed with chemical skinning (Harrison & Bers, 1989). In place of the mitochondria and SR, the activating solutions contained ATP and calcium (or strontium) to drive contraction. Since minimal differences were observed in the kinetics of activation of the isolated contractile proteins during the present study, it could be speculated that reported age-related changes in myocardial function ie. prolonged contraction and relaxation time (Andreu et al., 1998; Fraticelli et al., 1989; Hattori et al., 1991; Lakatta, 1993; Muscari et al., 1992; Paradies et al., 1994) are related to changes in mitochondria.

During the present study, the fact that minimal differences were observed during the contractile investigations indicates that perhaps the contractile apparatus changes very little with age. In contrast, a significant change in the volume of mitochondria and cytoplasm was observed across the age range in both the left and right side of the heart. These results support the reviewed literature that implicate mitochondria as an important contributor to myocardial (Hattori et al., 1991; Hayakawa et al., 1993; Ozawa, 1997; Wallace, 1992), and overall human ageing (Castellucio et al., 1994; Hattori et al., 1991).

Mitochondria provide the principle source of energy, in the form of adenosine triphosphate (ATP), via the process of oxidative phosphorylation (OXPHOS), for several organs, including the myocardium (Wallace, 1992). As previously mentioned, in the myocardium OXPHOS declines with age (Wallace, 1992) and mtDNA mutations increase (Hayakawa et al., 1993).

According to Kopsidas, Kovalenko, Heffernan, Yarovaya, Kramarova, Stojanovski, Borg, Islam, Caragounis & Linnane (2000), the cause of the age-related decline in the bioenergetic capacity of a cell or tissue, particularly in postmitotic tissues, is hypothesised to be due to mtDNA damage and mutation accumulation that may occur.
progressively with age until reaching a threshold level where the accumulations impinge on the bioenergetic capacity of the cell or tissue, often leading to cell death.

The role of mitochondria in cell death via apoptosis is widely acknowledged (Brown, 2000a & b; Cooper & Halestrap, 2000; Halestrap, 2000; Pollack & Leewenburgh, 2000; Wallace, 1999), as reviewed in the above literature review. Clearly mitochondria are involved in the overall physiological decline associated with advancing age, hence explaining why mitochondria were the focus of the present ageing related study. The question remains, to what extent are mitochondria involved.

The animals used during the present study were fed ad libitum, that is, they had free access to food and water whenever desired. According to Sohal and Weindruch (1996), "Ad libitum feeding of laboratory rodents promotes obesity and an earlier appearance of age associated pathologies" (p. 61). Calorie restriction (CR), without malnutrition, extends the maximum lifespan (MLS) of rodents by approximately 40% (Harman, 1998; Pollack & Leewenburgh, 1999; Sohal & Weindruch, 1996). The mechanism by which MLS is increased by CR is postulated to be due to a reduction in oxygen consumption with a simultaneous reduction in metabolic rate and body temperature, which lowered the known age-related increase in chronic oxidative stress (Pollack & Leewenburgh, 1999; Sastre et al., 1996; Sohal & Weindruch, 1996). Additionally, CR retards a variety of age-related detrimental biochemical and physiological changes, eg. diabetes and cancer development (Pollack & Leewenburgh, 1999).

Since the rats used in the present study were fed ad libitum, there is the possibility that this led to an age-related increase in chronic oxidative stress, and therefore increased free radical production and possible damage to mitochondria, particularly mitochondrial membranes (Sohal & Weindruch, 1996), which could have affected the volume of mitochondria.

Although these effects could not be monitored during the present study, as electron microscopic (EM) techniques are required to investigate such structural changes to mitochondria, a future study using EM techniques to investigate age-related changes in mitochondrial morphology, using two populations of rats, one population fed ad libitum
the other calorie restricted, is warranted. Concurrently, the effects of CR on MLS could also be investigated.

During the present study, obtaining senescent rats proved to be somewhat difficult, as it is highly likely that rats obtained from various breeding centres are fed ad libitum from birth and hence, this may reduce their MLS, leading to decreased populations of senescent rats ie. 2-2.5 years and older, available for such research. Ideally, breeding a population of rats to be fed a CR diet from birth would be the best solution, especially during a study such as the present, which seeks to investigate the effects of ageing on various physiological functions.

6 Future Research

Although beyond the scope of the present study, many interesting research possibilities arose that would warrant further investigation. Particularly an investigation to determine any sex differences that may alter the effects of ageing on the contractility and mitochondrial volume of myocardial tissue. To the experimenter's knowledge, the literature has not reported any sex-related differences in the function and structure of the myocardium with ageing in any species.

6.1 Contractile Investigations

The protocol for contractile investigations used during the present study was found to be limited for use on myocardial tissue. This protocol works successfully on skeletal muscle fibres, however, it was suspected that some myocardial preparations did not activate maximally.

Future work is needed to refine such protocols, especially regarding the effects of chemical skinning on the cardiac myofilaments, given the branching nature of cardiomyocytes. Also, the possibility that contractile apparatus proteins ie. TnC, are compromised by chemical skinning. (Gao et al., 1994), merits further investigation. It would be possible to conduct experiments using myocardial tissue, chemically skinned in various detergents eg. Triton X-100 or saponin, at various concentrations whilst monitoring force output. Such 'trial-and-error' experiments would determine the best
detergent to be used for the chemical skinning of cardiomyocytes, at the appropriate concentration and exposure time, to yield the best force output.

Further, the use of activating Ca$^{2+}$ and Sr$^{2+}$ solutions in concentrations appropriate for myocardial tissue requires further appraisal, to formulate a protocol that will generate maximal activation with minimal inhibitory effects.

Investigating the effect of temperature on the calcium sensitivity of cardiac myofilaments is another interesting aspect worth further study, given that the calcium sensitivity of cardiac myofilaments has been reported to decrease with decreasing temperature (Harrison & Bers 1989).

Additionally, the use of genetic techniques ie. PCR, as used by Fitzsimons et al. (1999), would be extremely useful for determining age-related changes in the composition of the contractile and regulatory proteins in the myofibrils. With this information, age-related contractile changes could be directly correlated with changes in the composition of the contractile and regulatory proteins.

Unfortunately, with the time constraints imposed, it was not possible to pursue these and other interesting observations further.

6.2 HISTOLOGICAL AND MORPHOLOGICAL INVESTIGATIONS

The use of electron microscopy would also increase the accuracy in determining mitochondrial volumes and profiles, as well as demonstrating structural alterations in mitochondrial morphology that are reported to occur with age (Goldstein et al., 1984; Bittles, 1989). Unfortunately the use of electron microscopic techniques was beyond the scope of the present study, in fact using EM techniques to investigate age related changes to mitochondrial numbers and morphology would constitute an Honours project on its own.
6.3 Other avenues of future research

Investigations into the extent of mtDNA accumulation in myocardial tissue with age, using genetic techniques i.e. PCR, and the physiological effects of myocardial tissue occupied by mutated mtDNA genomes, would be extremely useful, especially since mitochondria are so heavily implicated in cellular and overall ageing, as reviewed previously.

Another avenue for future investigation relates to calorie restriction (CR) and its effects on maximum lifespan (MLS) and chronic oxidative stress in laboratory rodents, as it is known that CR increases MLS and decreases age-related increases in oxidative stress (Pollack & Leewenburgh, 1999; Sohal & Weindruch, 1996).

The study could compare a population of rodents fed ad libitum to a population fed a CR diet, without malnutrition, from birth. MLS could be investigated, along with free radical production and the consequent structural changes to mitochondria.

The use of electron microscopic techniques to monitor changes in mitochondrial morphology would be useful in such a study, as oxidative stress leads to increased free radical production which damages mitochondrial membranes (Sohal & Weindruch, 1996), therefore altering mitochondrial functioning and contributing to the overall effects of ageing (see literature review).
REFERENCES


8 APPENDICES
8.1 APPENDIX I

8.1.1 Krebs-Henseleit Solution

(mMol/L)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>59 mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>14.2 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.38 mM</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.9 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.59 mM</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>10 mM</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.01 u/ml</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>25.4 ml of 100 mM</td>
</tr>
</tbody>
</table>
### 8.2 Appendices II

#### 8.2.1 Karnovsky’s Fixative

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0.2 M</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>10%</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>25%</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>to desired amount</td>
</tr>
</tbody>
</table>
### 8.3 APPENDIX III

#### 8.3.1 Activating and Relaxing Solutions

(Series 190 & 200 Calcium)  
(Series MA & NA Strontium)

<table>
<thead>
<tr>
<th></th>
<th>Solution I (high-relaxing)</th>
<th>Solution II (activating)</th>
<th>Solution III (low-relaxing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>60 mM</td>
<td>60 mM</td>
<td>60 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1 mM</td>
<td>1 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>HDTA</td>
<td>-</td>
<td>-</td>
<td>50 mM</td>
</tr>
<tr>
<td>EGTA</td>
<td>50 mM</td>
<td>50 mM</td>
<td>-</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>1 mM</td>
<td>1 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>Caffeine</td>
<td>10 mM</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>ATP</td>
<td>8 mM</td>
<td>8 mM</td>
<td>8 mM</td>
</tr>
<tr>
<td>CaCl₂ (or SrCl₂)</td>
<td>-</td>
<td>48.5 mM (36.0 mM)</td>
<td>-</td>
</tr>
</tbody>
</table>

**Abbreviations**

- **HEPES** = N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulphonic acid]
- **HDTA** = 1,6-Diaminohexane-N,N,N',N-tetraacetic acid
- **EGTA** = Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N-tetraacetic acid
- **ATP** = Adenosine 5’-triphosphate (Hill et al., 1996).
9  BUDGET

Animal costs:

Wistar Rats: $368-

Consumables including:

Chemicals, buffer, enzymes, Ringer chemicals: $500-
Surgical Instruments: $100-
Bedding and food for animals: $200-

TOTAL: $1,168-