THE ANTIOXIDANT PROPERTIES OF MELATONIN DURING CHEMICAL HYPOXIA AND REOXYGENATION INJURY IN RAT VENTRICULAR MYOCYTES

by

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PREFACE

I declare that the work described in this thesis: **The antioxidant properties of melatonin during chemical hypoxia and reoxygenation injury in rat ventricular myocytes**, is my own and was carried out at the MRC in the Experimental Biology Programme under the supervision of Prof. Amanda Lochner. All the sources I have used or quoted have been indicated and acknowledged by means of complete references.

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RUDUWAAN SALIE

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ABSTRACT

It is well known that ischaemia / chemical hypoxia followed by reperfusion is not always accompanied by recovery of myocardial function and often produces further cell damage (Hearse et al., 1973). This phenomenon commonly referred to as the oxygen paradox (Hearse et al., 1973), is usually characterised by extensive tissue damage and the formation of oxygen free radicals / reactive oxygen species (ROS).

As the work on free radicals progressed it became apparent that there is a fine balance between cellular systems that produce free radicals and those that sustain their tolerable levels, to maintain normal cell function (Ferrari et al., 1990). The relative importance of free radicals in the production of irreversible cell damage, as well as the mechanisms involved are not fully understood.

Using the rat model of myocardial infarction followed by reperfusion, many investigators employed various antioxidants / free radical scavengers in various combinations at different stages of hypoxia / reoxygenation with the intention of reducing free radical mediated damage (Meerson et al., 1981; Shlafer et al., 1982; Stewart et al., 1983; Hess et al., 1983; McCord and Fridovich, 1973). There is also an increasing interest in defining new antioxidants of high potency, low toxicity and good solubility properties in aqueous as well as organic phases.

Recently it has been shown that melatonin (n-acetyl-5-methoxytryptamine) has the ability to directly scavenge free radicals (Reiter, 1993).

It was therefore the aim of this study, to investigate oxidative stress during chemical hypoxia and reoxygenation in isolated rat ventricular myocytes and to study the antioxidant effects of melatonin, employed at different stages of chemical hypoxia and / or reoxygenation.

To reveal the phenomenon of oxidative stress using laser scanning confocal microscopy, myocytes were preloaded with tetramethylrhodamine (TMRM) together with one of the following fluorophores:

(i) dihydrochlorofluorescein diacetate (DCDHF) or dihydrorhodamine123 (DHR) to illustrate the formation of H_2O_2 and / or ROS; or

(ii) fluo-3, AM (fluo) to show changes in intracellular free calcium.

In superfused myocytes, chemical hypoxia was induced for 12.5 min by the addition of 1.5 mM KCN and 20 mM deoxyglucose to the superfusion buffer followed by 0, 1.5 or 15 min reoxygenation. Cells were also exposed to 27.5 min chemical hypoxia without reoxygenation. Melatonin (50 μM) was added at different time intervals during the experimental protocol.

All the myocytes (untreated and melatonin treated), indicated increased DCDHF but not DHR or fluo fluorescence under control oxygenated conditions (normoxia). This probably implicate normal physiological levels of H_2O_2 and / or ROS and presumably an intact endogenous antioxidant system, since none of the cells suffered any damage. It was also indicated that melatonin had no effect on H_2O_2 and / or ROS formation and the viability of any of the cells during normoxia.

Exposure of myocytes to chemical hypoxia for 12.5 min caused contracture and damage to 70 - 89 % of cells. This was associated with the generation of H_2O_2 and / or ROS, an increase in intracellular calcium but maintenance of membrane potential. Early reoxygenation did not exacerbate or improve the changes observed during chemical hypoxia. Melatonin protected myocytes from hypoxic induced damage and increased cell viability from 20 to 80 % ($p<0.05$).

Melatonin treated cells had reduced levels of ROS and intracellular calcium. The protection conferred by melatonin during exposure of cells to 12.5 min chemical hypoxia disappears if chemical hypoxia is prolonged to 27.5 min . After 27.5 min chemical hypoxia > 85 % of cells showed marked morphological changes and were considered to be irreversibly damaged.

Myocytes indicated increased DCDHF; DHR and fluo fluorescence, implicating increased H_2O_2 and / or ROS formation and increased intracellular calcium respectively, after 12.5 min chemical hypoxia. However, the application of melatonin, effectively scavenged H_2O_2 and / or ROS and protected cells only for the first 14.5 min of chemical hypoxia, after which all of the cells indicated increased H_2O_2 and / or ROS formation, increased intracellular calcium and suffered irreversible damage. Presumably, as chemical hypoxia progressed, the accumulation of H_2O_2, the formation of ROS and degradation of endogenous antioxidant enzymes transpired, which resulted in damage to the majority of cells by the end of this period.

CHAPTER 1

INTRODUCTION

1.1 *Introduction to oxygen toxicity*

All life forms are equipped to exist in their own well - defined oxygen environment. Lower oxygen tensions limit aerobic energy metabolism whereas higher values may cause oxygen toxicity. Mammalian cells require oxygen to sustain life but have to be equipped with those systems that rid the cell of oxygen - derived byproducts to ensure normal cell function and survival.

In 1969 McCord and Fridovich identified the enzyme superoxide dismutase that catalyses the production of hydrogen peroxide from the superoxide anion. They further observed that all mammalian cells contain this unique enzyme. These basic observations defined the new field of oxygen metabolism in biological systems and a potential role of oxidation / reduction reactions in both physiological and pathological conditions.

The byproducts of oxygen metabolism commonly referred to as oxygen derived free radicals / reactive oxygen species (ROS), and their metabolites have received much attention because they are known to play an important role in many biochemical reactions, which maintain or effect normal cell functions.

These free radicals are normally inactivated by endogenous scavenging systems; however the generation of large amounts of oxygen free radicals can overwhelm cellular defenses and induce tissue damage. Such tissue damage includes amongst others injury associated with inflammatory responses (Kuehl et al., 1979; Pryor, 1982), ischaemic - reperfusion injuries (Fridovich, 1978; McCord and Roy, 1982), and injuries resulting from intracellular metabolism of chemical and physical agents, such as drugs and radiation (Estabrook and Werringloer, 1977).

Oxygen - derived free radical involvement in ischaemic injury, have been hypothesised by Fridovich in 1978 and the evidence of many recent studies have suggested that oxygen – derived free radicals may be abundantly produced in ischaemic tissue, accounting for at least part of the damage that results (McCord et al., 1985).

Over the past decade, it has been shown in numerous studies have shown that reperfusion / reoxygenation after a period of ischaemia, is associated with the formation of large amounts oxygen free radicals. This phenomenon is accompanied by specific biochemical abnormalities (Zweier et al., 1987) and morphological changes associated with cell necrosis (frequently referred to as myocardial reperfusion injury). The damaged cells referred to during reperfusion injury are characterised by their square shape, contracture bands, slight myofibrillar disarray with blebs but with an intact membrane potential, which indicate that the cells are still viable.

Several significant observations have stimulated interest in developing sound approaches toward limiting the extent of irreversible tissue injury associated with ischaemia or ischaemia followed by reperfusion (Lucchesi, 1997).

Molecular oxygen, although essential for maintaining cell viability, is one of the primary factors involved in reperfusion injury. An early indication that molecular oxygen is involved in the development of reperfusion injury was provided by the observation that reperfusion of the anoxic heart with an oxygenated solution enhanced myocardial injury, while a solution relatively devoid of oxygen did not increase the extent of tissue injury (Hearse et al., 1975, Lucchesi , 1997).

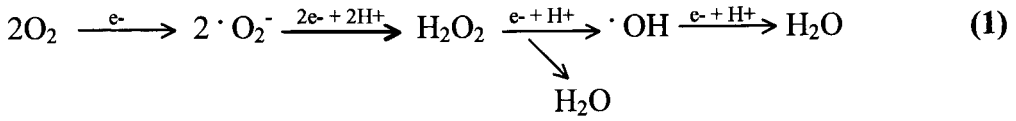
1.2 *The oxygen free radical system*

A free radical is defined as any chemical species that has one or more unpaired electrons. The unpaired electron in the free radical is conventionally represented by a superscript dot: $R^{\cdot}$

Molecular oxygen is relatively non - reactive because of its unusual structure: it is a paramagnetic biradical containing two unpaired electrons with parallel electron spin. The majority of organic compounds that may react with oxygen contains paired electrons. The insertion of two paired electrons into a molecule of oxygen would violate the rules of quantum mechanics (Thompson and Hess, 1986).

Incoming electrons prefer to enter the orbitals one at a time. This results in the formation of hydrogen peroxide (H_2O_2) or oxygen free radicals. The reduction of oxygen to water in normal living tissues can proceed by one of two pathways. Mitochondrial cytochrome oxidases reduces 95 % of oxygen to water by tetravalent reduction without the formation of any intermediates (Fridovich, 1978).

The remaining 5 % of oxygen is reduced by the univalent pathway in which several intermediates are produced:



(1)

· O_2^- = superoxide anion
H_2O_2 = hydrogen peroxide
· OH = hydroxyl radical

With the acceptance of the first electron, the superoxide anion (· O_2^-) is formed. The superoxide anion is the best studied free radical species. This free radical may act as a reducing agent donating its electrons or as an oxidizing agent in which case it is reduced to hydrogen peroxide.

In aqueous environments, · O_2^- is in equilibrium with its protonated form, · HO_2 . Since the pKa for this equilibrium reaction is 4.8, · O_2^- is the dominant species at neutral pH. However, relatively high concentrations of · HO_2 are favoured in acidic environments (such as ischaemia) (Ferrari et al., 1990).

Although · O_2^- is relatively non-reactive, several of its derivative compounds (including · HO_2) are capable of oxidising organic molecules such as polyunsaturated fatty acids of membrane phospholipids to form alkoxy radicals (RO ·) (Thomas et al., 1978). This reaction can be prevented in normal biological tissue by dismutation to hydrogen peroxide.

When the superoxide ($\cdot O_2^-$) and its protonated form ($\cdot HO_2$) approach equal molar concentrations, spontaneous dismutation occurs, and H_2O_2 is generated (Thompson and Hess, 1986). H_2O_2 can also form as a direct result of a double reduction of molecular oxygen (Thompson and Hess, 1986).

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad \textbf{(2)}$$

Superoxide dismutase (SOD) is present in varying concentrations in eukaryotic and prokaryotic cells. This enzyme, in the presence of normal intracellular concentrations of catalase and glutathione peroxidase, is responsible for scavenging oxygen free radicals, thereby serving as normal biological defenses against the formation and accumulation of reduced oxygen intermediates (see antioxidant mechanisms, page 19).

The H_2O_2 formed under these circumstances is catalytically reduced in the cell to H_2O by catalase (Equation 3B) or glutathione peroxidase (Equation 3C), the latter predominates in cardiac muscle.

$$2\cdot O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad \textbf{(3A)}$$

$$2H_2O_2 \xrightarrow{catalase} O_2 + 2H_2O \qquad \textbf{(3B)}$$

$$H_2O_2 + 2GSH \xrightarrow{glutathione\ peroxidase} GSSG + 2H_2O \qquad \textbf{(3C)}$$

These systems (Equation 3B and 3C) constitute the major intracellular hydrogen peroxide decomposition systems.

Although H_2O_2 may be a strong oxidant, it reacts slowly with most organic substrates. The major danger of H_2O_2 accumulation is the production of hydroxyl radical ($\cdot OH$) by the Haber - Weiss reaction (Equation 4A) or Fenton reactions (Equation 4B) (Thompson and Hess, 1986; Hess and Manson, 1984).

$$Me^{n+}\ \text{chelate} + \cdot O_2^- \longrightarrow Me^{(n-1)+}\ \text{chelate} + O_2 \qquad \textbf{(4A)}$$

$$Me^{(n-1)+}\ \text{chelate} + H_2O_2 \longrightarrow Me^{+}\ \text{chelate} + OH^- + \cdot OH$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + \cdot OH + OH^-$$

$$\cdot OH + H_2O_2 \longrightarrow H_2O + \cdot O_2^- + H^+$$

$$\cdot O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + \cdot OH \qquad \textbf{(4B)}$$

$$Fe^{2+} + \cdot OH \longrightarrow Fe^{3+} + OH^-$$

The potential for generating hydroxyl radicals (Equations 4A and 4B) is very real, but under normal physiological conditions, hydroxyl radicals would not be formed (Thompson and Hess, 1986).

Since $\cdot OH$ is a trivalent reduction product of O_2, the generation thereof, requires both $\cdot O_2^-$, which is normally scavenged by superoxide dismutase and H_2O_2, which is scavenged by the catalase / peroxidase system. Therefore large concentrations of hydroxyl radicals do not exist under physiologic conditions.

Since there is no physiologic defense or enzyme system that can scavenge excessive quantities of hydroxyl radicals (Hess and Manson, 1984; Thompson and Hess, 1986), tissue destruction would be extensive in pathological conditions known to generate hydroxyl radicals (Thompson and Hess, 1986).

The hydroxyl radical is a very reactive and unstable oxidising species that reacts with a large variety of organic compounds and biomembranes (Thompson and Hess, 1986). This extremely reactive species, has the ability to damage and degrade proteins, DNA, polysaccharides and can induce peroxidation of cell membrane lipids (Halliwell and Gutteridge, 1984).

Other oxygen - derived reactive metabolites that have been identified in biological systems include singlet oxygen (Fee and Valentine, 1977), hypohalous acids and a variety of n - chloramine compounds. Singlet oxygen has been suggested to be produced either during dismutation of $\cdot O_2^-$ or during an iron - catalaysed Haber - Weiss reaction.

The hydroxyl radical can also be formed in living cells as a result of ionising radiation (Hutchinson, 1966) or as a result of drugs such as adriamycin and alloxan (Estabrook and Werringloer, 1977).

1.3 *Biological sources of free radical formation*

As stated previously, the mitochondrial cytochrome oxidase catalyses the reduction of 95 % of oxygen to water by the tetravalent pathway without the production of oxygen intermediates, while several intermediates are produced from the remaining 5 % of oxygen.

1.3.1 *Xanthine oxidase*

This enzyme was the first documented biological source of the superoxide radical (McCord and Fridovich, 1968) and has been the subject of intensive study. It is widely distributed among tissues; the intestine, lung and liver are particularly rich sources in most species. The enzyme is synthesised as xanthine dehydrogenase (type D). This form appears to account for about 90 % of the total activity in healthy tissue (Roy and McCord, 1983). The dehydrogenase does not transfer electrons to molecular oxygen to form hydrogen peroxide or superoxide, but reduces NAD^+ (nicotinamide adenine dinucleotide), as follows:

$$\text{Xanthine} + H_2O + NAD^+ \xrightarrow{\text{Type D}} \text{uric acid} + NADH + H^+ \qquad \textbf{(5A)}$$

It has been illustrated in the rat heart (Roy and McCord, 1983) and myocardial mitochondria (Van Jaarsveld et al., 1988) that even brief episodes of ischaemia and reperfusion can cause the conversion of xanthine dehydrogenase (type D) to xanthine oxidase (type O). The oxidase can use molecular oxygen instead of NAD^+ to produce superoxide as follows:

$$\text{Xanthine} + H_2O + 2O_2 \xrightarrow{\text{Type O}} \text{uric acid} + 2\,{}^{\cdot}O_2^- + 2H^+ \qquad \textbf{(5B)}$$

Although it had been known for some time that this conversion was possible, early investigations produced no data suggesting that the process could occur in vivo or has pathophysiologic importance. Roy and McCord in 1983 have found that the conversion of xanthine dehydrogenase to xanthine oxidase does, in fact, occur in vivo in ischaemic tissue. What causes the conversion of type D activity to type O activity in vivo ? It was hypothesised that the process begins when the decrease in blood flow to a tissue is sufficient to limit oxygen availability for the production of ATP (McCord, 1985). As cellular energy charge drops, it is no longer able to maintain proper ion gradients across its membranes, and this precipitates a redistribution of calcium ions (McCord, 1985).

The elevated cytosolic calcium concentration activates a protease capable of converting the dehydrogenase to the oxidase (McCord, 1985). Concomitantly, the depletion of cellular ATP results in an elevated concentration of AMP which is catabolised to adenosine, inosine, and finally hypoxanthine.

The buildup of hypoxanthine in the ischaemic myocardium has been demonstrated (DeWall et al., 1971). Hypoxanthine, as well as xanthine, serves as an oxidisable purine substrate for xanthine dehydrogenase or xanthine oxidase. Hence, during ischaemia two important changes occur in tissue: a new enzyme activity appears, along with one of its two substrates. Molecular oxygen is the remaining substrate required for type O activity and is supplied during the reperfusion of tissue.

With it comes the burst of hydrogen peroxide and superoxide formation (McCord, 1985). This sequence of events is illustrated in the diagram below.

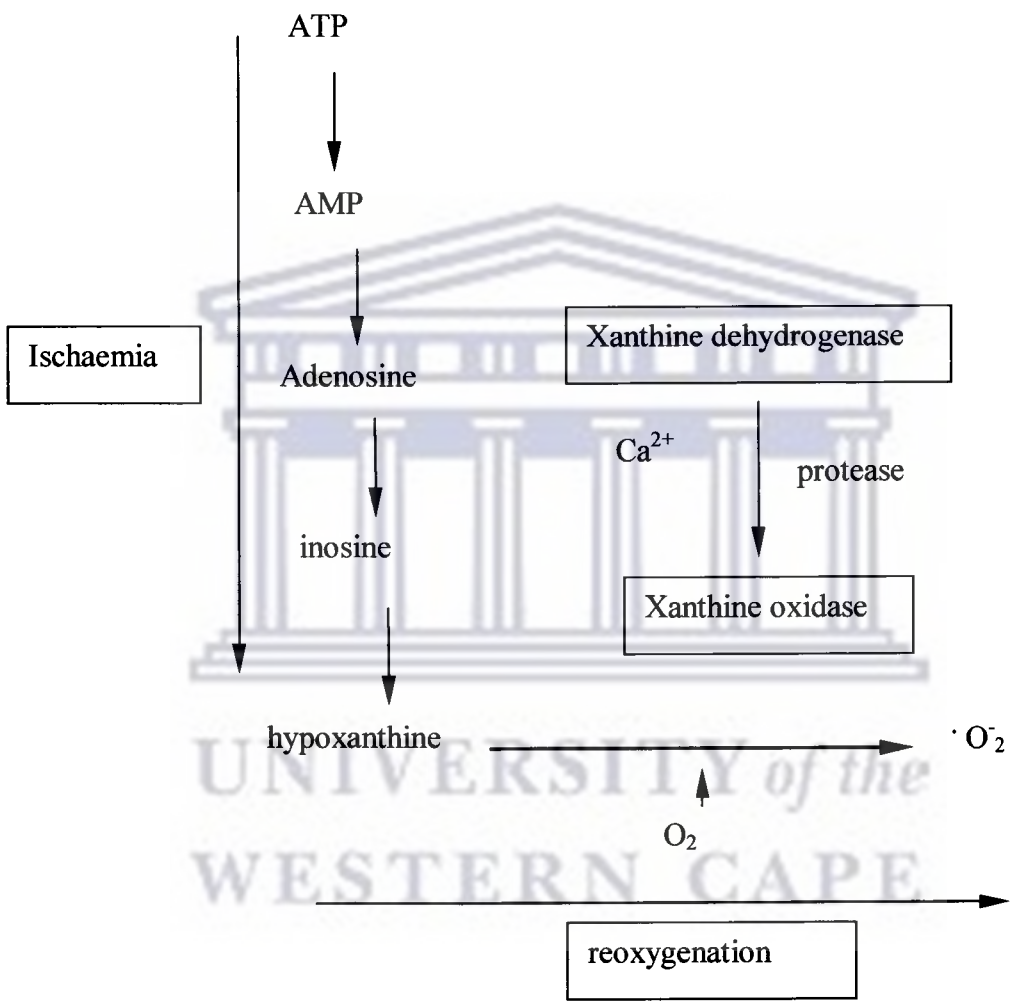


Fig. 1: Proposed mechanism for ischaemia - induced production of superoxide.

In normal rat ileum about 90 % of total enzyme activity is present as xanthine dehydrogenase. If the tissue is completely ischaemic for even a short period , there is a rapid increase in the amount of type O activity, with a corresponding decrease in the activity of type D. Similar but slower conversion occurs in other tissues (Roy and McCord, 1983). In the heart the oxidase content doubles after about eight minutes of nonperfusion, whereas in the liver, spleen, lungs and kidney the same increase requires about 30 min. However, the rabbit, pig and the human heart have little, if any, xanthine oxidase activity (Roy and McCord, 1983; Eddy et al., 1987).

Skeletal muscle is unique among the tissues examined in that its xanthine dehydrogenase is measurable but does not convert to type O upon nonperfusion. This observation correlates well with the clinically observable resistance of skeletal muscle to ischaemic injury, relative to other tissues (McCord, 1985).

The distribution of xanthine dehydrogenase / xanthine oxidase among various tissues and species shows that the enzyme activity varies widely and, indeed certain species show no activity (McCord, 1985).

1.3.2 *Mitochondria (electron transport chain)*

The mitochondrial electron transport system is undoubtedly, the most important site of free radical production under physiological conditions (Ferrari et al., 1990).

A small percentage of the oxygen utilised by intact, aerobic mitochondria is partially reduced by electrons which escape from electron carriers in the respiratory chain (Boveris and Chance, 1973).

The leakage forms the superoxide radical which was initially detected as its dismutation product, hydrogen peroxide (Boveris and Cadenas, 1975).

The primary site of superoxide production by cardiac mitochondria, responsible for 75 % of the superoxide generation, is the region between the ubisemiquinone and cytochrome b, on the inner mitochondrial membrane (Boveris and Cadenas, 1975). Superoxide anions are formed by auto - oxidation of semiquinones rather than as a direct catalytic product (Boveris and Cadenas, 1975). In addition, several other auto - oxidisable electron carriers exist in the inner mitochondrial membrane such as NADH dehydrogenase which are responsible for the remainder of the radical generation.

It may be that during ischaemia the adenine nucleotide pool is partially degraded, leaving the mitochondrial carriers in a fully reduced state when reperfusion occurs. Electrons that egress through the cytochrome oxidase will be diminished due to lack of ADP, even though oxygen is now available. As a result, the percentage of electron leakage will increase. At the same time, the mitochondria have been rendered less well equipped to deal with the increased radical flux (McCord, 1987).

Guarnieri et al, in 1978, showed in rabbit that during ischaemia there is a 50 % loss of heart mitochondrial superoxide dismutase and glutathione. Similar observations have been made in human skeletal muscle during shock (Corbucci et al., 1985).

Therefore, the increase in free radical production during ischaemia / reperfusion combined with deterioration of endogenous anti - oxidant mechanisms, will ultimately lead to injury to cytosolic components, cell membranes, generalised tissue injury and necrosis.

1.3.3 *Neutrophils*

There is a complex interrelationship among the various factors that contribute to reperfusion injury. Oxygen - derived free radicals produced by activated neutrophils that infiltrate ischaemic / reperfused myocardium, also contribute to this complexity. The poly - morphonuclear neutrophil and other phagocytic cells possess the capacity to produce reactive oxygen species when presented with appropriate stimuli (Babior, 1978) in a metabolic event referred to as the respiratory burst.

Oxidants produced by the activated phagocyte consist of superoxide ($\cdot O_2^-$), hydrogen peroxide, hypochlorous acid (HOCl), chloramines ($RNHCl^-$), and hydroxyl radical. The primary function of these oxidants is to provide a defense mechanism against invading microorganisms. The killing of microorganisms occurs within the phagocytic vacuole in which oxidants are contained. The reactive species become deleterious to the surrounding cells when they are released into the extracellular environment.

The primary reactant that serves as the ultimate source of each of the oxidants is $\cdot O_2^-$ which results from the one - electron reduction of oxygen catalysed by the enzyme NADH - oxidase. This enzyme, a membrane - bound flavoprotein, is dormant in the resting phagocytic cell, but becomes activated when stimulated by components (e.g. C5a) of the complement system. Neutrophils contain the enzyme myeloperoxidase, which catalyses a H_2O_2 dependent oxidation of halide ions that involves both Cl^- and Br^- ions to give rise to OCl^- or OBr^-. Each of these hypohalite anions can act as a powerful oxidant capable of attacking a wide variety of biomolecules including α_1-antiproteinase.

Leucocyte / endothelial cell interaction is essential for leucocyte migration across the endothelial surface. The superoxide anion as well as C5a have been demonstrated to increase adhesion of neutrophils to the endothelium.

Neutrophils are activated upon attachment to the vascular endothelium with the subsequent generation of toxic oxygen products and the release of destructive proteases capable of altering vascular permeability. The neutrophil contains two latent metallo - proteinases, collagenase and gelatinase, that are activated by hypochlorous acid and are capable of degrading collagen and lysing endothelial cells (Smedley et al., 1986). Activated neutrophils coming into contact with endothelial cells stimulate the conversion of xanthine dehydrogenase to xanthine oxidase (a rapid process that could occur within 5 - 10 min). This process leads to generation of superoxide and amplify the response to injury (Lucchesi, 1998).

Mediators present during myocardial ischaemia capable of activating neutrophils include complement activation products, leucotrine B_4, and platelet activating factor.

Several studies have documented the correlation between infarct size and the extent of neutrophil infiltration (Jolly et al., 1986; Romson et al., 1982). Neutrophils have been observed to infiltrate the damaged myocardium region beginning with the onset of ischaemic injury and to increase their numbers progressively for the first 24 hr post myocardial infarction (Fishbein et al., 1978). There is also a direct relationship between the duration of myocardial ischaemia and the extent of neutrophil infiltration and accumulation in the reperfused region as shown by Go et al., 1988.

1.3.4 *Other sources of free radical formation*

Electron transport mechanisms which operate within the endoplasmic reticulum and within the nuclear membranes of eukaryotic cells may also generate superoxide and hydrogen peroxide.

It should be mentioned that mixed - function oxidases and flavoproteins that contain cytochrome reductases have been identified in cell membranes, and all these compounds are capable of generating superoxide and hydrogen peroxide (Ferrari et al., 1990).

Peroxisomes contain oxidases which can generate hydrogen peroxide directly, without superoxide anion intermediates (Masters and Holmes, 1977). Up to 40 % of the hydrogen peroxide generated in the peroxisomes can diffuse in the cytoplasm and result in injury to the cytosolic components.

1.4 *Effects of oxygen - derived free radicals*

Oxygen free radicals or reactive oxygen species (ROS) react with a large variety of compounds and biomembranes. The polyunsaturated fatty acid side chains of cell membranes and proteins are some of the primary targets of free radicals and depending on the severity and duration of the injurious factors may lead to cell damage. The degradation of these organic matter can lead to further ROS formation and subsequent cell death.

1.4.1 *Lipid peroxidation*

The process of lipid peroxidation is started when a free radical species, for example ·OH, extract a hydrogen atom from the methylene group in the polyunsaturated fatty acid side chain. The removal of the hydrogen, leaves behind an unpaired electron on the carbon atom, which results in the formation of the carbon radical (Lipid ·) (Hess and Manson, 1984).

$$\text{Lipid} - \text{H} + \cdot\text{OH} \longrightarrow \text{H}_2\text{O} + \text{Lipid}\cdot \qquad \textbf{(6A)}$$

This lipid radical subsequently rearranges to produce a conjugated diene and in the presence of oxygen results in the formation of organic oxygen radicals.

$$\text{Lipid}\cdot + \text{O}_2 \longrightarrow \text{Lipid OO}\cdot \qquad \textbf{(6B)}$$

These organic radicals can abstract hydrogen from additional fatty acid side chains, resulting in a chain reaction and production of lipid peroxides.

$$\text{Lipid} - \text{H} + \text{Lipid OO}\cdot \longrightarrow \text{Lipid} - \text{OOH} + \text{Lipid}\cdot \qquad \textbf{(6C)}$$

These lipid peroxides are important components of polyunsaturated side chains of cell membranes and can decompose in the presence of transition metals such as iron and copper to produce alkoxy (lipid O ·) and peroxy (lipid OO ·) radicals (Thompson and Hess, 1986). Therefore, the presence of iron or copper salts will significantly increase the rate of lipid peroxidation, which may cause an increase in membrane fluidity, permeability and loss of membrane integrity (Thompson and Hess, 1986).

1.4.2 *Protein degradation*

Proteins are present inside and outside the cell in very high concentrations and because many are catalytic, modifications by free radicals may have an amplified effect. Proteins may thus be critical targets (Wolff and Dean, 1986).

Free radicals such as · OH and possibly alkoxy (RO ·) intermediates of lipid peroxidation can fragment and crosslink protein (Schuessler and Schilling, 1984; Wolff and Dean, 1986). In the absence of molecular oxygen, · OH induces crosslinks in protein which are often resistant to reduction, such as dityrosine (Halliwell and Gutteridge, 1984). Some crosslinking may also occur in the presence of oxygen but fragmentation is then much more pronounced. Steady - state gamma radiolysis permits (Wilson, 1983) generation of defined free radicals and shows that · OH in the presence of molecular oxygen, fragments many proteins efficiently whereas peroxy radicals (ROO ·), including · O_2^- and its conjugated acid, the hydroperoxy radical (HO_2 ·), are inert in this respect.

Protein fragmentation by · OH is a selective process: fragments of defined rather than random length are generated (Wolff and Dean, 1986; Orr, 1967; Gutteridge and Wilkins, 1983). The number of fragments varies from protein to protein, collagen giving a large number of fragments. Attack and modification by · OH is expected to depend on the relative concentrations and biomolecular rate constants of the reaction of the amino acid side chains with this radical.

However, although the initial site of · OH attack may be random, there can be rapid intra or intermolecular hydrogen / electron transfer which tends to locate the free electron (spin center) at the amino acid residue to its lowest free energy state (Levitzki and Anbar, 1967; Butler, 1984).

Proteins are fragmented and modified by H_2O_2 in the presence of transition metals or suitable chelates thereof (Wolff and Dean, 1986; Orr, 1967; Gutteridge and Wilkins, 1983). Chelated iron must possess an H_2O_2 – coordination site for these reactions to occur. The hydroxyl radical generated, may react with the chelating species rather than escape from the site of their generation. Thus generation of · OH by copper / histidinyl complexes seems to lead to the oxidation of the histidine residue to yield aspartate (Cooper et al., 1985).

The modification of amino acid residues by · OH and $\cdot O_2^-$, and subsequent changes in enzyme activity, have been used to identify residues crucial for protein function, such as methionine, tryptophan, histidine and sulphydryl groups (Wolff and Dean, 1986; Gee et al., 1985). In general the consequence of radical modification of enzymes is inactivation.

However, activation of some enzymes may occur, for example, by inactivation of an enzyme inhibitor such as α-1-protease by thymine peroxy or · OH radicals can lead to methionine oxidation (Wolff and Dean, 1986; Gee et al., 1985). Repair mechanisms may exist for several of the protein modifications (Wilson, 1983). Cellular reductants and antioxidants may replenish electron or hydrogen atom "holes" after · OH attack and cleave protein - disulphides.

1.5 *Protection against free radical formation (Antioxidant mechanisms)*

The aerobic myocardium is able to handle and survive continuous oxygen free radical production because of the delicate balance between cellular systems which generate the various oxidants and those that maintain the antioxidant defence mechanism (Ferrrari et al., 1990). In the heart, these defence mechanisms include the enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase plus other endogenous antioxidants such as vit. E, ascorbic acid and cysteine (Diplock and Lucy, 1972; Ferrari et al., 1986).

1.5.1 *Superoxide dismutase (SOD)*

The primary mechanism for clearance of the superoxide anions is the superoxide dismutase which catalyses the dismutation of superoxide to H_2O_2 and O_2.
(see formulas 3A on page 5). At least three separate forms of SOD have been characterised. The intracellular (CuZn) superoxide dismutase, which was discovered in 1969 by McCord and Fridovich, is present throughout the cytoplasm, nucleus, and to a lesser extent in the peroxisomes of all mammalian cells (Crapo et al, 1992; Oury et al, 1996).

It serves an important role as a bulk scavenger of superoxide produced in the cytoplasm and nucleus (Kinnula, et al 1995). Two other isozymes of SOD have also been described in mammals, the first being (Mn) SOD, which was described in 1973 (Weisiger and Fridovich, 1973a, 1973b). (Mn) SOD is present in very high concentrations in mitochondria (Weisiger and Fridovich, 1973a, 1973b; Oury et al, 1996), in which it plays a crucial role as an antioxidant.

The other isozyme was discovered by Sjoquist and Marklund in 1992 and is an extracellular (EC) - SOD, which contains both copper and zinc in its active site.

1.5.2 *Catalase and glutathione peroxidase*

Two enzyme systems are important in the breakdown of H_2O_2 produced by the univalent reduction of superoxide (Roos et al., 1980; Chance et al., 1979). The first is catalase, an enzyme, mainly present in cytosol, which catalyses the reduction of H_2O_2 to water. Catalase, however, is present in very low concentrations in the myocardium, whilst the second enzyme, glutatione peroxidase (GSH-Px)(a selenium dependent enzyme) is present at significant concentrations in the cytosol. (Chance et al., 1979) (see formulas 3B and C on page 5). There is much evidence to suggest that glutathione plays an important role in myocardial metabolism (Ferrari et al., 1988). Among other functions, glutathione is a key factor in the detoxification of electrophilic metabolites and reactive oxygen intermediates. More than 95% of cardiac glutathione is in the form of GSH.

Since SOD reduces $\cdot O^-_2$ to H_2O_2, which in turn can be converted to the highly toxic $\cdot OH$, it is important that the anti - oxidative enzymes catalase and glutathione peroxidase, both of which metabolise H_2O_2, work in concert with SOD (Chance et al, 1979).

The hexose monophosphate shunt produces through glucose - 6 - phosphate oxidation the reducing equivalents (NADPH) for the action of glutathione reductase (GSH-Rd) (fig. 2).

In the process of the conversion of H_2O_2 to water, reduced glutathione (GSH) is converted by glutathione peroxidase (GSH - Px) to its disulfide oxidised form, glutathione (GSSG) (see formulas 3C on page 5).

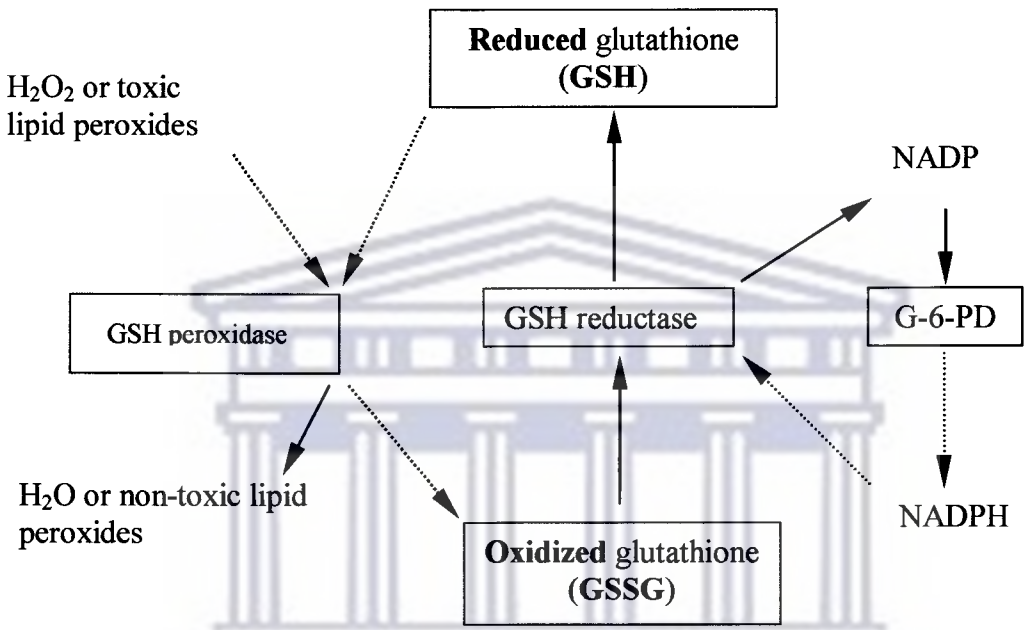


Fig. 2: GSH peroxidase metabolises H_2O_2 and lipid peroxides to non-toxic products and in doing so leads to the oxidation of GSH to GSSG. GSSG is recycled back to GSH in the presence of the enzyme glutathione reductase.

The GSH / GSSG ratio of aerobic myocardium is over 50 under normoxic conditions (Ferrari et al., 1988). As the determinant of the sulphydryl disulphide ratio (Ferrari et al., 1986), glutathione modulates the activity of a number of enzymes and is also involved in the transport of amino acids across the cell membrane (Ferrari et al., 1988).

GSH as a co - substrate of glutathione peroxidase provides essential protection against oxygen free radicals and prevents peroxidation of membrane lipids. This protective mechanism results in an increased formation of intracellular oxidised glutathione (GSSG). It follows that the changes of glutathione status provide important information in the cellular oxidative events, and tissue accumulation and / or release of GSSG in the coronary effluent is a sensitive and reliable index of oxidative stress (Ferrari et al., 1985; Ferrari et al., 1986).

1.5.3 *Vit E and C*

Vitamin E (alpha - tocopherol) has been demonstrated in significant concentrations in both myocardial cytosol and mitochondrial membranes. In vitro studies have shown vitamin E to function as a free radical scavenger and to protect cellular membranes from lipid peroxidation (Lucy, 1972; Ferrari et al., 1987).

Vitamin E functions synergistically with ascorbic acid (vitamin C) which can react with vitamin E radicals to generate vitamin E (Packer et al., 1979). Vitamin C radicals, in turn, can be reduced by NADH reductase \ cytochrome C (Packer et al., 1979). In the light of its lipophilic nature, vitamin E is likely to act as an antioxidant within membranes, while vitamin C, as a water - soluble electron - transport system, acts in the cytosol or in extracellular fluid. Although there is sufficient in vitro and in vivo evidence to support vitamin E as an important antioxidant, a protective role for this compound at physiological levels in humans has not been well documented.

1.6 *Detection of free radicals*

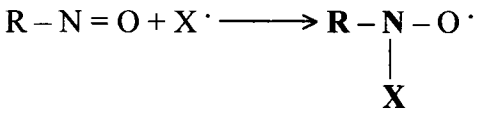
1.6.1 *Electron paramagnetic resonance spectroscopy*

Due to the extreme reactivity of free radicals, which is associated with a short life span and low concentrations under physiological conditions, experimental detection of free radicals has proved difficult. The most frequently used method of free radical detection in simple chemical systems is electron paramagnetic resonance spectroscopy (EPR) or electron spin resonance spectroscopy (ESR), which can be used with or without spin trapping agents.

When dealing with oxygen centered free radicals, produced in living tissue, detection of these free radicals may be possible by allowing interaction with a specific trapping agent to form relatively stable free radicals and subsequent detection of these components with EPR. The existence of free radicals in ischaemic reperfused hearts has been demonstrated directly using spin trapping agents (Arroyo et al., 1987; Baker et al., 1988; Zweier, 1988).

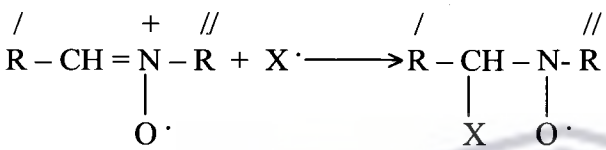
A spin trapping agent can be defined as an organic compound, which usually contains a nitroso or nitrone function. This can be used to convert reactive free radicals into more stable, detectable, radical adducts (reaction 7A and 7B).

The correct spin trap is of great importance since different spin traps have different specificities with respect to the radical and the biological system in which they will be used.



(7A)

Nitroso spin trap Nitroxide spin adduct



(7B)

Nitrone spin trap Nitroxide spin adduct

The method of using EPR / ESR, involves initial freeze clamping of tissue using Wollenberger tongs cooled to 77K in liquid nitrogen. The freeze - clamped tissue is then ground to a fine powder under liquid nitrogen, and transferred to precision EPR tubes. The EPR spectra are recorded at a temperature of 77K on a spectrometer. Quantitation of the signals must be done, for example, by comparison of the integrated signal area with that of the commonly used free radical standard potassium peroxylamine disulphonate in frozen aqueous solution in identical EPR tubes.

Direct electron paramagnetic resonance spectroscopy however has several limitations. Some studies have suggested that certain electron paramagnetic resonance spectra, initially thought to be due to oxygen radicals, were in fact due to freezing and mechanical manipulation of the tissue (Ogura et al., 1991; Khalid and Ashraf, 1993). Pulverisation of the frozen tissue can form artifactual radicals, giving rise to ambiguous electron paramagnetic resonance spectra (Pou et al., 1989;

1.7 *The development of new techniques*

Unfortunately, most of the evidence supporting the free radical hypothesis of ischaemic - reperfusion cell damage has been indirect, based on the use of inhibitors and activators of free radical generation. Consequently, a technique that could directly measure the generation of free radicals in the heart or in any other tissue is in great demand. Ideally, such a technique should allow characterisation of the radicals formed, as well as clarification of the mechanisms of their formation.

The use of high spatial and temporal resolution laser - scanning confocal microscopy combined with oxidant fluorescent probes, may fulfill some of the above criteria. Fluorescent probes are compatible with most fluorescent instrumentation, providing a powerful tool for the investigation of respiration, mitosis, substrate degradation and detoxification, intracellular transport and more. The fluorescent probes discussed below are examples of probes currently used in the study of free radical induced cell damage.

The probes can be used in live cells with minimal disruption of cellular functions. This approach, in conjunction with laser scanning confocal microscopy provides a means with minimal artifacts to study free radical generation in isolated ventricular myocytes during normoxic and ischaemic - reperfusion conditions. In order to comprehend the results obtained, it is imperative to understand the known actions of the fluorophores used.

1.7.1 *Tetramethylrhodamine (TMRM)*

Closely related to rhodamine 123, its methyl and ethyl esters tetramethyl - rhodamine (TMRM and TMRE) are currently the preferred dyes for determining membrane potential ($\Delta\Psi$) by quantitative imaging (Loew, 1993; Gross and Loew, 1989). TMRM and TMRE, like rhodamine 123, are cationic fluorophores that are taken up by mitochondria in response to the $\Delta\Psi$ (Ehrenberg et al., 1988). The fluorescence of TMRM is used to monitor sarcolemmal and mitochondrial membrane integrity and thus is an indicator of cell viability.

To specifically outline the TMRM or rhodamine mitochondrial fluorescence of a cell, the cell have to be loaded with another fluorophore like calcein which accumulates exclusively in the cytosol (Zahrebelski et al., 1995), since the inner mitochondrial membrane is quite impermeable to calcein (Nieminen et al., 1995). The TMRM or rhodamine 123 loaded mitochondria, fluoresces red and can thus be simultaneously imaged with the green - fluorescing calcein (Zahrebelski et al., 1995).

Specific potential - dependent fluorescence of mitochondria is also obtained by setting the extracellular K^+ concentration close to intracellular values (~137 mM), thereby depolarising the plasma membrane (Chen, 1989). The spatially resolved fluorescence of TMRM and TMRE presents an unbiased profile of their transmembrane distribution that can be directly related to the membrane potential (Loew, 1993).

Mitochondria, although abundant in TMRM loaded rabbit cardiac myocytes, could not readily be discerned and appeared as bright spheres and rods within the myocyte (Lemasters et al., 1993) giving the appearance of a red - orange stained myocyte.

TMRM is therefore used, not to specifically indicate mitochondrial membrane potential but to illustrate an unbiased distribution profile of membrane potential of all cellular and subcellular compartments, resulting in heterogenous fluorescence of the whole cell.

If the necessary precautions are taken to ensure that the fluorescence of TMRM is maintained for the duration of the experimental procedure, it can be concluded that the membrane system of the cell and specifically the mitochondrial and the sarcolemmal membrane system should still be unaltered. Cell damage would obviously result in changes in mitochondrial membrane potential and the subsequent TMRM fluorescence would change accordingly. Complete loss of membrane potential / cell viability would result in complete loss of fluorescence, which can be confirmed by positive propidium iodide (PI) nuclear staining.

1.7.2 *2,7-Dichlorodihydrofluorescein diacetate (DCDHF) and dihydrorhodamine 123 (DHR)*

Use of fluorogenic probes for the detection of H_2O_2 and / or ROS or overall oxidative stress is however, clouded by the fact that it may not easily discriminate between the various reactive oxygen species (molecular probes catalogue, section 21.4, 1999).

2, 7 Dichlorodihydrofluorescein (DCDHF) and dihydrorhodamine 123 (DHR) can be utilised for the detection of ROS in cells (Black and Brandt, 1974). These dihydro - derivatives, pass passively across cell membranes and accumulate in mitochondria in response to the negative inside $\Delta\Psi$ (Johnson et al., 1981; Ehrenberg et al., 1988). These fluorophores are readily oxidised back to the parent dye by reactive oxygen species and can thus serve as fluorogenic probes for the detection of oxidative activity in cells and tissues (Zhu et al., 1994; LeBel Ischiropoulos and Bondy, 1992).

The cell - permeant DCDHF commonly used to detect the generation of H_2O_2 in neutrophils and macrophages (Yuan et al., 1993) may also be extremely useful for assessing overall oxidative stress in toxicological phenomena (Oyama et al., 1994; LeBel, Ischiropoulos and Bondy, 1992).

DHR has always been used to investigate the production of reactive oxygen species in activated rat mast cells (Tsinkalovsky and Laerum, 1994) and cultured endothelial cells (Royall and Ischiropoulos, 1993).

Like DCDHF, DHR does not directly detect superoxide but rather reacts with H_2O_2 (Henderson and Chappell, 1993) in the presence of peroxidase, cytochrome c or Fe^{2+} (LeBel, Ischiropoulos and Bondy, 1992; Royall and Ischiropoulos, 1993). However, DHR has been reported to be more sensitive than DCDHF for detecting granulocyte respiratory burst (Vowels et al., 1995; Roth, Oser and Valet, 1988), ordinarily defined as the rapid formation and release of ROS and H_2O_2.

1.8 *The involvement of oxygen - derived free radicals in tissue damage*

Free radicals have become increasingly implicated in playing an important part in human disease, for example the pathology of cerebral ischaemia (Kontos et al., 1983), irradiation (Petkau et al., 1978), intestinal ischaemia (Granger et al., 1981; Parks et al., 1983), and pulmonary disorders (Fox et al., 1993; Johnson et al., 1981). A mounting body of evidence now indicates that free radicals may be responsible for some of the tissue damage seen in the ischaemic - reperfused heart as well.

With the progression of myocardial ischaemia, there is a continued increase in substrate concentrations for the production of oxygen - derived free radicals. Upon abrupt reoxygenation of an ischaemic vascular bed sets the stage for an explosion of free radicals and subsequent myocardial dysfunction (Thompson and Hess, 1986). This phenomenon has been termed the oxygen paradox (Hess and Manson, 1984; Hearse et al., 1978), which has been observed in the isolated myocyte as well (Altschuld et al., 1981).

The oxygen paradox is characterised by the development of myocardial contracture upon reoxygenation of the hypoxic or ischaemic myocardium, and is accompanied by the release of intracellular enzymes and irreversible myocardial cell injury (Lucchesi, 1998). Oxygen paradox / oxidative stress cannot only be the consequence of increased free radical production but can also be secondary to reduced endogenous antioxidant mechanisms (Ferrari et al., 1990).

All components within the cell are subject to attack by free radicals. Free radicals damage membrane lipids containing unsaturated double bonds. This will lead to the formation of lipid peroxides, lipid hydroperoxides, and aldehydes (Lucchesi, 1998). Another important site includes the membrane proteins involved in the transport of ions and the maintenance of cellular ionic homeostasis. This is especially true of proteins containing sulfhydryl groups, such as those with methionyl residues and peptides in which the amino acid has a critical role or enzymic function (Lucchesi, 1998). It is suggested that excessive oxidative stress, can lead to the disruption of essential components of the cell membrane systems and associated enzyme functions (Lucchesi, 1998).

These experimental findings has led to the hypothesis that clinical reperfusion injury, whether it be spontaneous, pharmacological, or mechanical, may be analogous to the oxygen paradox and has led several investigators to suggest that this phenomenon may be mediated by oxygen free radicals (Thompson and Hess, 1986). Although a number of potential sources of free radicals exist within the ischaemic heart, it is uncertain whether they are produced within the myocyte (Khalid and Ashraff, 1993). To avoid the contribution of extramyocardial factors, such as the presence of non - cardiac cells, neural stimulation, changes in coronary flow and circulatory catecholamines to reoxygenation injury, the isolated myocyte serves as an excellent model to investigate the phenomenon of oxidative stress within the contractile apparatus of the heart (Khalid and Ashraff, 1993).

1.8.1 *Protection against free radical mediated damage*

The recognition of a possible role of oxidative stress in the pathogenesis of heart failure has drawn attention to the potential of anti - oxidants or free radical scavengers as therapeutic agents (Ambrosio and Tritto, 1998). Much research is currently aimed at establishing whether prevention of oxidative stress in a clinical setting would translate into improved protection, and whether antioxidant therapy may represent a novel adjunct (Ambrosio and Tritto, 1998). Clinical applicable techniques and biological agents are currently being developed and used to remove free radicals from cellular sites where they are injurious. There is also an increasing interest in defining new antioxidants of high potency, low toxicity and good solubility properties in aqueous as well as organic phases.

It has been shown that oxidative stress can exert major consequences on various aspects of cardiac function, and that hearts receiving interventions aimed at preventing oxidant toxicity scored better on various indices used to measure cell injury (Ambrosio and Tritto, 1998). Amongst others, Guarnieri et al. (1978) found that perfusion with the antioxidant α-tocopherol reduced enzyme release, increased resting tension during the hypoxic perfusion period and attenuated the oxygen paradox during the reoxygenation period.

Further insight into the oxygen - derived free radical system was also provided by Guarnieri et al., in 1980. In this study, it was established that ischaemia is associated with a loss of intracellular scavenging enzymes (superoxide dismutase and glutathione peroxidase) and that with the reintroduction of molecular oxygen, these normal intracellular scavenging systems are depleted and significant lipid peroxidation damage occurs.

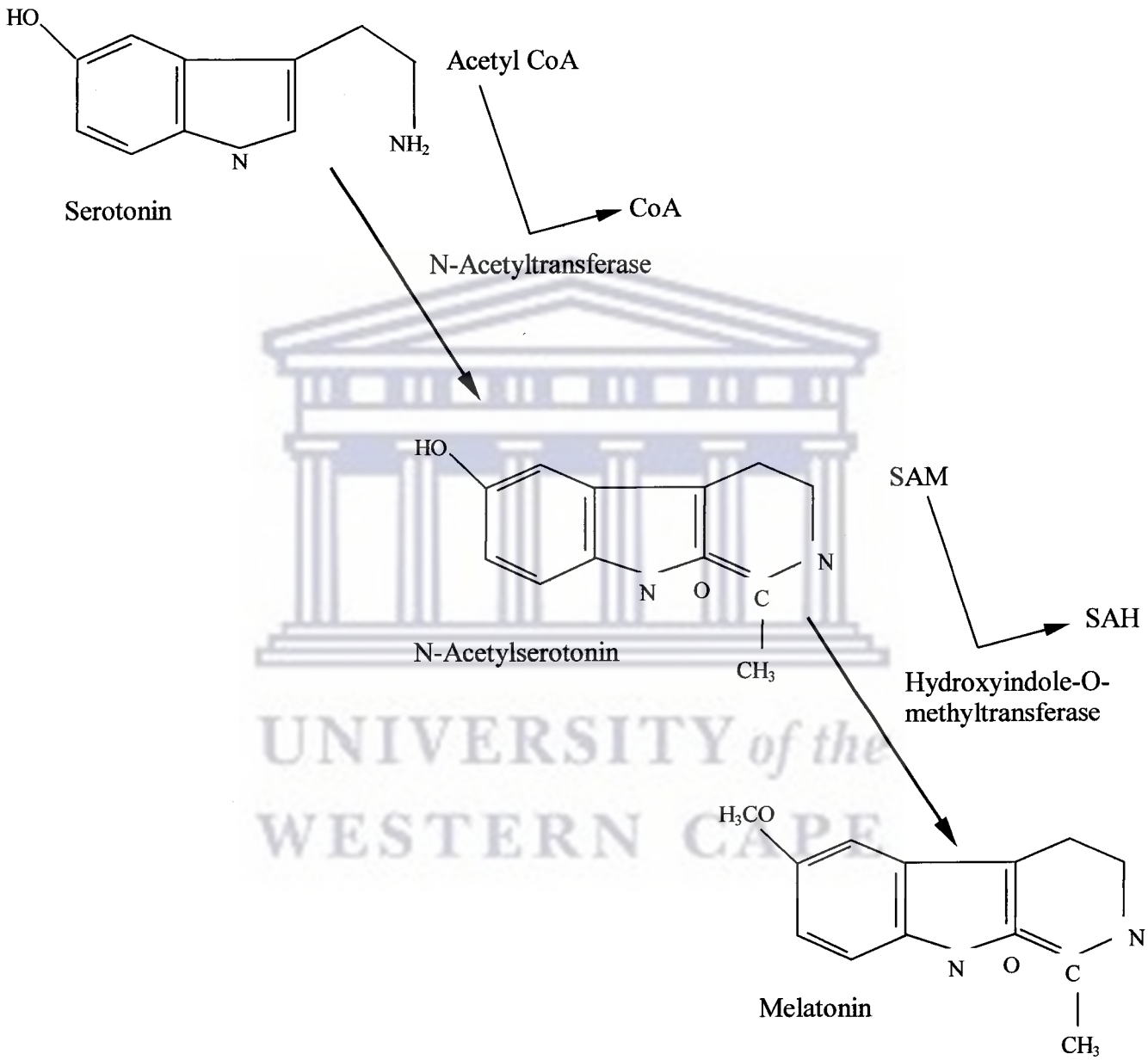


Fig. 3: Metabolism of 5HT to melatonin as it is known to occur in the pineal gland. CoA, co-enzyme A; SAM, S-adenosylmethionine; SAH, S-adenosyl homocysteine.

The highly lipophilic nature of melatonin, allows it to be rapidly released from pinealocytes and because of its high diffusibility, allows it to enter all cells and every sub - cellular compartment (Reiter, 1993). The discharge of melatonin into blood or cerebrospinal fluid (CSF), has been debated (Reiter et al., 1975) and there was universally agreed upon that it is released directly into the vascular systems and secondarily into other body fluids (Reiter, 1986).

The bulk of melatonin in the blood is bound to albumin (Partdrige and Mietus, 1980). The half - life of melatonin in the blood is rather short (10-40 min) (Kopin et al., 1961; Kveder and McIsaac, 1961) and during a single passage though the liver, 90 % of the melatonin is cleared (Partridge and Mietus, 1980). About 75 % of the melatonin taken up by hepatic cell is converted to 6-hydroxymelatonin by microsomal enzymes; this compound is subsequently conjugated to either sulphate (70 %) (6-sulfatoxy- melatonin) or glucoronide (6 %) (Kopin et al., 1961).

The existence of melatonin in unicellular organisms (Poeggeler et al., 1995) as well as its widespread action elsewhere (Reiter, 1991), in multicellular organisms led to the speculation that melatonin performed functions that did not require interaction with a receptor, particularly not a receptor located in the limiting membrane of the cell (Reiter et al., 1995). The recent demonstration that melatonin is also quite soluble in aqueous media is consistent with the intracellular actions of melatonin (Shida et al., 1994). The structural features of melatonin that makes it a potent free radical scavenger include the methyl group at position 5 and the acetyl group on the side chain of the molecule (fig. 3) (Tan et al., 1993a).

Melatonin works as an antioxidant via electron donation (fig. 4) (Scaiano, 1995; Hardeland et al., 1993 and Poeggeler et al., 1995). Since the interaction of a non - radical species (melatonin) with a radical (hydroxyl radical) must generate a radical, the radical produced in this reaction is the indolyl / melatonyl cation radical. This product may then scavenge the superoxide anion radical to produce 5-MAFK (N-acetyl-N-formyl-5-methoxykynuramine). Whether the indolylcation radical is re - cycled back to melatonin is unknown. These interactions have been proposed in part or in toto by Scaiano (1995); Hardeland et al. (1993) and Poeggeler et al. (1995).

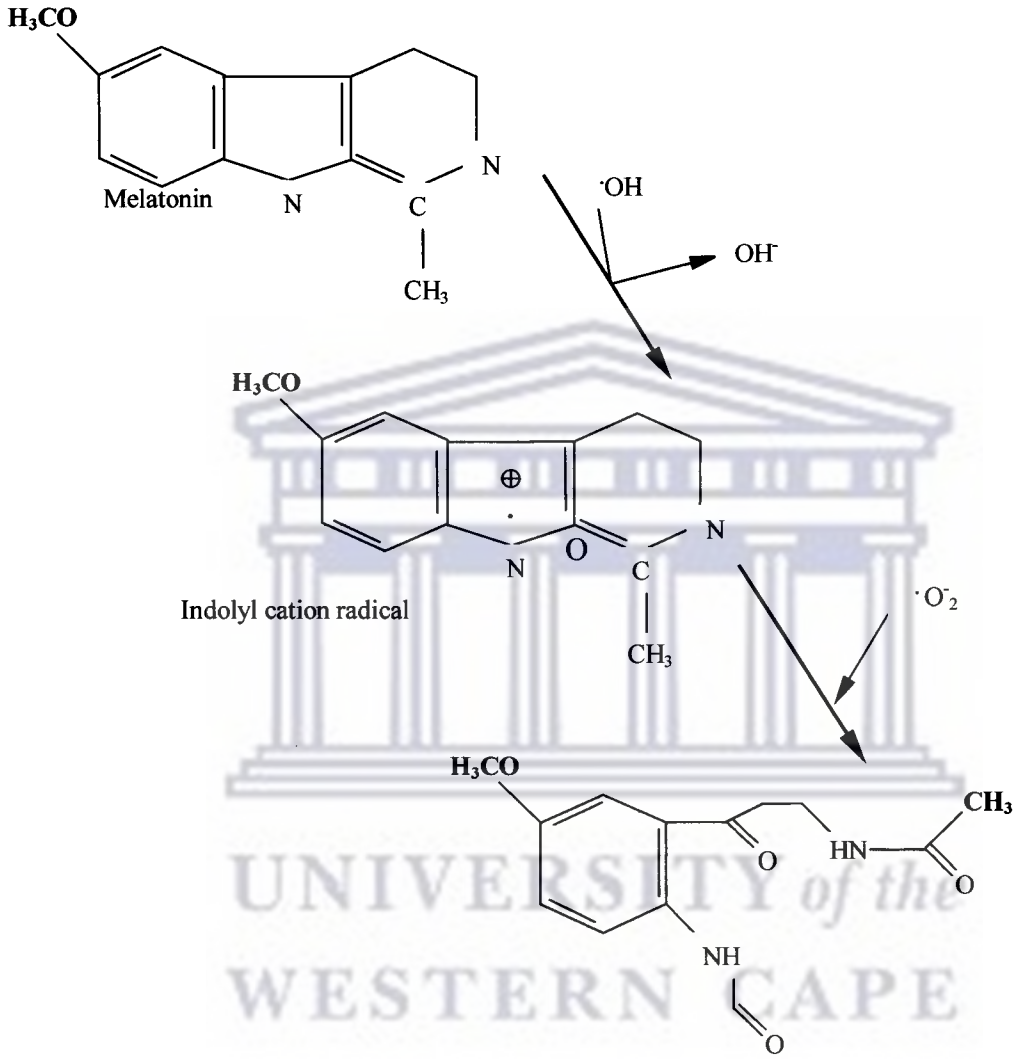
Several enzymes are important in antioxidative defence because they metabolise either free radicals or reactive oxygen intermediates to non - radical products. Some of the best known of these include a family of enzymes known as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd). Pharmacological levels of melatonin have been shown to stimulate the activity of GSH-Px (Barlow - Walden et al., 1995).

The initial in vitro findings suggest that melatonin is remarkably effective as free radical scavenger as indicated by the fact that when compared with the intracellular scavenger, glutathione (GSH), melatonin proved five times better in neutralising the · OH and, when compared to vitamin E, melatonin was twice as effective inactivating the ROO · (Reiter et al., 1995). GSH (Meister, 1992) and vitamin E (Packer, 1994) are considered to be premier antioxidants within the cell.

At molecular level, melatonin has been reported to increase tissue levels of mRNA for both (Mn) SOD as well as (Cu) SOD (Antolin et al., 1996); in the same study there was an indication that mRNA levels for GSH-Px were also augmented after melatonin administration.

Pierrefiche and Laborit (1995) demonstrated that melatonin stimulates the activity of glucose-6-phosphate dehydrogenase in both the liver and the brain. The importance of this lies in the fact that the enzyme re - supplies the cell with nicotinamide adenine dinucleotide phosphate, which is required for generating reduced glutathione from oxidised glutathione via the enzyme GSH-Rd.

Information about the potential role of melatonin in influencing the antioxidative defence systems via enzymes involved in free radical metabolism is incomplete (Reiter, 1991) and whether melatonin plays a pivotal enzyme modifying role in altering free radical damage requires further definition.



N-acetyl-N-formyl-5-methoxykynuramine (5-MAFK)

Fig. 4: The antioxidant mechanism by means of electron donation as proposed in part or in toto by Scaiano, 1995; Hardeland et al., 1993 and Poeggeler et al., 1995.

DNA damage resulting from either exposure to the chemical carcinogen safrole (Tan et al., 1993b, 1994) or to ionising radiation (Vijayalaxmi et al., 1995) was markedly reduced when melatonin was co - administered.

Physiological levels of melatonin have also been shown to inhibit the nitric oxide ($NO^{\cdot}$) generating enzyme, nitric oxide synthase (Pozo et al., 1994).

By inhibiting nitric oxide synthase, melatonin reduces the formation of the free radical, $NO^{\cdot}$. (Palmer et al., 1988). The reduction of $NO^{\cdot}$ production contributes to melatonin's antioxidant action since $NO^{\cdot}$ can generate the peroxynitrite anion ($ONOO^{-}$) by acting with $^{\cdot}O_2^{-}$, which can degrade into oxygen - based radicals such as the $^{\cdot}OH$ (Beckman, 1991; Radi et al., 1991).

For an antioxidant to function in this capacity, it must be at the site where free radicals are generated because of their fleeting half - life. Melatonin has the capability of entering cells because of its high lipophilic nature and it readily crosses all morphophysiological barriers.

1.9 *Aim of Study*

Myocardial cells are continuously exposed to physiological levels of oxygen free radicals and their metabolites which are neutralised by endogenous antioxidant mechanisms. However, these mechanisms can be overwhelmed / reduced and the resultant increased free radical production can bring about the phenomenon of oxidative stress (Hess and Manson, 1984; Sies and Cadenas, 1985).

It has been proposed that oxidation of cell constituents by oxygen radicals and their metabolites may represent a major mechanism for the specific form of myocardial injury that may occur in the setting of ischaemia - reperfusion / hypoxia - reoxygenation and heart failure (Hess and Manson, 1984; Becker and Ambrosio, 1987).

Much of the information regarding the above has been obtained using an indirect approach. In these studies the outcome of hearts exposed to standard conditions was compared to that of hearts that were treated with various antioxidants (Ambrosio and Tritto, 1998). By this approach, it has been possible to dissect out the relative component of myocardial injury due to oxygen deprivation during ischaemia from the possible alterations brought about by oxidation stress upon reflow (Ambrosio and Tritto, 1998). It was shown that hearts receiving interventions aimed at preventing oxidant toxicity scored better on the various indices (outlined below) of cell injury (Ambrosio and Tritto, 1998).

- Reduction of reperfusion arrhythmias
- Improvement of contractile recovery
- Reduction of vascular injury
- Reduction of cell damage and cell death

Thus far, direct evaluation of oxygen free radical mediated damage was done by methods, such as direct electron paramagnetic resonance spectroscopy or by identifying the products of lipid peroxidation, particularly malondialdehyde (Wolin and Belloni, 1985).

CHAPTER 2

MATERIALS and METHODS

2.1 *Preparation and culture of myocytes*

2.1.1 *Isolation of adult rat ventricular myocytes*

Adult male NEDH rats (200-250 g) were anaesthetised with thiopentone sodium (30 mg; intraperitoneal). The heart was excised and mounted onto the aortic cannula of a modified Langendorff perfusion apparatus.

The heart was initially perfused for 5 min with a calcium - free, HEPES - bicarbonate buffer, called buffer A containing (in mmol/l) NaCl 109; NaH_2PO_4 1.15; KCl 4.95; glucose 30.0; 2,3-butane-dione monoxime (BDM) 19.98; HEPES 25.0; $NaHCO_3$ 28.0; carnitine 1; taurine 1; creatine 1; adenosine 0.249; insulin[1] 0.5 ml (50 iu/L) and pen/strep[2] 1.0 ml (pen = 10 000 iu/L and strep = 10 mg/l), pH 7.4. The buffer was gassed with 95 % O_2 and 5 % CO_2 during the perfusion (temperature: 37 °C).

This was followed by a 10 min perfusion with 50ml buffer A, to which was added 25 µmol/l $CaCl_2$, collagenase (30 mg/50ml) (Boehringer Mannheim) and protease (3 mg/50 ml) (Sigma).

1 (100 iu/ml)
2 (pen 10.000 iu/ml; strep 10 mg/ml)

Thereafter the $CaCl_2$ concentration was increased to 50 μmol/l, and then to 100 μmol/l at 5 min intervals. The solution was gassed with 95 % O_2 and 5 % CO_2 throughout the isolation procedure.

The ventricles were cut off and the myocytes gently dispersed in 50 ml buffer A, containing 100 μmol/l calcium chloride (post digestion buffer). The $CaCl_2$ concentration was increased stepwise, at 10 min intervals to 1 mmol/l. The cells were allowed to settle; half of the buffer was decanted and replaced with nutrient medium containing M199[3] (9.82 g/L); $NaHCO_3$ (23.0 mmol/l); creatine (1 mmol/l); taurine (1 mmol/l); caprylic acid (1 mmol/l); carnitine (1 mmol/l); pen /strep[2] 1.0 ml (pen = 10 000 iu/l and strep = 10 mg/l) ; insulin[1] 0.5 ml (50 iu/l).

The cells were again allowed to settle and the buffer (25 ml) / nutrient medium (25 ml) mixture replaced by 50 ml nutrient medium. 3.5 - 4.7x10^6 rod - shaped, viable, calcium tolerant myocytes were obtained with each isolation from one heart. After gentle agitation, a fifth of this cell suspension was diluted to 50 ml with nutrient medium. The cells were now ready for attachment to coverslips and culturing.

2.1.2 *Preparation of coverslips*

At a laminar flow bench, 10 – 12 round coverslips (0.15 mm thick and 31 mm in diameter) were sterilised in 70 % ethanol for 1 hour. The coverslips were then placed in 35 mm petri dishes, under UV light for 1 hour, to dry.

1 (100 iu/ml)
2 (pen 10.000 iu/ml; strep 10 mg/ml)
3 Gibco BRL

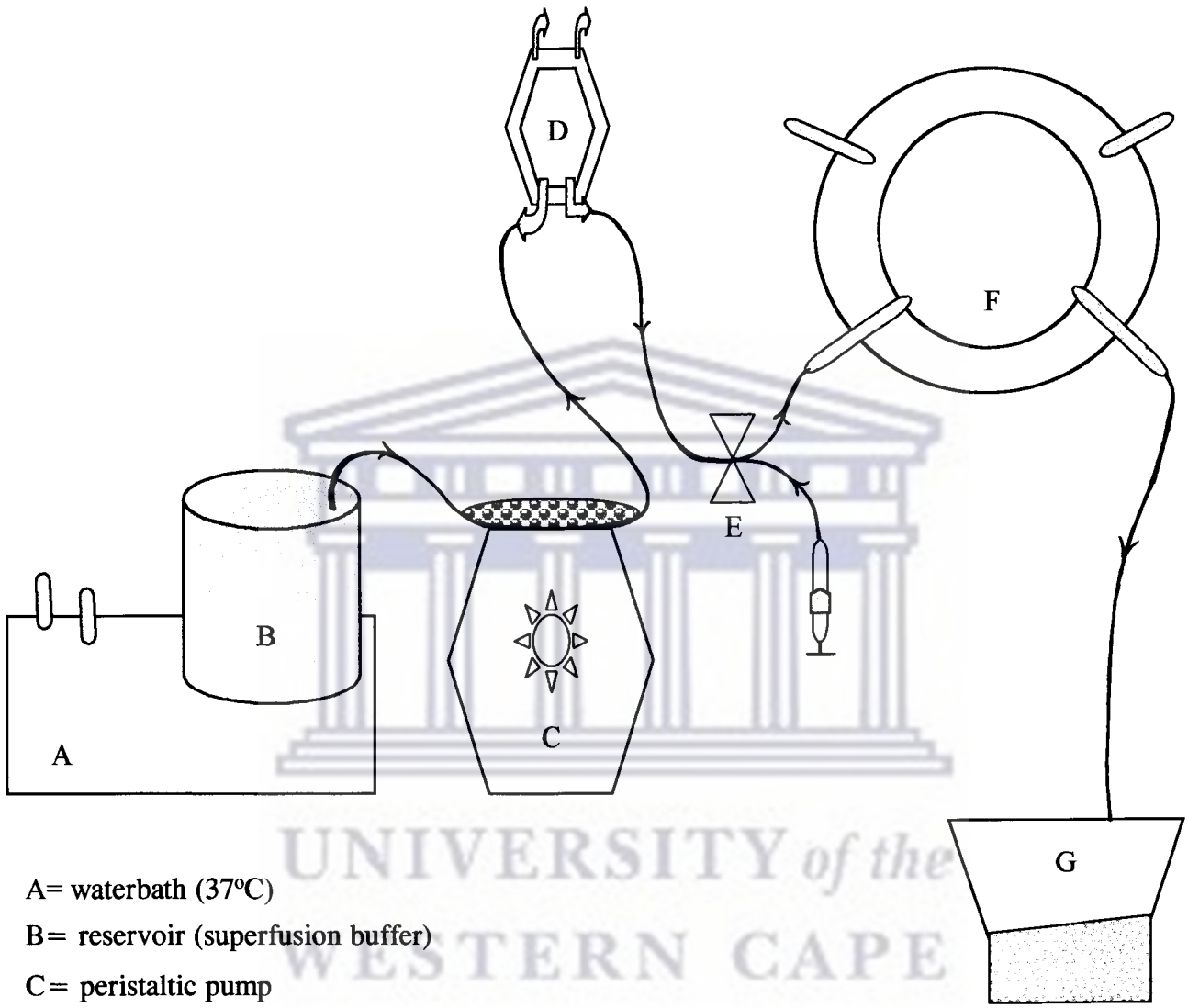
2.2 *The induction of chemical hypoxia and reoxygenation*

After loading the myocytes with the appropriate fluorophores, the coverslip was placed in an acetyl water jacketed chamber as indicated in fig. 5.

Chemical hypoxia was introduced by exposing the myocytes to 10 ml hypoxic buffer, pH 7.4 at 37 °C. The chemical hypoxic buffer consisted of buffer B in which glucose was replaced by 20 mmol/l 2-deoxyglucose and 1.5 mmol/l KCN. The buffer was gassed with 100 % nitrogen for 1h prior to the addition of KCN.

The cells were reoxygenated for 15 min by superfusing the cells with buffer B, pH 7.4 containing 5 mmol/l glucose.

PI (5 mmol/l) and a low concentration of TMRM (200 nmol/l) were also included in the hypoxic and reoxygenation buffers.



A= waterbath (37°C)

B= reservoir (superfusion buffer)

C= peristaltic pump

D= water-jacketed bubble trap

E= 3-way valve with attached syringe (KCN)

F= acetyl water-jacketed perfusion chamber

G= waste collection

Fig. 5: The experimental superfusion system

2.3 *Laser exposure, confocal parameters and image analysis*

2.3.1 *Photooxidation and photobleaching*

The circumstances under which the fluorophores were used, necessitated the investigation of the effects of laser activity on DCDHF and DHR. The use of low - intensity laser in scanning mode should minimise *photooxidation* of the probe as well as other artefacts associated with exposure of the cell to the laser beam (Reynolds and Hastings, 1995). Continuous illumination of DCDHF - loaded cells, even with low – intensity laser beam (0.15 mW total intensity), slowly increases dye fluorescence (Sarvazyan, 1996).

Preventative measures were therefore taken to ensure that laser activity does not lead to:

- the activation of the probes and the formation of free radical intermediates and / or ROS (photooxidation) and associated cell damage or
- fading of fluorescence (photobleaching).

In the initial experiments in this study, myocytes were preloaded with TMRM and DCDHF or DHR, subjected to oxygenated superfusion conditions (kept in buffer B) and monitored with ordinary brightfield microscopy for 27.5 - 30 min.

The loaded myocytes were then exposed to two, 8 sec laser scans, to visualise membrane potential (TMRM) and DCDHF or DHR fluorescence.

The cells were monitored for another 10 min with brightfield microscopy and again subjected to four to six, 8 sec laser scans with 5 minute intervals between each scan.

2.3.2 *Confocal parameters and fluorescent measurements*

The cells were observed through a 20x (0.5 NA) objective on a Zeiss 410 laser scanning microscope. Excitation of TMRM and PI was achieved using the 568 nM line of the 20 mW krypton - argon mixed gas laser, while excitation of DCDHF, DHR or fluo was achieved using the 488 nM line of the laser. The laser was attenuated using the 0.03 ND filter to prevent the loaded cells from being over - exposed and to reduce any potential probe - artefacts and laser injury. The chosen experimental field of view was exposed to 8 sec scans and the images stored at the time intervals as described in the experimental protocol (see 2.5).

The emitted light was collected on two optical channels:

Channel **1**, was used for TMRM imaging (590 nm long - pass filter).

Channel **2**, was used to detect emission of DCDHF (510 - 525 nm band pass filter), DHR (515 - 545 nm band pass filter) or fluo (515 - 545 nm band pass filter).

2.3.3 *Image acquisition and analysis*

Variation of parameters drastically changes the appearance of confocal images, either obscuring or emphasising the observed effects (Sarvazyan, 1996). These include parameters, such as intensity of the excitation beam, pinhole size, speed of scanning, photomultiplier tube settings.

Thus, depending on the probe with which the cells were loaded, it is absolutely imperative to have exactly the same settings each time the laser is used to acquire an image. This will ensure a valid comparison between the fluorophores used in the different experimental conditions.

2.3.4 *Fluorescence quantification*

Subsequent to the application of the various experimental conditions, the number of cells showing increase over baseline in DCDHF, DHR and fluo fluorescence was determined and expressed as a percentage of the total number of cells evaluated.

Fluorescence emission was detected by multiple photomultiplier tubes on the confocal microscope, digitised by an 8 bit AD converter, and hence provided 255 grey levels. Changes in cellular fluorescence were evaluated through application of color look - up tables (Lut's) to the greyscale images to assess qualitative changes and through histogramming analysis of intensity in individual cells.

To assess whether the observed changes in fluorescence differed from baseline values and if it were indeed significant, Scionimage software was used to evaluate the intensity of fluorescence obtained with the different fluorophores, at various stages of the experiments. Fluorescent line intensity profiles were used to calculate the mean fluorescence intensity (*greyscale value*) from a number of undamaged (grade 0) and damaged (Grade 1) myocytes respectively.

2.4 *Grading of myocytes*

Grade 0 = elongated / rod shaped cells, with strong mitochondrial accumulation of TMRM, indicating normal mitochondrial membrane potential

Grade 1 = square, contracted cells, slight myofibrillar dissary with blebs, membrane potential maintained indicated by strong TMRM fluorescence

Grade 2 = rounded, completely contracted cells with loss of membrane potential and positive PI nuclei staining

The chosen experimental field of view was exposed to 8 sec scans and the images stored at the following time intervals.

- at the beginning of chemical hypoxia;
- at the end of chemical hypoxia (12.5 min);
- after 60 - 90 sec of reoxygenation;
- after 5 min reoxygenation and again after 15 min reoxygenation.

2.6 *Statistical analysis*

For each experimental condition, 3 - 5 different myocyte preparations were used and ± 15 cells were evaluated per experiment. Thus, for each experimental condition 45 - 75 cells were studied.

Data are expressed as mean ± S.E.M.

Inter - group comparisons are done by using ANOVA and Bonferroni's correction.

P values < 0.05 are considered to be significant.

However, we did observe an increase in DCDHF fluorescence before exposure to the laser (fig. 6B). This was not accompanied by any change in cell morphology, and we assume this was a result of low endogenous levels of H_2O_2 and / or ROS.

These control experiments showed that, under the experimental conditions used in this study, exposure to laser did not cause photo - oxidation. The unchanged fluorescence of TMRM or DCDHF after repeated exposure to laser scanning, showed that no significant photo - bleaching was occurring.

3.2 *Evaluation of the effects of superfusion time under control oxygenated conditions (normoxia)*

3.2.1 *In the absence of melatonin*

To evaluate the effects of superfusion time under control oxygenated conditions (normoxia), myocytes were preloaded with TMRM and with one of the following fluorophores: DCDHF or DHR or fluo and kept in buffer B for 27.5 min (12.5 + 15). Confocal images were recorded at the beginning (0 min), after 12.5 min, 14 min (12.5 + 1.5) and again after 27.5 min (12.5 + 15) of superfusion as indicated in the figures below.

The pseudocoloured confocal micrographs of untreated myocytes, preloaded with TMRM and kept in buffer B for 27.5 (12.5 + 15) min indicated the maintenance of an intact membrane potential / cell viability. The majority of cells kept their rod - shaped morphology for the duration of the superfusion period (fig. 6A).

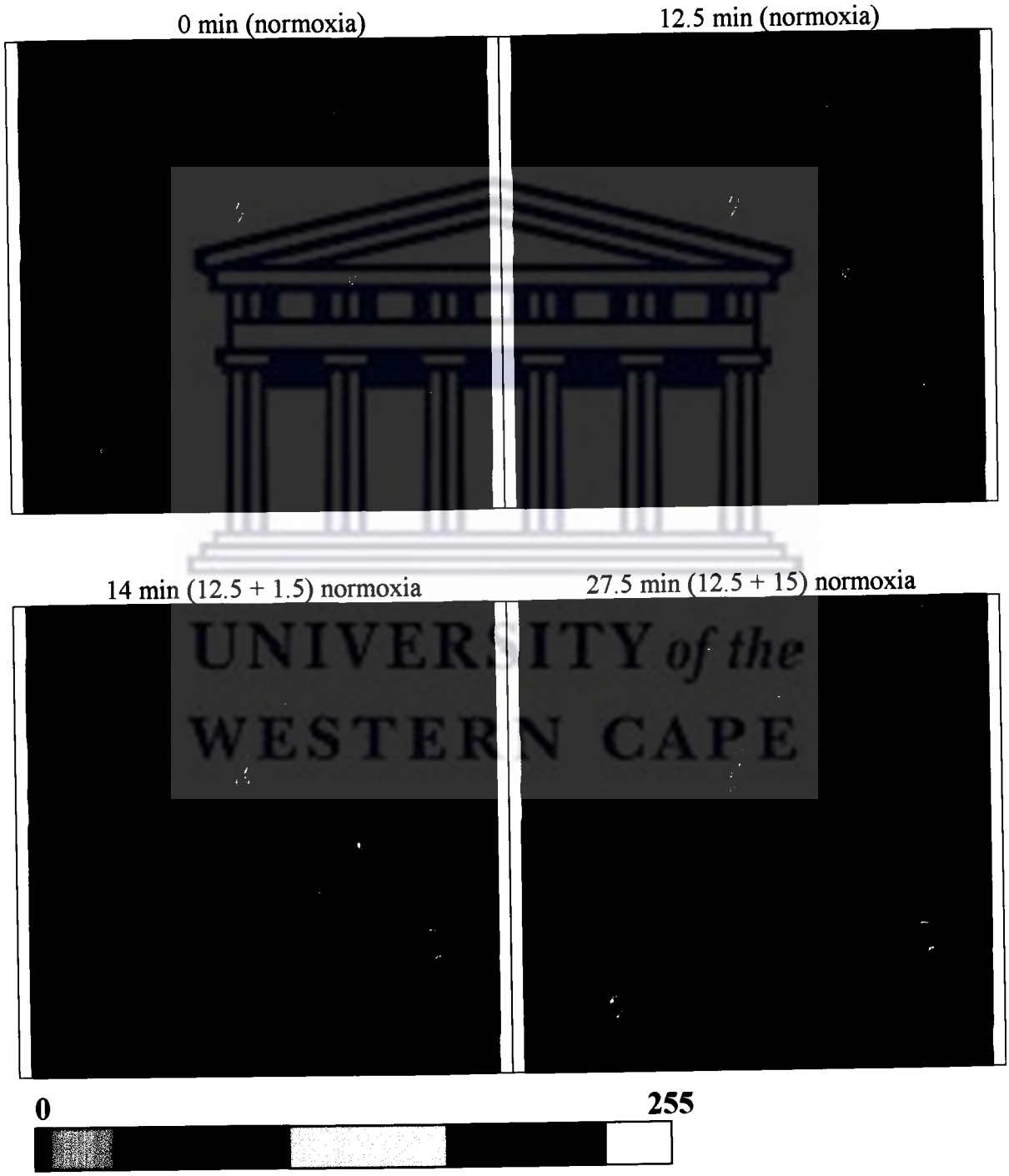
The recorded images of untreated myocytes pre - loaded with the fluorophore, DCDHF, showed an increase in the number of cells with increased DCDHF fluorescence after 12.5 min superfusion. The increased fluorescence, probably implicate the presence of H_2O_2 and / or ROS (fig. 6B). The viability and rod - shape morphology of the cells were not affected by the increased fluorescence, which remained the same after 27.5 (12.5 + 15) min superfusion (fig. 6B).

The faintly delineated cells showed no increases in fluorescence of the fluorophore, DHR, implicating the absence of H_2O_2 and / or ROS formation during this period. The cells remained viable and maintained their rod – shape morphology for the duration of the 27.5 min superfusion period (fig. 6C).

Investigating changes in intracellular calcium, untreated myocytes were pre - loaded with fluo. The cells were monitored and images recorded as before. The cells were faintly outlined and did not show damage or increased fluorescence, implicating normal intracellular calcium levels (fig. 6D).

Fig. 6: Confocal micrographs of untreated myocytes kept in buffer B for 27.5 min and loaded with TMRM (6A), DCDHF (6B), DHR (6C) and fluo (6D). The images show that the majority of myocytes remained viable and maintained their rod - shaped morphology for the duration of the superfusion period.

(A) Fluorophore: TMRM



(B) Fluorophore: DCDHF

(C) Fluorophore: DHR

(D) Fluorophore: Fluo

3.2.2 *In the presence of melatonin (50 or 100 μmol/l)*

Preliminary results indicated that 50 or 100 μmol/l melatonin had similar effects on morphology and cell viability (results not shown). Furthermore, the presence of melatonin had no effect on any of the parameters evaluated in oxygenated myocytes. All subsequent experiments were performed in the presence of 50 μmol/l melatonin.

Myocytes were pre - loaded with the same fluorophores as previously outlined and exposed to control oxygenated conditions (normoxia), in the presence of melatonin for 27.5 min. The myocytes were monitored and images recorded at the beginning (0 min), after 12.5 min, 14 min (12.5 + 1.5) and again after 27.5 min (12.5 + 15) of superfusion (fig. 7A-D).

The inclusion of melatonin in the superfusion buffer had no effect on the intense fluorescence of DCDHF observed after 12.5 min of superfusion (fig. 7B). The increased fluorescence of this fluorophore, remained the same after 27.5 (12.5 + 15) min superfusion and did not alter the cell viability or morphology of any of the myocytes.

The absence of cell damage and fluorescence with the fluorophores DHR (fig. 7 C) and fluo (fig. 7D) after 27.5 min of superfusion with buffer B, containing melatonin, indicated that no free radical species were formed or changes in intracellular calcium occurred. Secondly, melatonin did not promote free radical formation or affected intracellular calcium levels at any stage of the superfusion period.

(B) fluorophore: DCDHF

(C) fluorophore: DHR

(D) fluorophore: fluo

		A	B	C
	Superfusion (min)	Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **DCDHF** (% Cells)
Untreated	12.5	100	0	**100**
	14 (12.5+1.5)	100	0	**100**
	27.5	100	0	**100**
Melatonin treated	12.5	100	0	**100**
	14 (12.5+1.5)	100	0	**100**
	27.5	100	0	**100**

	Superfusion (min)	Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **DHR** (% Cells)
Untreated	12.5	100	0	**0**
	14 (12.5+1.5)	100	0	**0**
	27.5	100	0	**0**
Melatonin treated	12.5	100	0	**0**
	14 (12.5+1.5)	100	0	**0**
	27.5	100	0	**0**

	Superfusion (min)	Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **Fluo** (% Cells)
Untreated	12.5	100	0	**0**
	14 (12.5+1.5)	100	0	**0**
	27.5	100	0	**0**
Melatonin treated	12.5	100	0	**0**
	14 (12.5+1.5)	100	0	**0**
	27.5	100	0	**0**

Table 1: The effects of superfusion time and melatonin (50 μmol/l) on cell morphology, DCDHF, DHR, and fluo fluorescence of control oxygenated myocytes.
Results are expressed as percentage of the total number of cells evaluated.
n = 45 cells from three different myocyte preparations.

Column **A**: % viable, rod - shaped myocytes (grade 0 cells)

Column **B**: % viable, morphologically damaged cells (grade 1 cells)

3.3 *Effects of chemical hypoxia and reoxygenation on viability and free radical production in isolated myocytes*

In this series of experiments, the effects of chemical hypoxia (12.5 min) and reoxygenation (1.5-30 min) were evaluated. Confocal images were recorded at the beginning of chemical hypoxia, at the end of chemical hypoxia and after 1.5, 5, 15 and 30 min of reoxygenation.

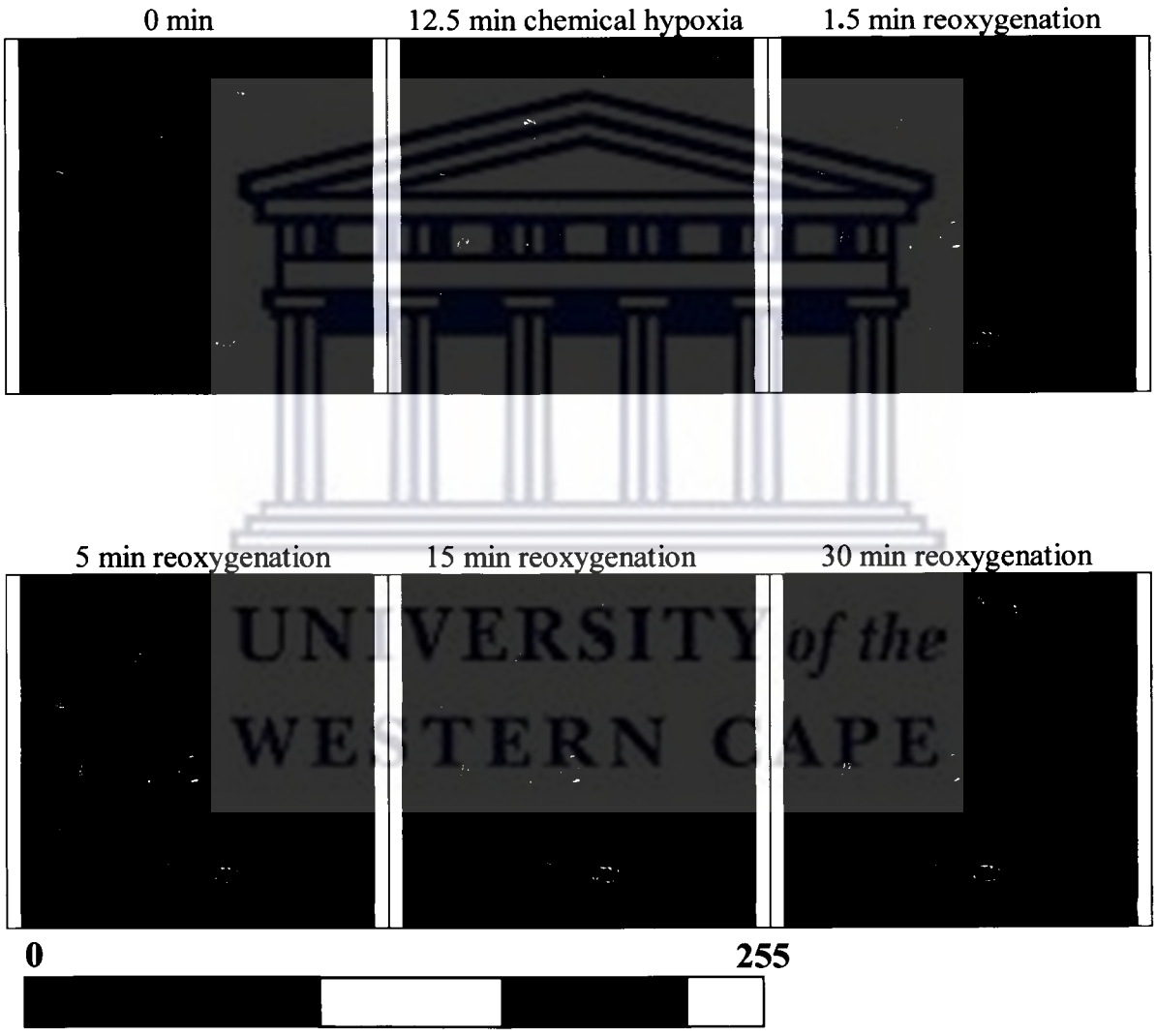
At the beginning of chemical hypoxia, the majority of cells were elongated and viable (grade 0 cells). After 12.5 min chemical hypoxia the larger percentage (70-89 %) of cells became contracted and damaged (grade 1 cells) but remained viable as indicated by the continued TMRM fluorescence (table 2 and fig. 9A), illustrating the maintenance of membrane potential. No further changes were observed during reoxygenation (fig. 9A).

Untreated myocytes were also pre - loaded with the fluorophore, DCDHF to indicate the formation of H_2O_2 and / or ROS. The majority of these cells became damaged (grade 1 cells) and showed high levels of fluorescence, following 12.5 min chemical hypoxia. The fluorescence did not decrease as reoxygenation progressed (fig. 9B).

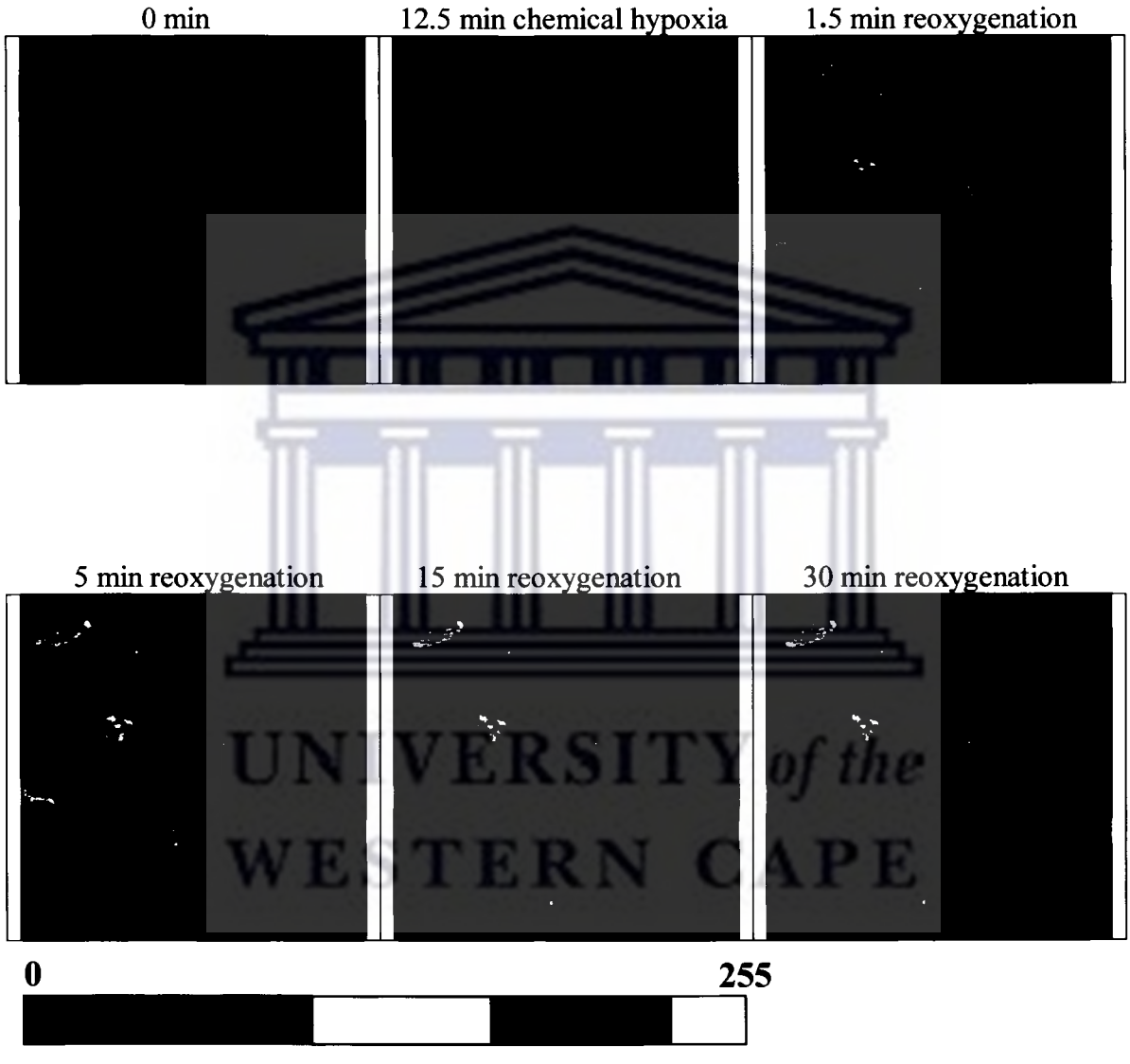
The majority of the untreated cells showed increased fluorescence of the fluorophore, DHR, implicating increased free radical intermediate and / or ROS formation, after 12.5 min chemical hypoxia. This fluorescence decreases rapidly with the onset and progression of reoxygenation (fig. 9C).

Fig. 9: Confocal images of untreated myocytes loaded with TMRM (9A), DCDHF (9B), DHR (9C) and fluo (9D) and exposed to 12.5 min chemical hypoxia followed by 30 min reoxygenation.

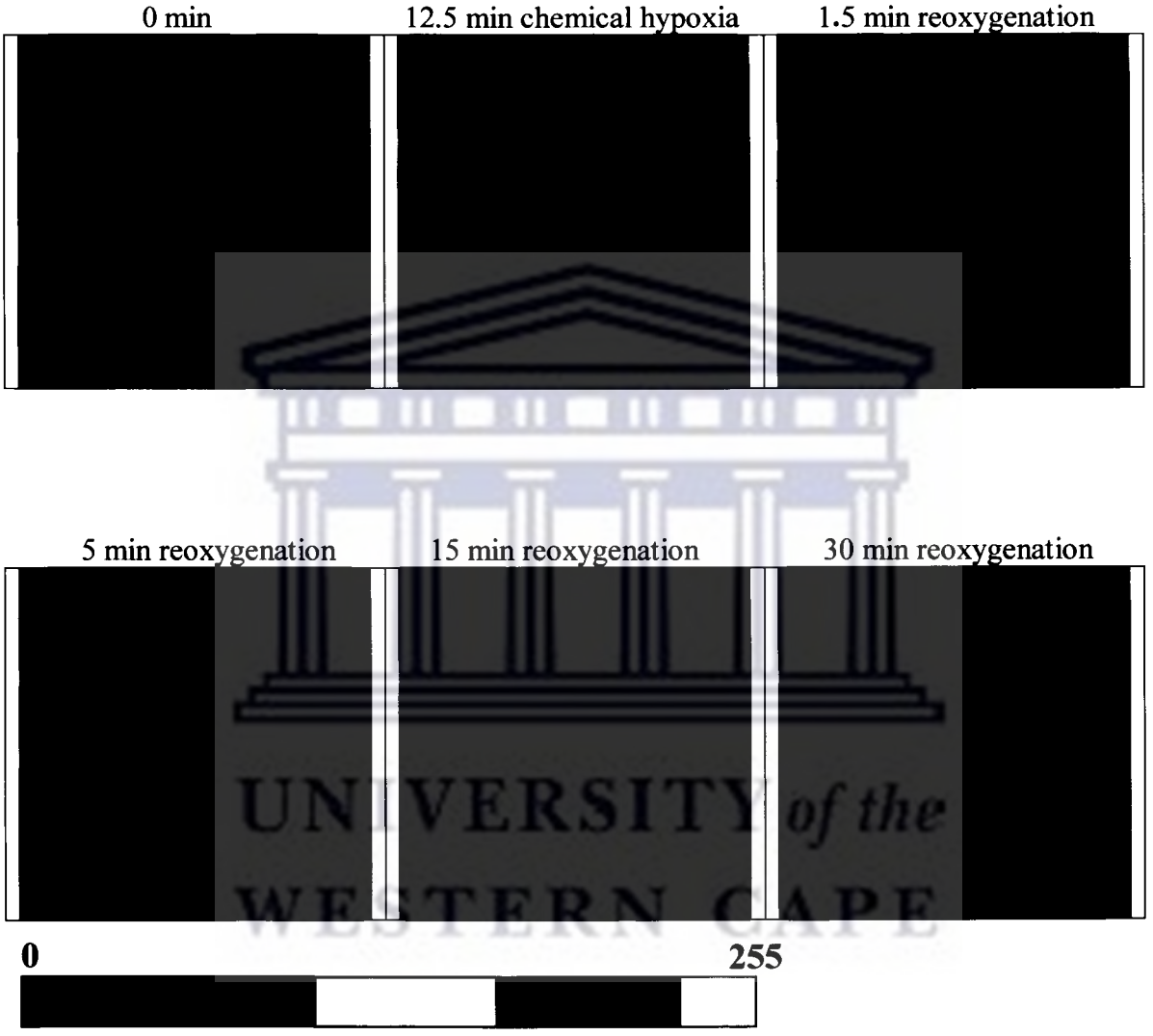
(A) Fluorophore: TMRM



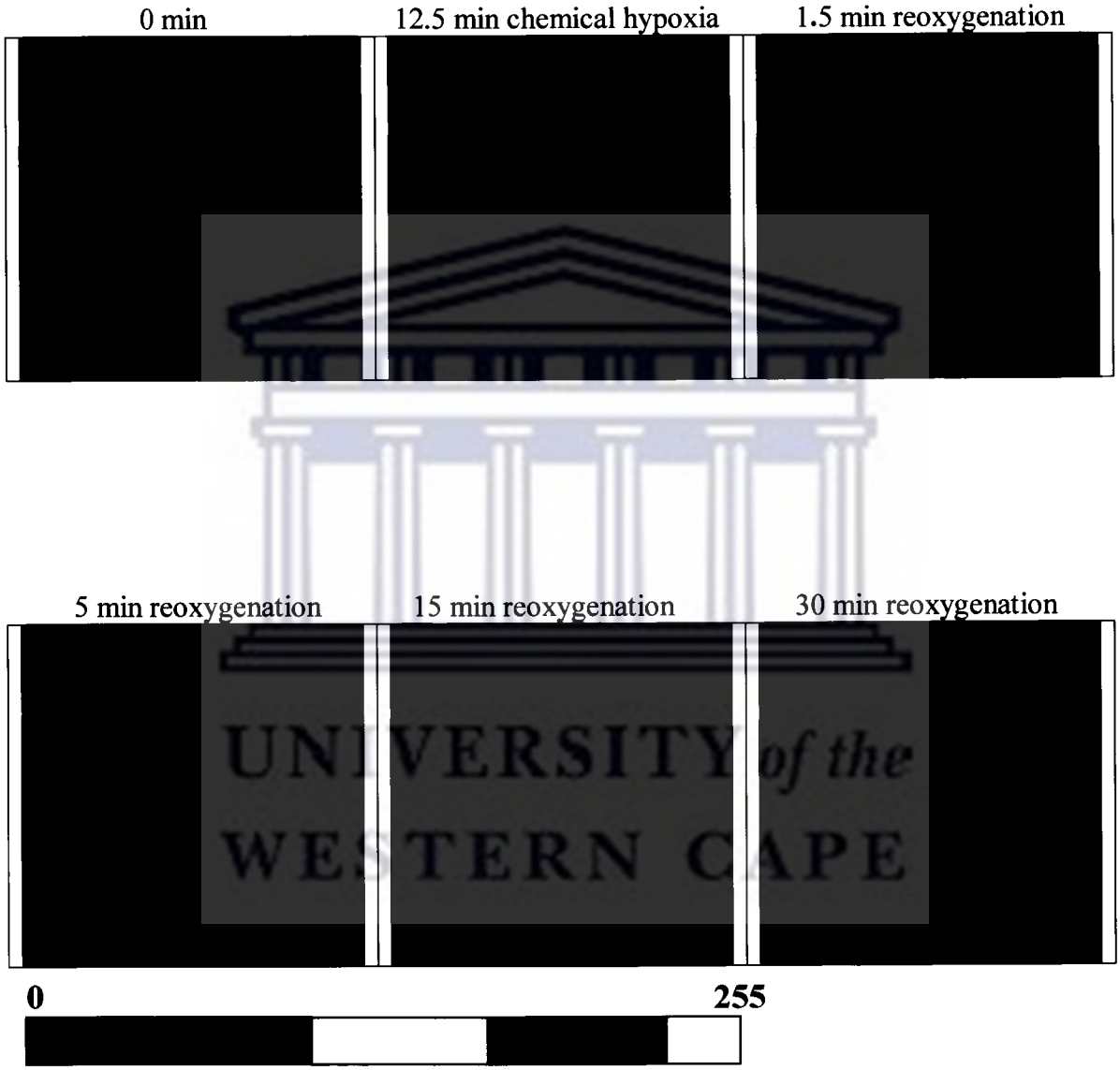
(B) fluorophore: DCDHF



(C) Fluorophore: DHR



(D) Fluorophore: Fluo



3.4 *The effects of melatonin (50 μM), applied during chemical hypoxia, on viability and free radical formation in myocytes exposed to chemical hypoxia and reoxygenation.*

In this series melatonin was applied during chemical hypoxia only. Melatonin treated myocytes, pre - loaded with the fluorophore, DCDHF showed minimal cell damage. However, all the cells (grade 0 and 1 cells) showed increased DCDHF fluorescence (table 2 and fig. 10B).

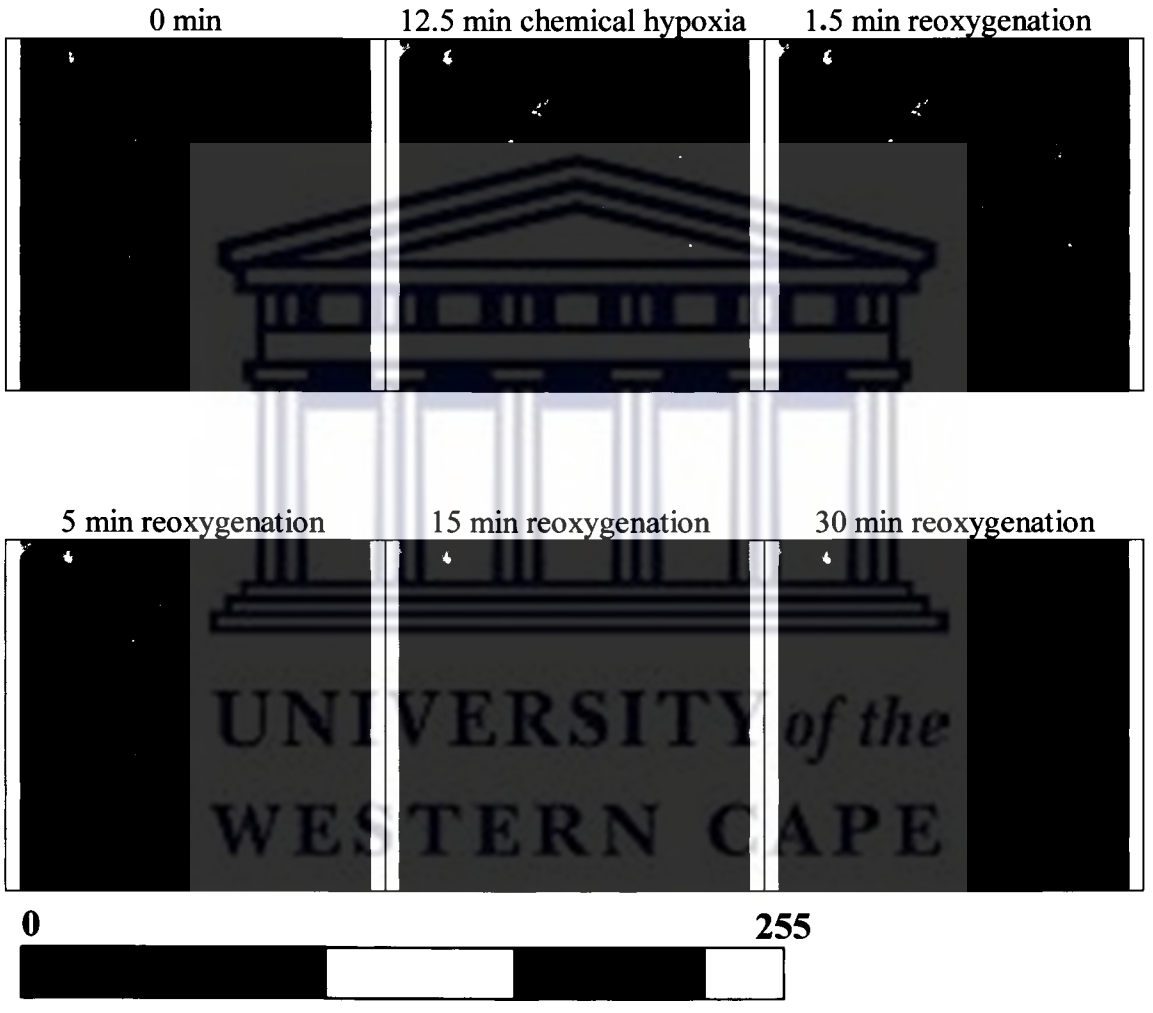
The presence of melatonin significantly reduced the marked fluorescence of DHR seen in the absence of melatonin (fig. 9C) and most cells maintained their viability and rod - shaped morphology.

Changes in intracellular free calcium were also investigated during chemical hypoxia and reoxygenation of melatonin treated cells using the fluorophore, fluo. The majority melatonin treated cells (70 %) illustrated low levels or no fluorescence by the end of chemical hypoxia. Only the few damaged cells showed increased fluorescence at the end of chemical hypoxia (12.5 min) / onset of reoxygenation (1.5 min), which eventually fades with the advancement of reoxygenation (fig. 10D).

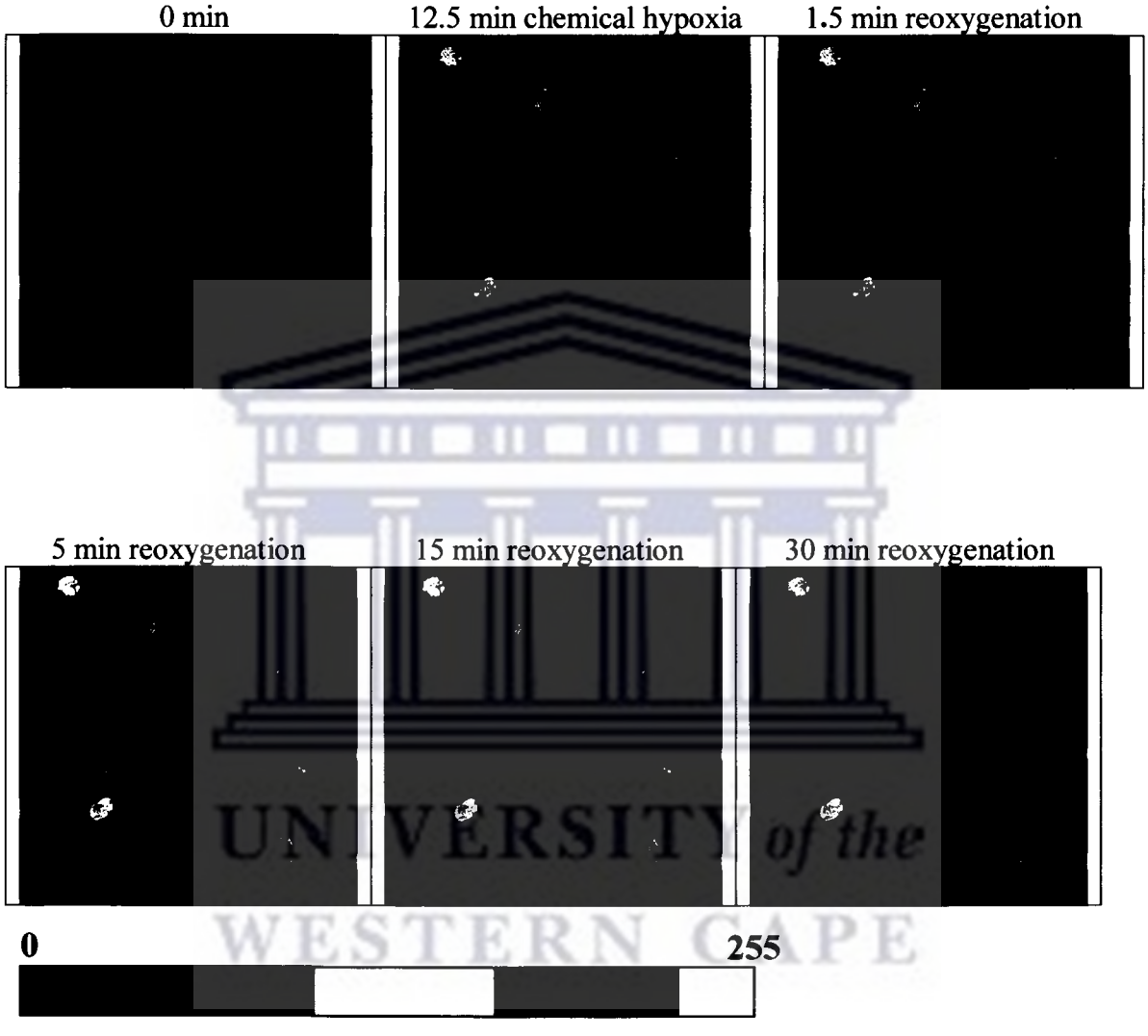
Quantification of the results showed that melatonin treatment significantly reduced the percentage grade 1 cells with a concomitant increase in grade 0 cells, regardless of the fluorophore used. With both DHR and fluo, melatonin not only reduced the number of grade 1 cells, but also caused a reduction in the intensity of fluorescence (fig. 11A and B). The number of cells showing increased fluorescence with DCDHF as well as the intensity thereof, remained unaltered in the presence of melatonin (table 2, fig. 11A and B).

Fig. 10: Confocal images of melatonin treated myocytes preloaded with TMRM (10A), DCDHF (10B), DHR (10C) and fluo (10D) and exposed to 12.5 min chemical hypoxia followed by 30 min reoxygenation. Cells were exposed to melatonin during chemical hypoxia only.

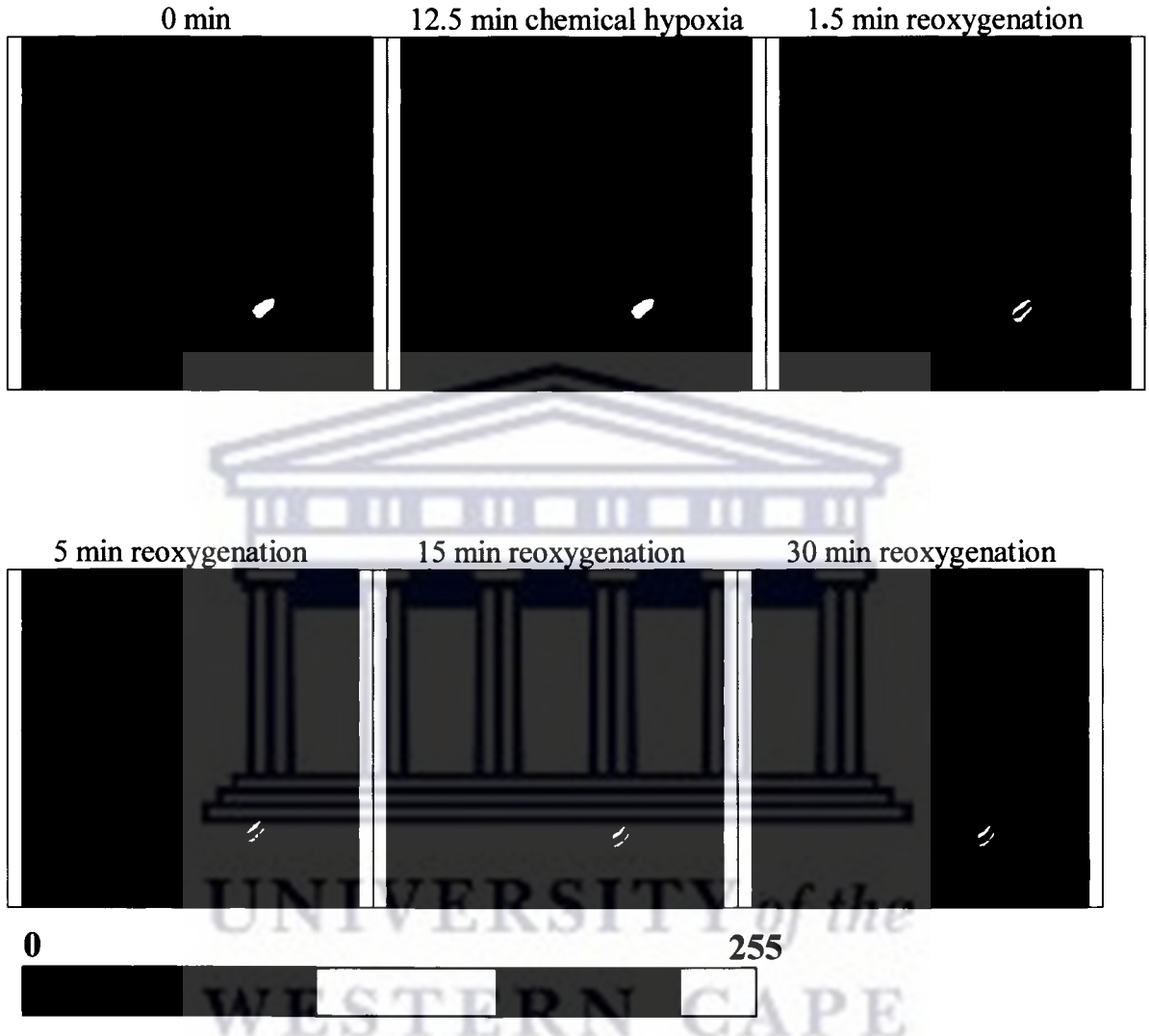
(A) Fluorophore: TMRM



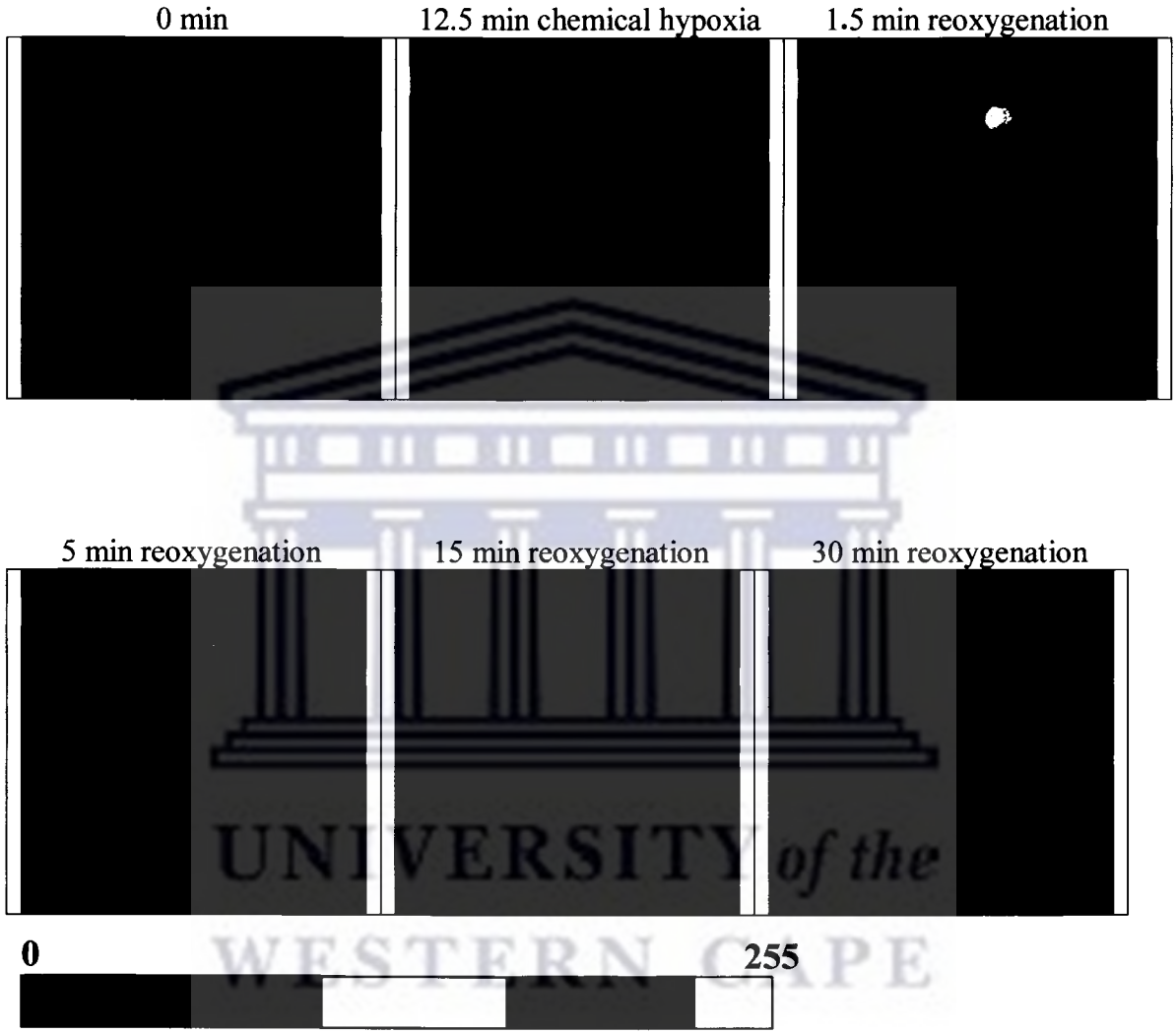
(B) Fluorophore: DCDHF



(C) Fluorophore: DHR



(D) Fluorophore: Fluo



		A	B	C
		Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **DCDHF** (% Cells)
Untreated	End Chemical hypoxia	22±2.8	78±2.3	100
	Onset Reoxygenation	22±2.8	78±2.3	100
Melatonin treated	End Chemical Hypoxia	80±3.1	20±3.1	90±3.6
	Onset Reoxygenation	80±3.1	20±3.1	96±3.2

		Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **DHR** (% Cells)
Untreated	End Chemical hypoxia	11±3.9	89±3.7	89±3.7
	Onset Reoxygenation	11±3.9	89±3.7	89±3.7
Melatonin treated	End Chem. Hypoxia	74±3.7	26±3.2	26±3.2
	Onset Reoxygenation	74±3.7	26±3.2	26±3.2

		Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **Fluo** (% Cells)
Untreated	End Chemical hypoxia	30±1.6	70±1.6	85±1.6
	Onset Reoxygenation	30±1.7	70±1.7	85±1.6
Melatonin treated	End Chemical hypoxia	70±1.6	30±1.6	30±1.6
	Onset Reoxygenation	70±1.6	30±1.6	30±1.6

Table 2: The effects of melatonin (50 μM) on viability and free radical formation in myocytes exposed to chemical hypoxia and reoxygenation. Melatonin was applied during chemical hypoxia only. Results are expressed as percentage of the total number of cells evaluated (mean ± sem).
n = 75 cells were evaluated from five separate myocyte preparations made on different days.

Column **A**: % viable, rod - shaped myocytes (grade 0 cells)

Column **B**: % viable, morphologically damaged cells (grade 1 cells)

3.5 *The effects of melatonin (50 μM), applied during chemical hypoxia and reoxygenation, on viability and free radical formation in myocytes exposed to chemical hypoxia and reoxygenation*

In this series of experiments melatonin was applied from the onset of chemical hypoxia and continued until the end of 30 min reoxygenation. Since prolongation of reoxygenation from 1.5 to 30 min had no detrimental or beneficial effects, quantification of data was made at the end of chemical hypoxia and after 1.5 min of reoxygenation.

As before, melatonin treated myocytes, indicated minimal cell damage after exposure to chemical hypoxia (12.5 min) and reoxygenation (30 min) (fig. 12A-12D and table 3).

All the cells (grade 0 and 1) showed increased DCDHF fluorescence after 12.5 min chemical hypoxia, which did not appear to have had any effect on the viability or rod - shaped morphology of grade 0 cells neither did it cause further deterioration of grade 1 cells. The fluorescence remained unchanged at the onset and for the duration of the reoxygenation period (fig. 12B and table 3).

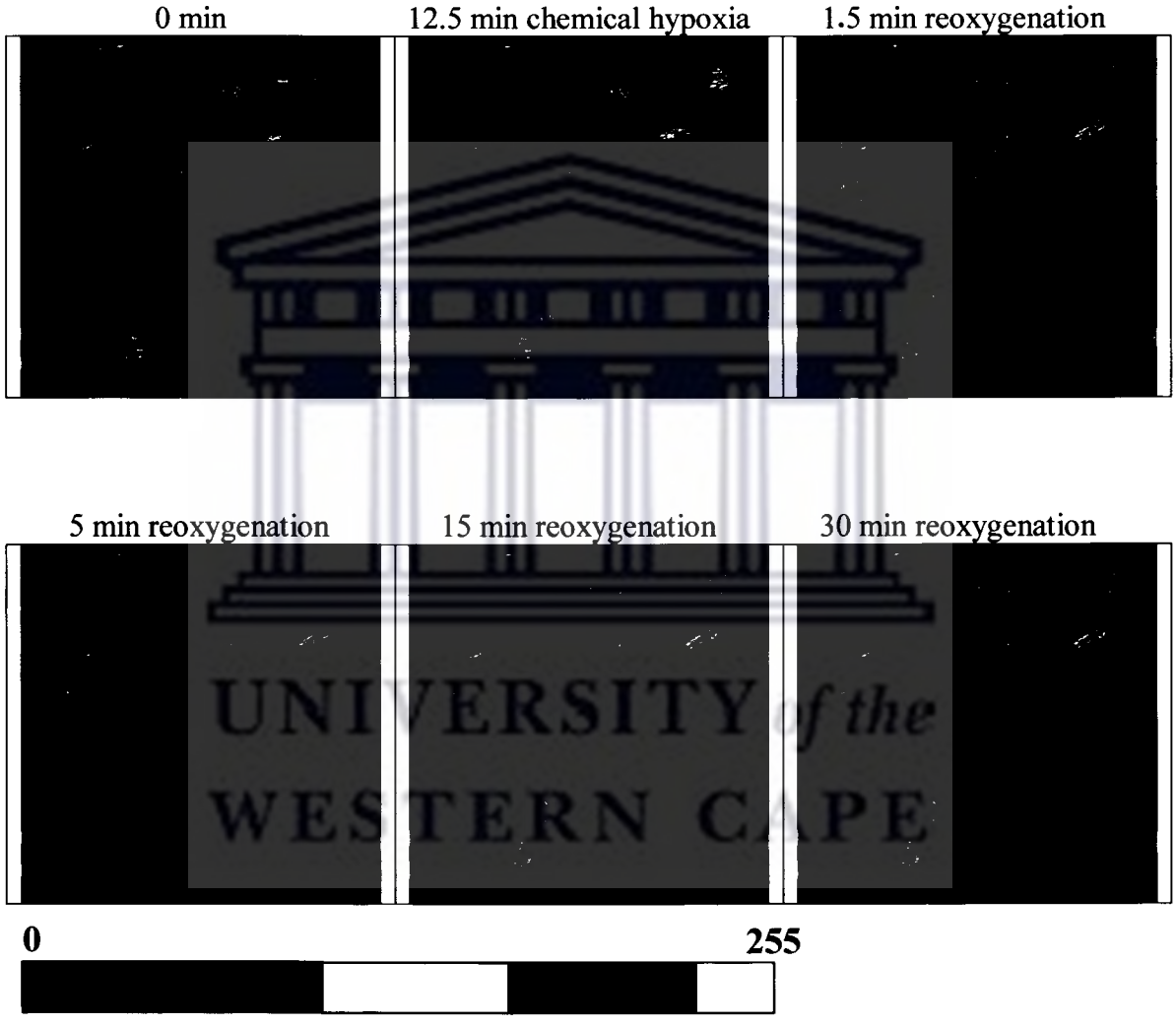
The few melatonin treated cells that were damaged during 12.5 min chemical hypoxia showed increased DHR or fluo fluorescence whereas the rest of the cells remained viable, maintained its rod - shaped morphology and showed very low levels or no fluorescence (fig. 12C, 12D and table 3). The fluorescence exhibited by the majority of myocytes, implicate no H_2O_2 and / or ROS formation and normal intracellular calcium levels respectively in the presence of melatonin.

Quantification of the results showed (table 2) that as before the presence of melatonin reduced the percentage grade 1 cells at the end of chemical hypoxia from ~ 80 to 25 %. Reoxygenation did not exacerbate cellular damage (fig. 13A).

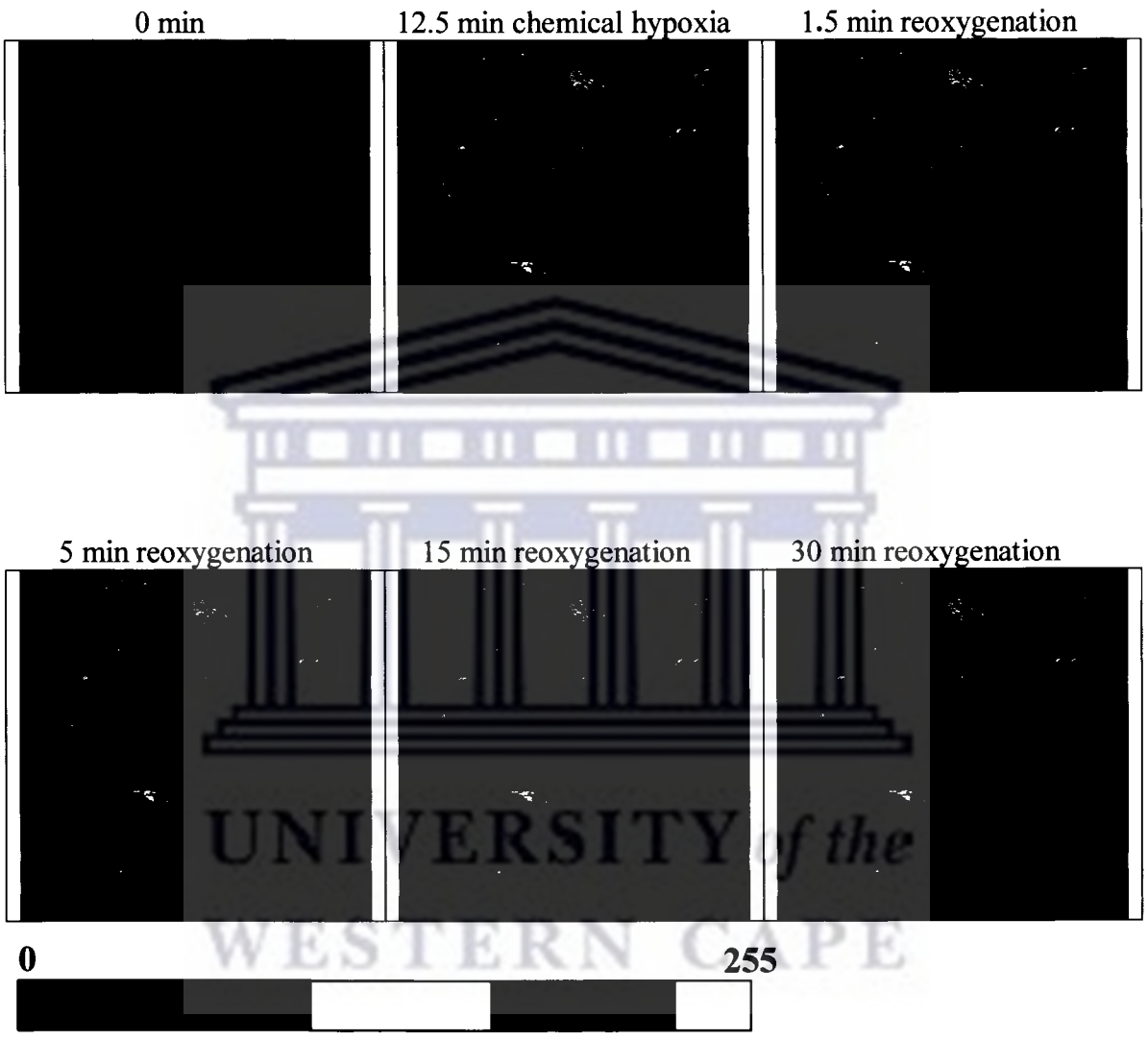
The intensity of fluorescence was also significantly reduced in the case of DHR and fluo (fig. 13C and 13D) at the end of chemical hypoxia and after 30 min reoxygenation. As before, the intensity of DCDHF fluorescence remained unaltered (fig. 13B).

Fig. 12: Confocal images of melatonin treated myocytes preloaded with TMRM (12A), DCDHF (12B), DHR (12C), fluo (12D) and exposed to 12.5 min chemical hypoxia followed by 30 min reoxygenation. Cells were exposed to melatonin throughout the experimental period.

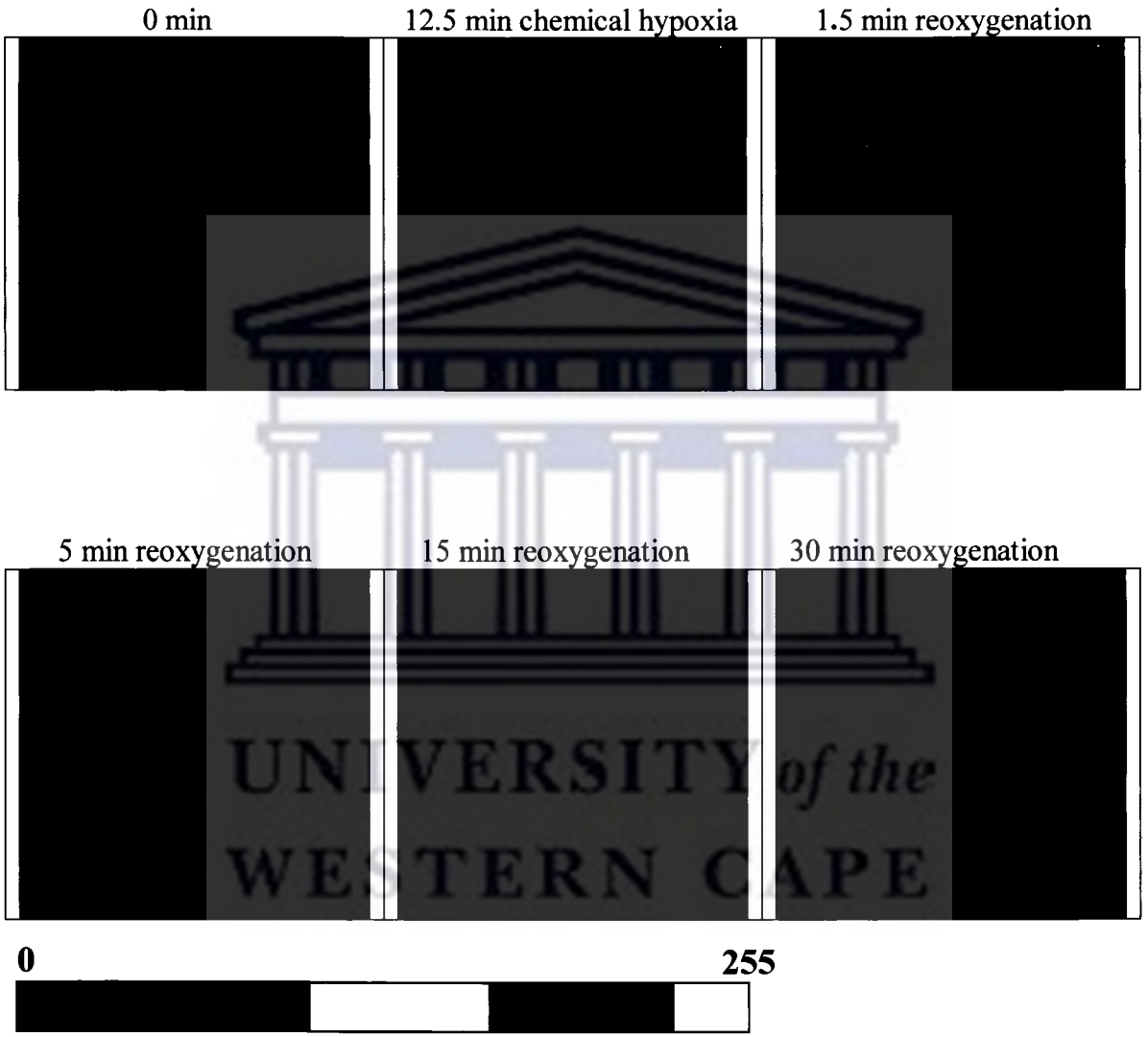
(A) Fluorophore: TMRM



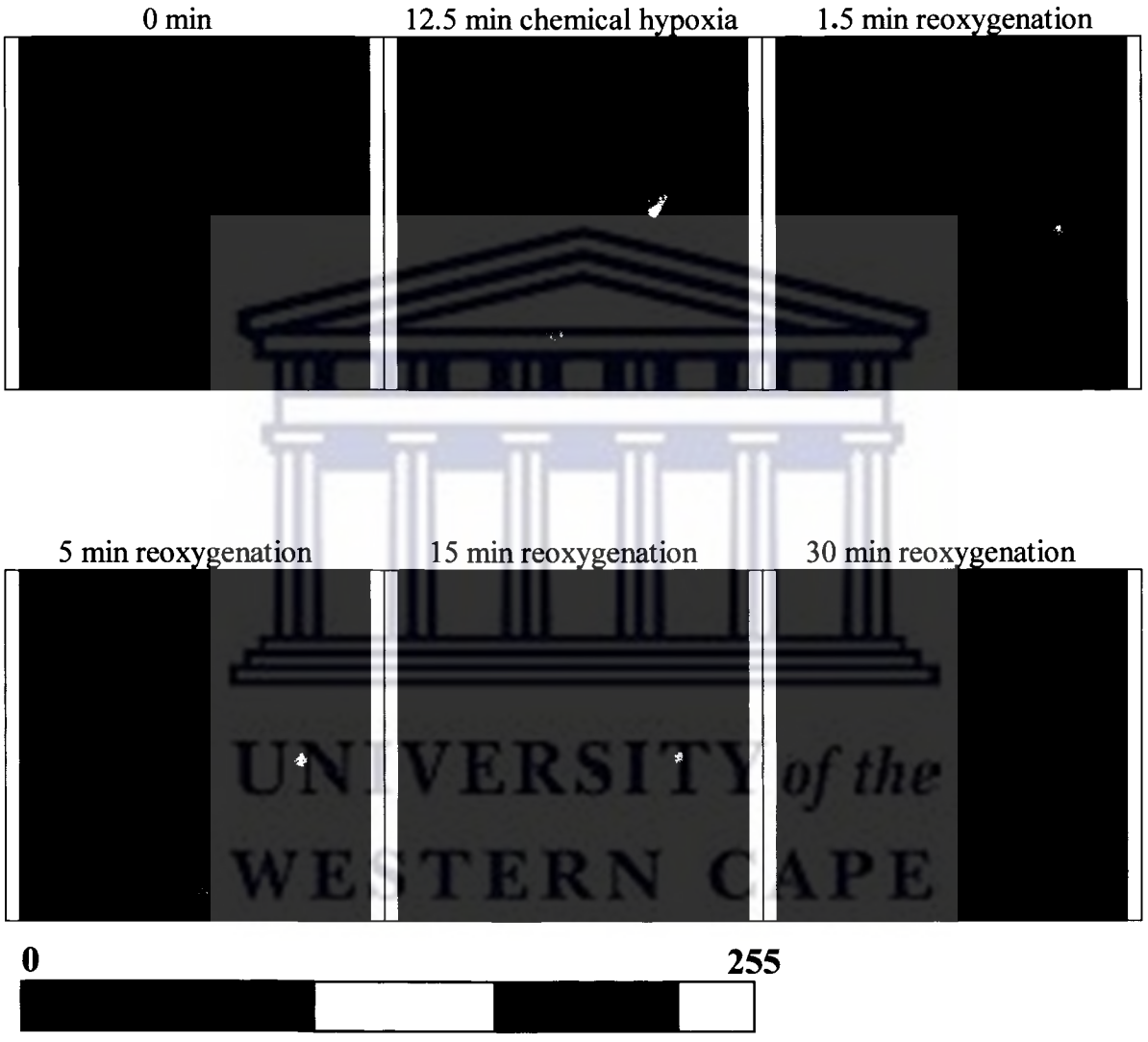
(B) Fluorophore: DCDHF



(C) Fluorophore: DHR



(D) Fluorophore: Fluo



		A	B	C
		Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **DCDHF** (% Cells)
Untreated	End Chemical hypoxia	25±3.8	75±3.4	100
	Onset Reoxygenation	21±2.8	79±3.5	100
Melatonin Treated	End Chemical Hypoxia	79±3.3	21±3.3	100
	Onset Reoxygenation	74±2.6	26±2.6	100

		Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **DHR** (% Cells)
Untreated	End Chemical hypoxia	25±3.5	75±3.5	75±3.5
	Onset Reoxygenation	21±3.2	79±3.5	79±3.5
Melatonin treated	End Chem. Hypoxia	76±3.8	24±3.6	24±3.6
	Onset Reoxygenation	75±3.8	25±3.6	25±3.6

		Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **Fluo** (% Cells)
Untreated	End Chemical hypoxia	28±2.5	72±2.5	72±2.6
	Onset Reoxygenation	19±2.5	81±2.5	81±2.6
Melatonin treated	End Chemical hypoxia	76±2.8	24±2.7	24±2.7
	Onset Reoxygenation	76±2.8	24±2.7	24±2.7

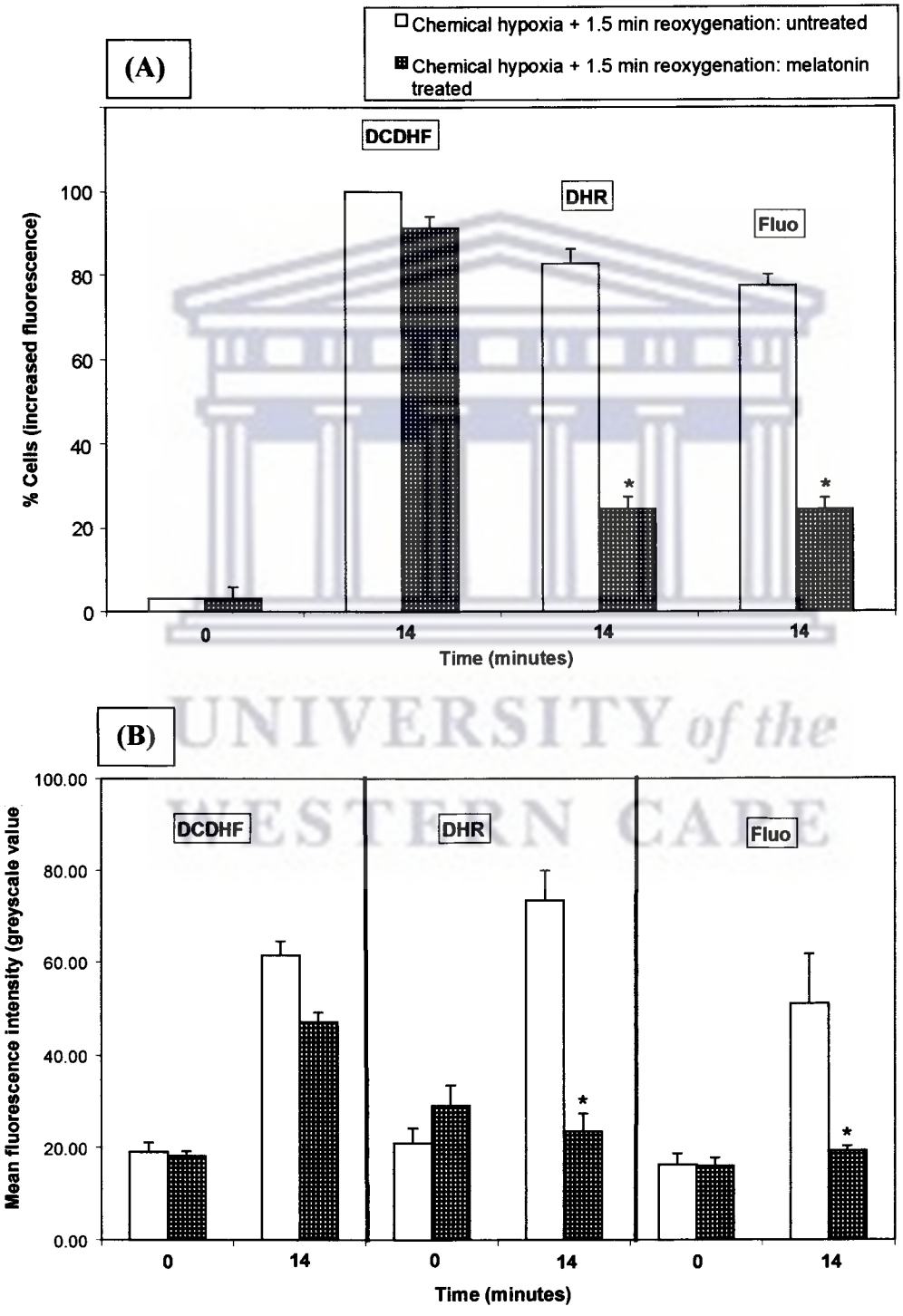
Table 3: The effects of melatonin (50 μM) on viability and free radical formation in myocytes exposed to chemical hypoxia and reoxygenation. Melatonin was applied from the onset of chemical hypoxia and continued until the end of reoxygenation. Quantification of changes was made at the end of chemical hypoxia and after 1.5 min reoxygenation. Results are expressed as percentage of the total number of cells evaluated (mean ± sem).
n = 75 cells were evaluated from five separate myocyte preparations made on different days.

Column **A**: % viable, rod - shaped myocytes (grade 0 cells)

Column **B**: % viable, morphologically damaged cells (grade 1 cells)

Fig. 13: Effect of melatonin treatment of myocytes on DCDHF, DHR and fluo fluorescence during chemical hypoxia and reoxygenation. Myocytes were exposed to melatonin throughout the experiment. (13A) indicates the percentage cells showing increased fluorescence. (13B) indicates the fluorescence intensity.

(*$p < 0.05$ vs untreated)



3.6 *The effects of prolonged chemical hypoxia (27.5 min) without reoxygenation on the viability and free radical formation in isolated myocytes.*

To determine the effects of increasing periods of hypoxia, a number of cell populations were exposed to hypoxic conditions for 27.5 min without reoxygenation. The majority of untreated myocytes pre-loaded with TMRM and DCDHF became contracted and damaged after 12.5 min chemical hypoxia. All the damaged (grade 1) and undamaged (grade 0) cells presented with increased DCDHF fluorescence, implicating increased H_2O_2 and / or ROS formation.

After exposure to 14 (12.5 + 1.5) min chemical hypoxia all cells were damaged and remained so until 27.5 min chemical hypoxia. At this point, some cells suffered irreversible damage, indicated by the loss of TMRM (fig. 14A) and DCDHF fluorescence (fig. 14B) and increased PI nuclear fluorescence (data not shown).

Untreated myocytes, pre - loaded with DHR or fluo, showed similar morphological damage, increased DHR fluorescence (increased H_2O_2 and / or ROS formation) (fig. 14C) and increased fluo fluorescence (intracellular calcium) (fig. 14D) after 12.5 min chemical hypoxia.

The DHR fluorescence indicated by contracted grade 1 cells after 12.5 min chemical hypoxia, decreased as chemical hypoxia progessed, while fluo fluorescence remained elevated.

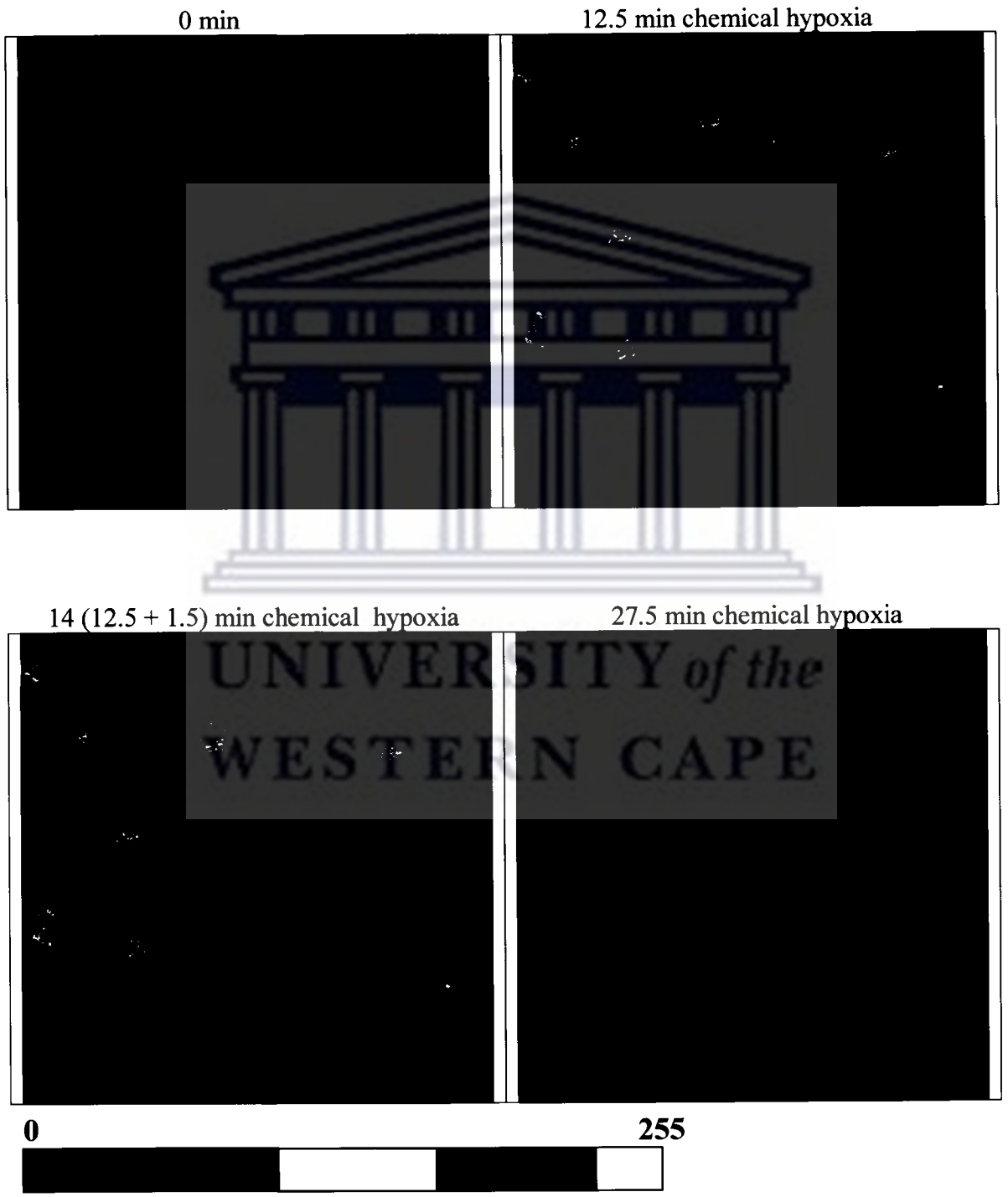
No further changes occurred until after 27.5 min of chemical hypoxia. At this time interval of chemical hypoxia and beyond, some cells suffered irreversible damage, indicated by the complete loss of fluorescence. Quantification of observations, showed that almost all of the cells (95 - 100 %) were grade 1 at 27. 5 min chemical hypoxia and the fluorescence intensity of each fluorophore was significantly higher at 27.5 min than at 12.5 - 14 min (table 4, fig. 16B and 16D).

Fig. 14: Confocal images of untreated myocytes preloaded with TMRM (14A), DCDHF (14B), DHR (14C) and fluo (14D) and exposed to prolonged (27.5 min) chemical hypoxia

(A) fluorophore: TMRM

(B) fluorophore: DCDHF

(C) Fluorophore: DHR



(D) Fluorophore: Fluo

3.7 *The effect of prolonged chemical hypoxia (27.5 min) without reoxygenation on the viability and free radical formation in isolated myocytes in the presence of 50 µmol/l melatonin.*

As previously observed, melatonin treated cells remained viable and morphologically unchanged but showed elevated levels of DCDHF fluorescence, after exposure to 12.5 min chemical hypoxia. However, after 14 min the majority cells were damaged, but maintained their fluorescence (fig.15 B). Similar observations were made after 27.5 min. At this point of chemical hypoxia some cells were irreversible damaged, as suggested by the loss of TMRM fluorescence (fig. 15A).

Melatonin treated myocytes pre - loaded with the fluorophore, DHR did not show any significant increases in fluorescence and the majority of the cells remained viable and morphological unchanged after 12.5 min chemical hypoxia (fig. 15 C). However, the majority of cells were damaged after 14 (12.5 + 1.5) min chemical hypoxia. The cells that were damaged at this stage of chemical hypoxia, showed increased fluorescence, which decreased as chemical hypoxia progressed. No further changes were seen after 27.5 min chemical hypoxia. However, some cells suffered irreversible damage, which was indicated by the loss of fluorescence.

The larger percentage of melatonin treated cells showed no damage or changes in fluo fluorescence (intracellular calcium) after 12.5 min chemical hypoxia (fig. 15D). The subsequent confocal images showed relatively the same changes, as previously found with DCDHF and DHR.

It is clear from the above that melatonin loses its protective effects at 14 min of chemical hypoxia, since at this stage the majority of cells were squared up and remained so until the end of the experiment.

Quantification of the results showed that whereas 23 - 29 % of melatonin treated cells showed increased fluorescence (grade 1) at 12.5 min chemical hypoxia, 100 % of cells (untreated and melatonin treated) were damaged after 27.5 min of chemical hypoxia (table 4, fig. 16A and 16C). In addition, the fluorescence intensity in untreated and melatonin treated myocytes was similarly increased at 27.5 min of chemical hypoxia (fig. 16D).

Fig. 15: Confocal images of melatonin treated myocytes preloaded with TMRM (15A), DCDHF (15B), DHR (15C) and fluo (15D) and exposed to chemical hypoxia.

(A) Fluorophore: TMRM

(B) Fluorophore: DCDHF

(C) Fluorophore: DHR

(D) Fluorophore: Fluo

		A	B	C
	Chemical hypoxia (min)	Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **DCDHF** (% Cells)
Untreated	12.5	20±4.3	80±4.3	100
	27.5	0	100	100
Melatonin Treated	12.5	77±3.5	23±3.5	100
	27.5	0	100	100

		A	B	C
	Chemical hypoxia (min)	Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **DHR** (% Cells)
Untreated	12.5	20±3.9	80±3.7	80±3.7
	27.5	0	100	100
Melatonin Treated	12.5	76±2.3	24±2.3	24±2.3
	27.5	5±2.8	95±2.8	95±2.8

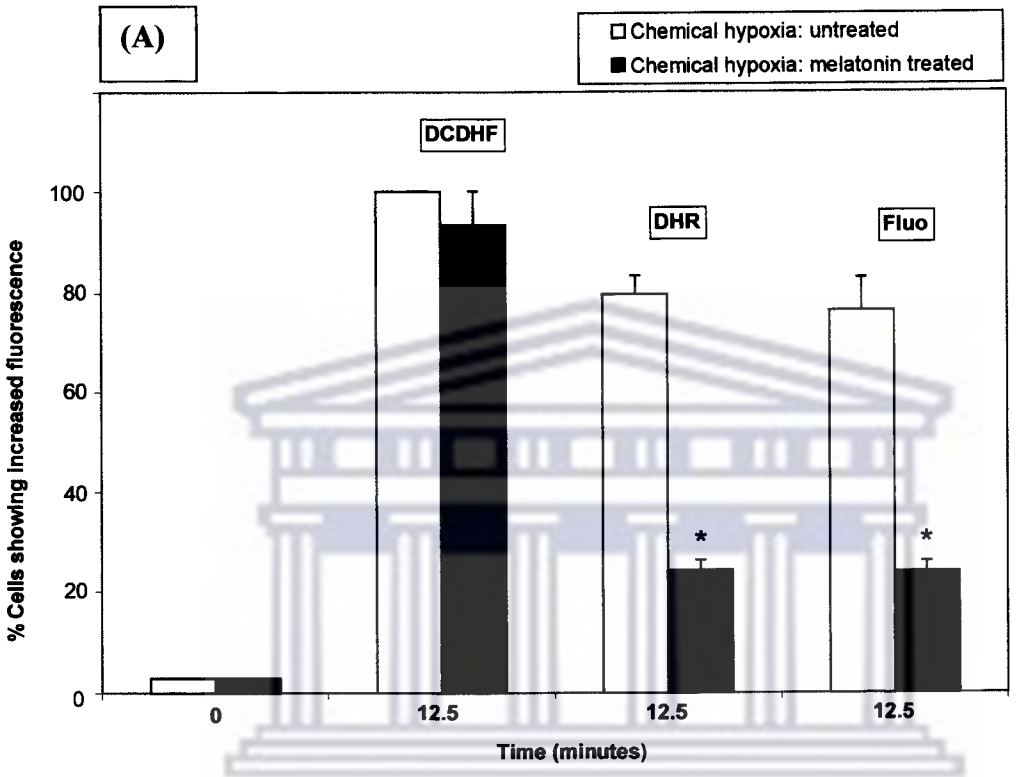
		A	B	C
	Chemical hypoxia (min)	Grade 0 (% Cells)	Grade 1 (% Cells)	Increased **Fluo** (% Cells)
Untreated	12.5	24±3.9	76±3.6	76±3.6
	27.5	5±2.4	95±2.4	95±2.4
Melatonin Treated	12.5	71±2.3	29±2.3	29±2.3
	27.5	0	100	100

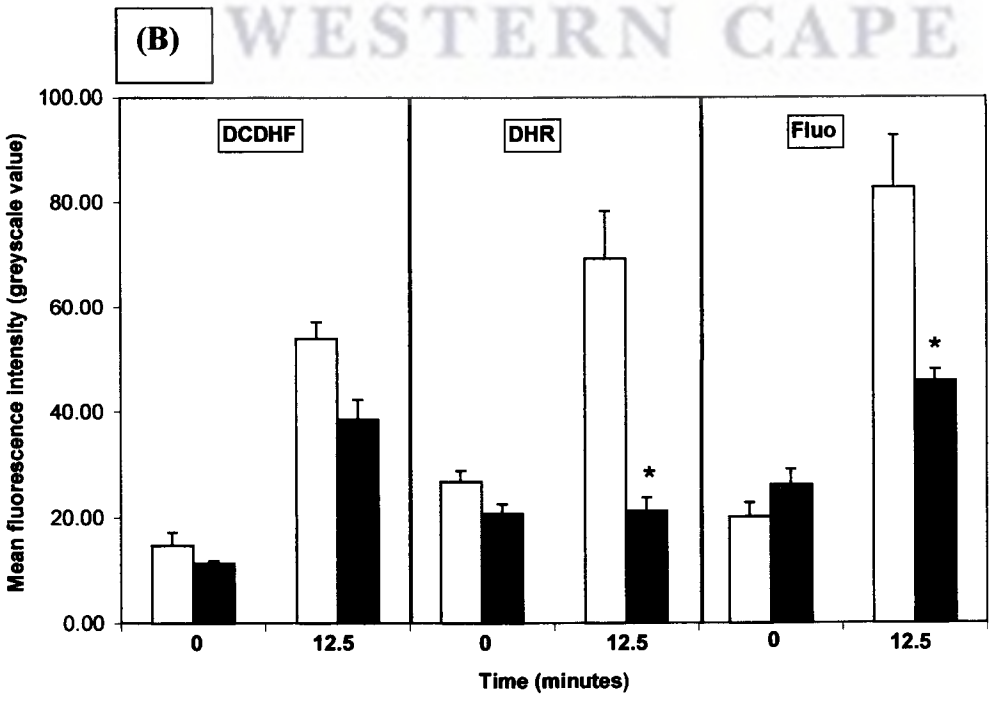
Table 4: The effect of prolonged chemical hypoxia (27.5 min) without reoxygenation on the viability and free radical formation in isolated myocytes in the absence and presence of 50 μmol/l melatonin. Results are expressed as percentage of the total number of cells evaluated (mean ± sem).
n = 45 cells were evaluated from six separate myocyte preparations made on different days.

Column **A**: % viable, rod - shaped myocytes (grade 0 cells)

Column **B**: % viable, morphologically damaged cells (grade 1 cells)

Fig. 16: Effect of melatonin treatment of myocytes on DCDHF, DHR and fluo fluorescence after 12.5 min (A and B) or 27.5 min chemical hypoxia (C and D). (A) and (C) indicates the percentage cells showing increased fluorescence. (B) and (D) show the fluorescent intensity (*$p < 0.05$ vs untreated).





CHAPTER 4

DISCUSSION

The expanse of pathological processes that indicate reactive oxygen species as the felons that contribute to the several forms of tissue damage continues to increase. Currently they include conditions such as apoptosis, ischaemia – reperfusion injury, ageing, inflammation, neuromuscular disorders, cancer, atherosclerosis, and many other phenomena (Jennings and Reimer, 1991; Reynolds and Hastings, 1995; Sarvazjan, 1996). In 1973, Hearse et al. described the phenomenon of hypoxic reoxygenation damage to the myocardium, the so - called oxygen paradox. They demonstrated that reoxygenation of the hypoxic heart resulted in significant damage rather than improvement.

Oxidative stress is described as the phenomenon in which oxidant metabolites can exert their toxic effect because of increased production, and / or because of an altered cellular defence mechanism (Hearse et al., 1973; Hess and Manson, 1984; Ferrari et al., 1990). Thus, increased free radical production cannot be managed by inadequate anti - oxidant mechanisms, which, in turn, greatly depends on the duration of the ischaemic episode (Adams et al., 1983; Curello et al., 1987). With increasing duration of ischaemia prior to reperfusion, the degree of recovery is less pronounced and, in many circumstances, there is no recovery at all with further exacerbation of the biochemical, mechanical and ultrastructural disarrangements induced by ischaemia (Ferrari et al., 1990).

Much research is currently aimed at establishing whether prevention of oxidative stress in a clinical setting would translate into better metabolism and function of myocytes, and whether antioxidant therapy may represent a novel adjunct to established clinical procedures (Ambrosio and Tritto, 1998). It has been shown that oxidative stress can exert major consequences on various aspects of cardiac function, and that hearts receiving interventions aimed at preventing oxidant toxicity scored better on various indices used to measure cell injury (Ambrosio and Tritto, 1998).

Much information has been obtained from a number of studies in which the outcome of hearts exposed to ischaemia - reperfusion was compared to that of hearts that were treated with various antioxidants (Ambrosio and Tritto, 1998). By this approach, it has been possible to dissect out the relative component of myocardial injury due to oxygen deprivation during ischaemia from the possible alterations brought about by oxidative stress upon reflow (Ambrosio and Tritto, 1998).

It was shown that hearts receiving interventions aimed at preventing oxidant toxicity scored better on the various indices (outlined below) of cell injury (Ambrosio and Tritto, 1998).

-Reduction of reperfusion arrhythmias

-Improvement of contractile recovery

-Reduction of vascular injury

-Reduction of cell damage and cell death

Using the rat model of myocardial infarction followed by reperfusion, many investigators employed various antioxidants / free radical scavengers in various combinations with the intention of reducing free radical mediated damage (Meerson et al., 1981; Shlafer et al., 1982; Stewart et al., 1983; Hess et al., 1983; McCord and Fridovich, 1973). There is also an increasing interest in defining new antioxidants of high potency, low toxicity and good solubility properties in aqueous as well as organic phases.

Recently it has been shown that melatonin fulfilled most of the foregoing criteria of an ideal antioxidant. Melatonin is described to be a highly efficient scavenger of the hydroxyl ion and peroxyradical, significantly reducing the generation of reactive oxygen species (Reiter et al., 1993; Reiter, 1995). The high solubility of melatonin in lipid and aqueous media allows the indole ready access to all the cellular and subcellular compartments (Reiter et al., 1993). In vitro studies of melatonin have shown that it effectively neutralised the hydroxyl radical, to be more efficient than glutathione and mannitol (Tan et al., 1993b) or vit E (Pieri et al., 1994). Melatonin also stimulates glutathione peroxidase (Barlow-Walden et al., 1995) and inhibits NOS (Pozo et al., 1994) activity.

4.1 *Experimental model*

Although a number of potential sources of free radical generation exist in the heart, the contribution of the myocyte per se to oxidative stress during hypoxia - reoxygenation is unknown.

To avoid extramyocardial factors contributing to reoxygenation injury, such as the presence of noncardiac cells, neural stimulation, changes in coronary flow and circulatory catecholamines, the isolated myocyte serves as an excellent model to investigate the condition of oxidative stress (Khalid and Ashraff, 1993).

Simulation of ischaemia in isolated myocytes can be achieved by exposure to cyanide and deoxyglucose which causes rapid and profound ATP depletion and respiratory inhibition as occurs during hypoxia or ischaemia (Zahrebelski et al., 1995). This type of metabolic inhibition, called chemical hypoxia, induces the condition of oxidative stress, which is usually associated with cell damage and ultimately, cell death.

The short episode of chemical hypoxia / metabolic inhibition and reoxygenation applied to isolated ventricular myocytes in this study, was used specifically to induce the condition of oxidative stress associated cell damage and not cell death (fig. 9A). After 27.5 min of chemical hypoxia, 95 - 100 % of cells showed marked morphological changes and were considered to be lethally and irreversibly damaged, but were still viable as indicated by the TMRM fluorescence (fig. 14A).

4.2 *Detection of free radicals*

In the study of oxygen free radical mediated damage, various methods, such as direct electron paramagnetic resonance spectroscopy or identification of the products of lipid peroxidation, particularly malondialdehyde have been used.

However, these methods have several limitations (Ogura et al., 1991; Khalid and Ashraf, 1993; Wolin and Belloni, 1985; Halliwell, 1984) and are prone to several artefacts and unable to accurately assess the subcellular patterns of free radical generation and changes in intracellular free calcium in living cells. Despite these shortfalls, sufficient evidence has accumulated indicating their role in the pathogenesis of cell injury under different pathological conditions.

Laser scanning confocal microscopy combined with oxidant-sensitive fluorescent probes, is perhaps preferable to the above techniques. Fluorescent probes can be used in living cells with minimal disruption of cellular functions and are compatible with most fluorescent instrumentation, providing a powerful tool for the investigation of respiration, mitosis, substrate degradation and more.

Despite the obvious advantages of monitoring events in a living cell, assessment of oxidative stress in living cells with fluorogenic chemiluminescent or chromogenic probes is complicated by the presence of multiple forms of reactive oxygen species in the same cell. In addition, experimental detection and quantitative analysis of free radicals using fluorescent probes has proved difficult due to:

(i) the extreme reactivity of free radicals, which is associated with a short life-span and low concentrations under physiological conditions;

(ii) the high intracellular concentration of glutathione which can reduce oxygen species;

(iii) the variable concentration of metals which can catalyse or inhibit free radical reactions;

(iv) the presence of other free radical-quenching agents such as spermine.

Intracellular damage would obviously result in changes in mitochondrial and subsequent sarcolemmal membrane potential and TMRM fluorescence would change accordingly. Complete loss of membrane potential / cell viability could result in complete loss of fluorescence, which would be confirmed by the positive PI nuclear staining.

The use of fluorogenic probes used for the detection of free radical intermediates and / or ROS or overall oxidative stress has been questioned (as shown in numerous studies below) since they may not easily discriminate between the various reactive oxygen species (molecular probes catalogue, section 21.4, 1999).

Both DCDHF and DHR can be used for the detection of ROS in cells (Black and Brandt, 1974). These dihydro - derivatives, pass passively across cell membranes and accumulate into mitochondria in response to the negative inside $\Delta\Psi$ (Johnson et al., 1981; Ehrenberg, 1986). They are readily oxidised back to the parent dye by reactive oxygen species and can thus serve as fluorogenic probes for the detection of oxidative activity in cells and tissues (Zhu et al., 1994; Lebel et al., 1992).

The cell - permeant DCDHF commonly used to detect the generation of reactive oxygen intermediates in neutrophils and macrophages (Yuan et.,1993) may also be extremely useful for assessing overall oxidative stress in toxicological phenomena (Oyama et al., 1994; LeBel et al., 1992).

DHR has been routinely used to investigate the production of reactive oxygen species in activated rat mast cells (Tsinkalovsky and Laerum, 1994) and cultured endothelial cells (Royall and Ischiropoulus, 1993).

Like DCDHF, DHR does not directly detect superoxide but rather reacts with hydrogen peroxide (Henderson and Chappell, 1993) in the presence of peroxidase, cytochrome c or Fe^{2+} (LeBel et al., 1992; Royall and Ischiropoulus, 1993). However, DHR has been reported to be more sensitive than DCDHF for detecting granulocyte respiratory burst (Vowells et al., 1995; Roth et al., 1988) ordinarily defined as the rapid formation and release of ROS and free radical intermediates. In contrast, DCDHF appears to be more sensitive for H_2O_2 than DHR: studies from our own laboratory showed that H_2O_2 at a concentration of 50 μM caused marked fluorescence of DCDHF while having no affect on DHR fluorescence. Therefore, although it is generally accepted that both these fluorophores react with H_2O_2, they definitely differ with regard to their specificity to this compound.

In this study it was not attempted to assess the specificity of these two probes for the different reactive oxygen species generated during simulated ischaemia in isolated myocytes, but rather to use them in a comparative study to assess the effects of chemical hypoxia and melatonin on these cells. The results obtained indicated definite differences between these probes: DHR does not appear to interact with H_2O_2, since the marked fluorescence observed with DCDHF as probe in oxygenated cells was completely absent when DHR was used.

The absence of increased H_2O_2 and reactive oxygen species generation during reoxygenation after chemical hypoxia, is in contrast with the generally accepted view that free radical generation occur mainly within the first seconds of reoxygenation (Ferrari et al., 1985; Jolly et al., 1984; Ambrosio and Tritto, 1986; Ferreira et al., 1989).

In 1993, Khalid and Ashraf also demonstrated increased fluorescence of DCDHF only during the first 90 seconds of reoxygenation of isolated anoxic adult myocytes. This may be due to the different models employed to simulate ischaemia - reperfusion (anoxia vs chemical hypoxia): 12.5 min chemical hypoxia caused a ~ 80 % reduction in the number of rods compared to only 20 % after 15 min anoxia. In a comparative study Vanden Hoek and co - workers (1996) also showed that loss of cell viability was significantly higher during chemical hypoxia than during hypoxia of neonatal myocytes, with less effect of reoxygenation in the former model.

4.6 *Hypoxia – reoxygenation and the application of melatonin*

To date, the ability of melatonin to protect against oxidative modification of lipids, proteins and DNA has been reported (Reiter, 1993; Reiter et al., 1995; Reiter et al., 1997). The bulk of these studies used pharmacological concentrations of melatonin. It appears that the role of physiological concentrations of melatonin in antioxidative defence has not been thoroughly investigated. Also in this study pharmacological concentrations of melatonin (50-100 μM) were used. In other in vitro studies, millimolar concentrations of melatonin were employed (Siu et al., 1988; Sewerynek et al., 1995).

The antioxidant mechanisms of melatonin seem different from classical antioxidants such as vitamin C, vitamin E and glutathione. Melatonin, as an electron - rich molecule, may interact with free radicals via an additive reaction to form stable end-products which are excreted in the urine. Melatonin can therefore be considered a terminal antioxidant (Tan et al., 2000).

In addition to its scavenging actions, melatonin also stimulates the antioxidant enzymes, glutathione peroxidase and superoxide dismutase (Okatani et al., 2000) while inhibiting NOS activity (Reiter et al., 1997). Evidence has also recently been presented to suggest involvement of melatonin in the oxidative status of the heart (Chen et al., 1994).

Although it is generally accepted that oxygen - derived free radicals are generated, amongst others, by mitochondria particularly during post - ischaemic reperfusion (Ferrari et al., 1985; Abrosio et al., 1986), the results obtained in this study showed significant amounts of free radicals generated during chemical hypoxia per se, while reoxygenation had no further effect (fig. 9B and C). Since mitochondrial respiration is completely abolished in the presence of KCN, the source of free radical generation in this particular model remains to be established.

Finally, although melatonin's ability to protect the hypoxic myocyte may involve as yet unidentified properties of the molecule, it is very likely that the results obtained in this study are due to its free radical scavenging properties. This is substantiated by a recent study where melatonin at a concentration of 100 μM effectively reduced hydroxyl radical generation and lipid peroxidation in ischaemic - reperfused isolated rat hearts (Kaneko et al., 2000).

4.7 Summary

Fluorescent intensity studies of the different fluorophores showed that the change in fluorescent signal from one time period to another in any experimental condition was indeed significant (fig. 11B; 13B; 16B and 16D). The fluorophores, DCDHF and DHR in preloaded adult myocytes illustrated the formation of H_2O_2 and / or ROS during chemical hypoxia / reoxygenation and prolonged chemical hypoxia without reoxygenation. It was also shown that DCDHF and DHR may illustrate different free radical entities because of the different fluorescent signals obtained under the same experimental conditions.

DCDHF indicated continuous, increased fluorescence during normoxic conditions, chemical hypoxia and reoxygenation. In addition, all cells (damaged and undamaged) indicated increased DCDHF fluorescence which is more indicative of H_2O_2 than ROS.

Increased DHR fluorescence, seen only during chemical hypoxia and early reoxygenation, was associated with morphological changes (fig. 9C). This could indicate the generation of ROS (rather than H_2O_2) under these conditions. However, the gradual disappearance of fluorescence during reoxygenation, suggest the generation of ROS is transient. The fluorescent pattern of DHR under these conditions could also be due to leakage of the fluorophore out of the cell, which is unlikely, or conversion of reactive oxygen species to other free radicals but this remains to be elucidated.

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