Chapter 6: Investigating the Cellular Effects of Triton X-100 and CoQ10, using Confocal Microscopy

6. 1 Introduction

Seeing a cell is an essential aspect of cell biology. To the small world of the cell, confocal microscopy is a major advance upon normal light microscopy since it allows one to visualize not only deep into cells and tissues, but to also create images in three dimensions (Hibbs, 2000). A confocal microscope creates sharp images of a specimen that would otherwise appear blurred when viewed with a conventional microscope (Semwogerere *et al*., 2005). The confocal microscope incorporates the ideas of point-by-point illumination of the specimens and rejection of out-of-focus light. One drawback with imaging a point onto the specimen is that there are fewer emitted photons to collect at any given instant (Semwogerere *et al*., 2005). Thus, to avoid building a noisy image each point must be illuminated for a long time to collect enough light to make an accurate measurement (Minsky, 1988). The solution is to use a laser light source. The ability of the microscope to create sharp optical sections makes it possible to build 3D renditions of the specimen. Data gathered from a series of optical sections imaged at short and regular intervals along the optical axis are used to create the 3D reconstructions.

Fluorescent probes were first used to reveal cell morphology and biochemistry in static conditions in the same manner as classical electron microscopy studies are used to assess cell ultrastructure (Bkaily *et al*., 1997). In recent years, numerous fluorescent probes have been developed which selectively label macromolecules or interact with specific sites within the cell. The advantage of these site selective probes is that they can be used to investigate structure and activity inside living cells with minimal disruption of cellular function (Lemasters *et al*., 1993). The advent of confocal microscopy has not only made it possible to determine biological structure in three dimensions, but more importantly, has enabled investigators to correlate dynamic processes or events in relation to specific subcellular structures through dual and/or multiple labelling (Bkaily *et al*., 1997). Among the most important aspects of fluorescence confocal microscopy is the choice of fluorescent probe (fluorophore). It is typically influenced by several factors

(Semwogerere *et al*., 2005). The fluorophore should tag the correct part of the specimen. It must be sensitive enough for the given excitation wavelength. For living specimens it should not significantly alter the dynamics of the organism; and an extra consideration is the effect of the specimen on the fluorophore – its chemical environment can affect the position of the peaks of the excitation and emission spectra (Sheppard *et al*., 1997). A major problem with fluorophores is that they fade irreversible when exposed to excitation light. Although the process is not completely understood, it is believed in some instances to occur when fluorophore molecules react with oxygen and/or oxygen radicals and become non-fluorescent (Becker , 1996 and Chen *et al*., 1995). The reaction can take place after a fluorophore molecule transitions from the singlet excited state to the triplet excited state. Although the fraction of fluorophores that transitions to the triplet state is small, its lifetime is typically much longer than that of the singlet state. This can lead to significant triplet state fluorophore population and thus to significant photobleaching (Semwogerere *et al*., 2005).

Biological laser scanning confocal microscopy relies heavily on fluorescence as an imaging mode, primarily due to the high degree of sensitivity afforded by the technique coupled with the ability to specifically target structural components and dynamic processes in chemically fixed as well as living cells and tissues. Many fluorescent probes are constructed around synthetic aromatic organic chemicals designed to bind with a biological macromolecule (for example, a protein or nucleic acid) or to localize within a specific structural region, such as the cytoskeleton, mitochondria, Golgi apparatus, endoplasmic reticulum, and nucleus. Other probes are employed to monitor dynamic processes and localized environmental variables, including concentrations of inorganic metallic ions, pH, reactive oxygen species, and membrane potential. Fluorescent dyes are also useful in monitoring cellular integrity (live versus dead and apoptosis), endocytosis, exocytosis, membrane fluidity, protein trafficking, signal transduction, and enzymatic activity. In addition, fluorescent probes have been widely applied to genetic mapping and chromosome analysis in the field of molecular genetics (Dunn *et al*., 2006).

In this study the cellular effect of Triton X-100 and CoQ10, alone, and in combination on cardiac and skeletal muscle cells were investigated with confocal microscopy. The following fluorescent probes were used in the study:

(i) Mito Tracker Red 580, a cationic dye that is selectively pumped into healthy respiring mitochondria in response to its negative membrane potential.

(ii) 4'6-diamino-2-phenylindole diydrochloride (DAPI), a dye that stains nuclei specifically with little or no cytoplasmic labelling.

(iii) diclorodihydrofluorescein diacetate (DCH_2FDA) is a fluorescent probe that detects the formation of ROS, specifically the ROS, H_2O_2 (only skeletal muscle cells were used).

In this Chapter the following research objectives were investigated:

- Investigate intracellular changes in cardiac and skeletal muscle cells in primary culture, evoked by Triton X-100 and CoQ10, alone and in combination, using Confocal Microscopy.
- Correlate the results obtained with SEM, with the results obtained with Confocal Microscopy.
- Determine whether Triton X-100 and CoQ10, alone and in combination produce reactive oxygen species (ROS), upon exposure, in cardiac and skeletal muscle cell cultures.

6.2 Materials and Methods

The primary chick embryonic cardiac and skeletal muscle cell cultures were prepared as for cytotoxicity testing in Chapter 4. To examine the cardiac and skeletal muscle cell morphology and structure of the primary cultures, the primary cultures at a concentration of at $5x10^4$cells/ml were plated onto the surface of Menzel Glazer glass coverslips coated with Poly-L-lysine (1ml Poly-L-lysine in 9ml sterile ddH_2O) positioned on the bottom of 6-well plates. After 72 hours of incubation the cell cultures were exposed to Triton X-100 (0.00005 – 0.5%) and CoQ10 (0.01 – 0.2mg/ml), alone and in combination, for 24 hours. To determine the effect of CoQ10 on cells exposed to Triton X-100, cell cultures were pre-treated with the different concentrations of CoQ10, 0.02 – 0.2mg/ml, two hours prior to exposure to Triton X-100 (0.005 – 0.05%). The cultures were maintained for the rest of the 24 hours at 37°C and 5% CO_2 before Mito Tracker® Red 580 (Invitrogen Molecular Probes™, Cat #: M22425), to identify cells with healthy respiring mitochondria and DAPI (Invitrogen Molecular Probes™, Cat #: D1306) to investigate possible alterations to the nuclei, were added to the cells. A stock solution of

1mM Mito Tracker was prepared in DMSO. DAPI was prepared at a concentration of 5mg/ml in ddH_2O and further diluted to a stock solution of 300nM. After the incubation period 1µl of Mito Tracker was added to the medium in each well and left in the dark for 25 minutes, 300µl of DAPI was then added to the medium and left in the dark for a further 5 minutes. The medium was removed from each well, and the attached cardiac and skeletal muscle cells were washed with DPBS. A 10x DPBS stock solution was used. Coverslips were removed from the 6-well plates and mounted onto microscope slides. The area between the coverslip and the microscope slide was wetted with DPBS and the edges were sealed. Cardiac and skeletal muscle cells were examined using a Zeiss LSM 510 META confocal microscope (Carl Zeiss Werke, Göttingen, Germany), with fluorescence excitation wavelengths 543 and 358nm for Mito Tracker and DAPI, respectively and emission wavelengths of 644 and 461nm for Mito Tracker and DAPI, respectively. Both Mito Tracker and DAPI were evaluated simultaneously, in order to produce a sharp contrast between nuclei labelling and active respiring mitochondria. Result images are presented in the form of an overlap of the two signals obtained at different excitation and emission wavelengths.

To determine the presence of ROS, skeletal muscle cells were kept unexposed in the incubator at 37ºC and 5% CO_2 for 86 hours. The skeletal muscle cells were only exposed to CoQ10, two hours before the fluorescent dye DCH_2FDA (Sigma-Aldrich, Johannesburg, South Africa) was added to indicate ROS formation. The cells were exposed to Triton X-100 10-15 minutes before DCH_2FDA was added and for cells exposed to Triton X-100 and CoQ10 in combination, the cells were exposed to CoQ10 two hours before Triton X-100 was added, and then exposed to the substances in combination for 15 more minutes, before DCH_2FDA was added to indicate ROS formation. Cells were exposed to the same concentrations as mentioned for Mitotracker Red and DAPI fluorescence. A 5µg/ml stock solution of DCH_2FDA was prepared in ddH_2O. After the exposure time, the medium from each well was removed and the attached skeletal muscle cells were washed with DPBS. A volume of 800µl DPBS was added to each well, together with 40µl of the stock solution of DCH_2FDA. After 7 minutes in the dark, the solution was removed and coverslips were mounted onto microscope slides (as described for Mito Tracker and DAPI), and evaluated with a Zeiss LSM 510 META confocal microscope to locate areas where ROS generation had occurred.

6.3 Results and Discussion

Confocal microscopy imaging studies in single cells and tissue sections confirm the importance of this non-invasive technique in the study of cell structure and function as well as the modulation of working living cells by various constituents of cell membranes, organelles and cytosol (Bkaily *et al*., 1997). Confocal laser scanning microscopy uses optical sections and digitized images to achieve a similar goal over tens of micrometers. These images were collected and can be processed for stereo viewing or presented in a collected form that provides extreme depth of focus (Borman *et al*., 1994).

6.3.1 Mito Tracker Red 580

In the mitochondrial respiratory pathway, an electrochemical gradient is generated by the active extrusion of protons from the mitochondrial matrix to the intermembrane space via complexes of the electron transport chain. This gradient is generated as reduced pyridine nucleotides are oxidized, protons are pumped, and electrons are passed down the chain. ATP is generated as Complex V (F_OF_1, ATP synthase) couples phosphorylation of ADP with NADH/$FADH_2$ oxidation, a process known as oxidative phosphorylation (OXPHOS). This inner mitochondrial membrane potential attracts some dyes to actively respiring mitochondria. Although conventional fluorescent stains for mitochondria, such as rhodamine 123 and tetramethylrosamine, are readily sequestered by functioning mitochondria, they are subsequently washed out of the cells once the mitochondrion's membrane potential is lost. This characteristic limits their use in experiments in which cells must be treated with aldehyde-based fixatives or other agents that affect the energetic state of the mitochondria. To overcome this limitation, Molecular Probes has developed the MitoTracker Orange and MitoTracker Red probes - dyes that accumulate in mitochondria in a membrane-potential–dependent manner and remain during subsequent processing steps for immunocytochemistry, *in situ* hybridization, or electron microscopy. In addition, MitoTracker Orange and MitoTracker Red reagents eliminate some of the difficulties of working with pathogenic cells because, once the mitochondria are stained, the cells can be treated with fixatives before the sample is analyzed (Invitrogen™). MitoTracker Red 580 dye is useful for staining mitochondria in fixed or live cells (staining is not membrane-potential sensitive), but the staining pattern is not retained following fixation and permeabilization (invitrogen™). Mito Tracker Red is a cationic dye that is selectively pumped into healthy respiring mitochondria in response

to its negative membrane potential. Once pumped into the mitochondria, the dye is covalently bound to proteins through its chloromethyl moiety and thus is retained after fixation of cells. Treatment of cells with an uncoupler of mitochondria such as carbonyl cyanide m-chloro phenylhydrazone (CCCP), resulted in a marked decrease in Mitotracker red fluorescence intensity that is indicative of a loss of membrane potential (Bova *et al*., 2005).

6.3.2 DAPI

4´,6-diamidino-2-phenylindole dihydrochloride (DAPI), is a popular nuclear and chromosome counterstain, DAPI emits blue fluorescence upon binding to AT regions of DNA. DAPI stains nuclei specifically with little or no cytoplasmic labelling. Although the dye is cell impermeable, higher concentrations will enter a live cell (InvitrogenTM). The blue-fluorescent DAPI nucleic acid stain preferentially stains dsDNA; it appears to associate with AT clusters in the minor groove. Binding of DAPI to dsDNA produces a ~20-fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove. DAPI also binds RNA, however in a different binding mode one thought to involve AU-selective intercalation (Invitrogen^{TM}). DAPI is due to its colour a very convenient stain for nuclei labelling during counterstaining experiments. In this study DAPI clearly differentiate the nucleus of muscle cells from the rest of the cell, making it possible to observe possible cell fusion.

6.3.3 Dichlorodihydrofluorescein diacetate (DCH_2FDA)

Dichlorodihydrofluorescein diacetate (DCH_2FDA) is a fluorescent probe that detects the formation of ROS, specifically the ROS, H_2O_2. This probe can easily enter the cell, where it is also cleaved by non-specific esterase into diclorodihydrofluorescein (DCFH). DCFH is oxidised by H_2O_2 to form diclorofluorescein (DCF), which is fluorescent and the cell exhibits a green fluorescence (Hipler *et al*., 2002). DCH_2FDA is a nonpolar, nonfluorescent probe that easily crosses the cell membrane due to two esterified acetate functional groups (Chini *et al*., 1997). On entering the cell, the diacetate group is cleaved enzymatically by esterases to form polar, dichlordihydrofluorescein that accumulates intracellularly (Scivittaro *et al*., 2000; Kim *et al*., 2000). In the presence of the ROS, H_2O_2, dichlordihydrofluorescein is oxidized to the highly fluorescent 2'7'-dichlorofluoresein. Consequently the fluorescent signal produced by 2'7'-

dichlorofluoresein is an index of oxidative stress in the biological system (Grishko *et al*., 2001).

No significant difference was seen between cardiac and skeletal muscle cells in the different groups on which the combined colorimetric cytotoxicity assays were performed. Neither did cardiac and skeletal muscle cells showed a drastic difference when cell structure was evaluated upon exposure to Triton X-100 and CoQ10, alone and in combination, using SEM. No difference was observed between the fluorescence produced by cardiac and skeletal muscle cells in each group when evaluated using confocal microscopy. Results of the different concentrations tested in this experiment are therefore shown irrespective of the cell culture/muscle cell type.

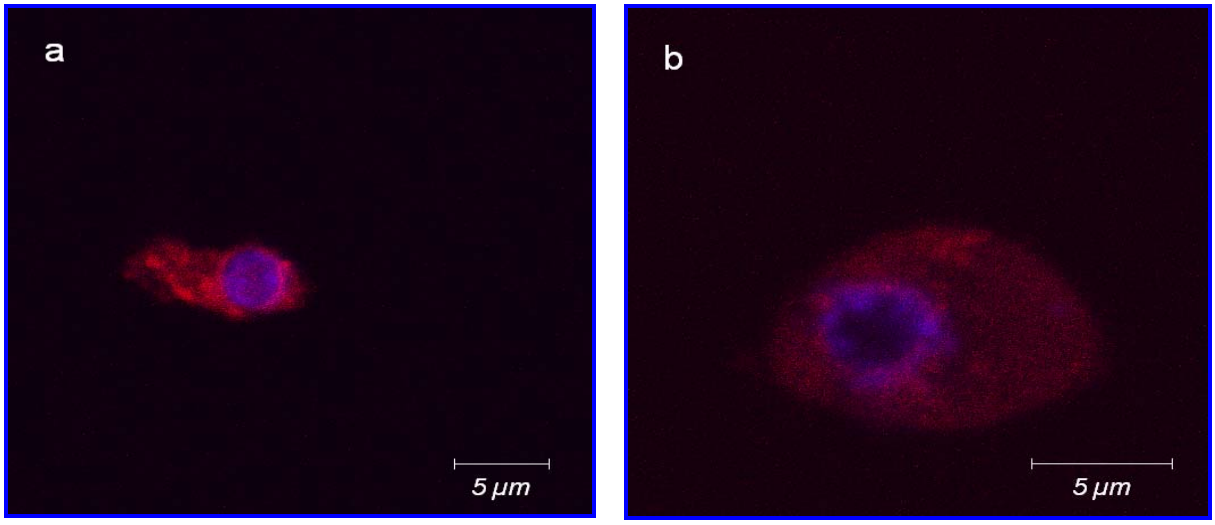

Figure 6.1: Muscle cells in the control group (**a** & **b**) stained with Mito Tracker Red and DAPI. A major problem with fluorophores (fluorescent probes) is that they fade irreversible (**b**) when exposed to excitation light (Semwogerere *et al*., 2005).

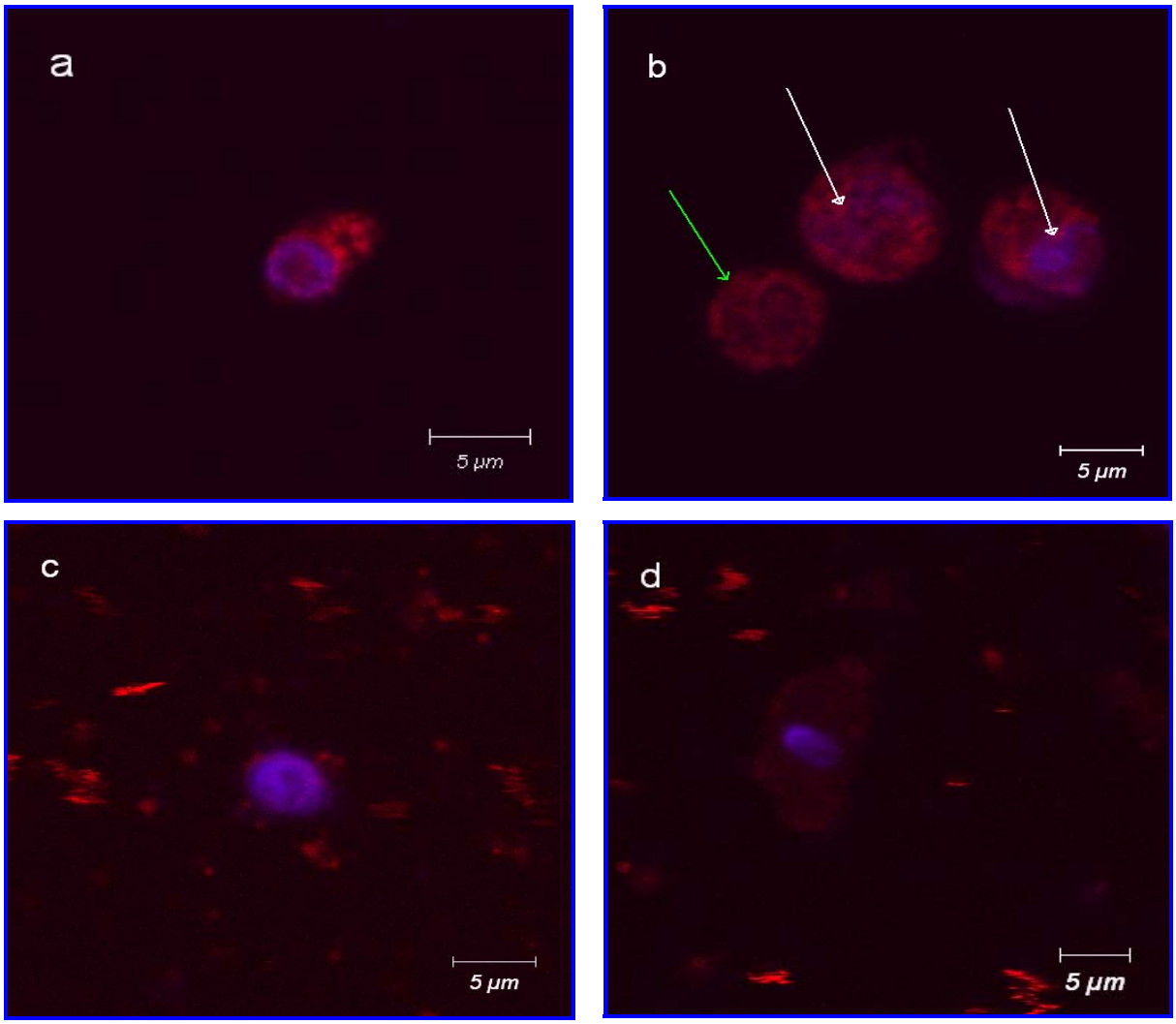

Figure 6.2 A: Muscle cells in the Triton X-100 group, stained with Mito Tracker Red and DAPI. **a**): 0.5% Triton X-100, little mitochondrial staining were seen, as the red signal produced by Mito Tracker Red was very weak. DAPI produced a weak blue signal, but the position indicates that the nucleus was not disrupted. **b**): Three muscle cells in the same culture as (**a**), the white arrows point to the distribution of the blue signal throughout the cell. The green arrow points to a cell where no blue signal was produced. **c**): 0.05% Triton X-100. **d**): 0.005% Triton X-100. In both (**c**) and (**d**) a relatively strong blue fluorescence was obtained, in disparity to the weak, almost absent Mito Tracker Red signal. Photos (**c**) and (**d**) point to the possibility that the nucleus was still intact, when mitochondrion were destroyed during the process of possible cell death caused by 0.05% and 0.005% Triton X-100.

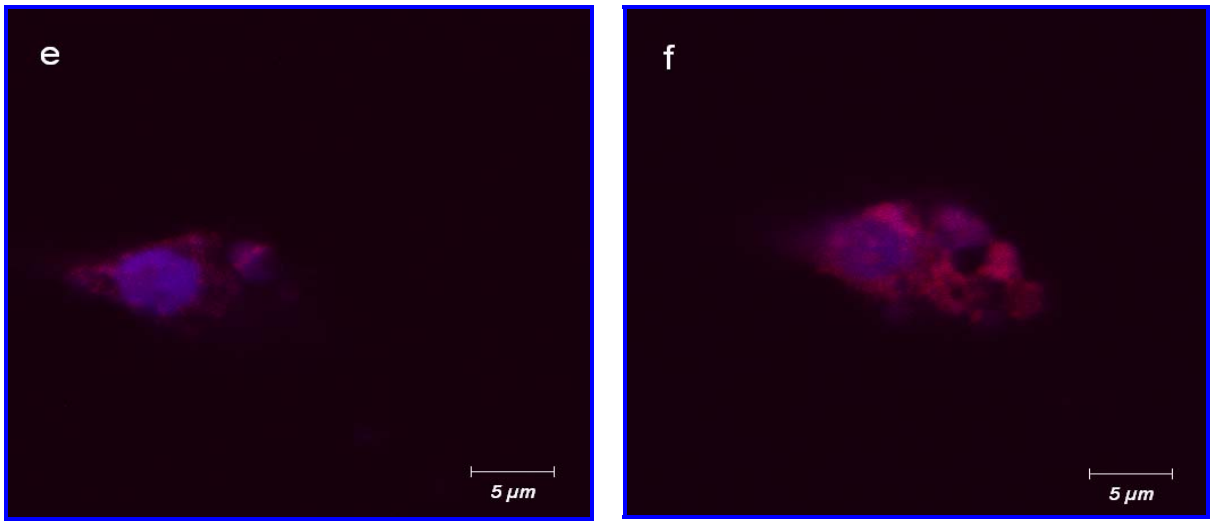

Figure 6.2 B: Muscle cells in the Triton X-100 group at the second lowest and lowest concentrations tested in the study. **e**): 0.0005% Triton X-100, a clear blue signal was observed indicating the position of the nucleus, which seems to be intact. A weak blue fluorescence can be seen outside the circular blue signal assumed to represent the nucleus. This phenomenon indicates that nucleic acid/nuclear material was also dispersed outside the boundaries of the nucleus. This was also observed in the muscle cell in (**f**), the dispersed blue signal was slightly stronger and the circular blue signal to the left of the cell was less pronounced than in (**e**).

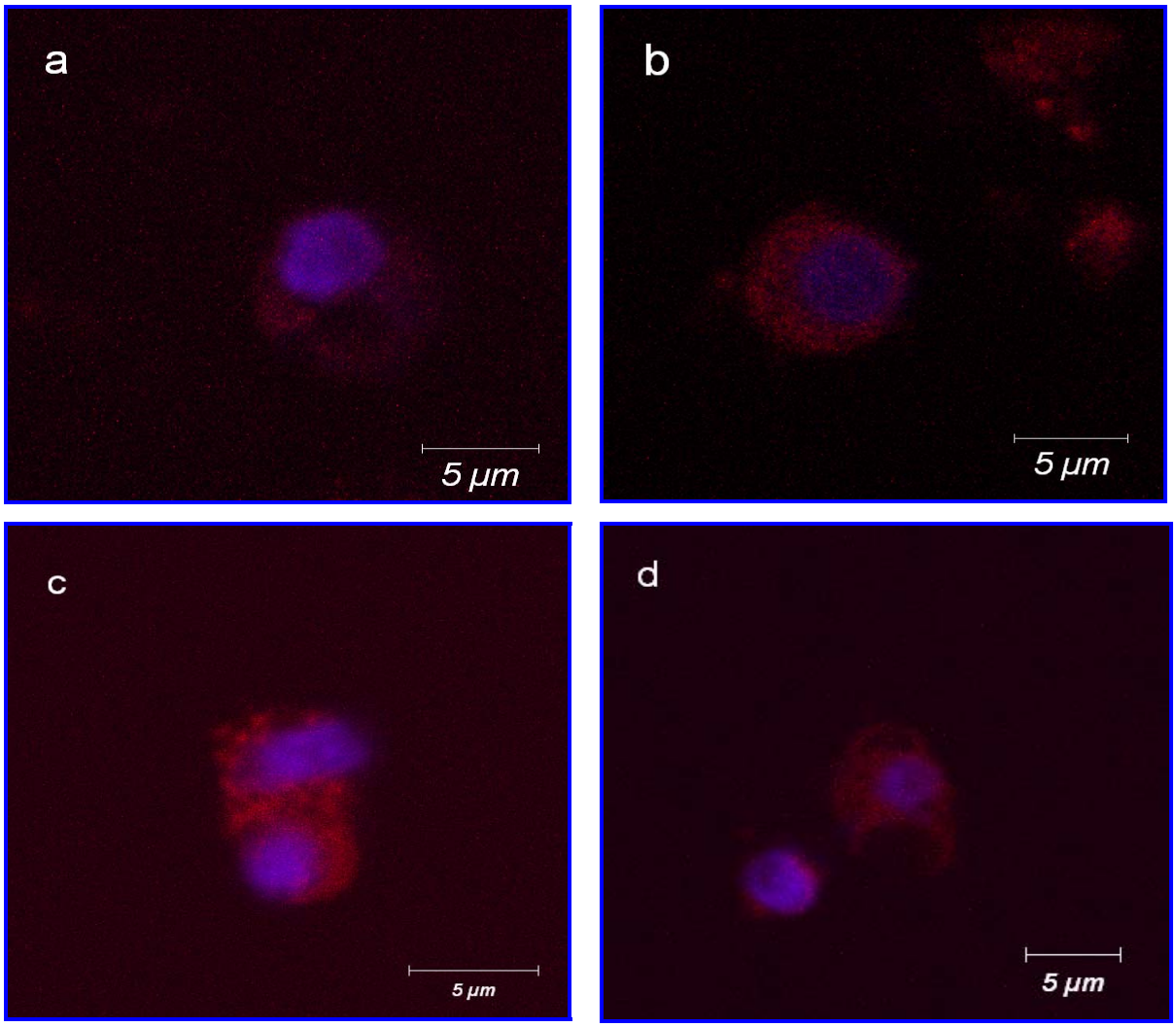

Figure 6.3 A: Muscle cells in the CoQ10 group stained with Mito Tracker Red and DAPI. **a**): 0.2mg/ml CoQ10. **b**): 0.1mg/ml CoQ10. In both the highest and second highest concentrations (**a** & **b**) of CoQ10 used in the study a definite circular blue signal was produced by DAPI, although the signal was very weak, it confirmed an intact nucleus. A very weak red signal was produced by Mito Tracker Red in both (**a**) and (**b**). Since Mito Tracker red is selectively pumped into healthy respiring mitochondria in response to its negative membrane potential, it might be possible that the membrane potential was altered, but since the blue signal produced by DAPI was also very weak, and not dependent on the membrane potential it can be safely assumed that the dye faded due to photobleaching, since "anti-fade" was not used in the study. **c** & **d**): 0.05mg/ml CoQ10. In both the photos shown, two very distinct blue signals in circular form, assumed to represent the nucleus was seen. This probably represents the process of fusion between two myoblasts to form a myotube, the mechanism whereby muscle cells form a syncitium in the process of muscle formation.

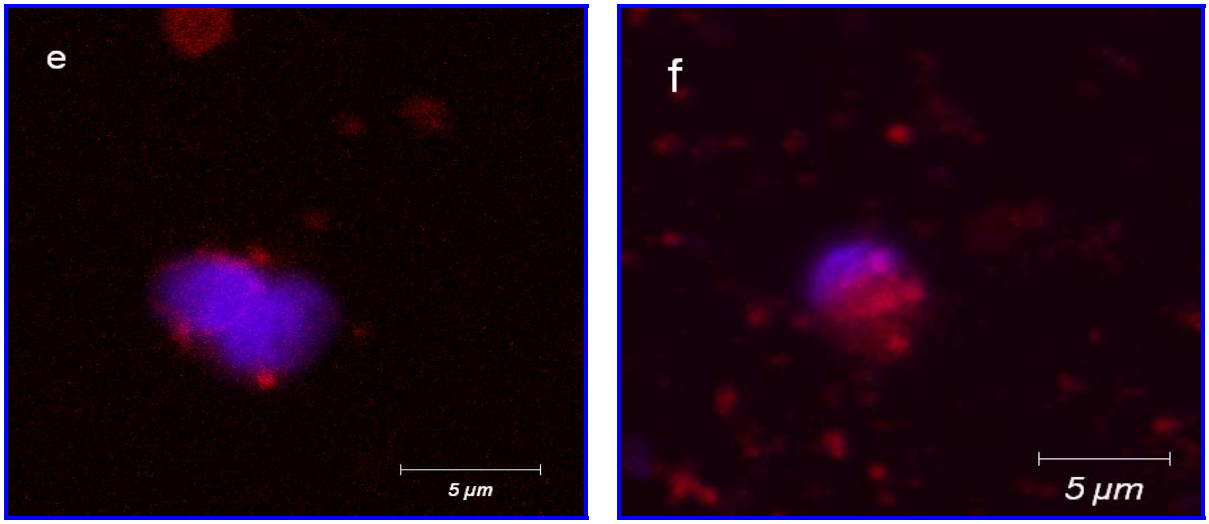

Figure 6.3 B: Muscle cells in the CoQ10 group at the two lowest concentrations of CoQ10 used in the study. **e**): 0.02mg/ml CoQ10. Two very distinct blue signal in circular form, very close to each other. Little, but intense red fluorescence was produced. It is possible that this cell is in the process to undergo proliferation, since there are two distinct nuclei. The little red fluorescence may point to the fact that cells in this phase minimize cytoplasmic contents in order to go through the proliferation process, thereafter the cytoplasmic contents and organelles are restored in order to maintain normal cellular metabolism. **f**): A clear blue fluorescence produced by DAPI at 0.01mg/ml. The nucleus appears to be intact. A bright red fluorescence was produced by Mito Tracker Red, indicating the healthy respiring mitochondria in the cells in both (**e**) and (**f**).

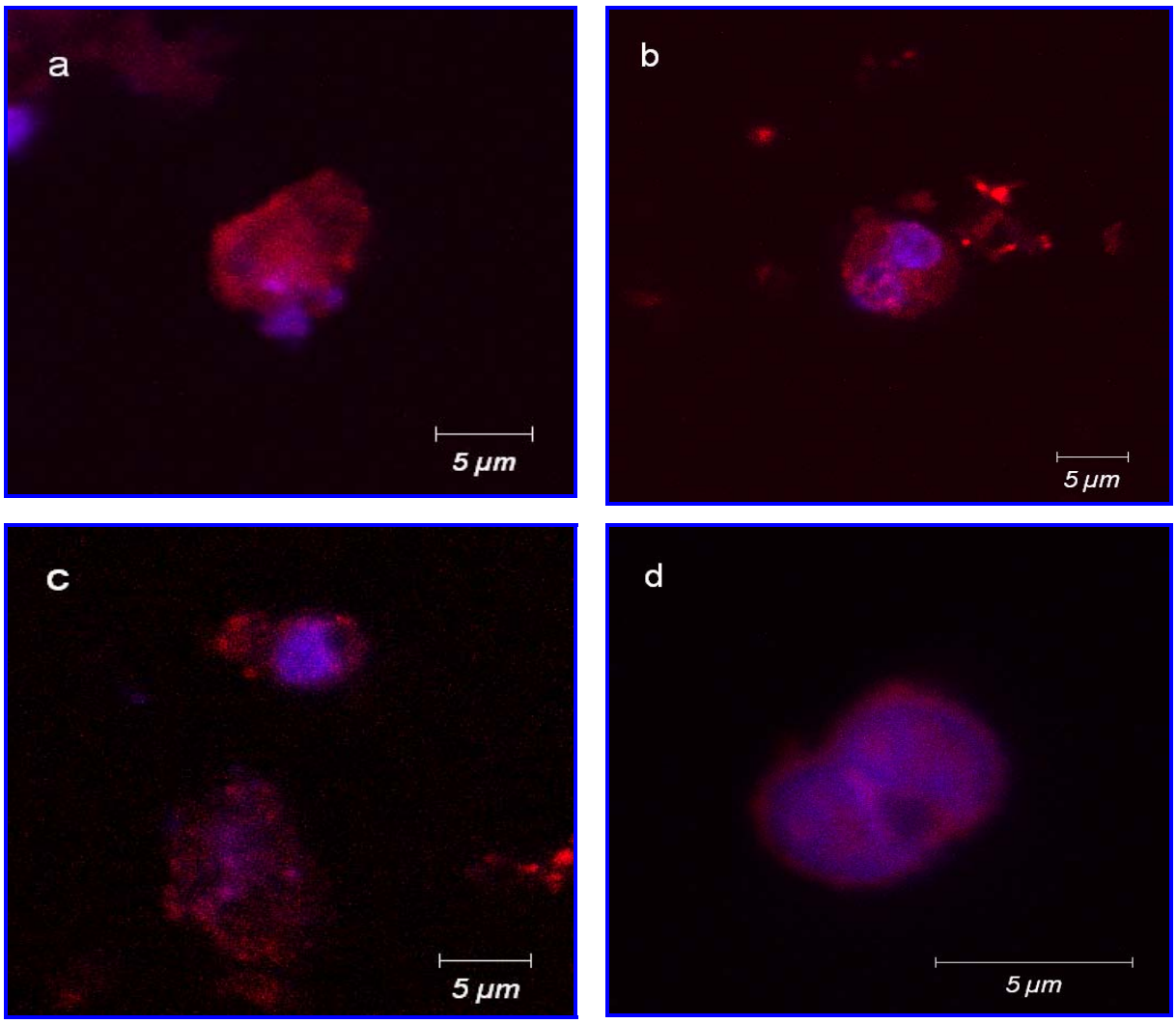

Figure 6.4 A: Muscle cells exposed to 0.05% Triton X-100 after two hours pre-treatment with CoQ10, stained with Mito Tracker Red and DAPI. **a** & **b**): Muscle cells pre-treated with 0.1mg/ml CoQ10. A definite blue signal was obtained in (**a**) with DAPI, the signal was in 3 distinct portions visible, indicating that the nucleus was not intact. The cell was probably in the process of undergoing apoptosis. The detectable morphological changes in the nucleus are chromatin condensation and, at a later stage, the fragmentation of the nucleus into several particles (Häcker, 2000). An intense red signal was produced by Mito Tracker Red, indicating active respiring mitochondria. In (**b**), two distinct blue signals can be observed, representing two intact nuclei, an intense red signal can be seen, with spreading of the red signal outside the direct vicinity of the nuclei, it is possible that membrane disruption occurred. **c** & **d**): Muscle cells pre-treated with 0.05mg/ml CoQ10. The blue signal in the top cell in **c** is intense and the shape indicate and intact nucleus, surrounded by respiring mitochondria. The blue signal in (**d**) is spread throughout the cell, with an invagination in the overall shape at the bottom part of the cell. This cell might be in the process of undergoing mitosis.

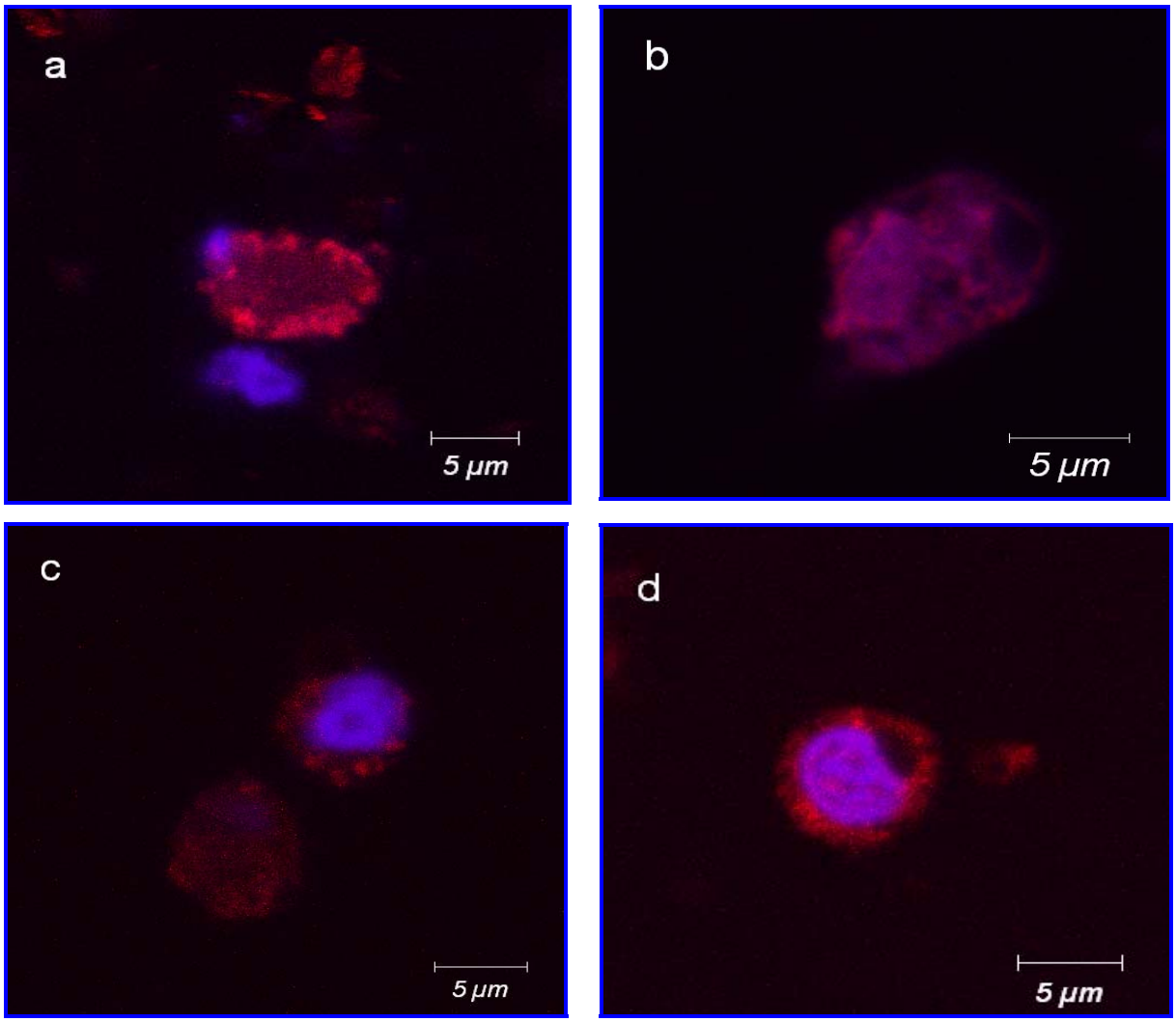

Figure 6.4 B: Muscle cells exposed to 0.005% Triton X-100 after two hours pre-treatment with CoQ10, stained with Mito Tracker Red and DAPI. **a** & **b**): Muscle cells pre-treated with 0.1mg/ml CoQ10. In (**a**) two distinct intense blue signals were obtained with DAPI, indicating intact nuclear morphology. Intense red fluorescence was also seen. In (**b**), the blue signal was spread in a non-condensed fashion throughout the cytoplasm merged with the red signal produced by Mito Tracker Red, indicating that the cell is probably in the process of undergoing mitosis. **c** & **d**): Muscle cells pre-treated with 0.05mg/ml CoQ10. In both (**c**) and (**d**), a very intense blue signal was obtained by DAPI staining indicating, intact nuclei. A red signal was obtained in both cells, indicating active respiring mitochondria. The more intense red signal in **d**, might be the result of a more negative membrane potential, as it compare well to the control group (Figure 6.1 **a**), the red signal in **c** might also be less intense due to photobleaching.

Wakelam, 1985, described skeletal muscle fibres as permanent multinucleated, non-mitotic cells. In 1983 Masuko *et al*., observed distinct changes in surface structure and form of chick myoblasts during the cell cycle. During the G1 and S phase, the cells were spindle-shaped with a relatively smooth surface, by late G2, cells have bulged in anticipation of rounding from mitosis (Masuko *et al*., 1983), and many microvilli, some blebs, and long slender filopodia appeared on cell surfaces. During M, the cells were generally spherical and their surface covered with numerous microvilli and some blebs. After cell division, microvilli and blebs disappear as the cell spread over the substrate (Masuko *et al*., 1983). The changes in surface structure observed by Masuko *et al*., 1983, seemed to them to be a "general phenomena related to mitosis". During embryonic development, several hundred myoblasts, precursors of skeletal muscle fibers, line up end to end, fusing with one another to form multinucleated cells known as myotubes. These newly formed myotubes manufacture cytoplasmic constituents as well as contractile elements, myofilaments, responsible for the contractile capability of the cell (Gartner *et al*., 2007). Myogenic differentiation involves extensive changes in cell morphology and subcellular architecture. Upon differentiation, myoblasts fuse to form multinucleated syncitia that eventually develop into terminally differentiated muscle cells, the myofibers (Pizon *et al*., 2005). When cells prepare to fuse, they have sparse skeletal structure, lightly cross-linked, and nearly empty, with a few major filamentous cables that terminate at the cell periphery (Fulton *et al*., 1981). Two crucial events occur during the commitment of the muscle cell precursor to myogenesis: (i) the cessation of proliferation of the precursor cell determined by the upregulated expression of myogenic regulatory factors (MRFs), Myf5 and MyoD, and the downregulation of Pax7, a transcription factor, and (ii) the terminal differentiation of the muscle cell precursor, triggered by myogenin and MRF4 (Kierzenbaum, 2007). In myoblasts preparing to fuse, both the surface lamina and the internal networks shows highly specific spatial rearrangement; in addition, the internal networks become more extractible. After fusion, both the internal networks and the surface lamina rapidly reorganize in a stable arrangement as the muscle cell begins to construct the extensive contractile apparatus (Fulton *et al*., 1981).

Satellite cells are a cell population distinct from the myoblasts. They attach to the surface of the myotubes before a basal lamina surrounds the satellite cell and myotube. Satellite cells are of considerable significance in muscle maintenance, repair, and regeneration in the adult. Satellite cells are mitotically quiescent in the adult, but can

resume self renewal and proliferation in response to stress or trauma. MyoD expression induces the proliferation of satellite cells. The descendants of the activated satellite cells, called myogenic precursor cells, undergo multiple rounds of cell division before they can fuse with existing or new myofibers. A population of stem cells in adult skeletal muscle, called side-population cells, has the capacity to differentiate into all major blood cell lineages as well as myogenic satellite cells. These cells are present in bone marrow and may give rise to myogenic cells that can participate in muscle regeneration (Kierzenbaum, 2007).

Yu *et al*., 1973, showed that Triton X-100 extraction of cells or membranes solubilizes the bilayer and most integral membrane proteins, leaving only the spectrin matrix and associated proteins in an insoluble and thus readily form (Yu *et al*., 1973). Apgar *et al*., 1985, found that extraction of intact cells (P815 tumour cells) with 1% Triton X-100 resulted in structures with a continuous layer of detergent-insoluble protein at the cell periphery. Confined within this peripheral layer was a nuclear remnant (also seen in Figure 5.4 b, thin white arrow in the present study), the cytoplasmic space was largely empty and clearly lacked filamentous cytoskeletal elements (as seen in Figure 5.3) (Apgar *et al*., 1985). Allen *et al*., 1964, incorporated Triton X-100 into reaction mixtures used for the visualization of esterases and acid phosphatases separated by electrophoresis in starch gels. The net effect was apparent enhancement of enzymatic activity with certain substrates and apparent inhibition of enzymatic activity with other substrates. It was shown by both quantitative and electrophoretic studies that Triton X-100 is an activator of certain esterases (Allen *et al*., 1964).

CoQ10 is a lipid-soluble component of virtually all cell membranes, and is located in the hydrophobic domain of the phospholipid bilayer of cellular membranes (Quinzii *et al*., 2007a; Sohal *et al*., 2007), it is also the only known lipid-soluble antioxidant that animal cells can synthesize *de novo*, and for which there exist enzymatic mechanisms which can regenerate it from its oxidized product formed in the course of its antioxidant function (Ernster *et al*., 1995). Free radicals are highly reactive molecules or chemical species containing unpaired electrons that cause oxidative stress, which can be defined as an imbalance between antioxidants in favor of the oxidants, potentially leading to damage (Sies, 1997). They are formed during normal physiological metabolism or caused by toxins in the environment. Oxidative stress can damage lipids, proteins,

enzymes, carbohydrates and DNA in cells and tissue, leading to membrane damage, fragmentation or random cross linking of molecules like DNA, enzymes and structural proteins and ultimately lead to cell death induced by DNA fragmentation and lipid peroxidation (Beckman *et al*., 1998). The physiological roles of CoQ10 in biological systems are most well characterized in the inner mitochondrial membrane, where three of its main functions are: (i) carrier of electrons from respiratory complexes I and II to complex III, (ii) generation of superoxide anion radical by autoxidation of ubisemiquinone and (iii) anti-oxidant quenching of free radicals (Sohal *et al*., 2007). Kagan *et al*., 1999, tested the use of CoQ10 as a generic anti-apoptotic compound and found that its ability to protect against apoptosis varies depending on both cell type and mode of cell death induction. It was established that the protective effect offered by CoQ10 was mediated by its effect on the mitochondrial function and viability (Kagan *et al*., 1999).

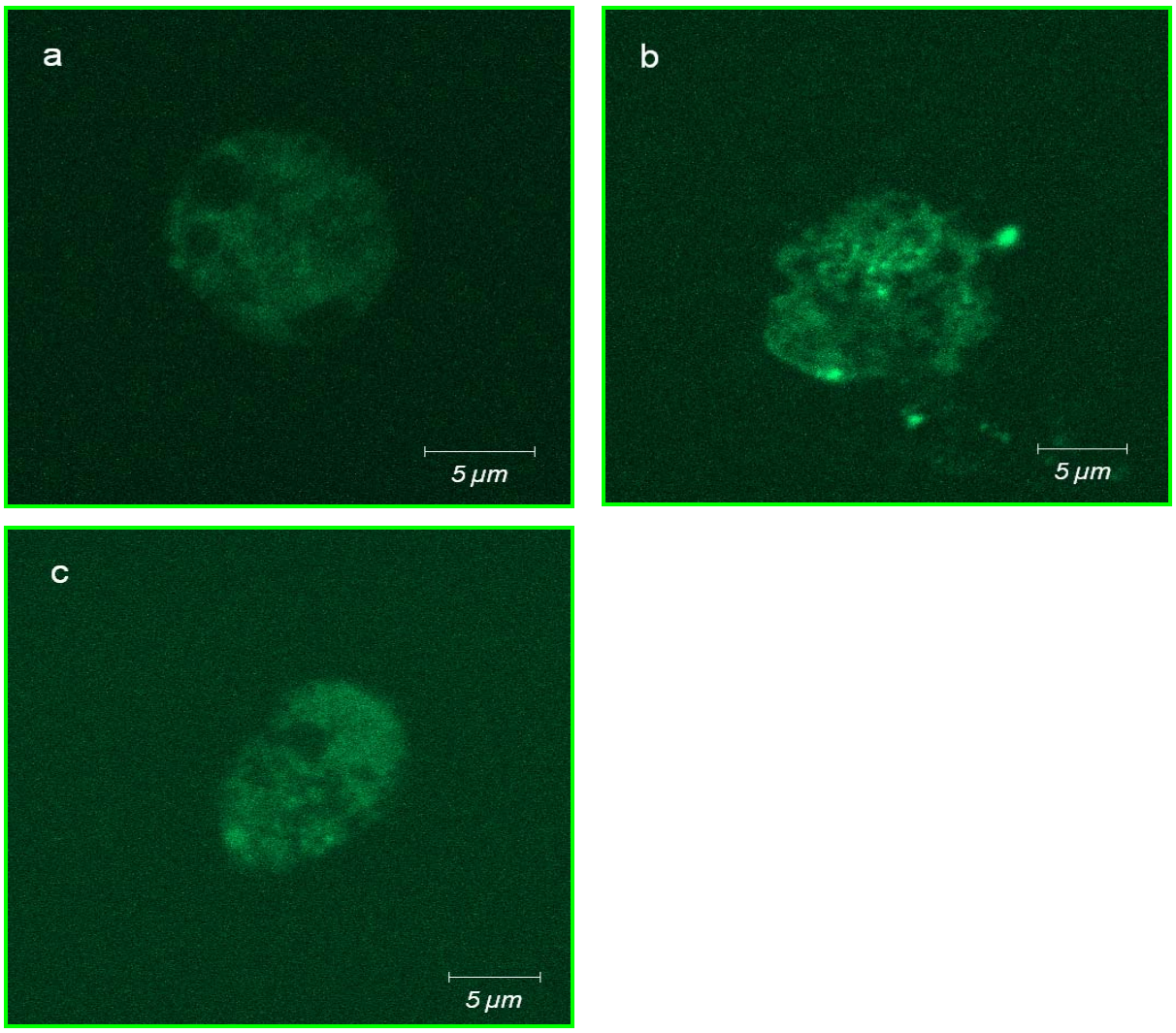

Figure 6.5: Muscle cells exposed to Triton X-100 stained with DCH_2FDA. **a**): 0.05% Triton X-100 produced a weak green fluorescence. It is possible that Triton X-100 induce cell death by a mechanism other than to produce ROS, or the weak signal may be due to photobleaching. **b**): 0.05% Triton X-100 produced a more intense green fluorescence, with the highest intensity localized to the boundary of the cell. **c**): 0.005% Triton X-100 produced a green signal throughout the whole cell, with background staining. In all the cells exposed to Triton X-100, the presence of ROS was detected with confocal microscopy.

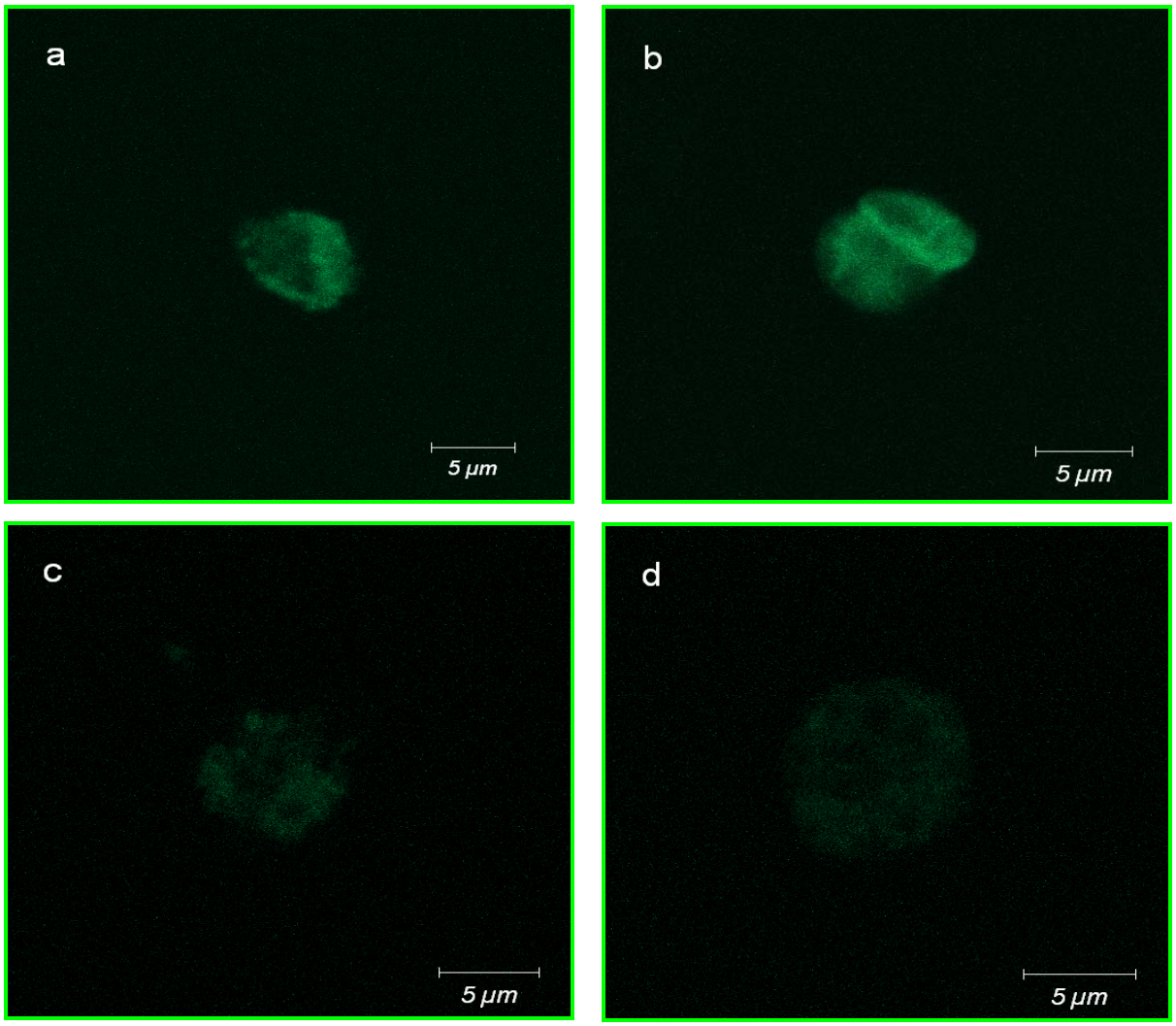

Figure 6.6 A: Muscle cells exposed to 0.05% Triton X-100, after two hours pre-treatment with CoQ10. **a** & **b**): Muscle cells pre-treated with 0.1mg/ml CoQ10. A mild green signal was obtained upon staining with DCH_2FDA in both cells, indicating the presence of ROS. **c** & **d**): Muscle cells pre-treated with 0.05mg/ml CoQ10. Almost no green signal was produced upon staining with DCH_2FDA, indicating the absence of ROS formation in cells exposed to 0.05% Triton X-100 after pre-treatment with 0.05mg/ml CoQ10.

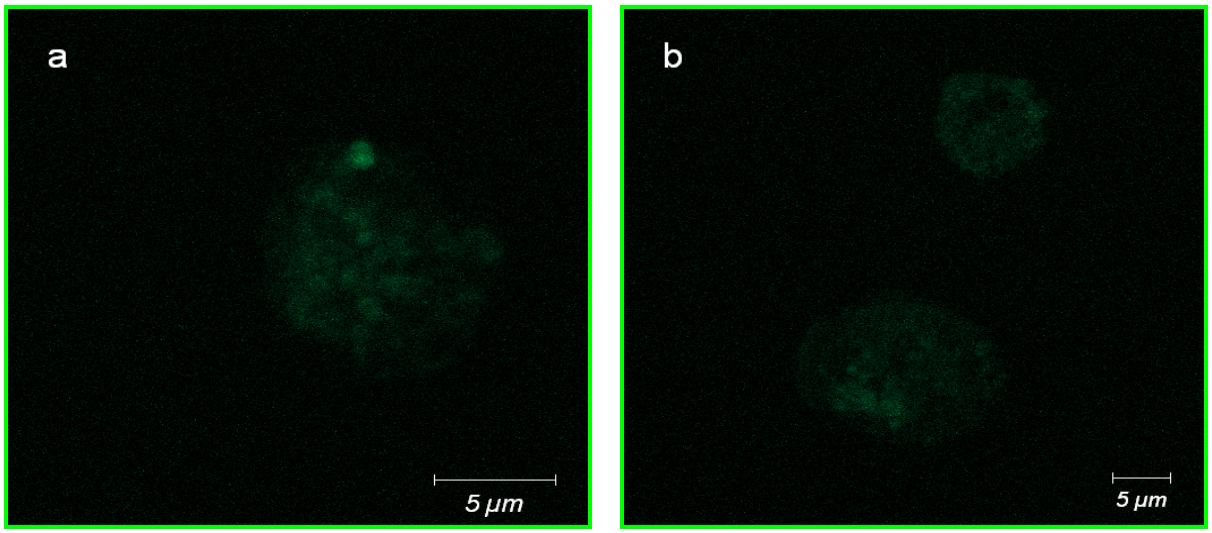

Figure 6.6 B: Muscle cells exposed to 0.005% Triton X-100, after two hours pre-treatment with CoQ10. Both (**a**) and (**b**) were pre-treated with 0.05mg/ml Triton X-100, almost no green fluorescence was detected upon staining with Dichlorodihydrofluorescein diacetate, indicating the absence of ROS and the absence in production thereof in the presence of 0.05mg/ml CoQ10 when cells are exposed to 0.005% Triton X-100.

6.4 Conclusion

A confocal microscope provides a significant imaging improvement over conventional microscopes. It creates sharper, more detailed 2D images, and allows collection of data in three dimensions. In biological applications it is especially useful for measuring dynamic processes (Semowegerere *et al*., 2005). The results obtained using confocal microscopy upon staining with Mito Tracker Red, DAPI and DCH_2FDA, confirmed the results obtained with SEM in Chapter 5 of this study. The counterstaining with Mito Tracker and DAPI offered great contrast and ease of interpreting the results. It was seen in Chapter 5, that solubilisation of muscle cells with Triton X-100 resulted in structures with a continuous layer of detergent-insoluble protein at the cell periphery, confined within this peripheral layer was a nuclear remnant, this was confirmed by DAPI staining of cells exposed to 0.5% - 0.005% Triton X-100 (Figure 6.2 A & B), nuclear material was present and produced a blue signal. CoQ10 cleary enhanced the process of cell proliferation in muscle cells, since myotube formation was only seen in this group. In Figure 6.3A, c & d, nuclei were very close together, characteristically of two mononucleated myoblasts fusing to form a multinucleated myotube. The process of fusion was seen with the SEM in Chapter 5, although nuclei could not be visualized using SEM, some cells had the surface and size characteristics of myotubes. Confocal microscopy confirmed the process of myotube formation in the CoQ10 group. In the presence of CoQ10, cells with dividing nuclei, characteristic of the process of mitosis were seen (Figure 6.3 B, e; and Figure 6.4 A, b & d), it is possible that CoQ10 promote mitotic formation of new cells in skeletal and cardiac muscle cells. Confocal microscopy also confirmed the possible protection CoQ10 offered to muscle cells exposed to Triton X-100. In Chapter 5, SEM images of a “membrane patch” in certain cells of which the membranes were injured by Triton X-100, after pre-treatment with CoQ10, suggested that CoQ10 offer some form of protection to the muscle cells. Confocal microscopy produced a blue signal indicating intact nuclei and active respiring mitochondria in muscle cells exposed to Triton X-100, after pre-treatment with CoQ10. Muscle cells in the Triton X-100 group, stained with DCH_2FDA produced a green signal, indicating the presence of ROS formation. In cells pre-treated with CoQ10, and exposed to Triton X-100 at concentrations 0.05% and 0.005%, little to no green fluorescence was obtained, indicating that CoQ10’s antioxidant function was able to scavenge the free radicals produced by Triton X-100 exposure. Muscle cells in the CoQ10 group, produced no

green fluorescence upon staining with DCH_2FDA, confirming that CoQ10 do not produce reactive oxygen species in cells but offer protection against it.

Chapter 7: Concluding Discussion

Coenzyme Q10 is a lipid-soluble coenzyme, synthesized in mammalian tissue to support energy production, and also act as an antioxidant, by scavenging free radicals present in the body. Because of the fact that CoQ10 synthesis becomes less efficient as people age, CoQ10 is thought to play a role in immune system modulation and disease. Certain medication and stress may deplete the body's endogenous CoQ10 store, by interrupting the biosynthetic pathway (Horowitz, 2003). Numerous disease conditions such as Parkinson's disease, AIDS, Alzheimer disease, autoimmune asthma, migraine, cancer, diabetes, mitochondrial disorders, muscle disorders, periodontal disease, male fertility and cardiac conditions, including hypertension, congestive heart failure, angina and cardiomyopathy has been shown to benefit from CoQ10 supplementation (Horowitz, 2003). Dr. John Ely at the University of Washington reported that CoQ10 and ascorbic acid (Vitamin C) are the two most important essential nutrients. They, along with other essential nutrients, have been rejected as unpatentable and unprofitable by certain "interests", according to expose's by Pauling and others (Pauling, 1987 and Ely, 1999). These were possibly the most lethal errors of modern medicine because no cell, organ, function, remedy, etc, can avoid failure unless essential nutrients, especially these two, are optimal. Supplementation of both is mandatory: for ascorbate, lifelong (since humans can't synthesize it); for CoQ10, it is increasingly necessary with age, stress, or disease (Ely, http://faculty.washington.edu/ely/turnover.html).

Results presented by Linnane *et al*., in 2002, indicate that CoQ10 functions as a major skeletal muscle gene regulator and as such, profoundly modulates cellular metabolism. Their data also suggested that CoQ10 treatment can act to influence the fibre type composition of muscle, toward the fibre type profile generally found in younger individuals (Linnane *et al*., 2002). Microarray gene expression analyses and differential gene display analyses demonstrated independently that the expression of a large number of genes is affected by CoQ10. Proteome analysis reflected the global gene response of CoQ10 supplementation on the protein expression profile of muscle tissue (Linnane *et al*., 2002).

Until recently, attention has been focused on requirements for CoQ10 in energy conversion in the mitochondrial compartment of cells or on the antioxidant properties of

CoQ10. New evidence shows that CoQ10 is present in other cell membranes. In the outer membrane it may contribute to the control of cell growth (Langsjoen, 1994). The membrane stabilizing property of CoQ10 has been postulated to involve the phospholipids-protein interaction that increases prostaglandin (especially prostacyclin) metabolism. It is thought that CoQ10 stabilizes myocardial calcium-dependent ion channels and prevents the depletion of metabolites essential for ATP synthesis (Greenberg *et al*., 1990). It is also thought that the isoprenoid side chain of CoQ10 may help to stabilize the lipid bilayer (Lenaz *et al*., 1999).

Triton X-100 is a well known membrane disrupter and is used extensively by cell biologists for that purpose (Macarulla *et al*., 1989). A concentration range of Triton X-100 between $5x10^{-5}$ to 0.5% has been selected in the present study to provide membrane disruption in various degrees of severity, ranging from complete cell lysis to slight membrane rupture. Triton X-100 offered the ideal membrane disrupter properties at the chosen concentration range to investigate whether CoQ10 might offer protection to cells in culture, more specifically to cell membranes exposed to disrupting agents. It was incidentally found that the chemical structure of Triton X-100 show correlation to that of nonylphenol, one of the most studied estrogen mimics that appear to interact with development in several organisms. Upon investigation, using the Recombinant Yeast Screen Assay (RCBA) for oestrogenic activity, it was found that Triton X-100 induced weak estrogenic activity. Due to the structural similarity to NPE the possibility of non receptor mediated estrogenic effects, triggered by alternative pathways in the cell that eventually elicit endocrine disruption, should be investigated. The variation in the dose-response curve shape were indistinct and obscure and were not interpretable, showing the inability to obtain repeatability with Triton X-100. The results confirmed high toxicity of Triton X-100. Further investigations should be done on the strange and indefinable properties elicited by Triton X-100.

Primary cell cultures of chick embryonic cardiac and skeletal muscle cells were successfully established using 13 day old chick embryos, as they provided significantly developed leg muscle and heart tissue. Most favourable results were obtained when the medium of the cells were not changed during the incubation period. To avoid fibroblast contamination, cells were pre-plated in flasks for 45 to 60 minutes. The advantage of using primary cultures include the ease of obtaining the tissue type that needs to be

studied, furthermore, primary cell cultures have the advantage in that they tend to retain the basic characteristics of the more complex *in vivo* system. The success of the culture establishment was confirmed by light microscopy used to investigate cell morphology. Well established populations of Crystal Violet stained cardiac and skeletal muscle cells, respectively, as well as myotube formation, a differentiation process, which arises from the fusion of mononucleated myoblasts, thought to be an irreversible process toward muscle formation (Hjiantoniou *et al*., 2007), were observed. The cell cultures provided a useful tool for studying the possible cytotoxic effects of Triton X-100 and CoQ10. Using the MTT, NR and CV assays in the form of a combined colorimetric assay, Triton X-100 showed to increase cell viability of ~ 9% in both cultures as measured by the MTT assay, to decrease lysosomal membrane integrity between 30 and 60% as measured by the NR assay, and appeared to have no significant effect on the cell number as evaluated by the CV assay. These results indicate that Triton X-100 might evoke its toxic response by mechanisms other than mitochondrial enzyme activity and cellular protein alterations. Coenzyme Q10 showed to slightly enhance mitochondrial enzyme activity, measured by the MTT assay, indicated by the average increase of ~10% in cell viability over the two cell cultures. The NR assay showed that CoQ10 disrupted an average of 47% of lysosomal membrane integrity, leading to lysosmal emptying of contents into the cytoplasm, and ultimately cell death. Coenzyme Q10 had no effect on cellular proteins as indicated by the CV assay. The results of NR assay evoked a suspicion about the reliability of the assay in the present application, since no known properties of CoQ10 indicate the ability of disrupting lysosomal membrane integrity to an extend where it will definitely induce cell death. Cytotoxicity was measured in cardiac and skeletal muscle cells pre-treated with CoQ10, two hours prior to Triton X-100 exposure at concentrations 0.05 and 0.005%, respectively, in both cell cultures by the NR assay. Cell viability was increased in both cell cultures and no effect was seen in cellular proteins, measured by the CV assay. These results indicate toxic insult to cardiac and skeletal muscle cells in culture by Triton X-100 and CoQ10, alone, and in combination.

Scanning electron microscopy is a useful method for studying the cell surface during muscle development in cell culture (Shimada, 1972). This very useful method was applied in the present study to observe the cellular alterations caused by the membrane disrupter properties of Triton X-100, at concentrations ranging form $5x10^{-5}$% to 0.5%. The results obtained reflect that of earlier studies describing the use of Triton X-100 to

extract the cytoskeletons of cells for morphological studies (Wallace *et al*., 1979 and Fulton *et al*., 1981). At the highest concentration (0.5%), we saw complete membrane lysis and, parallel to the findings of Vale et al., 1985, with PC12 cells, Triton X-100 solubilized membranes of cardiac and skeletal muscle cells and left behind a nucleus and an array of cytoskeletal filaments (Figure 5.2 b,) at a concentration of 0.5%. As the concentration of Triton X-100 decreased, the severity of membrane solubilization was decreased to where membrane tears, apoptotic blebs and membrane shrinkage was observed. At none of the concentrations of Triton X-100 tested in the study, after 24 hours of exposure, did cells in culture show any reparative actions. No remarkable differences between the cardiac and skeletal muscle cell alterations were observed. Triton X-100 is a useful tool for investigating cytoskeletal composition and properties, as indicated by the highest two concentrations (0.5 and 0.05%) used in the study, which enables the visualization of cytoskeletal components and variation in the cytoskeletal composition as the cell progress through the different phases of the cell cycle (as observed by Masuko *et al*., 1983).

No cellular or morphological damage to cardiac and skeletal muscle cells in culture were detected in the presence of the different concentrations of CoQ10 (0.2mg/ml – 0.02mg/ml) tested in the study. Cell membranes appeared smooth, intact, and most of the cells seemed to be in the process of fusion or postproliferative, which might indicate that CoQ10 might enhance the proliferation process in muscle cells. Fusion of myoblasts into myotubes were seen at 0.05 and 0.01mg/ml CoQ10 (Figure 5.17 & 5.18, and Figure 5.22 & 5.23), and not in any cultures exposed to Triton X-100 or in the control group, indicating that CoQ10 enhances the process of proliferation and syncytium formation in cardiac and skeletal muscle cells in culture. Membrane surfaces were remarkably smooth and intact, confirming the membrane stabilizing properties of CoQ10 discussed by Greenberg *et al*., 1990 and Dallner *et al*., 2000, with the presence of microvilli and small spherical protrusions, characterizing certain phases of the cell cycle. In all cells exposed to CoQ10, very distinct pores were visible (e.g. Figure 5.12; 0.2mg/ml), in some instances they appeared in larger quantities. These pores designated “ion channels” according to the literature, are clearly activated or their function is enhanced in the presence of CoQ10 to such an extend that they appear in larger numbers in an “open” state on the surface of the cell membranes. Ion channels were present at all the concentrations of CoQ10 being tested confirming the ability of

CoQ10 to maintain the integrity of myocardial calcium ion channels as described by Greenberg *et al*., 1990, Dallner *et al*., 2000, Shinde *et al*., 2005 and Terao *et al*., 2006.

In muscle cells pre-treated with the different concentrations of CoQ10 (0.02 – 0.2mg/ml) prior to exposure to 0.05% Triton X-100, membrane alteration were seen, correlating with that seen in the group of cells exposed to Triton X-100, alone. This indicates that the concentrations of CoQ10 tested, did not offer protection to muscle cells and their membranes exposed to the disrupting insult of 0.05% Triton X-100. In muscle cells exposed to 0.005% Triton X-100, a rare formation of a "membrane patch" was observed on the membrane surface of cells pre-treated with 0.05 and 0.1mg/ml CoQ10. Cell integrity of cells exposed to this concentration of Triton X-100 was retained, after pre-treatment with 0.05 – 0.1mg/ml CoQ10, indicating that CoQ10 is able to offer membrane protection to cardiac and skeletal muscle cells in culture, exposed to a concentration of 0.005% Triton X-100. Coenzyme Q10 also plays a role in the opening/activation of ion channels, and remarkably enhances the process of myotube formation in cardiac and skeletal muscle cells in culture.

The results obtained using confocal microscopy upon staining with Mito Tracker Red, DAPI and DCH_2FDA, confirmed the results on cellular morphology and structure obtained with SEM. DAPI staining of cells exposed to 0.5% - 0.005% Triton X-100, produced a blue signal showing that nuclear material was present, indicating the presence of the nuclear remnant seen with SEM upon solubilization of cells with Triton X-100. Coenzyme Q10 cleary showed to enhance the process of cell proliferation and differentiation in muscle cells, since myotube formation was only seen in the group of muscle cells treated with CoQ10. In the presence of CoQ10, cells with dividing nuclei, characteristic of the process of mitosis were seen (Figure 6.3 B, e; and Figure 6.4 A, b & d). It is possible that CoQ10 promotes mitotic formation of new cells in skeletal and cardiac muscle cells in culture. In Figure 6.3A, c & d, nuclei were very close together, characteristically of two mononucleated myoblasts fusing to form a multinucleated myotube. Confocal microscopy confirmed the process of myotube formation in the CoQ10 group. It also confirmed the possible protection CoQ10 offered to muscle cells exposed to Triton X-100. In Chapter 5, SEM images of a "membrane patch" in certain cells of which the membranes were injured by Triton X-100, after pre-treatment with CoQ10, suggested that CoQ10 offer some form of protection to the muscle cells.

Confocal microscopy produced a blue signal indicating intact nuclei, and a red signal indicating active respiring mitochondria, in muscle cells exposed to Triton X-100, after pre-treatment with CoQ10. Muscle cells in the Triton X-100 group, stained with DCH_2FDA produced a green signal, indicating the presence of ROS formation. In cells pre-treated with CoQ10, and exposed to Triton X-100 at concentrations 0.05% and 0.005%, little to no green fluorescence was obtained, indicating that CoQ10's antioxidant function was able to scavenge the free radicals produced by Triton X-100 exposure. Muscle cells in the CoQ10 group, produced no green fluorescence upon staining with DCH_2FDA, confirming that CoQ10 do not produce reactive oxygen species in cells but offer protection against it.

The study provides, in parallel with the literature, apparent evidence that CoQ10 offers protection to cardiac and skeletal muscle cells in culture after exposure to relatively low concentrations of the membrane disrupter, Triton X-100. Coenzyme Q10 also promotes the process of proliferation and differentiation in primary chick embryonic cultures of cardiac and skeletal muscle cells.