Chapter 1: Introduction

"Be the change you want to see in this world . . ."

Mahathma Gandhi, humanitarian

First identified in 1940, Coenzyme Q10 (CoQ10) discovery was not a simple accident, but the result of a long train of investigation into the mechanism of, and compounds involved in biological energy conversion (Horowitz, 2003 and Crane, 2007). It 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 and Sohal *et al*., 2007). Coenzymes are cofactors upon which the comparatively large and complex enzymes absolutely depend for their function (Langsjoen, 1994). Coenzyme Q10 is the only endogenously synthesized lipid with a redox function in mammals and exhibits a broad tissue as well as intracellular distribution (Dallner *et al*., 2000). 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). In the human CoQ10 is biosynthesized from tyrosine through a cascade of eight aromatic precursors, via the mevalonate pathway (Folkers, 1996). Cells generally rely on *de novo* synthesis for their supply of Coenzyme Q, and its levels are subjected to regulation by physiological factors that are related to the oxidative activity of the organism (Tran *et al*., 2007 and Ernster *et al*., 1995). Current knowledge about the biosynthetic pathway of CoQ10 is mostly derived from characterization of accumulating intermediates in Q-deficient mutant strains of *Saccharomyces cerevisiae*, and so far nine complementation groups of Q-deficient yeast mutants have been identified (*COQ1 – COQ9*) (Tran *et al*., 2007).

Coenzyme Q is composed of a tyrosine-derived quinone ring, linked to a polyisoprenoid side chain, consisting of 9 or 10 subunits in higher invertebrates and mammals (Ernster *et al*., 1995). The benzoquinone ring can assume three alternate redox states: the fully oxidized or ubiqunone (Q); the univalently reduced ($1e^- + 1H^+$) ubisemiquinone ($^{\cdot}QH$), a free radical; and the fully reduced ($2e^- + 2H^+$) ubiquinol (Sohal *et al*., 2007). The polyisoprenyl chain apparently facilitates the stability of the molecule within the hydrophobic lipid bilayer. The length of the chain seems to affect the mobility,

intermolecular interaction with membrane proteins, and autoxidizability (Sohal *et al*., 2007). There are a number of indications that CoQ10 is not always functioning by its direct presence at the site of action, but also using receptor expression modifications, signal transduction mechanisms and action through its metabolites (Turunen *et al*., 2004). The physiological roles of CoQ10 in biological systems are most well characterized in the inner mitochondrial membrane, where three of its main functions are: a) carrier of electrons from respiratory complexes I and II to complex III, b) generation of superoxide anion radical by autoxidation of ubisemiquinone and c) anti-oxidant quenching of free radicals (Sohal *et al*., 2007). Coenzyme Q10 is present in all cells and membranes and in addition to be a member of the mitochondrial respiratory chain it has several other functions of great importance in cellular metabolism (Turunen *et al*., 2004). Even greater significance was apparent when it was shown that reduced CoQ10 could restore antioxidant function to oxidized tocopherol. This is important because endomembranes have enzymes that can reduce CoQ10, but none for reduction of oxidized tocopherol directly (Crane, 2007).

The CoQ10 levels in the body increase from birth up to the age of 20 – 30 years, followed by a successive decrease to the initial birth level at around the age of 80 years. The final content of CoQ10 in human cells is a consequence of both synthesis rate and catabolism (Dallner *et al*., 2000 and Fernández-Ayala *et al*., 2005). The CoQ10 content is also altered in a number of diseases of which the decrease in cardiomyopathies and degenerative muscle diseases are the most studied (Turunen *et al*., 2004). Coenzyme Q10 is commonly used for treatment of cardiomyopathy and there is substantial evidence that heart function is improved upon administration of the lipid (Langsjoen *et al*., 1999). Coenzyme Q10 has been suggested to be involved in neurodegenerative diseases such as Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and Friedrich's ataxia (FRDA), where these disorders responded to oral CoQ10 supplementation (Turunen *et al*., 2004; Quinzii *et al*., 2007 and Quinzii *et al*., 2007b). Coenzyme Q10 has been seen to enhance the immune function in patients with AIDS (Mutter, 2003). Geromel *et al*., 2002, found that 200mg of CoQ10 per day, taken for 12 weeks, improved glycemic control and blood pressure in patients with type 2 diabetes. A deficiency of CoQ10 has been found in gingival biopsy tissue from patients with advanced periodontal disease (Horowitz, 2003). Dramatic improvement with topically applied CoQ10 was found in patients who could no longer eat solid food because of the severity of their gum disorder (Murray, 2000). In its role as an

antioxidant, CoQ10 acts to prevent damage caused by lipid oxidation in sperm cell membranes and thus, is pivotal to sperm motility (Horowitz, 2003). A study done by Gazdik *et al*., 2002, found decreased concentrations of CoQ10 in the blood of patients with autoimmune asthma. The complexity of the biosynthesis of CoQ10 suggests that defects in different biosynthetic enzymes or regulatory proteins may cause different clinical syndromes (Quinzii *et al*., 2007b).

The importance of CoQ10 in the life of living organisms is illuminated most clearly by the number of reports describing the genetic disorders in which CoQ10 synthesis is impaired. Lowered lipid content in organs causes serious metabolic disturbances, but CoQ10 supplementation reestablishes mitochondrial and other functions (Turunen *et al*., 2004). The molecular bases and pathogenic mechanisms of the various primary and secondary forms of CoQ10 deficiency remain largely unknown (Quinzii *et al*., 2007a). To date, primary CoQ10 deficiency has been genetically and biochemically proven in just a few patients with infantile multi-systemic severe diseases, where nephropathy and encephalopathy seems to be the most consistent feature. However, CoQ10 deficiency should be considered in the differential diagnosis of subacute exercise intolerance and weakness and of all genetically undefined adult-onset cases of cerebellar ataxia, as well as in patients with ataxia-oculomotor-aprataxia type 1 (AOA1), because CoQ10 supplementation seems to improve muscle weakness and other associated symptoms in some individuals (Quinzii *et al*., 2007a).

Since CoQ10 is essential to the optimal function of all cell types, it is not surprising to find a seemingly diverse number of disease states which respond favorably to CoQ10 supplementation. All metabolically active tissues are highly sensitive to a deficiency of CoQ10, and its function as a free radical scavenger only adds to the protean manifestations of CoQ10 deficiency (Lansjoen, 1994). It is therefore necessary to expand this field of research in order to increase awareness on this nonpatentable supplement, vital for normal physiological functioning and health. One aspect that appears to be sparsely researched is the effect of CoQ10 on cell membranes, which inspired the direction of this study to investigate the cellular effects of CoQ10 in the presence of Triton X-100 on primary chicken heart and skeletal muscle cell cultures. To gain a broader background regarding the numerous properties, advances, and physiological mechanisms of this coenzyme, the literature regarding these aspects will be reviewed in Chapter 2. It was incidentally, while reviewing the literature that it became

apparent that there exists a correlation between the structures of Triton X-100, chosen to produce the desired membrane disrupting effect in this study, and nonylphenol, an endocrine disruptor well known for its estrogen mimicking effect. The question arose whether Triton X-100, in addition to its properties as membrane solublizing detergent, might induce estrogenic activity. The outcome of the investigation gained insight on a substance that call for further investigation and is presented in Chapter 3.

In order to investigate the cellular effect of Triton X-100 and CoQ10 on heart and muscle cell cultures, from chick embryos, the establishment and optimization of an appropriate cell culture system was needed. The concentration range selected to represent the properties of CoQ10 and Triton X-100 in the cell culture, needed optimization, and was tested, alone, and in combination, for cytotoxicity, more specifically their effect on cell viability, lysosomal membrane integrity and cell number, in order to provide some parameter of expectation. The procedures and results that commence the outcome of this study are presented in Chapter 4. In order to address the limitations of our knowledge on the membrane protective properties of CoQ10 and to expand on the limited literature available on the membrane protective properties of CoQ10, its cellular effect alone and in the presence of the well known membrane disrupter, Triton X-100 was evaluated, by using scanning electron microscopy (SEM), which provided surface visualization of the three dimensional appearance of the extracellular cell surface, more specifically the morphology and structure of the cell membrane and alterations thereof. These results are presented in Chapter 5. In order to substantiate the findings in Chapter 5, confocal microscopy was done to provide insight on the intracellular effects brought about in the cell cultures upon exposure to CoQ10 and Triton X-100, and to correlate the intracellular information with the extracellular information obtained with SEM. The correlation is confirmed in Chapter 6.

Chapter 2: Literature Review

2.1 Introduction

"We are now in a position to witness the unfolding of the greatest medical tragedy of all time - never before in history has the medical establishment knowingly created a life-threatening nutrient deficiency in millions of otherwise healthy people."

- Peter H. Langsjoen, MD

2.1.1 History

Coenzyme Q10 was first isolated from beef heart mitochondria by doctor Frederick Crane (University of Wisconsin, USA), in 1957 (Crane *et al*., 1957). During the same year professor RA Morton from England, defined a compound obtained from vitamin A deficient rat liver to be the same as CoQ10 (Morton, 1957). He introduced the name ubiquinone, meaning “the ubiquitous quinone” (ubiquitous = found everywhere). In 1958, professor Karl Folkers and coworkers at Merck, Inc., determined the precise chemical structure of CoQ10, synthesized it, and became the first to produce it by the process of fermentation (Langsjoen, 1994). Professor Yamamura of Japan became the world’s first to treat a human disease, congestive heart failure, with coenzyme Q7, a compound related to CoQ10, in the mid-1960’s. Other practical uses then followed and in 1966, Mellors *et al*., showed that reduced Coenzyme Q6 revealed effective antioxidant properties. In 1972 the Italian, Gian Paolo Littarru and professor Karl Folkers documented a deficiency of CoQ10 in human heart disease (Littarru *et al*., 1972). By the mid-1970’s, the Japanese perfected industrial technology to produce pure CoQ10 in quantities sufficient for larger clinical trials (Langsjoen, 1994). In 1978 Peter Mitchell received the Nobel Prize for his contribution to the understanding of biological energy transfer that occurs at cellular level, through the chemiosmotic theory, which includes the vital role of CoQ10 in energy transfer systems (Langsjoen, 1994). In 1986 professor Karl Folkers received the Priestly Medal from the American Chemical Society and in 1990 the National Medal of Science from President Bush for his work with CoQ10 and other vitamins (Langsjoen, 1994). In the late 1980’s, promotion of CoQ10 supplements in capsule form began to reach health care professionals. In the 1990’s, direct marketing to

consumers became available to people who presumably might not meet their CoQ10 requirements via biosynthesis and normal dietary intake of 5 – 10 mg per day (Horowitz, 2003).

2.1.2 Background

Coenzyme Q10, also known as CoQ10, CoQ, Ubiquinone Q10, Ubidecarenone, Vitamin 10 or ubiquinone, is a naturally occurring quinone that is found in most aerobic organisms from bacteria to mammals. It is a natural human quinone, found naturally in the energy producing center of the cell, known as the mitochondria (Shinde *et al*., 2005), but it can be chemically synthesized (Collins *et al*., 1999). Coenzyme Q10 is not a drug, it is essentially a vitamin or vitamin-like, fat-soluble coenzyme found everywhere in the body. Coenzymes are cofactors upon which the comparatively large and complex enzymes absolutely depend for their function. Coenzyme Q10 is also a potent antioxidant, as is its reduced product, ubiquinol-10. It is the only known naturally occurring lipid soluble antioxidant for which the body has enzyme systems capable of regenerating the active reduced form, ubiquinol (Escolar *et al*., 2001). Antioxidants are substances that scavenge free radicals, damaging compounds in the body that alter cell membranes, tamper with DNA, and even cause cell death. Free radicals occur naturally in the body, but environmental toxins such as radiation, cigarette smoke, ultraviolet light and air pollution, can increase the number of these damaging particles (Shinde *et al*., 2005). Antioxidants such as CoQ10 can neutralize free radicals and reduce or even help prevent some of the damage they cause (Al-Hasso, 2001). Coenzyme Q10 is the coenzyme for at least three mitochondrial enzymes (complexes I, II and III) as well as enzymes in other parts of the cell. Mitochondrial enzymes of the oxidative phosphorylation pathway are essential for the production of the high-energy phosphate, adenosine triphosphate (ATP), upon which all cellular functions depend (Langsjoen, 1994). It is now believed that CoQ10 is the key nutrient for generating 95% of the total energy required by the human body (Fonorow, 2006).

The highest concentrations of CoQ10 have been measured in vital organs such as the heart and pancreas (Fonorow, 2006). Its biosynthesis from the amino acid tyrosine, is a complex, multistage process, requiring several vitamins and trace elements (Post, 2005). An age-dependent decrease in CoQ10 has been documented; peak serum levels occur at age 19 to 21 years of age, and drop by up to 65% by the age of 80 years

(Harmon, 1988). Other factors leading to CoQ10 deficiency include inadequate dietary intake, environmental stress, strenuous exercise, and selected drugs. Deficiencies has also been reported in various disease conditions, including congestive heart failure (CHF), cardiomyopathy, chronic obstructive pulmonary disease (COPD), acquired immunodeficiency syndrome (AIDS), cancer, hypertension, and periodontal disease (Fuke *et al*., 2002). When taken orally, it is slowly absorbed by the gastrointestinal tract and transported in serum within chylomicrons. Blood levels in normal individuals range from 0.6-1.0 ug/ml (Karlsson *et al*., 1993) and its plasma concentration mirrors the metabolic demand in various tissues (Escolar *et al*., 2001). Several studies have shown that exogenous supplementation of CoQ10 results in elevation of blood levels (Turunen *et al*., 1999), with two concentration peaks in serum, the first at 6.5 ±1.5 hr and the second at 24 hr. After its absorption, CoQ10 is deposited in the liver and packaged into VLDL lipoproteins that return to the circulation about 24 hr later. Coenzyme Q10 has a relatively long plasma half-life (33.19±5.32 hr) (Tomono *et al*., 1986).

2.2 Biosynthesis of CoQ10

2.2.1 The Chemical Structure and Chemical Properties

The chemical name for CoQ10 is 2,3-dimethoxy-5-methylbenzoquinone. It is the predominant human form of endogenous ubiquinone and is synthesized in the mitochondrial innermembrane (Quinzii *et al*., 2007a). Coenzyme Q10 is comprised of a ubiquinone head group (a quinone ring, capable of transferring electrons) attached to a tail of 10 five-carbon isoprenoid units, that anchor the molecule to the cytoplasmic or mitochondrial membranes (Turunen *et al*., 2004), and is structurally similar to vitamin K (Greenberg *et al*., 1990). The 10-isoprenoid side chain with 10-isoprene units and a total of 50 carbon atoms are biosynthetically derived from mevalonic acid (Folkers, 1996). In humans CoQ10 is found in relatively higher concentrations in cells with high energy requirements such as the heart, liver, muscle and pancreas (Fuke *et al*., 2002). Intracellularly, 40 – 50 % is found in the mitochondrial membrane. Peak blood levels occur 5 – 10 hours after ingestion, and the elimination half-life is 34 hours. It is preliminary excreted through the biliary tract (Collins *et al*., 1999).

CoQ10 can exist in three oxidation states (Misner, 2005):

1. Fully reduced ubiquinol form ($CoQH_2$)
2. Radical semiquinone intermediate ($CoQH^-$)
3. Fully oxidized ubiquinone form (CoQ)

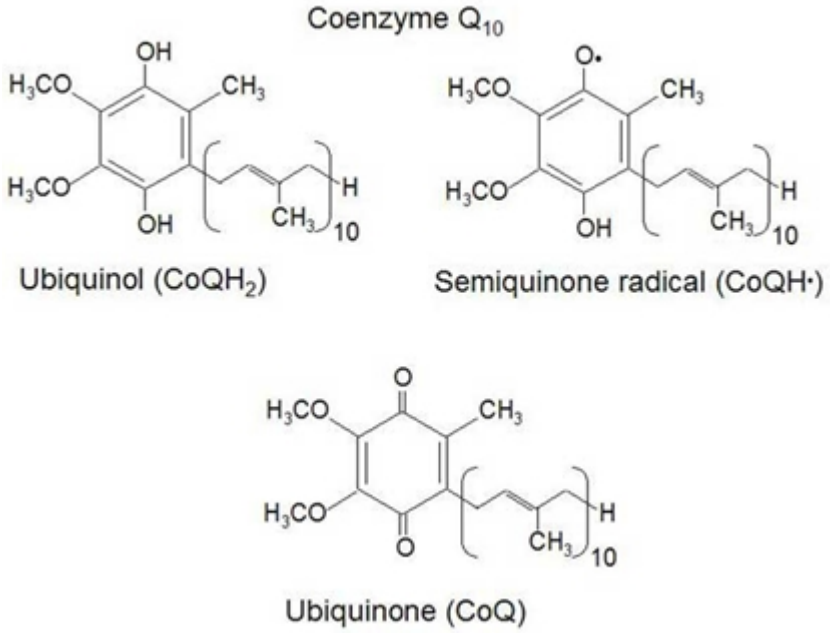


Figure 2.1: The chemical structure of CoQ10 (Shinde *et al*., 2005).

The all *trans* polyisoprene ensures an affinity for the interior of cell membranes. The two methoxy groups contribute to the specificity in enzyme action, as may the methyl group (Crane, 2001). The fully substituted quinone ring is the functional group and does not allow addition reactions with thiol groups in the cell such as thioctic acid, thioredoxin or glutathione. By reduction of the quinone to quinol, a carrier of protons and electrons is produced (Crane, 2001).

2.2.2 The Biosynthetic Pathway of CoQ10

CoQ10 can be produced by chemical synthesis, semi-chemical synthesis and microbial conversion. Biotechnological production has been done using various genera, such as *Agrobacterium*, *Rhodobacter* and *Paracoccus* (Choi *et al*., 2005). The pathway for CoQ10 is typically composed of three parts: the synthesis of a quinonoid ring, synthesis of decaprenyl diphosphate (decaprenyl diphosphate synthase produses a unique hydrophobic tail composed of ten isoprenoid units) and quinonoid ring modification (basically where the head and tail groups are combined and the reaction product are transferred to the membrane). There are differences in supplying precursors for prokaryotes and eukaryotes (Choi *et al*., 2005).

Intracellular synthesis is the major source of CoQ10, although a small proportion is acquired through diet. It has been shown that ubiquinone in animal cells occurs, in addition to the mitochondria, in the endoplasmic reticulum, the Golgi apparatus, the lysomes, the peroxisomes, and the plasma membrane (Ernster *et al*., 1995). According to evidence, ubiquinone synthesis begins in the endoplasmic reticulum and is completed in the Golgi membranes from where the quinone is transported to various other cellular locations (Ernster *et al*., 1995). It is also discharged, although to a limited extend, across the plasma membrane to the blood, where it binds serum lipoproteins (Ernster *et al*., 1995). The complexity of the biosynthesis suggests that defects in different biosynthetic enzymes or regulatory proteins may cause different clinical syndromes (Quinzii *et al*., 2007b). In order to understand the apparent effectiveness of CoQ10 in therapies, it is imperative to understand the basic questions that still exist regarding the *de novo* synthesis of CoQ10 and the mechanisms by which it is transported to and from mitochondrial sites (Clarke, 2000).

In the human, CoQ10 is biosynthesized from the amino acid, tyrosine, through a cascade of eight aromatic precursors. These precursors indispensably require eight vitamins, which are: 5,6,2,8-tetrahydrobiopterin, Vitamin B6, Vitamin C, Vitamin B2, Vitamin B12, Folic acid, Niacin and Pantothenic acid (Folkers, 1996). In 1971 Whistance *et al*., reported that the intermediates involved in the conversion of para-hydroxybenzoic acid to ubiquinones by higher animals have still to be defined, although there would appear to be evidence that they are the same as those which participate in the biosynthesis of ubiquinones by photosynthetic and some non-photosynthetic gram-

negative bacteria. In 2002 Kawamukai reported that several genes for the biosynthesis of ubiquinone in animals and plants have been reported, but that genes are basically similar to those from *Saccharomyces cerevisiae* (a yeast intensively studied for the biosynthesis of ubiquinone), and therefore it is considered that the eukaryotic type of biosynthesis is common.

Para-hydroxybenzoic acid is the first aromatic precursor from the essential amino acid, tyrosine, in the biosynthesis of CoQ10 in animals (Folkers, 1996). In yeasts, hydroxybenzoic acid is synthesized from chorismate via the shikimate pathway (Goewert, 1980). The building blocks for the synthesis of the polyisoprenyl chain are provided by dimethylallyl diphosphate and isopentenyl diphosphate (Tran *et al*., 2007). Isopentenyl diphosphate (IPP) is an important precursor in CoQ10 biosynthesis and in the biosynthesis of cholesterol, cartenoid and prenylated proteins (Choi *et al*., 2005). It is derived from acetyl-coenzyme A via the mevalonate pathway in humans, yeasts, and archaebacteria, and the non-mevalonate pathway used by eubacteria, green algae and chloroplasts of higher plants (Kuzuyama, 2002 and Eisenreich *et al*., 2004). This leads to the formation of farnesyl-PP, which after conversion to decarpenyl-PP condenses with 4-hydroxybenzoic acid to decaprenoyl-4-hydroxybenzoate, which is then converted to ubiquinone in a number of additional reaction steps (Ernster *et al*., 1995).

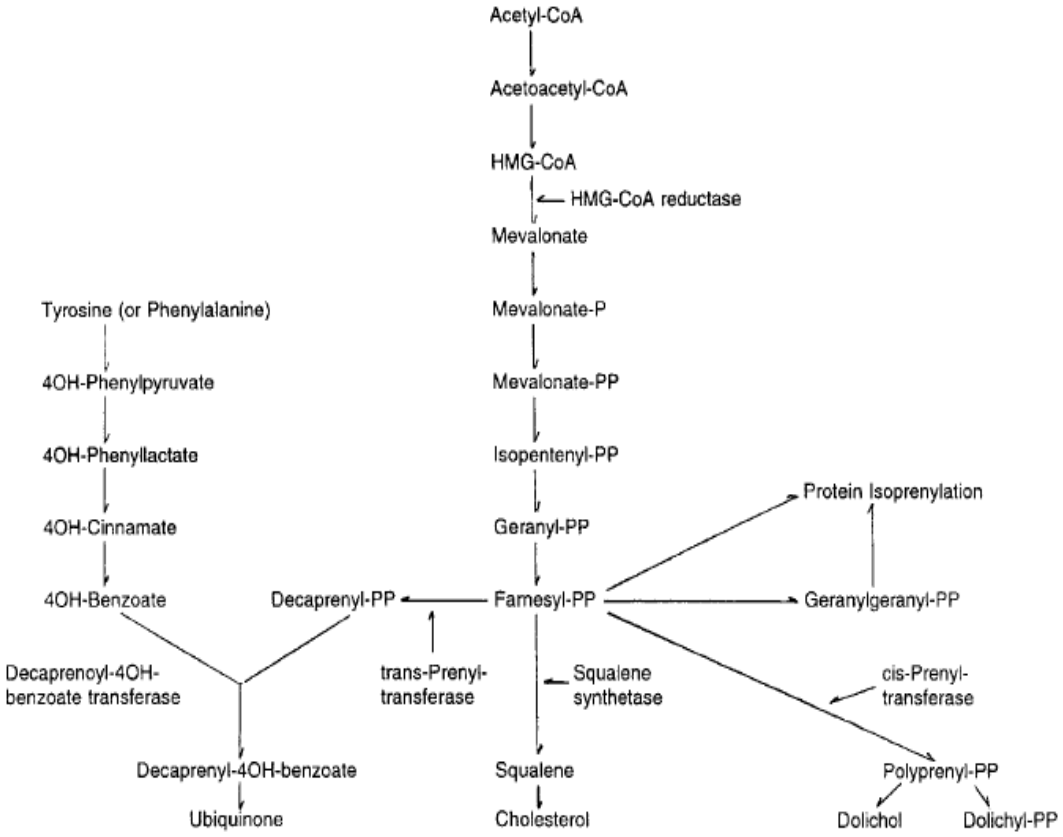


Figure 2.2: Reaction pathways of the biosynthesis of ubiquinone, cholesterol and dolichol (Ernster *et al*., 1995).

Clarke, 2000, reported evidence which suggested that the yeast *S. cerevisiae* provides an excellent prototype of a eukaryotic Q-biosynthetic pathway, and claimed that it can be used to answer fundamental questions about Q biosynthesis in higher eukaryotes. *S. cerevisiae* can synthesize Q from either chorismate or tyrosine (Meganathan, 2001). While in yeast the tyrosine is derived from chorismate *in vivo*, it has to be provided as an essential amino acid for mammals, which lack the shikimate pathway; therefore Q is not considered a vitamin (Meganathan, 2001). The methyl groups on the benzene ring are derived from S-adenosylmethionine (SAM). As stated, the isopentenyl diphosphate required for the formation of the polyprenyl side chain in eukaryotes, is derived from the mevalonate pathway. Grünler *et al*., 1994, described the mevalonate pathway as a sequence of cellular reactions that leads to farnesyl-PP, the common substrate for the synthesis of cholesterol, dolichol, dolichyl-P and ubiquinone as well as for prenylation of proteins.

2.2.2.1 The Tail

The starting precursor for the mevalonate pathway is acetyl-CoA (Meganathan, 2001). The pathway is initiated by the transfer of an acetyl group from acetyl-CoA (Begley *et al*., 1998) to the methyl carbon of a second acetyl-CoA, resulting in the formation of acetoacetyl-CoA (Eisenrieich *et al*., 2001). In the next step of the pathway, another molecule of acetyl-CoA condenses with acetoacetyl-CoA, resulting in the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (Lange *et al*., 2000). In a two step reaction, requiring NADPH, HMG-CoA is reduced to mevalonate (Boucher *et al*., 2000). Mevalonate is then converted to mevalonate-diphosphate by two phosphorylation steps, mediated by mevalonate kinase and phosphomevalonate kinase, respectively (Cunningham *et al*., 2000). Formation of IPP takes place in the subsequent step of the pathway where mevalonate diphosphate undergoes dehydration-decarboxylation, in an ATP requiring process (Cunningham *et al*., 2000). The isomerization of IPP to dimethylallyl diphosphate (DMAPP) then takes place and is catalyzed by an IPP isomerase (Grünler *et al*., 1994 and Qureshi *et al*., 1981). In the next step, the enzyme farnesyl-PP synthetase catalyzes the sequence tail 1'-4 coupling of IPP with DMAPP and Geranyl-PP (GPP) resulting in the formation of geranyl-PP and farnesyl-PP, respectively (Meganathan, 2001). In contrast with bacteria, the mammalian transprenyltransferase prefers geranyl-PP rather than farnesyl-PP as a substrate (Teclebrhan *et al*., 1993). Only one enzyme, farnesyl-PP synthase, is responsible for the formation of farnesyl-PP from dimethylallyl-PP and isopentenyl-PP (Poulter *et al*., 1981). Whether farnesyl-PP synthase is capable of forming geranyl-PP or if there is another enzyme responsible for synthesizing this intermediate, is not known (Dallner *et al*., 2000).

2.2.2.2 The Head

Ubiquinone contains a nonisoprenoid moiety namely the benzoate ring. This benzoate ring originates from the amino acid tyrosine, which can be synthesized from phenylalanine in animals, but the aromatic ring structure is always derived from dietary sources (Dallner *et al*., 2000). After condensation of the ring with the isoprenoid side chain, several substitutions and modifications of the benzoate ring occur. The sequence of enzymatic steps includes one decarboxylation, three hydroxylations, two O-methylations and one C-methylation to obtain the final product, a fully substituted benzoate ring, contributing to the properties of ubiquinone. The steps are not completely

understood in mammals, since most studies of ubiquinone biosynthesis have been performed on bacteria and yeast. In 2000 Dallner *et al*., reported that none of the enzymes involved have been either purified or cloned from mammals. In 2007, Tran *et al*., reported that nine complementation groups of Q-deficient yeast mutants (*COQ1* through *COQ9*) have been identified and that mammalian homologues of the yeast *COQ* genes have been identified via sequence homology.

CoQ10 is the dominant species in all human tissues, while only 2 – 7% of the total ubiquinone content is CoQ9 (Aberg *et al*., 1992), which is the main species in rats, while only 10-30% of the total ubiquinone content is CoQ10 (Dallner *et al*., 2000). In bacteria transprenyltransferase, which leads to two different products in the biosynthetic pathway, is the rate-limiting enzyme. Though it has been suggested that the hydroxylation or decarboxylation step in mammalian cells may be the rate-limiting factor, it should be emphasized that mammals, in contrast with bacteria and yeast, can not synthesize aromatic rings and that the pool of ring precursors could therefore be the rate-limiting factor. However, experiments support two other possibilities (Dallner *et al*., 2000). Polyprenyl-4-hydroxybenzoate may be a dead-end product, since it is considered a side reaction product resulting from the deacylation of a CoA derivative (Trumpower *et al*., 1974). 4-hydroxybenzoyl-CoA is a much better substrate for polyprenyl-4-hydroxybenzoate transferase than the nonactivated ring, since it was found to be 6-fold more effective as a precursor than p-hydroxybenzoate alone (Trumpower *et al*., 1974). Thus the CoA-derivatized polyprenyl-4-hydroxybenzoate may be required for the subsequent reaction leading to ubiquinone (Dallner *et al*., 2000). The second possibility is that 4-hydroxybenzoate may not be used as a substrate in mammals and instead, the more complete benzoate rings from catecholamine catabolism, protocatechuic and vanillic acid, could serve as precursors (Nambudiri *et al*., 1977). The possibility was raised that PHB, similar to fatty acids, must be activated by CoA to be able to condense with polyprenyl pyrophosphates. It was suggested that the benzoquinone ring may not be the only substrate (Forsgren *et al*., 2004). Rings modified by hydroxylation and methylation, which are supposed to take place later, may also serve as substrates in the condensation reaction (Forsgren *et al*., 2004). From a metabolic point of view these possibilities are of great interest, since some of these products are generated during catecholamine catabolism. The products could then be utilized for CoQ biosynthesis (Forsgren *et al*., 2004).

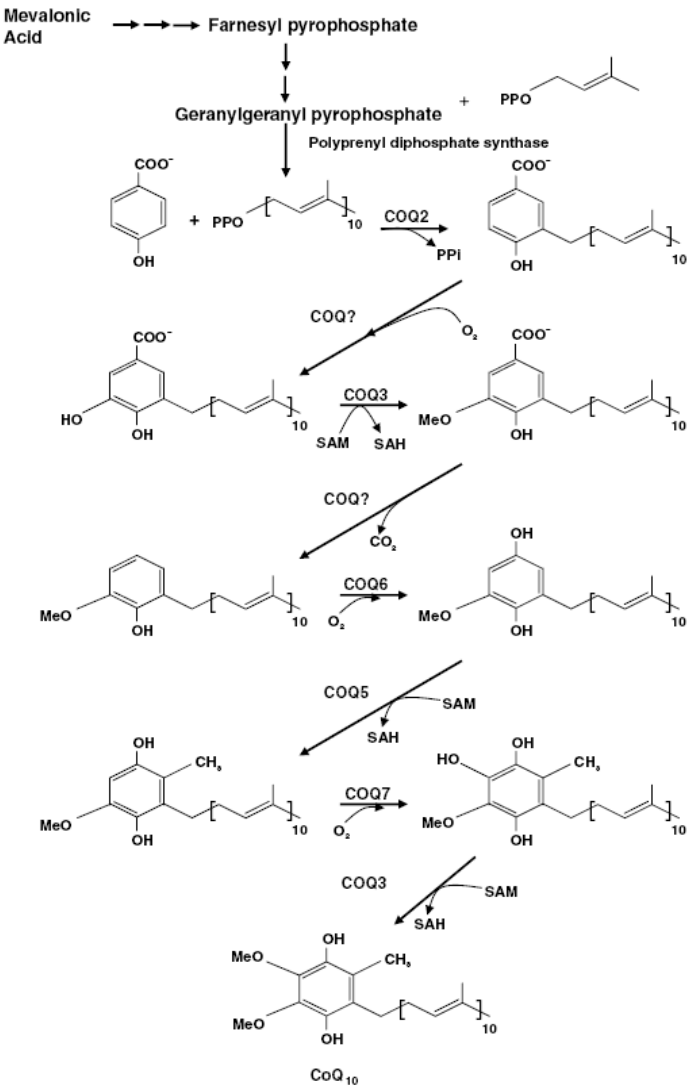


Figure 2.3: CoQ10 biosynthetic pathway with eight known biosynthetic enzymes denoted as polyprenyl diphosphosphate synthase (COQ1) and COQ2-8. Coenzyme Q10 is composed of a benzoquinone and a decaprenyl side chain. While the quinone ring is derived from amino acids tyrosine or phenylalanine, the isoprenoid side chain is produced by addition of isopentenyl pyrophosphate molecules to geranylgeranyl pyrophosphate (derived from mevalonate pathway) by decaprenyl diphosphate synthase. After para-hydroxybenzoate and decaprenyl pyrophosphate are produced, at least seven enzymes (encoded by COQ2-8) catalyze condensation, methylation, decarboxylation, and hydroxylation reactions to synthesize CoQ10 (Quinzii *et al*., 2007a).

2.3 The Genetic Link

So far nine complementation groups of Q-deficient yeast mutants (*COQ1* through *COQ9*) have been identified (Tran *et al*., 2007). Mammalian homologues of the yeast *COQ* genes have been identified via sequence homology. Human homologues of *CoQ2*, *CoQ3*, and *CoQ7* proteins were demonstrated to functionally complement the corresponding yeast null mutants, further indicating that the yeast Q-biosynthesis pathway is conserved in humans (Tran *et al*., 2007). While Coq1, Coq2, Coq3, Coq5, Coq6, and Coq7 proteins have known or proposed enzymatic functions in Q-biosynthesis, it is not clear whether other Coq proteins also possess enzymatic activities (Tran *et al*., 2007). Coq1 - Coq9 polypeptides localize to the mitochondria. *In vitro* mitochondrial import were investigated for seven of the yeast Coq polypeptides and demonstrated to be dependent on a mitochondrial membrane potential (Jonassen *et al*., 2001).

2.3.1 Function and Submitochondrial Localization of the Nine Coq Proteins (the following section refer to the study by Tran *et al*., 2007)

Coq1

Formation of the *trans*-polyprenyl diphosphate synthase tail in *S. cerevisiae* is catalyzed by the polypeptide encoded by the *COQ1* gene, which is responsible for determining the species-specific tail length of Q. The *Coq1* ortholog from the fission yeast *Schizosaccharomyces pombe* (Dps1) fails to complement the *S. cerevisiae coq1* null mutant, however, polyprenyl diphosphate synthases of fission yeast, mouse, and human, are each heterotetramers of two protein subunits, PDSS1 and PDSS2. Submitochondrial fractionation studies demonstrated that the *S. cerevisiae* Coq1 protein is peripherally associated with the inner mitochondrial membrane on the matrix side.

Coq2

The 4-HB polyprenyltransferase is a key enzyme catalyzing the attachment of the polyisoprenoid side chain to the 4-HB ring, generating the first membrane bound Q intermediate, 4-hydroxy-3-polyprenylbenzoic acid. The *S. cerevisiae* and *Homo sapiens*

gene encoding the enzyme is called *COQ2*. *In vitro* assays in isolated rat liver, demonstrated that the polyprenyl diphosphate: 4-HB activity is present mainly in the mitochondria. Polyprenyltransferases involved in Q biosynthesis generally display a lack of specificity for the chain length of the isoprenyl diphosphate substrate; however, the specificity was shown to be influenced by Mg^{2+} concentration in whole yeast extracts. Analysis of the predicted amino acid sequence of the *S. cerevisiae* Coq2 protein revealed two conserved putative substrate binding domains found in a family of polyprenyltransferases, six potential membrane spanning domains, and a typical mitochondrial targeting sequence. Coq2 protein behaves as an integral membrane protein associated to the inner mitochondrial membrane, facing the matrix side.

Coq3

Two O-methylation steps in the Q biosynthetic pathway are apparently catalyzed by the same enzyme encoded by *COQ3* gene. The amino acid sequences of the proteins encoded by these *COQ3* homologues all contain four regions that are conserved in a large family of methyltransferase enzymes utilizing S-adensymethionine (SAM or AdoMet) as the methyl donor and required a divalent cation. Like most of the other Coq polypeptides, the yeast Coq3 protein also contains a typical mitochondrial targeting sequence at the N-terminus. *In vitro* assays and subcellular localization studies showed that the Coq3 preprotein was imported and processed to the mature form in the mitochonria, in a membrane-potential-dependent manner. Submitochondrial fractionation demonstrated that it is a peripheral protein associated to the matrix side of the inner mitochondrial membrane.

Coq4

The enzymatic function of Coq4 protein, a peripheral protein associated with the inner mitochondrial membrane on the matrix side, has been a mystery. While it is appealing to speculate that Coq4 protein may serve as a hydroxylase or carboxylase in the yet-to-be-characterized step (designated Coq? In Fig 2.3), the amino acid sequence of Coq4 does not share significant homology with protein domains or motifs with known enzymatic activity. Steady-state levels of Coq3 and Coq7 proteins, which are diminished in *coq4* null mutants, are at wild-type levels in the *coq4-1* point mutation. This result together with recent work that demonstrates that the native Coq4 polypeptide co-migrates with

Coq3, Coq6, and Coq7 proteins as a high molecular mass complex indicates that the Coq4 protein has a structural role in the putative polypeptide Q biosynthetic complex.

Coq5

2-Methoxy-6-polyprenyl-1,4-benzoquinone methyltransferase catalyzes the only *C*-methylation step in the Q biosynthetic pathway, generating the 2-methoxy-5-methyl-6-polyprenyl-1,4-benzoquinone intermediate. In *S. cerevisiae*, the gene encoding this *C*-methyltransferase is designated *COQ5*. *In vitro* C-methyltransferase assays with the farnesylated analogs of the corresponding intermediates confirmed that Coq5 polypeptide is required for conversion of 2-methoxy-6-polyprenyl-1,4-benzoquinone to 2-methoxy-5-methyl-6-polyprenyl-1,4-benzoquinone in Q biosynthesis. The length of the polyisoprenoid tail does not play a crucial role in substrate recognition of Coq5 protein. Inclusion of NADH is essential for optimal enzymatic activity and is most likely required to convert the quinone to the hydroquinone, generating a nucleophile for the C-methylatransfer. Coq5 protein is peripherally associated with the inner mitochondrial membrane on the matrix side. Results suggested that Coq5 protein is essential for the stability and activity of at least two other Coq polypeptides, and provide genetic evidence for a complex of Coq polypeptides in yeast Q biosynthesis.

Coq6

COQ6 is a non-essential gene for viability, but is required for growth on non-fermentable carbon sources. The Coq6 protein is a mitochondrial protein, which is imported in a membrane-potential-dependent manner and peripherally associated with the matrix side of the inner membrane. *S. cerevisiae* Coq6 protein and its homologues in human, mouse, and *Canaerhobis. elegans*, each contains three conserved regions: an ADP-binding fingerprint, a motif with a putative dual function in FAD/NAD(P)H binding, and a consensus sequence that binds to the ribityl moiety of FAD. These conserved regions are common features of a large family of FAD-binding-aromatic hydroxylases. Consequently, Coq6 protein has been considered as a putative flavin-dependent monooxygenase responsible for adding the hydroxyl group to 4-hydroxy-3-polyprenyl benzoic acid and/or 6-methoxy-2-polyprenyl phenol, two uncharacterized hydroxylation steps in Q biosynthesis.

Coq7

Expression of *COQ7* homologues from *C. elegans*, rat, or human were shown to rescue the yeast *coq7* null mutant for growth on non-fermentable carbon resources, indicating functional conservation across species. Coq7 protein was shown to be required for the hydroxylation of 5-demethoxyubiquinol ($DMQH_2$). It was suggested that Coq7 protein is either involved in one or more mono-oxygenase steps or serves as an essential component of the putative multi-subunit enzyme complex. Biochemical function of Coq7 protein as a hydroxylase was further supported by the determination that it belongs to a family of di-iron binding oxidases containing a conserved sequence motif for the iron ligands, EXXH. It was indicated that yeast Coq7 protein functions in the hydroxylation of DMQ. Steady-state levels of the Coq3, Coq4, and Coq6 polypeptides were higher in the coq7E194K mutant than in the null mutant, suggesting that Coq7 protein and DMQ6 serve to stabilize other Coq polypeptides. Yeast Coq7 protein, like its homologues in mice, is peripherally associated to the inner membrane on the matrix side. The true nature of the Coq7 protein-membrane association awaits a structure determination for yeast Coq7p or one of its homologues.

Coq8

The *COQ8* gene was initially identified as *ABC1* for its ability to partially suppress, in multi-copy, the cytochrome b translation defect due to the cbs2-223 mutation in the *CBS2* gene, a nuclear gene encoding a translational activator of cytochrome b. Inactivation of *ABC1* resulted in respiratory defect and absence of NADH-cytochrome c reductase activity. It was subsequently shown that the respiratory complexes II, III and IV of the abc1 null mutant were thermo-sensitive, and addition of exogenous Q could partially compensate for the respiratory deficiency. These results led to a hypothesis that the *ABC1* gene product may function as a chaperone that is essential for proper conformation and activity of the bc1 and its neighboring complexes. Do *et al*., 2001 demonstrated that the *COQ8* gene required for Q biosynthesis, is the same as the *ABC1* gene and provided data indicating that Q deficiency is exclusively responsible for the pleiotropic defects of abc1/coq8 mutants. Although its biochemical function in Q biosynthesis is currently unknown, Coq8/Abc1 protein has been classified as putative protein kinase based on the identification of four kinase conserved motifs in its amino acid sequence.

Coq9

The *COQ9* gene was recently identified and characterized as a new gene that, when mutated, results in a Q-deficient phenotype, in a similar manner to other *COQ* genes. However, the function of Coq9 protein in Q biosynthesis is not yet known. Coq9 protein has no homology to proteins with known function. Based on the mobility in the SDS-PAGE, the molecular mass of Coq9 protein is about 25kDa, slightly smaller than the predicted precursor (29.9kDa), and is consistent with the removal of a putative mitochondrial targeting sequence. However, the native size of Coq9 protein estimated from its sedimentation on sucrose gradients is about three times larger, indicating that the protein is either a homo-oligomer or in a complex with other proteins, which may be Coq3 and Coq5 polypeptides, which were shown to co-sediment with the Coq9 protein. Submitochondrial localization analysis has demonstrated that Coq9 protein is a peripheral membrane protein associated with the matrix side of the mitochondrial inner membrane.

2.4 Physiological Functions of CoQ10

CoQ10 play five major physiological roles in the body (Collins *et al*., 1999):

1. It has an essential role in mitochondrial energy production through redox activity in the respiratory chain, transporting electrons between enzymes.
2. It functions as an antioxidant, inhibiting lipid peroxidation and scavenging free radicals.
3. It plays a role in extramitochondrial redox activity in the cell membrane and endomembranes.
4. It plays and important role in membrane stabilization and fluidity.
5. It has shown to prevent apoptosis by inhibiting the mitochondrial permeability transition pore (Papucci *et al*., 2003).

2.4.1 Mitochondrial Energy Production

CoQ10 is an essential part of the cellular machinery used to produce adenosine triphosphate (ATP), the energy on which the body runs. The major part of ATP production occurs in the inner mitochondrial membrane where CoQ10 is present (Crane, 2001). The unique function of CoQ10 is given by its ability to transfer electrons from the primary substrates to the oxidase system at the same time that it transfers protons to the outside of the mitochondrial membrane, resulting in a proton gradient across the membrane. As the protons return to the interior through the enzymatic machinery for making ATP, they drive the formation of ATP (Crane, 2001).

2.4.1.1 The Cellular Machinery

Energy generation in mitochondria occurs primarily through oxidative phosphorylation (OXPHOS), a process in which electrons are passed along a series of carrier molecules called the electron transport chain. The OXPHOS molecular system, which is embedded in the lipid bilayer of the mitochondrial inner membrane, consists of electron acceptors, coenzyme Q and cytochrome c, and five multisubunit protein complexes (complexes I-V) (Van den Heuvel *et al*., 2001). The electrons are generated from reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), which are produced by oxidation of nutrients such as glucose, and are ultimately transferred to molecular oxygen. The electron transport chain consists of four respiratory enzyme complexes arranged in a specific orientation in the mitochondrial inner membrane. The passage of electrons between these complexes releases energy that is stored in the form of a proton gradient across the membrane and is then used by ATP synthase to make ATP from adensine 5'-diphosphate (ADP) and phosphate by the fifth complex (Van den Heuvel *et al*., 2001). The ATP that is synthesized is then used for energy-requiring reactions in the matrix and is exported to the cytosol by the adenine nucleotide translocator, in exchange for cytosolic ADP.

Complex I (NADH:ubiquinone oxireductase):

The overall function of human complex I is the dehydrogenation of NADH and the transportation of electrons to coenzyme Q. This is the primary reductase where coenzyme Q is reduced by NADH (Crane, 2001). The electron transport is coupled to the translocation of protons across the mitochondrial inner membrane and creates a

proton gradient that is the proton-motive force for the production of ATP by F_1-F_0 ATP-synthetase (Van den Heuvel *et al*., 2001). During the reduction process, four protons are transported across the membrane for every coenzyme Q reduced (Crane, 2001). It has been proposed that coenzyme Q is reduced and reoxidized in the complex twice before electrons are transferred to a second loosely bound coenzyme Q, to form ubiquinol, which can travel through the lipid in the membrane to complex III, where the quinol is oxidized again, with transfer of protons across the membrane (Crane, 2001).

Complex II (succinate:ubiquinone oxidoreductase):

Human complex II catalyses the oxidation of succinate to fumarate during which electrons are transported from $FADH_2$ to the ubiquinone pool. The complex can be resolved into a soluble active succinate dehydrogenase (SDH), which is anchored to the matrix face of the inner mitochondrial membrane by two smaller subunits carrying cytochrome b558 and the ubiquinone-binding site, and a membrane-anchoring fraction (Van den Heuvel *et al*., 2001).

Complex III (decylubiquinol:cytochrome c oxidase):

Human complex III (cytochrome bc_1) catalyzes the electron transfer from ubiquinol to cytochrome c with the coupled translocation of protons across the inner mitochondrial membrane (Van den Heuvel *et al*., 2001). As in complex I, there is a cyclic oxidation-reduction-reoxidation with the oxidation and proton release step always on the outside, so that the proteins are released in the right direction (Crane, 2001). The mechanism by which electrons are transferred through the redox centers of complex III and the protons are translocated from the matrix side to the intermembrane space is called the protonmotive Q cycle (Van den Heuvel *et al*., 2001). The protonmotive Q cycle involves divergent oxidation of two molecules of ubiquinol (QH_2) and recycling of one electron from each oxidation through the bc_1 complex, while the second electron from each oxidation is passed through the Rieske iron-sulfur cluster and cytochrome c_1 *en route* to cytochrome c. Re-reduction of one molecule of ubiquinone via a stable semiquinone intermediate, is brought about by the two electrons that are recycled through the enzyme. The oxidation of two molecules of ubiquinol releases four protons to the intermembrane space, while re-reduction of ubiquionone results in the uptake of two protons from the matrix (Mitchell, 1975a; Trumpower, 1990; Hunte *et al*., 2003). The quinone cycle thus doubles the efficiency of coenzyme Q in building up the proton

charge across the membrane which allows twice as much ATP production than a simple one step oxidation of quinol (Crane, 2001). After the cycle is completed the oxidized quinone migrates through the membrane to be reduced at complex I (Crane, 2001).

Complex IV (cytochrome c oxidase):

Human complex IV (COX) is the last enzyme complex of the respiratory chain. It couples the transfer of electrons from reduced cytochrome c to oxygen, leading to a translocation of protons across the inner mitochondrial membrane. The resulting electrochemical gradient is used to drive the synthesis of ATP. The COX complex contains two copper atoms and two unique heme A iron porphyrins as redox centre, which are bound to a multisubunit protein frame that is embedded in the inner mitochondrial membrane (Van den Heuvel *et al*., 2001).

Complex V (F_0-F_1 ATP synthetase):

Human complex V catalyses the synthesis of ATP from ADP and P_i, using the energy of the proton motive force across the inner mitochondrial membrane (Van den Heuvel *et al*., 2001). The present consensus is that translocation of three protons drives the synthesis of one molecule of ATP, although it has sometimes been stated that only two protons are necessary (Brand, 1994). The F0 segment serves as a proton-conduction channel, allowing the transduction of the energy of the proton gradient and the membrane potential, through the connecting stalk subunits into the ATP-synthesizing F1 segment of the ATPase (Van den Heuvel *et al*., 2001).

The essential aspects of quinone function in energy transduction leading to ATP formation in the inner mitochondrial membranes, the plasma membrane of microbes, chromatophores, and chloroplasts, are control of the formation of hydroquinone and directed anisotropic hydroquinone movement to transfer protons across the membrane while electrons are transferred along a redox gradient (Mitchell, 1990).

2.4.2 Antioxidant Properties

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 favour 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 basis of our life on earth is the oxygen present in the atmosphere, but it can, under a number of conditions be a very toxic substance (Bentinger *et al*., 2007). Derivatives such as hydroxyl and superoxide radicals, hydrogen peroxide and singlet oxygen may be formed and are called reactive oxygen species (ROS). ROS appear not only in diseases but also under normal physiological conditions and interact with basic tissue components with consequences of disturbed function. Various types of antioxidant systems are available in all organisms for limitation and elimination of these unwanted species (Bentinger *et al*., 2007). The functional activity of the mitochondria greatly influences the extend of ROS formation, low levels of ADP and high mitochondrial membrane potential gives high levels of ROS, whereas high ADP levels and low membrane potential result in low production of ROS (Bentinger *et al*., 2007). Mitochondrial electron transport is accounted for two-thirds of the cellular oxygen consumption, and the observed limited leakage of electrons, 1 - 2%, is the largest contribution to the cellular O_2^- and H_2O_2 production (Papa *et al*., 1997).

Antioxidants are substances which counteract free radicals and prevent the damage caused by them (Venkat Ratnam *et al*., 2006). Antioxidants are enzymes and nonenzymatic agents that can prevent the formation of, or remove ROS (Turunen *et al*., 2004). There are four major groups of naturally occurring lipid soluble antioxidants, carotenoids, tocopherols, estrogens and coenzyme Q (Bentinger *et al*., 2007). Antioxidant enzymes include superoxide dimutase and various peroxidases such as glutathione peroxidase, catalase, thioredoxin reductase and peroxiredoxin. Nonenzymatic agents include vitamins C and E, cartenoids, glutathione, α-lipoic acid, flavinoids and the reduced form of CoQ, $CoQH_2$ that all rely on a mechanism of regeneration in the cell (Turunen *et al*., 2004). Mitochondrial $CoQH_2$ is efficiently regenerated by the respiratory chain and is normally kept in a highly reduced state (Aberg *et al*., 1992). Experiments on liposomes, mitochondria, microsomes, beef heart submitochondrial particles and lipoproteins of the blood, demonstrated that coenzyme Q in reduced form, ubiquinol, is an effective antioxidant and inhibits lipid peroxidation

(Bentinger *et al*., 2007). Bentinger *et al*., reported in 2007 that CoQ is our only endogenously synthesized lipid soluble antioxidant, and is mainly present in the activated (reduced) form. CoQ's effectiveness as lipid peroxidation inhibitor is based on its complex interaction during the process of peroxidation. The primary action is the prevention of lipid peroxyl radicals ($LOO^{\cdot}$) production during the initiation process, the first phase of the process, where an abstraction of a hydrogen atom from a methylene group of a fatty acid occurs, presupposing that it has several double bonds. $CoQH_2$ reduces the initiating perferryl radical with the formation of ubisemiquinone and H_2O_2. It is also possible that $CoQH_2$ eliminates $LOO^{\cdot}$ directly. The reduced lipid effectively regenerates vitamin E from the α-tocopheroxyl radical (Bentinger *et al*., 2007).

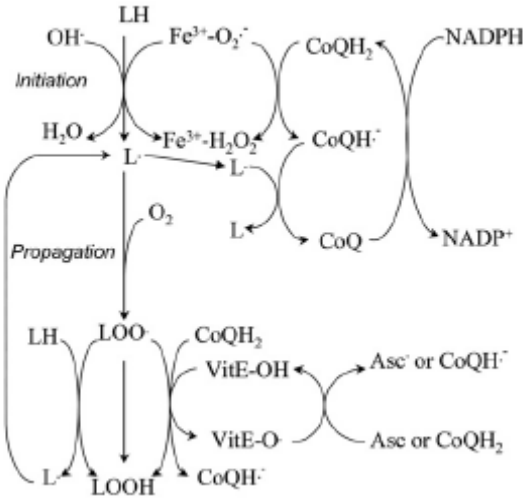


Figure 2.4: Sites of action of CoQ on lipid peroxidation. LH, polyunsaturated fatty acid; OH^{-}, hydroxyl radical; $Fe^{3}O_2^{\cdot -}$, perferryl radical; $CoQH_2$, reduced coenzyme Q; $CoQH^{\cdot -}$; ubisemiquinone; $L^{\cdot}$, carbon-centered radical; $LOO^{\cdot}$, lipid peroxyl radical; LOOH, lipid hydroperoxide; $VitE\text{-}O^{\cdot}$, α-tocopheroxyl radical; $asc^{\cdot}$, ascorbyl radical (Bentinger *et al*., 2007).

There are several mechanisms in protein oxidation and it appears that the most common is the direct oxidation of amino acid residues (Stadtman *et al*., 2000). Protein oxidation may also occur by lipid-derived free radicals and by breakdown products of phospholipid hyperperoxides. These compounds link covalently to basic amino acid residues and in the latter case also to sulfhydryl groups, causing intra- and intermolecular cross-linking (Bentinger *et al*., 2007). CoQ is effective in preventing protein oxidation by quenching the initiating perferryl radical and functioning as a chain-breaking antioxidant, thus preventing the process of propagation, the second phase in lipid peroxidation, where

$LOO^{\cdot}$ abstracts a hydrogen atom from an additional unsaturated fatty acid, leading to $L^{\cdot}$ and lipid hyperoxide (LOOH), which can be reoxidized to $LOO^{\cdot}$, which reinitiates lipid peroxidation (Bentinger *et al*., 2007). The sensitivity of proteins to oxidative stress depends on their structure, composition and localization. The close special relationship of CoQ to the neighboring membrane proteins is the main factor for its protective effect against protein oxidation (Bentinger *et al*., 2007).

CoQ also protect DNA against oxidative damage, which is of particular interest for mitochondrial DNA, since damage is not easily reparable (Bentinger *et al*., 2007). Oxidative stress may damage DNA by initiating a series of metabolic reactions in the cell leading to activation of nuclease enzymes that cleave the DNA backbone. A more common event is the interaction of H_2O_2 with metal ions bound to DNA which leads to the generation of hydroxyl radicals. DNA oxidation in isolated mitochondria takes place in the presence of ADP-Fe^{3+} and ascorbate, resulting in elevated content of 8-hydroxy-deoxyguanosine (8-OH-dG) (Ernster *et al*., 1995). Incubation in the presence of succinate and antimycin, which maximize the endogenous ubiquinol pool, eliminate the oxidative damage and decrease the increased strand breaks caused by ADP-Fe^{3+} (Bentinger *et al*., 2007). In mitochondria, ubisemiquinone radical is formed during respiration which is effectively reduced to ubiquinol by the “protonmotive Q cycle” described by Mitchell in 1975b. The large reducing capacity of the cell which is able to regenerate CoQ by reduction at all locations of the cell, is a very important property, contributing to the effectiveness of CoQ as an antioxidant (Bentinger *et al*., 2007). Supplemetation of CoQ is performed prophylactically to control oxidative events associated with oxygen radicals and therapeutically for the treatment of diseases related to impaired mitochondrial energy output (Mortensen, 1993).

2.4.3 Antiapoptotic Effect

Oxidative stress leads to mitochondrial dysfunction, which triggers the opening of the permeability transition pores (PTP), and the release of pro-apoptotic factors, causing cell death (Naderi *et al*., 2006). Physiological programmed cell death or apoptosis is essential for the maintenance of tissue homeostasis as it is responsible for the removal of damaged and infected cells. Apoptosis can be initiated through two major pathways; the extrinsic pathway (plasma membrane death receptor-dependent pathway) and the intrinsic pathway (mitochondrion-dependent pathway). Mitochondrial depolarization and

dysfunction play an important role in the intrinsic pathway, the initial steps in this pathway is represented by the opening of the PTP, followed by the collapse of inner mitochondrial membrane potential and release of pro-apoptotic factors such as cytochrome c and/or apoptosis inducing factor (AIF) (Green *et al*., 1998 and Reed, 2000). The mitochondrial permeability transition pore (PTP) is a complex, large conductance channel that plays a pivotal role in triggering apoptosis (Green *et al*., 1998). The opening of the PTP is responsible for the disruption of the mitochondrial transmembrane electrochemical gradient from -180 to 0 mV (Papucci *et al*., 2003). Ca^{2+} is the single most important factor for PTP opening *in vitro* (Walter *et al*., 2000). The inner membrane of the mitochondria has a low permeability to ions and solutes in order to permit energy conservation in the form of an electron and a pH gradient over the membrane. In order to facilitate trans-membranous transport, the inner membrane contains a number of macromolecule transporters and ion channels (Turunen *et al*., 2004). The mitochondria can undergo a generalized increase of permeability of the inner membrane under *in vitro* accumulation of Ca^{2+}. This is known as permeability transition (Turunen *et al*., 2004) and enables macromolecules with a size of approximately 1500 Da to cross the membrane, causing collapse of the protonmotive force, disruption of ionic status, loss of pyridine nucleotides, and hydrolysis of ATP (Fontaine *et al*., 1999). The PTP behaves like a voltage-dependent channel favoring a closed conformation at high membrane potentials and an open conformation at low membrane potentials. More than 40 classes of unrelated factors can modulate the PTP to open or close upon Ca^{2+} accumulation (Turunen *et al*., 2004). It is thought that the PTP serves as a fast Ca^{2+} release channel under physiological conditions (Turunen *et al*., 2004).

Walter *et al*., 2000, reported three classes of ubiquinone analogs that profoundly affect the PTP. PTP-inducers, PTP-inhibitors, and PTP-inactive quinones, which counteract the effect of the inhibitors and the inducers, were described. The structure-function correlation of the analogs has suggested that the methoxy groups are not essential for activity and that minor changes in the isoprenoid side chain can turn an inhibitor into an inducer. It was therefore concluded that the quinones modulate the PTP through a common binding site, whose occupancy would in turn modulate the PTP open-closed transitions through secondary changes of the PTP Ca^{2+} binding affinity, and that oxidation-reduction events were extremely unlikely (Walter *et al*., 2000). In a study done by Naderi *et al*., 2006, the ability of water-soluble CoQ10 to prevent apoptosis in

fibroblasts and transformed embryonic kidney cells, closely related to its ability to stabilize the mitochondrial membrane and decrease ROS generation, was shown. The results clearly indicated that mitochondrial membrane potential collapsed upon inducing oxidative stress, but if the cells were pre-treated with CoQ10 prior to oxidative stress induction, membrane potential remained intact. Their results also indicated direct inhibition of Bax activity on isolated mitochondria, suggesting that the protective effect of CoQ10 could be attributed to its ability to stabilize the mitochondrial membrane potential and blocking Bax activity (Naderi *et al*., 2006). Bax is a pro-apoptotic protein that moves from the cytoplasm to the mitochondrial membrane upon apoptotic stimulation (Naderi *et al*., 2006). Coenzyme Q10 was given to cell cultures simultaneous with cell death stimuli in a study done by Kagan *et al*., 1999. It was found that some cells were protected against cytotoxic insult by a general enhancement of mitochondrial viability and activity in the presence of CoQ10 in addition to a protection by CoQ10 of mitochondrial viability and activity after exposure to ceramide and ethanol (Kagan *et al*., 1999). They concluded that CoQ10 could interact with apoptotic machinery in the mitochondria, preventing the loss of mitochondrial potential by sequestering ROS generated in response to cell death signals and thus blocking cell death. The neuroprotective effect of CoQ10 may be due to antiapoptotic action that may vary depending on cell type and mode of cell death induction (Virmani *et al*., 2005). Alleva *et al*., 2001, pretreated Jurkat cells with $CoQ10H_2$- (the reduced form of CoQ10) and CoQ10, and then exposed the cells to α-TOS and hydrogen peroxide as well as immunological inducers of apoptosis. It was found that in $CoQ10H_2$- supplemented cells apoptosis was suppressed after exposure to α-TOS and hydrogen peroxide, but only a modest effect was exerted by these cells upon exposure to immunological agents. To clarify the mechanism by which $CoQ10H_2$- may suppress apoptosis induced by chemical and not immunological agents, the effect of CoQ10 supplementation on cellular ROS production was studied. ROS formation was suppressed in $CoQ10H_2$- but not CoQ10-enriched cells, suggesting that ROS are playing a major role in apoptosis induced by α-TOS, rather than by the immunological apoptogens, and therefore the redox function of $CoQ10H_2$- can modulate apoptosis induced by chemical stimuli. Thus, $CoQ10H_2$- may act as an antioxidant, blocking the actions of the oxidants implicated in the induction of apoptosis, which is supported by their finding that protection from apoptosis was not observed in CoQ10-enriched cells, confirming that the antioxidant potential of ubiquinol-10 is essential for its anti-apoptotic activity (Alleva *et al*., 2001). Brancato *et al*., 2000, have shown that

apoptosis of rabbit keratocytes induced by excimer lazer irradiation, a free radical-generating treatment, was prevented both *in vivo* and *in vitro* by CoQ10 (Brancato *et al*., 2000 & 2002). By using the same keratocyte cell line, Papucci *et al*., 2003, showed that CoQ10 prevent apoptosis also in response to apoptotic stimuli that do not generate free radicals. Coenzyme Q10 administration two hours prior to apoptotic stimuli prevents apoptosis not only in response to free radical-generating UVC irradiation, but also to antimycin A, C_2-ceramide and serum starvation, which do not generate free radicals, *i.e.* independently of the ability of apoptotic stimuli to trigger or not to trigger free radical generation (Papucci *et al*., 2003). The protective effect was clearly demonstrated by several evidences, including changes of cell morphology, quantification of living and apoptotic cells, and analysis of ATP cellular levels. Coenzyme Q10 significantly enhances the number of living cells, and lowers the number of cumulative apoptotic events. Coenzyme Q10 was also able to prevent massive reduction in ATP levels induced by all apoptotic stimuli, a phenomenon that is typically associated with the energy-consuming apoptosis execution and in particular with membrane potential collapse, consequent to PTP opening. The researchers suggested that the mechanism by which CoQ10 exerts its antiapoptotic activity, is associated with inhibition of PTP opening, and support their suggestion with the work of Fontaine *et al*., 1999 and Walter et al., 2000.

While in most tissues the large majority of CoQ is in reduced form, only 20% of the lipid is reduced in the brain. Since CoQ must be in the reduced form to function as an antioxidant, the large proportion of oxidized CoQ in the brain could therefore be a reflection of the high oxygen consumption in this tissue, causing an increased demand of antioxidants (Turunen *et al*., 2002). Cells require growth and survival factors that are provided by serum to maintain cell growth in culture; withdrawal of serum will induce cell growth arrest and some cells will develop the death program. Serum withdrawal causes a mild oxidative stress to cells in culture, leading to membrane damage and cell death (Ishizaki *et al*., 1995). Serum removal induces ceramide release to the cytosol by the activation of Mg^{2+} -dependent neutral sphingomyelinase (N-SMase) (Jayadev *et al*., 1995). Ceramide accumulation appears as a key component in the stress response pathway triggered by serum removal, since ceramides are able to induce cell death by activating proteases of the caspase family upon its intracellular accumulation (López-Lluch *et al*., 1999). López-Lluch *et al*., 1999, showed that the presence of CoQ10 in

serum-free cultures partially avoided the activation of apoptotic machinery through the inhibition of SMase pathway, and consequently prevented ceramide release and caspase-3 activation.

2.4.4 Extramitochondrial redox acitivity

Coenzyme Q10 is distributed among cellular membranes and it has a significant concentration at the plasma membrane. The plasma membrane contains a trans-membrane electron transport system, which is centered on CoQ10 (Gómez-Díaz *et al*., 2000). CoQ10 has access to enzymatic mechanisms able to regenerate its antioxidant reduced form, both in mitochondrial and extramitochondrial membranes (Ernster *et al*., 1995 and Kagan *et al*., 1996). CoQ10 is proposed to play a central role in antioxidant protection of the plasma membrane, either directly or through the regeneration of important exogenous antioxidants such as α-tocopherol and ascorbate (Gómez-Díaz *et a*l., 2000). CoQ10 can be considered as the central molecule in plasma membrane protection against lipid peroxidation because it is directly reduced by cytochrome b_5 reductase, and maintains both ascorbate and α-tocopherol in their reduced state (López-Lluch *et al*., 1999).

Coenyme Q10 is involved in the plasma membrane electron transport system, by which NADH in the cytoplasm transfers electrons through CoQ10 to electron acceptors such as iron or oxygen outside the cell (Crane, 2001). When this system is activated, the proton release through the H^+/Na^+ antiport is greatly increased, and when the system is inhibited by inhibitory CoQ10 analogs, the antiport is inhibited (Crane, 2001). If the H^+/Na^+ antiport is inhibited by amiloride, the transplasma membrane electron transport is accompanied by a slow release of protons equivalent to two protons released per quinol oxidized, indicating that the reduction oxidation of the CoQ10 in membranes is organized as in the lysosomes (Crane, 2001). The plasma membrane of eukaryotic cells contains an NADH oxidase (NOX) that is involved in the transfer of electrons across the membrane (Turunen *et al*., 2004). NOX is not a transmembranous protein, but is located at the external surface of the plasma membrane (DeHahn *et al*., 1997). The participation of CoQ10 in the plasma membrane electron transport was shown by the fact that the NOX activity was inhibited by the removal of CoQ10 with heptane and reconstitution of the activity by CoQ10 addition (Sun *et al*., 1992). In 1994, Villalba *et al*., 1995, showed that the participation of CoQ10 as an electron carrier is specific to trans-plasma

membrane electron transport in pig liver plasma membranes. Sun *et al*., 1992 reported additional evidence for CoQ10 function in trans-plasma membrane electron transport. Extraction of CoQ10 from the plasma membrane decreases NADH dehydrogenase, an enzyme shuttling reducing equivalents into the respiratory chain via complex I (Nohl *et al*., 2001), and NADH:oxidoreductase activity, and added CoQ10 partially restores activity. Ferricyanide reduction by transmembrane electron transport from HeLa cells is inhibited by CoQ10 analogs, which inhibit NADH dehydrogenase in plasma membranes, reduction of external oxidants by whole cells, and oxidant-induced proton release as well. The analog effects are reversed by CoQ10 (Sun *et al*., 1992). A relation between the transplasma membrane electron transport and control of the cytosolic redox state has been proposed (Crane, 2001). It is most dramatically revealed by the ability of external electron acceptors such as hexacyanoferrate to maintain Namalwa cell viability after mitochondrial energy production and substrate oxygen is lost. By maintaining cytosolic NAD, the transmembrane electron transport allows the production of ATP through glycolysis. The plasma membrane electron transport increases as cells lose their mitochondrial function (Larm *et al*., 1994).

Coenzyme Q10 can participate in several aspects of oxidation/reduction control such as signal origin and transmission in cells (Crane, 2001). The autoxidation of the semiquinone formed in various membranes during electron transport activity can be a primary basis for generation of H_2O_2 (Crane, 2001). The H_2O_2 in turn activates transcription factors such as NFκB, which protects against apoptosis, to induce gene expression (Crane, 2000 & 2001).

2.4.5 Membrane Stabilization

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 thought that the isoprenoid side chain may help to stabilize the lipid bilayer (Lenaz *et al*., 1999). Crane reported in 2001 that there is coenzyme Q floating in the phospholipids bilayer of the membranes. The free form may float with the all *trans* polyisoprene chain extended in the fatty acid chains of the lipid. Crane, 2001, also reported that there is evidence that in some cases the polyisoprenoid chain may be

folded into a shorter thicker structure. It is believed that the isoprenoid chain may help to stabilize the lipid bilayer (Lenaz *et al*., 1999). The quinone head group can be either oxidized (quinone), or reduced (quinol) (Crane, 2001). The quinol (hydroquinone) is more hydrophilic, so the head group can lie closer to the surface of the membrane. The change of position with oxidation and/or reduction may modify structural or enzymatic properties in the membrane. Crane, 2001, gave the redox state, which may control activity of phospholipases in the membrane, as an example. From the studies of Kagan *et al*., 1990, it appears that the membrane-stabilizing effects of ubiquinol are mainly based on recycling of α-tocopherol radicals resulting from an interference in autocatalytic lipid peroxidation. Besides α-tocopherol, ubiquinols may also react with lipid radicals; however, the rate constants of the reactions favor an interaction of lipid radicals with α-tocopherol. Irrespectively of the radical reacting with ubiquinol, the reaction product expected is the semiquinone (Nohl *et al*., 2001).

The aim of a study done by Hauβ *et al*., 2005, was the unequivocal determination of the location of CoQ10 in lipid bilayers, more precisely, the orientation and depth in the membrane profile. Neutron diffraction was employed, and data showed CoQ10 at the center of the hydrophobic core parallel to the membrane plane, and, not as might be expected, parallel to the lipid chains. This localization is of importance for its function as a redox shuttle between the respiratory complexes. Together with their results that squalane is in the bilayer center, it might be interpreted to show that all natural polyisoprene chains lie in the bilayer center. Thus, ubiquinone might also act as an inhibitor of transmembrane proton leaks (Hauβ *et al*., 2005). The distinct intramembranous localizations of lipids of the mevalonate pathway have considerable consequences on membrane properties. Polyisoprenoid chains of CoQ10, dolichol and dolichyl-P are present in the central hydrophobic region between the double layers of phospholipids fatty acids (Turunen *et al*., 2004). Turunen *et al*., 2004, suggested that they have a coiled formation and the three lipids saturate the available space. The functionally active groups, the benzoquinone ring of CoQ10 and the phosphorylated α-isoprene of dolichol-P, turn out to the outer or inner surface of the membrane depending on the functional requirement. This central localization is considered to destabilize membranes and results in an increased fluidity and permeability (Turunen *et al*., 2004). On the contrary, cholesterol is distributed between fatty acids on one side of the lipid leaflet, thereby causing stabilization with decreased fluidity and permeability. This

arrangement of isoprenoid-derived lipids has two major consequences. First, all the membranes have to be saturated with the appropriate lipid for optimal function and the level of saturation is dependent on the structural organization of the membrane type. Second, if a membrane is deficient in an isoprenoid, the consequences for membrane function, e.g. fluidity, will be deleterious (Turunen *et al*., 2004).

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). The study proposed that CoQ10 plays a key role in manipulating the redox potential poise, thereby affecting sub-cellular membrane potential changes, resulting in the differential regulation of sub-cellular membrane activities and compartments (Linnane *et al*., 2002). CoQ10 through the Q-cycle participates in the determination of mitochondrial membrane potential and in turn energy synthesis and mitochondrial substrate utilization (Linnane *et al*., 2002). CoQ10 has also been shown to be an essential co-factor of the uncoupling proteins which act to down-regulate mitochondrial membrane potential (Van Belzen *et al*., 1997). Gille *et al*., 2000, have demonstrated the occurrence of a lysosomal CoQ10 oxidoreductase system, which establishes a proton gradient across the membrane; such a system would contribute to the regulation and metabolite movement in and out of the lysosome.

2.5 CoQ10 Deficiency

Coenzyme Q10 deficiency may be caused by insufficient dietary CoQ10, impairment in CoQ10 biosynthesis, excessive utilization of CoQ10 or a combination of the three (Langsjoen, 1994). Significant lowered levels of CoQ10 have been noted in a wide variety of diseases in both animal and human studies (Langsjoen, 1994).

More than quarter a century has passed since Karl Folkers postulated that CoQ10 could have therapeutic potential for the treatment of cancer (Folkers, 1974). As CoQ10 was essential for normal cell respiration and function, any deficiency in its availability or biosynthesis could disrupt normal cellular functions, which could then lead to abnormal pattern of cell division that, in turn, might induce an oncogenic response (Folkers, 1974). The vitamins required for the biosynthesis of the four DNA bases, are also required for the biosynthesis of CoQ10 (Folkers, 1996). Mutations may be caused by the deficiencies in of one or more of these DNA bases and be considered only a dysfunction of genetics; however the initial biochemical dysfunction may actually be deficiencies of one or more of the three vitamins (vitamin B6, niacin and folic acid) required for the biosynthesis of the four DNA bases (Folkers, 1996). Although endogenous production is the body's primary source of CoQ10, it does not meet the requirements under certain pathological conditions (Bhagavan *et al*., 2005). Because of its crucial role in mitochondrial energy production, a number of systems are affected when the availability of CoQ10 becomes limiting, and tissues with high energy demands such as the heart are more readily affected (Bhagavan *et al*., 2005).

Primary CoQ10 deficiency is a clinically heterogeneous autosomal ressesive condition with a clinical spectrum that encompasses at least five major phenotypes: 1) encephalomyopathy, characterized by the triad of recurrent myoglobinuria, brain involvement and ragged red fibres; 2) severe infantile multisystemic disease; 3) cerebellar ataxia; 4) Leigh syndrome with growth retardation, ataxia and deafness; 5) isolated myopathy (Quinzii *et al*., 2007b). In most cases these disorders respond to CoQ10 supplementation.

Of the nine genes presumably involved in CoQ10 biosynthesis, and suspected of causing primary CoQ10 deficiency, three have been identified; *PDSS1*, *PDSS2* and

COQ2. There is good reason to believe that mutations in the six genes still at large may soon be found to underlie human disease (DiMauro *et al*., 2007). Mollet *et al*., 2007, reported an inbred family with CoQ10 deficiency manifesting as a multisystem disease with early-onset of deafness, encephaloneuropathy, obesity, livedo reticularis, and valvulopathy. Homozygosity mapping allowed the disease to be attributed to a homozygous missense mutation in *prenyldiphosphate synthase, subunit 1* (*PDSS1*), the enzyme that elongates the prenyl side chain of coenzyme Q. In the same study, direct sequencing of various genes involved in ubiquinone biosynthesis in an unrelated patient with fatal infantile multiorgan disease, detected a homozygous single base pair frameshift deletion in *OH-benzoate polyprenyltransferase* (*COQ2*), the gene encoding the enzyme involved in the second step of ubiquinone biosynthesis (Mollet *et al*., 2007).

López *et al*., 2006, reported the first pathogenic mutations in *PDSS2* which encodes *decaprenyl diphosphate synthase, subunit 2*, the first enzyme of the CoQ10 biosynthetic pathway, causing primary CoQ10 deficiency in an infant with fatal Leigh syndrome and nephrotic syndrome. Mutations in *PDSS2* should be considered as potential causes of CoQ10 deficiency in other patients with similar phenotypes as the patient in this study (López *et al*., 2006). Ataxia-oculomotor apraxia 1 (AOA1) is a newly identified autosomal recessive cerebellar ataxia associated with oculomotor apraxia, severe neuropathy, low levels of blood albumin, and increased levels of blood cholesterol (Le Ber *et al*., 2003). The *APTX* gene codes for aprataxin, a ubiquitously expressed protein that probably plays a role in single-strand break repair (Le Ber *et al*., 2007). A study by Le Ber *et al*., 2007, confirmed that aprataxin gene mutations are associated with decreased CoQ10 levels in muscle and that the decrease correlates with the genotype. Gempel, *et al*., 2007, described seven patients from five independent families with an isolated myopathic phenotype of CoQ10 deficiency. Coenzyme Q10 was significantly decreased in the skeletal muscles of all patients. Tandem mass spectrometry detected multiple acyl-CoA deficiency, leading to the analysis of the *electron-transferring-flavoprotein dehydrogenases* (*ETFDH*) gene, previously shown to result in another metabolic disorder, glutaric aciduria type II (GAII). All the patients in this study carried autosomal recessive mutations in *ETFDH*, suggesting that *ETFDH* deficiency leads to a secondary CoQ10 deficiency (Gempel *et al*., 2007).

CoQ10 deficiency can be also a secondary consequence of drugs, such as statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) (Quinzii *et al*., 2007). Statins have been used for the treatment of hypercholesterolemia and coronary artery disease and for the prevention of stroke. The mechanism of action by which statins elicit their effect is the inhibition of cholesterol synthesis at the level of mevalonic acid. Since the biosynthetic inhibition is not selective, statins also impair the synthesis of other compounds that share mevalonate as precursor, such as dolichol and CoQ10 (Quinzii *et al*., 2007a). For this reason, statin-related myopathy, manifesting as myalgia, muscle necrosis, and myoglobinura, has been hypothesized to be due to a partial deficiency of CoQ10 (Folkers *et al*., 1985 and Rundek *et al*., 2004).

Documented adverse effects associated with the use of CoQ10 in humans have been minor and include epigastric discomfort (0.39%), appetite suppression (0.23%), nausea (0.16%), and diarrhea (0.12%) (Greenberg *et al*., 1990).

2.6 Triton X-100

Triton X-100 is a nonionic detergent, 100% active ingredient, which is often used in biochemical applications to solubilize proteins. Triton X-100 has no antimicrobial properties and is considered a comparatively mild detergent and non-denaturing. The “X” series of Triton detergents are produced from octylphenol polymerized with ethylene oxide. The number “100” relates only indirectly to the number of ethylene oxide units in the structure. Triton X-100 has an average of 9.5 ethylene oxide units per molecule with an average molecal weight of 625, giving effective molarity of 1.7M. Any ethylene oxide polymer can form trace peroxides on exposure to oxygen. These impurities may interfere with biological reactions. For lysing cells, typically about 0.1% X-100 solution in water will be sufficient, and even up to 0.5% concentrations will usually not harm most enzymes being isolated. Many enzymes remain active in the presence of X-100; for example, a commonly used protease, Proteinase K, remains active in 1% (w/w) solutions of X-100 (Sigma Product Information Sheet).

An investigation by Deamer *et al*., 1967, has established that Triton X-100 is taken up by chloroplasts in a manner which involves a partition between the medium and chloroplast membranes, rather than by a strong, irreversible binding. This uptake causes

ultrastructural alterations consisting of a generalize swelling of all membrane structures followed by vesicle formation, at a concentration range of 50 – 200 µM, swelling initiated by Triton X-100 occurs over a period of several minutes (Deamer *et al*., 1967). Normally when Triton X-100 is added to chloroplast suspensions the chloroplast membranes become completely permeable to small ions, probably because uncharged "pores" are produced at the binding sites of Triton X-100. These pores are not necessarily true holes in the membrane, but probably represent points at which adsorbed Triton X-100 molecules have displaced normal membrane constituents (Deamer *et al*., 1967). Triton X-100 solubilizes membranes of PC12 cells and leaves behind a nucleus and an array of cytoskeletal filaments at a concentration of 0.5% (Vale *et al*., 1985), indicating the presence of proteins in the cytoskeleton that is insoluble to Triton X-100. A study done by Kőszegi *et al*., 2007, showed that Triton X-100 at alkaline pH is efficient in extracting total intracellular ATP in monolayer cell cultures. It was also shown that the detergent extract is suitable for protein determination, eliminating the need for subsequent protein-extraction steps (Kőszegi *et al*., 2007). A cross-linked form of Triton X-100, Triton WR-1339, has been shown to reduce the spread of tumour cells in laboratory animals (Picache *et al*., 2004). The outcome of a study by Picache *et al*., 2004, on the T24 bladder carcinoma cell line, provided a molecular basis for the antiproliferative effect of Triton X-100, namely its differential effects on various parts of the cell cycle machinery. Upon treatment of cells with Triton X-100, a potent antiproliferative effect resulting from the downregulation of the key cell cycle regulators, the cyclin-dependent kinases (CDK's), was seen. CDK activity was lost due to a twofold effect; the increased expression of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ in combination with the reduced expression of cyclin A, a regulatory CDK subunit, essential for CDK function (Picache *et al*., 2004). Triton X-100 seems to be a useful compound to analyze the structural and mechanistic features of complex I-associated ubiquinone oxidoreduction (Ushakova *et al*., 1999). Rapidly equilibrating Triton X-100 acts as a competitive inhibitor on Complex I and as non-competitive inhibitor on submitochondrial particles (SMP). Grivennikova *et al*., 1997, showed that rotenone binds more strongly to the active "turnover pulsed" enzyme than to its deactivated form. This appears to be also true for Triton X-100, and it is therefore expected that Triton X-100 solubilized Complex I would contain detergent molecule(s) bound at the Q-site(s), thus stabilizing the ubiquinone reactive state of the enzyme (Ushakova *et al*., 1999). The "unidirectional" preference of the inhibitory effect of Triton X-100 strengthens the proposal of the authors of different binding sites for both

pairs of the substrates (NADH/NAD^+ and ubiquinol/ubiquinone) which operates in the forward and reverse electron transfer catalyzed by the membrane-bound mammalian Complex I (Ushakova *et al*., 1999).

Biomembranes are not homogenous; they present a lateral segregation of lipids and proteins which leads to the formation of detergent resistant domains, also called “rafts”. These rafts are particularly enriched in sphingolipids and cholesterol (Kirat *et al*., 2007). Understanding the factors governing biomembranes' solubilization at the molecular level is essential in biophysics, biochemistry and cell biology. Especially in the case of rafts, examining the molecular determinants responsible for their insensitivity to Triton X-100 solubilization might clarify the mechanisms of membrane solubilization (Kirat *et al*., 2007). Kirat *et al*., 2007, reported two different Triton X-100 mediated solubilization pathways by doing real-time atomic force microscopy (AFM) study of model lipid bilayers exposed to Triton X-100 at different concentrations. It was concluded that (i) for non-resistant membranes, solubilization occurs by hole formation and the crumbling of the gel domains, and (ii) for resistant membranes, Triton X-100 erodes the bilayer patches visibly without affecting their center (Kirat *et al*., 2007).

2.7 Study Objectives

Therefore, the specific research objectives that directed this study were to:

1. Select a concentration range of Triton X-100, starting at a concentratin that will, according to the literature, cause membrane disruption and prepare a serial dilution from that.
2. Select a concentration range of CoQ10, where the recommended supplementation dosage, 100mg/day (Crane, 2001), will compare with the lowest two concentrations in the range
3. Establish and optimize methodologies used to isolate cardiac and skeletal muscle cells from chick embryos, and establish primary cultures of these chick embryonic cardiac and skeletal muscle cells.
4. Determine whether Triton X-100 and CoQ10 are cytotoxic to primary chick embryonic cardiac and skeletal muscle cell cultures by determining the effect of

Triton X-100 and CoQ10 on cell viability, lysosomal membrane integrity and cell number, using the MTT, NR and CV assays.

5. Choose two concentrations of Triton X-100, expected to cause cellular alterations, and test the cytotoxicity of the two concentrations on chick embryonic cardiac and skeletal muscle cell cultures, pre-treated with increasing concentrations of CoQ10, using the MTT, NR and CV assays.
6. Investigate the possibility that Triton X-100 might induce estrogenic activity, because of the similarity between the chemical structure of Triton X-100 and Nonylphenol, using the well established Recombinant Yeast Screen Assay (RCBA) for oestrogenic activity described by Routledge *et al*., 1996.
7. Investigate the ultrastructure of cardiac and skeletal muscle cells in primary culture, exposed to Triton X-100 and CoQ10, by SEM.
8. Determine whether CoQ10 offer any protection to cardiac and skeletal muscle cells in primary culture, ultrastructurally altered by the cell lysing properties of Triton X-100, by SEM.
9. 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.
10. Correlate the results obtained with SEM, with the results obtained with confocal microscopy.
11. 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.

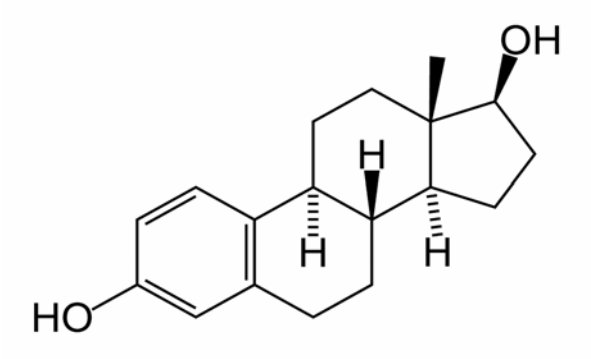
Chapter 3: Investigation of Possible Estrogenic Activity of Triton X-100

3.1 Introduction

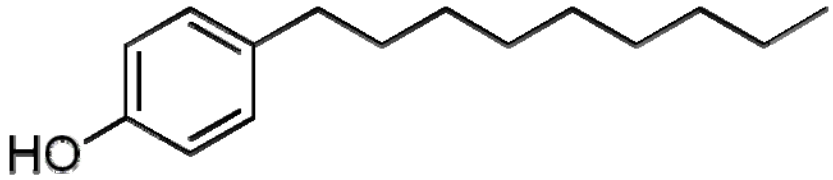
The endocrine system is a complex communication system between chemical signals and their targets responsible for regulating internal functions of the body. Any substance that alters the function of this system is termed an endocrine disruptor (Vazquez-Duhalt *et al*., 2004). An endocrine disruptor is a synthetic chemical that when absorbed into the body either mimics or blocks hormones and disrupts the body's normal functions. This disruption can happen through altering normal hormone levels, halting or stimulating the production of hormones, or changing the way hormones travel through the body, thus affecting the functions that these hormones control (Natural Resources Defense Council, 1998, http://www.nrdc.org/health/effects/qendoc.asp). Environmental chemicals that function as estrogens include, but are not limited to, chemicals that mimic the female sex hormone 17β-estradiol (Vazquez-Duhalt *et al*., 2004), and have been suggested to be associated with an increase in disease and dysfunctions in animals and humans (Klotz *et al*., 1996). The well-documented effects of environmental estrogens in animals and their potential for adverse effect in humans have led to the development of assays for identifying chemicals with estrogenic activity (Klotz *et al*., 1996). Nonylphenol ethoxylates (NPE) are surfactants used worldwide, and are transformed in the environment by microorganisms to form more toxic compounds, such as nonylphenol (NP) and short chain nonylphenol ethoxylates. These intermediates from microbial transformations, in addition to their intrinsic toxicity, seem to be able to mimic natural estrogens and disrupt the endocrine systems of higher organisms (Vazquez-Duhalt *et al*., 2004). Nonylphenol is one of the most studied estrogen mimics that appear to interact with development in several organisms (Vazquez-Duhalt *et al*., 2004). Analysis of the data compared to 17β-estradiol structure identified three structural criteria that were related to xenoestrogen activity and potency (Vazquez-Duhalt *et al*., 2004): (i) a hydrogen bonding ability of the phenolic ring mimicking the A-ring, (ii) a hydrophobic centre similar in size and shape to the B- and C-rings, and (iii) a hydrogen-bond donor mimicking the 17β-hydroxyl moiety of the D-ring, especially with an oxygen-to-oxygen

distance similar to that between the 3- and 17β-hydroxyl groups of 17β-estradiol. Moderately active compounds, such as NP, have a 4-hydroxyl substituted benzene ring with a hydrophobic moiety equivalent in size and shape to the B- and C-ring of 17β-estradiol (Vazquez-Duhalt *et al*., 2004).

a.



b.



c.

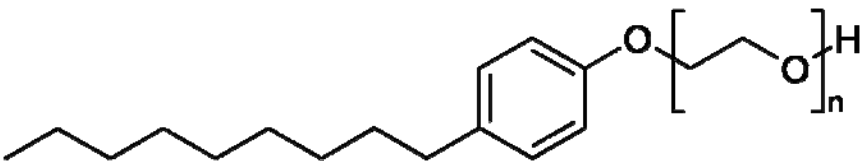


Figure 3.1: Chemical structures of **a**): 17β-estradiol, **b**): Nonylphenol and **c**): Nonylphenol Ethoxylates.

Triton X-100 is a polydisperse preparation of *p*-*t*-octylphenoxypolyethoxyethanols consisting of oxyethylene chain lengths averaging 9-10 oxyethylene units (Dennis, 1973). The hydrocarbon group is a 4-(1,1,3,3-tetramethylbutyl)-phenyl group. However, the general physical properties of this detergent are similar to those of the pure homogeneous compound having a chain length of 9 or 10 units (Becher, 1967). It is a nonionic detergent, 100% active ingredient, which is often used in biochemical applications to solubilize proteins. Triton X-100 has no antimicrobial properties. It is considered a comparatively mild detergent, non-denaturing, and is reported in numerous references as a routinely added reagent. It does absorb in the ultraviolet region of the spectrum, consequently, can interfere with protein quantitation (Sigma Product Information Sheet). Triton X-100 in aqueous solution forms micelles consisting of about 100-160 monomers corresponding to a molecular weight of about 63,000-105,000 (Dennis, 1973). Triton X-100 has a low critical micelle concentration (CMC) of 0.24 mM and can also be useful for the purification and the reconstitution of integral or lipid modified proteins in biomembranes (Kirat *et al*., 2007). Systems of nonionic polymer/surfactant possess many properties superior to those of ionic ones like higher stability, better biologic compatibility and lower toxicity, and these compounds have been widely used in material synthesis and biology simulation (Ge *et al*., 2007).

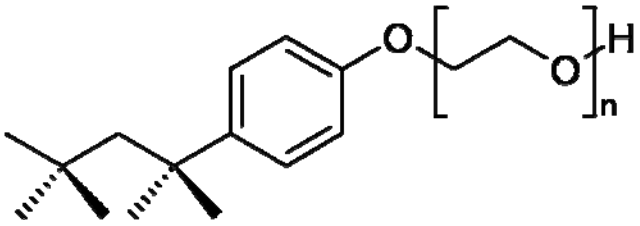


Figure 3.2: The chemical structure of Triton X-100.

a). **b).**

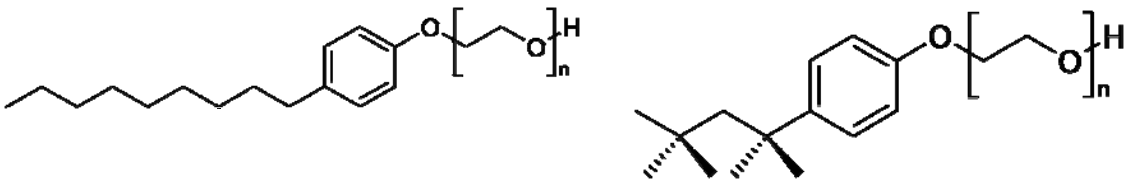


Figure 3.3: Comparing the structures of **a**): Nonylphenol Ethoxylates and **b**): Triton X-100

The clear resemblance between the chemical structures of Nonylphenol Ethoxylates and Triton X-100 (Figure 3.3) raised the question whether Triton X-100 might have estrogenic activity. In order to determine whether such properties do exist, the Recombinant Yeast Screen Assay (RCBA) for estrogenic activity was performed.

3.2 Materials and Methods

Triton X-100 (Cat. No. 9002-93-1), ethanol (Cat. No. 27,0741) and 17β-estradiol (Cat. No. E8875) were bought from Sigma-Aldrich (Pty) Ltd., Midrand, South Africa. Ninty six well, flat bottomed micro-titre plates (Cat. No. 95029780) were bought from Labsystems, Cape Town, South Africa. Chlorophenol red-β-d-galactopyranoside (CPRG) (Cat. No. 10884308001) was obtained from Roche Diagnostics, Randburg, South Africa.

The Recombinant Yeast Screen Assay (RCBA) for estrogenic activity (including details of medium components) previously described by Routledge *et al*., 1996 and Aneck-Hahn *et al*., 2005, was used. In this system, yeast cells transfected with the human estrogen receptor-α (ER-α) gene, together with expression plasmids, containing estrogen-responsive elements and the lac-Z reporter gene encoding the enzyme β-galactosidase, were incubated in a medium (minimal medium, pH 7.1) containing 17β-estradiol and the chromogenic substrate, chlorophenol red-β-d-galactopyranoside (CPRG). Active ligands, which bind to the receptor, induce β-galactosidase (β-gal) expression and these cause the CPRG (yellow) to change into a red product that can be measured by absorbance. The assay was carried out according to the standard assay procedure (Routledge *et al*., 1996) with minor adjustments (Aneck-Hahn *et al*., 2005) in a Type II laminar flow air

cabinet, to minimise aerosol formation. A 1% stock solution of Triton X-100 was prepared in ethanol and serial dilutions ranging from $1.56x10^{-11}$g/ℓ to $5.35x10^{-1}$g/ℓ were made and transferred to a 96 well micro-titre plate. Each plate also contained at least one row of blanks (assay medium and solvent ethanol) and a standard curve for 17β-estradiol ranging from 2.274µg/ℓ to 0.324pg/ℓ. After allowing the ethanol to evaporate to dryness on the assay plate, aliquots (200µℓ) of the assay medium containing the yeast and CPRG were then dispensed into each sample well. The plates were sealed and placed in a naturally ventilated incubator (Heraeus, B290) at 32°C for 3 to 4 days. After 3 days incubation the colour development of the medium was checked periodically at an absorbance (abs) of 540nm for colour change and 620nm for turbidity of the yeast culture. The absorbance was measured on a Titertek Multiskan MCC/340 (Labsystems) plate reader to obtain data with the best contrast. After incubation the control wells appeared light orange in colour, due to background expression of β-galactosidase and turbid due to the growth of the yeast. Positive wells were indicated by a deep red colour accompanied by yeast growth. Clear wells, containing no growth indicated lysis of the cells and colour varied from yellow to light orange. All experiments were performed in duplicate and repeated 6 times. The test absorbance of the samples was measured on day 4. The following equation was applied to correct for turbidity:

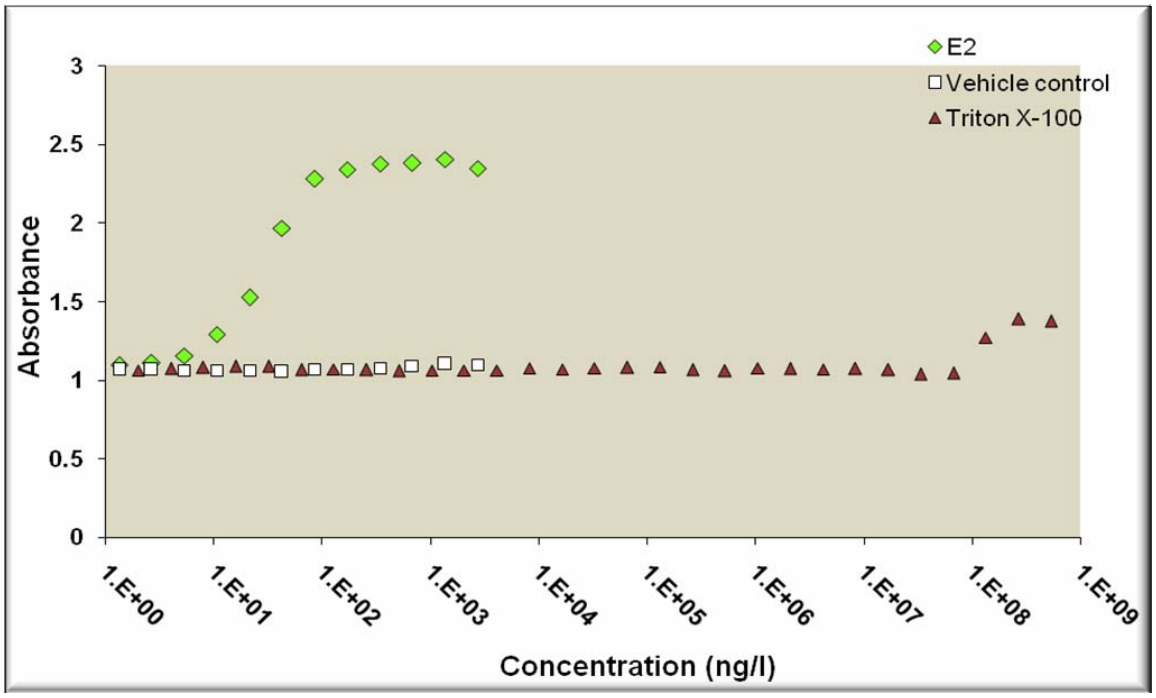
Corrected-value = test abs (540nm) - [test abs (650nm) - median blank abs (620nm)]

The 17 β-estradiol standard curve was fitted (sigmoïdal function, variable slope) using Graphpad Prism (version 2.01), which calculated the minimum, maximum, slope, EC50 value and 95% confidence limits. The detection limit of the yeast assay was calculated as absorbance elicited by the solvent control (blank) plus three times the standard deviation (SD).

3.3 Results and Discussion

A number of chemicals released into the environment are believed to disrupt normal endocrine function in humans and animals (Colborn *et al*., 1993 and Toppari *et al*., 1996). These endocrine disrupting chemicals (EDCs) have various endocrine and reproductive effects, believed to be due to their: (i) Mimicking effects of endogenous hormones such as estrogens and androgen, (ii) Antagonizing the effects of normal, endogenous hormone, (iii) Altering the pattern of synthesis and metabolism of natural hormones, (iv) Modifying hormone receptor levels (Soto *et al*., 1995). Chemical analysis on its own will rarely, if ever allow a confidant prediction of endocrine effects (Matthiessen *et al*., 1998). It has been shown that estrogen-mimicking chemicals are present in the aquatic environment. They are also accompanied by other substances, which can disrupt the endocrine system of aquatic fauna by alternative means (Aneck-Hahn *et al*., 2005). Bioassays and biomarkers that integrate these various endocrine disrupting processes, to achieve a more holistic picture of environmental impacts, are more cost-effective. Detection of activity can then be followed up with toxicity identification (Aneck-Hahn *et al*., 2005). One of the most widely applied *in vitro* assays uses genetically modified yeast strains that harbor an estrogen receptor expression cassette and a reporter construct (Breithofer *et al*., 1998, Arnold *et al*., 1996 and Coldham *et al*., 1997). Interaction of an estrogenic substance with the estrogen receptor causes a conformational change in the receptor, enabling the estrogen-estrogen receptor complex to bind to estrogen-responsive elements. The latter are located upstream of the *lacZ* reporter gene present on a reporter plasmid. Incubation of these recombinant yeasts with estrogenic chemicals triggers the expression of β-galactosidase (De Boever *et al*., 2001).

i.



ii.

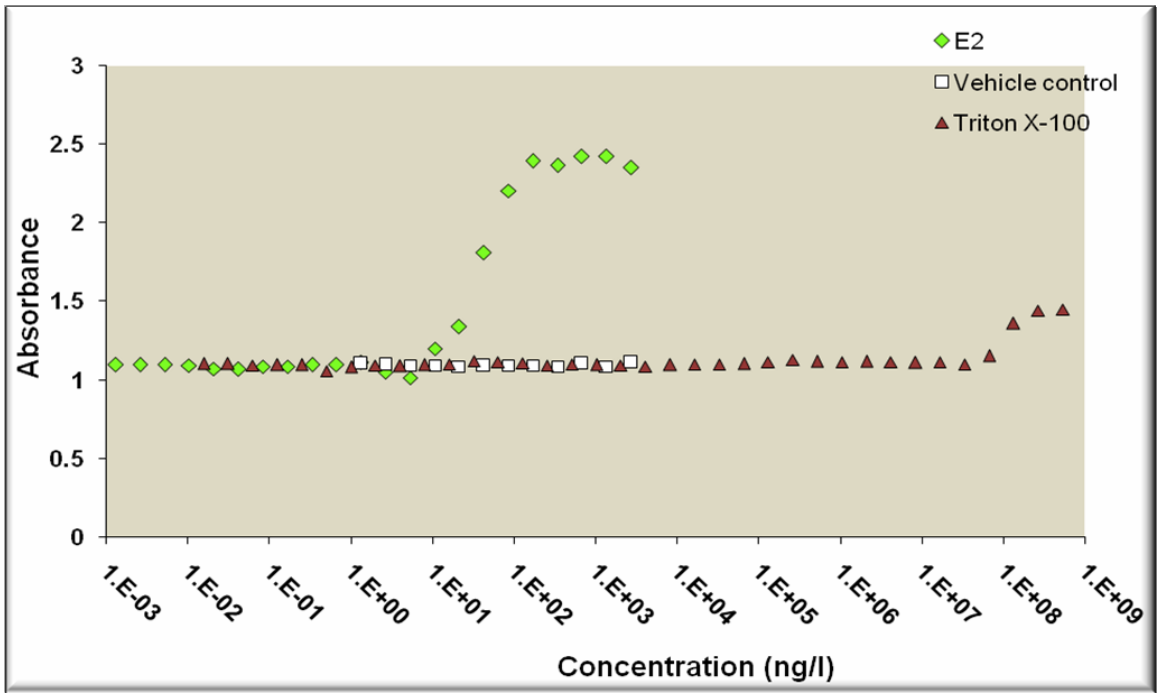
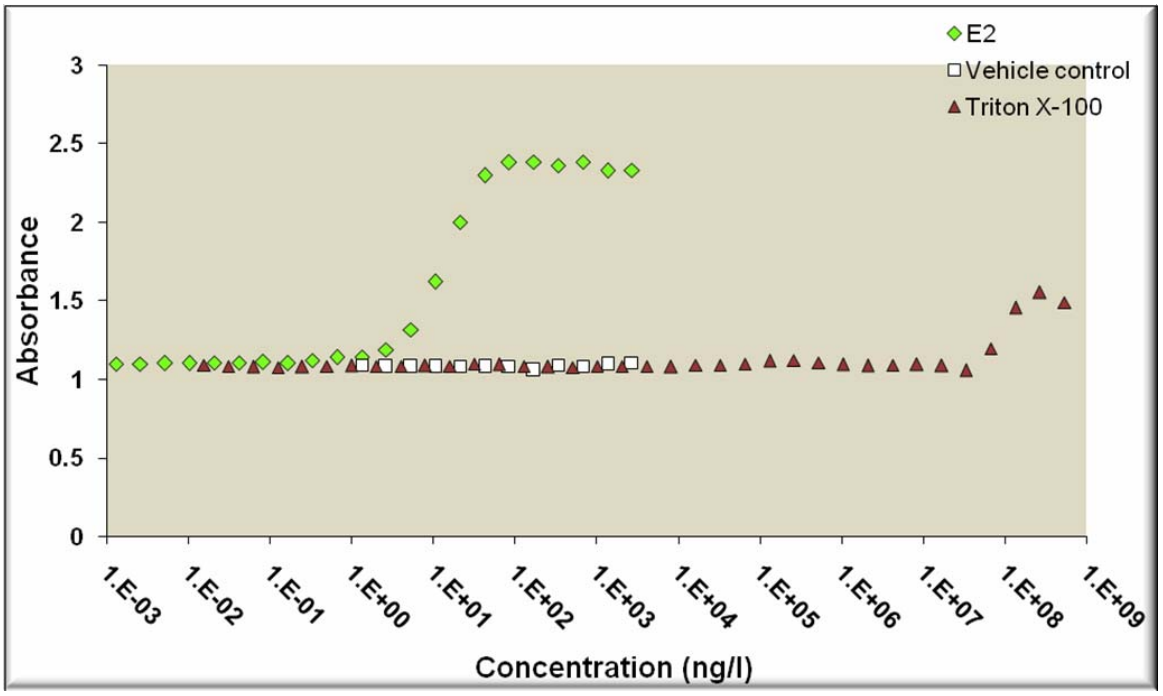


Figure 3.4 a: Log concentration of 17β-estradiol (E2) serially diluted from 2.72 x 10^{-6} g/l to 3.24 x 10^{-13} g/l and the log concentration for Triton X-100 serially diluted from 1.56x10^{-11} g/l to 5.35x10^{-1} g/l. Results for first and second repeats (**i**): sample 1 and (**ii**): sample 2 of the experiment.

i.



ii.

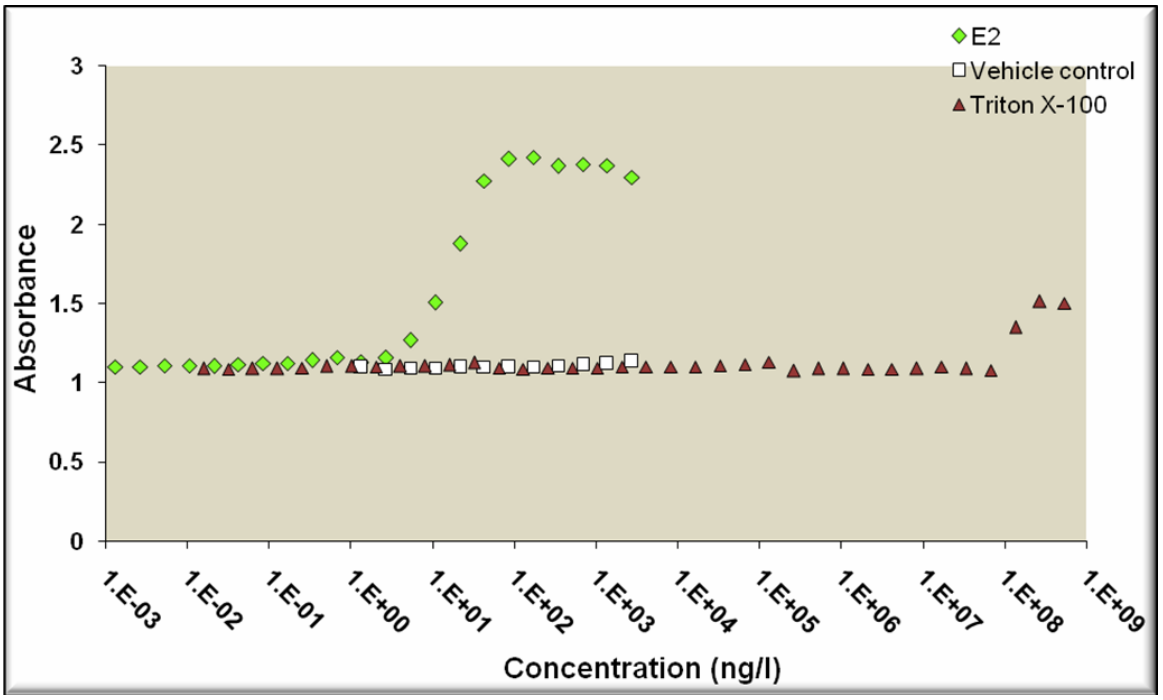
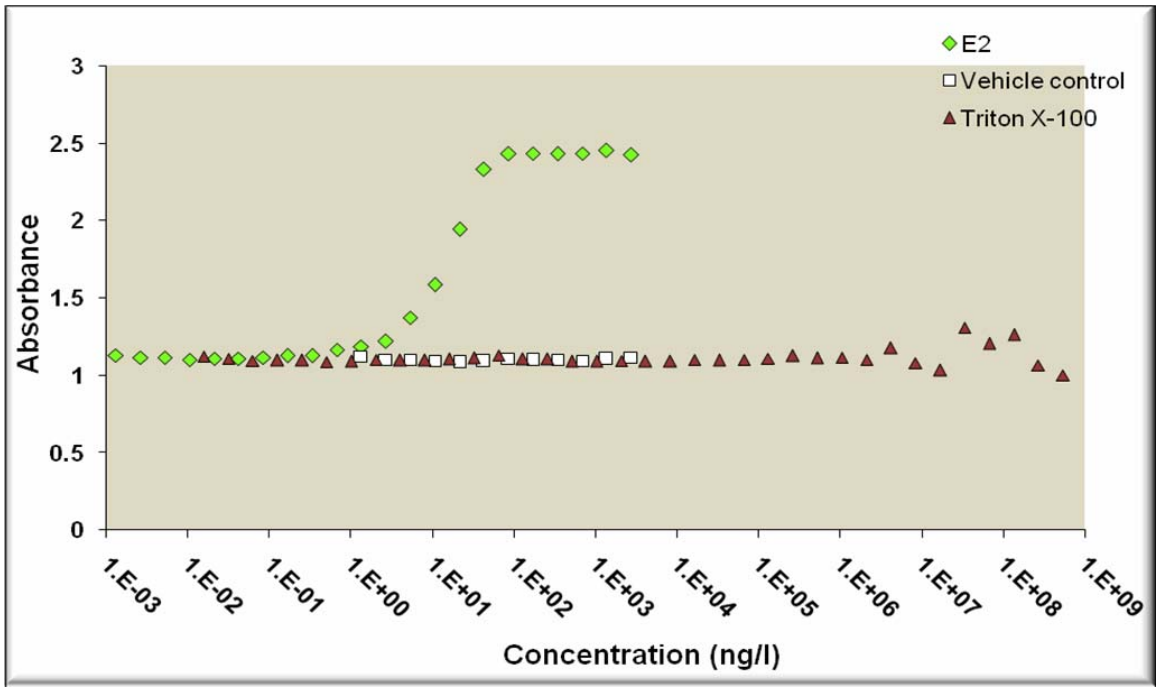


Figure 3.4 b: Log concentration of 17β-estradiol (E2) serially diluted from 2.72 x 10^{-6} g/l to 3.24 x 10^{-13} g/l and the log concentration for Triton X-100 serially diluted from 1.56x10^{-11} g/l to 5.35x10^{-1} g/l. Results for the third and fourth repeats; (**i**): sample 3 and (**ii**): sample 4 of the experiment.

i.



ii.

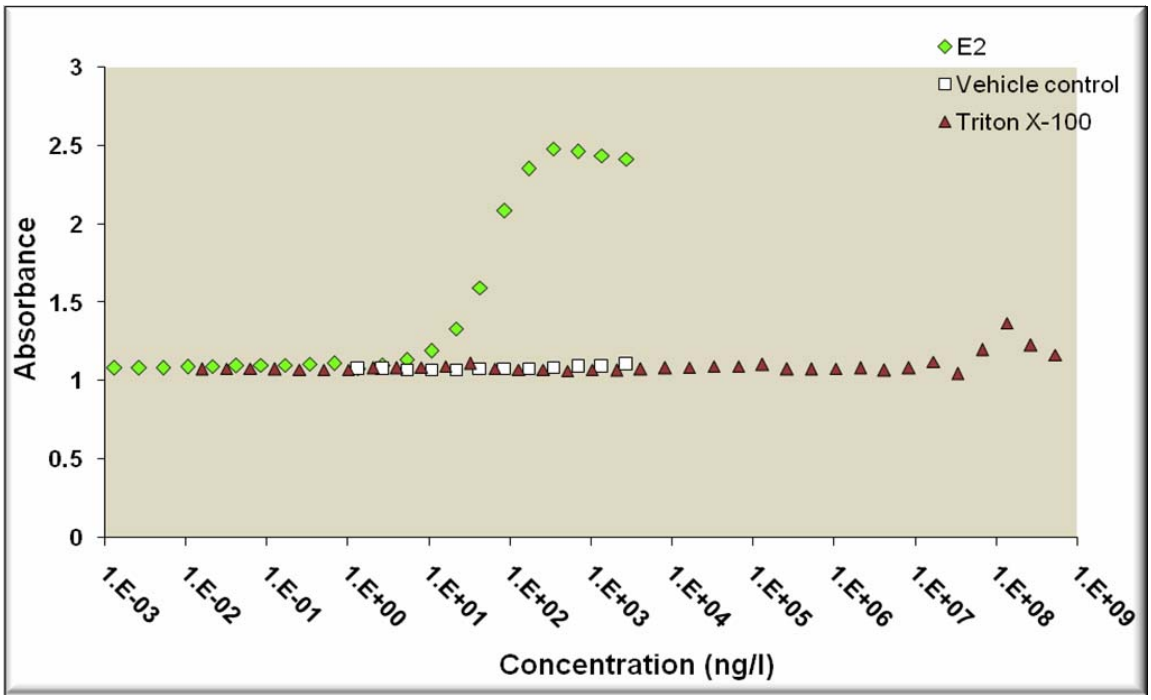


Figure 3.4 c: Log concentration of 17β-estradiol (E2) serially diluted from 2.72 x 10^{-6} g/l to 3.24 x 10^{-13} g/l and the log concentration for Triton X-100 serially diluted from 1.56x10^{-11} g/l to 5.35x10^{-1} g/l. Results for the fifth and sixth repeats; (**i**): sample 5 and (**ii**): sample 8 of the experiment.

Table 3.1: Summary of Results

Sample ID	YES result *	Type of response			Triton X-100 EC50 (ng/L)	17ß-Estradiol EC50 (ng/L)	Relative induction efficiency (RIE) %
		Toxic	Maximal	Submaximal			
1	3	x	-	x	14360	28.90	58
2	3	x	-	x	103100000	39.02	60
3	3	x	-	x	87010000	12.80	65
4	3	x	-	x	9901	16.08	64
5	3	x	-	x	2633000	14.67	53
6	2	x	-		N/Q	14.33	N/Q
7	2	x	-		N/Q	54.40	N/Q
8	3	x	-	x	64300000	52.59	55
Average					**42844544**	**29.10**	**59**
SD					**47597991**	**17.53**	**5**

Detection Limit (YES results)

* 0 Below detection limit

1 One point above detection limit

2 Two points above detection limit

3 Three or more points above detection limit (positive for estrogenic activity)

RIE = Relative induction efficiency. Max absorbance of sample/Max absorbance E2 x 100

N/Q = Not Quantifiable

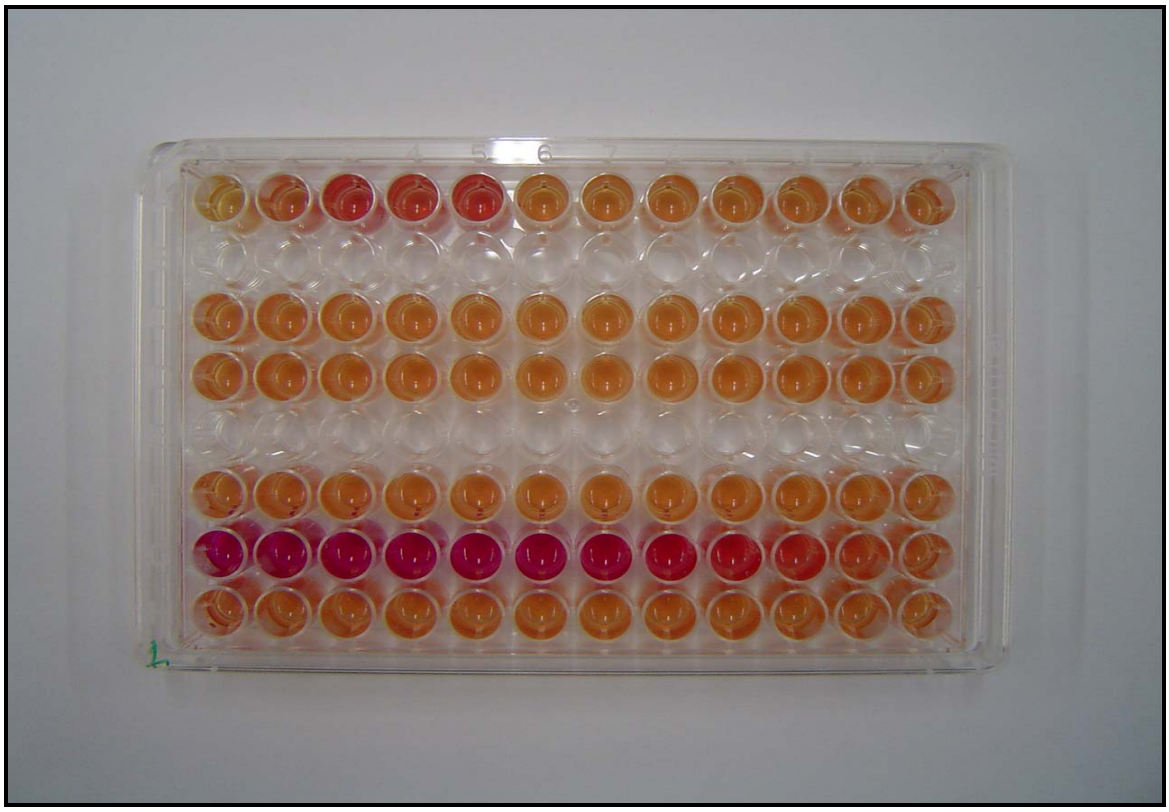


Figure 3.5: A 96-well plate used in the study photographed on day 4 of incubation. Rows 1, 3 and 4: a serial dilution of Triton X-100. Rows 2 and 5: left open, to prevent contamination due to possible creeping of the sample. Row 6: blank/control. Row 7 and 8: 17β-Estradiol. The increase in color intensity can be seen in the first (Triton X-100) row in the first 5 wells, indicating estrogenic activity and toxicity.

The recombinant yeast cells were designed and engineered for exquisite sensitivity to estrogens (McDonnell *et al*., 1991a, McDonnell *et al*., 1991b and Pham *et al*., 1991); over expression of human estrogen receptor, high amplitude frog vitellogenin estrogen response elements, and their tandem arrangement in the reporter plasmid all serve to amplify β-galactosidase production and hence sensitivity to 17β-estradiol (Klein *et al*., 1994).

A standard dose-response curve is defined by four parameters: the baseline response (bottom), the maximum response (top), the slope, and the drug concentration that provokes a response halfway between the baseline and maximum (EC50) (GraphPad Software, 1999). 17β-Estradiol produced dose response curves which occurred over the

whole of the absorbance range of the assay (full dose-response curves). A simple calculation, using EC50 values, showed that Triton X-100 is 496 times less potent than 17β-Estradiol and induced responses which were less than the maximum response obtained with 17β-Estradiol. Such curves that fail to reach the maximum response obtained with 17β-Estradiol are referred to as submaximal responses or submaximal response curves (Beresford *et al*., 2000). Parameter EC50 gives the transition center and equals the potency, which is the concentration that caused 50% efficiency (De Boever *et al*., 2001). The relative induction efficiency (RIE) is determined as the ratio of maximal β-galactoside activity induction with test compound to 17β-Estradiol x 100 (Coldham *et al*., 1997).

The detection limit of the yeast assay was calculated as absorbance elicited by the solvent control (blank) plus three times the standard deviation. When the absorbance values of the different samples of Triton X-100 were compared to the detection limit (three or more points above the detection limit is positive for estrogen activity), sample 6 and 7 had only two points above the detection limit. Sample 6 and 7 were not below the detection limit for estrogen activity and therefore, the EC50 values of Triton X-100 and the RIE were not quantifiable. The relative potency of Triton X-100 could not be determined, since Triton X-100 failed to induce β-galactosidase to 50% of the total 17β-Estradiol activity. Furthermore, Triton X-100 never provided the same induction of β-galactosidase activity as 17β-Estradiol at any concentration, as described by Coldham *et al*., 1997, and it was therefore not possible to calculate the relative potency of Triton X-100. Looking at the EC50 values of Triton X-100 (Table 3.1) it appears to be a weak estrogen despite limited repeatability. The yeast screen assay is a reliable assay to use to determine estrogenic activity. Good repeatability has been achieved when testing chemicals in this assay (Beresford *et al*., 2000). Considering that the six repeats (sample 1-5 and 8) were done in duplicate, the phenomenon of low repeatability is indistinct and obscure, and should be further investigated. Submaximal response curves in figure 3.4 c (sample 5 and 8), showed strange non-sigmoidal curves for Triton X-100. These results were strange and not interpretable, since the samples represented two repeats in duplicate, it is apparent that Triton X-100 has no repeatability. Although the relative potency of Triton X-100 was not consistent between repeats the RIE results for sample 1, 2, 3, 4, 5 and 8 were constant with a standard deviation (SD) of 5%. The maximum absorbance values of Triton X-100, ranged from 1.31 to 1.55 (control = 1.0) measured

on day 4, indicate the presence of weak estrogen activity (EEqs 0.04ng/L). One limiting factor of the recombinant yeast screen assay is that it can only measure estrogen activity if it is receptor-mediated. The weak estrogenic activity seen in sample 1 – 5 and 8 of the study must be receptor mediated. If the receptor had not been engaged there would have been no reaction. The reaction could be weak because it may function as an anti-estrogen. Alternatively Triton X-100 might possibly produce its estrogen properties by triggering other pathways in the cell contributing to endocrine disruption. Coldham *et al*., 1997, reported that competitive binding studies have shown that 2,3,7,8-tetrachlorodibenzo-p-dioxin does not bind to the estrogen receptor, but dioxins can evoke estrogenic and diverse potent antiestrogenic effects through various cellular mechanisms (Coldham *et al*., 1997, Birnbaum and Harris *et al*., 1990).

3.4 Conclusions

In conclusion, Triton X-100 showed weak estrogen activity. Upon comparison, the dose-response curves of Triton X-100 and 17β-estradiol in Figure 3.4 a and b i and ii, it was clear that Triton X-100 evoked an estrogenic response at much higher concentrations than the positive control 17β-estradiol. Similar results were obtained with Triton X-100 on the T47D-KBluc cell line (results not shown). The stable cell line, T47D-KBluc, constructed and described in a study by Wilson *et al*., 2004, resulted in the generation of a sensitive and responsive tool for the screening of chemicals for estrogenic activity. T47D human breast cancer cells, which contain both endogenous ERα and ERβ, were transfected with and ERE luciferase reporter gene construct. It provided an *in vitro* system that can be used to evaluate the ability of chemicals to modulate different assays (one for alpha and one for beta) would not be needed (Wilson *et al*., 2004). Triton X-100 showed tremendous variation in the results obtained in six duplicate repeats during the study. The results confirmed high toxicity of Triton X-100 however due to the results in YES and T47D-KBluc assay for estrogenic activity further investigations should be done on the strange and indefinable properties elicited by Triton X-100.

Chapter 4: The Toxic Effect of Triton X-100, CoQ10 Alone and in Combination on Primary Chicken Embryonic Cardiac and Skeletal Muscle Cell Cultures

4.1 Introduction

Triton X-100 is used in immunohistochemistry to make tissue permeable, to present certain antigens to antisera, and to prevent certain nonspecific interactions. This detergent is routinely dissolved in buffers at concentrations of 0.01 - 0.2% (Weruaga *et al*., 1998). Cimino *et al*., 2006, demonstrated that it is possible to successfully permeabilize mycobacteria with Triton X-100 in the concentration range 0.01% - 0.1% for intervals of 5, 10 and 15 minutes. These authors also showed that prolongation of the incubation time with Triton X-100 for more than 5 minutes generated cellular lyses (Cimino *et al*., 2006). In a study done by Fang *et al*., 1994, it was found that Triton X-100 non-enzymatically catalyzes the reduction of nitroblue diformazan (NBT) by NADPH to form formazan. Triton X-100 is also an activator of NADPH-diaphorase. Triton X-100 decreases the binding of formazan to cell membranes and dissolves the extracellular formazan. Triton X-100 also increases the permeability of cell membranes. These effects together can account for the improvement in the NADPH-diaphorase histochemical staining produced by Triton X-100 (Fang *et al*., 1994).

Coenzyme Q10 is present in the endomembranes of cells as well as in mitochondria, where it serves as a central component of the transmembrane electron transport system (Sun *et al*., 1992). The CoQ10 in endomembranes is concentrated in the Golgi apparatus and the plasma membrane (Kalin *et al*., 1987). The high concentrations of CoQ10 in these membranes raise the question of its function within the extramitochondrial membranes (Sun *et al*., 1992). Sun *et al*., 1992, listed four possible functions for CoQ10 in the extramitochondrial membranes: a) storage for transfer to mitochondria or the blood serum, b) action as a renewable antioxidant within the lipid bilayers, c) function as an electron carrier in these membranes. Plasma membranes contain oxidoreductase enzymes (Crane *et al*., 1988), including a trans-plasma

membrane electron transport system that influences the growth of cells (Crane *et al*., 1990), activates phosphorylation of membrane proteins (Harrison *et al*., 1991), and induces expression of *c-myc* and *c fos* protooncogenes (Crane *et al*., 1988 and Sun *et al*., 1992). Evidence is presented by Sun *et al*., 1992, that CoQ10 functions in transmembrane electron transport and that added CoQ10 stimulates growth of HeLa and BALB/3T3 cells in the absence of serum, by an unknown mechanism (Sun *et al*., 1992), which may indicate a possible role in cell growth (Kagan *et al*., 1999). In a study by Chopra *et al*., 1998, the relative bioavailability of typical commercially available forms of CoQ10 was compared with that of Q-Gel, a new solubilized form of CoQ10, in human subjects in two separate trials. The data from both the trials showed that Q-Gel is vastly superior to typical commercially available preparations of CoQ10, which means that much lower doses of Q-Gel will be required to rapidly reach and maintain adequate blood CoQ10 values than with any of the other currently available products (Chopra *et al*., 1998).

The heart is the first functional organ in the developing embryo (Lyons, 1996). The development of the chicken heart involves a series of cellular migrations, fusions, and tissue differentiation. The heart develops from the fusion of paired precardiac mesodermal tubes located on either side of the developing foregut. Between 25 and 30 hours of incubation, the paired heart vesicles begin to fuse at the anterior end and continue to fuse posteriorly to form one continuous tube. The heart begins to beat just after the paired heart rudiments begin to fuse, immediately before the bulbus cordis forms. Once the heart tubes have completely fused, the sinus venosus becomes the embryonic pacemaker. Eventually, when the atrium and ventricle each divide into a pair of chambers, and a typical four-chambered heart is present, the sinus venosus is incorporated into the right atrium where it gives rise to the sinoatrial node, the mature pacemaker (McCain *et al*., 1999). Contractions of cardiomyocytes can be observed in chicken embryos already after 36 hours *in ovo* (at the 9 somite stage) and after 12 further hours the entire blood flow is managed by a rhythmically contracting meshwork of myofibrils (Ehler *et al*., 1999). The limb buds are first identifiable at ~60 hours of embryonic development as small outgrowths of mesenchyme derived from both somatic and somatopleural areas adjacent to the limb, covered by body wall ectoderm (Chevallier *et al*., 1977). By 96 hours, cells of the limb mesoblast become developmentally restricted to the chondroblast, myoblast, or fibroblast lineages, and, as

development progresses, differentiated chondrocytes become evident in the core region of the limb and differentiated muscle fibers begin to form in the peripheral regions (Konieczny *et al*., 1983). All three muscle types, skeletal, cardiac and smooth muscles are composed of elongated cells specialized for contraction. Cardiac muscle cells have one nucleus but the ultrastructure is much like that of skeletal muscle cells. Skeletal muscle consists of a heterogenous population of multinucleated, striated myofibres, which are highly differentiated cells and therefore unique in structure, held together by connective tissue (Tortora *et al*., 2003).

Based on the known effects of Triton X-100, the cytotoxicity of this non-ionic detergent will be determined on primary chicken cardiac and skeletal muscle cell cultures, alone and in combination with CoQ10. The cytotoxic effects of CoQ10 on primary chicken cardiac and skeletal muscle cell cultures were determined. Cell culture is defined as growth of cells dissociated from the parent tissue by spontaneous migration or mechanical or enzymatic dispersal (Fresheny, 1994). Because of wide availability of chicken embryos and the ease of obtaining tissue from these embryos without resorting to sophisticated surgical procedures, chicken embryos are the ideal source of a variety of primary cell cultures. *In vivo* systems share the characteristic that they exclude the influence of other organs and of the circulatory and immune system, thus providing the possibility to study direct effects on a cell population (Sultan *et al*., 2001). Cell number, cell viability and lysosomal membrane integrity were determined using Crystal Violet (CV), MTT, and Neutral Red (NR) assays, respectively.

In this Chapter the following research objectives were investigated:

- Select a concentration range of Triton X-100, starting at a concentratin that will, according to the literature, cause membrane disruption and prepare a serial dilution from that.
- Select a concentration range of CoQ10, where the recommended supplementation dosage, 100mg/day (Crane, 2001), will compare with the lowest two concentrations in the range.
- Establish and optimize methodologies used to isolate cardiac and skeletal muscle cells from chick embryos, and establish primary cultures of these chick embryonic cardiac and skeletal muscle cells.

- Determine whether Triton X-100 and CoQ10 are cytotoxic to primary chick embryonic cardiac and skeletal muscle cell cultures by determining the effect of Triton X-100 and CoQ10 on cell viability, lysosomal membrane integrity and cell number, using the MTT, NR and CV assays respectively.
- Choose two concentrations of Triton X-100, expected to cause cellular alterations, and test the cytotoxicity of the two concentrations on chick embryonic cardiac and skeletal muscle cell cultures, pre-treated with increasing concentrations of CoQ10, using the MTT, NR and CV assays.

4.2 Materials

4.2.1 Primary Cell Cultures

Fertile chicken eggs used for the experiments were obtained from National Chicks (Pty) Ltd, and Eagles Pride Hatchery (Pty) Ltd, and were stored at 4˚C for a maximum of two weeks. For the embryo development the fertile eggs were placed in the Grumbach incubator for 13 days, at a temperature 37.5˚C until day of termination.

4.2.2 Triton X-100 and CoQ10

Triton X-100 (CAS number: 9002-93-1), was obtained from Sigma-Aldrich (Pty) Ltd., Midrand, South Africa. CoQ10 Q-Gel Mega, 100mg, used in this project was manufactured by the Nutraceutical Science Institute (NSI), and was obtained from *vitacost.com*. (Item number: NI 003723 or 835003003723), USA.

4.2.3 Media, Supplements, Reagents and Plasticware

Dulbecco's Modified Eagle's Medium (DMEM), Hank's Balanced salt solution (HBSS), Foetal Calf Serum (FSC), and penicillin-streptomycin-fungizone (100x) (PSF) were obtained from Highveld Biological, Lyndhurst, South Africa. Sartorius cellulose acetate membrane filters 0.22µm were from National Separations, Johannesburg, South Africa. 0.20µm Minisart-Plus filters were from Sartorius, Goettingen, Germany. Fixatives, acids and organic solvents, such as gluteraldhehyde, formaldehyde, hydrochloric acid (HCl), acetic acid, isopropanol, and formic acid were analytical grade and were purchased from Merck, Johannesburg, South Africa. Trypsin, ethylene diamine tetra acetate (EDTA),

dimethyl sulphoxide (DMSO), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium chloride, and sodium hydrogen carbonate ($NaHCO_3$) were from Merck, Johannesburg, South Africa.

MTT (1-(4,5-Dimethylthiazol-2-yl)–3,5 diphenylformazan), Crystal Violet (CV) powder and Neutral Red (NR) powder were form Sigma-Aldrich, Atlasville, South Africa. Water was double distilled and deionized (ddH_2O) with a Continental Water System and sterilized through a Millex 0.2µm filter. Glassware was sterilized at 140˚C in a HL-340 Series vertical Type Steam Sterilizer Autoclave.

Twenty four well plates and 96-well plates, $25cm^2$ and $75cm^2$ cell culture flasks, 5ml and 10ml pipettes, 15ml and 50ml centrifuge tubes, micro centrifuge tubes and eppendorf tubes were from NUNC™ supplied by AEC- Amersham, Johannesburg, South Africa.

4.3 Methods

4.3.1 Establishment of Chicken Cardiac and Skeletal Muscle Cell Cultures

For the establishment of primary cultures of chick cardiac and skeletal muscle cells, several parameters needed to be optimized, namely the stage of embryological development for successful establishment of primary cardiac and skeletal muscle cells. The eggs were incubated for six (E6), and thirteen (E13) days. After each incubation period, heart development and development of the leg muscle were evaluated and cells were isolated and plated as described below. The developmental phase between 4 and 9 days of incubation is characterized by rapid changes in the wings, legs, and visceral arches. From the 8^{th} to the 12^{th} day, feather-germs and eyelids provide the most useful criteria to describe and distinguish the developmental stages. The designation of stages during the last phase of incubation is difficult because practically no new structures are formed and there is mainly just growth of what already exists (Hamburger *et al*., 1951). Hamburger et al., 1951, described stage 39 (E13) of the limb development: "*Limbs:* Scales overlapping on superior surface of leg. Major pads of phalanges covered with papillae; minor pads are smooth. Length of third toe = 9.8 ± 0.3 mm". The tubular heart of chick embryos begins beating spontaneously at 36-45 hours, and its contractions are

coordinated by propagation of activity before the appearance of specialized conducting tissues. Desmosomes and intercalated discs are present at day 2. Gap junctions are not found. Myofibrils in young hearts are sparse, run in all directions, and are in various stages of formation. Sarcoplamic reticulum is present, but a transverse tubular system is absent (Sperelakis *et al*., 1972). On the 6th day of embryonic development, it was extremely difficult to obtain muscle tissue from the hind limbs, since the muscle tissue was still developing, and the limbs were very tiny. Development of the heart and leg muscle tissue were optimal on the 13th day of embryonic development (E13), since it was possible to remove the heart from the thoracic cavity without the risk of removing other tissue along with it. It was also possible to remove leg muscle tissue from the bones (cartilage), and remove the skin covering the limb from the muscle tissue without the risk of cartilage or epidermal cell contamination. Muscle tissue was of desired size and suitable for primary cell culture procedures. Embryonic day 13 was decided on to harvest tissue for culturing.

After each incubation period (13 days) eggs were removed from the incubator and sprayed with 70% ethanol. The wider side, containing the airspace was opened and the embryo was gently lifted out using a spoon spatula and placed into a sterile Petri dish, followed by immediate decapitation. Aseptic techniques were used. The leg muscle and heart were dissected from the embryo. The skin and bony structures were removed from the leg muscle, and the hearts were trimmed free of the pericardium and visible vessels. The heart and skeletal muscle tissues were then cut into small fragments in separate sterile Petri dishes, and washed thrice with Hanks Balanced Salt Solution (HBSS) (9.7g/L Hanks Balanced Salt Solution + 0.35g/L $NaHCO_3$ in 1L ddH_2O), in a 15ml test tube. The tissue was incubated in 0.025% trypsin solution (0.2g EDTA, 0.25g Trypsin in 100ml DPBS) prepared in Dulbecco's Phosphate Buffered Saline (DPBS), for 24 hours at 4˚C. A 10X DPBS stock solution was prepared by dissolving 2g/l KCl, 2g KH_2PO_4, 80g/l NaCl and Na_2HPO_4 in ddH_2O that was diluted 1:9 with ddH_2O prior to use. Thereafter, trypsin was removed, and the pellets were incubated for 20 minutes at 37.5˚C and 5% CO_2 in a NAUIRE^{TM} US Autoflow CO_2 water-jacketed incubator. After incubation Dulbecco's Modified Eagle's Medium (DMEM), containing 5% foetal bovine serum (FBS) and 2% antibiotics (PSF), (a commercially available antibiotic/fungus supplement prepared for cell culture media from Highveld Biological, Lyndhurst, South Africa who usually prepare the antibiotics at 1% each and the fungizone at 250ug/ml),

was added to inhibit digestion. The cells were then washed thrice with DMEM, by repeated resuspension and sedimentation using a Hermle Z300 centrifuge at 1250rpm for 2 minutes. The final pellet was resuspended in 4ml of DMEM containing 5% FBS and 2%PSF. Single cell suspension were prepared by mechanical trituration, accomplished by pippetting the suspension several times through a 5ml pipette, and then left for 1-2 minutes to allow any large fragments of cells to settle to the bottom of the 15ml test tube. A total cell count was performed by means of the Trypan Blue exclusion assay (0.4% Tryphan Blue solution in H_2O).

Heart cells were plated onto the surface of a 24-well plate. Skeletal muscle cells were plated onto the plastic surface of a 75cm^2 cell culture flasks to allow the attachment of fibroblasts, and then incubated for 45-60 minutes at 37.5˚C and 5% CO_2. Unattached cells were then replated onto the surface of a 24-well plate.

For each experiment, skeletal muscle cells and heart cells were plated at a cell concentration of 5 x 10^4 cells per ml in 24-well plates with a culture area of 1.9cm^2/well and were maintained at 37.5˚C and 5% CO_2 content for 72 hours to allow optimal cell development, attachment and differentiation. The medium was not changed during this period, as this causes loss of unattached cells.

4.3.2 Cellular Morphology and Cellular Structure of Primary Cultures

To examine the cardiac and skeletal muscle cell morphology and structure of the primary cultures, the primary cultures were plated onto to bottom of 24-well plates. Cells were not pre-plated to reduce fibroblast contamination. After 24hr exposure to the substances tested for cytotoxicity in this Chapter, the cells were fixed by adding 50µl of a 2% formaldehyde solution (in ddH_2O) for 30 minutes, washed with ddH_2O and left to dry overnight at room temperature. After the plates were completely dried, 300µl of Crystal Violet (CV) dye solution prepared in 200mM of formic acid, pH 3.5, was added to each well and left for 1 hour. The dye was removed and plates were washed with ddH_2O, and left to dry. Cell morphology and structure were studied by inverted light microscopy using a Zeiss Axiocam MRc5 microscope (Figure 4.2 to 4.7).

4.3.3 Preparation, Optimization and Exposure of Triton X-100 and CoQ10, Alone, and in Combination

100% Triton X-100 was obtained in liquid form and a filtered through a 0.20µm Minisart-Plus filter, before the concentrations tested were prepared. Fifty microliter of 100% Triton X-100 were dissolved in 10ml ddH_2O, a 0.5% Triton X-100 concentration in order to obtain the first concentration of five, labeled TX 1 in this study. A serial dilution of the 0.5% Triton X-100 solution was prepared and was: 0.05% (TX 2), 0.005% (TX 3), 0.0005% (TX 4), and 0.00005% (TX 5). One hundred microliter of the different Triton X-100 concentrations, TX 1 – TX 5, was added per well containing 500µl medium. Coenzyme Q10 was obtained in a 100mg gel capsule, and a stock solution was prepared using 1 x 100mg capsule dissolved in 10ml of ddH_2O, using an ultrasonic water bath. In order to obtain the five different concentrations used in the study (Q1 – Q5), 200µl, 100µl, 50µl, 20µl, 10µl, respectively were added to 5 test tubes each containing 10ml ddH_2O, making a concentration series of 0.2mg/ml, 0.1mg/ml, 0.05mg/ml, 0.02mg/ml and 0.01mg/ml. A volume of 100µl was added to each well, containing 500µl medium.

In order to study the combination effect of the substances, the cardiac and skeletal muscle cell cultures were exposed to TX 2 (0.05%) and TX 3 (0.005%), respectively, in combination with increasing concentrations of CoQ10 (Q4, Q3, Q2, Q1). After 72 hours, cells were treated with CoQ10 for two hours prior to Triton X-100 exposure.

4.3.4 The Toxic Effect of Triton X-100 and CoQ10, Alone, and in Combination

Cardiac and skeletal muscle cell cultures were established as described above. After 72 hours of maintaining the cells in culture the cells were well established and attached, and the cultures were exposed to Triton X-100 and CoQ10 alone and in combination. Following exposure to the various concentrations of the substances tested, cell number, cell viability and lysosomal membrane integrity was determined using the combined CV, MTT, and NR.

4.3.5 The Combined Colorimetric Cytotoxicity Assay

While the MTT assay is based on measurement of the mitochondrial dehydrogenase activity of cells, NR and CV stain primarily the lysosomes and the membrane of viable cells, respectively (Ishiyama *et al*., 1996). The combined NR/MTT/CV procedure was as follows. One hundred microliter of 0.15% NR prepared in ddH_2O was added to each well and then incubated for 90 minutes at 37.5˚C in a CO_2 water-jacketed incubator, then 100µl of a 0.1mg/ml 3-(4,5- Dimethylthiazol-2–yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well and the cell culture plates were maintained for a further 60 minutes at 37.5˚C. The medium was then carefully removed and plates were blotted dry. Cells were then fixed for 10 minutes with 200µl of a 1% acetic acid and 1% formaldehyde solution in ddH_2O. The fixative was removed and the NR was solubilized with 200µl of a 1% acetic acid and 50% ethanol solution prepared in ddH_2O. The dissolved NR was then transferred to a 96-well plate. The MTT formazan crystals were then dissolved with 200µl DMSO, by shaking the plates for 20 minutes on an electronic IKA® SCHÜTTLER MTS 2, before being transferred into a 96-well plate. Each well was washed once with DPBS and the plates were left to dry overnight. The cells attached to the bottom of the plates were stained by adding 300µl of a 0.1% (weight/volume (w/v)) CV solution prepared in 200mM of formic acid, pH 3.5, to each well, and left to stain for 60 minutes. After the plates were washed with ddH_2O and dried, the bound dye was dissolved in 10% acetic acid, prepared in ddH_2O. The solution was transferred into a 96-well plate, and the absorbance of each well was determined spectrophotometrically at 570 nm, using the EL900 plate reader. Data were processed using Microsoft Excel, and statistical analyses were performed.

4.3.6 Statistical Analysis

The CV, MTT and NR assays were analysed separately with an appropriate Analysis of Variance (ANOVA). The data was expressed as mean ± standard error of mean, and analysed for statistical significance using a one-way ANOVA for the Dunett 95% confidence interval, many samples against a control, was carried out for the Triton X-100 and CoQ10 studies, and a two-way ANOVA for the combination studies. Testing was performed at a 0.05 level of significance and pair wise comparisons were done using Tukey analysis when analyzing the effect between Triton X-100 and CoQ10. All statistical analysis was performed using the Microsoft Excel and Analyse-it program.

4.4 Results and Discussion

The study was undertaken in order to investigate the possible cytotoxic effects of Triton X-100 and CoQ10 alone, and in combination on 13 day-old chick embryo primary cardiac and skeletal muscle cell cultures' cell number, cell viability and lysosomal membrane integrity.

4.4.1 The Morphology and Structure of Cardiac and Skeletal Muscle Cells

Although many cells of multicellular organisms have limited contractile abilities, it is the capability of muscle cells, which are specialized for contraction that permits animals to move (Gartner, *et al*., 2007). These cells are therefore high energy consuming cells with a high number of mitochondria. It was decided to use muscle cells for the study, since the mechanism of action of CoQ10 are mainly located to the mitochondria. To study the cellular morphology a Crystal Violet (CV) dye solution prepared in 200mM of formic acid, pH 3.5 was added to each culture and left for 1 hour. The dye was removed and plates were washed with ddH_2O, and left to dry. Cell morphology and structure were studied by inverted light microscopy using a Zeiss Axiocam MRc5 microscope.

4.4.1.1 Skeletal Muscle Cells

During embryonic development, several hundred precursors of skeletal muscle fibers (myoblasts) line up end to end, fusing with one another to form long multinucleated cells known as myotubes, which matures into the long muscle cell with a diameter of 10 - 100 µm and a length of up to several centimeters. The newly formed myotubes manufacture cytoplasmic constitutents as well as contractile elements, called myofibrils, composed of specific arrays of myofilaments, the proteins responsible for the contractile capability of the cell (Gartner *et al*., 2007 and Kierszenbaum, 2007) Muscle cells or fibers form a long multinucleated syncytium grouped in bundles surrounded by connective tissue sheaths and extending from the site of origin to their insertion. The plasma membrane (sarcolemma) of the muscle cell is surrounded by a basal lamina and satellite cells. The sarcolemma projects long finger-like processes (transverse tubules/T tubules) into the cytoplasm (sarcoplasm) of the cell. T tubules make contact with membranous sacs or channels, the sarcoplasmic reticulum. The many nuclei of the muscle fiber are located at

the periphery of the cell, just under the sarcolemma. About 80% of the sarcoplasm is occupied by myofibrils surrounded by mitochondria (sarcosomes) (Kierszenbaum, 2007). Deep to the sarcolemma and interspersed between and among myofibrils are numerous and elongated mitochondria with many highly interdigitating cristae. The mitochondria may either parallel the longitudinal axis of the myofibril or wrap around the myofibril. Moreover, numerous mitochondria are located just deep to the sarcoplasm (Gartner *et al*., 2007). The sarcoplamic reticulum encases hundreds and sometimes even thousands of myofibrils. Myofibrils are cylindrical in shape and run in the length of the muscle fiber. Light microscopy shows that a myofibril has light and dark bands, responsible for the striated appearance of skeletal muscle. The striations are formed by the placement of protein filaments within the contractile units, called sarcomeres, which extend between two dark lines, called the Z lines (Mader, 2001). The sarcomere is the basic contractile unit of striated muscle. Sarcomere repeats are represented by myofibrils in the sarcoplasm of skeletal and cardiac muscle cells. The arrangement of thick (myosin) and thin (actin) myofilaments of the sarcomere is largely responsible for the banding pattern. Actin and myosin interact and generate contraction force. The Z disk forms a transverse sarcomeric scaffold to ensure the efficient transmission of generated force. During contraction the length of the sarcomere decreases because thick and thin filaments slide past each other (Kierszenbaum, 2007).

4.4.1.2 Cardiac Muscle Cells

Cardiac cells (cardiocytes) are branched cylinders, 85 – 100μm long, approximately 1.5 μm in diameter, with a single centrally located nucleus (Kierszenbaum, 2007). Cardiac muscle, the specialized muscle of the heart, shares features with both smooth and skeletal muscle. Like skeletal muscle fibers, cardiac muscle fibers are striated and have a sarcomere structure (Silverthorn, 2004). The organization of the contractile proteins are the same as found in skeletal muscle, however the cytomembranes exhibit some differences. Mitochondria are more abundant in cardiac muscle than in skeletal muscle and contain numerous cisternae (Kierszenbaum, 2007). Almost half the volume of the cardiac muscle cell is occupied by mitochondria, attesting to its great energy consumption (Gartner *et al*., 2007). Like single-unit smooth muscle, cardiac muscle fibers are electrically linked to each other. The cells are joined end-to-end by specialized junctional complexes called intercalated disks. Intercalated disks have a steplike arrangement, with transverse portions that run perpendicular to the long axis of the cell

and longitudinal portions running in parallel to the myofibrils (Kierszenbaum, 2007). Some cardiac muscle, like smooth muscle, exhibit pacemaker potentials. Cardiac muscle is under sympathetic and parasympathetic control, as well as hormonal control (Silverthorn, 2004).

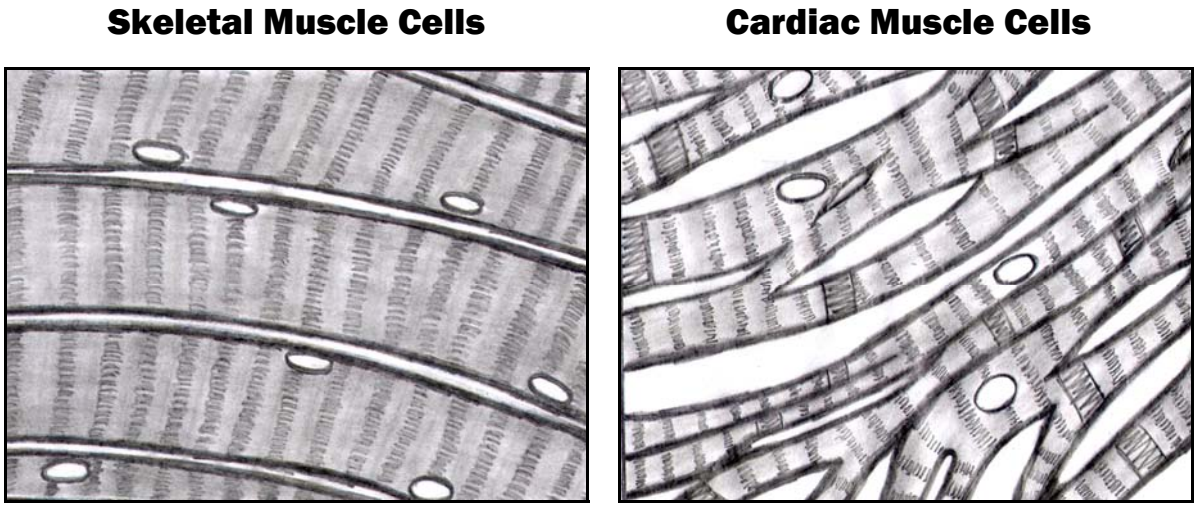


Figure 4.1: Schematic representation of skeletal and cardiac muscle.

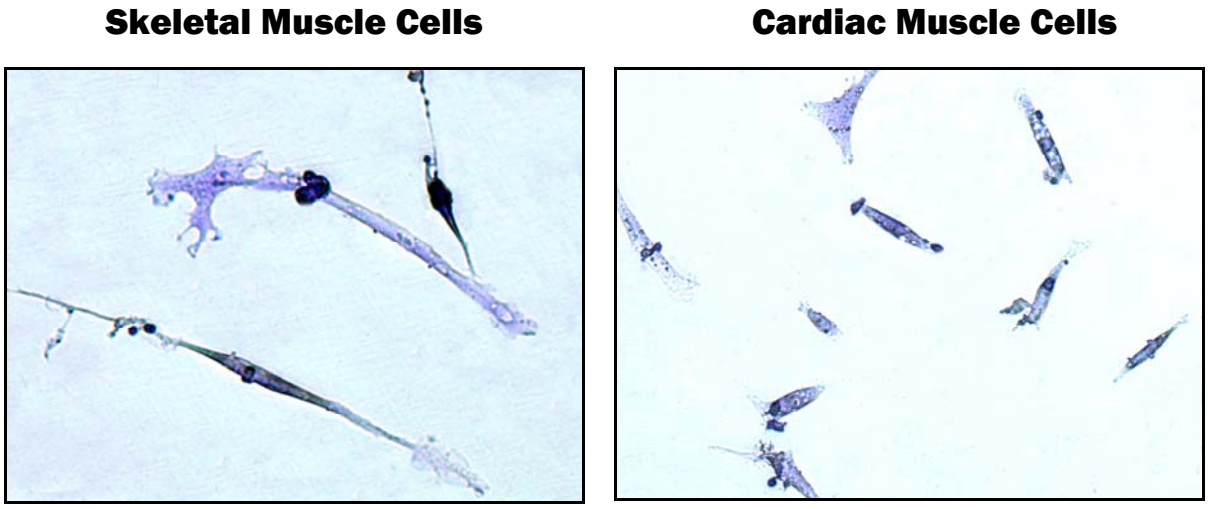


Figure 4.2: Skeletal and cardiac muscle cells of the control group at 20x long distance (LD) magnification, stained with crystal violet (CV).

Skeletal Muscle Cells

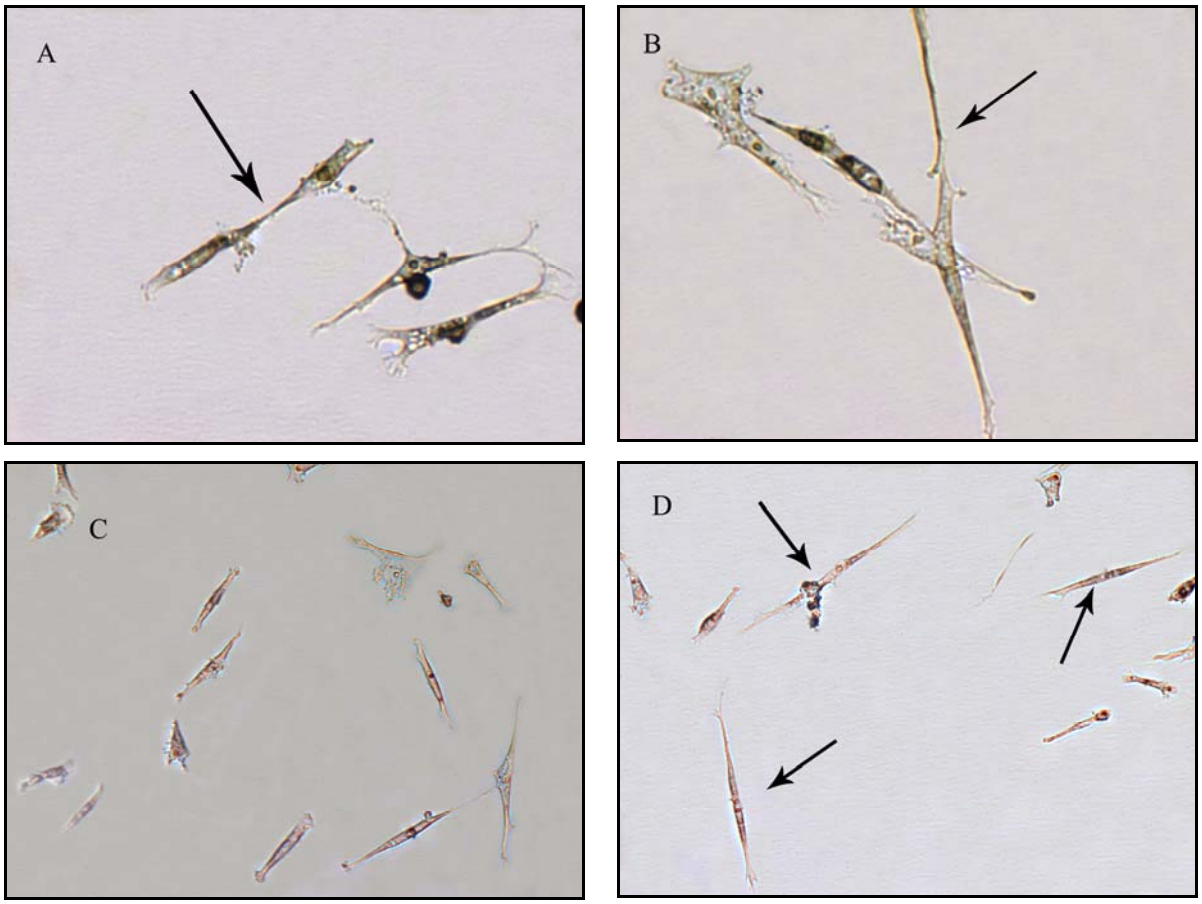


Figure 4.3: Skeletal muscle cells grown in a flask, stained with crystal violet to monitor the morphological characteristics of the cells, **A** & **B**): Skeletal muscle cells at 20x LD magnification. The black arrows indicate myoblasts that has fused to form a myotube. **C** & **D**): Skeletal muscle cells at 10x LD magnification. The black arrows indicate myotubes.

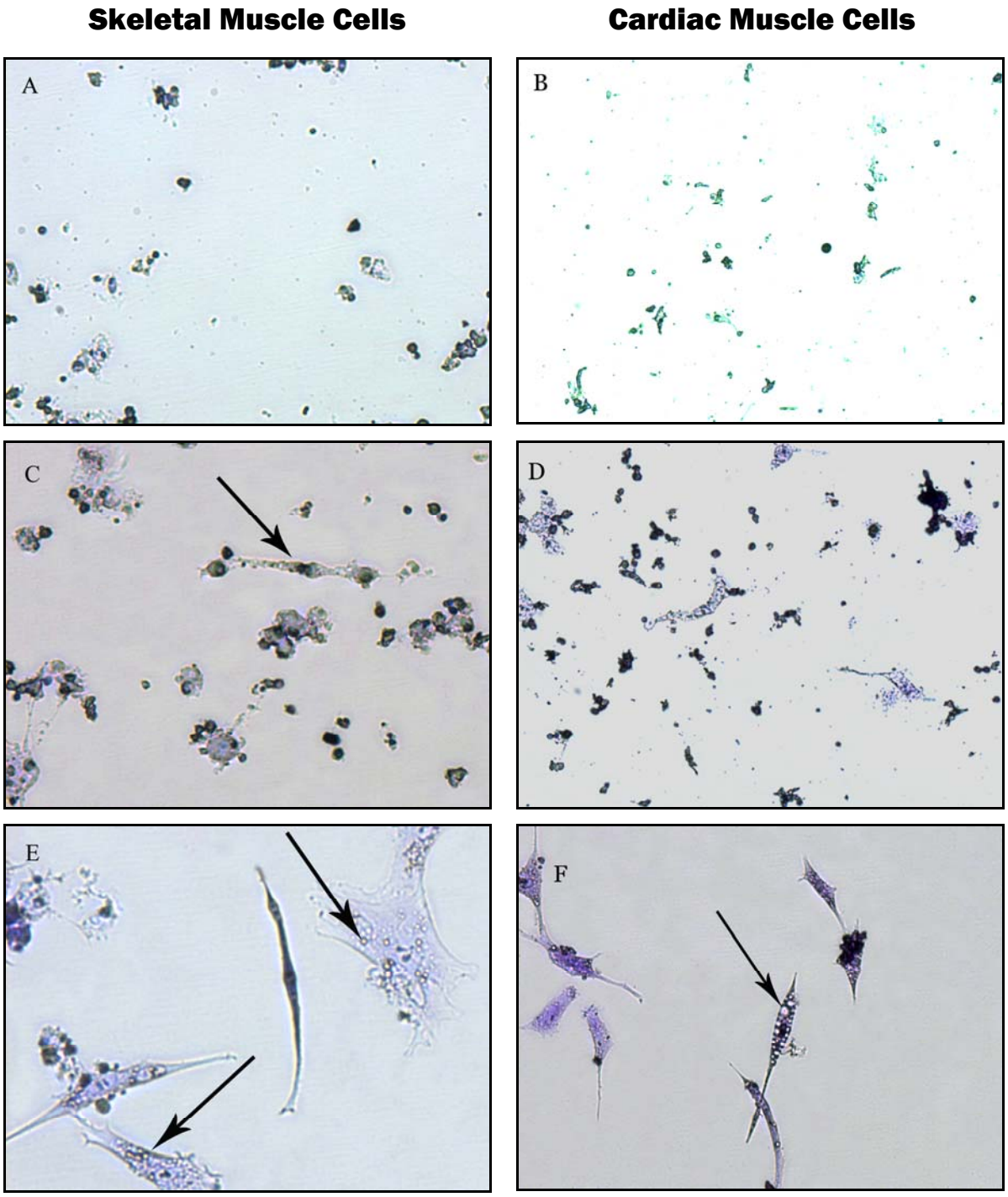


Figure 4.4 a: Skeletal and cardiac muscle cells exposed to Triton X-100. **A** & **B**): 0.5% Triton X-100, only cell fragments are present. **C** & **D**): 0.05% Triton X-100, cell fragments are present, the black arrow (**C**) indicate a skeletal muscle cell. **E** & **F**): 0.005% Triton X-100, cells seem to be intact, the presence of numerous vacuoles inside the cells was noticed (black arrows).

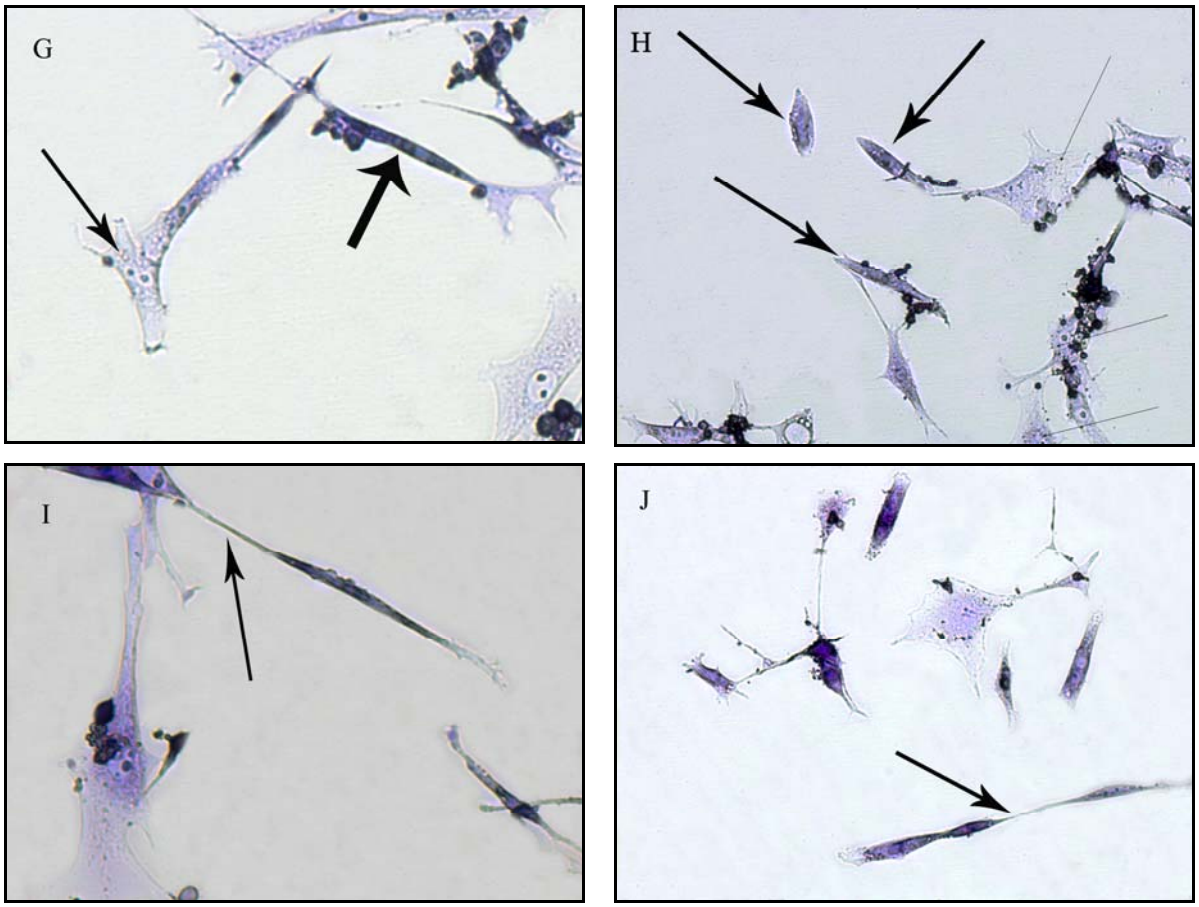


Figure 4.4 b: Skeletal and cardiac muscle cells exposed to Triton X-100. **G** & **H**): 0.0005% Triton X-100, cells appears to be structurally intact. Vacuoles present in **G** (thin black arrow). Characteristic light and dark bands of muscle cells were seen in **G** (thick black arrow). Muscle cells (thick black arrows) and fibroblasts (thin black arrows) were present in **H**. **I** & **J**): 0.00005% Triton X-100. Fusion of two myoblasts to form a myotube (indicated by black arrows). Cells are structurally intact.

Bader *et al*., 1976, found that chick embryonic cells transformed by the Bryan high titer strain of Rous sarcoma virus (RSV-BH) are heavily vacuolated. It was demonstrated that the vacuoles are cytoplasmic, bounded by membrane, and composed largely of water. Cells infected with the mutant form of the Rous sarcoma virus, RSV-BH-Ta, which induces reversible temperature-dependent transformation were used to investigate the physiological requirements for development of the vacuoles, and reversal of vacuolization. It was found that Na^+ was the only component of the cell culture medium found essential from both the development and reversal of vacuoles. Glucose depletion or dinitrophenol treatment inhibited vacuolization, suggesting a possible energy requirement in the vacuolization process. Ouabain, an inhibitor of Na^+/K^+ ATPase, enhanced vacuolization. Two sugars, glucosamine and mannosamin, prevented the disappearance of vacuoles. The observervations by Bader *et al*., 1976, suggested that cellular vacuolization may be a normal physiological response to an increase in water and Na^+.

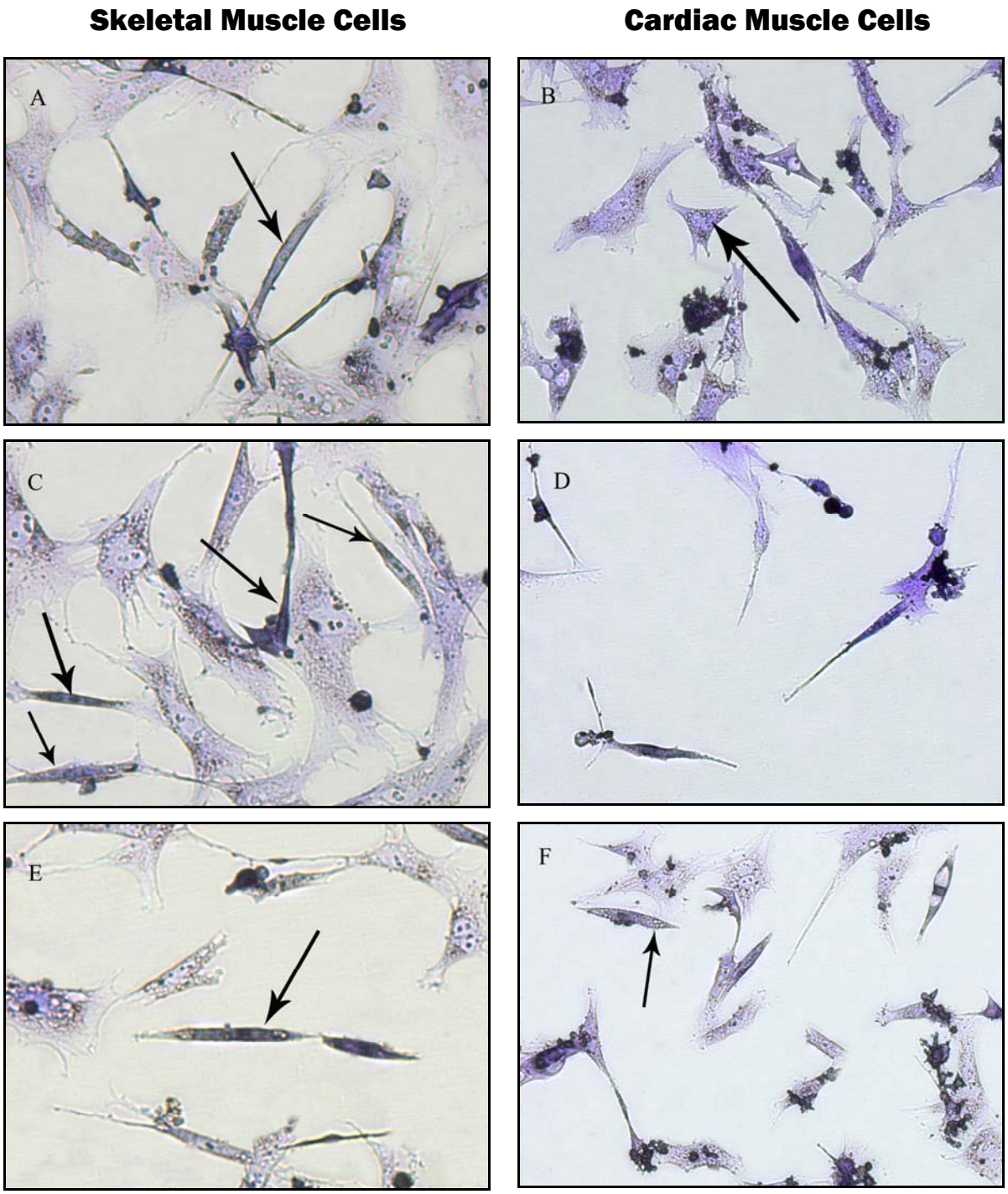


Figure 4.5 a: Skeletal and cardiac muscle cells exposed to CoQ10. **A** & **B**): 0.2mg/ml CoQ10; intact muscle cells (**A** – black arrow) were seen in both cultures. Numerous fibroblasts were present. Vacuoles were seen in some cells (**B** – Black arrow). **C** & **D**): 0.1mg/ml CoQ10; numerous intact muscle cells were seen in both cultures at this concentration, with notably more fibroblasts in the skeletal muscle cell culture. **E** & **F**): 0.05mg/ml CoQ10; Intact muscle cells in both cultures (black arrows).

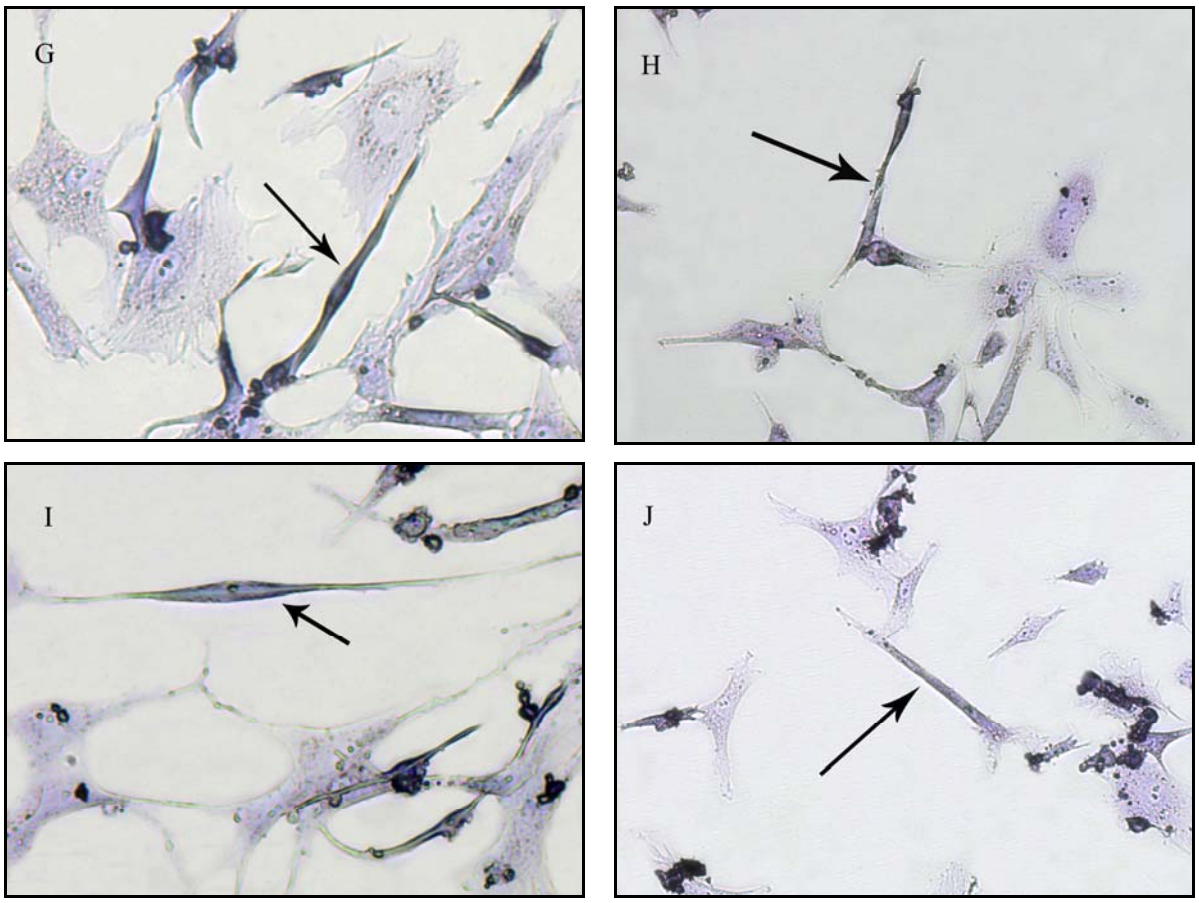


Figure 4.5 b: Skeletal and cardiac muscle cells exposed to CoQ10. **G** & **H**): 0.02mg/ml CoQ10; intact muscle cells were seen in both cultures, myoblast fusion (**H** – black arrow) and myotube formation (**G** – black arrow). **I** & **J**): 0.01mg/ml CoQ10; intact muscle cells were seen in both cultures, myotubes are indicated by the black arrows in both cultures.

Skeletal Muscle Cells

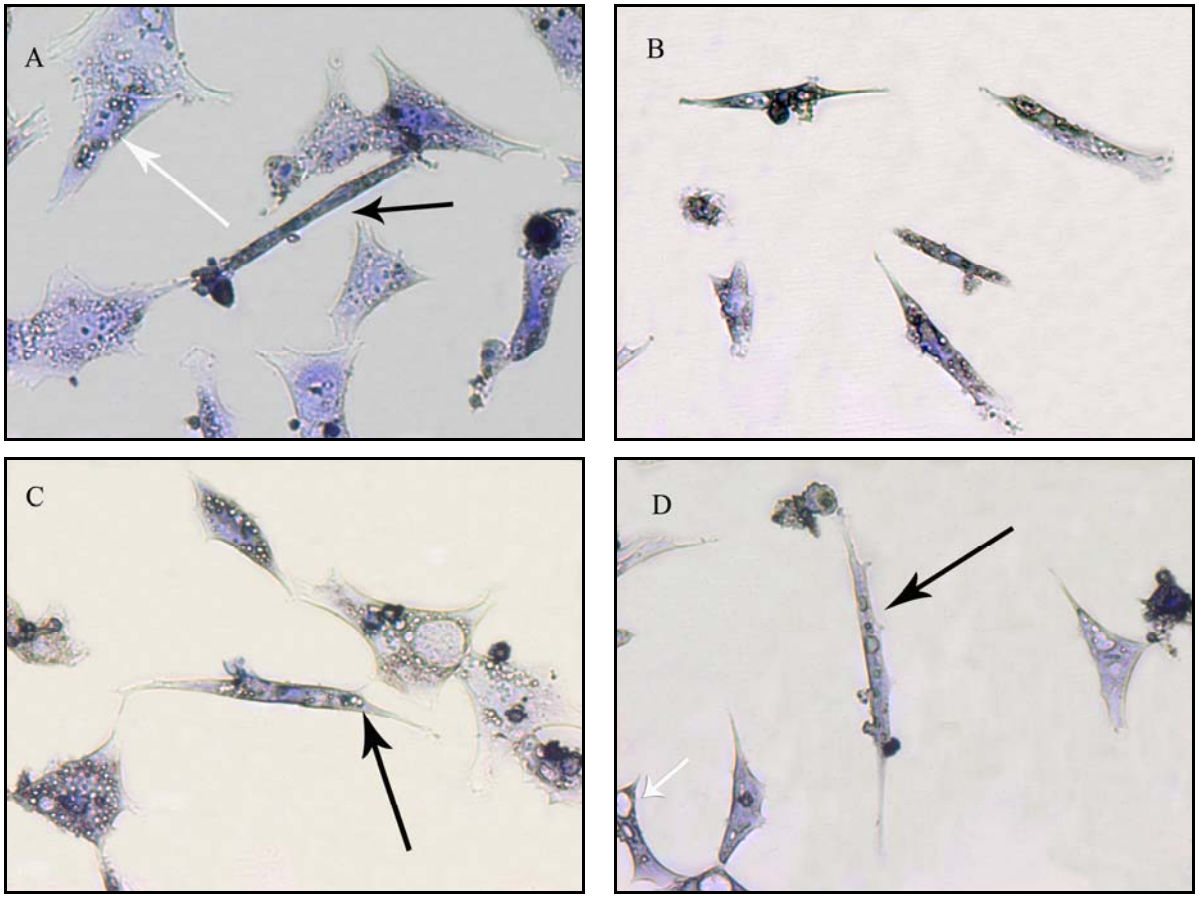


Figure 4.6: Skeletal muscle cells exposed to 0.05% Triton X-100, after two hours pre-treatment with different concentrations of CoQ10. **A**): 0.2mg/ml CoQ10; intact muscle cells were seen, myotubes were present at this concentration (black arrow), fibroblasts with numerous vacuoles were seen (white arrow). **B**): 0.1mg/ml CoQ10; muscle cells seemed less intact than the cells in **A**. **C**): 0.05mg/ml CoQ10; muscle cells presented with a disrupted with the presence of vacuoles. Fibroblasts with numerous vacuoles were present. **D**): 0.01mg/ml CoQ10; muscle cells (black arrow) showed bulging and instability of the membrane. Fibroblasts were present with vacuoles (white arrow).

Skeletal Muscle Cells

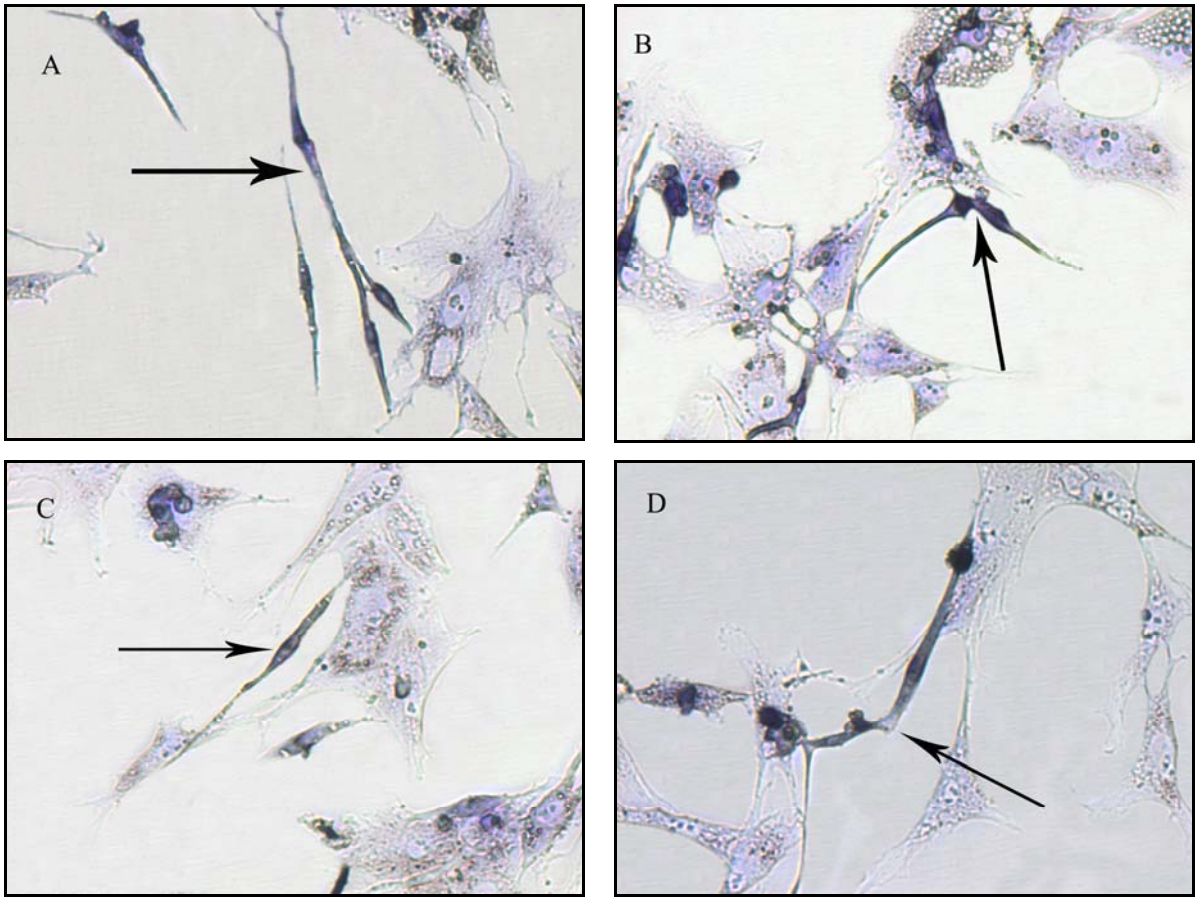


Figure 4.7: Skeletal and cardiac muscle cells exposed to 0.005% Triton X-100, after two hours pre-treatment with different concentrations of CoQ10. **A**): 0.2mg/ml CoQ10; **B**): 0.1mg/ml CoQ10; **C**): 0.05mg/ml CoQ10; **D**): 0.02mg/ml CoQ10. Myotube formation were seen in all the concentrations tested (**A** – **D** indicated by the black arrows).

4.4.2 The Effect of Triton X-100 on the Cell Number, Viability and Lysosomal Membrane Integrity of Chicken Embryo Primary Cardiac and Skeletal Muscle Cell Cultures

Considering the numerous protective effects of CoQ10 on cells, tissues, organs and systems, the question arose whether CoQ10 might have a protective effect on cells in culture, after exposure to the membrane disrupter Triton X-100. Before the effect of Triton X-100 at concentrations 0.05% and 0.005% in combination with increasing concentrations of CoQ10 (0.02mg/ml – 0.2mg/ml), on primary chicken cardiac and skeletal muscle cell cultures could be evaluated, it was necessary to determine the toxicity of each individual substance. The effect of each substance was measured using the NR, MTT, and CV assays. Since these dyes measure cell viability by different mechanisms, it was thought to be desirable to combine all these assays to obtain a better understanding of the interaction between toxicants and cells (Ishiyama *et al*., 1996). *In vitro* cytotoxicity assays with cultured cells are widely used in the sensitivity testing of chemicals because they are rapid, economical, and do not require the use of animals (Ishiyama *et al*., 1996). Ishiyama *et al*., 1995, reported that the combined assay gave similar IC_{50} values for SDS to those obtain with each independent assay, and also to the value reported in the MTT assay. At each concentration each assay was done in quadruple and triplicate experiments were done on the primary cultures.

The concentrations of Triton X-100 were decided on, after it became evident from the literature that Triton X-100 at a concentration of 0.1-0.2% are able to permeabilize biological membranes (Weruaga *et al*., 1998). Since there is no recommended daily allowance prescribed with NSI CoQ10 Q-Gel Mega, it was decided to start at a concentration of 0.2mg/ml of CoQ10 (highest concentration used in the study) and lower the concentration to 0.01mg/ml, compared to the 100mg/day required to significantly increase serum levels in humans, (Crane, 2001), 100mg/day falls between the range between the two lowest concentrations used in this study.

The cardiac and skeletal muscle cells were plated at a concentration of 5×10^4 cells per ml in 24 well plates with the culture area $1.9cm^2$/well and allowed to attach for 72 hours before being exposed to Triton X-100 and CoQ10 and a combination of Triton X-100 and CoQ10 ddH_2O was used as the carrier for both substances.

4.4.3 Assays

4.4.3.1 Neutral Red

The Neutral Red (NR) assay procedure is a cell survival/viability chemosensitivity assay, based on the ability of viable cells to incorporate and bind the supravital dye, Neutral Red. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion, accumulating intracellularly in lysosomes, where it binds with anionic sites in the lysosomal matrix. Alterations of the cell surface or the sensitive lysosomal membrane leads to lysosomal fragility and other changes that gradually become irreversible, resulting in a decreased uptake and binding of NR, (Ishiyama *et al*., 1996), making it possible to distinguish between viable, damaged or dead cells. Although the MTT and NR bioassays are often used to measure toxicity and are used to distinguish viable, damaged, and dead cells, these assays test different aspects of cell function and will therefore not always provide the same information regarding toxicity (Ishiyama *et al*., 1996 and Pariete *et al*., 2002).

4.4.3.2 1-(4,5-Dimethylthiazol-2-yl)-3.5 diphenylformazan

The 1-(4,5-Dimethylthiazol-2-yl)-3.5 diphenylformazan (MTT) assay measures the number of cells and mitochondrial activity of living cells (Gerlier *et al*., 1986). Toxicity causes mitochondrial damage, leading to changes in the structure and function of the organelle, leading to leakage of internal contents including enzymes into the cytoplasm. Contraction of the mitochondrial membrane may take place with increase in ATP/ADP ratio while prolonged contraction may lead to deterioration of the inner membrane that may eventually result in rupture and deterioration of the membrane. This process normally happens before cell death that is measured with the CV assay (Roche, Bedner *et al*., 1999; Marquardt 1999). This leakage of mitochondrial enzymes may ultimately trigger both apoptosis, via the intrinsic and extrinsic pathways, and necrosis. The MTT assay is a bioassay used to determine cell viability and/or the metabolic state of the cell by demonstrating the cell's oxidative systems. The assay is based on the capacity of the mitochondrial enzyme succinate dehydrogenase to transform the tetrazolium salt of MTT into a blue coloured product, MTT formazan. The reduction of the tetrazolium salt, however, does not occur as a direct effect of the specific dehydrogenase but most cellular MTT reduction occurs outside the mitochondrial inner membrane and involves NADH and NADPH-dependent mechanisms. The conversion of MTT takes place only in living cells, and the amount of formazan produced is proportional to the number of cells

present (Bounous *et al*., 1992, Slater *et al*., 1963, Mossman, 1983, Parish *et al*., 1983, Cook *et al*., 1989, Sgouras *et al*., 1990; Munetaka *et al*., 1996, Pariete *et al*., 2002).

4.4.3.3 Crystal Violet

Crystal Violet (CV) is a triphenylmethane dye also known as gentian violet. The most commonly used application for this dye is as the primary stain in the Gram-staining procedure. Gillies *et al*., 1986, used CV to quantify the cell number in monolayer cultures as a function of the absorbance of the dye taken up by the cells. CV stains cellular protein and the nuclei of fixed cells with a direct correlation between protein staining and cell number (Kueng *et al*., 1989). Toxic effects can cause cell detachment or cell damage with the resulting leakage of protein and therefore a corresponding decrease in protein staining (Timbrell, 2000; Kanduc, *et al*., 2002; Castro-Garza, 2007).

4.4.4 The Effect of Triton X-100

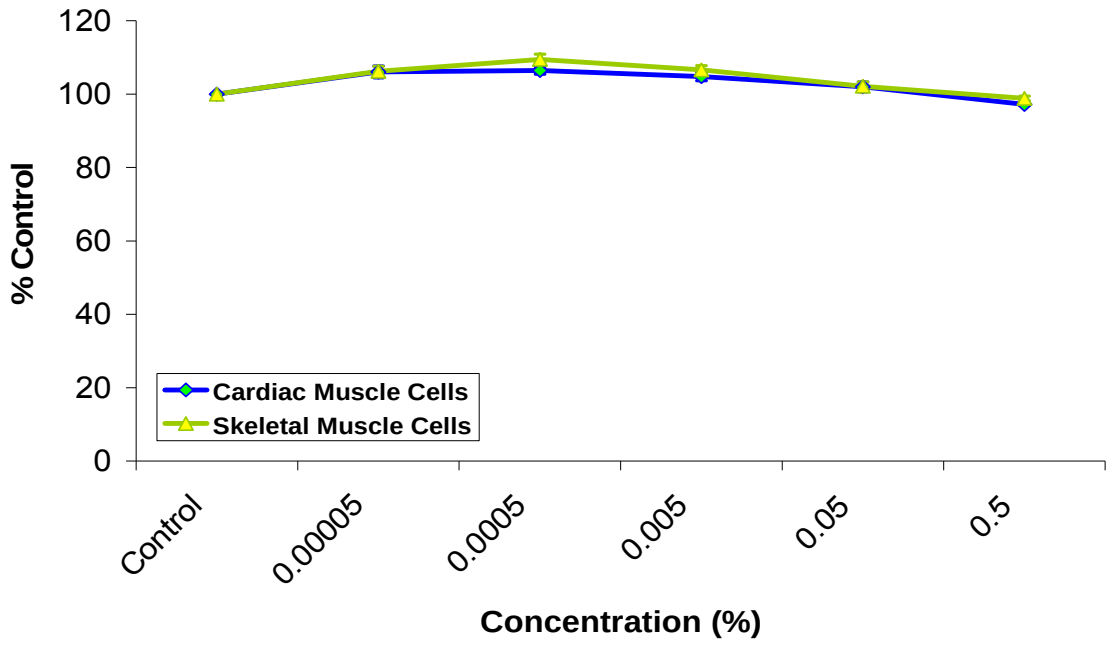


Figure 4.8 a: The effect of 0 – 0.5% of Triton X-100 on the cell viability of chick cardiac and skeletal muscle cells *in vitro,* measured using the MTT bioassay.

Using the MTT assay to determine changes in cell viability following exposure to 100µl of the five different Triton X-100 concentrations (TX 1 – TX 5), in a ddH_2O carrier dissolved in 500µl medium (Figure 4.8 **a**). Following 24 hour exposure to 0 – 0.5% of Triton X-100 an increase in cell viability was observed from 100% to 109% from control to 0.0005%. Cell viability then decreased to 97.2% in cardiac muscle cells (p= 0.0019), thus significant when compared to the increase of 9%, and 98.8% in skeletal muscle cells (p= 0.2232), thus not significant, at 0.5% Triton X-100. The increase in cell viability in both cultures was significant (cardiac muscle cells; p= 0.0022) and (skeletal muscle cells; p< 0.0001).

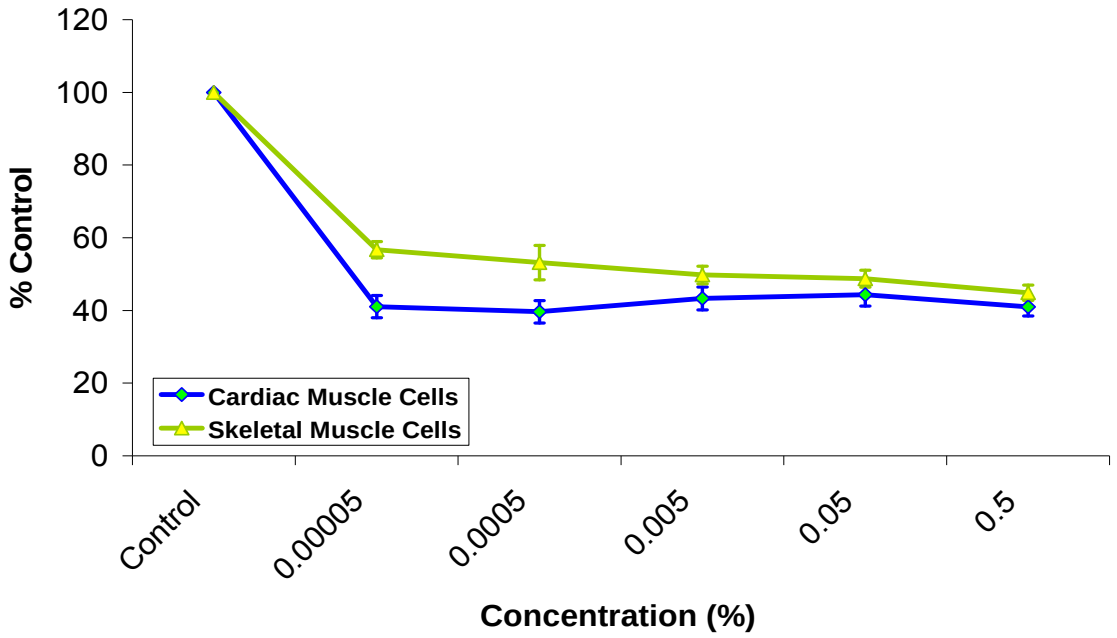


Figure 4.8 b: The effect of 0 – 0.5% of Triton X-100 on the lysosomal membrane integrity of chick cardiac and skeletal muscle cells *in vitro*, measured using the NR bioassay.

Using the NR assay to determine changes in lysosomal membrane integrity following exposure to 100µl of the five different Triton X-100 concentrations (TX 1 –TX 5), in a ddH_2O carrier dissolved in 500µl medium (Figure 4.8 **b**). Following 24 hour exposure to 0 – 0.5% of Triton X-100 a decrease in lysosomal membrane integrity was observed, both in cardiac (from 100 to 41%) and skeletal muscle cell cultures (from 100% to 57%) at 0.00005% Triton X-100. As the concentration of Triton X-100 increased, the lysosomal membrane integrity decreased significantly in both cell cultures, ($p<0.0001$), indicating that all the concentrations of Triton X-100 tested, caused alterations of the cell surface or the sensitive lysosomal membrane which lead to lysosomal fragility and other changes that gradually become irreversible, resulting in a decreased uptake and binding of NR. No significant difference in lysosomal membrane integrity was noted between the highest and lowest concentrations of Triton X-100 (cardiac muscle cell; $p=0.9900$) and (skeletal muscle cells; $p=0.3491$).

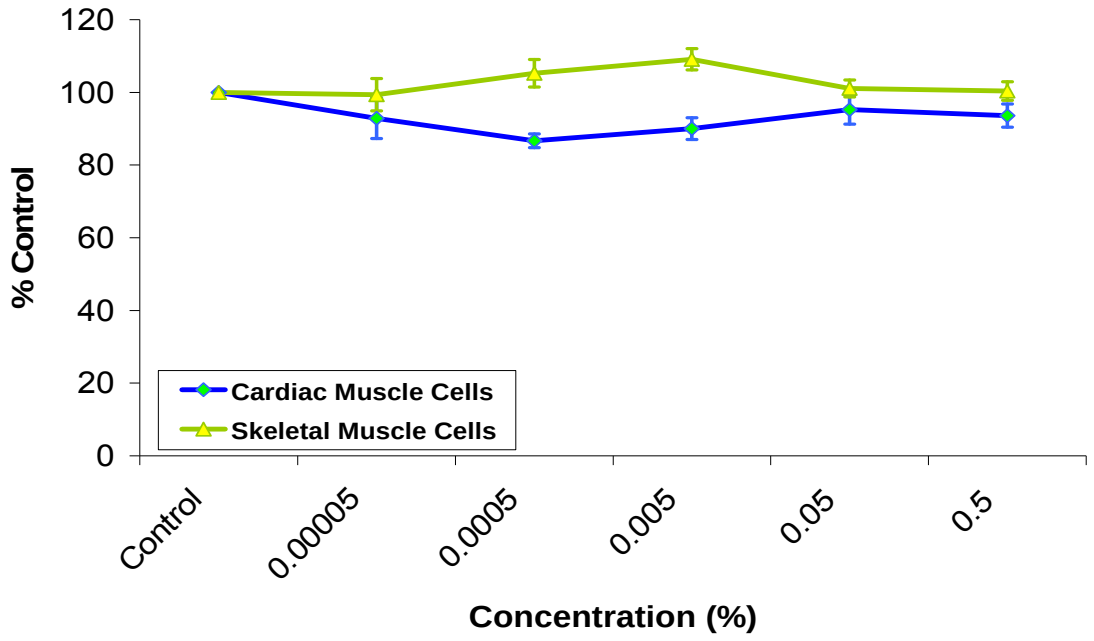


Figure 4.8 c: The effect of 0 – 0.5% of Triton X-100 on cell number of chick cardiac and skeletal muscle cells in vitro, measured using the CV bioassay.

Using the CV assay to determine changes in cell number following exposure to 100µl of the five different Triton X-100 concentrations (TX 1 –TX 5), in a ddH_2O carrier dissolved in 500µl medium (Figure 4.8 **c**). Following 24 hour exposure to 0 – 0.5% of Triton X-100, a slight but not significant decrease was seen in the cell number of cardiac muscle cells ($p=0.0189$). In contrast, the skeletal muscle cell culture revealed a slight increase in cell number at 0.0005 and 0.005% ($p=0.0071$). The increase might be an indication that the cells are trying to compensate for the toxic insult. Both cultures showed no significant difference in cell number at the highest concentration of Triton X-100 when compared to the control group, indicating that, with the exception of skeletal muscle cells at 0.005 and 0.0005%, neither of the Triton X-100 concentrations used in the study, altered the cellular proteins to such an extend that the cell number of either cultures was significantly decreased (cardiac muscle cells; $p=0.0189$) and (skeletal muscle cells; $p=0.3426$).

Dayeh *et al*., 2004, reported a loss of viability in cultures of three mammalian cell lines, two fish cell lines, and a ciliated protozoan, using three viability assays (Alamar Blue (AB), NR, and propidium iodide (PI) fluorescent dyes were used). After a 2 hour exposure to Triton X-100 (1mg/ml serially diluted) all the fluorescent dyes indicated a loss of viability in cultures. NR appeared to be the most sensitive, giving the lowest EC_{50} values and revealing no differences between the EC_{50} values for the five cell lines. The results with the animal cell lines suggested that lysosomal activity, measured as the uptake and retention of NR, is more susceptible to Triton X-100 than energy metabolism, as measured with AB, plasma membrane integrity, as measured with PI (Dayeh *et al*., 2004). 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).

4.4.5 The Effect of CoQ10

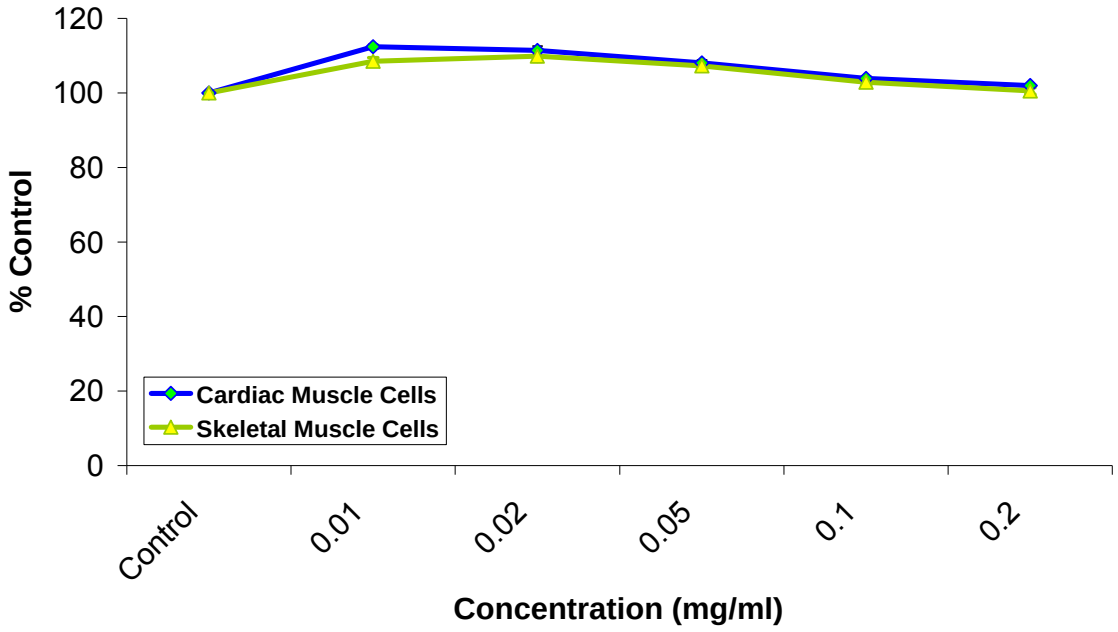


Figure 4.9 a: The effect of 0 – 0.2mg/ml of CoQ10 on the cell viability of chick cardiac and skeletal muscle cells in vitro, measured using the MTT bioassay.

Using the MTT assay to determine changes in cell viability following exposure to 100µl of the five different CoQ10 concentrations (Q1 - Q5), in a ddH_2O carrier dissolved in 500µl medium (Figure 4.9 **a**). Following 24 hour exposure to 0 – 0.2mg/ml of CoQ10, the MTT assay showed an increase in cell viability of both cardiac ($p<0.0001$) and skeletal ($p<0.0001$) muscle cells in culture. Compared to the control group, the highest concentration of CoQ10 revealed almost no increase or decrease in cell viability. The increase in cell viability (~10%) in both cultures at 0.01 and 0.02mg/ml, was significant, indicating that CoQ10 significantly enhanced mitochondrial enzyme activity, compared to the control group ($p<0.0001$).

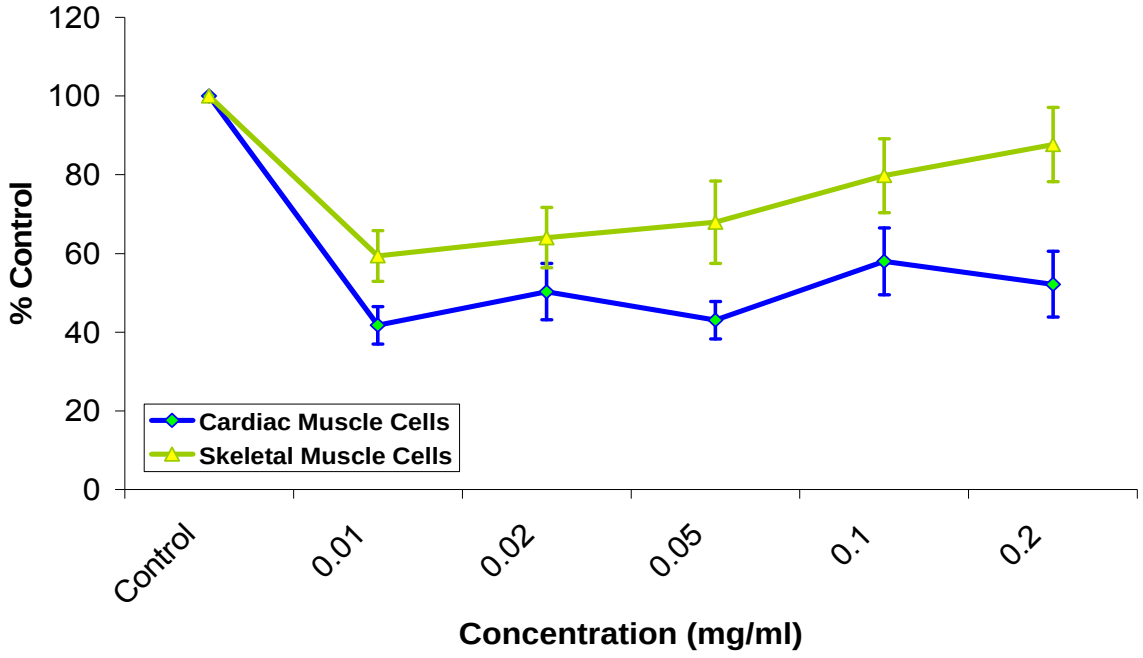


Figure 4.9 b: The effect of 0 – 0.2mg/ml of CoQ10 on the lysosomal membrane integrity of chick cardiac and skeletal muscle cells *in vitro*, measured using the NR bioassay.

Using the NR assay to determine changes in lysosomal membrane integrity following exposure to 100µl of the five different CoQ10 concentrations (Q1 - Q5), in a ddH_2O carrier dissolved in 500µl medium (Figure 4.9 **b**). Following 24 hour exposure to 0 – 0.2mg/ml CoQ10, lysosomal membrane integrity was altered significantly in both cell cultures. A decrease from 100 to 53% in skeletal muscle cells and 41% in cardiac muscle cells was seen at the lowest concentration of CoQ10. At the highest concentration, lysosomal membrane integrity in skeletal muscle cells decreased to 88% and 51% in cardiac muscle cells. This decrease in lysosomal membrane integrity in both cell cultures are significant (cardiac muscle cell; $p<0.0001$) and (skeletal muscle cells; $p=0.0011$), indicating that CoQ10 caused irreversible alterations, directly or indirectly, to the lysosomal membranes resulting in lowered NR uptake.

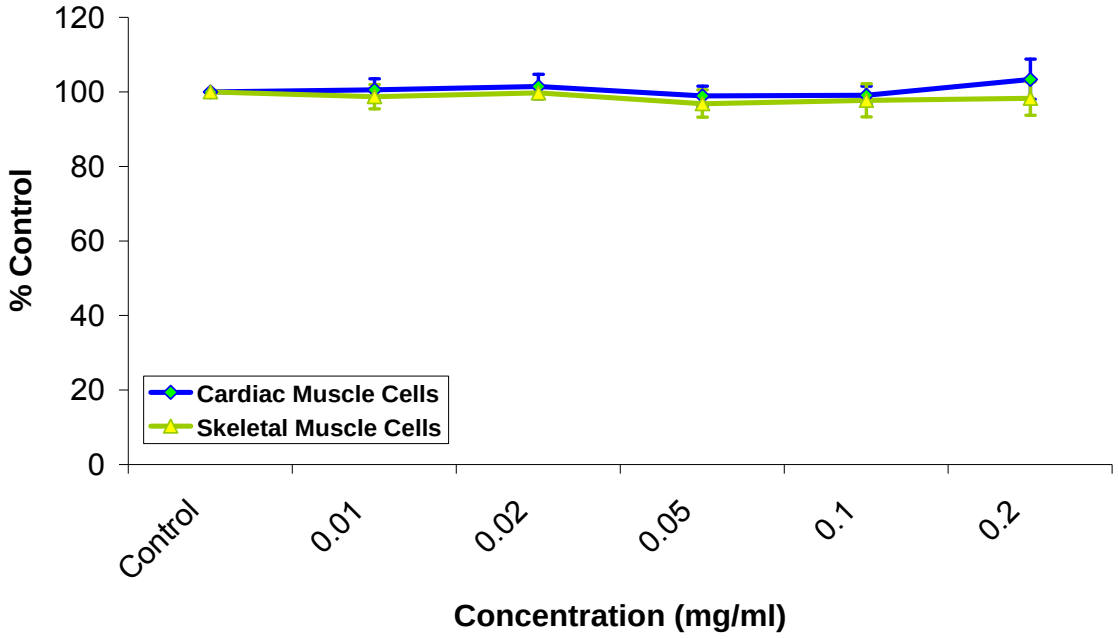


Figure 4.9 c: The effect of 0 – 0.2mg/ml of CoQ10 on cell number of chick cardiac and skeletal muscle cells in vitro, measured using the CV bioassay.

Using the CV assay to determine changes in cell number following exposure to 100µl of the five different CoQ10 concentrations (Q1 - Q5), in a ddH_2O carrier dissolved in 500µl medium (Figure 4.9 **c**). Following 24 hour exposure to 0 – 0.2mg/ml CoQ10, no significant increase or decrease was observed in cell number at any of the CoQ10 concentrations tested in the two cultures in the study (cardiac muscle cells; p=0.8392) and (skeletal muscle cells; p=0.6181). The results indicate that CoQ10 has no effect on cellular proteins, thus no difference in crystal violet colour intensity was observed when compared to the control group, following no exposure.

Alleva *et al*., 2001, linked the antioxidant capacity of redox-active compounds like CoQ10 and its analogues to their anti-apoptotic potential. The basis of the results suggested that ubiquinol-10 (the reduced form of CoQ10), but not ubiquinone-10, inhibits apoptosis induced by chemical apoptogens, which largely use the distal, mitochondrial signaling pathways, while apoptosis triggered by immunological agents, which rely on

their cognate receptors in apoptosis induction, appeared largely independent of the coenzyme Q status of the cell. The Jurkat T lymphoma cells were enriched with reduced or oxidized CoQ10, prior to apoptosis induction (Alleva *et al*., 2001). Papucci *et al*., 2003, demonstrated both *in vitro* and *in vivo* that CoQ10 prevents keratocyte apoptosis induced by laser irradiation more efficiently than other antioxidants. The administration of CoQ10 2 hours prior to apoptotic stimuli prevented apoptosis independently of the ability of apoptotic stimuli to trigger or not to trigger free radical generation. The protective effect was clearly demonstrated by several incidences, including changes of cell morphology detected by light microscopy and ultramicroscopy, quantification of living and apoptotic cells by the MTT colorimetric cytotoxicity assay, and analysis of ATP cellular levels by a bioluminescence assay. CoQ10 significantly enhanced the number of living cells evaluated by the MTT assay and lowered the number of cumulative apoptotic events scored by time-lapse videomicroscopy (Papucci *et al*., 2003). It was also able to prevent the massive reduction in ATP levels induced by all apoptotic stimuli. The suggested mechanism for its protective effect was associated with inhibition of the permeability transition pore (PTP) opening (Papucci *et al*., 2003). 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. Coenzyme Q10 at a concentration of 25-200μM was given to cell culture simultaneous with cell death stimuli and it appeared that in limited circumstances, CoQ10 can prevent apoptosis, where its effectiveness may be derived from directly stabilizing mitochondrial membrane potential or by indirectly protecting against loss in mitochondrial enzyme activity (CoQ10 at these concentrations did not have any adverse effects on cell death). Histiocytic lymphoma (U937) and mouse melanoma (B16F10) represented freely proliferative cells in the study, and were shown not to be protected by CoQ10. Differential rat adrenal pheochromocytoma (PC12) cells represented a differentiated non-proliferative phenotype, and exhibited the most consistent protection afforded by CoQ10 after exposure to cell death inducers (Kagan *et al*., 1999).

4.4.6 The Effect of Triton X-100 and CoQ10 in Combination

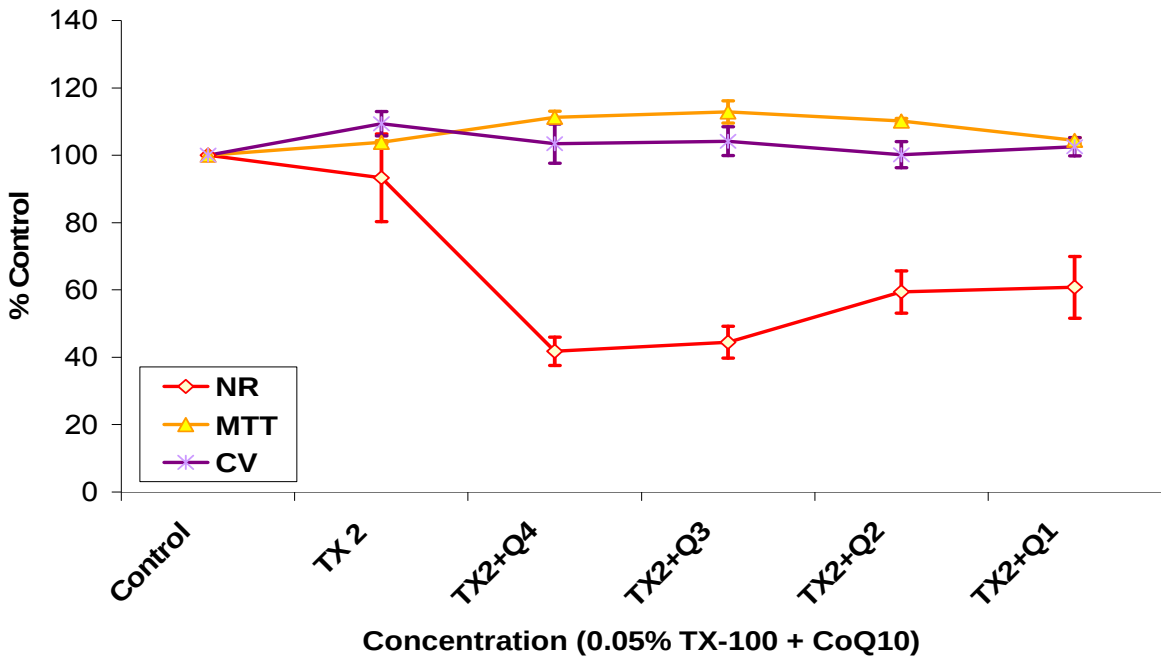


Figure 4.10 a: The effect of 0.05% Triton X-100 (TX2) in combination with increasing concentrations (0.02 – 0.2mg/ml) CoQ10 on chick cardiac muscle cells, as measured by the MTT, NR, and CV bioassays.

A slight increase was seen in cell viability of chick cardiac muscle cells upon exposure to Triton X-100 at a concentration of 0.05% in combination with increasing concentrations of CoQ10 (0.02 – 0.2mg/ml), a significant increase on cell viability (an average of 109%) (Figure 4.10 a) as indicated by the MTT assay ($p<0.0001$) was seen. No significant changes in cell number as indicated by the CV assay was measured ($p=0.3319$), indicating that no alterations to cellular proteins was caused upon exposure. The NR assay indicated a significant decrease in lysosomal membrane integrity, upon exposure of 0.05% Triton X-100 in combination with 0.02mg/ml CoQ10 ($p<0.0001$). A difference in the percentage decrease in lysosomal membrane integrity was noted as the concentration of CoQ10 increased from 0.05 to 0.2mg/ml ($p=0.0143$). The results reflect toxic insult to cardiac muscle cells caused by 0.05% Triton X-100 in combination with increasing concentrations of CoQ10, when the lysosomal membrane integrity was measured.

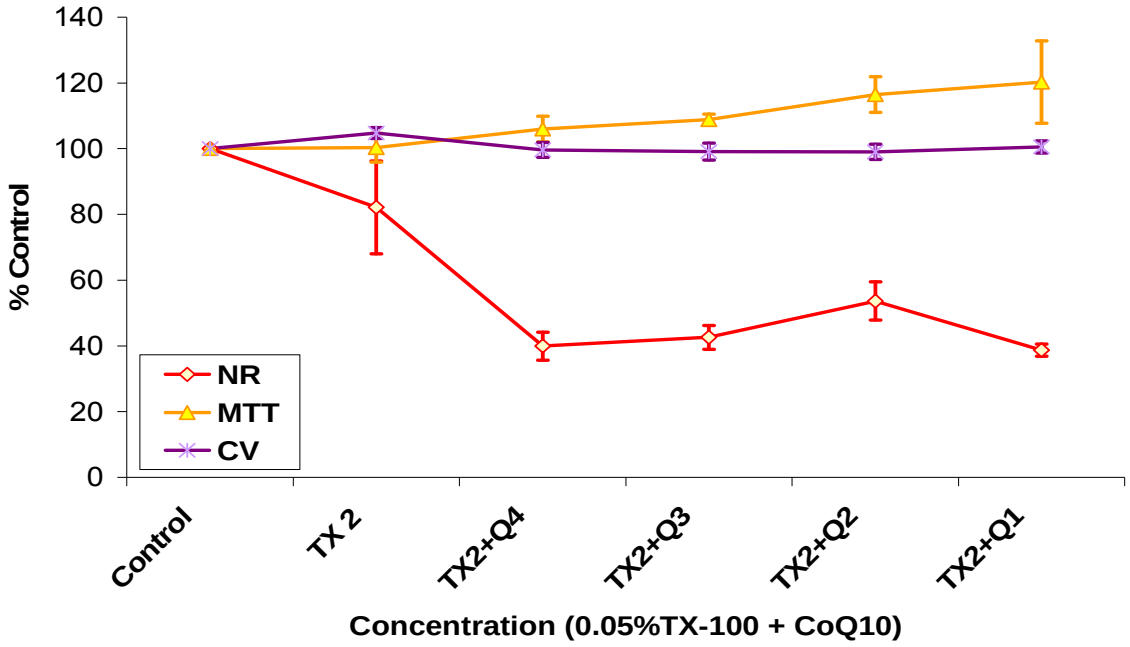


Figure 4.10 b: The effect of 0.05% Triton X-100 (TX2) in combination with increasing concentrations (0.02 – 0.2mg/ml) CoQ10 on chick skeletal muscle cells, as measured by the MTT, NR, and CV bioassays.

Cell viability in skeletal muscle cell cultures, as indicated by the MTT assay was significantly increased ($p<0.0001$), following exposure to 0.05% Triton X-100 in combination with increasing concentrations of CoQ10 (0.02 – 0.2mg/ml) (Figure 4.10 b). The cell viability increased from 106 to 120% as the concentration of CoQ10 increased from 0.02 to 0.2mg/ml, which indicate an increase in mitochondrial activity in the presence of 0.05% Triton X-100 when cells were pretreated with increasing concentrations of CoQ10. No significant changes was noted in cell number as measured by the CV assay ($p=0.7695$). In disparity to the cell viability increase, the lysosomal membrane integrity was significantly decreased following the combination exposure of Triton X-100 and CoQ10 ($p<0.0001$). No significant differences was observed between lysosomal membrane damage between the highest and lowest concentrations of CoQ10 ($p=0.7657$).

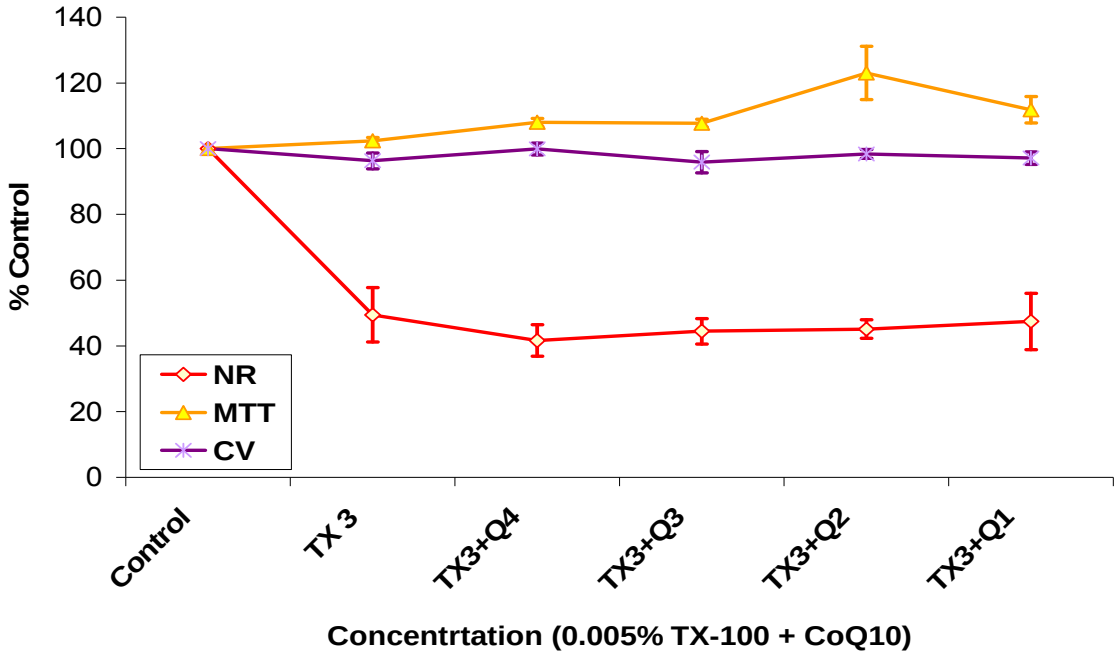


Figure 4.10 c: The effect of 0.005% Triton X-100 (TX3) in combination with increasing concentrations (0.02 – 0.2mg/ml) CoQ10 on chick cardiac muscle cells, as measured by the MTT, NR, and CV bioassays.

An increase in cell viability (an average of 112.6%) as measured by the MTT assay upon exposure of chick cardiac muscle cells to 0.005% Triton X-100 in combination with increasing concentrations of CoQ10 (0.02 – 0.2mg/ml) was seen ($p=0.0006$) (Figure 4.10 c). A definitely significant increase in cell viability was observed when cells were pretreated with 0.1mg/ml CoQ10 ($p<0.0001$), when compared to the other concentrations of CoQ10. No significant increase or decrease was seen in cell number as measured by the CV assay upon exposure to 0.005% Triton X-100, when pretreated with increasing concentrations of CoQ10, indicating no alterations caused to cellular proteins ($p=0.2505$). Contradictory to the increased cell viability, the lysosomal membrane integrity was significantly decreased from 100 to 49% ($p<0.0001$) in the presence of 0.005% Triton X-100 when pretreated with increasing concentrations of CoQ10. No significant difference was seen in the decreased lysosomal membrane integrity of different CoQ10 concentration in the presence of 0.005% Triton X-100 ($p=0.2093$).

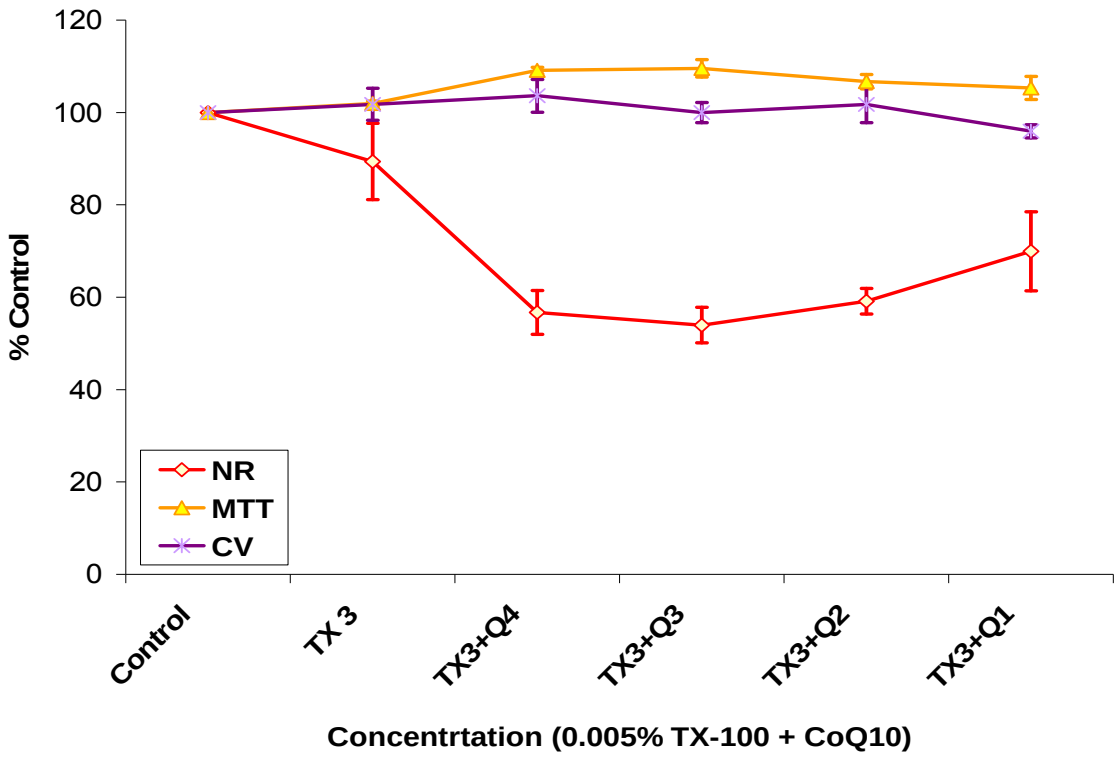


Figure 4.10 d: The effect of 0.005% Triton X-100 (TX3) in combination with increasing concentrations (0.02 – 0.2mg/ml) CoQ10 on chick skeletal muscle cells, as measured by the MTT, NR, and CV bioassays.

Cell viability, as measured by the MTT assay, of chick skeletal muscle cells, in culture increased (with an average of 107.7%) when pretreated with increasing concentration of CoQ10 in the presence of Triton X-100 at 0.005% ($p<0.0001$) (Figure 4.10 d). The CV assay measured no significant increase or decrease in cell number of the cells ($p=0.8382$), indicating no alterations to cellular proteins were caused upon the combination exposure. The lysosomal membrane activity of the skeletal muscle cells was significantly decreased upon exposure to 0.005% Triton X-100 after pretreatment with increasing concentrations of CoQ10 ($p<0.0001$). A difference in the decrease in the lysosomal membrane activity was noted between the different concentrations of CoQ10 ($p=0.0080$).

In conclusion, Triton X-100 and CoQ10, at all the concentrations tested in the study, is toxic to cardiac and skeletal muscle cells when evaluated by the NR assay. A slight increase in cell viability as measured by the MTT assay was measured in both cardiac and skeletal muscle cell cultures upon exposure to both Triton X-100 and CoQ10. Upon exposure to 0.05% and 0.005% Triton X-100, CoQ10 offered protection to the cell viability of cardiac and skeletal muscle cells in culture after pre-treatment with CoQ10, as determined by the MTT assay. No alterations in cellular proteins or cell number were measured by the CV assay. Toxicity was seen when cells were exposed to Triton X-100 and CoQ10, in combination as measured by the NR assay indicating that Triton X-100 and CoQ10, alone and in combination, have an irreversible effect on lysomal membrane integrity.