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Oxidative Stress- A Key Emerging Impact Factor in Health, Ageing, Lifestyle and Aesthetics

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Abstract

Oxidative stress is the resultant damage that arises due to redox imbalances, more specifically an increase in destructive free radicals and reduction in protection from antioxidants and the antioxidant defence pathways. Oxidation of lipids by ROS can damage cellular structures and result in premature cell death.
At low levels, ROS-induced oxidative stress can be prevented through the action of antioxidants, however, when ROS are present in excess, inflammation and cytotoxicity eventually results leading to cellular oxidative stress damage. Increasing evidence for the role of oxidative stress in various diseases including neurological, dermatological, and cardiovascular diseases is now emerging.
Mitochondria are the principal source (90%) of ROS in the cell, with superoxide radicals being generated when molecular oxygen is combined with free electrons. Given the key role of mitochondria in the generation of cellular oxidative stress it is worth considering this organelle and the process in more detail and to provide methods of intervention.

Oxidative Stress

Oxidative stress is the resultant damage that arises due to redox imbalances, more specifically an increase in destructive free radicals and reduction in protection from antioxidants and the antioxidant defence pathways [1]. Free radicals are molecules with an unpaired electron [1, 2]; there are many types of free radicals; relevant ones are reactive oxygen species (ROS), peroxides, superoxide anion, hydroxyl radical and singlet oxygen. Free radicals, in particular ROS, contribute to oxidative stress through a variety of mechanisms. Interaction with nucleic acids (both in the mitochondria (mtDNA) and nucleus (nDNA), results in mutations that predispose to DNA strand breaks [2]. Oxidation of lipids by ROS can damage cellular structures, for example the phospholipid cellular membranes, and result in premature cell death [2]. Interaction with proteins may lead to oxidation of individual amino acids thereby altering their structure and impairing enzymatic function of the protein [2]. ROS are often a by-product of mitochondrial respiration (see below in detail) and are involved in numerous physiological functions within cells including redox signalling [3-5].

At low levels, ROS-induced oxidative stress can be prevented through the action of antioxidants, however, when ROS are present in excess, inflammation and cytotoxicity eventually results leading to cellular oxidative stress damage [5, 6]. Increasing evidence for the role of oxidative stress in various diseases including neurological, dermatological, and cardiovascular diseases is now emerging [7-11]. Furthermore, over time antioxidant levels naturally decline which can result in an accumulation of oxidative damage, this manifests as ageing which affects multiple tissues and is described in more detail below [4].

Apart from being known as the “powerhouse of the cell”, mitochondria are the principal source (90%) of ROS in the cell, with superoxide radicals being generated when molecular oxygen is combined with free electrons as they “leak” from the electron transport chain (ETC) complexes in the mitochondrial inner membrane[12]. Although not particularly reactive itself, superoxide can be converted to other more hostile reactive species which are more likely to cause damage to macromolecules such as lipid, protein and DNA. Therefore, given the key role of mitochondria in the generation of cellular oxidative stress it is worth considering this organelle and the process in more detail and to provide methods of intervention.

Mitochondria are the major source of oxidative stress

Mitochondria are dynamic, double membrane bound organelles originating from the symbiosis of an α-proteobacteria and Archaea host cell [13, 14]. They are found in numerous copies within a cell and each carries between 2-10 copies of the mitochondrial genome [15]. Mitochondria were once believed to be individual entities however it is now known that they constantly interact with one another inside cells through a process known as fission-fusion [15]. This enables the transfer of contents between mitochondria allowing toxic waste products to be transferred into a single mitochondrion, which can then be targeted for destruction by mitophagy [15]. This continuous process helps to maintain a healthy and functioning mitochondrial pool within cells [15].

An important role of mitochondria is in the production of cellular energy through a process termed oxidation phosphorylation (OXPHOS) [16]. This is enabled by the electron transport chain (ETC), located on the highly folded inner mitochondrial membrane, which is the principal site of both OXPHOS and endogenous reactive oxygen species (ROS) generation [17-21]. The ETC consists of five protein complexes (Complexes I-V), each of which contributes to the generation of adenosine triphosphate (ATP) and release of energy [18-21] (Figure 1).

Molecules of nicotinamide adenine dinucleotide (NADH) and flavin-adenine dinucleotide ($FADH_2$), produced during glycolysis and the Krebs cycle, are utilised by Complexes I and II respectively within the ETC [19]. Oxidation of these molecules releases electrons, which are subsequently transferred further down the ETC through each protein complex in turn [18-22]. Complex 1, also known as NADH-ubiquinone oxidoreductase, oxidises NADH and subsequently reduces ubiquinone, a small mobile electron carrier also located within the inner mitochondrial membrane [21, 22]. This allows the release of energy that can be harnessed to pump protons across the inner mitochondrial membrane into the intermembrane space (IMS) allowing the formation of a proton gradient [21]. Complex II (succinate-ubiquinone oxidoreductase) similarly oxidises $FADH_2$ allowing further electron transfer to ubiquinone but is not involved in proton transfer [22]. Complexes III and IV do however additionally contribute to the accumulation of protons in the IMS [21]. Complex III (ubiquinone-cytochrome *c* oxidoreductase) enables the transfer of electrons from ubiquinol, the reduced form of ubiquinone, to another mobile carrier, cytochrome *c* [21]. Finally, Complex IV (cytochrome *c* oxidase) encompasses the final redox reaction involving the reduction and transfer of electrons to oxygen which comprises the terminal electron acceptor [19-21].

The proton gradient generated by complexes I, III and IV is utilised by complex V (ATP synthase) to synthesise molecules of ATP [18, 21]. The dissipation of the gradient by complex V releases energy which rotates the upper part of the complex; this mechanical energy is converted and used to synthesise ATP from inorganic phosphate and adenosine diphosphate (ADP) [21] with a concomitant utilisation or consumption of oxygen. This oxygen consumption is readily and accurately detected by

the Seahorse Bioscience XF^{e}96 Extracellular Flux Analyser [23] which enables *in vitro* measurement of cell metabolism. Specifically, it is able to simultaneously measure oxygen consumption rate and the rate of extracellular acidification which together provide a measure of intracellular processes including; OXPHOS, glycolysis, and oxidation of fatty acids [23]. Cells exposed to different ROS-inducing agents can therefore be compared using the Seahorse analyser allowing comparison of levels of oxidative stress.

Traditionally complexes I and III were believed to be responsible for electron leakage from the ETC; however, more recently complex II has also been found to contribute [24-33] (Figure 1). These electrons are able to react with oxygen and form one of the primary ROS, the superoxide free radical anion ($O_2^{\cdot-}$) [5]. ROS are capable of causing damage to intracellular structures including proteins, lipids, and nucleic acids, and collectively contribute to oxidative stress [4, 34-36]. Other ROS include hydrogen peroxide (H_2O_2) and the even more highly reactive free radical species, the hydroxyl radical ($^{\bullet}OH$) [1, 2, 4, 37].

Figure 1

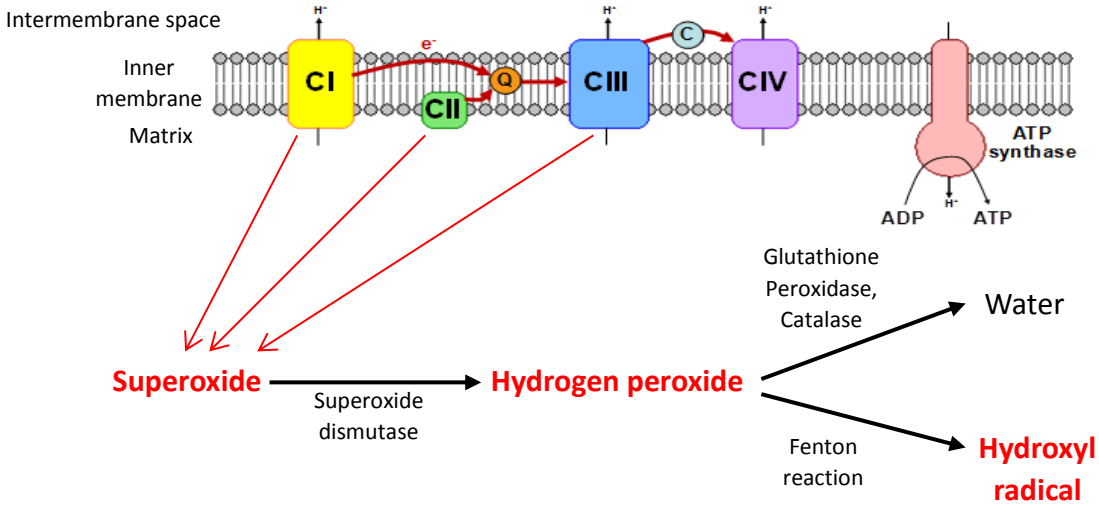

Figure 1. Mitochondrial electron transport chain and ROS generation. Five protein complexes, situated within the inner mitochondrial membrane, form the electron transport chain. Complex I (CI) and II (CII) transfer electrons (e^{-}) to ubiquinone (Q) which subsequently reduces complex III (CIII). Cytochrome *c* (C) acts as a mobile electron carrier between complexes III and IV (CIV). ATP synthase then generates ATP utilising the proton gradient formed from complexes I, III and IV. Also shown is the formation of superoxide from the electron transport chain and its subsequent removal or conversion to a more toxic by-product.

Sources of ROS

Ultraviolet Radiation

In addition to mitochondrial production of ROS, exogenous sources can also contribute to ROS formation [2]. Ultraviolet radiation (UVR), is a potent initiator of ROS within the skin and hair; it forms 6% of total solar radiation and can be further subdivided into UVA (320 to 400nm), UVB (280 to 320nm) and UVC (100 to 280nm) [4]. Most UVC is filtered out by the ozone and thus has little biological significance. Cutaneously UVA is able to penetrate deeper however the shorter wavelengths of UVB are found to be more damaging [17]. Upon absorption by nucleic acid, UVB-induced mutations arise and unless repaired can predispose to the development of skin

malignancies [17]. Specifically within mitochondrial DNA (mtDNA) the 4977 and 3895 base pair (bp) deletions and T414G point mutation are most common [17, 38-40].

MtDNA consists of a double-stranded circular molecule, formed from both an outer (heavy) and inner (light) strand of nucleic acid [12]. Contained within the genome are two ribosomal RNA genes, 22 transfer RNA genes and 13 genes coding for subunits of complexes I, III, IV and V of the ETC [12]. In comparison to nuclear DNA (nDNA), mitochondria do not possess efficient DNA repair mechanisms; furthermore mtDNA lacks the protection from histone organisation [4, 12, 17]. Consequently, following exposure to mutagenic agents such as UVR, mtDNA is very vulnerable to damage, and due to the polyploid nature of the mitochondrial genome however the level of damage can accumulate within a mitochondrion for some time before functional decline is seen [12, 17]. However, these factors do allow mtDNA to be used as a useful biomarker of UV-induced damage in skin [17, 41]. Eventually, damage to mtDNA can result in impairment of the ETC and subsequently increase ROS production, and in turn this may induce further mtDNA damage resulting in a vicious cycle of events [17]. UVR is therefore able to increase oxidative stress by either direct or indirect means, via mtDNA mutations, stimulation of ROS levels, or by an induction of an imbalance in cellular antioxidant defences [17]. The phenotypic manifestations of this in skin is commonly termed photoageing [38].

Within hair, UVR damage is both wavelength and hair colour dependent [42]. As with skin, UVA similarly penetrates deeper into the cortex of hair shafts but in comparison it results in up to five times the level of damage when compared to UVB. UVA-induced biochemical damage causes hair colour changes, whereas UVB-induced protein loss causes more superficial morphological damage to the cuticle of hair shafts [42]. Subsequent changes include dryness, reduced strength and graying of hair (canities) described in more detail below [42-44].

Pollution

Pollution is another major environmental cause of ROS production [45]. Particles of less than 0.1µm diameter, defined as ultrafine particles, are particularly harmful with vehicle exhaust emissions being a major source [6, 37]. Ultrafine particles specifically are able to penetrate tissues more easily and have been found to localise in mitochondria within epithelial and macrophage cells [6]. The geographical location, temperate climate, and chemical content all affect the nature of the particulate matter; however, most environmental pollutant agents have the ability to induce ROS [37, 46]. Adsorption at the surface of ultrafine particles leads to ROS generation which then induces oxidative stress; ultrafine particles that adsorb transition metal ions on their surface are able to produce ROS via the Fenton reaction described below [37].

Lifestyle - Exercise

A variety of lifestyle factors such as exercise and diet have also been identified as influencers of ROS-mediated oxidative stress. Exercise has been shown to induce ROS and oxidative stress and although the exact mechanism remains elusive it is suspected to be both tissue and exercise type and intensity dependent [10, 47, 48]. Mitochondria have been proposed as the primary source of exercise-induced ROS, possibly due to elevated energy demands and oxygen consumption within the ETC, however this is debated [1, 48]. Furthermore, whether or not the elevation of ROS in tissues post-exercise is beneficial or harmful is also debated [48].

Lifestyle - Diet

Interest in diet has increased in recent years especially due to the diet being an important source of exogenously derived antioxidants [49]. Carotenoid substances, found in fruit and vegetables, as well as vitamins A, C, and E are said to be most protective and correlate negatively with levels of oxidative stress [4, 5, 49]. Various mechanisms have been identified, for example citric and ascorbic

acid behave as metal chelating agents and work by reducing availability of free metal ions for reactions such as the Fenton reaction [4]. Alternatively, carotenoids work by appeasing free singlet oxygen molecules, thereby reducing the likelihood of formation of oxygen free radicals [1, 4]. Moreover, inducers of ROS, for example smoking and UVR exposure, can reduce levels of carotenoid antioxidants and thereby further potentiate levels of oxidative stress [4, 49]. In addition, the study showed that tomato paste can protect human skin against UVR-induced effects partially mediated by oxidative stress such as erythema (skin redness) and extracellular changes.

Alcohol

Adverse lifestyle behaviours such as alcohol and smoking have also been associated with an increase in oxidative stress levels [2]. Both acute and chronic alcohol consumption leads to an increase in ROS which is partly related to acetaldehyde, the product of ethanol metabolism [2]. Bailey *et al*., (1999) investigated individual responses of ETC complexes in hepatocytes in response to ethanol exposure and found the greatest ROS production arising from complexes I and III [50]. Alcohol also raises iron levels in the body further contributing to the Fenton reaction and $^{\bullet}OH$ production, and additionally it reduces antioxidant levels creating a further imbalance that promotes oxidative stress [2].

Smoking

Cigarette smoke is a particular hazardous substance to health and is a well-established cause of premature ageing of the skin and hair [45, 51, 52]. In addition to possessing the ability to induce intracellular ROS, cigarette smoke contains free radical substances within it, including O_2^-, H_20_2 and $^{\bullet}OH$, that are inhaled directly into the respiratory tract [46, 51]. Similar to alcohol, cigarette smoke reduces antioxidant availability again leading to higher oxidative stress levels [46, 51].

Antioxidants

As described above, mitochondria are responsible for the production of the majority of ROS within a cell, and these potentially harmful radicals are modulated by the expression of endogenous antioxidants. Endogenous antioxidants include superoxide dismutase (SOD), produced both in the cytoplasm and the mitochondria, which is able to convert O_2^- to H_20_2 via dismutation (Figure 1). Although H_20_2 is not itself a free radical, it is able to pass through cellular membranes and cause damage throughout the cell. H_20_2 is then able to be converted to harmless water by the antioxidants catalase or glutathione. However, this conversion is not entirely efficient, and the highly reactive $^{\bullet}OH$ can be produced via the Fenton reaction in the presence of metal ions (Figure 2).

Antioxidants are produced to counteract the harmful effects of ROS, in an attempt to prevent a chain-reaction of damage caused by the cascade of free radical reactions. If ROS levels become too great they can overwhelm the antioxidant defences. Oxidative stress can occur via the cascade of free radical reactions which although tend to be short lived can extend beyond the site of initiation (i.e. propagation of oxidative stress) and can lead to damage to biological structures, and accumulation of which is thought to contribute to the ageing process [53] and can be carcinogenic [20]. In addition, within this scenario and within the context of hair, it has been shown (described fully by Dr James Schwartz in one of the following articles in this mini-series) that there can be propagation of oxidative damage from one tissue (scalp) to adjacent tissues (hair). However, ROS are not always detrimental, as they act as important signalling molecules in processes such as apoptosis.

Figure 2

$$H_20_2 + Fe^{2+} + H^+ \rightarrow {}^{\bullet}OH + Fe^{3+} + H_20$$

Figure 2. The Fenton reaction. Schematic diagram of the Fenton reaction; a reaction between H_2O_2 and a metal ion resulting in the production of the $^{\bullet}OH$ free radical species.

The Nrf2-Keap1 Pathway (endogenous)

The expression of endogenous antioxidants is regulated primarily by the nuclear factor erythroid 2-related factor 2 (Nrf2)- kelch-like ECH-associated protein 1 (Keap1) pathway [54], which mediates transcriptional expression of antioxidants via the antioxidant response element (ARE) found in the promotor region of genes encoding antioxidants [55]. Under non-stressed conditions, Nrf2 is inactivated via its constitutive degradation by ubiquitination mediated by the Keap1 protein to which it is tethered [54, 56]. In response to oxidative stress, the Nrf2 protein is able to dissociate from Keap1 and translocate to the nucleus where it binds to ARE to induce the expression of downstream genes involved in antioxidant production as well as mitochondrial protection [56, 57] (Figure 3). Because of its key role in the upregulation of antioxidants, Nrf2 has been investigated as a potential therapeutic target in the treatment of oxidative stress. Recent work has demonstrated that targeting Nrf2 could be used as a potential treatment for chronic obstructive pulmonary disease (COPD) [58], liver disease [59], and neurodegenerative disease [57], as well as potentially in other diseases involving oxidative stress.

Figure 3

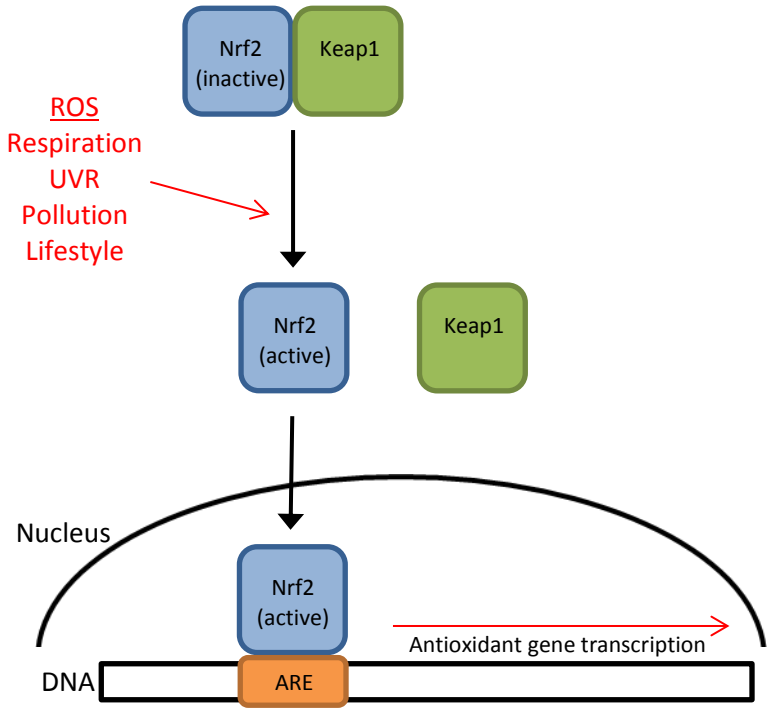

Figure 3. The Nrf2-Keap1 pathway. Antioxidant induction induced by ROS is mediated by the Nrf2-Keap1 pathway. During non-stressed conditions, Nrf2 remains tethered to Keap1 which targets its degradation. ROS allow for the dissociation of Nrf2 and Keap1, after which Nrf2 is able to translocate to the nucleus where it binds to ARE to induce the expression of downstream genes involved in antioxidant production.

Antioxidant Supplementation (exogenous)

Due to the exacerbation of oxidative stress by external stressors such as UVR, pollution, and lifestyle factors, people have begun to supplement their diets with antioxidants obtained exogenously. Many examples of past work have shown that diets rich in fruit and vegetables have a protective effect against a variety of diseases, via their high antioxidant capacities. For example, the Mediterranean diet increases the total antioxidant capacity, and is associated with a lower incidence of cardiovascular disease and cancer [60]. Consumption of the powerful antioxidant lycopene, which is found in tomato paste, has been shown to protect human skin against UVR-induced effects mediated by oxidative stress [61]. Consumption of blueberries, which have an extremely high antioxidant capacity, has been shown to decrease oxidative DNA damage [62], improve memory [63], and improve arterial stiffness and blood pressure [64]. In addition, supplementation with the antioxidants selenium, coenzyme Q10, and vitamin E has been shown to decrease the higher oxidative stress levels associated with patients with psoriasis, and improve disease severity [65]. A vast array of additional papers have also shown possible beneficial effects of antioxidants; however, conflicting results have also been received in terms of antioxidants, with some past work showing little or no benefit [66], possibly due to the antioxidant being unable to get to the appropriate location in the body, an inefficient dose of antioxidant with possible toxicity at high levels, and differences in the genetic backgrounds of individuals.

The Mitochondrial Free Radical Theory of Ageing

Ageing describes the progressive functional decline of an organism over time, leading to an increased susceptibility to age-related diseases such as cancers, and eventually to the death of the organism. Despite a vast repertoire of ageing studies performed over the past century, the exact causes of ageing remain unknown. Many theories as to how and why we age have been proposed over the past century, with the most promising theory proposed almost 60 years ago by Denham Harman, which was the Free Radical Theory of Ageing [53]. This theory suggested that free radicals can affect and are possibly the cause of the ageing process of animals. ROS were suggested to be able to cause damage to biological structures leading to a gradual decrease in cellular and tissue function over time resulting in ageing. As mitochondria are responsible for the production of the majority of ROS, this theory was later refined to the Mitochondrial Free Radical Theory of Ageing (MFRTA) in 1972 [67], which suggested that mitochondria play a key role in the ageing process, and it remains one of the most widely accepted theories of ageing to this day [68, 69]. In support of this theory, it has been observed that pigeons live on average 9-fold longer than rats, despite similar masses and metabolic rates [70-72]. Pigeons were found to have a much lower rate of H_2O_2 production which could indicate that a lower level of ROS generation results in a longer lifespan. In addition, oxidative damage has been shown to be increased with age in human heart tissue [73], and longer-lived animals generally have lower levels of oxidative mtDNA damage [74, 75]. High levels of mtDNA mutations have also been shown to be causative in terms of ageing, for which mice with accelerated accumulation of mtDNA mutations (via a mutated mtDNA polymerase) have been shown to have increased mitochondrial dysfunction, reduced longevity, and accelerated onset of ageing phenotypes [76, 77].

Within the MFRTA, another theory has been suggested entitled the Vicious Cycle Theory of Ageing [78] (Figure 4). This theory suggests that ROS production from the mitochondrial ETC is able to cause damage to the mtDNA found in close proximity. MtDNA encodes the majority of mitochondrial proteins, so errors in gene expression of mtDNA may result in dysfunctional mitochondrial subunits. Dysfunctional mitochondria are then thought to contribute to further ROS leakage due to their inefficiency, which could then exacerbate mtDNA damage in a continuing vicious cycle. ROS may also cause damage to nDNA [79] resulting in the altered expression of complex II mitochondrial subunits, as well as other nuclear-encoded mitochondrial proteins. In addition to this, ROS may have more

detrimental effects in older individuals due to the lower levels of antioxidants found with age [80]. It is likely that the Vicious Cycle Theory is not the sole cause of ageing and is instead a contributor to ageing, as ageing phenotypes have been shown to occur without certain aspects of the cycle taking place, such as ROS production [76, 77, 81]. This could suggest that each stage of the vicious cycle is able to contribute to the ageing process, but does not necessarily result in a continuing vicious cycle [82]. For example, it could be that not all mtDNA mutations resulting in mitochondrial dysfunction also induce ROS [76, 77], yet these mutations could be damaging by other mechanisms and contribute to ageing without a vicious cycle of damage. In accordance with this, mtDNA mutations are capable of inducing age-related diseases independently from ROS production [83, 84]. The exact role of ROS, mtDNA damage, and mitochondrial dysfunction in the ageing process remain unknown, and whether they interact in a vicious cycle of increasing damage remains to be seen. However, mitochondria are still very likely to play a role in the ageing process [69] even if not necessarily via a vicious cycle of damage.

Figure 4

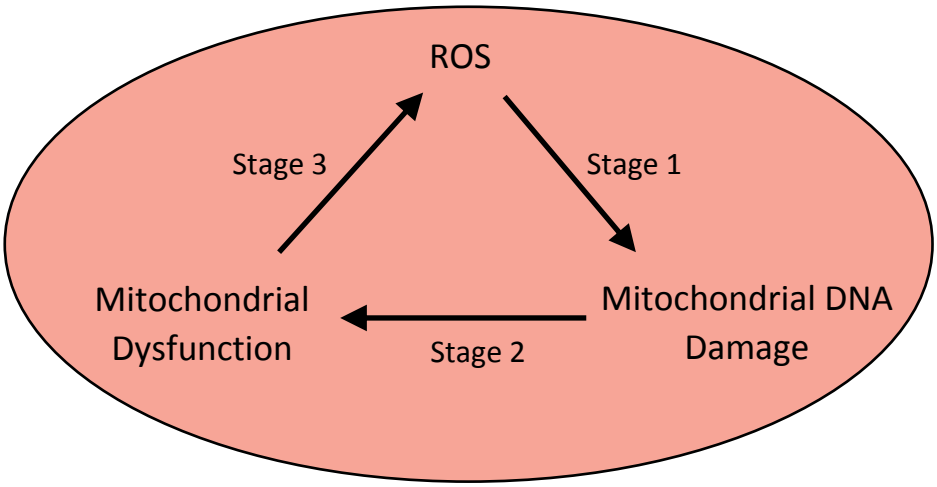

Figure 4. The vicious cycle theory of ageing. The vicious cycle theory of ageing suggests that ageing may be caused or exacerbated by a continuing cycle of increasing damage, due to the generation of ROS at the ETC causing damage to mtDNA found in close proximity (stage 1). This in turn may result in the altered expression of mitochondrial subunits and mitochondrial dysfunction (stage 2). Dysfunctional mitochondria may then release even more ROS (stage 3), in a continuing vicious cycle resulting in oxidative damage to biological structures and lower mitochondrial efficiency with age.

"Free radical theory of graying"

Analogous to the above, a "free radical theory of graying" has been proposed to characterise the ageing process taking place in hair [52]. Hair follicles are unique in that growth and development are characterised by three stages; hair shaft growth and melanogenesis, the synthesis of melanin pigment within small melanosome organelles, takes place during the anagen phase [44, 85]. Melanocyte apoptosis occurs second during the catagen phase followed by the resting-state telogen phase [85]. As hair ages a reduction in antioxidant enzymes, such as catalase, and an increase in ROS, particularly H_2O_2 has been found suggesting redox imbalance and an increase in oxidative stress but the exact pathophysiology remains elusive [44, 85]. Furthermore, ROS production naturally occurs

during melanin production [52]. Proposed mechanisms of ageing hair include, nuclear and mtDNA damage, with the mitochondrial 4977bp DNA common deletion being seen at a greater frequency in graying hair follicles, reduced antioxidant defence mechanisms and loss of melanosome enzymes [44, 85]. Ultimately this reduces melanin synthesis causing loss of hair colour. Moreover, ageing hair also demonstrates reduced anti-apoptotic proteins such as Bcl-2 phenotypically manifesting as hair thinning and eventual loss (alopecia) [44, 85]. Given the psychosocial impacts of ageing in both hair and skin a better understanding of the underlying ageing processes and the role of oxidative stress is required to help develop targeted therapeutics which could moderate the phenotypic manifestations of ageing.

Antioxidant supplementation to modulate lifespan

As the MFRTA is one of the most accepted theories as to why we age, and because many age-related diseases appear to be linked to oxidative stress, there have been many attempts to modulate longevity using exogenous antioxidants from plant and food sources. However, these studies have produced conflicting results with some showing an increase in longevity and others not, thought to be due to the antioxidant being unable to reach the source of ROS at the mitochondria. The dose of the antioxidant is also likely to be important, as low levels of vitamin E were shown to increase the lifespan of Drosophila, yet high levels did not [86], implying possible toxicity at higher levels. Since ROS are important signalling molecules [87, 88], the dose and target of the administered antioxidant would have to be considered to prevent the alteration of cellular homeostasis.

Conclusion

This article has described:

- The fact thatmitochondria are the major (i.e. 90%) source of cellular oxidative stress as electrons leak from the electron transport chain and combine with molecular oxygen. This aspect of mitochondrial function and its metabolic consequences can be measured using a variety of semi-automated instruments.
- The sources of oxidative stress, namely UVR, pollution, lifestyle (exercise and diet), alcohol and smoking
- The nature of oxidative stress and the free radicals including the cascade of reactions where immediate consequences of oxidative damage in one tissue (e.g. scalp) can affect adjacent tissues (e.g. hair) to be discussed more fully in one of the following articles (Dr Schwartz).
- The external and internal sources and inducers of ROS have been described together with the exogenous and endogenous antioxidant defence system that is available to help abrogate the increased oxidative stress.
- Finally, the vicious cycle theory of ageing is described which is applicable not only to hair (free radical theory of graying) and skin but to other body tissues, and its understanding provides further means by which to intervene and attempt to slow down the ageing process.

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