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Recharging mitochondrial batteries in old eyes. Near infra-red increases ATP

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ABSTRACT

Progressive accumulation of age related mitochondrial DNA mutations reduce ATP production and increase reactive oxygen species output, leading to oxidative stress, inflammation and degradation. The pace of this is linked to metabolic demand. The retina has the greatest metabolic demand and mitochondrial density in the body and displays progressive age related inflammation and marked cell loss. Near infra-red (670 nm) is thought to be absorbed by cytochrome c oxidase (COX), a key element in mitochondrial respiration and it has been demonstrated that it improves mitochondrial membrane potentials in aged eyes. It also significantly reduces the impact of experimental pathology and ameliorates age related retinal inflammation. We show ATP decline with ageing in mouse retina and brain. Also, in these tissues that ATP is significantly increased by 670 nm exposure in old mice. In the retina this was associated with increased COX and reduced acrolein expression. Acrolein, being a free radical marker of retinal oxidative stress, is up regulated in Alzheimer's and retinal degeneration. This is the first demonstration of ATP manipulation *in vivo* and may provide a simple non-invasive route to combating age related tissue decline.

The retina suffers with ageing. Mitochondria are the major source of adenosine triphosphate (ATP) that provides energy for cellular metabolism. The mitochondrial theory of ageing argues that progressive accumulation of mutations in mitochondrial DNA (mtDNA) reduces ATP output and increases reactive oxygen species production driving oxidative stress, inflammation and cell loss (Harman, 1981). This is marked in regions of high metabolic demand such as the retina that suffers progressive inflammation, extra cellular debris accumulation and cell loss (Bonnel et al., 2003; Hoh Kam et al., 2010; Jarrett et al., 2008; Xu et al., 2009), such that by 70 years approximately 30% of central rod photoreceptors are lost in the human retina and a similar number in rats at 2 years (Jackson et al., 2002; Cunea and Jeffery, 2007). This can develop into disease in humans as there are links between mitochondrial dysfunction and age related macular degeneration (AMD. Jarrett et al., 2008). Inflammation is an early hall mark of AMD, partly driven by deposition of pro-inflammatory amyloid beta in the outer retina where this disease is initiated (Hoh Kam et al., 2010; Hoh Kam et al., 2013; Johnson et al., 2002).

It had been thought that it was not possible to ameliorate age related ATP decline or reduction due to disease. But it is known that near infra-red light is absorbed by cytochrome c oxidase (COX), complex IV of the respiratory electron transport chain needed for ATP production (Review Fitzgerald et al., 2013). Also, these relatively long wavelengths partially reverse age related decline in retinal mitochondrial membranes potentials and significantly reduce age related retinal inflammation and that associated with ocular immune vulnerability arising from the absence of complement factor H (CFH. Begum et al., 2013; Kokkinopoulos et al., 2012). Further, there is now evidence that near infra-red light has the ability to preserve mitochondrial integrity in terms of ultrastructure in response to inflammatory damage in the visual system (Cummins et al., 2013). Experiments that have explored this have commonly used 670 nm as a source in brief administration in a wide range of induced pathologies both in the visual system and other organs (Review Fitzgerald et al., 2013). Here we chart ATP decline in the ageing murine eye and brain. We then show that 670 nm light exposure is directly associated with a significant increase in ATP. Exposure to near infra-red may represent a simple direct route to combating age related disease in the eye.

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A total of 79 C57/Bl mice were used throughout. Those at 2 ($N = 7$), 4 ($N = 14$) or 12 ($N = 25$) months were used to chart retinal and brain age related ATP decline. 12 months mice were used to assess the impact of 670 nm on ATP ($N = 23$), COX and acrolein expression ($N = 10$). COX is a key element in the ATP respiratory chain. Acrolein has been used as a marker of oxidative stress and is an end product of lipid peroxidation (Kehrer and Biswal, 2000). It is also associated with Alzheimer's disease and retinal degeneration (Cimini et al., 2009; Huang et al., 2013; Jia et al., 2007; Komeima et al., 2006), and with oxidative stress and mitochondrial dysfunction in retinal cells (Feng et al., 2010; Shen et al., 2005). 670 nm exposure was undertaken by scruffing mice individually and holding them approximately 15 cm from the source (CH electronics UK) for 90 s 7 times spaced evenly over 84 h. The energy delivered was 40 mW/cm^2. For ATP measurements, mice were killed by decapitation and heads placed directly in ice chilled Krebs solution where the eyes were removed. Retinae and brains were removed in this in <1 min, with a mean extraction time for retinae of approximately 25 s. All tissues were then placed in cell lysis reagent (ATP bioluminescence Assay Kit; Roch UK) at 1 °C. Brains used for age related changes and 670 nm treatment were cut into small sections prior to placing in lysis reagent. This included dividing tissue into cortex and thalamus. Visual cortex was avoided to exclude direct visual effects. ATP was measured with a standard luciferase protocol. Immunohistochemistry: eyes were enucleated and fixed in 4% paraformaldehyde for 1 h, washed, the cornea and lens removed and cyro-protected in 30% sucrose before embedding in OCT compound. These were cryosectioned at 10 μm and mounted onto slides before for staining with standard protocols for COX and Acrolein. The following primary antibodies were used: COX subunit VIb (rabbit monoclonal 1:200, Abcam, Cambridge, UK) and Acrolein (rabbit polyclonal 1:200, Abcam, Cambridge, UK), followed by incubation with the appropriate Alexa fluor secondary antibody, donkey-anti rabbit 568 (1:2000, Invitrogen, Paisley, UK). Negative controls were with primary antibody omitted. Intensity of label was measured using standard procedures, which have recently been shown to be tightly correlated with independent measurements of the same features using qPCR and Western blot analysis validating this method in mice retinae (See Fig. 3. Begum et al., 2013). Statistical analysis was undertaken using a single tailed Mann–Whitney *U* test.

Retinal and brain ATP concentrations were measured in mice at 2, 4 and 12 months of age. The respective ATP concentrations in the retina with ageing were approximately 22,000, 18,000 and 15,000 pm/mg at 2, 4 and 12 months. The decline in retinal ATP with age was statistically significant between 2 and 4 months and between 2 and 12 months ($P < 0.05$ and $P < 0.01$ respectively. Fig. 1A). When 12 month old mice were exposed to 670 nm for 90 s 7 times spaced over 84 h at an energy level of 40 mW/cm^2 there was significant increase in retinal ATP of just under 20% from around 14,000 to 17,000 pm/mg ($P < 0.05$. Fig. 1B).

In the brain ATP concentrations showed a similar age related decline as the retina, being approximately 7000, 5000 and 3000 pm/mg at 2, 4 and 12 months. The content of ATP was significantly different between all ages (Fig. 1C. $P < 0.01$, 2–4 months. $P < 0.001$, 2–12 months, $P < 0.01$, 4–12 months). Additionally, there was a more than 3-fold greater concentration of ATP in the retina compared with the brain at all ages (Fig. 1). These results are consistent with the very high metabolic demand of the retina and its high density of mitochondria in photoreceptor inner segments. Again 670 nm exposure significantly increased ATP in both the cortex ($P < 0.01$ Fig. 1D) and the thalamus ($P < 0.05$ Fig. 1E).

COX is essential for mitochondrial function. Retinal ATP increase via 670 nm exposure was correlated with a significant increase in COX expression in the outer retina where mitochondrial density is at its greatest (Fig. 2A,B and E $P < 0.01$). COX was also up-regulated in the outer plexiform layer (OPL), but this was not measured because it remained relatively diffuse. Adjacent sections were also stained for acrolein, a molecule associated with free radicals and oxidative stress (Calingasan et al., 1999; Cingolani et al., 2006). This showed a significant decrease (Fig. 2C,D and F. $P < 0.01$) in the outer retina in the same regions where COX was elevated.

This is the first study showing ATP modulation *in vivo* by an external stimulus. However, near infra-red wavelengths have been used by many labs in diverse tissues to ameliorate induced

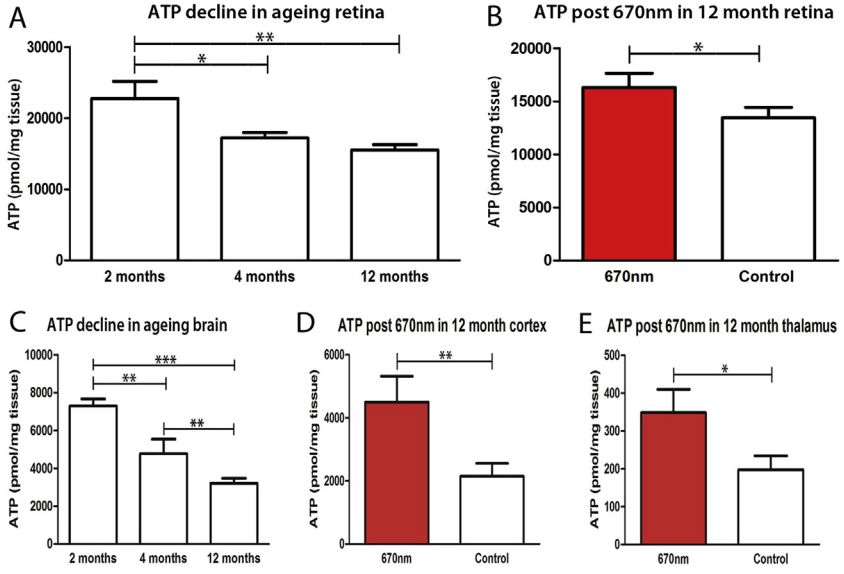

Fig. 1. ATP content in the retina and brain. **A**. Retinal ATP levels at 2, 4 and 12 months. ATP showed a significant decline between 2 and 4, and 2 and 12 months. The decline between 4 and 12 months was not significant. Two months was selected as the initial time point as eye size is approximately adult like. **B**. 12 month old mice were given seven 90 s 670 nm exposures at 40 mW/cm^2. This significantly increased retinal ATP compared with aged matched controls. **C**. Brain ATP levels at 2, 4, and 12 months. ATP shows a significant decline between 2 and 4 month and between 2 and 12 months. There was also a significant difference between 4 and 12 months. **D**. 12 months old mice were exposed to 670 nm, which significantly increased ATP levels in the cortex compared to aged matched controls. **E**. In the same mice the thalamus was examined and 670 nm exposure also significantly increased ATP levels here, although to a lesser extent than in the cortex. The number of mice used were at 2 ($N = 7$), 4 ($N = 14$) or 12 ($N = 25$) months were used to chart retinal and brain age related ATP decline. Additionally, to assess the impact of 670 nm on ATP further 23 mice were used.

pathology, and it had been thought that elevated ATP or its preservation in pathology may have been the mechanism, but this has never been demonstrated *in vivo* (Review Fitzgerald et al., 2013). However, as studies in different fields have often used different key words and phrases in their manuscript titles, the magnitude of publications in this field of research is largely underestimated. In spite of this, our results go some way to confirming that up regulation of ATP may be part of the mechanism common to many of these experiments.

We have also shown that 670 nm retards normal age related retinal inflammation, potentially via improved mitochondrial membrane potentials (Kokkinopoulos et al., 2012). We have also shown that it reduces inflammation in $CFH^{-/-}$ mice, which are immune-compromised and suggested as a model of AMD (Begum et al., 2013). A further link between mitochondrial function and 670 nm comes from Cummins et al. (2013) who have shown that 670 nm partially blocks degenerative changes induced by optic nerve section probably by halting decline in mitochondrial ultrastructure and preserving elements of the citric acid cycle.

The *in vivo* demonstrations of the impact of 670 nm are supported by *in vitro* studies on liver mitochondria and neuronal cultures (Liang et al., 2008; Passarella et al., 1984). Also, cellular exposure of lymphocytes with 632.8 nm results in significant changes to mitochondrial morphology in 1 h (Manteifel et al., 1997). Similar results have been found in yeast cells where exposure expanded the mitochondrial matrix that may be associated with increased energy transfer (Manteifel and Karu, 2005). While the main aim of our study has been to show changes in ATP as a result of 670 nm in old mice we have also shown that this treatment increases COX content and reduces acrolein in very similar regions of the outer retina where mitochondrial density is the greatest in the body. We have previously demonstrated that COX expression is elevated in the outer retina following 670 nm exposure using immunohistochemistry, qPCR and Western blots, with each independent method revealing an approximate doubling in COX content. This confirms that measurements of density of immunohistochemical staining do accurately reflect changes in protein levels (Begum et al., 2013).

COX is a highly conserved soluble protein mainly localized to mitochondria that is a component of the mitochondrial electron transport chain. It is capable of undergoing oxidation and reduction. It plays an essential role in the generation and maintenance of mitochondrial transmembrane potentials that are critical for ATP production via oxidative phosphorylation. Its release when membrane potentials decline is associated with cell death. It is thought that COX, which acts as the terminal enzyme in the electron transport chain, is a light absorbing chromophore for deep red light. When COX absorbs proton energy the redox state of the mitochondria is changed and there is an increase in ATP production along with intra-cellular calcium. However, it is unlikely to be the only absorbing chromophore. There is much to explore in the relationships between different wavelengths, their patterns of absorption and their subsequent impacts in cellular energy production. Further metrics undoubtedly reside in how this energy is applied in terms of differential delivery patterns (Borutaite et al., 2013; Siletsky, 2013).

The relatively long wavelengths used at the energies applied penetrate tissue deeply and we found similar ATP up regulation in brains following light exposure. However, the degree of up regulation depended on the depth, with a smaller increase in the

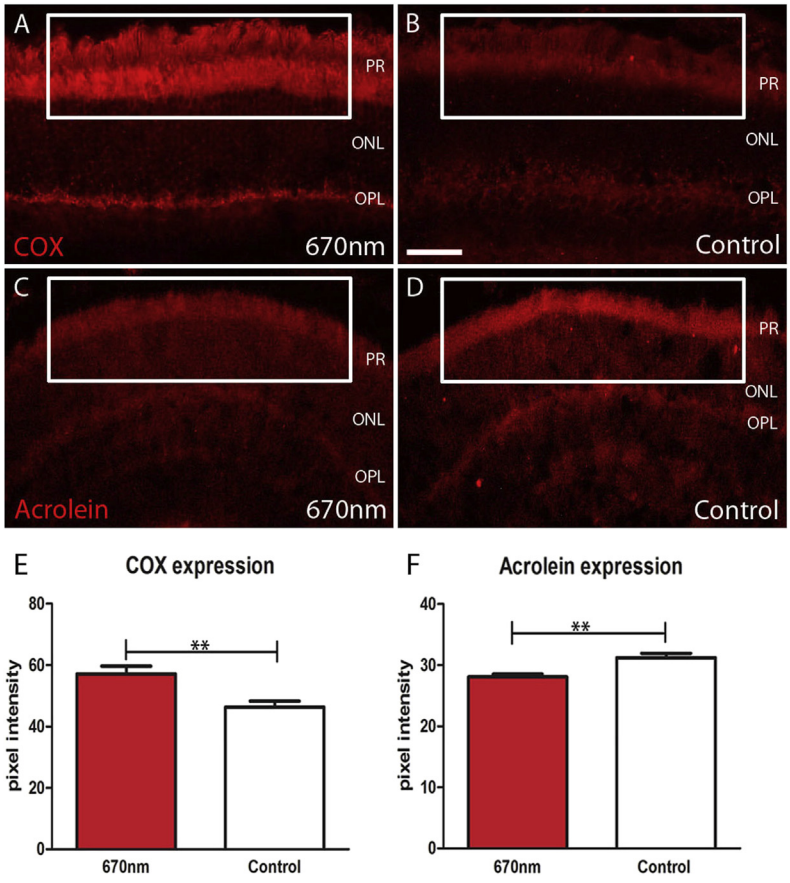

Fig. 2. COX and Acrolein expression in 670 nm treated mice and controls. **A and B**. 670 nm resulted in COX up regulation in the outer retina (**E**) where mitochondrial density is greatest (represented in boxed region). COX label was also observed in the OPL and appeared elevated following 670 nm. **C and D**. In the same areas Acrolein expression was significantly reduced (**F**). Abbreviations. PR Photoreceptors. ONL Outer nuclear layer. OPL Outer plexiform layer. Scale bar for all images = 50 μm. For COX and acrolein expression 10 mice were used.

thalamus than the cortex. In the case of the thalamus the more modest increase may be due to less light penetrating to these deeper tissues. That up regulation occurs in the brain excluding the visual cortex is consistent with the notion that the effect of 670 nm is independent of visual function. The energy applied was 40 mW/cm^2 in 90 s bursts, which are standard, simply because it was the setting on earlier commercial devices. To place this energy level in context, indirect light energy on a clear spring morning measured outside our lab was about 400 W/cm^2 across the spectrum. Hence, therapeutic energy levels are log units lower than direct daylight. Also, they are from a safe part of the spectrum being confined to longer wavelengths, avoiding the shorter wavelengths associated with tissue damage. Interestingly, Begum et al. (2013) found evidence for significant impact of 670 nm on retinal inflammation when environmental light was supplemented with this wavelength and mice were free to engage in normal activities rather than hand held in front of the source. Here many animals slept through some exposures or were not consistently oriented towards them when switched on. This may suggest that the actual energy at this wavelength that can have a therapeutic impact may be much less than 40 mW/cm^2. This is supported by our finding of increased ATP deep in the brain where light is heavily filtered by the fur, skin, skull and more superficial cortical regions.

Mitochondrial dysfunction is associated with AMD. Barron et al. (2001) revealed that aged foveal photoreceptors have increased mtDNA deletions and COX deficiencies, and mtDNA instability is viewed as a critical risk factor in photoreceptor defence (Barot et al., 2011; Jarrett et al., 2008). Photoreceptors suffer added stress as their oxygen dependent activities are compromised by progressive extra cellular deposition along Bruch's membrane, which separates them from their blood supply (Hoh Kam et al., 2010). In light of all these factors, it is likely that this treatment will benefit retinal disease. But this need not be confined to the eye, as declining ATP is a feature of all ageing tissues. In light of this it is interesting to note that one minor clinical trial for dry AMD presented positive results for 670 nm at ARVO 2012 (Merry et al., 2012). Hence, there are reasons to believe that this potential therapy has gained some traction. The results we present here may go some way to explaining the underlying mechanism of action for this.

Author contributions

DG, RB, TS, GL, CH KYC and GJ undertook experimental work. AS provided facilities. All authors reviewed the manuscript.

Conflicting interests

Authors declare no competing financial interests or conflict of interest.

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