Photostimulation of mitochondria as a treatment for retinal neurodegeneration

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Abstract

Absorption of photon energy by neuronal mitochondria leads to numerous downstream neuroprotective effects. Red and near infrared (NIR) light are associated with significantly less safety concerns than light of shorter wavelengths and they are therefore, the optimal choice for irradiating the retina. Potent neuroprotective effects have been demonstrated in various models of retinal damage, by red/NIR light, with limited data from human studies showing its ability to improve visual function. Improved neuronal mitochondrial function, increased blood flow to neural tissue, upregulation of cell survival mediators and restoration of normal microglial function have all been proposed as potential underlying mechanisms of red/NIR light.

Keywords

Red light; near infrared light; retina; neurodegeneration; photostimulation; low-level light therapy; photobiomodulation

Abbreviations

Near infrared (NIR)

Light emitting diode (LED)

The U.S. Food and Drug Administration (FDA)

Cytochrome *c* oxidase (COX)

Retinal ganglion cell (RGC)

Electroretinogram (ERG)

Nitric oxide (NO)

Reactive oxygen species (ROS)

Reaactive nitrogen species (RNS)

Nuclear factor (NF)

Interleukin (IL)

1.0 Introduction

The therapeutic properties of light have been known since antiquity, as far back as 1400 BC, where it was used by Hindus to treat skin disorders [1]. The ancient Egyptians, Greeks and Romans were also reportedly aware of the beneficial effects of sunlight which they used to treat various ailments [2]. The evidence for the use of phototherapy in those time, however, is purely anecdotal. It was not until 1903 that the therapeutic power of light gained scientific recognition, when Niels Finsen was awarded the Nobel Prize in medicine for the discovery of UV light as a treatment for skin tuberculosis (*lupus vulgaris*) [3].

Red light was, later, found to have biostimulatory effects; an unintentional discovery made by Endre Mester, in 1967, who wanted to assess the ability of 694 nm lasers to cause carcinogenesis in mice [4]. The mice in both the light-treated and untreated groups were shaved prior to laser exposure. The results found that the light-treated group did not develop cancer, but more intriguingly, the hair grew back on the laser treated mice at a faster rate than the untreated group.

In more recent times, there has been a surge in the use of red and near infrared (NIR) lasers and LEDs in clinical and preclinical research [5]. As red and NIR light have relatively long wavelengths, they have the advantage of a greater penetration depth over shorter wavelengths, making them an ideal choice for the treatment of neural tissue [6]. In addition to light being able to penetrate into the tissue of interest, another requirement is that the photon energy corresponds to the absorption characteristics of the chromophores responsible for triggering the beneficial effects upon photoexcitation. It appears that red and NIR light correspond to the absorption maxima of such chromophores as will be discussed later. For various reasons, LEDs are most commonly used as the light source in these studies. Most importantly, red/NIR LED therapy has been approved for use in humans and has been

deemed as a non-significant risk by the U.S. Food and Drug Administration. Although shorter wavelengths of visible light and UV light are also employed for therapeutic purposes, their safety for use in humans, especially for the eye, is less clear [7, 8]. With the ultimate objective of exploring the efficacy of phototherapy as a treatment for neurodegeneration in the human retina, this review will focus only on the use of wavelengths that are least likely to cause adverse effects, that is, red and NIR light[9].

2.0 The potential of red/NIR light as a treatment for neurodegeneration

2.1 Evidence from in vitro studies

Red and NIR light have been shown to provide protection against the deleterious effects of mitochondrial electron transport chain inhibitors and excitotoxic cell death in neurons *in vitro* [10-12]. Since impaired mitochondrial function and excitotoxicity are common causes of cell death in neurodegenerative conditions, the ability of red/NIR light to protect against these challenges *in vitro* has emphasized the potential of this therapy in various neurodegenerative conditions.

2.2 The effects of red/NIR light in models of neurodegeneration

Red/NIR light therapy has shown great potential in the treatment of acute neurodegenerative conditions, showing neuroprotective effects in rodent models of spinal cord injury, traumatic brain injury and stroke [13-18].

Furthermore, red light has been shown to have beneficial effects in animal models of some of the most prevalent neurodegenerative diseases. A reduction in cell loss and other markers of disease severity was seen with red/NIR light treatment, in rodent models of multiple sclerosis, Alzheimer's and Parkinson's disease [19-26].

2.3 The potential of red/NIR light as a successful treatment for neurodegeneration in humans

While transcranial red/NIR light therapy is yielding remarkable results in numerous rodent models of neurodegeneration, the real question is how well these results will translate when applying this therapy to human patients.

Interestingly, in a neurotoxin-induced monkey model of Parkinson's disease, 670 nm light was delivered directly to the macaque midbrain using an implanted optical fibre which was activated over the period of time of 5-7 days when the neurotoxin precursor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was injected [27]. The study found a reduction in clinically-assessed behavioural impairment with this method of red light delivery in this primate model as well as neuroprotection to the dopaminergic neurons of the substantia nigra. Although a more invasive method of delivery than transcranial red light treatment, no major adverse effects were observed following surgical implantation of the optical fibre. However, it would have been of great interest if the effects of transcranial light delivery were also tested in this model, for comparison.

3.0 Red light treatment in retinal degenerative diseases

Since the retina is an extension of the CNS, the neuroprotective effects of red/NIR light, as discussed above, should also be observed in this tissue. In fact, irradiating the retina with red or NIR light seems more likely to be successful as a non-invasive treatment for human patients as the issue of tissue penetration is avoided.

3.1 The safety of light treatment on the retina

The greatest concern arising when aspiring to use red light therapy to treat retinal degeneration, is the potential retinal damage that may occur upon direct exposure of the

retina to light with high levels of irradiance. The dangers of high levels of irradiance on the retina is highlighted in a study on anesthetised monkeys [28]. It was found that white light with a retinal irradiance of 270 mW/cm^2 caused irreversible damage to the photoreceptors and retinal pigment epithelium. White light is made up of light of all wavelengths in the visible light spectrum, with light of shorter wavelengths and higher frequencies having a greater damaging effect on photoreceptors. Blue light, with a relatively short wavelength, was found to cause irreversible damage to S cones [29]. While exposure to green and red light caused damage to M and L cones, respectively, the damage to these cones was reversible, with a full recovery of function seen after a few weeks. More recent studies on macaque monkey, however, have demonstrated that yellow light of 568 nm wavelength can cause retinal damage manifested as disruption of the retinal pigment epithelium at the dose below the Maximal Permissible Exposure established by the American National Standard Institute's (ANSI) as a standard for the safe use of lasers [30].

Albeit transient and less severe than light of shorter wavelengths, damage to L cones upon exposures to high levels of red light would be a cause for concern when considering red light as a treatment for retinal degeneration. This concern has been addressed with numerous *in vivo* studies. These studies have shown that therapeutic effects were achieved, in the absence of retinal damage, when rodent retinas were exposed to 670 nm light with a therapeutically effective irradiance and exposure times [31-33]. Further, this included irradiance of 60 mW/cm2 which is the highest irradiance level found in studies of *in vivo* models of retinal degeneration where positive results were achieved using 670 nm light. This demonstrates the safety of using 670 nm light as a treatment for retinal degeneration.

In addition to photoreceptor damage, the possibility of photothermal damage to the retina and surrounding ocular structures evokes further concern when considering using light to treat retinal degeneration [34]. Comparing the effects of green, red and NIR laser light exposure on the temperature rise in the human choroid, it was found that the longer wavelengths led to a smaller degree of choroidal heating, due to the decrease in absorption by melanin with increasing wavelength [35]. The variation in choroidal heating between green and red wavelengths was minor compared with the difference between green and NIR wavelengths.

Still the question remains as to which would be the optimal wavelength for use as a neuroprotective agent in the retina. Addressing this, the efficacy of red and NIR light were compared in a model of partial optic nerve transection [33]. It was found that although protective effects were seen in retinas treated with both red and NIR light, 670 nm light was more effective in improving visual function compared with 830 nm light. However, in a rat model of light induced retinal degeneration protective effects were observed with 670 nm light treatment, but no protection was seen with 830 nm light [33]. It is therefore not surprising that most studies testing the effectiveness of phototherapy on neurodegeneration in the retina use red light at 670 nm.

3.2 670 nm light therapy in models of photoreceptor damage

As discussed above, exposing the retina to bright light can cause photoreceptor damage, an event that can occur with excessive sunlight exposure or accidental exposure to high intensity artificial light sources. Models of light induced photoreceptor damage are also used to simulate retinal degenerative diseases, involving photoreceptor specific death. Emphasising the vast and diverse effects of light on biological tissue, irradiating the retina with red light

provided protection against the structural damage to the outer retina and loss in photoreceptor function in a rat model of light induced photoreceptor degeneration [32]. Methanol can also induce damaging effects on the retina causing photoreceptor toxicity due to the ability of its metabolite, formic acid, to inhibit cytochrome *c* oxidase, the terminal enzyme of the electron transport chain. In a rat model of methanol induced retinal toxicity, red light treatment brought about a significant recovery of rod and cone mediated function, in addition to preventing methanol induced changes to outer retinal morphology [36]. These studies show the ability of red light to protect against loss of photoreceptor function, an event that would cause severe visual impairment and would otherwise be irreversible.

3.3 The effects of red light on inflammation in the outer retina

Inflammation in the retina has been implicated in many retinal diseases including age related macular degeneration (AMD) and diabetic retinopathy, and as such, looking at ways to alleviate inflammation in these conditions is thought to reduce the severity of symptoms associated with these diseases [37]. Also, an upregulation in inflammatory proteins has been observed following light damage in rats, demonstrating pathological features similar to “dry” AMD [38]. In addition to its ability to protect against light induced photoreceptor damage, red light treatment was found to reduce the complement propagation that occurs in the retina following light damage. Red light treatment was also tested in an aged genetic mouse model of AMD, the complement factor H knockout, which, likewise, presents with reduced retinal function and increased inflammation [39]. In contrast to most studies with red/NIR light, where the light source was held directly in front of the animal, the environmental light was supplemented with red light, for this study. Even though the red light exposure was indirect,

the levels reaching the retina were sufficient to reduce inflammation in the outer retina, in this model.

In addition to inflammation associated with disease pathology, inflammation increases in the retina with age. Red light was effective in reducing proinflammatory cytokines and a chronic marker of inflammation in the aged mouse retina, demonstrating the potential of red light to also alleviate the visual decline associated with normal aging [40]. These studies draw attention to the ability of red light to produce anti-inflammatory effects associated with outer retinal degeneration, whether by induced damage, disease pathology or normal aging.

3.4 The effects of red light on neurodegeneration in the inner retina

Retinal ganglion cell (RGC) death and optic nerve degeneration are hallmarks of optic neuropathy, a frequent cause of vision loss, of which the causes are many. Red light therapy has been trialled in a rat model of diabetic retinopathy and positive outcomes have been reported. There was a significant reduction in the diabetes induced RGC death and a 50% improvement in the diabetes-induced reduction in ERG amplitude with exposure to red light [41]. Highlighting the beneficial effects of red light on the retinal vasculature, red light also prevented the diabetes-induced increase in leukostasis in the retinal vasculature, an event which is implicated in the pathogenesis of diabetic retinopathy. Further, the diabetes-induced increase in retinal expression of an adhesion molecule, essential for leukostasis, ICAM-1, was also prevented with red light. Similarly, in a model of secondary degeneration of RGCs, resulting from traumatic injury to the optic nerve, red light was found to be protective [42]. The secondary damage, following partial optic nerve transection, normally leads to further loss of RGCs and visual function; however, normal visual function was restored with red light treatment[42]. Furthermore, treatment with 670 nm light in a rat model of partial optic nerve

transection, resulted in improved vision, 7 days post injury [33]. Additionally, dendropathy of retinal ganglion cells, an event found to be associated with visual loss in experimental models of glaucoma and autosomal dominant optic atrophy, was partially prevented, in an axotomy model of neurodegeneration, with red light treatment [43]. Protection against RGC and optic nerve degeneration, arising from different conditions, appears to be possible with red light treatment. Since RGC dysfunction and optic nerve degeneration are common features among numerous other types of optic neuropathies red light has the potential to provide protection in these conditions also.

However, the successful outcomes achieved in the discussed experimental models were seen when red light was administered immediately after induced optic nerve injury, a treatment strategy that would be unachievable in a real life clinical setting. Although the therapeutic window of opportunity for red/NIR light therapy in models of traumatic optic neuropathy has not been explored, it has been assessed in other CNS injury models. NIR light therapy improved motor function in a rabbit model of embolic stroke when treatment was administered 6 hours post-embolization but was ineffective when administered 12 hours post embolization [44]. The findings show that neuroprotection can be achieved when red/NIR light is administered for up to 6 hours after the induced injury, showing the therapy to be applicable to a clinical setting. Other studies on animals demonstrated effectiveness of near-infrared light in a mouse model of traumatic brain injury when administered 4 hours after injury, with additional treatments administered at one day and two days post injury[45, 46]. The transcranial treatment upregulated brain-derived neurotrophic factor (BDNF), improved neurological functions, reduced the size of the lesion, stimulated formation of new neurons and synaptogenenesis. There is also a growing body of evidence suggesting that people

affected by chronic traumatic brain injury or after stroke can benefit from transcranial irradiation with red/near-infrared light [47, 48]

3.5 Red/NIR light as a therapy for patients with retinal disease

The protective effects seen with red light treatment, in the absence of adverse effects, in numerous *in vivo* models of retinal degeneration, strongly suggest this non-invasive treatment should be trialled in patients with retinal degeneration. Progress to this end has commenced with trials of red and NIR light therapy yielding promising results in patients with AMD. In one such study, a brief exposure of NIR light (780 nm), from a semiconductor laser diode, to AMD patients, twice per week for two weeks, resulted in a significant improvement in their visual acuity [49]. This improvement in vision was seen in patients with both wet and dry AMD and was maintained for 3-36 months after treatment. Moreover, no adverse effects of the treatment were seen. In this study, the laser was applied transconjunctivally, to the macula, when the eye was in adduction. In another study looking at the effects of photobiomodulation on patients with dry AMD, the retina was irradiated through the pupil with red light (670 nm), from the FDA approved Warp 10 LED light source, 3 times per week for 6 weeks [50]. The visual acuity and contrast sensitivity remained significantly improved for 12 months after treatment; however, the improvement in visual acuity began to decline after 4 months. The results provide vital information on the time at which patients may benefit from re-treatment, in addition to providing pilot data on the safety and effectiveness of 670 nm light from an LED source. Red/NIR light has also been trialled in diabetic macular edema and Leber's hereditary optic neuropathy, however, there are no results available from these studies. A summary of all the studies using red/NIR light as a treatment intervention in conditions associated with neurodegeneration in the retina is available in Table 1. From all

the studies listed, red/NIR light is showing the greatest potential as a treatment for AMD. Since positive outcomes have been observed in patients with AMD, this paves the way for the application of this therapy in other retinal degenerative conditions, particularly those where positive outcomes have been seen in preclinical studies.

Responsible party	Last verified on ClinicalTrials.gov	Condition	Duration of treatment period	Number of patients	Duration of improved vision after treatment	Status
Harry T Whelan	01/09/2013	Diabetic Macular Edema	3 months	20	No results available	Completed
Ivandic and Ivandic 2008	N/A	Wet and dry AMD	2 weeks	203	Up to 36 months	Completed and published{Ivandic, 2008}
Merry, Graham	01/11/2011	Non-exudative Age-related Macular Degeneration	6 weeks	9	Up to 12 months	Completed and published{Merry, 2013}
Merry et al., 2016	N/A	Non-exudative Age-related Macular Degeneration	3 weeks	24	3 months	Completed and published{Merry, 2016}
University of Sydney	01/06/2014	Diabetic retinopathy	4 weeks	N/A	N/A	Planned
LumiThera, Inc.	01/04/2016	Age-related Macular Degeneration	3 weeks	30	N/A	Planned
Harry T Whelan	01/09/2014	Leber's hereditary optic neuropathy	3 months	4	N/A	Terminated (0/4 patients completed the study)

Table 1: A summary of the completed, planned and terminated clinical trials, using red/NIR light as a treatment intervention in conditions associated with retinal neurodegeneration.

4.0 The effects of red/NIR light on mitochondrial dysfunction

Although the therapeutic benefits of red/NIR light therapy have been demonstrated in a number of different disease models, in addition to AMD patients, the underlying molecular mechanisms are less well understood [5]. The question is no longer whether or not light has biological effects, it is rather how these effects are mediated at a cellular and molecular level [51].

4.1 Cytochrome c Oxidase: the photoacceptor for red/NIR light

Endeavors to uncover the underlying molecular mechanisms suggest a major role for cytochrome *c* oxidase (COX) which is the terminal enzyme of the electron transport chain, transferring electrons from cytochrome c to molecular oxygen [52], [53].

COX is a large multicomponent protein, containing two copper centers (Cu_A and Cu_B) and two heme iron containing centers (heme *a* and heme *a3*), which absorbs photons in the red to NIR region of the electromagnetic spectrum [53]. These transition metals are also the intermediate redox sites in the electron transfer pathway from cytochrome *c* to oxygen, a process which is coupled to the pumping of protons across the inner mitochondrial membrane. The electrons pass from cytochrome *c* to Cu_A then passed to heme *a*, from heme *a* to heme a3-Cu_B and finally to molecular oxygen.

4.2 The absorption of photons by photoacceptors in COX

One theory proposed to explain how photon energy is absorbed by COX, centers on its heme molecules [54]. Heme is comprised of a porphyrin ring with an iron atom at its center that can continuously switch its oxidation states between ferrous (Fe^{2+}) and ferric (Fe^{3+}) by accepting or donating an electron. The porphyrin ring is made up of four pyrrole rings that are

connected through their carbon atoms via π bonds. The electrons in these π bonds are delocalised, moving back and forth from one configuration to another, creating resonance. Electrons, like photons, have a dual nature, behaving like particles or electromagnetic waves, thereby creating a resonating electromagnetic cloud in the porphyrin ring. Photons with similar wavelengths are absorbed by this cloud, increasing its energy. The energy from these photons causes photoexcitation of electrons of Fe^{2+}, bringing them to an unstable higher energy level. Upon absorption of sufficient energy, these electrons are released from the orbitals of Fe^{2+} causing the oxidation of Fe^{2+} to Fe^{3+}. The oxidised iron atom can then accept electrons from cytochrome c, thus increasing the electron flux through the electron transport chain.

Experimental evidence shows that red/NIR light has the ability to upregulate the enzymatic activity of complex IV, increase the mitochondrial membrane potential and increase ATP production [39, 40, 51, 55-58]. These mitochondrial specific effects of photobiomodulation may offer a partial explanation for its beneficial effects in neurodegenerative diseases associated with mitochondrial dysfunction. However, in genetic or toxin induced models of Parkinson's disease, where loss of dopaminergic cells was triggered by complex I dysfunction, neuroprotective effects were seen with red/NIR light treatment. The absorption of photon energy by the heme group in complex IV may explain how photobiomodulation can increase ATP production when complex IV is inhibited but fails to explain how this effect can be achieved in models with complex I inhibition.

It has been hypothesised by Zielke *et al*. that the electrons released in this oxidation process are free to reduce NAD^+ and FAD, providing substrates for complex I and II, respectively, creating a closed circuit of electron transfer [54]. In the case of aberrant functioning of COX

the flux of electrons through this closed circuit would maintain the proton pumping functions of complex I and III, thereby maintaining the electrochemical gradient across the mitochondrial membrane required for ATP synthesis. However, since oxygen is very electronegative it would be most likely that the released electrons would be readily accepted by the oxygen molecule bound to the reduced heme a3-Cu_B component of COX, reducing it to water. In the event of aberrant COX activity the released electron would most likely react with unbound oxygen molecules, forming reactive oxygen species. Therefore, how red/NIR provides neuroprotection against complex I dysfunction is not explained by its direct action on the COX.

5.0 The nitric oxide theory of red/NIR light therapy

5.1 The role of nitric oxide in mitochondrial respiration

Nitric oxide ($^{\bullet}NO$) has important roles in the regulation of blood pressure and vasculature tone, however, excessive $^{\bullet}NO$ production, as seen in neurodegenerative diseases, can cause impairment of mitochondrial respiration and apoptosis [59]. Mitochondria harbor nitric oxide synthase (NOS) to produce NO, which they use to hinder respiration, as an intrinsic mechanism to prevent oxygen from reaching precariously low levels [9]. $^{\bullet}NO$, at low concentrations, competes with oxygen to bind to the reduced heme a3-Cu_B component of COX [52]. This prevents COX from reducing molecular oxygen, thus impairing the proton pumping abilities of the enzyme and essentially the energy production ability of the mitochondria. Additionally, $^{\bullet}NO$ was found to cause inhibition of complex I, II and IV-dependent respiration in mitochondrial suspensions, where the inhibitory action of $^{\bullet}NO$ was found to be more profound at lower oxygen tensions [60]. Interestingly, this inhibitory action of $^{\bullet}NO$ was overcome by reoxygenation of mitochondrial suspensions for a mere 10 seconds,

resulting in complete recovery of complex I and IV dependent oxygen consumption and a 50% recovery of complex II dependent respiration. This shows that the inhibitory actions of •NO are almost completely reversible upon restoration of normal oxygen levels. Therefore, this intrinsic mechanism would protect a tissue if the depleted oxygen supply were temporary, by putting the mitochondria in a state of conservation until the return of normal oxygen levels. However, prolonged inhibition of respiration would deplete ATP levels and bring about cell death [61].

5.2 The effects of red/NIR light on •NO-mediated mitochondrial dysfunction

Red/NIR light has been proposed to influence the photodissociation of •NO from COX, thereby allowing oxygen to reclaim its binding site, permitting the ATP production process to resume [52]. This would be most beneficial in pathological situations where •NO levels are higher than normal physiological amounts, favouring the binding of •NO rather than O_2 to COX. Experiments have shown that irradiating cells with red/NIR light increases COX activity in normal healthy neurons and restores the activity in COX inhibited neurons [10]. However, the increase in COX activity appears to be mediated by an increase in the expression of the proteins in the COX complex, suggesting that a mechanism, additional to the disinhibition of COX, may also be involved[62]. Furthermore, exposure of cells to an irreversible COX inhibitor was overcome by NIR light treatment, providing further support to the claim that restoration of activity is mediated by upregulating the expression of COX proteins [10].

An indirect effect of red/NIR light on COX activity helps explain how beneficial effects are also seen in neurodegenerative conditions that are associated with impairment of other electron transport chain complexes. The absorption of photon energy from red/NIR light by COX may indeed have direct effects on the enzyme itself, however, this initial event may trigger further

downstream events, which may have more far-reaching effects. As •NO is widely known for its function as an intercellular signaling molecule, the release of •NO upon red/NIR light exposure would increase its bioavailability allowing it to function as a signaling molecule [63]. It has been suggested that •NO intracellular signaling could play a role in the upregulation of COX proteins upon red/NIR light exposure. Intracellular signaling from the mitochondria to the nucleus may be triggered by other byproducts of mitochondrial respiration, levels of which may be altered by red/NIR light, therefore, will be discussed in more detail later in this review.

5.3 The indirect effects of Red/NIR light on mitochondrial dysfunction

The findings by Cassina and Radi suggest that increasing the delivery of oxygen to the mitochondria, as would occur with increased blood flow to the tissue, would improve mitochondrial function in tissues with •NO-mediated mitochondrial electron transport chain inhibition [60]. It has been found that the exposure of blood vessels to red light from an LED source can induce photorelaxation of blood vessels, an event that would increase blood flow and, in turn, oxygen delivery to the irradiated tissue [64]. Exposure of porcine coronary arteries to red light caused their vasodilation, as measured by wall tension in the exposed vessels. Since nitric oxide is known to have a primary role in the regulation of vasculature tone, and NOS is activated upon absorption of visible light, the vasodilation effect seen upon red light exposure is thought to be mediated through nitric oxide [65]. Additionally, red/NIR light can trigger the photodissociation of •NO from nitrosyl hemoglobin and nitrosyl myoglobin[63]. The •NO released from hemoglobin in the blood would contribute to the vasodilation effects of red/NIR light. Depending on the physiological situation, photobiomodulation can either reduce or increase •NO levels, however, the molecular events

determining whether the effect will be an inhibitory or a stimulatory one are yet to be identified [66]. An increase in •NO upon red light exposure would provide beneficial effects in conditions such as TBI, where increased cerebral blood flow could increase mitochondrial function in hypopxic cells (Figure 1) [67]. As mentioned earlier in the review, hypoxia can trigger inhibition of respiration through the binding of •NO to COX. The phototdissociation of NO from COX in hypoxic tissue may not cause a significant improvement in mitochondrial function since there would be limited oxygen available to reclaim the binding site on COX. However, if combined with an increase in cerebral blood flow, the associated increase in oxygen levels would lead to a more substantial improvement in mitochondrial function. This proposes a partial explanation for the neuroprotective effects seen upon red/NIR light exposure.

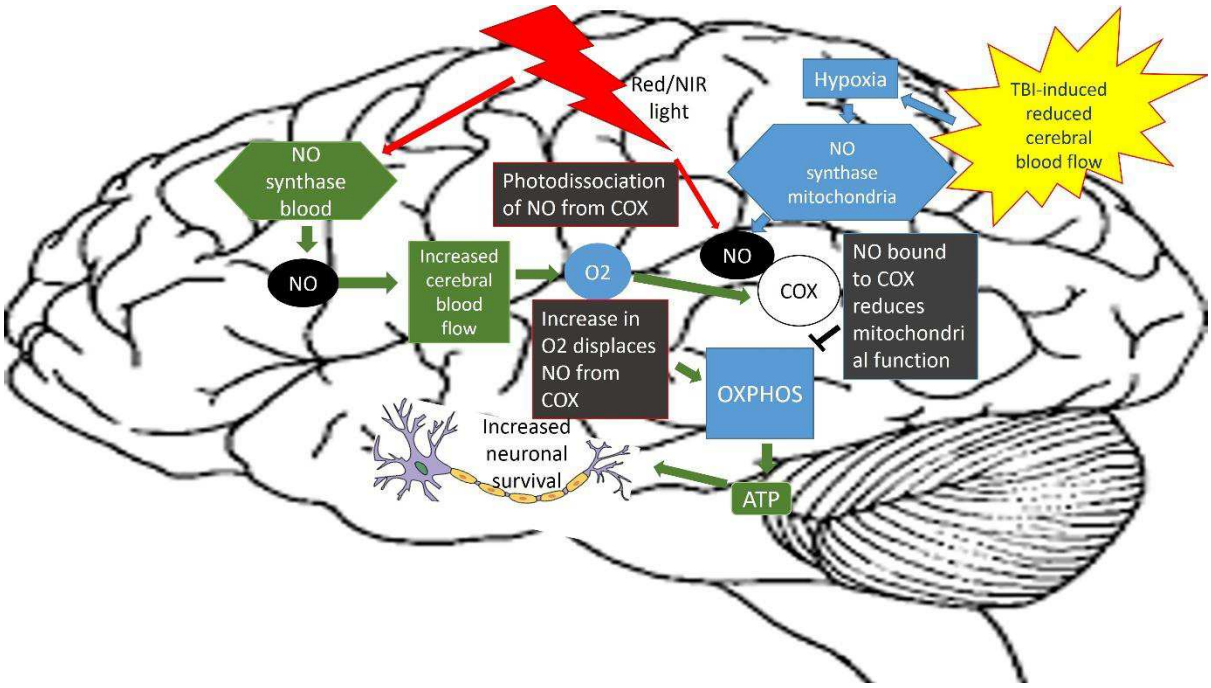

Figure 1: The direct and indirect actions of red/NIR light on nitric oxide improve mitochondrial function in neurons vulnerable to degeneration, in acute neurodegenerative conditions such as TBI.

5.4 The effects of red/NIR light on cellular function

As mentioned above, the ability of red/NIR light to increase free •NO can have beneficial effects in cells where mitochondria are dysfunctional. Providing further support to this theory, it was found that red/NIR light was protective against hypoxia and re-oxygenation injury in cultured cardiomyocytes. The observed protection was dependent on an increase in •NO as the protective effects were abolished in the presence of •NO scavengers [68]. Further, it was observed that the increase in NO seen upon red/NIR exposure was partially prevented by the non-selective inhibition of all isoforms of NOS. Of note, there are three isoforms of NOS: Neuronal NOS (nNOS), which is expressed in the central nervous system and plays a role in synaptic plasticity and central regulation of blood pressure, endothelial NOS (eNOS), which is mostly expressed in endothelial cells and primarily functions in controlling blood pressure, and inducible NOS (iNOS), which can be expressed in many cell types in response to cytokines and other agents to generate large amounts of •NO[69]. The findings show that the increase in •NO by red/NIR light is mediated in part by its action on NOS, however, the exact isoform of NOS responsible for the protective effects has not been determined. The source of the remaining NO could be that which is released during the photodisociation of •NO from COX in the mitochondria as discussed above.

This *in vitro* model of cardiac ischemia provides useful insight into how red/NIR light mediates its effects via •NO in ischemic conditions. Since the experiment was done *in vitro,* the observed protective effects most likely arose from the local effects of an increase in intracellular •NO rather than an indirect effect of •NO by increasing blood flow to the ischemic tissue. Although reperfusion is essential to limit cell death after hypoxia, paradoxically, this event itself causes further cell death due to excess ROS production [70]. The increase in free

•NO upon red/NIR light exposure would increase its availability to bind to COX and cause the reversible S-nitrosation of Complex I, events which would slow down the reactivation of the electron transport chain during the crucial initial stages of reperfusion [71]. In this particular pathological situation red/NIR light, administered upon reoxygenation, could reduce the harmful levels of ROS produced during reperfusion injury by reversibly inhibiting the electron transport chain.

Contrastingly, in an *in vivo* model of cerebral ischemia, where excess •NO production is said to be associated with neurotoxic effects, red/NIR light has been shown to have the ability to reduce the levels of •NO by down-regulating the activity and expression of all isoforms of NOS [72]. Both studies focus on •NO to explore the underlying mechanism responsible for the protective effect of red/NIR light in models of ischemia, yet, in these examples, the effects on •NO were found to be conflicting. The respective increase and decrease in •NO levels seen upon red/NIR light irradiation in the discussed models, was dependent on the respective activation and inhibition of NOS. Although theories have been proposed to explain the increase in NOS activity in response to red/NIR light irradiation, how red/NIR light inhibits NOS activity is less clear. Since the expression of the three isoforms of NOS showed a similar trend to the specific activities of the NOS enzymes in response to red/NIR light, red/NIR light must be somehow suppressing the expression of NOS, but the mechanism responsible for this effect is unknown[72].

This observed dual effect of red/NIR light on intracellular •NO levels has great relevance in the field of neurodegenerative conditions. •NO at physiological amounts confers neuroprotection, however, if produced in excess, •NO has neurotoxic effects [73]. In the *in vivo* model of cerebral ischemia mentioned above the light was administered immediately

after middle cerebral artery occlusion but the levels of NOS activity were not measured until 4 days post injury, the time at which the NOS levels peaked before returning to pre-injury levels. In the *in vitro* model of cardiac ischemia the light was also administered immediately after hypoxia but the •NO levels were measured after just 2 hours of reoxygenation. It is possible, therefore, that red/NIR light triggers an initial increase in •NO levels, sufficient to reduce ROS production and bring about the observed cytoprotective effects. Furthermore, as the cell is then in a state of elevated •NO levels and reduced ROS levels this may be sufficient to switch off the endogenous trigger that induces the increased expression of NOS and the subsequent delayed surge in •NO levels, which only contribute to the toxic effects at that late stage of ischemia. However, as shown in Figure 2, low levels of •NO are produced during the early stages of ischemia to induce neuroprotective effects in the absence of red/NIR light, yet an increase in •NO is responsible for the neuroprotective effects achieved with red/NIR light in the *in vitro* model of ischemia. Therefore, how does the •NO produced by red/NIR light provide further neuroprotective effects in the early stages of ischemia? Also unanswered is how the increase in •NO by red/NIR light and the associated reduction in ROS production would downregulate the delayed surge in NO production in the later stages of ischemia when the •NO produced in the absence of red/NIR light fails to do so.

There is much evidence to show that •NO acts as a neuroprotective agent through its various cellular effects. One such effect is the induction of the signaling molecule cyclic guanosine 3',5'-monophosphate (cGMP), a molecule with a key role in vasodilation and mitochondrial biogenesis [74]. Mitochondrial biogenesis has been found to occur in response to red/NIR light irradiation [75]. Diseases with a mitochondrial origin such as Leber's hereditary optic

neuropathy, retinitis pigmentosa and autosomal dominant optic atrophy would benefit from the associated increase in mitochondrial biogenesis, as a way of supporting neuronal survival.

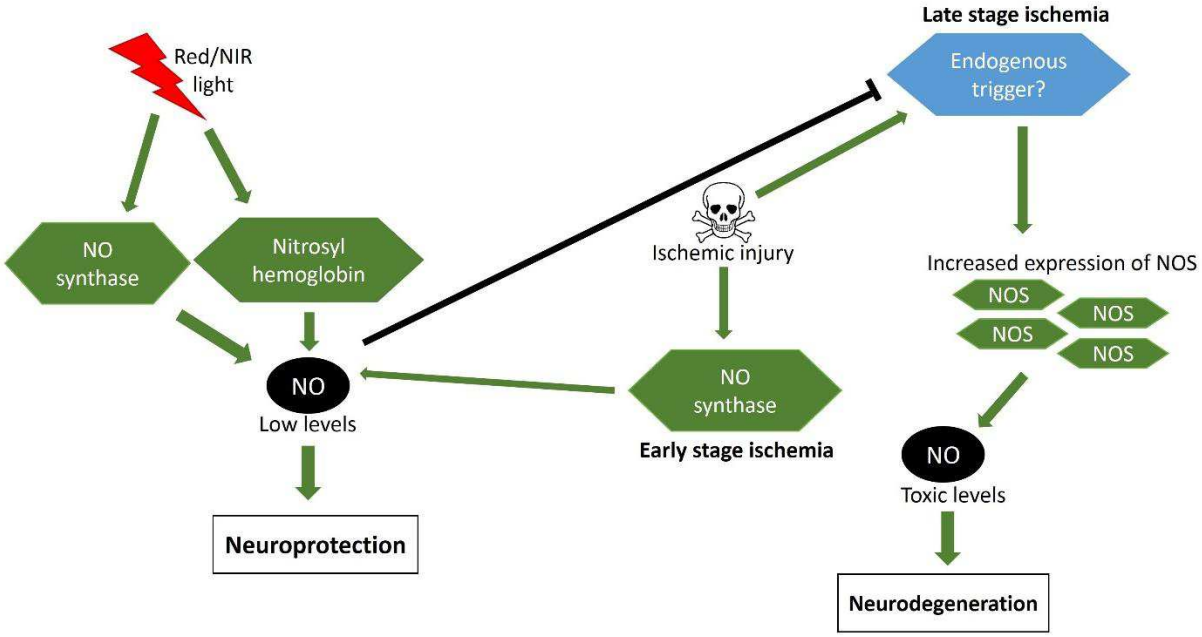

Figure 2: A proposed explanation for the observed dual effect of red/NIR light on NO in models of ischemia.

5.5 The effects of reactive nitrogen species on mitochondrial function

There is much evidence to show that •NO, at higher doses, is toxic to neurons; however, this NO-mediated toxicity is not produced by •NO alone, but by the formation of reactive nitrogen species (RNS) [76]. As a by-product of mitochondrial respiration the superoxide radical anion ($O_2^{\bullet -}$) is formed when electrons from Complex I or III are transferred to oxygen molecules instead of their respective substrates: ubiquinone and cytochrome c [77]. If the amount of superoxide produced in the cell increases to a level that would exceeds the antioxidant capacity of the cell, oxidative stress will result. In that pro-oxidant state, the production of •NO can cause the generation of additional cytotoxic compounds. The reaction of NO with the superoxide radical anion ($O_2^{\bullet -}$) leads to the generation of the powerful oxidant peroxynitrite ($ONOO^-$), which causes detrimental effects in the cell, through its interactions with lipids, proteins and DNA [76].

Nitric oxide, superoxide and peroxynitrite are often generated in excess during inflammatory and pathological conditions, contributing to the associated toxic effects [78]. Peroxynitrite can induce mitochondrial dysfunction and cell death in neurons by its ability to inhibit many mitochondrial proteins including complex I, II and IV, and ATP synthase. It can also increase mitochondrial proton permeability, an effect which may be caused by lipid peroxidation [79]. Impairment of normal mitochondrial function causes depletion of ATP and generation of free radicals, causing cellular dysfunction and further oxidative stress. Peroxynitrite has been found to be involved in the pathogenesis of many neurodegenerative disorders [80]. Since the formation of peroxynitrite depends on the availability of $^{\bullet}NO$, inhibition of nitric oxide synthase activity, as was found to occur in cerebral ischemic-rats irradiated with red light, may reduce the amount of peroxynitrite produced and the associated deleterious effects in neurodegenerative disorders [72].

6.0 Red/NIR light in the mitochondrial signaling pathway

Causing further controversy in the efforts to uncover the underlying molecular mechanism of red/NIR light therapy, is the effect that it has on ROS and RNS production. It is also uncertain whether an increase or a decrease in these molecules, in the cell, in response to red/NIR light exposure would be the most therapeutically beneficial. In some physiological situations red light mediates its therapeutic effects by increasing levels of free radicals but in other circumstances by reducing the levels of free radicals. Consequently, a further look at the molecular effects of free radicals in neurodegeneration is required to uncover the underlying mechanism of red/NIR therapy.

6.1 The role of ROS and RNS in neurodegeneration

Postmortem analysis of the brains of patients with various neurodegenerative diseases shows an increase in ROS and RNS in the affected brain regions [81, 82]. It is known that these reactive species can cause oxidative and nitrative damage to cellular components thereby having toxic effects on the cell [83]. It may be deduced from this association that the increase in ROS and RNS is contributing to the cellular death and that an antioxidant may be beneficial in such conditions. Since red/NIR light has been found to be protective in models of such conditions it could, therefore, be possible that the therapeutic benefits of red/NIR light could be due, in part, to an antioxidant effect. One possible mechanism of providing the antioxidant protection is the photodissociation of $^{\bullet}NO$, which at low concentrations can exert an antioxidant effect [84-87].

Because red/NIR light has been found to increase the activity of the electron transport chain, and ROS/RNS production is a byproduct of such activity, sometimes it is presumed that red/NIR light increases ROS production. It can be argued, however, that by removing NO-mediated inhibition of COX, the electron flow is restored and therefore the likelihood of donating an electron from Complex I or III to oxygen (which results in generation of superoxide radical anion) is reduced [77]. Thus by enabling the electron flow in the electron transport chain, the risk of formation of superoxide is decreased.

Investigation into the effects of red/NIR light on ROS production by various groups provides inconsistent results as some found a reduction in ROS upon irradiation while others found that ROS was, in fact, upregulated [83]. Regardless of the effect of red/NIR light on ROS levels, it is unclear whether an antioxidant or pro-oxidant effect would be most beneficial when employing red/NIR light as a neuroprotective agent. Regulated ROS production could trigger signaling pathways involved in cell protection, but unregulated ROS production could result

in cellular damage and cell death [88]. In addition, when ROS levels are too low, this also has detrimental effects for the cell. The concept that lower, non-toxic levels of ROS are essential for promoting cell survival by inducing an adaptive responses is called mitochondrial hormesis or mitohormesis [89]. Exposure to red/NIR light may trigger a transient increase in ROS levels, sufficient to induce this adaptive response and provide neuroprotective effects. This explanation seems plausible in situations where light is administered as a pre-treatment as the cells could employ this adaptive response to protect against a subsequent injury-induced increase in ROS levels. However, if the cell is already in a state of elevated ROS levels, as in many pathological situations, it is unclear as to how an additional increase in ROS by red/NIR light would provide neuroprotective effects. From the current literature, it is clear that the mechanism for the effect of red/NIR light on ROS production, in addition to the cellular mechanisms responsible for balancing the ROS levels required for maintaining optimal mitochondrial and cellular function are not fully understood. However, since maintaining redox homeostasis is paramount for the optimal functioning of the cell and neuronal survival, we suggest that the exposure to red/NIR light may restore redox homeostasis in pathological conditions where it is perturbed. Therefore, in cells with elevated ROS levels, red/NIR light may cause mild cellular stress by an unknown mechanism that may induce an adaptive response, which includes the upregulation of genes with a role in redox homeostasis. We suggest that the observed increase in $^{\bullet}$NO upon red/NIR light irradiation, by the various mechanisms discussed in section 5, may facilitate the production of peroxynitrite through the reaction of $^{\bullet}$NO with ROS. The elevated RNS levels may induce an adaptive response by triggering a different signaling pathway to that which is triggered by ROS.

6.2 ROS/RNS as signaling intermediates

The upregulation of ROS and RNS, such as peroxynitrite, are thought to promote cell viability and increase proliferation owing to their ability to function as signal intermediates (Figure 3). ROS, which is produced by the ETC complexes as a byproduct of cellular respiration permits communication from the mitochondria to the rest of the cell. This mitochondrial signal transduction can activate various signaling pathways resulting in the expression of a plethora of genes including those involved either directly or indirectly in the suppression of apoptosis, cell survival or cell proliferation. Curiously, among the genes affected by red/NIR light irradiation, were those with roles in anti-oxidation[83].

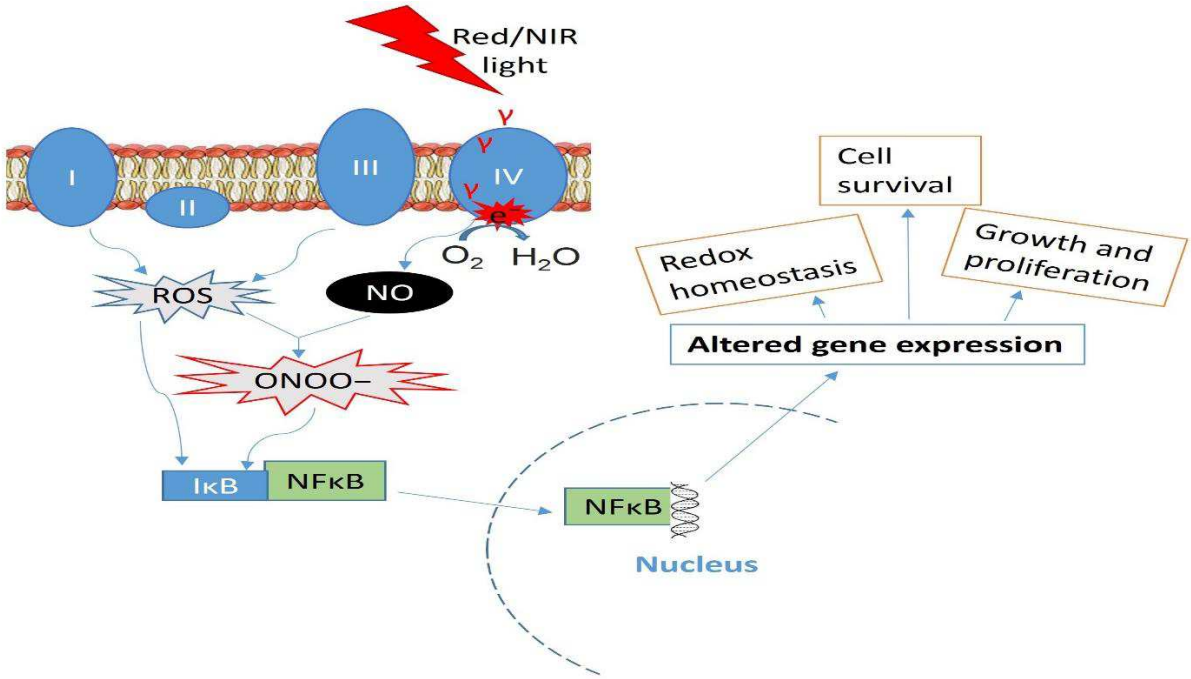

Figure 3: The upregulation of ROS or RNS by red/NIR light triggers the translocation of the transcription factor NFκB to the nucleus, enabling NFκB to alter gene expression.

6.3 The mitochondrial signaling pathway

ROS is thought to mediate its protective effect via the activation of the redox sensitive transcription factor nuclear factor (NF)-κB which is proposed to be sensitive to an increase in ROS generation. In support of this theory it was found that an increase in ROS production by mitochondrial inhibitors brought about a concurrent increase in NFκB, while the exposure to

antioxidants reduced the NFκB activation [90]. NFκB can both induce and repress gene expression by binding to κB elements in the promoter and enhancer regions of the gene. This transcription factor has been shown to induce the expression of numerous genes with functions in cell survival, the stress response and inflammation [90]. When superoxides are produced in the mitochondria they are metabolized to H_20_2 which is thought to activate NF-κB by triggering the dissociation of inhibitor of κB kinase from NFκB. This allows NF-κB to translocate to the nucleus where it can alter gene expression (Figure 3) [91].

The redox sensitive signaling pathways however differ with cell type making it difficult to predict the outcome of red/NIR light therapy if attempting to find a general cellular mechanism [91]. It is no surprise that the results obtained appear to be inconsistent with varying cell type. This may provide some explanation for the differential results that have been observed with the use of red/NIR light therapy in different cell types. For example, irradiation of traumatized muscle tissue with red light therapy reduced the levels of ROS and NFκB activation and the associated increase in the expression of proinflammatory genes [92]. On the other hand NIR light has been shown to induce ROS production and NFκB activation in murine embryonic fibroblasts [90].

In a similar fashion, peroxynitrite production triggers this stress response, causing the up or downregulation of cell signaling cascades in a cell dependent manner [93]. Peroxynitrite signaling is mediated by tyrosine nitration of proteins, particularly those involved in phosphotyrosine-dependent signaling. The oxidant is also involved in signaling via mitogen activated protein kinase, protein kinase B and C, NFκB and the insulin receptor. Of note, these pathways which converge on the upregulation of mediators of cell survival, growth and proliferation are also activated by other stress stimuli such as ROS.

In support of the theory that red/NIR light causes upregulation in the expression of genes associated with cell survival, is the increased neuroprotective effects observed when red/NIR light is administered before the induced injury in models of neurodegeneration, compared to when administered after injury [94]. This suggests that red/NIR light could be mediating its protective effects by instigating the production of ROS/RNS at levels sufficient to cause the upregulation of stress response genes, with negligible damage to the irradiated tissue. If the same tissue was subsequently exposed to a toxic agent, the cell would be equipped with the appropriate defense mechanisms to cope with such an insult. The destructive effects to the tissue in such an instance would be remarkably less, such as is seen in various pretreatment models [31, 38, 95]. This knowledge could be exploited in neurodegenerative diseases with a pre-symptomatic phase. The observation demonstrates that red/NIR light therapy would be most effective as a preventative therapeutic treatment, administered before the onset of clinical symptoms and irreversible damage. With respect to retinal degenerative disease this preventative treatment approach would be particularly relevant in inherited optic neuropathies such as Leber's hereditary optic neuropathy. Individuals with a LHON-causing mtDNA pathogenic variant could be monitored so that treatment with red/NIR light could begin upon detection of pre-symptomatic abnormalities as a pre-treatment for the clinical symptoms. In the event of a more acute neurodegenerative condition such as TBI, exploiting the enhanced therapeutic effects of red/NIR light observed when used as a pretreatment would not be feasible. Therefore, the neuroprotective potential of red/NIR light therapy, as seen in the pre-treated animal models, may be somewhat limited in a real life clinical setting.

7.0 The effects of red/NIR light on the anti-inflammatory response

Peroxynitrite and ROS are also produced by macrophages during inflammation, which are responsible for the cytotoxic effects [96]. As mentioned above, red/NIR light has shown the ability to reduce the presence of these reactive species in irradiated tissues, thereby attenuating some of the cytotoxic effects of the inflammatory response. Furthermore, modulation of the immune response, itself, was seen in many studies with red/NIR light treatment [39, 40, 66]. Yet, how red/NIR light mediates this anti-inflammatory effect is poorly understood.

Mitochondria play a major role in the activation of the inflammasome, a molecular platform that activates proapoptotic proteins, cytokines and other mediators of inflammation upon detection of infectious agents or cellular damage [97]. ROS can similarly activate the inflammasome. The production of mitochondrially-derived ROS is known to increase when mitochondria are dysfunctional. Decreased electron flux through the electron transport chain reduces the ability of COX to fully reduce oxygen to water, thereby increasing the formation of ROS. The action of red/NIR light in improving the functions of the mitochondrial electron transport chain could reduce the amount of ROS generated, which may reduce the activation of the inflammasome. Alternatively, the mitochondrial signal transduction triggered by an increase in ROS/RNS as discussed above, could also upregulate the expression of anti-inflammatory or antioxidants proteins, to either directly or indirectly dampen the inflammatory response. However, it has been found that low level laser irradiation (LLLI) can reduce the gene expression of anti-inflammatory cytokines as well as pro-inflammatory cytokines [66]. In this particular situation LPS was used to elicit an inflammatory like phenotype, triggering the expression of pro-inflammatory cytokines in addition to the anti-

inflammatory cytokine, IL-10. The anti-inflammatory cytokine is also triggered during the inflammatory response to limit the host immune response, thereby minimising the damage caused to the affected tissue during the inflammatory response [98]. The findings demonstrate that LLLI is able to dampen the entire inflammatory response as oppose to specifically downregulating proinflammatory cytokines, suggesting that red/NIR light may be acting further upstream, influencing factors governing the inflammatory response. Also, there appears to be a correlation between the inflammatory level in the tissue and the extent of the inhibition of the inflammatory response [66]. It was found that LLLI had little effect on the inflammatory response at low levels of inflammation, but, produced a potent anti-inflammatory effect at high levels of inflammation[66]. This shows an ability of red/NIR light to restore homeostasis in the tissue. In fact, ROS is produced by the microglia itself, and plays an important role in the induction of pro-inflammatory genes [99]. Therefore, restoring the redox balance in microglia may facilitate the transition of the microglia from an activated to a resting state.

It has been suggested that the mitochondrial dysfunction seen in many neurodegenerative diseases not only affects neurons, but also the microglia [100]. Experimental findings have shown that complex I inhibition in microglia inhibited the IL-4 mediated reduction in pro-inflammatory cytokines and the secretion of the neuroprotective insulin-like growth factor-1. The inflammatory response triggered by pathogens or damaged neurons functions to protect the neural tissue, but if this response is not attenuated the response would switch from a protective one to a deleterious one [101]. When activated, microglia secrete anti-inflammatory cytokines in addition to pro-inflammatory cytokines to control the inflammatory response, preventing unnecessary damage to neural tissue. Electron transport deficiencies in microglia, appear to perturb the anti-inflammatory arm of the immune

response [100]. Since red/NIR light has been shown to improve the function of the electron transport chain in neurons, it can be suggested that a similar effect would be seen in microglia with mitochondrial dysfunction.

8.0 Conclusion

Wavelengths of light in the red to NIR region of the electromagnetic spectrum are optimal for the photostimulation of mitochondria as a treatment for neurodegeneration in the retina. The photon energy of red/NIR light appears to correspond to the absorption maxima of chromophores present in complex IV of the electron transport chain, triggering biostimulatory effects. Additionally, red and NIR light have an increased ability to penetrate tissue and are associated with significantly less safety concerns than light of shorter wavelengths. The potential for this treatment to be a success in patients is high. This is supported, firstly, by the potent neuroprotective effects demonstrated in various models of retinal damage. Secondly, data from human studies, albeit limited, shows the ability of red and NIR light to improve visual function. Furthermore, no adverse effects were observed in these previously published studies. The findings suggest that red/NIR light therapy is safe and effective as a non-invasive treatment for retinal neurodegeneration.

Much evidence has been gathered in efforts to elucidate the underlying mechanism responsible for the neuroprotective effects of red/NIR light, but the mechanism remains unclear. Experimental findings suggest that there are many possible molecular and cellular effects of red/NIR light, which could all contribute to the observed neuroprotective effects, when explored separately, but when taken collectively some effects appear to contradict

others. Improved neuronal mitochondrial function, increased blood flow to neural tissue, an increase in •NO levels, slowing down of the reactivation of the mitochondrial electron transport chain during reperfusion, increased mitochondrial biogenesis, a reduction in ROS levels, a reduction in •NO levels, upregulation of cell survival mediators and restoration of normal microglial function have all been proposed as potential underlying mechanisms of red/NIR light. The increased ability of the neuron to survive during challenging conditions may be due to the resulting net effect of a number of red/NIR light induced molecular and cellular events. The effects that prevail in a particular cell/tissue may depend on the state of the cell/tissue at the time of irradiation and the subsequent challenges to which the cell is exposed to.

Aside from the fact that the mechanism of action is unclear, the lack of consensus surrounding the optimal parameters of red/NIR light, such as irradiance, radiant exposure and wavelength, for different conditions is a cause for concern. Although beneficial effects have been found using several different parameter combinations, efforts to find the best possible effect with the least possible risk are, for the most part, not done. This unconventional experimental approach has been established based on the assumption that red/NIR light therapy is safe, but this approach would not be tolerated for any other therapeutic interventions. In order for advances to be made in this field a much more detailed collection of experiments needs to be done to establish the optimal parameters for each condition with a potential for treatment with red/NIR light.

Acknowledgements

This research was funded by Fight for Sight UK.

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