Inhibiting Mitochondrial Cytochrome c Oxidase Downregulates Gene Transcription After Traumatic Brain Injury in Drosophila

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Traumatic brain injuries (TBIs) caused by a sudden impact to the head alter behavior and impair physical and cognitive function. Besides the severity, type and area of the brain affected, the outcome of TBI is also influenced by the patient’s biological sex. Previous studies reporting mitochondrial dysfunction mainly focused on exponential reactive oxygen species (ROS) generation, increased mitochondrial membrane potential, and altered mitochondrial dynamics as a key player in the outcome to brain injury. In this study, we evaluated the effect of a near-infrared (NIR) light exposure on gene expression in a Drosophila TBI model. NIR interacts with cytochrome c oxidase (COX) of the electron transport chain to reduce mitochondrial membrane potential hyperpolarization, attenuate ROS generation, and apoptosis. We subjected w¹¹¹8 male and female flies to TBI using a high-impact trauma (HIT) device and subsequently exposed the isolated fly brains to a COX-inhibitory wavelength of 750 nm for 2 hours (hr). Genome-wide 3′-mRNA-sequencing of fly brains revealed that injured w¹¹¹8 females exhibit greater changes in transcription compared to males at 1, 2, and 4 hours (hr) after TBI. Inhibiting COX by exposure to NIR downregulates gene expression in injured females but has minimal effect in injured males. Our results suggest that mitochondrial COX modulation with NIR alters gene expression in Drosophila following TBI and the response to injury and NIR exposure varies by biological sex.

Keywords: traumatic brain injury, sex-differences, gene expression, near-infrared light, mitochondria

INTRODUCTION

Traumatic brain injury (TBI) results from a violent blow or jolt to the head causing a wide range of physical and psychological effects (Finnie and Blumbergs, 2002; Ciuffreda et al., 2016; Dale Horne, 2018). There are estimated to be about 2.5 million TBI cases every year that require emergency department visits or result in death (Taylor et al., 2017). The most common causes of TBI include sports, motor vehicle accidents, falls, and violence (Hiebert et al., 2015) with the symptoms of...
the injury depending on the type and area of the brain affected (Morries et al., 2015). TBI is a heterogeneous disorder consisting of primary damage resulting from direct mechanical forces followed by secondary damage centered on mitochondrial dysfunction leading to neuronal death (Cheng et al., 2012).

Mitochondria are membrane-bound organelles distributed throughout the brain cytosol and responsible for energy production using the electron transport chain (ETC) (Kim et al., 2017). The ETC is a series of protein complexes made of NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinone, bc1-complex (complex III), cytochrome c (Cyt c), and cytochrome c oxidase (COX; complex IV) (Hüttemann et al., 2008). Complexes I, III, and IV pump protons across the inner mitochondrial membrane to generate the mitochondrial membrane potential ($\Delta\Psi_m$) (Hüttemann et al., 2008) which is utilized by ATP synthase (complex V) to synthesize ATP from ADP and phosphate (Hüttemann et al., 2008; Sanderson et al., 2013). An optimal physiological $\Delta\Psi_m$ between 120–140 mV allows efficient ATP production and minimal reactive oxygen species (ROS) generation from complexes I and III (Sanderson et al., 2013). Disruption of $\Delta\Psi_m$ is considered as an indicator of mitochondrial damage causing decreased respiration, decreased ATP production, increased ROS generation and induction of apoptosis by efflux of macromolecules like Cyt c and caspase-3 cascade activation (Singh et al., 2006; Watts, 2016).

Mitochondrial impairment has been shown to play a key role in several neurodegenerative disorders including Alzheimer’s disease (AD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), ischemic brain injury, and stroke (Reddy, 2009; Sanderson et al., 2013, 2018; Strubakos et al., 2020). Following TBI using a controlled cortical impact model, mitochondrial dysfunction and calcium perturbation was observed in male rats (Xiong et al., 1997). A significant decrease in mitochondrial oxidative phosphorylation and calcium buffering capacity was observed in male mice 3 hr post-TBI (Singh et al., 2006). These mice also show structural damage to isolated mitochondria and an increase in oxidative stress (Singh et al., 2006). A Drosophila model of TBI also showed significant decrease in ATP production which was observed 24 hr after injury (Sen et al., 2017). It is evident that production of ROS, hyperpolarization of $\Delta\Psi_m$ beyond the physiological range, and caspase activation induce mitochondrial damage following TBI (Lifshitz et al., 2004; Kim et al., 2017). Additionally, biological sex has been shown to influence mitochondrial function (Demarest and McCarthy, 2015; Ventura-Clapier et al., 2017) and the outcome to TBI (Leitgeb et al., 2011; Gupte et al., 2019) but the role of sex-differences in mitochondrial function in response to TBI has not been studied. In a previous study, we observed sex differences in mitochondrial gene transcription and oxidation in Drosophila subjected to trauma using the high-impact trauma device (HIT-device) (Katzenberger et al., 2015; Shah et al., 2020). Thus, efforts to attenuate mitochondrial damage have been increasingly studied in TBI, as mitochondrial maintenance could possibly preserve brain function (Watts, 2016). However, most pharmacological approaches to circumvent mitochondrial damage suffer from a critical issue: the ability of drugs to cross the blood-brain barrier and attain effective concentrations in the injured tissue (Sanderson et al., 2013).

In the current study, we explore a non-pharmacological approach of preserving mitochondrial damage in TBI using near-infrared (NIR) light. Photobiomodulation (PBM) or the use of NIR has been studied as a therapeutic alternative in animal and human TBI to protect tissue from dying, increase mitochondrial function, improve blood flow, stimulate healing and tissue oxygenation (Naeser et al., 2011; Hamblin, 2018). PBM involves shining red or NIR light onto the head where the light penetrates into the brain and is absorbed by specific chromophores (Hamblin, 2018). NIR has been shown to interact with cytochrome c oxidase (COX), the terminal complex of the ETC (Sanderson et al., 2018; Strubakos et al., 2020). COX contains several chromophores including two copper centers that act as photoacceptors for NIR and absorb light in the range of 700–1000 nm (Karu and Afanas’eva, 1995). Many studies in animal models of TBI using NIR from light-emitting diodes (LEDs) have found improved neurological and cognitive function and reduced inflammation and cell death in brain post-exposure (Naeser et al., 2011; Hamblin, 2018). Drosophila exposed to 670 nm NIR showed increased ATP production and reduced inflammation with age (Begum et al., 2015) whereas irradiating Drosophila pink1 mutants at 808 nm rescued mitochondrial defects (Vos et al., 2013). Recent studies in animal models of stroke and ischemia/reperfusion injury have found that NIR exposure at 750 nm inhibits COX activity (Sanderson et al., 2018), reduces mitochondrial respiratory and $\Delta\Psi_m$, and prevents ROS generation (Sanderson et al., 2018; Strubakos et al., 2020). In line with this evidence, we sought to investigate the effect of modulating mitochondrial COX activity with 750 nm NIR exposure at 750 nm in both sexes of a Drosophila model of mild traumatic brain injury (mTBI). Drosophila brain is enclosed in an exoskeleton consisting of chitin which strongly absorbs NIR and although previous studies found other wavelengths to penetrate fly and mammalian tissues effectively, we found 750 nm NIR penetration through the exoskeleton ineffective. Thus, we isolated fly brains from $w^{1118}$ male and female flies inflicted with trauma using the HIT-device at control, 1, 2, and 4 hr post-TBI and exposed them to NIR at 750 nm for 2 hr. Post-treatment, we assessed change in gene expression separated by sex and found an overall downregulation in transcription in $w^{1118}$ females in response to NIR exposure. As compared to $w^{1118}$ females, we saw fewer changes in $w^{1118}$ males in response to TBI and NIR exposure. These data suggest that outcome to TBI differs between sexes in Drosophila exposed to NIR, which could be a result of sex-differences in mitochondrial function. This is the first study to investigate the response of mitochondrial COX inhibition using NIR light at a single wavelength in both sexes post-TBI within 4 hr.

**MATERIALS AND METHODS**

**Fly Stocks and Crosses**

$w^{1118}$ stock were obtained from the Bloomington Drosophila Stock Center and repo-GFP stock was a gift from Dr. Laura...
Buttitta (University of Michigan). Fly stocks were stored at 25°C at constant humidity and fed with standard sugar/yeast/agar medium. All assays were performed on adult mated flies (10–14 days old).

**Traumatic Brain Injury**

Both sexes of *w^{1118}* and repo-GFP flies were subjected to a single strike full body trauma using a modified HIT device with the impact arm constrained to a 45° angle (Katzenberger et al., 2013; Sen et al., 2017). This device inflicts mild TBI to the flies. No more than 50 flies were placed in a plastic vial before being confined to the bottom quarter of the vial by a stationary cotton ball. Upon deflection and release of the spring, the vial rapidly contacts a styrofoam pad delivering a mechanical force to the flies as they contact the vial wall and rebound causing closed head trauma.

**NIR Light Emitting Diodes and Exposure**

Diodes (epoxy lens type infrared illuminator LED750-66-60, Roithner Lasertechnik, Vienna, Austria) were mounted on heat sinks (black aluminum, 47 × 20 for LED array 60 chips) together with a small fan (EC3010M05X; Evercool, New Taipei City, Taiwan) operated in reverse mode to avoid any heating of the condition and placed in a 35 mm petri dish containing cold Schneider’s media. The petri dish was then placed under the 750 nm diode within 2 cm distance and exposed to NIR for 4% PFA (paraformaldehyde). Fixed brains were mounted using 1X PBS (phosphate-buffered saline) and fixed for 5 min with 4% PFA (paraformaldehyde). Fixed brains were mounted using Prolong gold antifade mounting media to visualize changes in GFP expression using a confocal microscope (Zeiss LSM 800) at the Microscopy, Imaging and Cytometry Resources Core at Wayne State University, School of Medicine. Average fluorescence intensity for all brains in each condition was calculated using ImageJ (Schneider et al., 2012) in a blinded study. The fluorescence intensity for all brains in each condition was indicated in black text.

**RNA Isolation**

Total RNA was extracted from *w^{1118}* single fly brains using QIAzol® lysis reagent and Direct-zol™ RNA MicroPrep kit (Zymo Research) following manufacturer’s instructions.

**3′-mRNA Expression Analysis**

Expression analysis was conducted in collaboration with the Wayne State University Genome Sciences Core. Three biological replicates were used for each condition (Shah et al., 2020).

QuantSeq 3′-mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen) was used to generate libraries of sequences close to the 3′ end of polyadenylated RNA from 15 ng of total RNA isolated from single fly brain in 5 μl of nuclease-free water following the low-input protocol. Library aliquots were assessed for overall quality using the ScreenTape for the Agilent 2200 TapeStation and quantified using Qubit® 1X dsDNA HS Assay kit (Invitrogen). Barcoded libraries were normalized to 2 nM before sequencing at 300 pM on one lane of a NovaSeq 6000 SP flow cell. After de-multiplexing with Illumina’s CASAVA 1.8.2 software, the 50 bp reads were aligned to the *Drosophila* genome (Build dm3) with STAR_2.4 (Dobin et al., 2013) and tabulated for each gene region (Anders et al., 2015). Differential gene expression analysis was used to compare transcriptome changes between conditions using edgeR v.3.22.3 (Robinson et al., 2010) and transcripts were defined as significantly differentially expressed at absolute log2 fold change (|log2 FC| > 1) with an false discovery rate (FDR) < 0.05. Significant gene expression changes were submitted for gene ontology (GO) analyses using RDAVID (Fresno and Fernandez, 2013) for the following categories: GOSTERM_BP_ALL, GOSTERM_MF_ALL, UP_KEYWORDS, GOSTERM_BP_DIRECT, and GOSTERM_MF_DIRECT.

**RESULTS**

**Gene Transcription in TBI Inflicted Flies Is Downregulated After Exposure to NIR**

Several studies in experimental models of brain injury have shown structural and functional damage to mitochondria being an early event in TBI which leads to activation of cell death pathways (Fischer et al., 2016). In a previous study, we demonstrated an upregulation in gene transcription involved with immune response, cytoskeleton organization and apoptosis after injury in *Drosophila* (Shah et al., 2020). Moreover, we have also shown sex-differences in mitochondrial stress and...
gene transcription in response to TBI (Shah et al., 2020). To identify gene expression changes after attenuation of mitochondria response by COX-inhibitory NIR exposure, we generated 3′-mRNA-Seq libraries from isolated w¹¹¹⁸ male and female fly brains at control and 1-, 2-, and 4-hr post-injury (single strike) time points. Differential gene expression analysis shows significant changes in both sexes after TBI and NIR exposure (Figure 1) with females (Figures 1A–C) exhibiting more transcriptional changes than males (Figures 1D–F). Gene expression changes in response to TBI were less pronounced in both sexes exposed to NIR as compared to flies not treated with NIR (Shah et al., 2020) at all 3 time-points (\(| \log FC| > 2; p\text{-value} < 0.05\)).

Significant genes identified from 3′-mRNA-Seq were classified for their biological functions using RDAVID (Huang et al., 2009; Fresno and Fernandez, 2013) and several GO categories were found to be changed in both sexes exposed to NIR as compared to flies not treated with NIR (Shah et al., 2020) at all 3 time-points (\(| \log FC| > 2; p\text{-value} < 0.05\)). A subset of GO categories significantly altered in both sexes are indicated in Figure 2 wherein it is observed that females have more processes affected than males at all three time-points. In w¹¹¹⁸ females exposed to NIR, the highest number of significant categories (FDR < 0.05) were altered 2 hr after injury (41 GO terms) (Figure 2A) and we also observed significant changes in GO terms for “Humoral immune response,” “Defense response,” “Response to stress,” and “Detection of light stimulus” (Figure 2A, Table 1, and Supplementary Data 1). In our previous study, we observed GO processes including “Immune response,” “Mitochondrial organization,” and “Programmed cell death” significantly altered after TBI in w¹¹¹⁸ females untreated with NIR (Shah et al., 2020). Here, we found no change in any of these processes in females treated with NIR after TBI indicating a positive effect of the COX-inhibitory NIR exposure that could help minimize aberrant gene transcription after brain injury. However, for w¹¹¹⁸ males exposed to NIR, there were fewer significant changes observed than for females, and gene expression related to “nervous system development” and “neurogenesis” was altered after NIR exposure (Figure 2B, Table 2, and Supplementary Data 1). Previous studies involving the use of PBM for TBI patients have also reported downregulation of immune response and upregulation of neurogenesis as an effect of NIR treatment (Hennessy and Hamblin, 2017; Santos et al., 2018). There was no overlap in biological processes affected across all three TBI time-points shared by both sexes. Additionally, we also exposed control fly brains to NIR for both sexes and found that female controls had more significant GO enrichment (18 categories) as compared to male controls (7 categories) (Supplementary Data 1). For female controls, GO terms like “Neurogenesis,” “Oxidation-Reduction Process,” and “Intracellular Transport” were altered whereas for male controls “Nuclear Transport” and “Nucleic Acid Metabolic
Process” were changed in response to NIR exposure. Neither sexes showed alteration in immune response or mitochondrial organization (Supplementary Data 1).

These data suggest that while NIR exposure could be neuroprotective in brain injury, the effect of this exposure varies by biological sex in Drosophila. Male and female flies show differences in gene transcription after injury and such differences are also prevalent after exposure to COX-inhibitory NIR. Overall, both sexes exhibit fewer transcriptional changes in response to TBI after exposure to COX-inhibitory NIR.

NIR Treatment Downregulates Immune Gene Transcription in Injured Flies

Brain trauma triggers immune system activation, which helps protect tissue against damage (Plesnila, 2016), but long-term inflammation may contribute to neurological deterioration and cognitive decline (Henry et al., 2020). TBI induced neuroinflammation and pathology have also been linked to an increased risk of developing neurodegenerative disorders like AD, PD, and chronic traumatic encephalopathy (CTE) (McKee and Lukens, 2016). We have previously reported an upregulation of immune gene expression in female flies within 4 hr of injury (Shah et al., 2020) and there have been several reports of an upregulated immune response in male flies days, weeks or months after injury (Katzenberger et al., 2016; van Alphen et al., 2018; Swanson et al., 2020). In animal models of TBI, exposure to NIR light has been shown to downregulate pro-inflammatory cytokines and upregulate anti-inflammatory cytokines (Moreira et al., 2009; Khuman et al., 2012; Quirk et al., 2012; Zhang et al., 2014). Based on this evidence, we aimed to explore the effect of modulating mitochondrial COX using NIR exposure at 750 nm on immune gene expression in male and female fly brains inflicted with TBI.

The Drosophila immune system is regulated by the Toll, Immunodeficiency (Imd) and Janus Kinase protein and the Signal Transducer and Activator of Transcription (JAK-STAT) pathways (West and Silverman, 2017). We looked at transcriptional changes in genes involved with these pathways and observed that w1118 flies exposed to NIR post-injury exhibit fewer alterations to immune genes than those untreated with NIR (Figure 3). In females, we have previously seen a significant upregulation of genes encoding anti-fungal peptide Drs (Drosomycin) and anti-bacterial peptides Diptericin (Dpta, DptB), Cecropin (Ceca1, CecA2, CecB, and CecC), and Attacin (Att, AttB, and AttC) (Figure 3A) after injury. w1118 females exposed to NIR show an upregulation in transcript levels of CecB, AttC, DptB, CecA1, and Dro in the immediate time frame after injury and are mostly unchanged by 4 hr. The Drosophila NF-kB transcription factor Rel (Relish) (Hetru and Hoffmann, 2009), a downstream component of the immune deficiency pathway, was significantly upregulated in injured females not exposed to NIR but unchanged after NIR treatment. The NF-κB pathway functions in the host defense of Drosophila to control the expression of genes encoding immune-responsive peptides and proteins (Hetru and Hoffmann, 2009). Expression of Mtk (Metchnikowin), an antimicrobial peptide, was seen to be significantly increased in females after TBI but is unchanged in injured females exposed to NIR. Loss of Mtk has been shown to reduce mortality and behavioral deficits and improve lifespan in TBI exposed flies (Swanson et al., 2020).

w1118 males show no transcriptional change in response to injury with or without exposure to NIR for immune response (Figure 3B). We have observed significant upregulation in CecB in injured males exposed to NIR 4-hr after injury. Although not significantly induced after injury, we have seen consistently high transcription of AttA and DptB in injured males not exposed to NIR.

Trauma-induced changes in glial gene expression is a conserved feature of mammalian (Allen and Barres, 2009; Chung et al., 2015) and Drosophila models (van Alphen et al., 2018; Swanson et al., 2020). In flies, glia are able to perform immune related functions and dysregulation of immune signaling in glial cells has been implicated in neurodegeneration (Petersen et al., 2012, 2013). In this study, we utilized the Gal4/UAS system to drive GFP expression in glia using the glial marker Repo driving...
Gal4 and the reporter gene UAS-GFP. Thus, in addition to gene transcription, we looked at change in GFP reporter expression in glial cells after injury and exposure to NIR in both sexes. We have observed differences in GFP expression and repo transcription in both sexes post-TBI (Figure 4). In females (Figures 4A, B) and males (Figures 4D, E), we saw a significant increase in GFP expression 1, 4, and 24 hr after injury but no change at 2 hr compared to control. At all three time-points, the observed

| Rank | GOBPID | Term | Fold enrichment | FDR   |
|------|--------|------|----------------|-------|
| A: Selected GO terms differentially regulated in w^{1118} females treated with NIR after 1 hr of injury |
| 1    | UP_KEYWORDS | Antibiotic | 66.29 | <0.01 |
| 2    | UP_KEYWORDS | Secreted   | 7.11  | <0.01 |
| 3    | UP_KEYWORDS | Antimicrobial | 38.67 | <0.01 |
| 4    | GO:0019731 | Antibacterial humoral response | 36.78 | <0.01 |
| 5    | GO:0006959 | Humoral immune response | 35.24 | <0.01 |
| 17   | UP_KEYWORDS | Polymorphism | 9.28  | <0.01 |
| 18   | GO:0006508 | Proteolysis | 3.92  | <0.01 |
| 30   | GO:0009617 | Response to bacterium | 14.71 | 0.0289 |
| 32   | GO:0045087 | Innate immune response | 8.13  | 0.0374 |
| 33   | GO:0006952 | Defense response | 3.71  | 0.0380 |

| B: Selected GO terms differentially regulated in w^{1118} females treated with NIR after 2 hr of injury |
| 1    | GO:0048583 | Regulation of response to stimulus | 1.76  | <0.01 |
| 2    | GO:0010646 | Regulation of cell communication | 1.78  | <0.01 |
| 3    | GO:0023051 | Regulation of signaling | 1.78  | <0.01 |
| 4    | UP_KEYWORDS | Vision | 5.32  | <0.01 |
| 5    | GO:0009966 | Regulation of signal transduction | 1.84  | <0.01 |
| 19   | GO:0009583 | Detection of light stimulus | 4.01  | <0.01 |
| 21   | GO:0071482 | Cellular response to light stimulus | 4.92  | <0.01 |
| 27   | GO:0050794 | Regulation of cellular process | 1.23  | 0.0158 |
| 28   | GO:0016056 | Rhodopsin mediated signaling pathway | 7.50  | 0.0168 |
| 32   | GO:0050789 | Regulation of biological process | 1.21  | 0.0196 |
| 37   | GO:0030162 | Regulation of proteolysis | 2.82  | 0.0376 |

| C: Selected GO terms differentially regulated in w^{1118} females treated with NIR after 4 hr of injury |
| 1    | UP_KEYWORDS | Protease | 4.31  | <0.01 |
| 2    | UP_KEYWORDS | Disulfide bond | 3.16  | <0.01 |
| 3    | UP_KEYWORDS | Hydrolyase | 2.04  | <0.01 |
| 5    | GO:0006508 | Proteolysis | 3.04  | 0.0133 |
| 10   | GO:0006040 | Amino sugar metabolic process | 5.63  | 0.0230 |
| 13   | GO:0008152 | Metabolic process | 1.26  | 0.0273 |
| 17   | GO:0042252 | Serine-type endopeptidase activity | 3.25  | 0.0447 |
| 18   | GO:0006022 | Aminoglycan metabolic process | 4.89  | 0.0484 |

GO terms were sorted based on FDR and ranked accordingly. Tables show selected GO terms changed in females after injury. GOBPID is the ID of the biological process in GO database.
increase in GFP expression is significantly decreased after NIR exposure in both sexes. We did observe a significant decrease in GFP expression at 2 hr in males but the regulation of this phasic pattern is not understood. Sex-differences were also observed in repo transcription with injured females (Figure 4C) exhibiting significant upregulation 1 hr after injury whereas injured males (Figure 4F) showed significant downregulation 2 hr after injury.

Overall, male and female flies exhibit differences in immune response activation after brain injury with females exhibiting a more immediate alteration in gene transcription than males. Injured males do exhibit an apparent upregulation of immune response as seen by repo-GFP expression, but this response is discordant with repo gene transcription which did not change in males, likely due to changes in protein dynamics (degradation or turnover) not captured by transcriptional data. Inhibiting COX-activity after injury prevents the aberrant activation of immune response gene transcription in females but has no effect on males. It is likely that sexual dimorphism in immune response contributes to the differences in response to injury and NIR exposure.

Injured Flies Exposed to NIR Exhibit Downregulation in Mitochondrial Gene Transcription

From meeting the increased demand for ATP production to initiating apoptotic signals for clearance of cell debris, mitochondrial function is crucial to the repair process after brain injury (Lifshitz et al., 2004; Hiebert et al., 2015). However, this upregulation in mitochondrial process is also accompanied by an increased generation of ROS, hyperpolarization of $\Delta \Psi_m$ and mitochondrial dysfunction leading to neurodegeneration (Liesa et al., 2009; Shah et al., 2019, 2020). Sexual dimorphism in mitochondrial metabolism is also found to play a crucial role in development of pathologies following injury (Gupte et al., 2019). We have previously shown sex-differences in mitochondrial stress and gene transcription after TBI in Drosophila (Shah et al., 2020) and we here wanted to explore the effects of inhibiting mitochondrial COX activity on gene expression in both sexes of injured flies.

Transcription of genes involved in mitochondrial oxidative phosphorylation, biogenesis, transport and translation is significantly upregulated in injured $w^{1118}$ females (Figure 5). We have seen increased expression of SdhA and SdhB (Succinate dehydrogenase, subunit A and B), subunits of the succinate dehydrogenase complex of the ETC after injury in females. Surf1 (Surfeit 1), involved in the assembly of COX is also upregulated in injured females. Increased transcription of ETC genes could indicate upregulation of mitochondrial activity to increase production of ATP (Sen et al., 2017). There is significant upregulation of mitochondrial ribosomal genes like mRP43, mRP25, mRP46, mRP11, mRP21, and mRP35 after injury in females. Upregulation in transcription of these genes could indicate alterations in mitochondrial dynamics to clear damage and dysfunctional mitochondria after injury and restore homeostasis. Interestingly, COX inhibition with NIR exposure in females did not have significant transcriptional effects after TBI. There was significant downregulation observed only in mRP43 after injury in NIR exposed females. Similar to immune processes, we observed limited changes in injured $w^{1118}$ males with or without NIR exposure (Figure 5). mRP43 was significantly downregulated in injured males but unchanged after NIR exposure whereas mRP35 was significantly upregulated after NIR treatment only. It is possible that in Drosophila, similar to humans,
mitochondrial function and metabolism vary by sex, which may explain the variations observed between both sexes in response to injury.

These data suggest that modulating mitochondrial COX within the immediate early period following brain injury could prevent mitochondrial damage. However, additional studies that determine ΔΨₘ, oxygen consumption, ATP production and superoxide production after NIR exposure will be useful to support the observed changes in gene transcription reported here.
Cytoskeletal Gene Transcription Is Downregulated in Injured Flies After NIR Exposure

Mitochondria move along the axons in both directions using the microtubules and motor proteins (Bartolak-Suki et al., 2017). These interactions between the cytoskeleton and mitochondria are essential for maintaining mitochondrial morphology (Boldogh and Pon, 2006). The twisting and shearing of axons caused by the movement of brain within the skull during an injury is known to cause mechanical deformation on the neuronal cytoskeleton (Hill et al., 2016). Following TBI, Tau, a microtubule associated protein is found to be hyperphosphorylated and impact cytoskeletal integrity and mitochondrial transport (Sivanandam and Thakur, 2012; Edwards et al., 2019). Thus, we wanted to look at the effects of exposure to COX-inhibitory NIR on tau and cytoskeletal gene transcription in injured flies of both sexes.

As observed in immune response and mitochondrial gene transcription, we saw significant upregulation in expression of cytoskeletal genes in injured females (Figure 6). w^{1118} injured females exhibit significant upregulation of kinases involved in Tau phosphorylation like lok (loki) and Cdk5alpha (Cdk5 activator-like protein) after injury (Figure 6). Cyclin-dependent kinases were shown to be involved in mitophagy in cell culture models (Moskal et al., 2020). Microtubule integrity depends largely on tubulin polymerization (Bartolak-Suki et al., 2017) and we found a significant upregulation in alphaTub84D (α-Tubulin at 84D) and betaTub97EF (β-Tubulin at 97EF), both involved in polymerization of microtubules. In w^{1118} injured males (Figure 6), we observed very little transcriptional change with only pbl (pebble), involved in actin cytoskeleton reorganization being significantly downregulated whereas pbl was upregulated in injured females. Exposure to NIR resulted in significant alteration in Tip60 (Tat interactive protein 60kDa), cher (cheerio) and Cdk5alpha in females. DCTN5-p25 (Dynactin 5, p25 subunit), a motor protein involved in retrograde axonal transport was significantly upregulated in injured females but unchanged after NIR exposure. DCTN5-p25 was significantly upregulated after NIR exposure in injured males.

Our data suggests that brain injury affects cytoskeletal integrity differently in male and female flies and NIR exposure limits the damage sustained by trauma in both sexes.

DISCUSSION

Pharmacological therapies for brain injury are primarily focused on modulating major neurotransmitter processes to enhance the neurocognitive sequelae of TBI (Morries et al., 2015). Unfortunately, little has been found to reverse TBI damage caused by mitochondrial dysfunction, a well-known cause of neuronal death (Hiebert et al., 2015; Morries et al., 2015). In this study we employed NIR light, a non-pharmacological approach to minimize mitochondrial damage after TBI in Drosophila. NIR treatment has been shown to provide benefit in animal models of ischemia/reperfusion injury (Sanderson et al., 2018), spinal cord injury (Giacci et al., 2014), stroke (Strubakos et al., 2020), optic nerve injury (Giacci et al., 2014), and in human trials of TBI (Naeser et al., 2014). Sex differences in outcome to TBI have been observed in flies (Shah et al., 2020) and there exist strong evidence indicating sex differences in mitochondria could also have an effect on TBI response (Conley et al., 2014; Gupte et al., 2019). Hence, we assessed the effects of modulating
mitochondrial COX using NIR exposure on gene transcription in the immediate time frame after brain injury in both sexes. The fly brain is enclosed in a cuticle composed of chitin which strongly absorbs the 750 nm infrared radiation (Klocke et al., 2011), so we sequenced 3′mRNA libraries of isolated w1118 fly brains at control, 1, 2, and 4 hr after TBI. Our results suggest that inhibiting COX activity downregulates gene expression in injured females whereas injured males exhibit minimal changes with or without NIR treatment.

Different studies have suggested a positive effect of PBM treatment in TBI patients (Naeser et al., 2014; Poiani et al., 2018; Carneiro et al., 2019). NIR between 700–1000 nm readily penetrates the scalp and skull and has the potential to improve cellular activity of compromised brain tissue (Santos et al., 2018). Most studies employing NIR treatment have focused on evaluating the efficacy of this exposure on neuropsychological defects like impaired cognition and mood arising from brain injury (McKee and Lukens, 2016; Plesnila, 2016; Dinet et al., 2019). Efforts to manipulate genes or pathways that can either trigger anti-inflammatory responses or inhibit pro-inflammatory processes have been underway as means to curb this extensive brain damage (Griffin, 2011; McKee and Lukens, 2016). A growing body of evidence also links mitochondrial dysfunction with increased incidences of immune activation (Walker et al., 2014; Breda et al., 2019). Here, we see an increased expression of genes involved in the Drosophila immune system after injury in females and most of these upregulated genes are unchanged after NIR exposure. Previous studies have also noted protective effects of PBM on immune system in TBI models (Moreira et al., 2009; Hennessy and Hamblin, 2017) but the underlying cause for differences between males and females as observed here remains unknown. Drosophila males have been shown to have increased immune gene transcription days or weeks after injury (Katzenberger et al., 2016; Swanson et al., 2020), so it is likely that an upregulated gene expression could be observed if longer time points are assessed. Sexual dimorphism in mitochondria (Demarest and McCarthy, 2015; Gupte et al., 2019) could also be influencing the secondary damage cascades after brain injury but further studies are required to explore such differences in flies. It should also be noted that the NIR wavelength (COX-inhibiting) and energy density employed in this study is different to previously published reports (Weinrich et al., 2017), which could also have an impact on the response. Weinrich et al. (2017) showed that lifelong 670-nm exposure in Drosophila extends lifespan and improves aged mobility.
However, our initial assessments determined that chitin absorbed NIR at the 750-nm exposure, thus raising the possibility that different wavelengths have varying penetration through tissues. Due to this we were unable to assess behavioral function or survival post-TBI in NIR-exposed flies, however our focus was to assess gene expression changes in the brain after injury.

One of the most pronounced effect of TBI is axonal damage which impacts the cytoskeletal integrity and structure (Fitzpatrick et al., 1998; Saatman and Burkhardt, 2009). The cytoskeleton regulates mitochondrial positioning and transport and this interaction of mitochondria with microtubules is a tightly regulated process (Anesti and Scorrano, 2006; Boldogh and Pon, 2006). An additional level of regulation is accomplished by microtubule-associated proteins (MAP's) like Tau (Ebner et al., 1998) and pathological Tau, a common finding in TBI patients and animals models of brain injury (Collins-Paino and Corrigan, 2017; Castellani and Perry, 2019), inhibits mitochondrial transport (Shahpasand et al., 2012). In this study, we observed an upregulation in several cytoskeletal genes in injured females but no change in tau transcription in both sexes after TBI. NIR exposure appears to show an immediate response in females but minimal change in males. Similar trend in gene expression is also observed in mitochondrial gene expression for both sexes after injury and subsequent NIR exposure. Since tau transcription was unaffected by brain injury, we also exposed TBI inflicted Tau knockout flies to NIR and observed mostly downregulation or no change in gene expression in both sexes (Supplementary Data 1). The synergistic effect of absence of tau and NIR exposure in injured flies highlights the role of tau expression and mitochondrial dysfunction in TBI outcome.

In conclusion, we have shown that exposure to COX-inhibitory NIR light within the immediate timeframe after brain injury downregulates gene expression in injured flies. The response to TBI and the subsequent modulation of mitochondrial COX by exposure to NIR varies by biological sex with females exhibiting a more pronounced effect than males in Drosophila. Although the cause of these transcriptional differences remains unknown, the presence of metabolic tissues, sex-specific genes or brain architecture could be potential factors requiring further studies. In this study, we were unable to assess lifespan or behavioral measures in NIR-exposed flies due to the absorbance property of chitin, but we present genome-wide transcriptional changes in Drosophila brains to decipher gene networks or pathways that are affected by TBI and NIR. Finally, we propose that a detailed understanding of the disease mechanism at the mitochondrial level after acute stress (Sanderson et al., 2013, 2018) will make possible use of COX-inhibitory and -activating NIR at the early and late time periods following tissue injury, respectively, to limit Δψm hyperpolarization and thus ROS early to suppress cell death, whereas at later chronic stages of the disease, COX-activating NIR may be useful to enhance tissue remodeling and repair.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material. Gene expression data are available in the GEO database under accession number GSE140663 (w1118 without NIR exposure) and GSE158061 (w1118 with NIR exposure).

**AUTHOR CONTRIBUTIONS**

DR, MH, and KG conceived the project. ES and KG conceptualized the content. ES performed the data analysis and wrote the manuscript. All authors edited the manuscript and assisted with data analysis.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.628777/full#supplementary-material

**Supplementary Data Sheet 1** | Gene Ontology tables.

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Conflict of Interest: MH and TS are co-founders of Mitovation Inc., that develops infrared light therapy for ischemia/reperfusion injury applications.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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