The effect of different bands of LED exposure on vitreous metabolites and retinal architecture.

Nagarajan TN (✉ nagarajan.t@manipal.edu)

Manipal College of Health Professions <https://orcid.org/0000-0003-1028-5807>

Manjunath B Joshi

Manipal Academy of Higher Education School of Life Sciences

Manna Valiathan

Kasturba Medical College Manipal

Shonraj BG Rao

Manipal College of Health Professions

Shailaja Bhat

Kasturba Medical College Manipal

Sudarshan Surendran

Melaka Manipal Medical College <https://orcid.org/0000-0003-0973-8856>

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Abstract

The accumulating experimental evidence indicates that exposure to blue and white LED light leads to damage in the visual system against short-term exposure. Chronic exposure, adaptive responses to light and self-protective mechanisms against LED light exposures need to be explored and would be essential to know the repercussions of LED radiations on vitreous metabolites. A total of 24 male *Wistar rats* were used in our study, which was divided into four groups ($n = 6$ in each group). Three groups were exposed to either blue, white, or yellow LED light for 90 days (12:12 light-dark cycle routine) with uniform illumination (450–500 lux). Control rats were maintained under standard laboratory conditions. Post-exposure the vitreous was removed for mass spectrometry and retinal tissues for immunofluorescence and H&E staining. The thickness of the retina decreased in blue and white light exposure animals compared with controls, whereas the yellow light exposure group showed an increase in thickness ($p < 0.001$). The number of apoptotic cells was significantly lower in controls compared to light-exposed groups ($p < 0.001$) and ($p < 0.001$). Altered metabolites were observed in light exposure groups particularly in D-alanine, taurine, D-serin ($p < 0.05$ and lysine ($p < 0.001$). The self-protective or reworking system in the chronic light exposure could be dazed and drop the ability to compensate for the defending mechanism. This might fail to maintain the metabolomic structural integrity of the vitreous and retina.

Introduction

Light plays a crucial role in visual perception from a non-ionizing visible light spectrum between 380-700nm [1]. Environmental light plays a significant role in zeitgebers [2, 3]. The retinal ganglion cell layer and photoreceptors provide vital information about ambient illumination and light-dark cycles to visual and non-visual higher cortical centres [4]. Non-natural light is an essential source in contemporary life, a large segment of the population is exposed to a different type of light-emitting diodes (LEDs), and it is cast off globally for interior and exterior lighting and rudimentary lighting components of digital devices.

The blue range of the visible spectrum has high energy that can penetrate tissues, which is associated with the occurrence of malignant melanoma compared to other wavelengths of the visible spectrum in animal models [5]. LED radiation diminishes the capability of cellular mechanism and increases apoptosis and it damages DNA by excessive production of ROS [6, 7]. These mechanisms are activated by chromophores within the retinal cells, they are intrinsically sensitive retinal ganglion cells [8]. These LEDs can alter ocular (retinal) and other biological tissues [4, 6, 9, 10]. Excessive exposure to light can alter physiological rhythms like sleepiness, alertness and increase cortisol levels [11, 12].

Radiation-induced retinal damage can occur either through photo-vaporization or photochemical mechanisms [13, 14]. Photo-vaporization results in disruption of tissue by short-wavelength light exposure with high irradiance level [15]. The photochemical injury occurs when tissue temperature increases due to the entrapment of thermal energy into a tissue molecule which could result in retinal damage [16]. Biological chromophores of retinal pigmented epithelial cells can captivate the photons of light from visible spectrum 380–780nm. These absorptions are necessary for visual functions, but peak energetic radiations (violet to blue 400-500nm) can lead to a lethal consequence to retinal cells [17, 18]. These absorbed excited states of electrons can return to the inhibited state by dissipating the superfluous energy, and the energy molecules can break a bond in another molecule through a direct exchange of electron or hydrogen-producing reactive oxygen species (ROS)[19, 20]. The interaction of these chromophores with photons of light might generate ROS which might damage the amino acids, lipids, and DNA [21].

The visual system is exposed to a limited time to LED lights, of different spectra as its technology are blooming now to decrease the energy consumption and CO₂ emission. These technologies eventually replaced standard lighting systems of indoor and outdoor. The white and blue LEDs have high energetic characteristics compared with other traditional light sources, so the potential ocular and general risks of these LEDs are widely explored [4, 19, 22–26]. Inference from these studies cannot be generalized because few studies suggested that low levels of blue light for a longer duration (few months to years) are needed to determine the effects of blue light on the visual system [27]. Considering this we have hypothesized that chronic exposure might induce a compensatory mechanism, stimulated by an increased insult to ocular tissues and fluid. Hence, we aimed at understanding the relationship between chronic light exposure and alterations in the vitreous metabolites and retina structures in *Wistar rats*.

Methodology

The Manipal Academy of Higher Education, Kasturba Medical College animal ethics committee approved the experimental protocol (IAEC/02/2017). Animal handling and investigational procedures were carried out as per IAEC and CPCSEA (No:94/PO/Re Bi/5/99/CPCSEA) guidelines. The healthy animals (n=24) are divided into three experimental groups (n=6 in each group), one serving as an (unexposed) control group (n=6). We have included healthy rats for the study and the control rat's retinas were selected from the same pool of rats. However, it's safe to assume that the experimental control rats will also not have any degeneration. Both eyes of rats were exposed to blue, white, and yellow LED light with the maintenance of homogeneousness and intensity of light. The light standard was measured and standardized using Asensetek Lighting Passport Pro, New Taipei City, Taiwan[28]. The spectral sensitivity of different wavelengths of light visible spectrum 400-780nm) used for this experiment (450-500 lux) light. The spectral properties of light transmittance of the front surface of light, scotopic sensitivity, flicker index and melatonin suppression of each light were measured. The total light exposure per day of (450-500 lux). The height from the source to the animals (cage) was 50 cm and the exposure time (12:12 dark & light cycle routine for 90 days) was matched with the nocturnal time of the rodents. The light exposure group animals were subjected to the blue LED (400 - 490nm), white LED (380-780nm) and yellow LED (400-780nm) light exposure (Ack LED Panels, 3W, Epistar, ES-EMBCF22L-A, InGan -series Blue LED chip, ES-SMYLPX42C, UHB-PX, AlGaInP PN-series LED Chip). The light source was fitted on the top of the cage (L=100cm, W=70cm and H=50cm) the mounting of the light kept it at 50 cms (100 % system level light output at standard operating voltage range, the total light output of the cage at the testing area (510 lumens). Post-exposure, all animals were sacrificed with a lethal dose of pentobarbital intraperitoneal injection (i.p. 100 mg/kg) (Euthasol®) and xylazine (10 mg/kg) (Proxylaz®). Then eyes were enucleated by using watchmaker forceps (number 5) and Sklar's blunt enucleation scissors. Immediately after enucleation, the vitreous was extracted by aspiration (21-gauge hollow needle) technique and stored in -20° C for LC-MS and the retinal tissue stored in 4% paraformaldehyde (7 days) for immunofluorescence (IF) and Hematoxylin and eosin (H&E) stain.

Rat whole vitreous:

For the analysis of the chronic light exposure model, the whole vitreous sample was collected by aspiration technique. The samples were sonicated and stored at -20 °C until LC-MS analysis.

Vitreous Metabolomic Analysis

Untargeted quantitative metabolomics was used to analyze the vitreous fluid samples using an LC-MS Agilent LC-QTOF system (Agilent Technologies, Santa Clara, CA, USA). It consisted of an Agilent 1200 LC system coupled online with an Agilent 6520 time-of-flight mass spectrometer. The vitreous samples were defrosted on ice, vortexed, and centrifuged at 12,000 rpm for 15 min. An 8 µl aliquot was injected into an Agilent 1290 LC system coupled to an ESI-Q-TOF instrument (Agilent 6520, Agilent Technologies, Santa Clara, CA, USA). HPLC column (Phenomenex, Torrance, CA, USA (P/No:00G-4601-E0; Desc: Kinetex 5µm C18 100A; Size: LC Column 250X 4.6 mm; S/No H18-343854; B/No 5701-0060) was maintained was at 25°C. The injected vitreous data were collected in the positive mode of electrospray ionization (ESI) technique. Basic and neutral metabolites were eluted in positive mode using subsequent gradient at 400 µl/min using mobile phase A: 0.1% formic acid in water and mobile phase B: 0.1% formic acid in 90% acetonitrile (2% to 98% B in 25 minutes, 98% B for 10 minutes and equilibrated to 2% B for 10min). ESI spray voltage was maintained at 3.5kV with MS interface capillary at 350°C. Fragmentor was set at 140V. Drying gas and nebulizer were maintained at 8 litres/minute and pressure of 40 psig respectively. Data was acquired at an acquisition rate of 2Hz in a range of 50-1700 m/z. Based on precise mass value and retention time, an untargeted mass spectrometer approach was employed for confirmation of the most abundant and significant metabolites.

Data processing and analysis:

The raw data obtained from each run was processed using a molecular feature extraction tool in Qualitative Mass Hunter Analysis Software B.04.00 (Agilent Technologies). The data files containing monoisotopic mass, respective abundance and retention time were used for data alignment and filtering in Mass Profiler Plus software (MPP) (Agilent Technologies, version B.12.5). Raw data files were segregated aligned, transformed to log10, and baselined to the median of all samples in MPP. Features that were present in at least 75% of individuals in each group were considered for further analysis. Partial least square discriminant analysis (PLS-DA) was performed on metabolome data to discriminate the study groups. Compounds were identified in METLIN and HMDB databases based on isotopic pattern distribution and accurate mass within a specified tolerance (15 ppm error). Peaks obtained

from raw MS data were aligned and subjected to bioinformatics and statistical analysis. The metabolite peaks were attained in NC, BLE, WLE and YLE groups separately (Figure 1:).

Retinal Immunostaining:

The twenty-four retinal tissues were embedded in 4% paraformaldehyde immediately after the following enucleation. The retinal tissues were sectioned after calibrating the cryostat for 40 μ thickness and the sections were mounted on the gelatin-coated slides serially. Frozen sections (Leica cm3050 s) of 40 microns thickness were made in the sagittal plane and kept at -80°C before further processing. Sections were fixed with 2% paraformaldehyde in 0.01M phosphate buffer solution at 24°C for 1 hour, followed by incubation with 1% H₂O₂, 2% sodium azide, 0.1% saponin, 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in EBSS -saponin for 36 hours at 24°C in the dark. Sections were then rinsed and incubated in a moist chamber overnight at 24°C with primary rabbit polyclonal to active + pro Caspase 3 antibody directed against cleaved caspase-3 (1:200 aliquoted 4 μ l of antibody is added to 1.6ml of PBS.TX) with working buffer and approximately 200 μ l of diluted (Abcam, Cambridge, UK). The retinal tissue sections were washed and incubated with a secondary antibody (1:100, Anti-Rabbit IgG (whole molecule) F(ab)2 fragment-Cy3 antibody, Abcam) for 16 hours at 2°C.

Immunofluorescence imaging and quantification: Caspase-3 (active + pro Caspase 3) stained retinae were imaged using a Dmi8-SP8 Confocal Microscope (Leica Microsystems) using a 63 X objective. The laser power offset and gain, and other acquisition parameters were determined by using isotopic control samples. The values of these parameters were then fixed across all retinal samples. Retinal mosaics were created in Adobe Photoshop 7.0 (Mountain View, CA, USA). The area of each retinal layer was segmented by a masked observer, and the average intensity was calculated after subtracting the background using ImageJ software (To overcome the background intensity staining we have accounted brightness in each layer, and it was expressed relative intensity to the entire retina, and the total brightness was averaged). A total of 12 images, 2 from each eye were analyzed in each group and the intensity of IF was quantified in a masked method.

Measurement of hematoxylin and eosin-

Measurement of hematoxylin and eosin-stained retinal layers was conducted as follows. Images of a sagittal plane containing the optic nerve on paraffin-embedded blocks of H&E images were taken using a Motic Images Plus 3.0 for Windows 32 (2021-10-29) Microscope (Leica Microsystems, Germany) at 40 $\times$ magnification then Retinal mosaics were created in Adobe Photoshop 7.0 (Mountain View, CA, USA). Images were analyzed with the built-in measuring tool in *Image-J* software. The thicknesses of the whole retina (excluding the outer segments) and the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL) were measured for each layer in each picture in three different areas. Data were collected in Excel and then imported to R software (version 3.6.3) for statistical analysis

Data preparation and statistical application

The LC-MS/MS spectral region 5.00 ppm was segmented into bins of 0.05 ppm width Agilent pro software. The region anything above 5.00 ppm error was excluded from the analysis as it might have a remnant noise signal. A total column (bins) of metabolites were obtained and the integrated area within each bin was normalized to a constant sum of 500 for each range to minimize the effects of variable concentration among different samples.

Results

Different band of LED persuaded retinal insult: As we showed in figure 2: after 90 days of light exposure the retinal layers showed depletion with marked proliferation, increased cellularity and enlarged cells with vacuolation of ganglion cell layer (GCL), and it became thinner and disorganized, and more hyperchromatic nuclei cells were observed in BLE and WLE groups. The GCL of the retinas in YLE was slightly disarrayed with nuclear enlargement and outer nuclear layer (ONL) thickness was increased with a focal enlargement (Figure 2:). The ONL thickness was decreased in BLE and WLE groups (Table 1:). We found there were varying notches of decreased thickness in the outer nuclear layer (ONL) in the BLE and WLE groups in contrast with YLE and NC. There was no significant difference was found in the INL thickness in all groups ($p < 0.01$). As shown in (Figure 2:) the nucleolar damage,

including pyknosis, was distinct with detached IS and OS structures and irregular arrangements of the photoreceptors in groups BLE and WLE when compared with the YLE and NC groups.

The stained retinal sections were obtained in the dorsal-ventral axis for immunofluorescence (Figure 3A:) (IF) quantification. The whole retinal tissue section was selected at different peculiarities, for cell count and the total number of stained nuclei was counted in a 200- μ m-long tissue section. For every retinal location, the amount of apoptosis in the NC group of retinae was significantly lower than in the light-exposed groups (ANOVA) (Figure 3B:). Besides, statistically significant differences were found in light exposure groups of rats ($p < 0.01$) (Figure 3B:).

The retina showed a significant change after 90 days across the light exposure groups (Figure 3A:). IF analysis of CASPASE-3 expression showed a reorganization of morphological structures in BL and WL in comparison with NC whereas no obvious morphological changes were noticed in inner retinal layers and significant damage occurred in ONL in the YLE group using Cy-3 staining. The percentage of apoptotic cells was increased BLE and YLE in GCL, INL, ONL ($p < 0.001$) and inner and outer segments of photoreceptor and IPL are relatively reduced in the YLE and NC group. However, the apoptosis was 86%, 84%, and 66% for BLE, WLE and YLE -light exposure tissues respectively ($p < 0.001$) (Figure 3B:). Four regions of interest in every retinal tissue section were selected at different peculiarities, for cell count and the total number of stained nuclei was counted in a 200- μ m-long tissue section. The amount of apoptosis in the NC group of retinae was significantly lower than in the light-exposed group's rats ($p < 0.001$) (Figure 3B:). Besides, statistically significant differences were found in light exposure groups of rats ($p < 0.001$) and ($p < 0.001$) (Figure 3B:). However, no self-protective or recovery was observed based on the hypothesis. The acquired images from the posterior pole within 1 mm of the optic nerve and the phototoxicity also were observed in the region of interest (ROI).

Metabolomic amendment of chronic light exposure and reprogramming.

A total of 24 vitreous samples, withdrawn from light exposure (BLE, WLE and YLE) and control group (Time 10:00 AM-10:30 AM IST) were analyzed. We got 20884 hits across the groups and these molecules were filtered out by frequency out of these 459 molecules which were presented across the samples. These molecules were considered to perform further analysis (one-way ANOVA) to observe the significance.

243 molecules were statistically significant ($p < 0.05$) for the entity of 2.0-fold change (FC). Further, we did differential expression analysis using a t-test against zero, kept the p-value as asymptotic across the samples with an FC of 3.1. Out of total (20884) molecules 1521 were statistically significant to specific 907 molecules ($p < 0.05$), 348 molecules ($p < 0.02$), 169 molecules ($p < 0.001$), 82 molecules ($p < 0.0001$) and 20 molecules were ($p < 0.00001$) exhibited definite statistical alteration.

PCA and PLS-DA

We steered Principal component analysis (PCA) on all detected metabolites. The parting of the NC, BLE, WLE and YLE groups demonstrated their difference in metabolomic expression. Most metabolites with extreme abundance were certainly isolated in light groups. Further, we did a summary of frequency normalization for abundance values ($\log_{10}$) for across the groups out of it 99% of samples were normally distributed and outliers were removed in each group. Only significant metabolites into account, the molecules in the same group clustered to each other which stated less difference among the light exposure group.

Through partial least square discriminant analysis, we found the pattern of metabolomics, with a sensitivity of 90% and a specificity of 87%. Notably, the amino acids and small molecule levels were elevated in the BLE and WLE groups. A clear difference in metabolomic expression of the BLE and WLE, YLE groups and the control group is displayed where there is a significant cluster of metabolites were observed between the groups.

Further to comprehend we heat map (Figure 4:) representation for the significant (243 molecules ($p < 0.05$)) metabolites and we observed a clear upregulation of molecules in BLE, WLE and YLE where these molecules were in normal range/downregulated in NC. Having three groups of light exposure (BLE, WLE and YLE) from age-matched controls (NC). The BLE&WLE exposure groups have been vital to show the specificity of these biomarkers to vitreous. Some metabolites were perturbed in all groups. Untargeted metabolomics by LC-MS/MS revealed 20 metabolites that are biologically relevant to ocular fluid. Thus, we next sought to validate our findings using the targeted analysis to quantify the concentrations of these metabolites. As well as targeting the metabolites dysregulated in BLE, WLE and YLE samples, we expanded the targeted analysis to include metabolites from related metabolic

pathways, to determine the biological relevance of our findings using METLIN, Agilent pro and HMDB databases. The focused analysis was carried out using authentic standards to obtain accurate fold changes of the metabolites in the extracted samples. It was confirmed that 20 metabolites were statistically significant.

To further understand the perturbed metabolomic alterations, develop over the light exposure period of 90 days in Wistar rats, we performed one-way ANOVA and these values were compared across the groups. The p-values <0.05 considered as significant and significantly altered metabolites between the groups are plotted in Table 2: using Tukey HSD post-hoc test.

Pathway enrichment (figure 5:) analysis was performed for the very significant 20 metabolites in the NC, BLE, WLE and YLE groups using the MetaboAnalyst 4.0 program. The analysis revealed that arginine, aspartate glutamate and taurine metabolites were the most perturbed pathways in light exposure conditions (Figure 5:). The analysis revealed that alanine, aspartate, and glutamate ($p=0.0600$), Pathway analysis of the significant metabolites identified for the comparison among BLE revealed significant enrichment for aspartate ($p=0.0068$), glutathione ($p=0.0263$), glycine ($p=0.00436$), glutamate ($p=0.054$), leucine and taurine ($p=0.007$) and taurine and hypotaurine degradation (Figure 5:) were the most perturbed pathways in BLE and WLE conditions. This augments a growing frame of evidence to suggest that photo-chemical metabolism is particularly compromised in LE animals that potentially disrupts the taurine and hypotaurine dysregulation pathway.

Discussion

This study reveals 90 days of (12:12 hours) cyclic LED light exposure to rat's eye resulted in vitreous metabolomic changes and retinal structure alterations. These observations might be related to oxidative stress within the retinal tissue consistent with the aforementioned findings of short exposure especially a few days to months [6, 13, 29, 30].

LC-MS showed obvious changes in the vitreous metabolites after 90 days of light exposure (BLE, WLE and YLE) and some of these changes, like upregulated amino acids (AA) and lipids were more intense in BLE and WLE groups while others, like the increase in levels of AA, were observed from YLE. Light exposed vitreous showed several notable differences in the spreading silhouettes of the perceived metabolites between LED light exposure groups and normal controls of the vitreous fluid, predominantly, amino acids and proteins. Inadvertently, the role of altered metabolites and excitotoxic potential to light-induced damage in the vitreous is not well documented. It has been involved in a few ischemic induced ocular disease conditions including retinal vessel occlusion, glaucoma, and diabetic retinopathy [31, 32]. The glutamate levels were altered in BLE and WLE groups which could also be a triggering factor for retinal apoptosis [33, 34]. Glutamate is primarily mediated by overstimulation of the NMDA subtype of glutamate receptor, triggering an increase in intracellular calcium and initiating a cascade of events that finally lead to cell death/apoptosis, depending on glutamate levels [35]. The eccentricities in glutamate metabolism result in elevation of the extracellular concentration of glutamate which might increase the risk of excitotoxicity [33]. This indicates that failure in upholding glutamate homeostasis might lead to ocular toxicity [33].

The ganglion cell layer (GCL) thickness was drastically reduced in BLE and WLE groups, which might be due to the elevation of vitreal glutamate levels [36]. We noticed that metabolites of the vitreous fluid were dysregulated in the light exposure (BLE and WLE) groups, which could be due to a shift of glycolysis' products, such as fructose 1,6 bisphosphate (F1,6BP; a product of glycolysis) and citrate, to the inner retina, while glucose 6 phosphate (G6P) was detected at high intensities both in the inner and outer retina [37]. The up-regulated metabolites in BLE and WLE groups could be due to metabolomic requirements to withstand or protect from light-induced damage. Studies have demonstrated the importance of cytokines induced protective mechanism of photoreceptors in light-induced damage against constant exposure [38, 39] and stated that this could be due to survival-promoting genes and proteins that might alter during retinal light adaptation to a new bright light environment in the BLE and WLE groups. The identified molecules from Alvarez et al., 2001 which can protect against light-induced damage were up-regulated contemporaneously [40].

Interestingly, de la Barca et al., 2017 studied the effect of light stress in the retinal metabolomes and found that the mechanisms of light preconditioning (pre-exposure to moderate light before intense light) remained unknown, even though they seemed to have a self-protective effect. The light-induced changes in vitreous metabolites such as amino acids and lipids could likely involve nitric oxide-related signalling pathways [14, 37]. Post-acute bright light exposure, the retinal proteins expressions were up-regulated in particular glial @brillary acidic protein, heme oxygenase and heat-shock protein as well as cytokines [39, 41–43]. Beneath a

constant light exposure condition, the endurance of the alteration of vitreous metabolites and its concentration revealed that this could be an endogenous oscillator, it correlates with previous experiments on aqueous proteins[44]. The upregulated metabolites concentration noticed in light exposure groups (BLE and WLE) (Fig. 4:) could be related to light responses in ocular bounded tissues or linked cellular restoration[44] and antioxidant protection to retinal tissues[45]. Abundant proteins remained to play a major role in modifying such light damage[46]. Our current experiment suggests that the degree of susceptibility to light damage in tissues adjoining to the vitreous may also be contingent upon time and light dependence.

The increase in period of cumulative and constant light exposure for 90 days on vitreous fluid, the self-protective or reworking system in light (12:12 light-dark cycle) period could be dazed and might drop the ability to compensate for the defending mechanism, and thus might fail to maintain the metabolomic structural integrity. Such mechanisms increase post LED exposure if the compensatory mechanism is failed due to light damage and might lead to lethal consequences to the biochemical composition of vitreous fluid.

With the effects of blue, white, and yellow LEDs on the whole-mount retina, the significant structural alterations and damage leading to the disruption of the retina, could be due to disruption of blood-retinal barriers by increased production of ROS[47]. We found that the BLE and WLE exposed retinal tissues exhibited higher levels of CASPASE-3 immunostaining mostly in the INL, ONL and GCL of the retina (Fig. 5:) and this is consistent with previous findings [6, 30]. Furthermore, few studies found that post light exposure with higher intensity of blue and white LEDs inhibited the increased proliferation of retinal cells with apoptosis compared with yellow LED [6]. Unfortunately, studies have not been conducted for chronic light exposure (Beyond 28 days). The relationship between long-term light exposure and retinal damage was unidentified. Few studies have indicated that lipofuscin could be a possible mediator of the risk associated with long-term exposure to blue light-induced retinal damage [48, 49]. When lipofuscin absorbs blue light, it produces ROS that eventually leads to oxidative stress and retinal damage [27]. This mechanism (lipofuscin) is directly related to the type of light and its spectral composition [50]. Thomas et al reported that chronic blue light exposure could accelerate photoreceptor degeneration in an animal model in a retinal degeneration study [51]. The lipofuscin levels in retinal pigmented epithelium may alter with age, with levels being low in young and high in old animals [52]. This could also be one of the confounding factors to accelerate the retinal damage along with chronic exposure to the blue and white component of the light spectrum. The presence of a self-protective mechanism diminishes with age [53]. Light exposure leads to oxidative stress eventually resulting in the visual system no longer being able to protect the retinal architectures against chronic light, thus triggering irreversible vagaries in the visual system. Overall, the attained results can suggest the following sequences of events the ROS produced by the high-energy light from a blue and white LED[54] and the altered vitreous metabolites by inducing protein and AA modification affecting the hypotaurine pathway. This observation suggests that photo-chemical metabolism is compromised in light exposure rats potentially disrupting taurine and hypotaurine metabolism that might impact neural signals in the retina Fig. 5. This study has some limitations as we used only (CASPASE-3) immunohistological markers, which make little difference to the blue and white light delivered to the animals used in this study. A more detailed study, with more time point sampling and specific cell death assay (e.g. western blot), further reveal understated anatomical changes to use OCT (real-time imaging) in chronic light exposure. The results shown here were obtained in rats with cumulative and continuous LED light exposure, their eyes are different from humans because they are nocturnal animals and do not have a macula this cannot be directly applicable to humans.

Conclusion

Our findings state that chronic exposure to blue and white LED light damages the retina and alters the vitreous metabolites. The prolonged acute cumulative blue and white LED light exposure causes indemnities to the structural integrity of the vitreous and retinae. The self-protective or reworking system in the light-exposed visual system is dazed and deteriorate the ability to compensate for the self-protective mechanism.

Declarations

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Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Nagarajan T], [Manjunath B Joshi], [Sudarshan S], [Shonraj BG Rao], [Manna Valiathan] and [Shailaja S]. The first draft of the manuscript was written by [Nagarajan.T] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Ethical Statement: All experimental procedures in the present study were approved by the institutional animal ethics committee (IAEC) at Kasturba Medical College, Manipal Academy of Higher Education (IAEC/KMC/02/2017, dated 21 January 2017), India. Animal handling and investigational procedures were carried out as per CPCSEA (No:94/PO/Re Bi/5/99/ CPCSEA) and ARRIVE guidelines.

Informed Consent Statement: Not applicable.

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Tables

Table 1: The different wavelength of LED light exposure of individual retinal layer thicknesses stained with hematoxylin and eosin were measured using ImageJ software (RPE to RGC) layer thickness were measured at quarterly points for each retinal cross-section and averaged. Averaged retinal thickness was converted to the percentage of the thicknesses from keeping standard against controls. Also, the layers are examined with different parameters (Atrophy, vacuolation, pyknosis and morphological alteration) by histopathologist.

Layer	Damage	BLE n = 12	WLE n = 12	YLE n = 12
Ganglion Cell Layer	Atrophy	2%	8%	2%
	Vacuolation	4%	6%	4%
	Proliferation	7%	1%	0
	Focal enlargement	8%	10%	6%
Inner Plexiform Layer	Atrophy	7%	0	0
Inner Nuclear Layer	Focal enlargement	4%	0	4%
	Atrophy	2%	0	2%
	Decreased thickness	2%	6	0
Outer Nuclear Layer	Atrophy	0	0	4%
	Focal enlargement	0	0	3%
	Attenuated	0	0	1%
	Decreased thickness	12%	12%	10%
Inner segment - Outer Segment	Atrophy	8%%	6%%	5%
	Decreased thickness	8	7%	6%

Table 2: Distinguish altered vitreous metabolites in controls, BL, WL and YL Metabolite intensities of most abundant and significantly altered amino acids, small molecules and lipids detected in vitreous of control (n=6), BL (n=6), WL (n=6) and YL (n=6). Statistically significant changes in metabolite intensity between the control and lens protection groups are represented by indicating (**p < 0.01, * p < 0.05).

S.no	Mass	Metabolites	P-Value - ANOVA	Tukey HSD post-hoc analysis					
				NC-BLE	BLE-WLE	BLE-YLE	NC-WLE	NC-YLE	WLE-YLE
1	89.0932	D-Alanine	0.013	0.02	1	0.753	0.021	0.146	0.773
2	105.0926	D-Serin	0.009	0.091	0.016	0.014	0.84	0.811	1
3	119.1192	L-Threonine	<0.001	0.002	0.503	0.629	<0.001	<0.001	0.997
4	125.147	Taurine	0.006	0.861	0.006	0.16	0.032	0.509	0.401
5	128.172	lysine	0.001	0.014	0.733	0.89	0.001	0.003	0.989
6	132.1577	L-Asparagine	0.011	0.051	0.987	0.943	0.025	0.016	0.996
7	133.1027	D-aspartic acid	0.012	0.122	0.975	0.021	0.25	0.818	0.05
8	147.13	L-glutamic acid	<0.001	<0.001	0.509	0.857	0.009	0.002	0.926
9	153.1784	Dopamine	0.009	0.091	0.016	0.014	0.84	0.811	1
10	175.188	citrulline	<0.001	<0.001	0.734	0.995	0.002	<0.001	0.596
11	219.237	Pantothenate	0.017	0.096	0.92	0.94	0.026	0.03	1
12	228.245	Hydroxyprolyl-Proline	0.004	0.053	0.619	0.975	0.004	0.022	0.851
13	231.249	L-Asparaginyl-L-valine	0.004	0.979	0.994	0.02	0.999	0.008	0.012
14	231.296	α -(γ -Aminobutyryl)-lysine	0.02	0.338	0.414	0.778	0.017	0.064	0.923
15	284.272	L-Glutamyl-L-histidine	0.038	0.22	0.849	0.46	0.049	0.956	0.131
16	307.32	Glutathione	0.012	1	0.019	0.743	0.02	0.757	0.144
17	317.3398	L-Hydroxyprolyl-L-tryptophan	<0.001	0.002	0.965	0.999	0.001	0.001	0.989
18	364.5619	2-Arachidonylglycerol	0.041	0.067	0.999	0.87	0.05	0.263	0.802
19	420.0956	inositol 1,4,5-trisphosphate	0.029	0.176	0.826	0.935	0.033	0.059	0.993
20	579.7895	Lysophosphatidylcholine	0.011	0.819	0.01	0.139	0.066	0.514	0.598

Figures

4

Figure 1

Represents the spectra features of metabolite peaks were obtained in NC, BLE, WLE and YLE groups separately. The X-axis represents the count versus acquisition of metabolites time in minutes and Y-axis represents the generated distinct peaks on the total ion chromatogram (TIC) traces ($\times 10^6$)

Figure 2

Retinal Histology of normal controls (NC), blue light exposure (BLE), white light exposure (WLE) and yellow light exposure (YLE) groups after 90 days of light exposure. Representative images of Hematoxylin and Eosin-stained retinal cross-sections from the Controls (NC.), blue light (BLE), white light (WLE) as well as yellow light (YLE) groups. Scale bar: 100 µm. Photoreceptor, Ganglion cell layer (GCL), inner nuclear layer (INL), inner plexiform layer (IPL), outer plexiform layer (OPL), and outer nuclear layer (ONL).

Figure 3A:

Figure 3B:

Figure 3

A: Different wavelengths of LED light-induced caspase-3 immunostaining in retinal layers. Representative images of retinal cryosections from normal control (NC top right), light exposure (BLE, top left) and WLE groups second row right and YLE second row left) immunolabeled with cleaved caspase-3 antibody (Immunofluorescence), IgG (whole molecule) F(ab)2 fragment-Cy3 antibody). At 90 days after light exposure, BLE and WLE eyes showed increased apoptosis. This was less evident of caspase-3 levels in NCs and YLE groups.

B: Effect of different LED light exposure on retinal caspase immunofluorescence. Bar plots showing immunofluorescence intensity relative to total retinal intensity (%) for the ganglion cell layer. Error bars show the standard error of the mean. P-values in each panel indicates the significance of one-way ANOVA comparison between groups. * Indicates significant ($p < 0.05$) post-hoc difference between pairs.

Figure 4

Hierarchical clustering analysis (HCA) by R version. **a.** HCA of all vitreous metabolites. **b.** HCA of significant Metabolites across the group. The colour gradient illustrates the fold change (Fc3.1) of protein abundance between the four groups. A clear separation of the metabolite's alteration in the three exposure and control group conditions is shown and the alteration becomes more ostensible when significant metabolites have been considered.

Figure 5

Metabolite set enrichment analysis: The Aspartate, Glycine, Serine, and glutathione metabolic pathways are the most significantly affected in the upper panel; 4 Upregulated FDR = 2.99E-01 and 4/20; FDR = 1.00E+00) and in the lower panel; 4 dysregulated features out of 20; False discovery rate (FDR) = 1.00E+00)