

# 17 $\beta$ -Estradiol Ameliorates Light-Induced Retinal Damage in Sprague–Dawley Rats by Reducing Oxidative Stress

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**Abstract** Oxidative stress is considered as a major cause of light-induced retinal neurodegeneration. The protective role of 17 $\beta$ -estradiol ( $\beta$ E2) in neurodegenerative disorders is well known, but its underlying mechanism remains unclear. Here, we utilized a light-induced retinal damage model to explore the mechanism by which  $\beta$ E2 exerts its neuroprotective effect. Adult male and female ovariectomized (OVX) rats were exposed to 8,000 lx white light for 12 h to induce retinal light damage. Electroretinogram (ERG) assays and hematoxylin and eosin (H&E) staining revealed that exposure to light for 12 h resulted in functional damage to the rat retina, histological changes, and retinal neuron loss. However, intravitreal injection (IVI) of  $\beta$ E2 significantly rescued this impaired retinal function in both female and male rats. Based on the level of malondialdehyde (MDA) production (a biomarker of oxidative stress), an increase in retinal oxidative stress followed light exposure, and  $\beta$ E2 administration reduced this light-induced oxidative stress. Quantitative reverse-transcriptase (qRT)-PCR indicated that the messenger RNA (mRNA) levels of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (Gpx) were downregulated in female OVX rats but were upregulated in male rats after light exposure, suggesting a gender difference in the regulation of these antioxidant enzyme genes in response to light. However,  $\beta$ E2 administration restored

or enhanced the SOD and Gpx expression levels following light exposure. Although the catalase (CAT) expression level was insensitive to light stimulation,  $\beta$ E2 also increased the CAT gene expression level in both female OVX and male rats. Further examination indicated that the antioxidant proteins thioredoxin (Trx) and nuclear factor erythroid 2-related factor 2 (Nrf2) are also involved in  $\beta$ E2-mediated antioxidation and that the cytoprotective protein heme oxygenase-1 (HO-1) plays a key role in the endogenous defense mechanism against light exposure in a  $\beta$ E2-independent manner. Taken together, we provide evidence that  $\beta$ E2 protects against light-induced retinal damage via its antioxidative effect, and its underlying mechanism involves the regulation of the gene expression levels of antioxidant enzymes (SOD, CAT, and Gpx) and proteins (Trx and Nrf2). Our study provides conceptual evidence in support of estrogen replacement therapy for postmenopausal women to reduce the risk of age-related macular degeneration.

**Keywords** Neuroprotection · 17 $\beta$ -estradiol · Retinal light damage · Oxidative stress · Intravitreal injection · Antioxidation

## Abbreviations

$\beta$ E2	17 $\beta$ -estradiol
LD	Light damage
IVI	Intravitreal injection
OVX	Ovariectomized
AMD	Age-related macular degeneration
ERG	Electroretinogram
MDA	Malondialdehyde
CAT	Catalase
Gpx	Glutathione peroxidase

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SOD	Superoxide dismutase
Trx	Thioredoxin
Nrf2	Nuclear factor erythroid 2-related factor 2
HO-1	Heme oxygenase-1

## Introduction

17 $\beta$ -Estradiol ( $\beta$ E2) plays an important role in the development, growth, and differentiation of both female and male secondary sex characteristics. The protective effect of  $\beta$ E2 has been identified in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Blanchet et al. 1999; Amtul et al. 2010; Zhang et al. 2012). The neuroprotective effects of  $\beta$ E2 include the following: increased neuronal survival, antioxidant activity, suppression of neuronal apoptosis, and promotion of synaptic sprouting and axonal regeneration (Garcia-Segura et al. 2001; Ba et al. 2004; Quintanilla et al. 2005). Our previous *in vitro* and *in vivo* data demonstrated that  $\beta$ E2 protected retinal nerve cells from H<sub>2</sub>O<sub>2</sub>-induced apoptosis and rat retinas from light-induced apoptosis via the activation of the PI3K/Akt signaling pathway (Yu et al. 2005; Feng et al. 2013; Li et al. 2013; Mo et al. 2013). Aside from its anti-apoptotic effects, there is emerging evidence suggesting an antioxidative effect of  $\beta$ E2 on many types of neurons. For example,  $\beta$ E2 attenuated ethanol-induced neurotoxicity and oxidative stress in the developing male rat cerebellum (Ramezani et al. 2011), suppressed tributyltin-induced reactive oxygen species (ROS) production and lipid peroxidation in rat hippocampal slices by activating Akt (Ishihara et al. 2014), and provided neuroprotection against MPTP-induced oxidative stress by increasing SOD1 and SOD2 immunoreactivity in the nigral neurons of male mice (Tripanichkul et al. 2007).

Age-related macular degeneration (AMD) is the most common cause of loss of central vision in elderly individuals, especially postmenopausal women. The severity and progression of AMD are exacerbated following excessive exposure to environmental light (Taylor et al. 1990), as photoreceptors are sensitive to a wide range of visible light conditions and intensities, leading to photoreceptor degeneration and cell death (Noell et al. 1966). Previous studies showed that the pathogenesis of retinal light damage involves the generation of ROS and the accumulation of oxidatively modified lipids, nucleic acids, and proteins (Jarrett et al. 2008; Mandal et al. 2009). Therefore, antioxidant nutrients might be useful for reducing light-induced cell damage and preventing AMD. However, the mechanisms underlying the antioxidative effect of  $\beta$ E2 on light-induced retinal damage remain to be elucidated.

Because oxidative stress is an important mechanism involved in light-induced retinal damage, the activity of

antioxidant enzymes may be an essential factor that protects against neurological damage. Whether  $\beta$ E2 alters the activity of antioxidant enzymes during light-induced retinal damage has yet to be examined. In the present study, we sought to elucidate the mechanism by which  $\beta$ E2 reduces light-induced retinal damage via antioxidation. We used the female OVX and male rats to generate a light-induced retinal damage model and then administered  $\beta$ E2 to these rats via intravitreal injection to investigate the underlying neuroprotective mechanism of  $\beta$ E2. The results indicated that  $\beta$ E2 alleviated light-induced retinal damage, and the underlying mechanisms involved the reduction of the oxidative stress via the upregulation of antioxidant enzymes (SOD, CAT, and Gpx) and proteins (thioredoxin (Trx) and nuclear factor erythroid 2-related factor 2 (Nrf2)).

## Materials and Methods

### Animals

Male and female Sprague–Dawley rats (SD rats, each weighing 180–230 g) were obtained from the Experimental Animal Center of Xi'an Jiaotong University College of Medicine. All animals were cared for and handled according to the Xi'an Jiaotong University Guidelines for Research and the Association for Research in Vision and Ophthalmology statement for the use of animals in vision and ophthalmic research.

### IVI

The male and female rats were anesthetized via intraperitoneal injection of ketamine (120 mg/kg) and xylazine (6 mg/kg) after 18-h dark adaptation. One drop of lidocaine hydrochloride was applied to each eye as a topical anesthetic prior to intravitreal injection (IVI). The rats were intravitreally injected with  $\beta$ E2 (Sigma, St. Louis, MO) or control as described previously (Chiu et al. 2007; Mo et al. 2013). IVI was performed under red light illumination in a darkened room to preserve dark adaptation. After IVI, the rats were recovered for 4 h prior to damaging light exposure.

### LD

The male and female rats were dark-adapted for 24 h before exposure to light. Light damage (LD) was induced by exposing the eye to diffuse, cool, constant, white light at an intensity of 8,000 lx for 12 h, as previously conducted (Mo et al. 2013). Preceding light exposure, the pupils of the rats were dilated using 1 % tropicamide. After light exposure, the rats were placed in darkness for another 24 h and were subsequently returned to the standard light/dark cycle.

## ERG

Electroretinogram (ERG) was performed 1 day after LD and was recorded using an ERG recording system (TGC-350v, Chongqing Tektronix Corp., Chongqing, China). Prior to examination, the rats were dark-adapted for 24 h and were deeply anesthetized via intramuscular injection of sodium pentobarbital (50 mg/kg) and xylazine (6 mg/kg). At 30 min before anesthesia, the pupils were dilated using 1 % tropicamide and 0.5 % (w/v) proparacaine HCl. Full-field ERGs were recorded from both eyes using gold wire electrodes placed centrally on the cornea. All electrodes were connected to a two-channel amplifier. All procedures were performed in dim red light, and the rats were kept warm throughout the procedure. The Rod-ERG and Max-ERG recordings were performed as described previously. The amplitude (a) from the baseline to the negative trough of the a-wave, and the amplitude of the b-wave measured from the trough of the a-wave to the following peak of the b-wave.

## H&E Staining

Hematoxylin and eosin (H&E) staining was performed 7 days after LD. The rats were sacrificed following intraperitoneal injection of sodium pentobarbital (50 mg/kg). The eyeballs were harvested and fixed via immersion in 4 % (w/v) paraformaldehyde in PBS overnight. Then, the eyeballs were placed successively in 55, 65, 75, and 85 % (v/v) ethanol at room temperature for 1 h. Subsequently, the cornea and the lens were removed. The eyecups were placed successively in 95, 95, and 100 % (v/v) ethanol at room temperature for 1 h. The samples were immersed in chloroform for 10 min and then embedded in paraffin for sectioning. Subsequently, continuous serial sections (4  $\mu$ m thick) of the entire retina, including the optic nerve head, were stained with H&E. For each section, digitized images of the entire retina were captured using an Olympus microscope coupled to a digital camera at  $\times$ 400 magnification.

## ELISA

Total protein lysates of retinal tissue and blood plasma were collected and assayed using a Rat Estradiol (E2) Enzyme-Linked Immunosorbent Assay (ELISA) Kit according to the manufacturer's instructions (MyBioSource, San Diego, California, USA).

## MDA Assay

The malondialdehyde (MDA) levels in the retinal lysates were measured using an MDA assay kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

## qRT-PCR

Total RNA was extracted from the rat retina using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed (RT) using superscript reverse transcriptase and oligo(dT) primers. Quantitative reverse-transcriptase (qRT)-PCR was performed using an iQ5 instrument (BIO-RAD, Hercules, CA, USA) and SYBR<sup>®</sup>Premix Ex Taq<sup>™</sup> II (TaKaRa, Ohtsu, Shiga, Japan). The primer sequences for rat SOD1, SOD2, CAT, heme oxygenase-1 (HO-1), Gpx1, Gpx2, Gpx4, Nrf2, Trx1, Trx2, and  $\beta$ -actin are shown in Table 1. The gene expression levels were normalized to those of  $\beta$ -actin.

## Statistical Analysis

All data were expressed in the figures as the means  $\pm$  standard error of the mean (SEM). One-way ANOVA or Student's *t* test was performed to determine the statistical differences between the groups using SPSS16.0 software (SPSS, Chicago, IL, USA). *p* < 0.05 was considered to be statistically significant.

## Results

*Exposure to White Light for 12 h Induced LD* To rule out an effect of endogenous  $\beta$ E2, the female rats were ovariectomized (OVX) or sham-operated (see experiment overview in Fig. 1) as described previously (Green et al. 1999). A previous study reported that the body weight increased more in OVX than in sham-operated female Wistar rats (Hertrampf et al. 2006). To investigate whether ovariectomy induces a rapid body weight gain in our model, we measured the body weight after OVX or sham operation from 0 to 14 days. As shown in Fig. 2a, there was no significant difference in the rate of body weight gain between the sham-operated and OVX rats during these 14 days. However, this examination period after the ovariectomy operation may have been too short to reflect a change in the rate of body weight gain, and a significant difference may appear during an extended examination period.

Next, the female OVX rats were exposed to 8,000 lx white light for 12 h, and the changes in retinal function and morphology were assayed via ERG (1 day later) and H&E staining (7 days later), respectively. H&E staining showed that in the absence of light exposure, there was no clear change in the retinal morphology of OVX rats compared with sham-operated rats. However, after light exposure, the photoreceptors began to display destructive retinal changes, resulting in massive photoreceptor loss, pyknosis of the retinal cells, and disappearance of the outer plexiform layer (OPL) between the

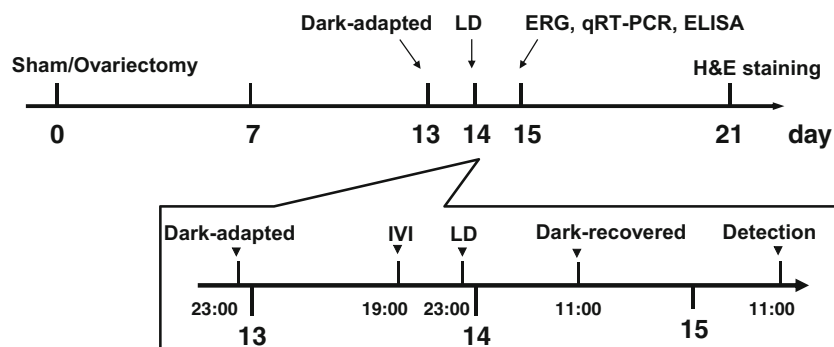
**Table 1** Information of primers for RT-qPCR

Primer	Forward (5'-3')	Reverse (5'-3')
SOD1	GCGGTCCAGCGGATGAAGAG	CCAATCACACCACAAGCCAAGC
SOD2	GCCTCCCTGACCTGCCTTAC	TGATAGCCTCCAGCAACTCTCC
CAT	GAGATGGCACACTTTGACAGAGAG	TGGAGAATCGGACGGCAATAGG
HO-1	GAACCTTTCAGAAGGGTCAGGTGTC	TGGCTGGTGTGTAAGGGATGG
GPx1	GGCTCACCCGCTCTTTACCTTC	CGCACTGGAACACCGTCTGG
GPx2	GCAACCAGTTCGGACATCAGGAG	AGAATGGGTCGTCATAAGGGTAGG
GPx4	AATTCGCAGCCAAGGACATCG	CCAGGATTTCGTAAACCACACTCG
Nrf2	TGCCATTAGTCAGTCGCTCTC	ACCGTGCCTTCAGTGTGC
Trx1	GCTTGTGGTAGTGGACTTCTCTG	GCAACATCCTGGCAGTCATCC
Trx2	TCGGTTAGAGAAGATGGTAGC	GGTAGGCACAGCAGACAC
$\beta$ -actin	GAGGGAAATCGTGCCTGAC	GCATCGGAACCGCTCATT

outer nuclear layer (ONL) and the inner nuclear layer (INL). Moreover, the thickness of the ONL and the INL were significantly reduced (Fig. 2b). These results indicated that the retinal structure was seriously disrupted after light exposure. Based on the ERG assay (Fig. 2c, d), there was also no functional damage in OVX rats compared with the sham-operated group, although the amplitudes of the a- and b-waves on the Rod-ERG and the Max-ERG were all significantly decreased after light exposure. Rod-ERG a-waves primarily correspond to the electrophysiological activity of retinal rod cells, whereas Rod-ERG b-waves, Max-ERG a-waves, and Max-ERG b-waves typically include the electrophysiological activities of multiple types of retinal neurons. These results demonstrated retinal function damage after light exposure. In summary, these results demonstrated that exposure to 8,000 lx for 12 h significantly disrupted retinal morphology and function.

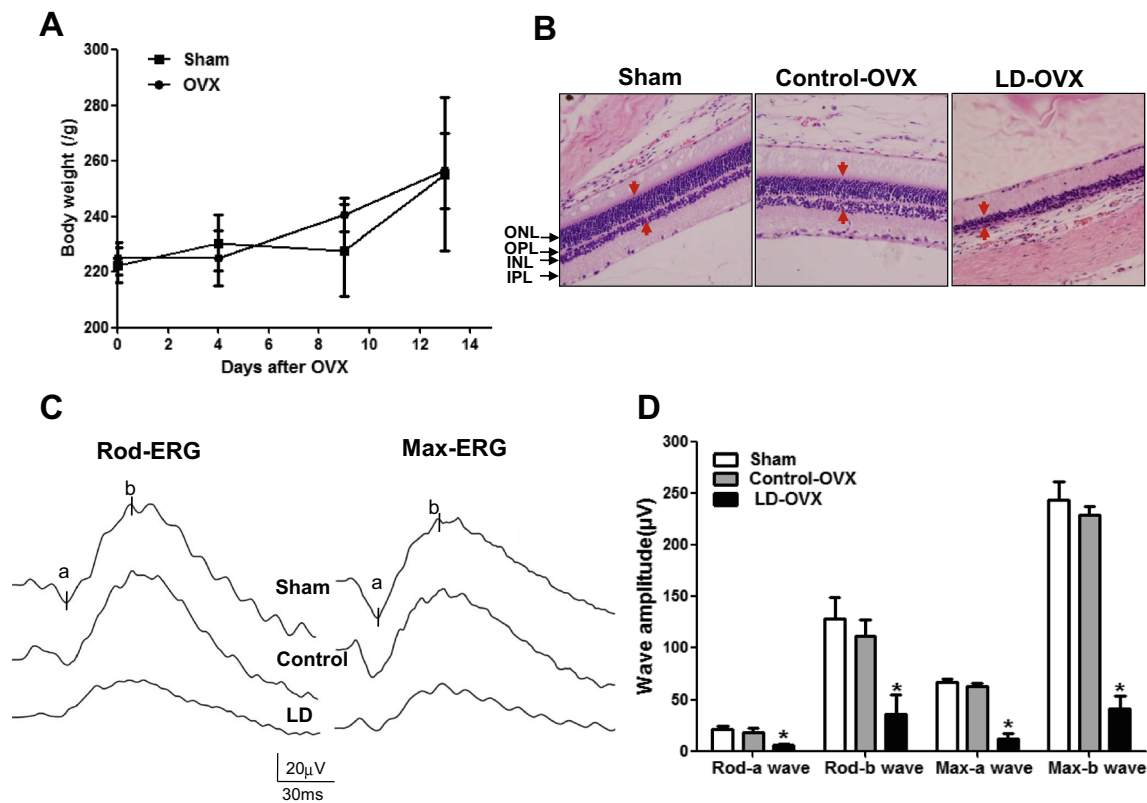
*The  $\beta$ E2 Concentration was Increased Following IVI* To evaluate the protective effect of  $\beta$ E2 on light-induced retinal damage, we administered  $\beta$ E2 (final concentration 10  $\mu$ M)

via IVI. To examine the efficacy of IVI, the concentration of  $\beta$ E2 in retinal lysates from each group was measured via an ELISA assay (1 day after injection). As shown in Fig. 3a, in the control OVX group, the  $\beta$ E2 concentration was significantly decreased compared with the sham-operated group; however, after IVI of  $\beta$ E2, the  $\beta$ E2 concentration was restored. To determine the sex-related differences, we also used male rats to examine the protective effect of  $\beta$ E2 on light-induced retinal damage. Consistent with the results using female rats, the  $\beta$ E2 concentration also increased following IVI into the male rats (Fig. 3b). In addition, we also measured the  $\beta$ E2 level in blood plasma, and the results indicated that the concentration of  $\beta$ E2 in plasma was also increased following IVI into both female and male rats (Fig. 3c, d). These results suggested that  $\beta$ E2 circulates throughout the blood plasma after IVI.  $\beta$ E2 is a sex hormone that plays an essential role in the growth and differentiation of the uterus, the cervix, the vagina, and the breasts. A previous study reported that  $\beta$ E2 in blood modulates uterine morphology and growth (Kang et al. 1975). To further validate that  $\beta$ E2 enters the bloodstream, we examined the effect of  $\beta$ E2 injection on the



**Fig. 1** Overview of the experimental design. After ovariectomy/sham surgery on day 0, the rats were subjected to weighing. On day 13 at 2300 hours, all animals were dark-adapted for 24 h. On day 14 at 1900 hours, the rats were intravitreally injected with  $\beta$ E2 (10  $\mu$ M) or control. After recovery for 4 h, the rats were exposed to light for 12 h and

then placed in darkness for another 24 h. The ERG assay, qRT-PCR, and ELISA were performed at 1100 hours on day 15; H&E staining was performed at 1100 hours on day 21. *IVI* intravitreal injection, *LD* light damage, *ERG* electroretinogram, *H&E* hematoxylin and eosin staining



**Fig. 2** Light-induced retinal damage. **a** The body weight gain of female OVX rats from day 0 to 14. **b** The morphological changes in the retina were measured via H&E staining following exposure to 8,000 lx white light for 12 h after 7-day dark recovery ( $\times 400$ ). ONL outer nuclear layer, INL inner nuclear layer, OPL outer plexiform layer, IPL inner plexiform layer.

layer. **c** The disruption of retinal function was measured via ERG after 1-day dark recovery. **d** Statistical analysis of the mean amplitudes of the a- and b-waves on Rod-ERG and Max-ERG. The data represent the means  $\pm$  SEM. \* $p < 0.05$  vs. control;  $n = 6$  (eye samples for each group)

uterine weight. The uterine weight was significantly reduced in the OVX rats compared with the sham-operated rats, and this difference was alleviated by  $\beta E2$  administration (Table 2). Taken together, these results demonstrated that  $\beta E2$  administration via IVI was effective. Notably, we found that following IVI,  $\beta E2$  circulates throughout the blood plasma.

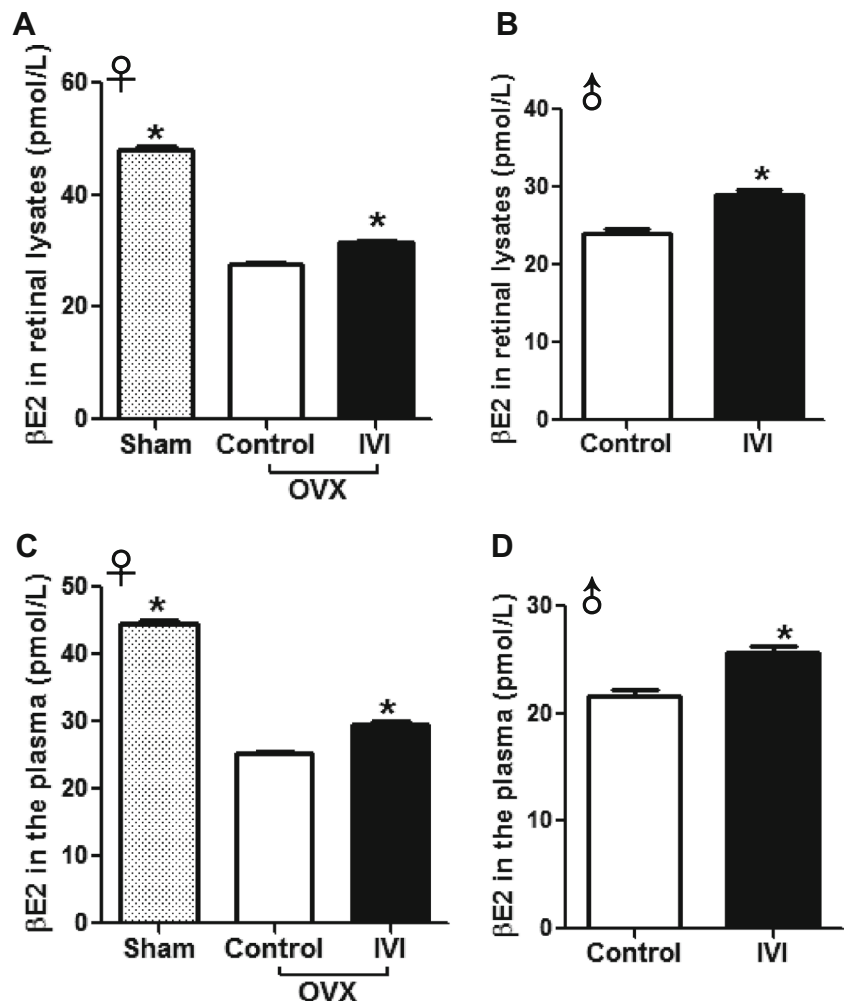
*$\beta E2$  Administration Protected the Retina from LD* To determine the protective effect of  $\beta E2$  on light-induced retinal damage, we administered  $\beta E2$  via IVI for 4 h prior to light exposure. As shown in Fig. 4a, a severe disruption of retinal morphology was detected in LD-induced rats, as described in Fig. 2b. However,  $\beta E2$  administration rescued this disruption in retinal structure by preventing the loss of photoreceptor cells and maintaining the thickness of the ONL and the INL. Based on the ERG assay, LD led to an excessive reduction in the mean amplitudes of the a- and b-waves on the Rod-ERG and Max-ERG. However,  $\beta E2$  administration protected retinal function by markedly increasing the mean amplitudes of the a- and b-waves (Fig. 4b). We also used male rats to examine the protective effect of  $\beta E2$ . In line with the results using female OVX rats, the male rats also displayed damaged retinal function and morphology, and  $\beta E2$  administration

improved retinal function based on ERG and H&E analyses (Fig. 4c, d). According to the above experimental results, we concluded that  $\beta E2$  mediates neuroprotection against retinal LD in both female OVX and male rats.

*$\beta E2$  Administration Decreased Light-Induced Oxidative Stress by Upregulating Antioxidant Enzymes* MDA, a product of lipid peroxidation, is considered as a biomarker of cellular oxidative stress. As oxidative stress increased following with exposure to light, we measured MDA generation using the thiobarbituric acid (TBA) test. As shown in Fig. 5a, b, MDA production was increased following light exposure in both female OVX and male rats, whereas administration of  $\beta E2$  significantly blocked the light-induced generation of MDA.

Previous studies indicated that  $\beta E2$  exerts its antioxidative effect by upregulating the expression of antioxidant genes. To further determine whether  $\beta E2$  affects antioxidant gene expression in our experiments, we first assessed the mRNA levels of the antioxidant enzymes superoxide dismutase (SOD)1, SOD2, catalase (CAT), and glutathione peroxidase (Gpx)1, Gpx2, and Gpx4 via qRT-PCR. As shown in Fig. 6a, in female OVX rats, light exposure led to a significant

**Fig. 3** The  $\beta$ E2 concentration was increased following IVI. ELISA was performed to measure the  $\beta$ E2 concentration in retinal lysates (a and b) and blood plasma (c and d) after IVI into female OVX (a and c) and male rats (b and d). \* $p$ <0.05 vs. control;  $n$ =4. The data are presented as the means $\pm$ SEM. ♀ female rats, ♂ male rats



reduction in SOD1, Gpx2, and Gpx4 expression, which were restored by  $\beta$ E2 administration. Although the expression of SOD, CAT, and Gpx1 was not reduced in response to light,  $\beta$ E2 administration also increased the expression of these genes. In male rats, LD did not reduce the mRNA level of these enzymes but exerted an increasing effect on the expression of Gpx1, Gpx2, and Gpx4, which was enhanced by  $\beta$ E2 administration (Fig. 6b). The expression of SOD1, SOD2, and CAT was also increased by  $\beta$ E2 administration. These results indicated that the antioxidant enzymes SOD, CAT, and Gpx are involved in the  $\beta$ E2-mediated protection against LD.

**Table 2** Effect of 17 $\beta$ -estradiol on uterine weight

Treatment group	Uterine weight (g)
Sham	0.54600 $\pm$ 0.719050
Control-OVX	0.20325 $\pm$ 0.102337*
$\beta$ E2-OVX	0.34675 $\pm$ 0.152281*#

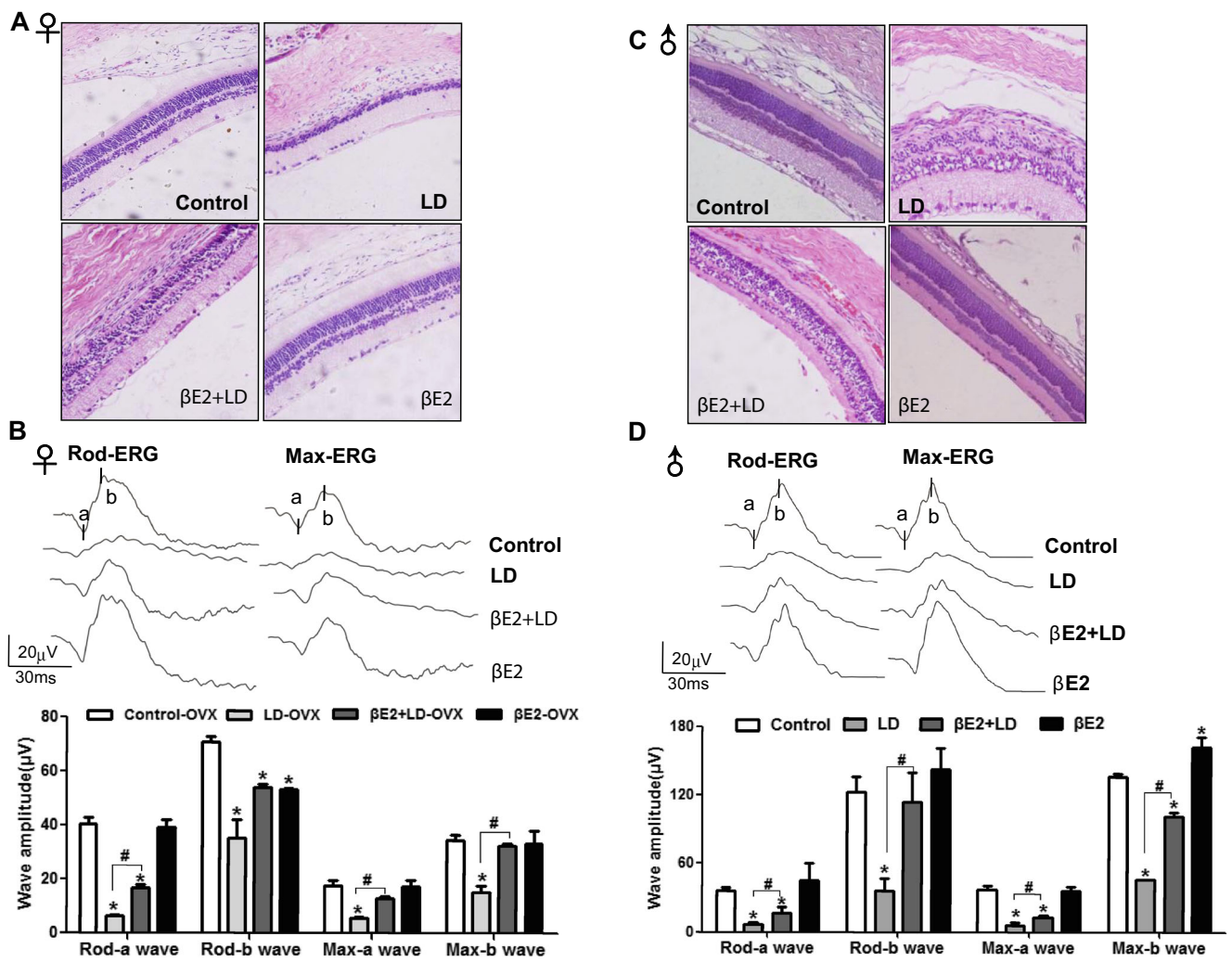
Data shown are as mean $\pm$ SEM

\* $p$ <0.05 vs. Sham; # $p$ <0.05 vs. control ( $n$ =6)

Moreover, there is a gender difference in the regulation of these antioxidant enzymes in response to light.

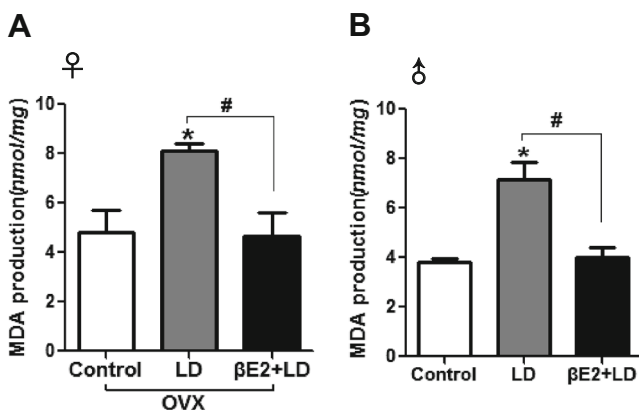
*The Antioxidant Proteins Trx and Nrf2, but not HO-1, Contribute to the Antioxidative Effect of  $\beta$ E2* Trx, thioredoxin reductase (TrxR), and nicotinamide adenine dinucleotide phosphate (NADPH) comprise the Trx system. Trx is a defense-related protein that is induced by various stresses and exerts antioxidative, anti-apoptotic, and anti-inflammatory effects. To examine whether Trx is involved in  $\beta$ E2-mediated antioxidation, the mRNA levels of Trx1 and Trx2 were determined via qRT-PCR. As shown in Fig. 7a, b, Trx2 expression, which decreased after light exposure, was more sensitive to light than Trx1 expression.  $\beta$ E2 administration blocked the downregulation of Trx2 expression and induced the expression of both Trx1 and Trx2.

HO-1 is a stress response-related enzyme that is induced by various oxidative agents. The activation of HO-1 appears to reflect an endogenous defense mechanism used by cells to reduce inflammation and tissue damage in several injury models. Unsurprisingly, the HO-1 expression level was



**Fig. 4**  $\beta$ E2 administration protects against light-induced retinal damage. The female OVX (a and b) or male rats (c and d) were treated with  $\beta$ E2 (10  $\mu$ M) via IVI for 4 h and then exposed to 8,000 lx white light for 12 h. The morphological and functional changes were determined via H&E

staining (a and c) and ERG assays (b and d), respectively. Bar graph represents statistical analysis of the mean amplitudes of the a- and b-waves on Rod-ERG and Max-ERG. The data represent the means  $\pm$  SEM;  $n=6$  (eye samples in each group). \* $p<0.05$  vs. control; # $p<0.05$

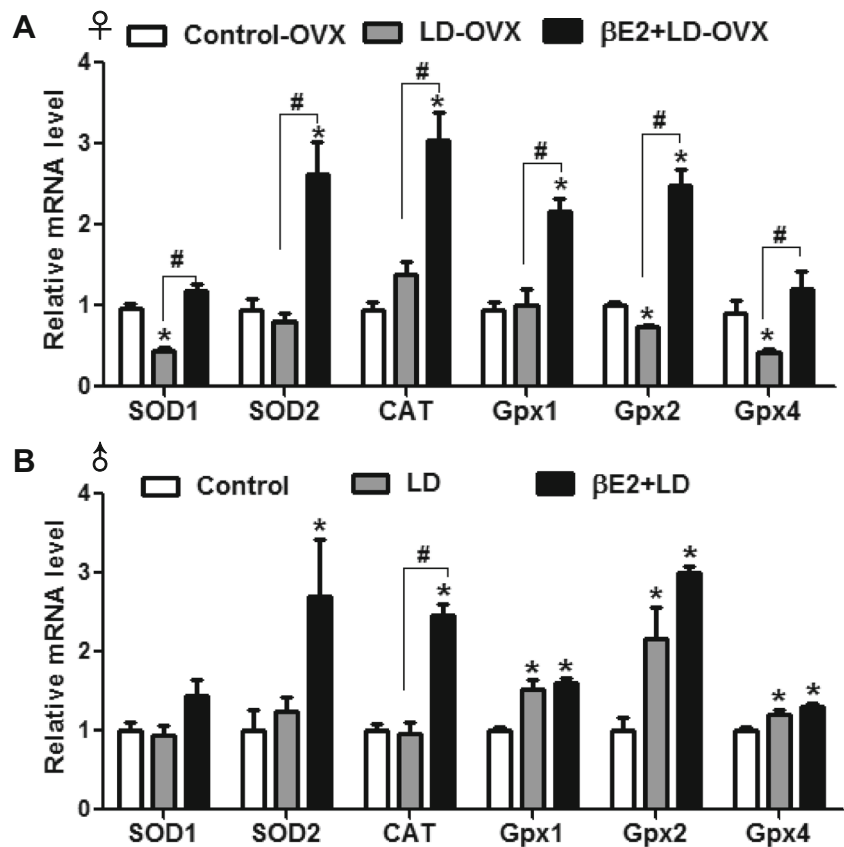


**Fig. 5**  $\beta$ E2 administration reduces MDA production. The generation of MDA in the retinal lysates from female OVX (a) and male (b) rats was assessed using an MDA assay kit. The data are presented as the means  $\pm$  SEM of four rats per group. \* $p<0.05$  vs. control; # $p<0.05$

significantly increased following LD to female OVX and male rats. However, during light exposure,  $\beta$ E2 administration did not affect HO-1 expression (Fig. 7c, d). These results suggested that HO-1 may be involved in an endogenous defense mechanism but not in the antioxidative effect of  $\beta$ E2.

Nrf2 is a key transcription factor that plays a central role in the cellular defense against oxidative stress. Several recent studies demonstrated that Nrf2 is also a key transcription factor in regulating the expression of a variety of cytoprotective genes in various types of cells/tissues. To investigate whether Nrf2 is also involved in the antioxidant effect of  $\beta$ E2, the Nrf2 expression level was assessed. There was no significant difference in Nrf2 expression after light exposure in female OVX rats, but Nrf2 expression was increased after light exposure in male rats (Fig. 7e, f). However, Nrf2 expression was increased following  $\beta$ E2 administration to all of the groups. Taken together, our results demonstrated

**Fig. 6**  $\beta$ E2 upregulates the expression of the antioxidant enzymes SOD, CAT, and Gpx. The female OVX (a) and male rats (b) were intravitreally injected with  $\beta$ E2 and then subjected to LD. The mRNA levels of SOD1, SOD2, CAT, Gpx1, Gpx2, and Gpx4 were quantified via qRT-PCR. The results are presented as the means  $\pm$ SEM of five rats per group and are expressed as the fold-change in expression after normalization to the expression of  $\beta$ -actin. \* $p$ <0.05 vs. control; # $p$ <0.05



that the antioxidant proteins Trx and Nrf2, but not HO-1, are involved in the antioxidative effect of  $\beta$ E2 against LD.

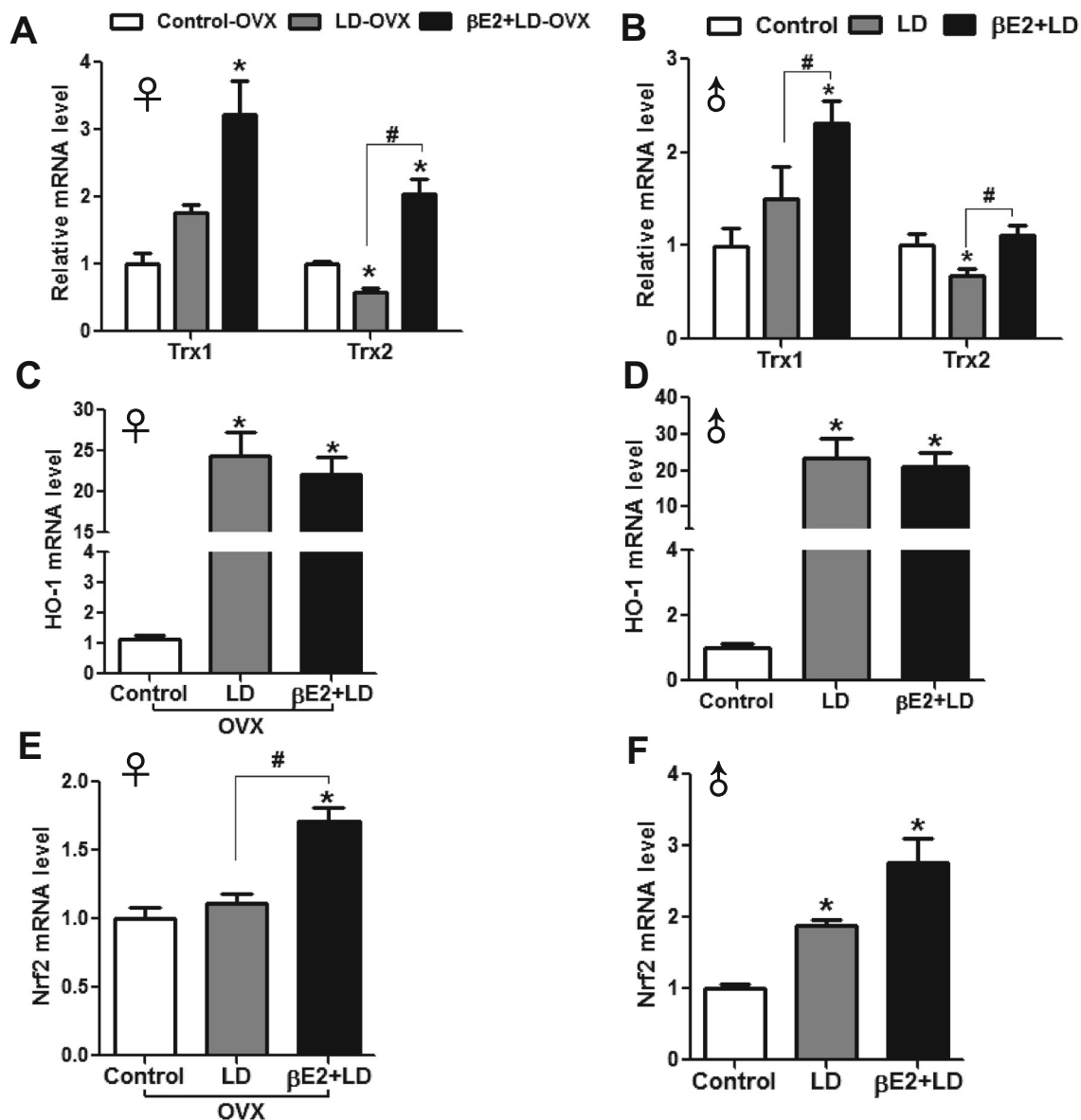
## Discussion

In the present study, we demonstrated an antioxidative effect of  $\beta$ E2 on light-induced retinal damage.  $\beta$ E2-mediated antioxidation is supported by several lines of evidence. First, exposure to white light for 12 h disrupted retinal morphology and function, and administration of  $\beta$ E2 prevented this disruption of retinal function (Figs. 2 and 4). These results suggest that  $\beta$ E2 mediates neuroprotection in this light-induced retinal damage model. Further analysis revealed that  $\beta$ E2 exerts its protective effect by decreasing MDA production, which is mediated by upregulation of the expression of the antioxidant genes SOD, CAT, and Gpx. Furthermore, the antioxidant proteins Trx and Nrf2 also contribute to this  $\beta$ E2-mediated antioxidation.

$\beta$ E2 is considered to display neuroprotective properties via anti-apoptosis and antioxidation. We previously reported that  $\beta$ E2 protects retinal neurons from light-induced apoptosis by inhibiting caspase-3 cleavage (Mo et al. 2013). Here, we found that  $\beta$ E2 protects retinal neurons against LD via its antioxidative effects. During light-induced retinal damage,

lipid peroxidation (MDA production) was increased in the retinal lysates, which resulted in a range of serious retinal oxidative injuries. However, IVI of  $\beta$ E2 abrogated this light-induced MDA production (Fig. 5). The homeostasis of ROS in the body is achieved largely by antioxidation via an intricate antioxidant system. On one hand, human antioxidants include enzymes, such as SOD, CAT, and Gpx (Wang and Chau 2010). On the other hand, human antioxidants also consist of low molecular weight antioxidants, such as reduced glutathione (GSH) and vitamins C and E, as well as non-catalytic antioxidant proteins, such as Trx and glutaredoxin (Grx). In our study, we demonstrated that  $\beta$ E2 mediated antioxidation by increasing the expression of both antioxidant enzymes (SOD, CAT, and Gpx) and non-catalytic antioxidant proteins (Trx and Nrf2) (Figs. 6 and 7). Trx, the transcript of a cytoprotective gene, is a regulator of cellular functions in response to stress, such as infectious agents, UV radiation, or  $H_2O_2$  (Watanabe et al. 2010). Here, we found that Trx1 expression was not changed due to light exposure, although Trx2 expression was reduced due to light exposure, suggesting differential roles of Trx1 and 2 in light-induced retinal damage. Furthermore, after administration of  $\beta$ E2, the expression levels of both Trx1 and Trx2 were increased compared with LD alone (Fig. 7a, b). HO-1 is a member of the heat shock protein family that can be upregulated in response to multiple stressful stimuli, such as heavy metals, endotoxin,





**Fig. 7** βE2 upregulates Trx and Nrf2 expression. The mRNA levels of Trx1 and Trx2 (a, b), HO-1 (c, d), and Nrf2 (e, f) were quantified via qRT-PCR in female OVX (a, c, and e) and male rats (b, d, and f). The data are

presented as the means±SEM and are expressed as the fold-change in expression after normalization to the expression of β-actin; n=5. \*p<0.05 vs. control; #p<0.05

heat shock, inflammatory cytokines, and prostaglandins. HO-1 is reported to be a cytoprotective enzyme, and its induction commonly occurs under conditions of increased cellular stress to help maintain physiological homeostasis (Stocker and Perrella 2006). In our study, HO-1 expression was significantly increased due to LD but was not increased following βE2 administration (Fig. 7c, d), and increasing HO-1 expression initiates an intrinsic defense system to protect against LD. The transcription factor Nrf2 is an important regulator of oxidative stress (Kensler et al. 2007). Nrf2 plays a key role in the transcriptional induction of various antioxidants, including SOD, CAT, Gpx, and HO-1(Kensler et al. 2007; Dong et al. 2008). Trx is also a target gene of Nrf2 (Kim et al. 2003). We

further indicated that Nrf2 also contributed to βE2-mediated antioxidation (Fig. 7e, f) in line with the report that βE2 can upregulate Nrf2 in nuclear extracts and increase the expression of HO-1, Cu/Zn-SOD, and GST during H/R injury (Yu et al. 2012). Several investigations reported that PI3K/Akt activate Nrf2 expression in many cell types (Wang et al. 2008; Mitsuishi et al. 2012). Our previous study indicated that 17β-estradiol protects against light-induced retinal damage via the PI3K/Akt signaling pathway. According to this result, we speculate that the PI3K/Akt-Nrf2 pathway may contribute to the antioxidative effect of βE2, and further examination is required to verify this hypothesis.

Another important finding in our study was the sex differences in the levels of these antioxidant enzymes and proteins in response to LD to male and female OVX rats. LD caused a reduction in SOD1, SOD2, Gpx2, and Gpx4 expression in female OVX rats, whereas LD caused the induction of Gpx1, Gpx2, and Gpx4 expression in male rats (Fig. 6a, b). Nrf2 expression did not change in the female OVX rats but was increased in male rats following light exposure (Fig. 7e, f). However, all of these antioxidant genes were upregulated in female OVX and male rats following administration of  $\beta$ E2. Despite their destructive activity, ROS are well-described second messengers in a variety of cellular processes, including tolerance to environmental stresses (Desikan et al. 2001), such as LD in our study. Whether ROS acts as a damaging or signaling molecule, it depends on the delicate equilibrium between ROS production and scavenging. In female rats, the expression levels of the antioxidant enzymes SOD1, SOD2, CAT, Gpx2, and Gpx4 were all decreased after ovariectomy compared with sham operation (data not shown), indicating decreased antioxidant activity in the female OVX rats. These decreases in antioxidant activity disrupt the equilibrium between ROS production and scavenging, hindering the compensatory response to light damage. However, in male rats, which were not subjected to ovariectomy, LD-induced ROS production may initiate the endogenous defense system and mediate the compensatory upregulation of antioxidant proteins (Gpx1,2,4 and Nrf2) in response to injury-related stresses. Consistent with our results, another report indicated that visible light may increase oxidative stress and cause the compensatory upregulation of antioxidant enzyme (SOD, CAT, and GSHPx) activities in frog eyes (Yusifov et al. 2000). In summary, in this study, we suggest that in male rats, the LD defense mechanism involves a dual mechanism consisting of, on one hand, the light-mediated initiation of the endogenous defense system (upregulation of Gpx, HO-1, and Nrf2 expression) and, on the other hand, the antioxidative effect of exogenous  $\beta$ E2 administration via IVI on LD. However, in female rats, exogenous  $\beta$ E2 is the predominant factor that induces the increase in SOD, CAT, Gpx, Trx2, and Nrf2 expression, and only HO-1 is involved in the endogenous defense mechanism in response to LD.

Aside from the antioxidative effect of  $\beta$ E2 on light-induced retinal damage, we found that 1 day after IVI, the  $\beta$ E2 concentration was increased in both retinal lysates and plasma (Fig. 3) and that the uterine weight was maintained in the OVX rats (Table 2), suggesting that  $\beta$ E2 circulates throughout the blood after IVI. IVI-mediated administration of various pharmacological drugs is commonly used to treat retinal diseases, such as diabetic retinopathy, AMD, macular edema, and retinal vein occlusion. The advantage of the IVI technique is its ability to maximize the intraocular levels of medications (Peyman et al. 2009), whereas elevated intraocular pressure and glaucoma are the most common side effects (Jonas et al.

2004; Sampat and Garg 2010). The blood–vitreous barrier and the blood–retinal barrier (BRB) might block drug distribution and diffusion following IVI of drugs; however, some drugs can permeate the BRB, such as anti-VEGF compounds (Semeraro et al. 2014). Here, we found that  $\beta$ E2 may also permeate the BRB and enter the blood circulation after IVI. However, this  $\beta$ E2 did not cause any systemic toxicity because the  $\beta$ E2 concentration in plasma was not higher than that from the sham group. Intravitreally injected  $\beta$ E2 rapidly attains a higher local drug concentration in the vitreous cavity and provides effective neuroprotection. Then,  $\beta$ E2 enters the circulation, which decreases the intraocular pressure, avoiding this side effect of IVI.

In conclusion, the results of this study demonstrated that  $\beta$ E2 protects against light-induced retinal damage via its antioxidative effect. These results provide a new approach to address the increasing AMD rate among postmenopausal women and estrogen replacement therapy.

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**Conflict of Interest** None.

## References

- Amtul Z, Wang L, Westaway D, Rozmahel RF (2010) Neuroprotective mechanism conferred by 17 $\beta$ -estradiol on the biochemical basis of Alzheimer's disease. *Neuroscience* 169:781–786
- Ba F, Pang PK, Davidge ST, Benishin CG (2004) The neuroprotective effects of estrogen in SK-N-SH neuroblastoma cell cultures. *Neurochem Int* 44:401–411
- Blanchet PJ, Fang J, Hyland K, Arnold LA, Mouradian MM, Chase TN (1999) Short-term effects of high-dose 17 $\beta$ -estradiol in postmenopausal PD patients: a crossover study. *Neurology* 53:91–95
- Chiu K, Chang RC, So KF (2007) Intravitreal injection for establishing ocular diseases model. *J Vis Exp* 313
- Desikan R, Mackerness SA-H, Hancock JT, Neill SJ (2001) Regulation of the Arabidopsis transcriptome by oxidative stress. *Plant Physiol* 127:159–172
- Dong J, Sulik KK, Chen SY (2008) Nrf2-mediated transcriptional induction of antioxidant response in mouse embryos exposed to ethanol in vivo: implications for the prevention of fetal alcohol spectrum disorders. *Antioxid Redox Signal* 10:2023–2033
- Feng Y, Wang B, Du F, Li H, Wang S, Hu C, Zhu C, Yu X (2013) The involvement of PI3K-mediated and L-VGCC-gated transient Ca<sup>2+</sup> influx in 17 $\beta$ -estradiol-mediated protection of retinal cells from H<sub>2</sub>O<sub>2</sub>-induced apoptosis with Ca<sup>2+</sup> overload. *PLoS One* 8:e77218
- Garcia-Segura LM, Azcoitia I, DonCarlos LL (2001) Neuroprotection by estradiol. *Prog Neurobiol* 63:29–60
- Green PG, Dahlqvist SR, Isenberg WM, Strausbaugh HJ, Miao FJ, Levine JD (1999) Sex steroid regulation of the inflammatory response: sympathoadrenal dependence in the female rat. *J Neurosci Off J Soc Neurosci* 19:4082–4089
- Hertrampf T, Degen GH, Kaid AA, Laudenbach-Leschowsky U, Seibel J, Di Virgilio AL, Diel P (2006) Combined effects of physical activity,

- dietary isoflavones and 17beta-estradiol on movement drive, body weight and bone mineral density in ovariectomized female rats. *Planta Med* 72:484–487
- Ishihara Y, Fujitani N, Kawami T, Adachi C, Ishida A, Yamazaki T (2014) Suppressive effects of 17beta-estradiol on tributyltin-induced neuronal injury via Akt activation and subsequent attenuation of oxidative stress. *Life Sci* 99:24–30
- Jarrett SG, Lin H, Godley BF, Boulton ME (2008) Mitochondrial DNA damage and its potential role in retinal degeneration. *Prog Retin Eye Res* 27:596–607
- Jonas JB, Kreissig I, Degenring RF (2004) Retinal complications of intravitreal injections of triamcinolone acetonide. *Graefes' archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle. Ophthalmologie* 242:184–185
- Kang YH, Anderson WA, DeSombre ER (1975) Modulation of uterine morphology and growth by estradiol-17beta and an estrogen antagonist. *J Cell Biol* 64:682–691
- Kensler TW, Wakabayashi N, Biswal S (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* 47:89–116
- Kim YC, Yamaguchi Y, Kondo N, Masutani H, Yodoi J (2003) Thioredoxin-dependent redox regulation of the antioxidant responsive element (ARE) in electrophile response. *Oncogene* 22:1860–1865
- Li H, Wang B, Zhu C, Feng Y, Wang S, Shahzad M, Hu C, Mo M, Du F, Yu X (2013) 17beta-estradiol impedes Bax-involved mitochondrial apoptosis of retinal nerve cells induced by oxidative damage via the phosphatidylinositol 3-kinase/Akt signal pathway. *J Mol Neurosci* 50:482–493
- Mandal MN, Patlolla JM, Zheng L, Agbaga MP, Tran JT, Wicker L, Kasus-Jacobi A, Elliott MH, Rao CV, Anderson RE (2009) Curcumin protects retinal cells from light-and oxidant stress-induced cell death. *Free Radic Biol Med* 46:672–679
- Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, Yamamoto M, Motohashi H (2012) Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell* 22:66–79
- Mo MS, Li HB, Wang BY, Wang SL, Zhu ZL, Yu XR (2013) PI3K/Akt and NF-kappaB activation following intravitreal administration of 17beta-estradiol: neuroprotection of the rat retina from light-induced apoptosis. *Neuroscience* 228:1–12
- Noell WK, Walker VS, Kang BS, Berman S (1966) Retinal damage by light in rats. *Investig Ophthalmol* 5:450–473
- Peyman GA, Lad EM, Moshfeghi DM (2009) Intravitreal injection of therapeutic agents. *Retina* 29:875–912
- Quintanilla RA, Munoz FJ, Metcalfe MJ, Hirschfeld M, Olivares G, Godoy JA, Inestrosa NC (2005) Trolox and 17beta-estradiol protect against amyloid beta-peptide neurotoxicity by a mechanism that involves modulation of the Wnt signaling pathway. *J Biol Chem* 280:11615–11625
- Ramezani A, Goudarzi I, Lashkarbolouki T, Ghorbanian MT, Salmani ME, Abrari K (2011) Neuroprotective effects of the 17beta-estradiol against ethanol-induced neurotoxicity and oxidative stress in the developing male rat cerebellum: biochemical, histological and behavioral changes. *Pharmacol Biochem Behav* 100:144–151
- Sampat KM, Garg SJ (2010) Complications of intravitreal injections. *Curr Opin Ophthalmol* 21:178–183
- Semeraro F, Morescalchi F, Duse S, Gambicorti E, Romano MR, Costagliola C (2014) Systemic thromboembolic adverse events in patients treated with intravitreal anti-VEGF drugs for neovascular age-related macular degeneration: an overview. *Expert Opin Drug Saf* 13:785–802
- Stocker R, Perrella MA (2006) Heme oxygenase-1: a novel drug target for atherosclerotic diseases? *Circulation* 114:2178–2189
- Taylor HR, Munoz B, West S, Bressler NM, Bressler SB, Rosenthal FS (1990) Visible light and risk of age-related macular degeneration. *Trans Am Ophthalmol Soc* 88:163–173, discussion 173–168
- Tripanichkul W, Sripanichkulchai K, Duce JA, Finkelstein DI (2007) 17Beta-estradiol reduces nitrotyrosine immunoreactivity and increases SOD1 and SOD2 immunoreactivity in nigral neurons in male mice following MPTP insult. *Brain Res* 1164:24–31
- Wang CY, Chau LY (2010) Heme oxygenase-1 in cardiovascular diseases: molecular mechanisms and clinical perspectives. *Chang Gung Med J* 33:13–24
- Wang L, Chen Y, Sternberg P, Cai J (2008) Essential roles of the PI3 kinase/Akt pathway in regulating Nrf2-dependent antioxidant functions in the RPE. *Invest Ophthalmol Vis Sci* 49:1671–1678
- Watanabe R, Nakamura H, Masutani H, Yodoi J (2010) Anti-oxidative, anti-cancer and anti-inflammatory actions by thioredoxin 1 and thioredoxin-binding protein-2. *Pharmacol Ther* 127:261–270
- Yu X, Tang Y, Li F, Frank MB, Huang H, Dozmorov I, Zhu Y, Centola M, Cao W (2005) Protection against hydrogen peroxide-induced cell death in cultured human retinal pigment epithelial cells by 17beta-estradiol: a differential gene expression profile. *Mech Ageing Dev* 126:1135–1145
- Yu J, Zhao Y, Li B, Sun L, Huo H (2012) 17beta-estradiol regulates the expression of antioxidant enzymes in myocardial cells by increasing Nrf2 translocation. *J Biochem Mol Toxicol* 26:264–269
- Yusifov EY, Kerimova AA, Atalay M, Kerimov TM (2000) Light exposure induces antioxidant enzyme activities in eye tissues of frogs. *Pathophysiol Off J Int Soc Pathophysiol* 7:203–207
- Zhang X, Wang J, Xing Y, Gong L, Li H, Wu Z, Li Y, Wang Y, Dong L, Li S (2012) Effects of ginsenoside Rg1 or 17beta-estradiol on a cognitively impaired, ovariectomized rat model of Alzheimer's disease. *Neuroscience* 220:191–200