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# 17 $\beta$ -estradiol ameliorates oxygen-induced retinopathy in the early hyperoxic phase



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## ABSTRACT

Retinopathy of prematurity (ROP) is a major and leading cause of blindness in premature infants. It has been realized that early treatment for ROP is important. However, all the early treatments of ROP are focusing on peripheral retinal ablation which does not surmount the limit of extinguishing retinal neovascularization and protecting the retinas of children with ROP from the injury of ablation. In this study, we investigated the morphological changes of retina and oxidative stress alterations in the early phase of oxygen-induced retinopathy (OIR) and tested the effects of 17 $\beta$ -estradiol (17 $\beta$ -E2), a nonselective estrogen receptor (ER) agonist, on early phase OIR development. We found that large central capillary-free areas were induced in the retinas of pups exposed to hyperoxia on postnatal day 9 (P9), whereas vascularization was almost complete in the retinas of pups exposed to normoxia at the same age. The concentrations of malondialdehyde (MDA), an end-product of oxidative stress, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a major enzyme producing free radicals, as well as the activity of NADPH oxidase were significantly elevated in the retinas of pups exposed to hyperoxia on P9 and postnatal day 13 (P13) compared to those in age matched pups exposed to normoxia. Treatment with 17 $\beta$ -E2 decreased not only the percentage of the central capillary-free area to total retina area but also the concentrations of MDA and NADPH oxidase as well as the activity of NADPH oxidase in a dose-dependent manner in pups exposed to hyperoxia on p9 and P13. The concentration of VEGF was significantly decreased on P9 but increased on P14 in the retinas of pups exposed to hyperoxia, whereas it was significantly elevated on P9 but decreased on P14 in the retinas of pups treated with 17 $\beta$ -E2. The effect of 17 $\beta$ -E2 could be reversed by the co-treatment with ICI182780, a high affinity estrogen receptor antagonist, which suggested that 17 $\beta$ -E2 might exert its effect on early hyperoxic phase of OIR through estrogen receptor. Our results suggest that treatment with antioxidant drugs at early hyperoxic phase of ROP even before the appearance of retinal neovascularization may be more effective than their application to ROP at late phase, which may abolish the deleterious factors that contribute to retinal neovascularization and promote retinal blood vessels to develop healthily.

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## 1. Introduction

Retinopathy of prematurity (ROP) is a disease that occurs in premature infants and is one of the most common causes of visual

loss in childhood and can lead to lifetime vision impairment and blindness [1]. It was found that the relatively high levels of oxygen routinely given to premature infants were an important risk factor, and that reducing the level of oxygen given to premature babies reduced the incidence of ROP [2,3]. Although newer technology and methods have been applied to monitor the oxygen levels when rescuing premature infants with oxygen, it is unavoidable for the premature infants to be exposed to relative high levels of oxygen. Therefore, for understanding the mechanisms of ROP, oxygen-induced retinopathy (OIR) is still used to generate ROP in animal models [4].

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Although different therapeutic treatments have been discovered, ROP remains as a major cause of blindness in premature infants, and the incidence of ROP is increasing in light of the rising numbers of preterm deliveries and improved neonatal care [1]. At present, most of the therapeutic treatments, including cryotherapy, photocoagulation and powerful anti-neovascular drugs, are focusing on depressing retinal neovascularization [1]. However, residual anatomical changes, the high prevalence of strabismus, amblyopia and high ametropia in those infants, and injury to the vulnerable retina caused by treatments themselves have made it difficult to restore impaired vision function appreciably by inhibiting retinal neovascularization [5–8]. Therefore, optimal treatment for ROP should be prior to retinal neovascularization, indicating that the best time for treating ROP should be in the early hyperoxic phase, not during the hypoxic phase.

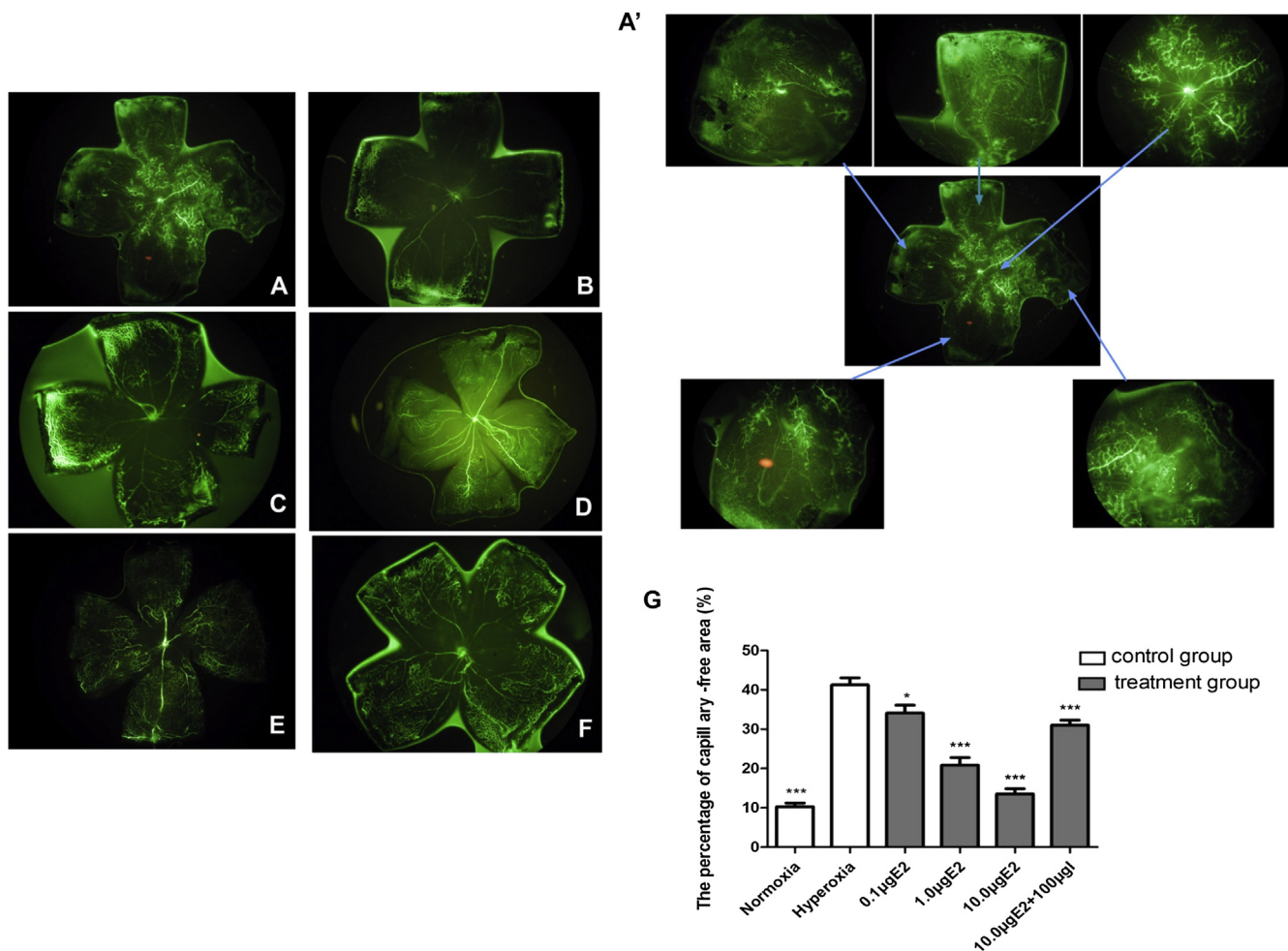
OIR in animal models can be divided into an early hyperoxic phase and a late hypoxic phase like ROP [9]. The morphological changes of retina in the early phase of OIR are more obvious than those in the late phase [10], indicating that to explore the mechanism of hyperoxia-induced vascular loss of OIR in the early phase, which is closely related to primary retinal vascular loss and late

neovascularization, may help to develop better therapeutic approaches for treating ROP. Oxidative stress plays a pivotal role in OIR. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a major enzyme producing free radicals, is crucial for OIR [11,12]. We and others have found that  $17\beta$ -estradiol ( $17\beta$ -E2), a nonselective estrogen receptor (ER) agonist, could effectively ameliorate later-phase OIR and decrease the concentration of malondialdehyde (MDA), an end-product of oxidative stress, on postnatal day 17 [13,14]. However, the role of  $17\beta$ -E2 on early hyperoxic phase of OIR has not been investigated. In this study, we examined the morphological changes of retina and oxidative stress alterations in the early hyperoxic phase of OIR and tested the effects of  $17\beta$ -E2 on OIR development during this phase.

## 2. Materials and methods

### 2.1. Oxygen exposure to induce retinopathy in pups

OIR was induced as described by Smith et al. and our previous publication [4,15]. In brief, 7-day-old C57BL/6j pups were randomly assigned to six experimental groups: Group 1, room air with vehicle



**Fig. 1.** Retinal flat mounts and the percentage of retinal avascular area on P9. A. Retinal vascularization was distributed to almost the whole retinas of normoxia control pups. The mature retinal vessels were found in the peripheral region while the immature retinal vessels were found in the central region as showed by the stained vessel wall. B. Large capillary-free areas were found in the retinas of hyperoxia alone exposure pups. C–E. The percentage of capillary-free area was decreased in the retinas of pups treated with hyperoxia plus different concentrations of  $17\beta$ -E2. F. The capillary-free area was broadened in the retinas of pups treated with hyperoxia plus 10.0  $\mu$ g of  $17\beta$ -E2 and 100.0  $\mu$ g of ICI182780. No neovascular tufts or clusters were found on the border of the avascular region and the vascular region in any of the retinal flat mounts. A'. Magnification of retinal flat mounts of normoxia control pups on P9. The blurred mature vessels in the whole retinal flat mounts of normoxia control pups on P9 obviously appeared under magnification ( $\times 4$ ). G. Statistical analysis of the difference in retinal avascular area represent with percentage of the percentage of the capillary-free area to total retina area between normoxia and hyperoxia groups as well as between hyperoxia alone exposure group and hyperoxia plus different concentrations of  $17\beta$ -E2 with or without ICI182780 groups ( $n = 6$  in each group). \* $P < 0.05$ , \*\*\* $P < 0.01$ . I is the abbreviation of ICI182780.

injection (control); Group 2, hyperoxia ( $75\% \pm 2\% \text{ O}_2$ ) with vehicle injection; Group 3, hyperoxia ( $75\% \pm 2\% \text{ O}_2$ ) plus  $0.1 \mu\text{g}$  of  $17\beta\text{-E}_2$  (Sigma–Aldrich, St. Louis, MO, USA) injection; Group 4, hyperoxia ( $75\% \pm 2\% \text{ O}_2$ ) plus  $1.0 \mu\text{g}$  of  $17\beta\text{-E}_2$  injection; Group 5, hyperoxia ( $75\% \pm 2\% \text{ O}_2$ ) plus  $10.0 \mu\text{g}$  of  $17\beta\text{-E}_2$  injection; and Group 6, hyperoxia ( $75\% \pm 2\% \text{ O}_2$ ) plus  $10.0 \mu\text{g}$  of  $17\beta\text{-E}_2$  and  $100.0 \mu\text{g}$  of ICI182780 (an antagonist of estrogen receptors) (Tocris Bioscience, Ellisville, IL, USA) injection. In general, pups were injected subcutaneously with different doses of  $17\beta\text{-E}_2$  (dissolved in ethanol and diluted in  $0.05 \text{ ml}$  PBS (phosphate-buffered saline)/pup) or vehicle alone from postnatal day 7 (P7) to P13 daily (14), whereas pups treated with the hyperoxia and  $17\beta\text{-E}_2$  were exposed to hyperoxia ( $75\% \pm 2\% \text{ O}_2$ ) plus  $17\beta\text{-E}_2$  from P7 to P12 and then exposed to room air from P12 to P13. The pups for normoxia treatment were kept in room air from P7 to P13. The pups were sacrificed on P9 and P13, respectively.

## 2.2. Retinal flat mounts and quantification of the avascular area

Retinal flat mounts were obtained by perfusing the pups' left ventricles with high-molecular-weight dextran ( $2,000,000 \text{ Da}$ ) conjugated with fluorescein (Sigma–Aldrich, St. Louis, MO, USA) as previously described [4,15,16]. A fluorescence microscope was applied to capture the images of the superficial blood vessel layers. The total retinal and central capillary-free areas were measured with Optimas software, version 5.2 (Meyer Instruments, Inc., Houston, TX, USA), and the central capillary-free area was represented as a percentage of the total retinal area.

## 2.3. Measurement of retinal lipid peroxidation concentrations

The retinal lipid peroxidation concentrations on P9 and P13 were determined with MDA assay kit (Nanjing Jiancheng Bioengineering Ins, Nanjing, China) as described in our previous publications [15,17,18], and were represented as nanomole of MDA per mg proteins.

## 2.4. Measurement of retinal NADPH oxidase concentrations

The retinal NADPH oxidase concentrations on P9 and P13 were assayed with EnVision immunohistochemistry kit (Genmed Scientifics Inc., Wilmington, DE, USA) as described in our previous publication [15].

## 2.5. Measurement of retinal NADPH oxidase activity

The NADPH oxidase activity in the retinas was determined on P9 and P13 with the NADPH oxidase activity assay kit (Genmed Scientifics Inc., Wilmington, DE, USA) as described in our previous publications [15,19], and was represented as millimoles of NADPH per minute per milligram of proteins.

## 2.6. Measurement of retinal VEGF concentrations

VEGF concentrations in the retinas were measured on P9 and P14 with enzyme linked immunosorbent assay (ELISA) kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as described in our previous publication [15].

## 2.7. Statistical analysis

Data were analyzed with GraphPad Prism software, version 5.01 (GraphPad Software Inc., La Jolla, CA, USA). For each test, a value of  $P < 0.05$  was considered statistically significant.

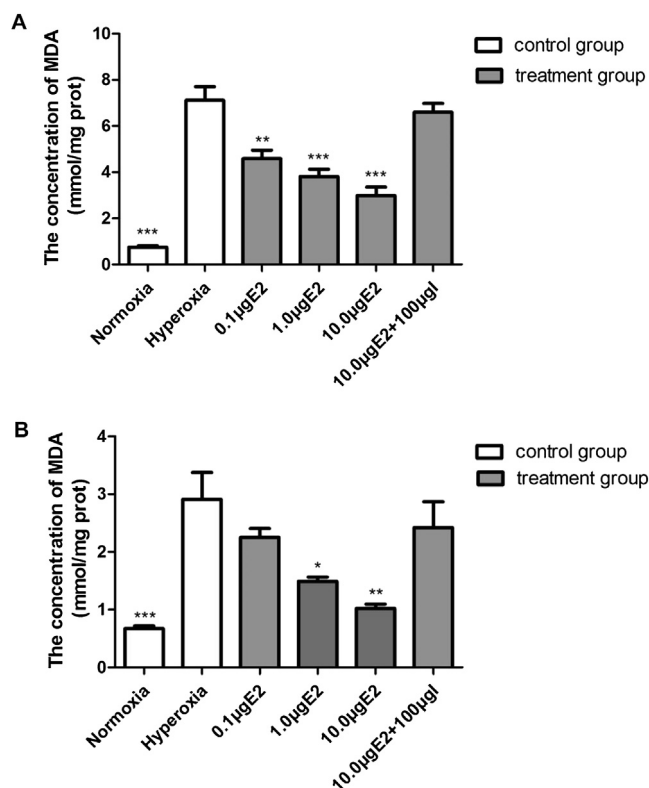
## 2.8. Ethics statement

All the experiments were approved by the Eye Institutional Committee of Shaanxi Province for Animal Use in Research and Education and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

## 3. Results

### 3.1. Oxidative stress induces retinopathy in the early hyperoxic phase and $17\beta\text{-E}_2$ treatment blocks oxygen induced OIR at this phase

To investigate whether oxidative stress was able to induce retinopathy in the early hyperoxic phase, we exposed pups to normoxia and hyperoxia conditions from postnatal day 7 (P7) to 9 (P9). We found that pups exposed to hyperoxia induced considerable vascular changes in retina on P9 compared to that in pups exposed to room air. In the normoxia (room air) treated pups (group 1), retinal vascularization on P9 was almost completed characterized by its maturation in the peripheral zone and partial immaturation in the central zone, (Fig. 1A and 1A'), whereas large retinal capillary-free areas were found in the central zones of retina in pups exposed to hyperoxia ( $75\% \pm 2\% \text{ O}_2$ ) condition (group 2) (Fig. 1B). This result suggested that oxidative stress can induce retinopathy in the early hyperoxic phase. To investigate the effect of  $17\beta\text{-E}_2$  on hyperoxia induced OIR, we treated the pups with hyperoxia plus different concentrations of  $17\beta\text{-E}_2$ . We found that in



**Fig. 2.** MDA concentration in the retina on P9 and P13. A and B. Retinal MDA concentration was significantly increased in hyperoxia alone exposure P9 (A) and P13 (B) pups compared to that in normoxia control pups, and was significantly decreased upon hyperoxia plus different concentrations of  $17\beta\text{-E}_2$  treatment at P9 (A) and P13 (B) pups. No significant difference was found between hyperoxia alone exposure pups and hyperoxia plus  $10.0 \mu\text{g}$  of  $17\beta\text{-E}_2$  and  $100.0 \mu\text{g}$  of ICI182780-treated pups. \* $P < 0.05$ , \*\* or \*\*\* $P < 0.01$ . I is the abbreviation of ICI182780.

the hyperoxia plus 17 $\beta$ -E2 treated groups (group 3, 4, and 5), the percentage of the central capillary-free area to total retina area was decreased in a dose-dependent manner, in which treatment with hyperoxia plus 10.0  $\mu$ g of 17 $\beta$ -E2 had the most striking effect on reducing the central capillary-free area (Fig. 1C, D and E) ( $n = 6$  per group,  $p < 0.05$  between any two groups). However, the decrease of the capillary-free area induced by treatment with hyperoxia plus 10.0  $\mu$ g of 17 $\beta$ -E2 could be blocked in the retinas of the pups co-treated with 100.0  $\mu$ g of ICI182780, a high affinity estrogen receptor antagonist (group 6) (Fig. 1F), which suggested that 17 $\beta$ -E2 may be through estrogen receptor to ameliorate oxygen induced OIR at this phase. In addition, no neovascular tufts or clusters were found on the border of the avascular zone and the vascular zone in any of the retina flat mounts. Our statistical analysis indicated that the percentage of the capillary-free area to total retina area between normoxia and hyperoxia groups as well as between hyperoxia alone exposure group and hyperoxia plus different concentrations of 17 $\beta$ -E2 with or without ICI182780 groups were significant changed ( $n$  in each group = 6,  $P < 0.05$ ) (Fig. 1G).

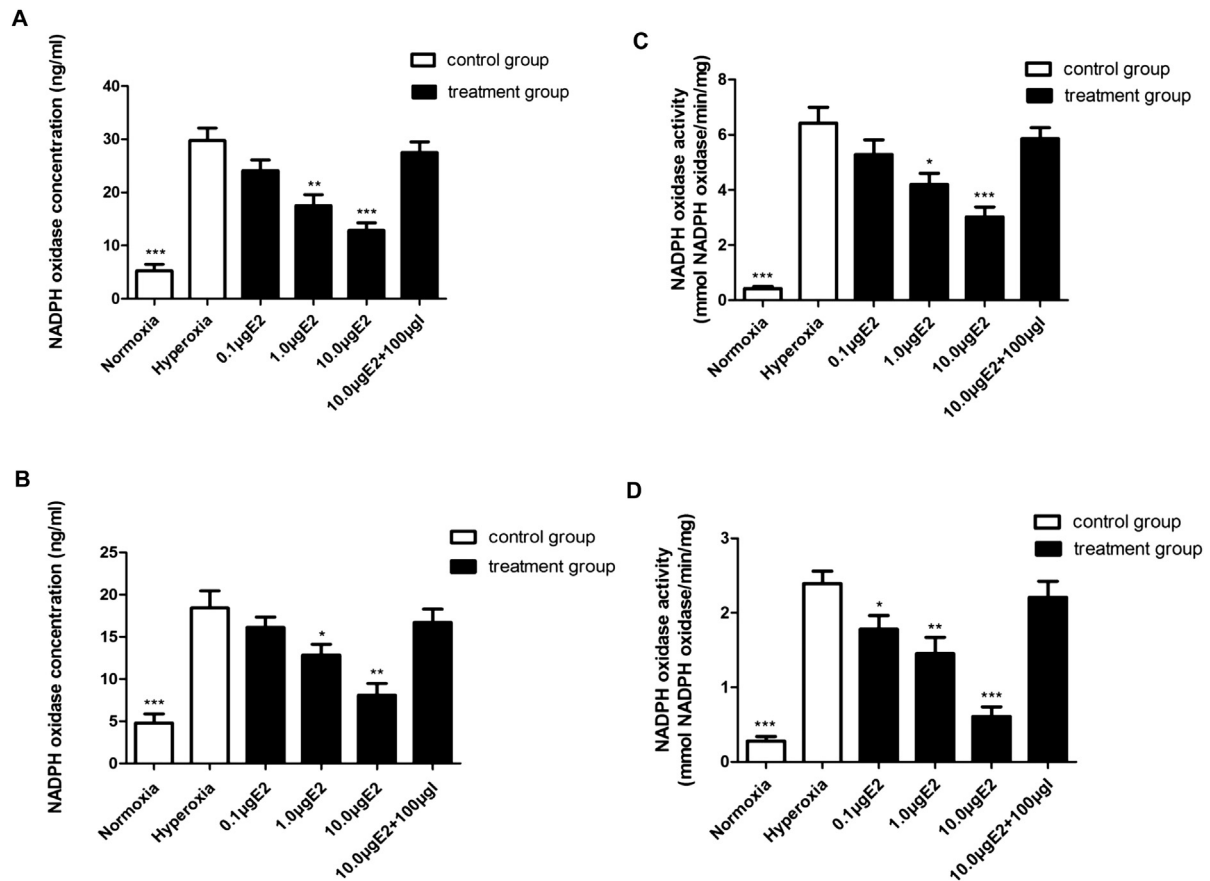
### 3.2. Retinal lipid peroxidation characterized by MDA levels was increased in early hyperoxic phase of OIR and could be decreased by 17 $\beta$ -E2 treatment

To evaluate the retinal lipid peroxidation in early hyperoxic phase of OIR, we measured the concentrations of malondialdehyde

(MDA), an end-product of oxidative stress [13,14], in different experimental groups. We first found that MDA levels were significantly increased in the retinas of the hyperoxia-exposed pups compared to those in age-matched normoxia control pups ( $P < 0.01$  on P9 and  $P < 0.05$  on P13) (Fig. 2A and B), whereas MDA levels in the retinas of the normoxia control pups were not significantly changed as analyzed on P9 and on P13 ( $P > 0.05$ ). Treatment with different concentrations of 17 $\beta$ -E2 significantly decreased the levels of MDA in the retinas compared to those in age matched pups treated with hyperoxia alone, and the effect of 17 $\beta$ -E2 was more significant on P9 pups than that on P13 pups ( $F = 38.56$ ,  $P < 0.05$  on P9 and  $F = 10.07$ ,  $P < 0.05$  on P13) (Fig. 2A and B). As expectation, the lowest MDA level was found in the retinas of hyperoxia plus 10.0  $\mu$ g 17 $\beta$ -E2 treated pups ( $P < 0.01$  on P9 and P13) compared to the hyperoxia alone exposed pups although it was still higher than that in the retinas of the normoxia-exposed pups ( $P < 0.01$ , on P9 and P13) (Fig. 2A and B). The effect of 17 $\beta$ -E2 on reducing the levels of MDA in hyperoxia plus 17 $\beta$ -E2 treated pups could be markedly reversed by co-treatment with ICI182780 ( $P < 0.01$  on P9 and  $P < 0.05$  on P13, respectively) (Fig. 2A and B).

### 3.3. NADPH oxidase concentrations and activity were increased in early OIR and could be normalized by 17 $\beta$ -E2 treatment

It has been reported that NADPH oxidase, a major enzyme producing free radicals, is crucial for OIR [11,12]. We further found



**Fig. 3.** NADPH oxidase concentration and activity in the retina on P9 and P13. A and B. Retinal NADPH oxidase concentration was significantly increased in hyperoxia alone exposure P9 (A) and P13 (B) pups compared to that in normoxia control pups, and was significantly decreased upon hyperoxia plus different concentrations of 17 $\beta$ -E2 treatment at P9 (A) and P13 (B) pups. No significant difference was found between hyperoxia alone exposure pups and hyperoxia plus 10.0  $\mu$ g of 17 $\beta$ -E2 and 100.0  $\mu$ g of ICI182780-treated pups. \* $P < 0.05$ , \*\* or \*\*\* $P < 0.01$ . I is the abbreviation of ICI182780. C and D. Retinal NADPH oxidase activity was significantly increased in hyperoxia alone exposure P9 (C) and P13 (D) pups compared to that in normoxia control pups, and was significantly decreased upon hyperoxia plus different concentrations of 17 $\beta$ -E2 treatment at P9 (C) and P13 (D) pups. No significant difference was found between hyperoxia alone exposure pups and hyperoxia plus 10.0  $\mu$ g of 17 $\beta$ -E2 and 100.0  $\mu$ g of ICI182780-treated pups. \* $P < 0.05$ , \*\* or \*\*\* $P < 0.01$ . I is the abbreviation of ICI182780.



that the concentration and activity of NADPH oxidase was not significantly changed in the retinas of the normoxia group on P9 and P13 ( $P > 0.05$ ) whereas the concentration and activity of NADPH oxidase was increased significantly in the retinas of the hyperoxia alone exposed pups both on P9 and on P13 versus the normoxia control pups ( $P < 0.01$ ) (Fig. 3). The concentration and activity of NADPH oxidase were markedly reduced in the retinas of pups treated with hyperoxia plus 17 $\beta$ -E2 compared to that in hyperoxia alone exposed pups. The lowest level of NADPH oxidase was found in the retinas of the pups treated with hyperoxia plus 10.0  $\mu$ g of 17 $\beta$ -E2, while it could be significantly reversed by co-treatment with ICI182780 on P9 and P13 pups ( $P < 0.01$  both on P9 and on P13) (Fig. 3).

### 3.4. VEGF concentrations were decreased in early stage of early hyperoxic phase of OIR but increased in later stage of early hyperoxic phase of OIR and could be normalized by 17 $\beta$ -E2 treatment at both stages

There was no significant difference of VEGF concentrations was found in the retinas of the normoxia group on P9 and P14 ( $P > 0.05$ ), whereas VEGF concentrations were decreased on P9 but increased on P14 in the retinas of the hyperoxia alone exposed pups versus

the normoxia control pups ( $P < 0.01$ ) (Fig. 4A and B). Treatment with different concentrations of 17 $\beta$ -E2 gradually increased VEGF concentrations in retinas in P9 pups compared to that in hyperoxia alone exposed pups, and the highest level was found in 10.0  $\mu$ g 17 $\beta$ -E2-treated P9 pups ( $P < 0.05$ ) (Fig. 4A and B). However, increased VEGF concentrations in retinas of pups induced by hyperoxia alone exposure from P7 to P14 could be decreased by the co-treatment with different concentrations of 17 $\beta$ -E2 and the lowest level of VEGF in retinas was found in pups with hyperoxia plus 10.0  $\mu$ g 17 $\beta$ -E2 treatment versus the hyperoxia alone exposed pups ( $P < 0.01$ ) (Fig. 4A and B). The VEGF concentrations in the retinas of the 10.0  $\mu$ g of 17 $\beta$ -E2-treated pups on P9 were not significantly reversed by ICI182780 ( $P > 0.05$ , versus the 10.0  $\mu$ g of 17 $\beta$ -E2-treated group), while the decreased VEGF concentrations in the retinas of the 10.0  $\mu$ g of the 17 $\beta$ -E2-treated pups on P14 could be significantly reversed by ICI182780 on P14 ( $P < 0.01$ , versus the 10.0  $\mu$ g of 17 $\beta$ -E2-treated group) (Fig. 4A and B).

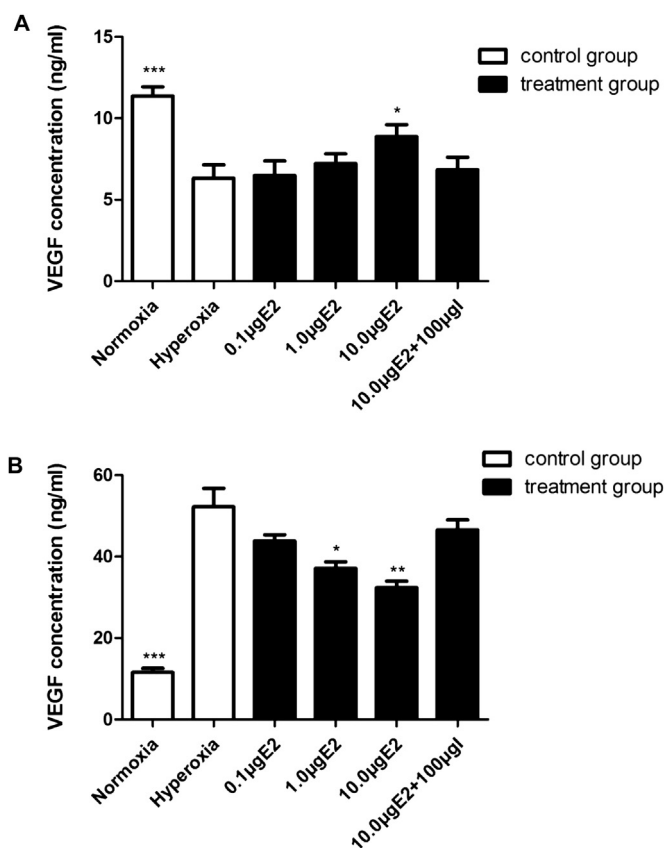
## 4. Discussion

Due to the complications with the traditional treatment of ROP, researchers have begun to explore the feasibility of early treatment for ROP, and exciting results have been achieved in terms of structural outcomes and graded visual acuity [20]. However, all the early treatments of ROP are focusing on peripheral retinal ablation which does not surmount the limit of extinguishing retinal neovascularization and protecting the retinas of children with ROP from the injury of ablation. We propose that an effective treatment of ROP should be started earlier than the appearance of retinal neovascularization, which may abolish the deleterious factors that contribute to retinal neovascularization and promote retinal blood vessels to develop healthily.

In this study, we found that the retinal vascularization was almost complete on normoxia-exposed pups at P9 while large capillary-free areas were found in the retinas of hyperoxia-exposed pups at the same age, indicating that marked morphological and pathological retinopathy could occur during the first two days of OIR and is consistent with the chronological development of retinal capillary-free areas found by others [10]. The capillary-free areas in the retinas of hyperoxia-exposed pups on P9 were overlapped with the central zones with immature retinal vessels of the retinas of normoxia-exposed pups on P9, suggesting that hyperoxia might only damage immature vessels but not mature vessels during the development of early hyperoxic phase of OIR.

The obvious retinal morphological alterations of OIR in the early hyperoxic phase encouraged us to explore the mechanism of OIR during this phase. We found that MDA and NADPH oxidase concentrations and NADPH activity were increased in the retinas of P9 pups exposed to hyperoxia, which was closely related to oxidative stress and resulted in the formation of capillary-free areas. Although antioxidant drugs were used to treat ROP many years ago [21], however, unsatisfactory clinical results discouraged their further application to ROP [22,23]. According to our results and other relevant studies [22–24], improper treating time may be responsible for those unsatisfactory results. Thus, our results suggest that treatment with antioxidant drugs at early hyperoxic phase of ROP may be more effective than their application to ROP at late phase.

To support this notion, we present for the first time that 17 $\beta$ -E2 as a nonselective ER agonist can ameliorate early hyperoxic phase of OIR (P9 and P13), as it does on late phase (P17) of OIR [13,14,25]. Since capillary-free areas in the early hyperoxic phase are crucial for neovascularization in late phase, early 17 $\beta$ -E2 administration which decreases the percentage of the central capillary-free area to



**Fig. 4.** VEGF concentration on P9 and P14. A. Retinal VEGF concentration on P9 was significantly decreased in the hyperoxia alone exposure pups compared to that in the normoxia control pups, and was significantly increased upon hyperoxia plus 10.0  $\mu$ g of 17 $\beta$ -E2 treatment compared to that in hyperoxia alone exposure pups. \* $P < 0.05$ , \*\*\* $P < 0.01$ . I is the abbreviation of ICI182780. B. Retinal VEGF concentration on P14 was significantly increased in the hyperoxia alone exposure pups compared to that in the normoxia control pups, and was markedly decreased in the hyperoxia plus 1.0 and 10.0  $\mu$ g of 17 $\beta$ -E2 treated pups compared to that in hyperoxia alone exposure pups. No significant difference was found between hyperoxia alone exposure pups and hyperoxia plus 10.0  $\mu$ g of 17 $\beta$ -E2 and 100.0  $\mu$ g of ICI182780-treated pups. \* $P < 0.05$ , \*\* or \*\*\* $P < 0.01$ . I is the abbreviation of ICI182780.

total retina area should be more effective in treating OIR than its late administration.

17 $\beta$ -E2 can bond with estrogen receptor (ER)  $\alpha$  and  $\beta$  which are distributed not only in the cytosol but also on the cell membrane to exert its rapid membrane-initiated steroid signaling (MISS), such as anti-oxidative stress, and its durable nuclear-initiated steroid signaling (NISS), such as the transcription and expression of the VEGF gene [26,27]. It is highly possible that 17 $\beta$ -E2 can exert its role in ameliorating OIR in early hyperoxic phase by rapidly bonding with ER $\alpha$  and  $\beta$  on the cell membrane to exert its anti-oxidative stress function and promote retinal vessel development through MISS, and in late hypoxic phase through NISS to modulate oxidative stress and retinal neovascularization.

In sum, we found that striking alterations in retinal morphology and oxidative stress occurred in early phase of OIR. It should be more efficient to treat ROP with antioxidant drugs during the early phase than that during the late phase.

### Conflict of interest

All authors have non-financial interest in the subject matter or materials discussed in this article.

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