

Original Research Article

Effect of early exogenous supplementation of rhIGF-1 on oxygen-induced retinopathy in a mice model of prematurity, and on expressions of IGF-1 and VEGF

Xiangjun Wu, Hui Ye, Qiao Cai, Yuanxiang Ke, Danying Wang*

Department of Pediatrics, Taizhou First People's Hospital, Taizhou 318020, People's Republic of China

*For correspondence: **Email:** zjchs7@163.com

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Abstract

Purpose: To investigate the effect of early exogenous supplementation of recombinant human insulin-like growth factor (rhIGF-1) on oxygen-induced mouse model of retinopathy of prematurity (ROP).

Methods: Three groups of healthy SPF grade C57BL/6 mice were used in this study, with 20 mice in each group. Hyperoxia saline (HS) and hyperoxia rhIGF-1 (HrGF) groups were placed in a closed oxygen chamber for one week and returned to the normal environment on the 15th day. The hyperoxia rhIGF-1 (HrGF) group was intraperitoneally injected with rhIGF-1 (1.5 mg/kg), while mice in high-oxygen saline (HS) group received normal saline. The air group (AG) was untreated. Changes in retinal blood vessel distributions, expression levels of serum IGF-1 and VEGF, and retinal IGF-1 and VEGF were determined.

Results: On day 20, pronounced neo-vascularization was observed, but the distribution was disordered. Serum IGF-1 levels in AG and HrGF were significantly higher than that in HS group, but VEGF level was lower in HS mice ($p < 0.05$). VEGF level in hyperoxia rhIGF-1 group on days 11 and 15 decreased, relative to control value, while retinal IGF-1 and VEGF in AG and hyperoxia rhIGF-1 mice were elevated, relative to corresponding values in HS mice ($p < 0.05$).

Conclusion: Early exogenous supplementation of rhIGF-1 exerts a therapeutic effect on ROP. Thus, rhIGF-1 may be a potential drug regimen for ROP in clinics.

Keywords: Oxygen-induced retinopathy, rhIGF-1, Premature infants, IGF-1, VEGF

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INTRODUCTION

Retinopathy of prematurity (ROP) is also known as post-lens fiber hyperplasia (RLF). It refers to immature retinal blood vessels in premature infants which result in retinal neovascularization and fibrous tissue proliferation, the major causes of blindness in children [1,2]. The incidence of ROP in China is increasing year by year, and it

seriously affects quality of life of the patients. Studies have found that ROP is closely related to premature birth, low birth weight and high concentration of oxygen. Exogenous recombinant human insulin-like growth factor-1 (rhIGF-1) is a multifunctional cell proliferation regulator that induces and promotes cell differentiation [3].

Vascular endothelial growth factor (VEGF) promotes angiogenesis and inhibits the proliferation of abnormal neovascularization in the eye [4]. At present, ROP is treated through direct inhibition of angiogenesis. However, apart from not providing a fundamental solution to ROP, this treatment results in irreparable damage to the patient [5]. In the present study, the effects of early exogenous rhIGF-1 supplementation on oxygen-induced mice model of ROP and the expressions of IGF-1 and VEGF were investigated.

EXPERIMENTAL

Laboratory animals

Sixty newly-littered and healthy SPF grade C57BL/6 mice pups weighing 16 ± 4 g were provided by Shanghai Ruitai Moss Biotechnology Co. Ltd. [production license SCXK (Shanghai) 2016-0001]. The pups were fed freely in our laboratory by lactating does, at a temperature of 20 ± 3 °C and humidity of 58 ± 12 %. Feed and drinking water were freely provided.

This work was approved by the Animal Ethical Committee of Taizhou First People's Hospital (approval no. 20180911), and was implemented in line with "Principles of Laboratory Animal Care" [6].

Instruments and reagents

The instruments and reagents used (with their suppliers and models/specifications shown in brackets) were: Oxygen box (US SHELLAB Company, model: BACTRO X-2); Oxygen concentration analyzer (Shenzhen Shandun Technology Co. Ltd, model: Smart Pro10); Oxygen cylinder, nitrogen bottle (Shandong Huasheng High Pressure Container Co. Ltd, specification: 40 L); Sanmu microscope (Shanghai Yuanhao Testing Equipment Co. Ltd., model: YRS-32); Constant temperature water bath (Shanghai Hetian Scientific Instrument Co. Ltd, specification: HH-US), and refrigerator (Qingdao Haier Group, model: BCD-470WDPG).

Others were immunohistochemistry kit (Shanghai Meixuan Biotechnology Co. Ltd); 0.9 % sodium chloride injection (Chengdu Qingshan Likang Pharmaceutical Co. Ltd, specification: 100 mL: 0.9 g, production batch number: 2011120917); rhIGF-1 (Jiangsu Prosci Biotechnology Co., Ltd., Brand: AbZyme); Rabbit IGF-1 Monoclonal Antibody (Beijing Yiqiao Shenzhou Technology Co. Ltd, Specification: 50 µg); Mouse VEGF Monoclonal Antibody (PeproTech, USA, specifications: 500 µg); IGF-1 test kit (Qingdao

Jieshikang Biotechnology Co., Ltd., specification: 48T); VEGF test kit (Shanghai Enzyme Technology Co. Ltd, specification: 48T), and ketamine hydrochloride injection (Zhejiang Jiu Xu Pharmaceutical Co. Ltd, approval number: National Medicine quasi-word H20173609, specification: 10 mL: 0.1 g).

Mouse pup groups

Sixty (60) pups were randomly divided into three groups, with the random number table method: air group (AG), hyperoxic saline (HS) group, and hyperoxia rhIGF-1 HrGF) group (20 pups per group). The mice were raised together with lactating does. The HS saline mice and the hyperoxia rhIGF-1 mice groups were placed in a closed oxygen chamber. The air inlet was connected to an oxygen cylinder and a nitrogen bottle, and the outlet was connected to an oxygen concentration analyzer to maintain the oxygen partial pressure at 74 ± 1 % or thereabout. The controlled oxygen chamber temperature was maintained at 25 ± 2 °C, and the humidity was 57 ± 6 %.

The HS and HrGF mice were placed in oxygen box for one week, and the litter, the lactating doe and the feed were changed daily. On the 15th day, they were returned to the normal breeding environment with a relatively low oxygen state so as to establish an ROP model. The hyperoxia rhIGF-1 group was intraperitoneally injected with 1.5 mg/kg rhIGF-1, while the hyperoxia saline group was intraperitoneally injected with an equivalent volume of normal saline in place of rhIGF-1. The air group was normally reared without treatment. All mice were sacrificed at the end of the experiment.

Study indices

Five mice in each group were weighed and intraperitoneally injected with 1 - 2 mg/kg ketamine hydrochloride for anesthesia on the 7th, 11th, 15th, and 20th days. The eyeballs were removed and fixed in 5 % formaldehyde so as to remove the cornea, lens, and vitreous humor tissues. The retina was separated to avoid damage to the optic papilla. It was rinsed after staining, and the distribution characteristics of retinal blood vessels were examined.

During the removal of eyeball from the mice, 1 mL of blood was taken from each mouse, and the serum obtained after centrifugation was used for the assay of IGF-1 and VEGF. The levels of serum IGF-1 and VEGF were determined using enzyme linked immunosorbent assay (ELISA).

The expressions of IGF-1 and VEGF in the retina were determined using immunohistochemistry.

Statistical analysis

Statistical analysis was performed using SPSS21.0 software package. Statistical comparison of measurement data was carried out with independent sample *t*-test, while chi square test was used for comparison of count data. Ranking data were compared with the Redit test. Statistical significance was fixed at $p < 0.05$.

RESULTS

Body weight changes in mice

On the 7th day, there was no significant difference in body weight amongst the groups ($p > 0.05$). However, on days 11, 15 and 20, the mice in the hyperoxia saline group had the lowest body weight, and at the same period, body weight of the high oxygen rhIGF-1 mice differed significantly from that of hyperoxia saline group ($p < 0.05$). In contrast, body weight was comparable between the air and hyperoxia rhIGF-1 mice groups on day 11 ($p > 0.05$). However, on days 15 and 20, the difference between the two groups was statistically significant ($p < 0.05$). These results are shown in Table 1.

Table 1: Mouse body weight changes (g; mean \pm SD, n =5)

Group	Day 7	Day 11	Day 15	Day 20
Air	5.95 \pm 0.46	7.84 \pm 0.93	12.68 \pm 1.38	16.54 \pm 0.98
Hyperoxia saline	5.88 \pm 0.54	6.97 \pm 0.61	8.79 \pm 1.21	10.29 \pm 0.79
Hyperoxia rhIGF-1	6.02 \pm 0.42	7.93 \pm 0.79	10.78 \pm 0.95	13.08 \pm 1.04
<i>F</i>	0.43	9.06	5.15	220.62
<i>P</i> -value	0.651	<0.001	<0.001	<0.001

Vascular distribution characteristics of mice

On days 15 and 20, retinal blood vessels in the air group were basically mature and evenly distributed, and on day 15, the retinal vessel diameter in the HS mice group was markedly thinner and unevenly distributed, showing wide occlusion areas. On the 20th day, extensive neovascularization was present, but the distribution was disordered. In addition, the retinal vessel diameter and blood vessel distribution in the hyperoxia rhIGF-1 group were similar to those in the air group. These results

are shown in Figures 1 A, 1B and 1C, and in Figures 2 A, 2B and 2C.

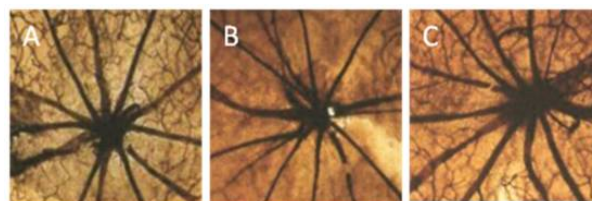


Figure 1: Distribution of retinal blood vessels in each group on day 15. A: air group; B: high-oxygen saline group; C: high-oxygen rhIGF-1 group

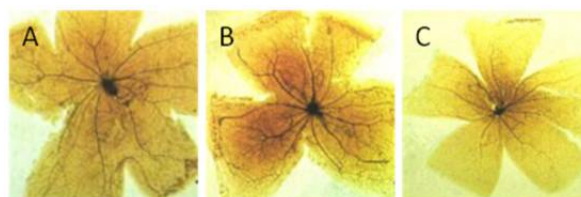


Figure 2: Distribution of retinal blood vessels in each group on day 20. **Note:** A, air group; B, high-oxygen saline group; C, high-oxygen rhIGF-1 group

Serum IGF-1 and VEGF levels in mice

As the mice aged, the serum IGF-1 level gradually increased, with the air group showing the fastest increase, followed by the hyperoxia rhIGF-1 group, while the hyperoxic saline group was the slowest. In contrast, VEGF levels gradually decreased with age, and the hyperoxic saline group decreased the fastest, while the decrease in the air group was the slowest. However, after 15 days, VEGF level in the hyperoxic saline group increased rapidly, while the VEGF level in the hyperoxia rhIGF-1 group increased only slightly. On the day 7, there were no significant differences in levels of IGF-1 and VEGF amongst the groups ($p > 0.05$).

On days 11 and 15, the serum levels of IGF-1 and VEGF in AG and the HrGF mice groups were markedly elevated, relative to their corresponding levels in HS mice. On the 20th day, serum IGF-1 levels were markedly higher in AG and HrGF mice groups than in HS mice, but the VEGF level was significantly lower than that in the hyperoxia saline group ($p < 0.05$). On days 11, 15 and 20, although serum IGF-1 of the hyperoxia rhIGF-1 group was less than control value, the values were comparable ($p > 0.05$). The VEGF level of hyperoxia rhIGF-1 group on days 11 and 15 were markedly lower than that in the hyperoxia saline group ($p < 0.05$). Serum VEGF level of hyperoxia rhIGF-1 group was lower than that of the control group on day 20, but the two values were comparable ($p > 0.05$). These results are shown in Table 2.

Table 2: Serum IGF-1 and VEGF levels of mice (mean \pm SD)

Group	Time	Air group	Hyperoxia saline group	Hyperoxia rhIGF-1 group	t	P-value
IGF-1 (n=5)	Day 7	22.47 \pm 7.91	22.51 \pm 4.49	23.41 \pm 6.92	0.03	0.968
	Day 11	64.67 \pm 11.66	28.97 \pm 3.19	59.03 \pm 13.68	16.57	<0.001
	Day 15	74.12 \pm 12.58	39.71 \pm 8.42	67.64 \pm 13.87	11.91	0.001
	Day 20	90.58 \pm 11.45	53.51 \pm 9.84	83.82 \pm 6.79	21.33	<0.001
VEGF (n=5)	Day 7	2093.25 \pm 224.41	2021.48 \pm 202.44	2030.89 \pm 213.27	0.17	0.848
	Day 11	1748.74 \pm 172.59	610.09 \pm 114.11	1306.26 \pm 181.12	65.36	<0.001
	Day 15	1295.74 \pm 148.83	332.66 \pm 49.88	812.98 \pm 139.08	79.08	<0.001
	Day 20	820.98 \pm 109.43	1744.83 \pm 136.55	820.03 \pm 148.84	120.45	<0.001

Table 3: Retinal expressions of IGF-1 and VEGF in mice (mean \pm SD)

Group	Air group	Hyperoxia saline group	Hyperoxia rhIGF-1 group	t	P-value
IGF-1 (n=10)	0.13 \pm 0.03	0.13 \pm 0.05	0.12 \pm 0.03	0.23	0.794
	0.23 \pm 0.04	0.14 \pm 0.03	0.22 \pm 0.05	14.61	<0.001
	0.24 \pm 0.03	0.15 \pm 0.04	0.23 \pm 0.03	21.47	<0.001
	0.31 \pm 0.04	0.18 \pm 0.03	0.28 \pm 0.05	23.56	<0.001
VEGF (n=10)	0.39 \pm 0.07	0.36 \pm 0.06	0.38 \pm 0.07	0.52	0.599
	0.33 \pm 0.03	0.17 \pm 0.04	0.27 \pm 0.06	32.13	<0.001
	0.26 \pm 0.05	0.12 \pm 0.03	0.21 \pm 0.03	35.12	<0.001
	0.21 \pm 0.03	0.31 \pm 0.02	0.22 \pm 0.03	41.36	<0.001

Expressions of IGF-1 and VEGF in retina of mice

As the mice aged, the level of retinal IGF-1 gradually increased, with the increase being fastest in AD mice, followed by HrGF mice. The hyperoxia saline group was the slowest. In contrast, VEGF level gradually decreased, and the decrease was slowest in the hyperoxic saline group, and fastest in the air group. On day 15, VEGF level of the mice in the hyperoxia group was rapidly increased, while in the hyperoxia rhIGF-1 group, VEGF was only slightly increased. On the 7th day, the expressions of IGF-1 and VEGF were comparable amongst the three groups ($p > 0.05$). However, on days 11 and 15, retinal IGF-1 and VEGF levels in the air and hyperoxia rhIGF-1 groups were markedly elevated, when compared with levels in HS mice.

On day 20, retinal IGF-1 levels in AG and hyperoxia rhIGF-1 group were markedly higher than IGF-1 in hyperoxia mice, and the VEGF level was markedly less than that in the hyperoxia group ($p < 0.05$). On days 11, 15 and 20, retinal IGF-1 levels in the hyperoxia rhIGF-1 control groups were comparable ($p > 0.05$). The VEGF levels in the hyperoxia rhIGF-1 group on days 11 and 15 were significantly lower than those in the control group ($p < 0.05$). There was no significant difference between the levels of retinal VEGF in the hyperoxia rhIGF-1 group and the control group on day 20 ($p > 0.05$). These results are presented in Table 3.

DISCUSSION

Retinopathy of prematurity (ROP) occurs in premature infants with low birth weight and long-term oxygen inhalation, whose fibroangioma of the non-vascularized retina develops and contracts, leading to retinal detachment and blindness [7,8]. In the present study, newly-littered mice pups aged one week were exposed to high-oxygen environment for a period of time and then returned to the normal environment for further feeding. The results showed that mice in the hyperoxia saline group had the lowest body weight on days 11, 15 and 20. There were significant differences in body weight between the high-oxygen rhIGF-1 group and the hyperoxia saline group, but there was no significant difference in body weight between the air group and the hyperoxia rhIGF-1 group on day 11.

However, body weight differed markedly between mice in both groups on days 15 and 20. Retinal blood vessels in the air group were basically mature and evenly distributed on days 15 and 20. On day 15, retinal vessel diameter of the mice in the hyperoxia group was significantly thinner and unevenly distributed, showing a wide occlusion area, and on day 20, intensive neo-vascularization was evident, although the distribution was disordered. On day 15, retinal vessel diameter and blood vessel distribution in the hyperoxia rhIGF-1 group and the air group were comparable. These results indicate that early exogenous supplementation of rhIGF-1

promoted mice growth and significantly reduced angiogenesis.

Studies have shown that the incidence of ROP may be related to a variety of cytokines. In normal people, pro-angiogenic factors and angiogenesis inhibitors are in a relatively balanced state. However, in ROP patients, this homeostasis is impaired due to predominance of pro-angiogenic factors, resulting in enhanced neovascularization rate [9]. It is known that VEGF is a homodimeric protein and a specific heparin-binding growth factor for vascular endothelial cells, and that it effectively promotes angiogenesis [10]. Some researchers are of the view that the production of ROP is closely related to oxygen concentration [11]. Due to the fact that the retina of premature infants is under-developed, their retinal blood vessels stop growing or grow at reduced rates at high oxygen concentrations. However, once the hyperoxia condition is removed, the retina reverts to a relatively low oxygen state, leading to significant increases in VEGF levels which ultimately lead to ROP [12].

It has been reported that VEGF levels significantly higher than normal promote abnormal blood vessels in the retina and vitreous humor [13]. Premature infants have immature lungs. High oxygen environments inhibit expression of VEGF mRNA, but VEGF expression/secretion is accentuated when the hyperoxia environment is relieved [14]. This occurs at the late pregnancy stage in order to meet the needs of the rapid developments in various tissues and organs. Thus, there is need for high VEGF levels, but with the continuous improvements in the development of various tissues and organs, VEGF levels are gradually reduced [15]. The important role of VEGF in ROP has been demonstrated.

It is known that IGF-1 is a broad-spectrum growth-promoting factor that increases the synthesis and activity of VEGF by binding to the IGF-1 receptor. This promotes the migration, differentiation, and maturation of retinal vascular endothelial cells, and ultimately induces vascular lumen formation. It has been reported that IGF-1 levels are significantly reduced in ROP. Moreover, IGF-1 plays a very important role in the development of the retina and brain [16].

In the present study, IGF-1 and VEGF levels in AG and the hyperoxia rhIGF-1 group were markedly elevated, when compared with corresponding values in HS mice on days 11 and 15. On day 20, IGF-1 levels in the air and the hyperoxia rhIGF-1 groups were markedly

increased, relative to hyperoxia mice, and the VEGF level was markedly less than that in the hyperoxia mice. However, on days 11, 15 and 20, IGF-1 in the control and hyperoxia rhIGF-1 groups were comparable. The levels of VEGF in the hyperoxia rhIGF-1 group were significantly lower than those in the control group on days 11 and 15. Serum VEGF in the hyperoxia rhIGF-1 and control groups were comparable on day 20. These results indicate that early supplementation with exogenous rhIGF-1 results in marked increases in serum IGF-1 and retinal IGF-1 levels. At the same time, IGF-1 promoted VEGF production and retinal VEGF expression, and significantly enhanced the development of retinal blood vessels, thereby reducing the formation of new blood vessels.

CONCLUSION

Early supplementation with exogenous rhIGF-1 in the treatment of oxygen-induced ROP in a mouse model produces desirable outcomes. It promotes the growth and development of ROP mice, and reduces retinal neovascularization. Moreover, it has a positive effect on the expressions of IGF-1 and VEGF. Further investigations are required to determine its suitability for the clinical management of ROP.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

This work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. Danying Wang designed the study and interpreted the results. Xiangjun Wu, Hui Ye, Qiao Cai, Yuanxiang Ke, Danying Wang collected data and drafted the manuscript. Xiangjun Wu performed the experiments.

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