·Basic Research·

Effects of aminoguanidine on retinal apoptosis in mice with oxygen-induced retinopathy

An-Jie Du[1,2], *Bing Ren*[1], *Xiao-Wei Gao*[1], *Lei Yang*[1], *Yan Fu*[1,3], *Xu-Dong Zhao*[1]

[1]Ophthalmic Center, No. 474 Hospital of Chinese PLA, Urumqi 830013, Xinjiang Uygur Autonomous Region, China
[2]Department of Ophthalmology, Yuncheng Central Hospital, Yuncheng 044000, Shanxi Province, China
[3]Department of Ophthalmology, Baoding First Central Hospital, Baoding 071000, Hebei Province, China
Correspondence to: Bing Ren. Ophthalmic Center, No. 474 Hospital of Chinese PLA, Urumqi 830013, Xinjiang Uygur Autonomous Region, China. Rb54391@sohu.com
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Abstract

• AIM: To explore the protective effects of amino-guanidine (AG) on retinal apoptosis in mice with oxygen-induced retinopathy (OIR).

• METHODS: A total of 80 C57BL/6J mice, aged 7 days, were randomly divided into four groups: normal, high oxygen, high oxygen saline and high oxygen treated with AG. In the normal group, mice were housed in normoxic conditions from postnatal day P7 to P17. Mice in the other 3 groups were placed under hyperoxic conditions (75±2% O_2) in an oxygen-regulated chamber for 5 days and subsequently placed in normoxic conditions for 5 days. Mice in the AG group were treated once daily, from P12 to P17, with AG hemisulfate (100mg/kg body weight, intraperitoneally) dissolved in physiological saline. An equivalent amount of 0.9% physiological saline was administered, as above, to mice in the high oxygen saline group. Ten mice were randomly selected from each group on P14 and on P17, euthanized and the retinas examined. Apoptotic cells in the retina were detected using the terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) method. The expression of nitric oxide synthase (iNOS) in the retina was detected by immunohistochemistry and changes in rod cells were observed using electron microscopy.

• RESULTS: TUNEL-positive cells and iNOS immuno-reactive neurons were present in the inner nuclear and ganglion cell retinal layers of mice in the high oxygen group. The number of TUNEL-positive cells was significantly greater in the high oxygen group compared with the normal group (t=-20.81, P_{14d}<0.05; t=-15.05, P_{17d}<0.05). However, the number of TUNEL-positive cells in the AG treatment group was significantly lower (t=-13.21, P_{14d}<0.05; t=-6.61, P_{17d}<0.05) compared with the high oxygen group. The expression of iNOS was significantly higher in the high oxygen group compared with the normal group (t=-21.95, P_{14d}<0.05; t=-17.30, P_{17d}<0.05). However, the expression of iNOS in the AG treatment group was significantly lower (t=-12.17, P_{14d}<0.05; t=-10.30, P_{17d}<0.05) compared with the high oxygen group. The outer segments of the rods were disorganized and short in the high oxygen group. Rod morphology appeared to be slightly improved in the AG group.

• CONCLUSION: AG may protect retinal neurons in OIR by inhibiting apoptosis. The mechanism may be related to iNOS.

• KEYWORDS: aminoguanidine; retinopathy of prematurity; apoptosis; inhibitor of nitric oxide synthase
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INTRODUCTION

Retinopathy of prematurity (ROP) is a serious complication in premature infants and is a leading cause of blindness in children. Improvements in neonatal care have led to a significant increase in the survival rates of premature infants, however, this has also resulted in higher rates of ROP. The incidence of ROP is increasing in countries with rapidly developing economies, such as China[1]. In addition to neovascularization (NV), studies suggest that retinal apoptosis may play a major role in ROP [2]. Aminoguanidine (AG) is a highly selective inhibitor of nitric oxide synthase (iNOS) and has been confirmed to have preventive effects on retinal neuron apoptosis[3].

MATERIALS AND METHODS

Materials Pregnant female C57BL/6J mice were provided by the Laboratory Animal Center of Xinjiang Medical University (Xinjiang, China). The JEM-100 CXII-electron microscope was also provided by Xinjiang Medical University. AG hemisulfate was purchased from Sigma Aldrich (Wuhan, China) and the gas analyzer (model CY-12C) was obtained from Electrochemical Analytical Instruments, Inc. (Meicheng, China). The rabbit anti-human iNOS polyclonal antibody was purchased from Santa Cruz Biotechnology (Shanghai, China) and the Colorimetric

terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) Apoptosis Assay Kit was obtained from BIOBOX, Inc. (Nanjing, China).

Methods All experiments were conducted in accordance with the Animal Care and Use Committee and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. This mouse model of oxygen induced retinopathy (OIR) was first described by Smith *et al* [4]. A total of 80 C57BL/6J mice, aged 7 days and weighing 3.5±0.5g, were used. The mice were randomly divided into four groups, 20 mice per group (40 eyes/group), as follows: group 1 (normal), group 2 (high oxygen), group 3 (high oxygen saline), and group 4 (AG treatment). Mice in group 1 were housed in normoxic conditions from P7 to P17. In the other 3 groups, mice were placed in an oxygen-regulated chamber, under hyperoxic conditions (75±2% O_2), for 5 days and subsequently placed in normoxic conditions for 5 days. In the AG treatment group, mice were treated once daily, from P12 to P17, with AG hemisulfate (100 mg/kg body weight, intraperitoneally) dissolved in physiological saline; an equivalent amount of 0.9% physiological saline was administered, *via* the same method, to mice in the high oxygen saline group. Ten mice from each group were anesthetized with 10% chloral hydrate (500mg/kg, intraperitoneally) and euthanized on P14 and another 10 mice were from each group were euthanized, as above, on P17. After euthanization, both eyes of all mice were enucleated. Left eyes (n=10) were fixed for 24h in 10% paraformaldehyde fixative. The fixed eyes were then dehydrated using ascending concentrations of alcohol prior to being embedded in paraffin wax. Left eyes were serially sectioned at 4μm and placed on poly-L-lysine-coated slides for staining by immunohistochemistry and TUNEL. Right eyes (n=10) were fixed for 24h in 4% glutaraldehyde fixative. The retina was then removed under a microscope and the eyes fixed for an additional 2h in 1% osmium tetroxide fixative for observation by electron microscopy.

TUNEL The samples were deparaffinized and rehydrated according to kit specifications. A volume of 50μL of Terminal Deoxynucleotidyl Transferase (TdT) was added to each slice and slices were then kept at room temperature for 60min. Sections were subsequently washed 3×5 minute with 0.01mmol/L phosphate buffered solution (PBS). A 100μL volume of Streptavidin-HRP was added, the sections were kept at room temperature for another 30min and then washed with PBS as above. A 100μL volume of DAB was added and sections were kept at room temperature for an additional 10min. After washing with PBS as above, sections were stained with Mayer hematoxylin and dehydrated with alcohol. Using light microscopy (400× magnification), 5 discontinuous high power fields were selected from each section and the number of positive cells (those with brownish-yellow granules deposited in the cytoplasm or nuclei) were counted.

Immunohistochemistry Immunohistochemistry was done according to kit specifications. The sections were stored at 4℃ overnight after adding iNOS rabbit polyclonal antibody (1:100). All sections were then washed 3×5min with PBS. A 50μL volume of rabbit anti-goat biotinylated IgG was added and sections were kept at room temperature for 30min. Sections were again washed with PBS, as above, 50μL streptavidin-biotin peroxidase complex was then added, and sections were kept at room temperature for another 30min. A 100μL volume of DAB was added and sections were kept at room temperature for an additional 10min. Sections were subsequently stained with Mayer hematoxylin and dehydrated with alcohol. Using light microscopy (400X magnification), 5 discontinuous high power fields from each section were selected and the number of iNOS immunoreactive neurons (those with yellow or brownish-yellow granules deposited in the cytoplasm or nuclei) were counted.

Electron Microscopy The slices were fixed, dehydrated using an ethanol series, infiltrated and embedded in JB-4 medium. Sections were then cut to thicknesses of 40-60nm and stained with uranyl acetate for 30min. After uranyl acetate staining, all sections were washed 3×5min with PBS, stained with lead citrate for 30min and washed with PBS as above. Changes in the rod outer segments were observed using electron microscopy.

Statistical Analysis SPSS 17.0 for windows statistical software was used in this study. All data are expressed as mean± SD. One-way analysis of variance (ANOVA) and the LSD-test were employed to compare differences between groups, in the number of TUNEL-positive cells and iNOS immunoreactive neurons. A P valve <0.05 was considered statistically significant.

RESULTS

Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling No TUNEL-positive cells were found in the normal mice group. A large number of TUNEL-positive cells was observed in both the high oxygen and high oxygen saline groups. TUNEL-positive cells in the high oxygen group were mainly located in the inner nuclear layer. TUNEL-positive cells were also present in the ganglion cell layer in this group, though they were fewer in number than in the inner nuclear layer. There were significantly more TUNEL-positive cells in the high oxygen group on P14 compared to P17. The number of TUNEL-positive cells in the AG treatment group was significantly lower compared to the number in the high oxygen group (P<0.05). However, there was no difference in TUNEL-positive cells when comparing the high oxygen and high oxygen saline groups to each other (P>0.05) (Table 1, Figure 1).

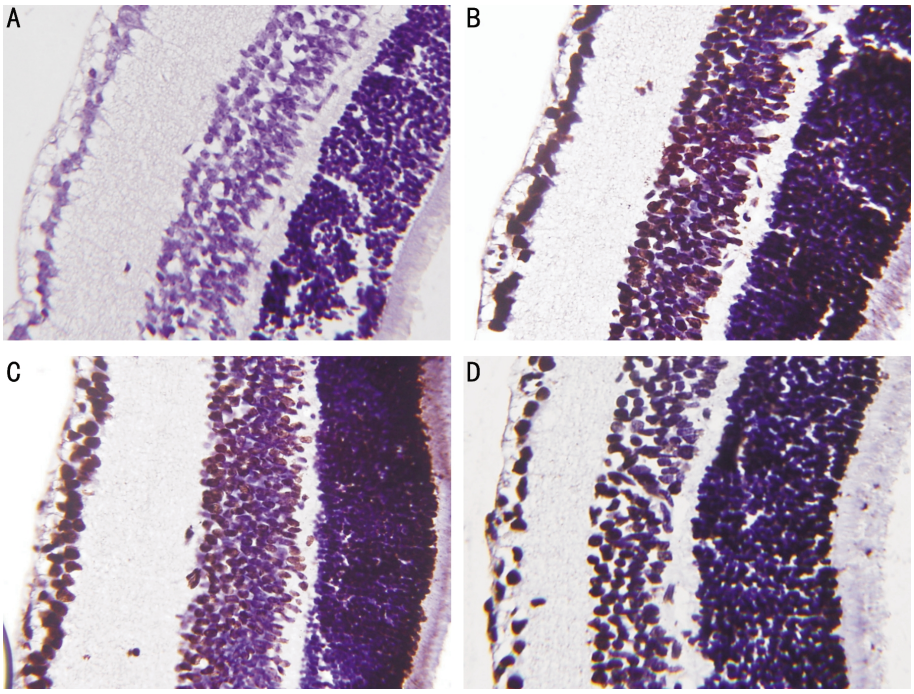

Figure 1 TUNEL staining of the retinal tissue of mice on day 14 A: Normal group; B: High oxygen group; C: High oxygen saline group; D: AG treatment group (×400). There was a large number of TUNEL-positive cells in the high oxygen group. The number of TUNEL-positive cells in the AG treatment group was significantly lower compared to the high oxygen group (P<0.05).

Table 1 The number of TUNEL-positive cells among the retinal nerve cells of mice with OIR ($\bar{x} \pm s$, n=10)

Groups	TUNEL staining	
	14 days	17 days
Normal group	0.0000±0.0000	0.0000±0.0000
High oxygen group	29.4000±4.2560[a]	7.7333±1.5796[a]
High oxygen saline group	28.6667±4.3861	8.1333±1.3020
AG treatment group	10.7333±1.6242[c]	4.3333±1.0465[c]
F	309.065	161.656

[a]P<0.05 *vs* normal group; [c]P < 0.05 *vs* high oxygen group (ANOVA, LSD-test).

Table 2 Effects of AG on expression of iNOS in retinal nerve cells of mice with OIR ($\bar{x} \pm s$, n=10)

Groups	Expression of the iNOS	
	14 days	17 days
Normal group	0.0000±0.0000	0.0000±0.0000
High oxygen group	22.7333±3.1952[a]	12.2000±1.9712[a]
High oxygen saline group	23.2667±2.7894	12.0000±2.2360
AG treatment group	10.1333±1.5976[c]	4.9333±1.0328[c]
F	363.230	210.985

[a]P<0.05 *vs* Normal group; [c]P<0.05 *vs* High oxygen group (ANOVA, LSD- test).

Immunohistochemistry The iNOS immunoreactive neurons were mainly located in the inner nuclear layer and the ganglion cell layer of mice in the high oxygen group. There were significantly more iNOS immunoreactive neurons present on P14 than on P17. There were no iNOS positive cells in the normal group. iNOS expression was significantly higher in the high oxygen group as indicated by the significantly higher the presence of yellowish-brown granules, with deep color, in the cytoplasm compared to the normal group. The expression of iNOS in the AG treated group was significantly lower than in both the high oxygen and high oxygen saline groups (P<0.05) as indicated by the reduced presence of yellowish brown granules of lighter color, in the cytoplasm. However, there was no significant difference in iNOS expression when comparing the high oxygen and the high oxygen saline groups (P>0.05) (Table 2, Figure 2).

Electron Microscopy In the normal group, the rod outer segment was arranged in neat rows and the structure was clear. However, in the high oxygen group, the rod outer segment was disorganized and was significantly shorter. The outer rod segment morphology was slightly less disorganized and longer in the AG treatment group compared with the high oxygen group (Figure 3).

DISCUSSION

In the current study we investigated the potential for AG to prevent apoptosis of retinal neurons by observing the effect of AG on the expression of iNOS in retinal nerve cells of mice with OIR. Changes in rod cells were also examined using electron microscopy.

ROP can lead to retinal detachment and blindness in premature infants and is a significant clinical problem. In addition to physical problems, ROP also places social and financial burdens on communities. Until recently, inhibiting the growth of neovascularization was the main treatment for ROP. However, despite effective treatment of vascular injury, many children still suffer visual impairment, suggesting that there may be a neuronal disruption in ROP[5].

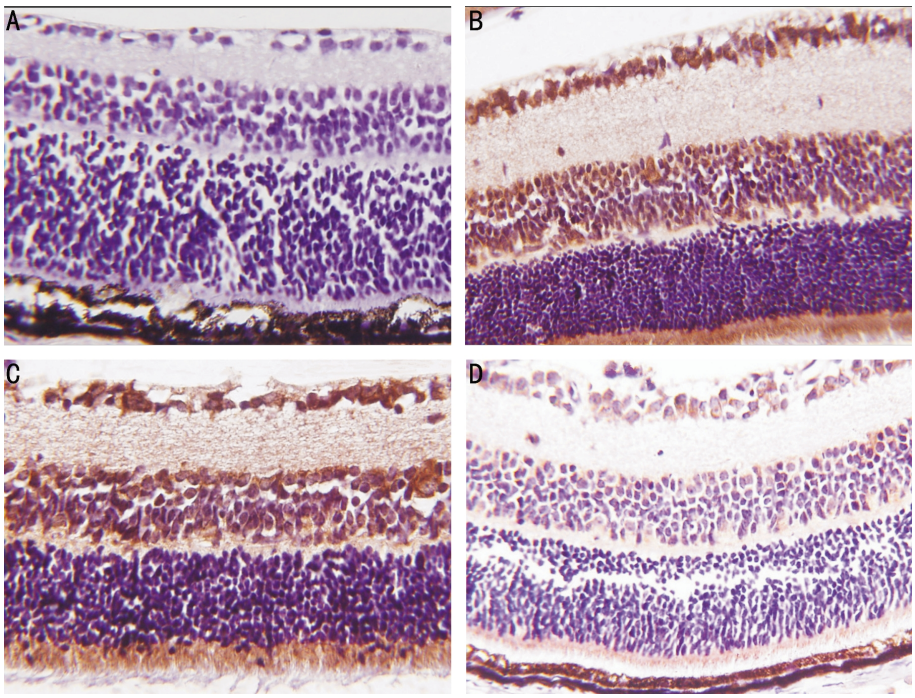

Figure 2 Expression of iNOS on day 14 A: Normal group; B: High oxygen group; C: High oxygen saline group; D: AG treatment group (×400). There were no iNOS positive cells in the normal group. There was significantly increased iNOS expression in the high oxygen group. The expression of iNOS in the AG treatment group was significantly lower than in the high oxygen group.

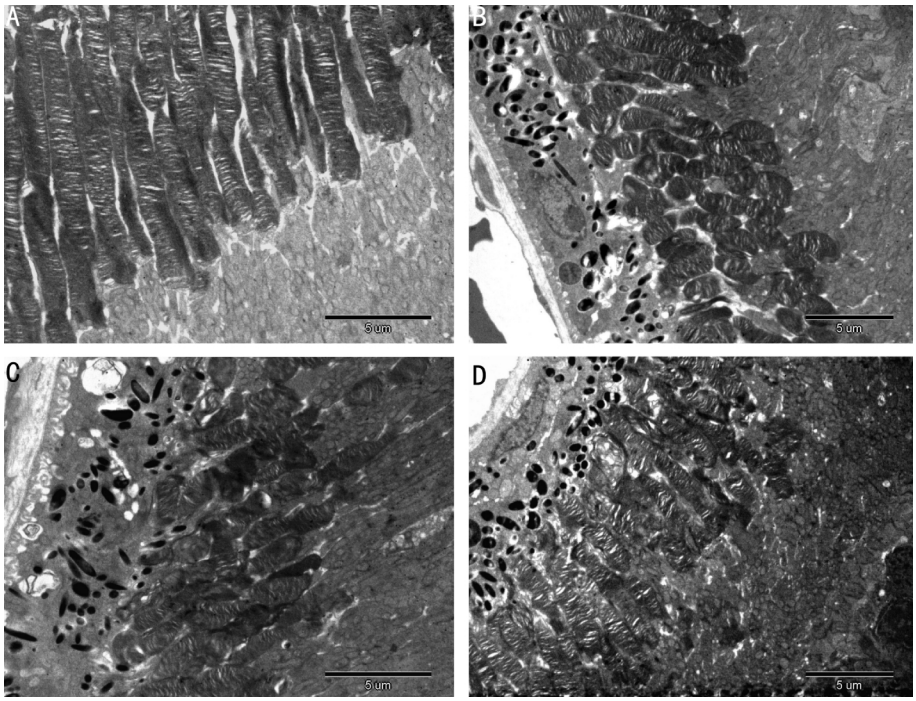

Figure 3 The changes in the rod outer segment as seen with electron microscopy on day 17 A: Normal group; B: High oxygen group; C: High oxygen saline group; D: AG treatment group (5μm Scale bars). Compared to the normal group, the rod outer segment was disorganized and was significantly shorter in length in the high oxygen group. The outer rod segment morphology was slightly less disorganized and longer in the AG treatment group compared to the high oxygen group.

In this study, mice in the high oxygen group had rod outer segments that were disorganized and significantly shorter. This observation is consistent with data from Fulton *et al*[6] indicating that subtle alterations in the rod outer segment may damage the mechanisms controlling the assembly of the outer segment disc membranes thus slowing the kinetics of the molecular processes in the activation of photo-transduction. It has been shown that ROP is a neurovascular disease and that the age of onset of ROP coincides with that of photoreceptor development[7]. ROP has been found, not only to alter the structure and function of photoreceptors, but also to lead to deficits in rod sensitivity[8-10]. Furthermore, even when conspicuous neurovascular abnormalities disappear, subtle structural anomalies and

functional deficits may persist for years after clinical ROP resolves [9, 11]. In rat models, rod dysfunction precedes ROP and predicts subsequent ROP vascular status. Early pharmaceutical interventions that target fundamental rod function have been found to improve vascular outcomes[12].

Pathological neovascularization, retinal degeneration and glial activation have been demonstrated to be the main mechanisms involved in ROP [13] and apoptosis has been reported to be the major factor in retinal degeneration [2]. In addition, Sennlaub *et al* [2] demonstrated that apoptotic cells were mainly located in the inner nuclear layer and reported that the major mechanism may be related to the expression of iNOS. A decrease in the number of bipolar cells has been reported in the rat model of OIR [14]. Previous ERG studies have found that the amplitude and sensitivity of a-waves were decreased, suggesting photoreceptor dysfunction, and reported reductions in b-wave amplitudes as well, indicating that retinal interneurons are affected [15]. In the rat model of OIR, Narayanan *et al* [16] showed that the amplitudes of a-waves and b-waves were significantly higher and that bipolar function was markedly impaired when apoptosis was inhibited. These observations are consistent with data from Sennlaub *et al* [2] showing that apoptosis may be one of the mechanisms of ROP.

Apoptosis in ROP may be related to the toxic effects of nitric oxide (NO). A small amount of NO can cause vascular dilation and protect the retina. However, excessive quantities of NO can react with superoxide to produce peroxynitrite which can then undergo further decomposition, producing oxygen free radicals such as OH^- and OH·. These free radicals attack other biomolecules, such as protein and DNA, triggering apoptosis [17]. NO can affect and regulate apoptosis by stimulating the production of cGMP *via* guanylate cyclase activation in smooth muscle cells, thus stimulating the release of glutamate [18]. There are three isoforms of NOS in human body, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS and eNOS are constitutive isoforms (cNOS). cNOS is the key enzyme that produces NO under physiological conditions, however, iNOS is the key enzyme that produces NO under pathological conditions. The expression of iNOS is increased in the late stage of retinal ischemia and iNOS releases excessive quantities of NO leading to serious damage of the retinal tissue [19]. AG is a non-toxic, small molecule that is a highly selective inhibitor of iNOS. It has been widely used in studies of NO [20]. Compared with other inhibitors of NOS, AG has little effect on cNOS, so the NO produced by cNOS is protected. The mechanism for apoptosis in ROP is complex and remains unknown. The expression of Bax, caspase, P53 and HIF-1 are raised in OIR and may also play important roles in the apoptotic process[16,21,22].

This study showed that the structure of rod outer segments was slightly better in the AG group compared with the high oxygen group. Cell apoptosis was also significantly lower and the expression of iNOS was significantly weaker in AG group. It is possible that AG protects retinal nerve cells by inhibiting the expression of iNOS, thereby reducing apoptosis. Additionally, it has been reported that AG may inhibit the growth of retinal neovascularization in the mouse model of OIR through a decrease in the expression of VEGF, COX-2 or HIF-1 [23-25]. AG has been reported to have low toxicity and to be well-tolerated by animals. However, side effects have been encountered in human patients treated with AG during clinical trials. Thus, further studies are necessary to explore methods for reducing the risk of side effects in patients. Research on AG is in the beginning stages and additional and more detailed experimental studies are needed to determine the appropriate dosage of and time of administration for AG.

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