·Basic Research·

Potential involvement of nitric oxide synthase but not inducible nitric oxide synthase in the devel - opment of experimental corneal neovascularization

Yuan Chen, Gao-Qin Liu, Pei-Rong Lu

Foundation items: National Natural Science Foundation of China (No. 30771978 and 30972712); Natural Science Foundation of Jiangsu Province (BK2006528); Qing-Lan Project of Education Bureau of Jiangsu Province (Lu PR)

Department of Ophthalmology, the First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu Province, China
Co-first authors: Yuan Chen and Gao-Qin Liu
Correspondence to: Pei-Rong Lu. Department of Ophthalmology, the First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu Province, China. lupeirong@yahoo.com.hk

Received:2011-07-18 Accepted:2011-07-29

Abstract

• AIM: To investigate the effect of nitric oxide and its synthetase on experimental corneal neovascularization (CRNV).

• METHODS: CRNV was induced by alkali injury in mice, nitric oxide synthetase (NOS) was inhibited by NG-nitro-L-arginine (L-NAME) and inducible nitric oxide synthetase (iNOS) was inhibited by aminoguanidine hemisulfate salt (AG). The inhibitory effect was detected at day 2 and 4 after corneal alkali injury by reverse transcription polymerase chain reaction (RT-PCR). CRNV was compared between the control and the treated mice by microscopic observation and corneal whole mount CD31 immunostaining.

• RESULTS: The inhibition of L-NAME to NOS and AG to iNOS after corneal injury was confirmed by RT-PCR ($P<0.05$). Compared with control mice, L-NAME treated mice exhibited significantly decreased CRNV areas ($P<0.05$). In contrast, AG treatment failed to attenuate alkali induced CRNV ($P>0.05$).

• CONCLUSION: Our findings suggest that NOS but not iNOS plays a critical role in alkali injury induced CRNV.

• KEYWORDS:corneal neovascularization; nitric oxide synthase; inducible nitric oxide synthase
DOI:10.3980/j.issn.2222-3959.2011.04.03

Chen Y, Liu GQ, Lu PR. Potential involvement of nitric oxide synthase but not inducible nitric oxide synthase in the development of experimental corneal neovascularization. *Int J Ophthalmol* 2011;4(4): 343-348

INTRODUCTION

Cornea is characterized by the absence of blood vessels and hematopoietic cells including erythrocytes and leukocytes under physiological conditions [1]. Corneal avascularity is required for optical clarity and maintenance of vision. Corneal neovascularization (CRNV) arises from many causes including corneal infections, misuse of contact lens, chemical burn, and inflammation, and can lead to severe impaired vision. We previously observed in the early phase after alkali injury induced experimental CRNV, leukocyte including neutrophils and macrophages recruited to the injured corneas, and then produced serials of chemokines and proinflammatory factors. The intracorneal infiltrated neutrophils and macrophages exert complicated roles, by utilizing different chemokine receptor and proinflammatory signals in the development of experimental CRNV [2-6]. We also found the time kinetic expression of iNOS during the experimental CRNV and the murine macrophage expressed iNOS, which suggest the potential role of nitric oxide and its synthetase on experimental CRNV[6].

Nitric oxide (NO) is a pleiotropic regulator and plays a role in a variety of models of angiogenesis. The family of nitric oxide synthases (NOS) comprises inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Nitric oxide (NO) seems to have proangiogenic or anti-angiogenic effects depending upon the setting. Ando *et al* [7,8] observed that deficiency of eNOS suppresses retinal but not CNV, whereas deficiency of nNOS or iNOS suppresses choroidal, but not retinal neovascularization. She *et al* [9] observed the effect of NOS on PDT treated experimental CNV and they found there was no difference in CNV size and leakage between L-NAME treated to control rats. These data suggest that NO contributes to both retinal and CNV and that different isoforms of NOS are involved in different settings and different disease processes.

Thus, it still remains to be investigated on the roles of the NOS family in CRNV. To further address the roles of nitric oxide and its synthetase on experimental CRNV, in the

current study, we used L-NAME and AG to inhibit NOS and iNOS expression during alkali injury and compared the alkali injury induced experimental CRNV.

MATERIALS AND METHODS

Reagents and antibodies Rat anti-mouse CD31 (MEC13.3) mAbs were purchased from BD Pharmingen (San Diego, CA). Alexa Fluor 488 donkey anti-rat IgG was purchased from Invitrogen Life Technologies(Carlsbad,CA). NG-nitro-L-arginine (L-NAME), aminoguanidine hemisulfate salt (AG), sodium hyaluronate (HA), and Avertin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Mice Specific pathogen-free 6-7 week old male BALB/c mice weighing 20-25g were obtained from Shanghai SLAC Laboratory Animal Co. Ltd and were kept in our animal facility under specific pathogen-free conditions. All animal experiments were done in accordance with the Guideline for the Care and Use of Laboratory Animals on the Chinese Medical Academy and the Soochow University Animal Care Committee, and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were kept in groups of 5 and fed regular lab chow and water ad libitum. A 12-hour day and night cycle was maintained.

Alkali –induced corneal injury model Alkali-induced corneal injury model was prepared for target genes detection and CRNV areas analysis. Six to seven week-old 40 BALB/c mice were divided into 4 groups. Each group contained 10 mice. Among them, two groups were treated with L-NAME or AG respectively. Another two groups were saved as control for each treated groups. Corneal injury was induced by placing a 2-mm filter disc saturated with 1N NaOH onto the left eye of the mouse for 50 seconds in L-NAME treated study or 40 seconds in AG treated study (50 seconds treatment resulted in similar but not impaired CRNV, to ruled out the possibility of enhanced CRNV in AG treated group, we next selected 40 seconds) as previously described [2-6]. L-NAME (100mg/kg/day) in drinking water was given in treated group 1 week before alkali injury. In AG related experiments, the alkali-treated eyes received 5μL of AG in 0.2% HA at a concentration of 60mg/mL to inhibit iNOS expression, or 5μL of 0.2% sodium hyaluronate as vehicle three times a day for 14 days immediately after the alkali injury. In the experiment for CRNV area detection, at the indicated time intervals, the treated or the control mice were sacrificed, and corneas were removed and fixed in acetone for 20 minutes. The corneas were used for whole mount CD31 staining. In other experiments for target gene detection, at the indicated time after alkali injury, the corneas were removed and placed immediately into RNALate (Qiagen, Tokyo, Japan), and kept at -86℃ until total RNA extraction was performed. Each experiment was repeated at least three times.

Biomicroscopic examination Eyes were examined with a slit lamp from Haag Streit (BQ 900®, Swiss made), and results were photographed on day 14. In brief, under anesthesia, photographs of the corneas were obtained using a digital camera (Nikon, Tokyo, Japan) linked to the slit lamp.

Enumeration of corneal neovascularization Corneal whole mount staining with CD31 was performed and blood vessels in the corneas were measured according to previous reports [10]. In brief, Corneal flat mounts were rinsed in PBS, fixed in acetone, rinsed in PBS, blocked in 2% bovine serum albumin, stained with rat anti-mouse CD31 (1:100; BD Pharmingen) at 4℃ overnight, washed, the corneas were then incubated with Alexa Fluor 488 donkey anti-rat IgG (1/100) for 1 hour at room temperature in the dark and detected by microscope. Digital pictures of the flat mounts were taken. Then, the area covered by CD31 was measured morphometrically on these flat mounts using NIH Image software (National Institutes of Health, Bethesda, MD). The total corneal area was outlined using the innermost vessel of the limbal arcade as the border. The total area of neovascularization was then normalized to the total corneal area, and the percentage of the cornea covered by vessels was calculated. The relative neovascular area was compared between treated group and control group. We used Student's t test to statistically analyze the difference between treated and control group.

Semi –quantitative reverse transcription (RT) – polymerase chain reaction (PCR) Total RNAs were extracted from the corneas with the use of RNeasy Mini Kit (Qiagen, Tokyo, Japan). The resultant RNA preparations were further treated with ribonuclease-free deoxyribonuclease(DNase) I (Life Technologies Inc., Gaithersburg, MD) to remove genomic DNA. 2μg of total RNAs were reverse-transcribed at 42℃ for 1 hour in 20μL of reaction mixture containing mouse Moloney leukemia virus reverse transcriptase and hexanucleotide random primers (Qiagen). Serially two-fold diluted cDNA was amplified for GAPDH to estimate the amount of transcribed cDNA. Then, equal amounts of cDNA products were amplified for the target genes using the primers under the following conditions; denaturation at 94℃ for 2 minutes, followed by the optimal cycles of 30 seconds at 94℃, 45 seconds at 56-58℃, 1 minute at 72℃, and a final 10 minutes extension step at 72℃. Primers and PCR conditions used were shown in Table 1. The amplified PCR products were fractionated on a 1.0% agarose gel and visualized by ethidium bromide staining. The band intensities were measured and their ratios to GAPDH were determined with the aid of NIH Image Analysis software.

Table 1 Specific sets of primers and conditions of PCR

Primers	Nucleotide sequence(5'→3') sense/anti-sense	Annealing temperature(℃)	PCR Cycles
NOS	5'-CTGGCTCGCTTTGCCACGGA-3' 5'-TGCGACAGCAGGAAGGCAGC-3'	58	35
iNOS	5'-CTGCTGTACCTCCACCATGCCAAGT-3' 5'-CTGCAAGTACGTTCGTTTAACTCA-3'	56	35
GAPDH	5'-ACCACAGTCCATGCCATCAC-3', 5'-TCCACCACCCTGTTGCTGTA-3'	58	25

Statistical Analysis The means and standard error of the mean (SEM) were calculated for all parameters determined in the study. Values were processed for statistical analyses (Student's t test) with statistic software SPSS13.0. A value of $P<0.05$ was considered statistically significant.

RESULTS

Effect of NOS on alkali induced corneal neovascularization In the present result, we used oral administration of L-NAME to inhibit NOS expression after alkali injury. To observe the inhibitory effect of L-NAME to NOS, we detected the NOS expression after alkali injury by RT-PCR. RT-PCR result showed that L-NAME treated mice exhibited decreased intra-corneal NOS mRNA ($P<0.05$) expression in the early phase (day 2, day 4) after alkali injury compared with control group (Figure 1). The result revealed that the administration of L-NAME in drinking water could efficiently inhibit the NOS expression in the cornea after alkali injury.

Next, we observed the effect of NOS on alkali induced CRNV. Micro-observation about the CRNV 2 weeks after alkali injury showed the impaired CRNV after NOS inhibition compared to control (Figures 2A and 2B). Corneal whole mount staining by CD31, a neo-vascular marker, further indicated the impaired CRNV in L-NAME treated mice compared to those of control mice (Figures 2C and 2D). The corneal neovascularization area in L-NAME treated group was significantly decreased compared to control mice as shown in Figure 2E.

Effect of iNOS on alkali injury induced experimental corneal neovascularization To observe the effect of iNOS on CRNV, we next inhibited the iNOS expression after alkali injury by AG topical administration. We detected the iNOS expression after alkali injury by RT-PCR and the result as shown in Figure 3 indicated the iNOS expression was significantly decreased in AG treated mice compared to the control mice. These results would confirm the inhibitory effect of AG on iNOS expression.

We further observed the effect of iNOS on alkali induced CRNV. Micro-observation about the CRNV 2 weeks after alkali injury showed no difference between AG treated mice to control mice (Figures 4A and 4B). Corneal whole mount staining further indicated the similar alkali induced CRNV in AG treated mice compared to those of control mice (Figures 4C and 4D). There was no significant difference of corneal neovascularization area evaluated by corneal whole mount CD31 staining between AG treated mice to control mice as shown in Figure 4E.

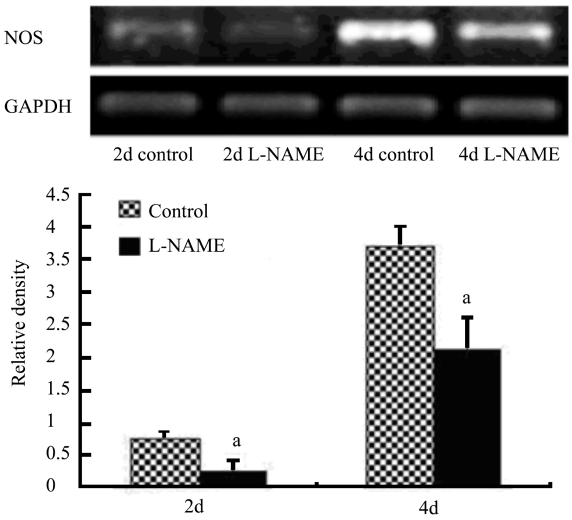

Figure 1 The NOS gene expression in the control and L-NAME treated mice at day 2 and 4 after alkali injury RT-PCR shows that L-NAME treated mice exhibited decreased intra-corneal NOS mRNA expression in the early phase (day 2, day 4) after alkali injury compared with control group. A representative result from three experiments is shown ($^aP<0.05$ *vs* control)

DISCUSSION

Normal corneas are avascular and this state is maintained by the balance between angiogenic factors and anti-angiogenic factors. In some conditions such as infection, trauma, edema and so on, the expression balance of these factors was broken and angiogenic factors was dominated, CRNV would be inevitably formed. To our knowledge, nitro oxygen is characteristic by its chemical activation being biological messenger and plays an important role in series of physiological and pathological progress. In this study, we investigated the role of NO and its synthetase in alkali injury induced inflammatory CRNV.

According to our previous report [2], in alkali injury induced CRNV model, CRNV was evident at 7 days, peaked at 14 days after injury and declined gradually there after as detected by immunostaining with anti-CD31 antibodies. Many inflammatory cells infiltrated into the corneas and peaked at 2 days to 4 days after injury, after which inflammatory cell infiltration declined gradually and was

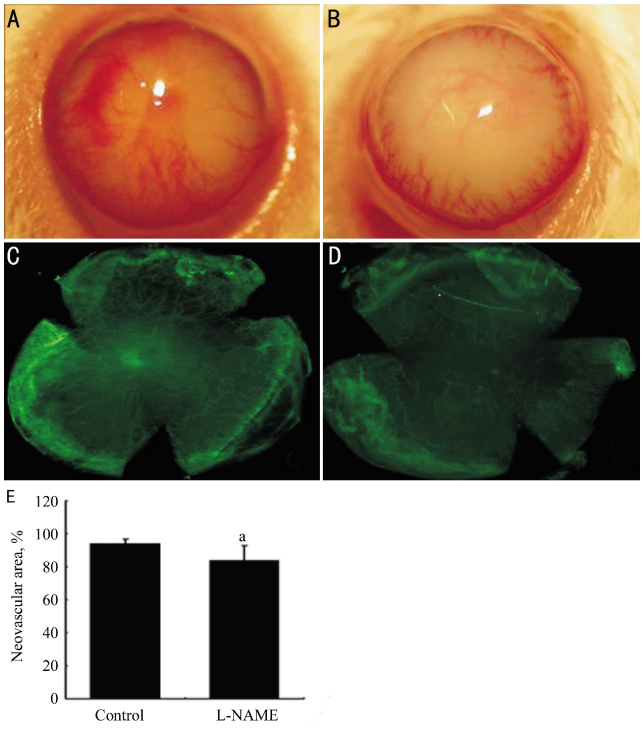

Figure 2 The observation of CRNV at day 14 after alkali injury A: microscopic observation shows that new vessels have arrived pupillary zone of the corneas in control group; B: new vessels have just passed the corneal limbus in L-NAME treated group; C: immunofluorescence shows the new vessels have spread over the cornea in control group; D: immunofluorescence shows the new vessels have arrived pupillary zone of the cornea in L-NAME treated group; E: the L-NAME treated mice exhibited significantly decreased CRNV area compared to the control (t = 2.322, ^{a}P<0.05 *vs* control)

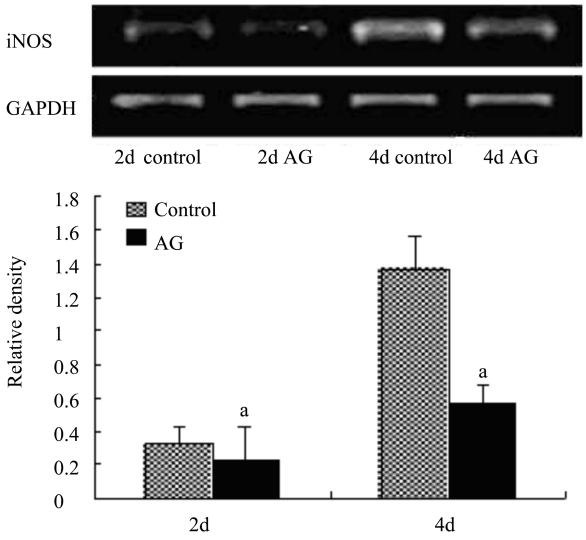

Figure 3 The iNOS gene expression in the control and AG treated mice at day 2 and 4 after alkali injury RT-PCR shows that AG treated mice exhibited decreased intra-corneal iNOS mRNA expression in the early phase (day 2, day 4) after alkali injury compared with control group. A representative result from three experiments is shown (^{a}P<0.05 *vs* control)

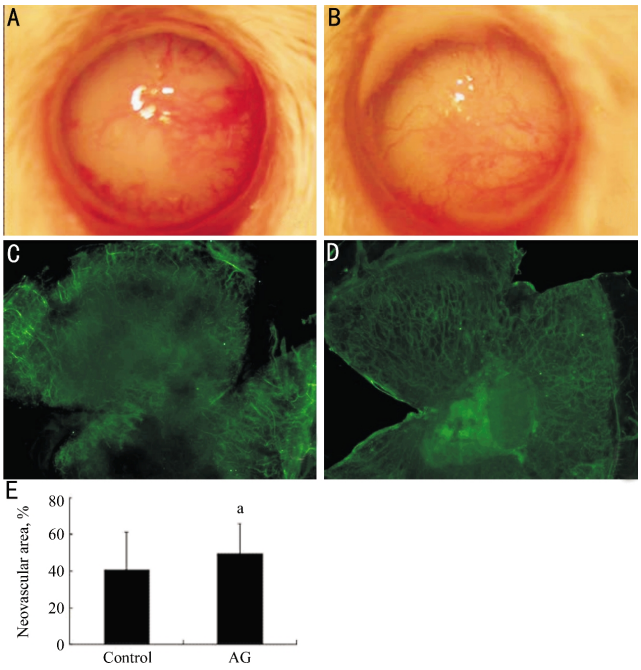

Figure 4 iNOS on CRNV day 14 after alkali injury A and B: microscopic observation shows that new vessels of AG treated and control mice have arrived pupillary zone of the corneas; C and D: immunofluorescence indicated the new vessels of AG treated and control mice have arrived pupillary zone of the corneas; E: no significant difference was observed by statistical analysis (t = -1.852, ^{a}P<0.05 *vs* control)

marginal at 14 days after the injury. CRNV formation was promoted by angiogenic factors that secreted by corneal tissue and intra-corneal infiltrated inflammatory cells. In this study, we detected NOS and iNOS expression at day 2 and day 4 after alkali injury. We detected the cornea neovascularization 14 days after injury, and compared the difference between control group and intervention group.

NO is synthesized from L-arginine catalyzed by NOS. It is a kind of short-life active molecules, which is hard to be detected. In the current study we indirectly observed the expression of NO by detecting NOS. As we know, NOS can be divided into three types according to their different sources: nNOS, eNOS and iNOS. The first two were constitutive types which were physiologically provider for NO while the iNOS was expressed mainly under inflammatory circumstance. L-NAME is a non-selective NOS inhibitor, which can inhibit all types of NOS [11,12]. In a Cerebral ischemia and colitis animal model, inhibited NOS by L-NAME resulted in the significantly inhibited development of new blood vessels[13,14]. In our study of alkali injury induced CRNV, we treated mice with L-NAME by oral administration. Our RT-PCR results showed that the NOS expression were decreased in corneal tissue of treated mice and the CRNV area were markedly reduced, indicating

that NOS plays an important role of angiogenesis in alkali injury induced CRNV.

NO was mainly catalyzed by iNOS in inflammatory pathological conditions. In our study we found that inhibit iNOS couldn't inhibit CRNV. It seems to be contradictory with the previous reports. NO was generated from L-arginine by the catalyzing of NOS. iNOS is the main but not only provider of NO in inflammatory pathological conditions. Under the inflammatory response, eNOS and nNOS are also catalyzed to generated NO. Different types of NOS may play different role in angiogenesis. Ando *et al*[7,8] observed that deficiency of eNOS suppresses retinal but not CNV, whereas deficiency of nNOS or iNOS suppresses choroidal, but not retinal neovascularization. Using ischemic induced limb angiogenesis model, Kimura *et al*[15] observed eNOS knockout mice and iNOS knockout mice resulted in different angiogenesis. L-NAME is a kind of NOS inhibitor, our RT-PCR result showed that in alkali injury induced CRNV, the expression of NOS in L-NAME treated group was inhibited, which suggesting decreased NO generation. Our result of impaired CRNV in experiment group suggests that NO plays a role of promoting angiogenesis in alkali induced CRNV. Different to L-NAME, AG is a selective iNOS inhibitor and it can inhibit iNOS gene expression thereby inhibit NO production. By topic administration of AG, we did not found CRNV area change between treated and control group. This result suggests that iNOS does not play a key role in CRNV development. Based on above findings, we concluded that in the process of alkali induced CRNV NO but not iNOS promote the formation and development of CRNV. In addition, we observed that the VEGF expression in corneal tissue have a trend of increase after intervention with iNOS (data not shown) although the CRNV area was not significantly increased. However, the possibility that the increased anti-angiogenic factor expression after the iNOS intervention and the rebalance of angiogenic and anti-angiogenic factors can not be ruled out. We will further explore it in future research.

The roles of iNOS in angiogenesis are still obscure. There are conflicting reports on the roles of iNOS in angiogenesis. In chick chorioallantoic membrane study, Pipili *et al*[16] used a large number of LPS to induce iNOS expression and found that the formation and development of new blood vessels was inhibited. In oxygen induced-retinopathy study, Sennlaub *et al*[17] found that iNOS can inhibit the expression of VEGF to inhibit the development of new blood vessels. On the other hand, in the study of gastric cancer animal model, iNOS inhibition by AG resulted in impaired tumor angiogenesis [18]. In gastric ulcer study, ulcer repair function by exogenous VEGF was completely blocked by inhibiting the expression of iNOS [19]. More over, iNOS has also been reported to have no effect on angiogenesis, such as in herpes simplex virus-induced experimental viral keratitis [20]. In our experiment we found that in alkali induced CRNV, iNOS did not promote angiogenesis. This may be due to different organization or different pathological environment and the effect of iNOS on angiogenesis may depend on the environment.

As we know, CRNV developed under the extremely complex and dynamic network. At present the effect of iNOS in inflammatory CRNV are not fully understood yet. What factors contributed to the different roles of iNOS in different environment? What is the relationship between eNOS and nNOS in the inflammatory CRNV? More investigative work needs to be done to clarify the role of nitric oxide and its synthetase in CRNV and it will provide experimental basis for clinical treatment of neovascular eye disease.

REFERENCES

1 Ambati BK, Nozaki M, Singh N, Takeda A, Jani PD, Suthar T, Albuquerque RJC, Richter E, Sakurai E, Newcomb MT, Kleinman ME, Caldwell RB, Lin Q, Ogura Y, Orecchia A, Samuelson DA, Agnew DW, St. Leger J, Green WR, Mahasreshti PJ, Curiel DT, Kwan D, Marsh H, Ikeda S, Leiper LJ, Collinson JM, Bogdanovich S, Khurana TS, Shibuya M, Baldwin ME, Ferrara N, Gerber H-P, DeFalco S, Witta J, Baffi JZ, Raisler BJ, Ambati J. Corneal avascularity is due to soluble VEGF receptor-1. *Nature* 2006;443(7114):993-997

2 Lu P, Li L, Mukaida N, Zhang X. Alkali-induced corneal neovascularization is independent of CXCR2-mediated neutrophil infiltration. *Cornea* 2007;26 (2): 199-206

3 Lu P, Li L, Kuno K, Wu Y, Baba T, Li YY, Zhang X, Mukaida N. Protective roles of the fractalkine/CX3CL1-CX3CR1 axis against alkali-induced corneal neovascularization through enhanced anti-angiogenic factor expression. *J Immunol* 2008;108(6):4283-4291

4 Lu P, Li L, Wu Y, Mukaida N, Zhang X. Essential contribution of CCL3 to alkali-induced corneal neovascularization by regulating vascular endothelial growth factor production by macrophages. *Mol Vis* 2008;14:1614-1622

5 Lu P, Li L, Liu G, van Rooijen N, Mukaida N, Zhang X. Opposite roles of CCR2 and CX3CR1 macrophages in alkali-induced corneal neovascularization. *Cornea* 2009;28(5):562-569

6 Lu P, Li L, Liu G, Zhang X, Mukaida N. Enhanced experimental corneal neovascularization along with aberrant angiogenic factor expression in the absence of IL-1 receptor antagonist. *Invest Ophthalmol Vis Sci* 2009;50(10):4761-4768

7 Ando A, Yang A, Mori K, Yamada H, Yamada E, Takahashi K, Saikia J, Kim M, Melia M, Fishman M, Huang P, Campochiaro PA. Nitric oxide is proangiogenic in the retina and choroid. *J Cell Physiol* 2002;191(1):116-124

8 Ando A, Yang A, Nambu H, Campochiaro PA. Blockade of nitric-oxide synthase reduces choroidal neovascularization. *Mol Pharmacol* 2002;62(3):539-544

9 She H, Nakazawa T, Matsubara A, Hisatomi T, Young TA, Michaud N, Connolly E, Hafezi-Moghadam A, Gragoudas ES, Miller JW. Reduced photoreceptor damage after photodynamic therapy through blockade of nitric oxide synthase in a model of choroidal neovascularization. *Invest Ophthalmol Vis Sci* 2007;48 (5): 2268-2277

10 Ambati BK, Anand A, Joussen AM, Kuziel WA, Adamis AP, Ambati J. Sustained inhibition of corneal neovascularization by genetic ablation of CCR5. *Invest Ophthalmol Vis Sci* 2003;44(2):590-593

11 Kubo-Inoue M, Egashira K, Usui M, Takemoto M, Ohtani K, Katoh M, Shimokawa H, Takeshita A. Long-term inhibition of nitric oxide synthesis increases arterial thrombogenecity in rat carotid artery. *Am J Physiol Heart Circ Physiol* 2002;282(4):H1478-H1484

12 Kazanski VE, Kamkin AG, Makarenko EY, Lysenko NN, Sutiagin PV, Kiseleva IS. Role of nitric oxide in the regulation of mechanosensitive ionic channels in cardiomyocytes: contribution of NO-synthases. *Bull Exp Biol Med* 2010;50 (2): 263-267

13 Chi OZ, Hunter C, Liu X, Weiss HR. Effects of VEGF and nitric oxide synthase inhibition on blood-brain barrier disruption in the ischemic and non-ischemic cerebral cortex. *Neurol Res* 2005;27(8):864-868

14 Aoi Y, Terashima S, Ogura M, Nishio H, Kato S, Takeuchi K. Roles of nitric oxide (NO) and NO synthases in healing of dextran sulfate sodium-induced rat colitis. *J Physiol Pharmacol* 2008;59(2):315-336

15 Kimura K, Goto T, Yagi K, Furuya H, Jujo S, Itoh J, Sawamura S, Koide S, Mori H, Fukuyama N. Biphasic action of inducible nitric oxide synthase in a hindlimb ischemia model. *Journal of Clinical Biochemistry and Nutrition* 2006;38 (2): 95-102

16 Pipili-Synetos E, Kritikou S, Papadimitriou E, Athanassiadou A, Flordellis C, Maragoudakis ME. Nitric oxide synthase expression, enzyme activity and NO production during angiogenesis in the chick chorioallantoic membrane. *Br J Pharmacol* 2000;129(1):207-213

17 Sennlaub F, Courtois Y, Goureau O. Inducible nitric oxide synthase mediates the change from retinal to vitreal neovascularization in ischemic retinopathy. *J Clin Invest* 2001;107(6):717-725

18 Wang GY, Ji B, Wang X, Gu JH. Anti-cancer effect of iNOS inhibitor and its correlation with angiogenesis in gastric cancer. *World J Gastroenterol* 2005;11 (25):3830-3833

19 Dudar GK, D'Andrea LD, Di Stasi R, Pedone C, Wallace JL. Vascular endothelial growth factor mimetic accelerates gastric ulcer healing in an iNOS-dependent manner. *Am J Physiol Gastrointest Liver Physiol* 2008;295(2): G374-G381

20 Keadle TL, Morris JL, Stuart PM. The effects of aminoguanidine on primary and recurrent ocular herpes simplex virus infection. *Nitric Oxide* 2005;13(4):247-253