• Basic Research •

Beta-alanine promotes angiogenesis in laser-induced choroidal neovascularization mice models

Jia-Li Wu[1], *Min Zhang*[1], *Xiao-Dong Sun*[1,2,3,4,5]

[1]Department of Ophthalmology, Shanghai General Hospital (Shanghai First People's Hospital), Shanghai Jiao Tong University, School of Medicine, Shanghai 200080, China
[2]National Clinical Research Center for Ophthalmic Diseases, Shanghai 200080, China
[3]National Clinical Research Center for Eye Diseases, Shanghai 200080, China
[4]Shanghai Key Laboratory of Fundus Diseases, Shanghai 200080, China
[5]Shanghai Engineering Center for Visual Science and Photomedicine, Shanghai 200080, China
Co-first authors: Jia-Li Wu and Min Zhang
Correspondence to: Xiao-Dong Sun. Department of Ophthalmology, Shanghai General Hospital (Shanghai First People's Hospital), Shanghai Jiao Tong University, School of Medicine, Shanghai 200080, China. xdsun@sjtu.edu.cn
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Abstract

● **AIM:** To investigate the effect of β-alanine (BA) on laser-induced choroidal neovascularization (CNV) mice models.

● **METHODS:** Laser-induced CNV mice models were established, and BA was administrated for one week and two weeks in advance, separately. Furthermore, retinal pigment epithelium (RPE)-choroid flat mounts were separated, and immunohistochemical staining was performed. The laser-induced CNV lesion areas were measured and compared. In addition, liver and kidney morphologies were observed to identify potential hepatorenal toxicity.

● **RESULTS:** Enlarged CNV lesion areas were observed in the BA treated group. No significant differences were observed in the liver and kidney sections between groups.

● **CONCLUSION:** BA treatment increase CNV lesion areas, suggesting the detrimental effects of BA as a nutritional supplement in age-related macular degeneration (AMD) population.

● **KEYWORDS:** age-related macular degeneration; choroidal neovascularization; β-alanine; angiogenesis
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INTRODUCTION

Age-related macular degeneration (AMD) is one of the leading causes of irreversible blindness in the elderly population. The expected number of AMD patients reached approximately 196 million by 2020 globally, and it is anticipated to increase to 288 million by 2040 due to the aging of the population[1]. Clinically, AMD can be classified into two types: dry (atrophic) and wet (neovascular). Choroidal neovascularization (CNV) is a major pathological manifestation of neovascular AMD. CNV is characterized by abnormal vessel growth from the choroid, disrupting Bruch's membrane and the retinal pigment epithelium (RPE) layer, resulting in vision loss[2]. Anti-vascular endothelial growth factor (VEGF) therapy is the first-line treatment for CNV; however, repeated long-term injections are reportedly associated with diminished effectiveness. As many as 60% of neovascular AMD patients failed to achieve excellent functional visual acuity even after sustained anti-VEGF treatment for 1y[3-4]. In addition, frequent injections may cause adverse events, such as endophthalmitis and fibrosis. Therefore, further investigation of AMD pathology is warranted to develop novel or complementary therapies.

As a multifactorial disorder, AMD interferes with multiple pathological mechanisms, predominantly aging. The prevalence of AMD in people over 80 years of age has reached approximately 32.3% in the Iran population[5]. Senescent cells have been detected in the retinas and RPEs of older individuals and primates[6]. Moreover, the elimination of senescent RPE cells decreased CNV volume in laser-induced CNV mouse models[7]. Therefore, aging is a potential therapeutic target for AMD treatment. Metabolic dysfunction is also associated with aging. Small-molecule metabolites are downstream products of genetic transcription, and are influenced by both the environment and diet. The identification of metabolites that participate in or regulate the aging process is critical for developing novel therapeutic approaches. Recently, Singh *et al*[8] reported that a deficiency of taurine, which is the most

abundant nonessential amino acid, drives aging and affects the lifespan of an individual. Taurine depletion could be induced by β-alanine (BA) supplementation, and BA-induced taurine deficiency has been found to cause retinal ganglion cell loss and impair axonal transporting[9]. BA is a widely used nutrient supplement that improves exercise. A large number of patients with AMD may take BA supplements as well. However, the role of BA in AMD remains to be elucidated.

In the present study, we investigated the role of BA intake in AMD. By systematically administering BA to laser-induced CNV mice, we found that the lesion areas were aggravated. Our findings suggest a potential proangiogenic effect of BA in AMD.

SUBJECTS AND METHODS

Ethical Approval The animal experiments were approved by the Animal Care and Use Committee at Shanghai Jiao Tong University (Approval number: 2019AW055), and the procedures were conducted according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

β-alanine Functional Analysis The Comparative Toxicogenomics Database (CTD, https://ctdbase.org/) was applied to study the structure of BA, its association with eye diseases, pathway analysis, Gene Ontology (GO) analysis, and phenotype analysis.

Animals and Treatments Six- to eight-week-old C57BL/6J male mice were purchased from Shanghai Slac Laboratory Animal Company and housed in a controlled atmosphere with a 12-hour light-dark cycle. Laser-induced CNV models were generated as reported previously[10]. Briefly, the mice were anesthetized, and pupils were dilated with tropicamide. Four laser spots were created in each eye. If hemorrhage or laser drag occurred, the eye was excluded. Seven days after laser treatment, eyeball, liver and kidney samples were dissected for the experiments described below.

β-alanine Supplementation BA (S5526, Selleck Chemicals) was dissolved in 0.9% NaCl solution. The dosing concentration was 3%. BA was administrated 1 mg/kg•d, corresponding to approximately 100 μL of BA solution, by oral gavage.

Immunofluorescence Staining Immunofluorescence staining assay was conducted on RPE-choroid complexes seven days after laser injury. After embedded with 4% paraformaldehyde, the complexes were blocked by phosphate buffer solution with 0.1% Triton X-100 and 5% bovine serum albumin for 1h at room temperature, followed by primary antibody against isolectin-B4 (Santa Cruz Biotechnology, 1:500) overnight. The flat mounts were visualized *via* confocal microscope (Leica, United States), and we further quantified the isolectin B4-positive area as CNV lesion area with Image J software (NIH, United States).

Hematoxylin and Eosin Histological Staining The mice were sacrificed, and then the liver and kidney samples were enucleated and fixed in fix solution (G1101; Servicebio, China). After wax embedding, tissue sections were then dewaxed and stained with hematoxylin and eosin (H&E) staining solutions (Servicebio). Briefly, the slides were stained in hematoxylin solution for 5min. After treated with hematoxylin differentiation solution and rinsed with water, the slides were further treated with hematoxylin scott tap bluing and rinsed with water again. Thereafter, the slides were dehydrated sealed with neutral gum. Tissue morphology was observed under a microscope.

Statistical Analysis Statistical analysis was performed with SPSS 21.0 software (IBM, Chicago, IL, United States) and presented with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Data were showed as the mean±standard deviation (SD). The differences between groups were analyzed by a Student's *t*-test. P value <0.05 was considered statistically significant.

RESULTS

Functional Analysis of β-alanine The functional analysis of the BA is shown in Figure 1. Early onset macular degeneration, AMD, and retinal diseases were all correlated with BA (Figure 1B). Metabolism, signal transduction, and the immune system were the top three pathways, whereas cellular anatomical entity, binding, and cellular processes were the most significantly enriched in GO analyses (Figure 1C, 1D). The reported phenotypes of BA include the regulation of nucleotide metabolic processes, pyrimidine nucleotide metabolic processes, metabolic processes and amino acid metabolic processes, as presented in Table 1[11-16].

β-alanine and Angiogenesis in Choroidal Neovascularization Mice Models Laser-induced CNV mouse models were established to evaluate the effects of BA. The administration process lasted for 1 and 2wk, separately. One week after laser induction, RPE-choroid flat mounts were isolated and stained with isolectin-B4. The effects of BA on angiogenesis were assessed by quantifying laser-induced lesion sizes. The timeline of the procedure is shown in Figure 2.

As shown in the representative flat mounts, laser treatment induced the formation of angiogenesis spots. RPE-choroid flat mounts in the group with consecutive BA supplementation for seven days showed significantly larger CNV lesion areas, compared to sections from the control group (P=0.02; Figure 3A, 3B). In the 14-day group, BA dietary administration also increased spot sizes compared to those in the control group, although the difference was not statistically significant (P=0.08; Figure 3A, 3C). These results indicate that BA aggravated angiogenesis in the CNV models.

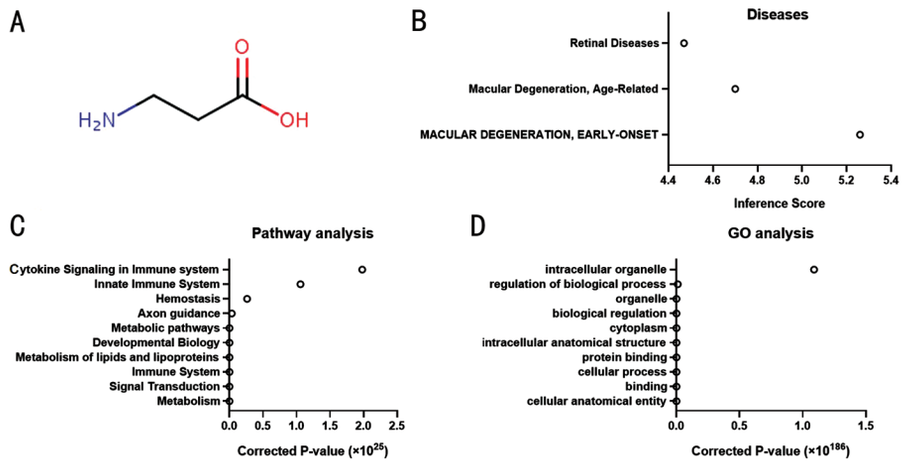

Figure 1 Functional analysis of BA A: Structure of BA; B: Correlation between ocular diseases and BA; C: Pathway analysis of BA; D: GO analysis of BA. BA: β-alanine; GO: Gene Ontology.

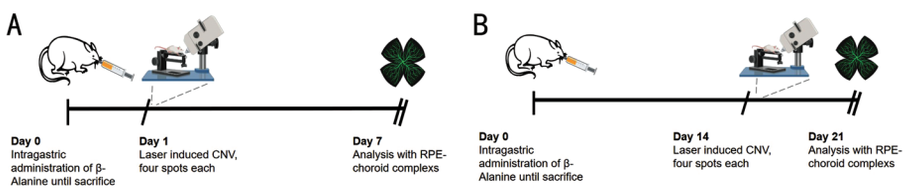

Figure 2 Experimental grouping and processing A: Laser was performed the day after intragastirc administration of BA. BA supplementation was given once a time until sacrifice at day 7. B: BA dietary supplementation started 14d before laser treatment, and continued for another 7d until sacrifice. BA: β-alanine.

Table 1 Summary on phenotypes of BA

Phenotype	Co-mentioned terms	Interaction	References
Regulation of nucleotide metabolic process	Dichlorodiphenyl-dichloroethylene	Dichlorodiphenyl-dichloroethylene affects regulation of nucleotide metabolic process which results in decreased abundance of BA	11
Pyrimidine nucleotide metabolic process	Methylmercuric chloride	Methylmercuric chloride affects pyrimidine nucleotide metabolic process which results in increased abundance of BA	12
Metabolic process	Niclosamide	Niclosamide analog results in increased metabolic process which results in increased abundance of BA	13
Amino acid metabolic process	Saccharin	Saccharin affects amino acid metabolic process which results in increased abundance of BA	14
Amino acid metabolic process	Diquat	Diquat affects amino acid metabolic process which results in decreased abundance of BA	15
Amino acid metabolic process	Benzo(a)pyrene	Benzo(a)pyrene affects amino acid metabolic process which results in increased abundance of BA	16

BA: β-alanine.

β-alanine and Hepatic or Renal Damage in Choroidal Neovascularization Mice Models Subsequently, we examined the potential hepatorenal toxicity of BA. Liver and kidney tissues were acquired, and pathological examinations were performed using H&E staining. As shown in Figure 4, no apparent pathological changes were observed in the BA group.

DISCUSSION

BA is one of the most popular supplements for sportsmen. General views have focused on its benefits in improving exercise performance; however, its side effects have rarely been studied. In this study, we investigated the promotion of CNV formation based on laser-induced CNV mice model for the first time.

As one of the leading causes of irreversible visual impairment, AMD poses a substantial medical and economic burden on geriatric populations[17]. Anti-VEGF treatment was introduced to target the CNV process, a critical pathological event in AMD, and has become the first-line therapy. However, non-responsiveness and resistance remain unresolved. Thus, the identification of other pathological factors is important. Several mechanisms have been shown to promote AMD progression, including oxidative stress, and metabolic dysregulation. As discussed in our previous study, aging has a cause-and-effect relationship with these multiple etiologies[10]. Several aging-related genes were significantly altered in CNV mouse models, suggesting that aging could be a promising target for further therapeutic exploration[10].

Metabolic dysregulation contributes to aging and age-related diseases, including AMD. Novel untargeted metabolomics has enabled the assessment of metabolic changes in the AMD

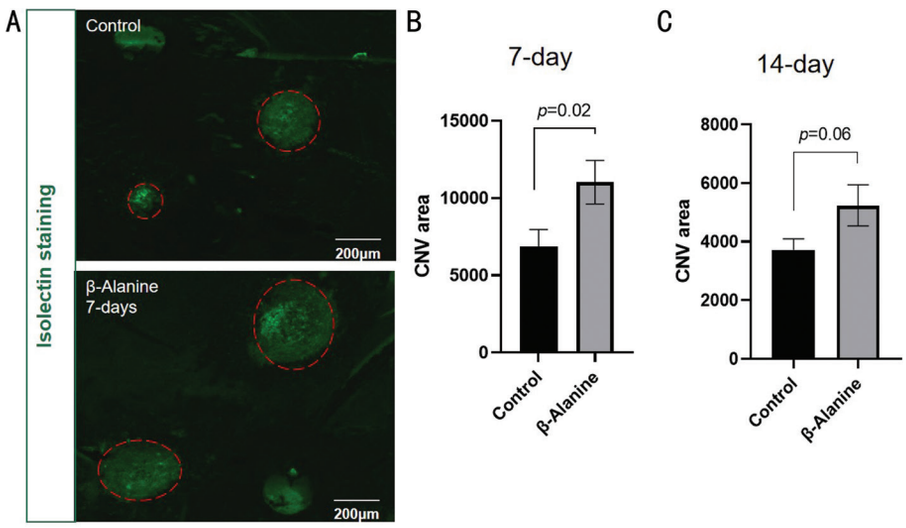

Figure 3 CNV lesion area increased in BA treated group A: Isolectin-B4 staining of RPE-choroid flat mounts with either 0 (Control) and BA supplementation for 7d. B: Quantitative evaluation of 7-day result. C: Quantitative evaluation of 14-day result. CNV: Choroidal neovascularization; RPE: Retinal pigment epithelium; BA: β-alanine.

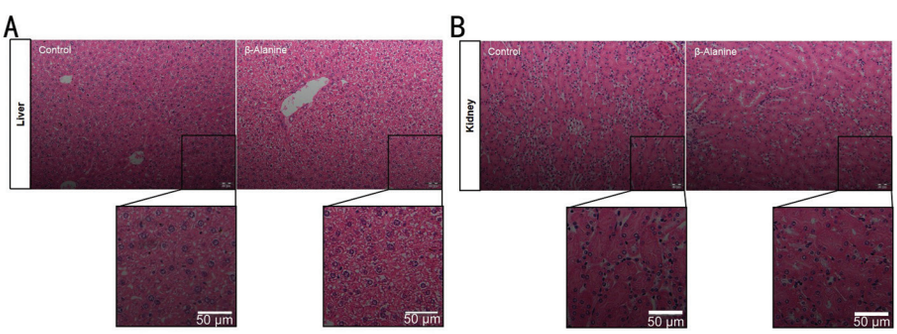

Figure 4 H&E staining was used to observe morphology of liver and kidney in control and BA-treated group A: Liver; B: Kidney. H&E: Hematoxylin and eosin; BA: β-alanine.

process. A previous metabolic study using CNV models identified altered amino acid metabolism, such as proline metabolism[18]. Proline is a predominant nutrient in RPE cells, and can mediate metabolic communication between the RPE and retina[19]. Another metabolic study conducted in an AMD population suggested that significantly decreased levels of excreted amino acids were common features of AMD[20]. As a non-essential amino acid synthesized in the liver, BA increases carnosine levels in human skeletal muscles and delayes blood lactate accumulation[21]. Previous evidence had shown that BA supplementation contributes to improvements in all exercise measures[22]. For example, BA had been reported to increase the performance of the ventilatory threshold by as much as 13.9%[23]. However, the adverse effects of BA have rarely been investigated. In our study, its use as a nutritional supplement may present potential challenges in certain pathological conditions for certain populations, such as AMD.

BA-related metabolic dysregulation had been reported in patients with fundus disorders and neovascular pathologies. For example, alanine, aspartate, and glutamate metabolism was enriched in oxygen-induced retinopathy[24]. Genes involved in L-alanine fermentation were enriched in intestinal microbiomes of patients with AMD[25]. In addition, D-alanine levels are decreased by ocular hypertension in rat retinas[26]. Histidine and BA signaling was revealed in diabetic retinopathy[27]. The BA trafficking pathway was also required for histamine homeostasis and visual neurotransmission in Drosophila[28]. Furthermore, anti-VEGF treatment of glioblastoma resulted in increased levels of alanine metabolites[29]. These results shed insight into the potential mechanisms of BA in neovascular AMD.

However, the general effects of alanine on neovascularization remain unclear. The available evidence focuses mainly on materials. Aleem *et al*[30] loaded alanine into chitosan/collagen hydrogels and demonstrated their angiogenic potential using a chorioallantoic membrane assay. Compared to the control group, increased growth of blood vessels was observed in the alanine group. Debats *et al*[31-32] used alanine as a placebo when studying the effects of intravenous and oral arginine administration on human skin graft donor site healing, and reported that enteral arginine supplementation did not improve angiogenesis in the wound healing of skin donor

Table 2 Study summary of BA function in oxidative stress and retinal degeneration

Model	Dosage	Phenotype	Mechanism	References
Light exposure in rats	3% in drinking water for 2mo	Photoreceptor outer segment degeneration; retinal thickness decrease	Cause oxidative stress to photoreceptors; activate glial cell; impair phagocytic capacity of RPE	9
Light exposure in rats	3% in drinking water for 2mo	Retinal degeneration; photoreceptor degeneration	NA	35
	2% in drinking water for 2mo	Photoreceptor light-sensitivity reduction; outer nuclear layer thinning; bisretinoid formation reduction	NA	36
	3% in drinking water for 1mo	Decrease ventricular wall thickness, left ventricle dry weight, myocyte sectional area, left ventricle posterior wall thickness and ventricular geometry	Increase oxidative stress	37
I/R of the small intestine in rats	10, 30, and 100 mg/kg intravenously	Intestinal I/R-induced injury amelioration	Diminish macrophage accumulation	38
Myocardial I/R in rats	Water containing BA from conception until weaning	Cardiac I/R injury exacerbation; arterial pressure dysregulation	NA	39
21-day-old Wistar rats	Peritoneal injections of BA (300 mg/kg) twice a day at 12-h interval, from the 7th to 21st postpartum day	NA	Cause cellular oxidative damage; change energy metabolism	40
T-butyl hydroperoxide treated murine erythrocytes	3% in aqueous medium for 14d	NA	Increase phosphatidylserine externalization and reactive oxygenspecies formation	41

BA: β-alanine; I/R: Ischemia and reperfusion; RPE: Retinal pigment epithelium; NA: Not available in the study.

sites. Considering the significant superiority of arginine-loaded chitosan/collagen hydrogels in angiogenesis[30], the negative results reported by Debats *et al*[31] might contribute to the angiogenic potential of the placebo, rather than the neutral role of arginine. However, Zhou *et al*[33] reported that BA-functionalized gadofullerene nanoparticles exhibited superior antitumor activity through the destruction of tumor vasculatures *in vivo*. Our results showed that the CNV mouse models with consecutive BA gavages exhibited larger neovascularization lesion areas. Therefore, the involvement of other BA-related pathologies in AMD development and CNV formation should be further explored.

By summarizing the function of BA in previous studies, we found that it played a major role in oxidative stress and retinal degeneration (Table 2[9,34-40]). Martínez-Vacas *et al*[9] found that BA supplementation caused cell damage to multiple retinal layers, including the RPE, and increased glial cell reaction and oxidative stress. Moreover, Baek *et al*[41] found that BA treatment of endothelial cells resulted in increased vessel density and cell proliferation through phosphorylation of extracellular regulated protein kinases (ERK) and protein kinase B (Akt). We speculate that the pro-angiogenic effect observed in our study could be the result of oxidative stress activation.

Our study has some limitations. First, BA concentration was not detected in patients with AMD. Second, alanine exhaust was not used to verify its effects on CNV. A detailed illustration of BA-induced neovascularization is required. In summary, our study found that BA increased the CNV lesion areas for the first time, suggesting the potential risk of using BA as a nutritional supplement in the AMD population.

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