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Original Paper

N-Acetylcysteine Suppresses LPS-Induced Pathological Angiogenesis

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Key Words
N-acetylcysteine • NAC • Lipopolysaccharide • LPS • Pathological angiogenesis • Chick CAM • Oxidative stress

Abstract
Background/Aims: Angiogenesis is a key feature during embryo development but is also part of the pathogenesis of cancer in adult life. Angiogenesis might be modulated by inflammation. ***Methods:*** We established an angiogenesis model in chick chorioallantoic membrane (CAM) induced by the exposure of lipopolysaccharide (LPS), and analyzed the effects of the antioxidant N-acetylcysteine (NAC) on angiogenesis in this model as well as on the expression of key genes known to involved in the regulation of angiogenesis. ***Results:*** Treatment with NAC was able to normalize LPS induced angiogenesis and restore the LPS-induced damage of vascular epithelium in chick CAM. Using quantitative PCR, we showed that NAC administration normalized the LPS induced expression of Keap1-Nrf2 signaling and oxidative stress key enzyme gene expressions (SOD, GPx and YAP1). ***Conclusion:*** We established a LPS-induced angiogenesis model in chick CAM. NAC administration could effectively inhibit LPS-induced angiogenesis and restore the integrity of endothelium on chick CAM. LPS exposure caused an increased expression of genes involved in oxidative stress in chick CAM. NAC administration could abolish this effect.

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Introduction

During embryogenesis the cardiovascular system develops and reaches full functional status at birth. This developmental of the cardiovascular system is caused by epigenetic mechanisms that are sensitive to eviromental challenges [1, 2] as well as direct effects of various drugs on embryonic development [3]. Normal vasculature development occurs typically at three different morphological stages, which includes vasculogenesis, angiogenesis and vascular remodeling [3]. Both vasculogenesis and angiogenesis are accomplished during the prenatal period. The formation of the primitive vascular plexus derived from hemangioblasts in extra-embryonic yolk sacs or from within the embryois the first step of vasculogenesis. Angiogenesis involves the expansion and remodeling of the vascular plexus through endothelial sprouting and intussusceptive microvascular growth [4]. It is a complex process and regulated by a many factors. An important stimulating factor of angiogenesis is VEGF, acting through VEGFR. VEGF is a specific mitogen which stimulates vasculogenesis and angiogenesis [5]. Another significant growth factor are the angiopoietins (Ang1 and Ang2) which are involved in the secondary stages of vessel growth and are required for the formation of mature blood vessels [6]. Fibroblast growth factor 2 (FGF-2), known for its angiogenic potential, is also of great importance in angiogenesis. One of the most important functions of FGF-2 is the promotion of endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures, thus promoting angiogenesis [7].

Pathological angiogenesis is characterized by aberrant proliferation of blood vessels, seen for example during cancer formation. Another example of pathological angiogenesis is important for vision loss in diabetic patients. Here pathological angiogenesis is seen in the retina and may cause blindness [8]. Hypoxia is considered as an important factor in pathological angiogenesis, since hypoxia can activate the crucial angiogenesis mediators such as transcription factors hypoxia-inducible factor (HIF) and vascular endothelial growth factor (VEGF), which in turn promote tumor dissemination, invasion and metastasis [9, 10]. Therefore, either laser photocoagulation or intravitreal injection of therapeutic antibodies binding VEGF is currently used for the treatments of pathological angiogenesis in diabetic patients [11]. However, there are many drawbacks for those therapeutic approaches because the consistent application is of inconvenience, low or variable efficacy, and costly. Thus, it is necessary to look for alternative treatment strategies, especially noninvasive and efficacious natural agents.

Pollet et al. reported that bacterial lipopolysaccharide (LPS) stimulated endothelial sprouting to initiate angiogenesis *in vivo* and *in vitro* through activating TRAF6-dependent signaling pathways [12]. Endotoxin and/or some cytokines stimulate pathological angiogenesis and the aberrant formation of new blood vessels [13]. Simon et al. reported that LPS exposure evoked cell death in umbilical vein endothelial cells through triggering ROS production [14]. *In vivo* studies demonstrated that LPS exposure caused large amounts of ROS production in activated microglia, which, in turn, cause neuronal damage [15]. Many compounds have been found to possess the inhibitory effect on LPS-stimulated reactive oxygen species (ROS) production [16].

Reactive oxygen species (ROS), as a component of oxidative phosphorylation, play an important role in the redox control of various signaling pathways [17-19]. However, excessive ROS generation in the body is associated with the pathogenesis of many diseases [17-19]. Excessive ROS accumulation could interfere with cellular and physiological functions through deleterious oxidization of macromolecules including proteins, lipids, DNA and signal transduction [20]. ROS can act as primary or secondary messengers to promote cell growth or death, and oxidative stress could initiate crucial reactions that either positively or negatively influence embryonic development. Therefore, a fine balance between ROS production and degradation is key for normal physiological functions of the cell [21, 22]. In this context, oxidative stress is regarded as a result of an imbalance between ROS generation

and degradation. We have been interested in N-acetylcysteine (NAC) since it could act as an antioxidants to restore intracellular levels of glutathione (GSH), the one of the body's most powerful antioxidant defenses [23].

NAC is a cysteine pro-drug, which has been used in therapeutic practices on the treatment of various disorders such as paracetamol intoxication for several decades [24]. As an antioxidants, NAC could also commendably diminish oxidative stress in patients with chronic obstructive pulmonary disease (COPD) [25, 26]. The function of NAC as anti-oxidation is achieved via its reaction with free radicals as well as the restitution of reduced glutathione (GSH) in numerous cells of the body [24, 26]. In this study, we investigated the biological effect of NAC on the pathological angiogenesis using an *in vivo* chick ACM model.

Materials and Methods

Avian embryos and treatments

Fertilized chick eggs were obtained from the Avian Farm of the South China Agriculture University. For early gastrula embryos, HH0 (Hamburger and Hamilton stage 0) [27] chick embryos were prepared and incubated in absence/presence of N-acetylcysteine (NAC) (Sigma, USA) or/and lipopolysaccharides (LPS, also called lipoglycans and endotoxins) (Sigma, USA) using early chick culture (EC culture) [28]. For later stage embryos, various concentrations of NAC (200/400/800/1000 μg/ml), LPS (1/5/10/20/100 μg/ml), Vitamin C (Vc) (5mg/ml) or saline (control) were directly injected into the air chamber at the blunt end of the fertilized egg.

The embryos were harvested at desired time based on the experimental requirements after incubation at 38°C. All of the embryos were photographed using a stereomicroscope (Olympus MVX10, Japan) before they were fixed with 4% paraformaldehyde for analysis of morphology and gene expression. Only the surviving embryos were used for further research. For the histological analysis, the treated embryos or yolk sacs were dehydrated, embedded in paraffin wax and serially sectioned at 5 μm using a microtome (Leica RM2126RT, Germany). And then the sections were de-waxed in xylene, rehydrated and stained with either hematoxylin and eosin dye or immunofluorescent staining. The sections were photographed using a fluorescent microscope (Olympus IX50) with the NIS-Elements F3.2 software package.

Assessment of angiogenesis using chick chorioallantoic membrane (CAM)

As previously described [29], chick embryos were incubated until day 7 when chick CAM is well developed. The embryos were treated with NAC, LPS, or saline (control) for 48 hours and all surviving embryos were harvested for analysis. The CAM and accompanying blood vessels in the control, NAC and/or LPS-treated embryos were photographed using a Canon Powershot SX130 IS digital camera (12.1M Pixels). Ten embryos in each experimental group were examined. CAM tissues from eight embryos in each group were embedded, sectioned and stained with hematoxylin & eosin. The blood vessel density (BVD) were quantified using an IPP 5.0 image analysis program. The blood vessel density was expressed as the percentage of area occupied by blood vessel as previously described [30]. The chick CAM tissues were also harvested for biochemical assays as described below.

The chick embryo aortic arch assay

The aortic arches assay was done with minor modifications as reported previously [31]. Briefly, aortic arches of day 13 chick embryos are isolated and cut into small piece of rings tissues. The outgrowth of cells including the formation of vessel-like structures occurs mainly within 24-48 hours. The distance between cell emigrating from the aortic arches and the aortic arches is a parameter describing cell migration.

Assessment of blood vessel integrality

The assay of Evans blue (EB) (Sigma, US) leakage was used to evaluate blood vessel disruption following LPS, NAC, Vitamin C and saline treatment as described previously. The EB measurement was performed according to the previous study [32]. Briefly, 2 % EB (4 ml/kg) was injected via YSM. At 2h after EB injection, YSM were harvest. The embryos for detecting the Evans blue contents were reperfused with PBS before

harvest. YSM samples were then homogenized by formamide (1 ml formamide/100 mg tissue) and incubated for 24 h at 60°C The absorbance of supernatants was measured at 620 nm [33] with microplate reader (Biotek, ELX800, US).

Semiquantitative RT-PCR

Total RNA was isolated from the gastrula chick embryos or chick CAM tissuesusing a Trizol kit (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized to a final volume of 25 µl using SuperScript RIII first-strand (Invitrogen, USA). Following reverse transcription, PCR amplification of the cDNA was performed. The sets of primers used for semiquantitative RT-PCR are provided in the Table 1. The PCR reactions were performed in a Bio-Rad S1000TM Thermal cycler (Bio-Rad, USA). The final reaction volume was 50 µl composed of 1 µl of first-strand cDNA, 25 µM forward primer, 25 µM reverse primer, 10 µlPrimeSTARTM Buffer ($Mg2^+$ plus), 4 µl dNTPs Mixture (TaKaRa, Japan), 0.5 µlPrimeSTARTM HS DNA Polymerase (2.5U/µl TaKaRa, Japan), and RNase-free water. cDNA was amplified for 30 cycles. One round of amplification was performed at 94°C for 30 s, 30 s at 58°C, and 30 s at 72°C. The PCR products (20 µl) were resolved using 1% agarose gels (Biowest, Spain) in 1× TAE buffer (0.04 MTrisacetate and 0.001 M EDTA) and 10, 000x GeneGreen Nucleic Acid Dye (TIANGEN, China) solution. The resolved products were visualized using a transilluminator (SYNGENE, UK), and photographed using a computer-assisted gel documentation system (SYNGENE). Each of these experiments was replicated at least three times.

Table 1. Primers sets used in the RT-PCR analysis

Primers	Forward	Reverse
VEGFA	ACAAGAAAATCACTGTGAGCCT	TGCTCACCGTCTCGGTTTTT
VEGFC	TTGCGATCTGTGTCCAGTGT	GAGCTGGAGTGTTCCCTGTT
VEGFD	CTTCTGGACCACGGATGGATT	ATGCAGCTCCACTCTCACAAG
VEGFR2	TTGGCTGGCGGTATTCACAT	GTCTCTGGAGGAAAGCACCC
VEGFR3	ATGTTCTCTCGACGCAGCCTTAG	TGTACATGGCTGAGACGTTGG
ANG1	ACAAAAGCGGCGTCTACACT	AGCCAGTGTTCACCTGATGG
ANG2	ACTGAATCCCAAGAAGTGAAGA	TCTACTGCAACCATGCTCAGA
ET1-1	GGAGCTGTTTACCCCCACTC	GTGCCCTTTTAACGGGGAGA
ET1-2	GAGCTGTCCAAGTCAGACGC	TCAGCCCAAGTGCCCTTTTA
ETA	ACTAGACGGCCTCCGGTATC	TCGGGCCATTCCTCATACAC
ETB	GTGTCATGCCTGGTGTTCGT	GCCAGTCCTCTGCAAGTAGC
GAPDH	GTCAACGGATTTGGCCGTAT	AATGCCAAAGTTGTCATGGATG
SOD1	CAGATAGGCACGTGGGTGAC	CCATGGTACGGCCAATGATG
SOD2	ACTGTTGTGCGACAAAGGGA	CACAAAGTGTGCGTTTCCACT
GLRX	CTGCCCTTACTGCAAGAATGC	AAACACACGAGGGACGGTTC
GPX1	GGGTACCGAAAGTCACTGGG	GTTCACGCGGGACAGTTTTC
SRXN1	CTGTGTTGGAGCCGGAGAAA	GTAGAAGTAGTCCCCGCCCT
Yap1	GTGTCATGGGTGGGAGTAGC	TGGCTACGGAGAGCCAATTC
SIRT3	AGTGCAGGACTGGCTGC	CTTCCGAATGAGCTCTGCCA
HIF1α	GGCAGTACTTCCATCCTGCTC	GCTACAATGCACTGCTGCAAAG
HIF-1β	TGGTGCCTCCAGTGAACATC	CACCTGGGCTGCGAACG
HIF2α	CGATGACAGCTGACAAGGAGAAG	AGGTGGGAACTGACATTGTGG
Fgf2	AGAAACTGCATGGTGGCAGA	CCTTCAGTGATTCCGGGGAC
FgfR	ACTGCGCAGACAGGTAACAG	GGCAGCTCATACTCGGAGAC

Data analysis

Statistical analysis for all the experimental data generated was performed using a SPSS 13.0 statistical package program for windows. The data were presented as mean ± SE. Statistical significance were determined using paired t-tests, independent samples t-test or one-way analysis of variance (ANOVA). *p<0.05, **p<0.01 and ***p<0.001 indicate significant difference between control and ethanol-treated specimens.

Results

Selecting the optimal concentration of NAC using gastrula chick embryos

To choose the optimal concentration of NAC, we incubated HH0 chick embryos in EC culture containing 0 (control), 200, 400, 800, 1000 µg/ml NAC, until HH12 (Fig. 1A). NAC showed a trend of a dose dependent increase in mortalitz, however, this effect was not significant (Fig. 1G); interestingly, exposing 800 µg/ml NAC (3461±214.9µm) did not significantly alter the length of HH12 chick embryo compared to control (3557±86.71µm), although other concentrations of NAC stimulated the growth of gastrula chick embryos to some extent (Fig. 1F-H). Therefore, 400 and 800 µg/ml NAC are used in the following experiments.

400 and 800 µg/ml NAC administration on its own did not affect angiogenesis on chick CAM

Using the angiogenesis model on chick CAM, we studied the effect of 400 and 800 µg/ml NAC on the embryonic angiogenesis. NAC did not significantly affect the angiogenesis on CAM (Fig. 2B-D) as well as the blood vascular densities of the vascular nets on chick CAM (Fig. 2E). The expressions of oxidative stress-related key genes on chick CAM were assessed using quantitative PCR following the exposure of 400 and 800 µg/ml NAC (Fig. 2G-L).

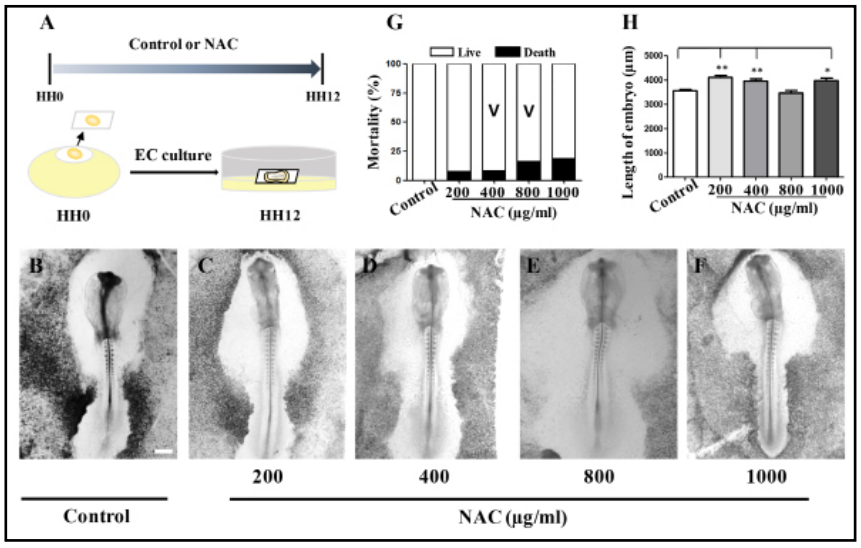

Fig. 1. Assessment of gastrula chick embryo development and mortality in absence/presence of various concentrations of NAC. A: The sketches illustrate to incubation of HH0 chick embryos until HH12 in EC culture in absence/presence of NAC (see also material and method for more details). B-F: Representive bright-field images of HH12 gastrula chick embryos were taken from saline treated control embryos (B), as well as embryos treated with 200 μg/ml NAC (C), 400 μg/ml NAC (D), 800 μg/ml NAC (E), 1000 μg/ml NAC (F) ,respectively. G-H: Bar graphs showing the comparison of mortality (G) and lengths (H) of the HH12 chick embryos either with saline (B) or with 200 μg/ml NAC (C), 400 μg/mlNAC (D), 800 μg/ml NAC (E), 1000 μg/ml NAC (F) , respectively. (n>3 chick embryos in each group). (*P<0.05, **P<0.01, ***P<0.001 versus controls). Scale bars = 300 μm in B-F.

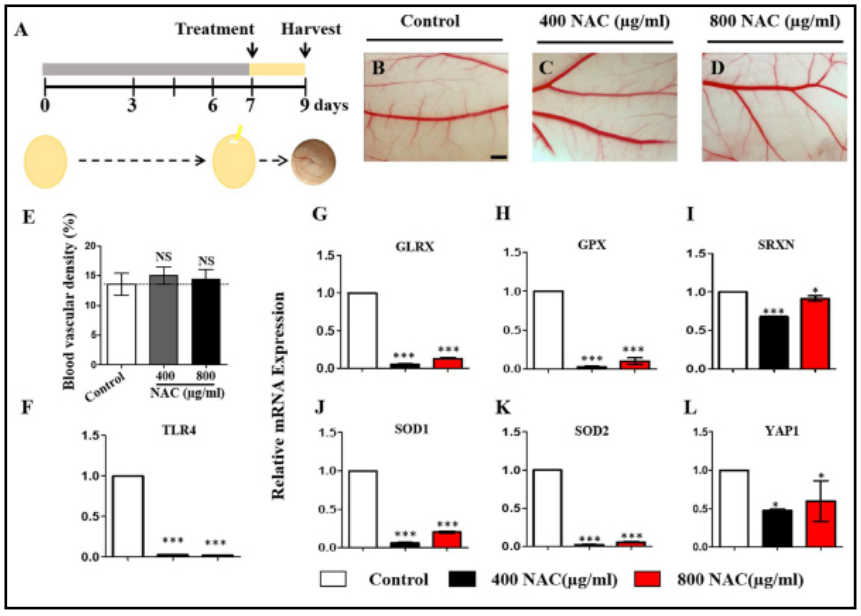

Fig. 2. Assessment of NAC effects on angiogenesis and mRNA expression of key enzymes involved in ROS production in chick CAM. A: The sketches illustrate the experimental design of the experiment. NAC or saline were given at day 7 of embryo development. Angiogenesis and gene expression was assed at day 9. B-D: Representative bright-field image of angiogenesis on CAM exposed to saline (Control, B), 400 μg/ml NAC (C) and 800 μg/ml NAC (D), respectively. E: Bar graph showing blood vascular densities on the CAM in control embryos, embryos treated with 400 μg/ml NAC and 800 μg/ml NAC groups. n>3 CAMs in each group. F-L: Quantitative PCR data show the gene expressions of TLR4 (F), GLRX (G), GPX (H), SRXN (I), SOD1 (J), SOD2 (K) and YAP1 (L) in control – saline treated - embryos, embryos treated with 400 μg/ml NAC and 800 μg/ml NAC , respectively. (Each of these experiments was replicated at least three times.). (*P<0.05, **P<0.01, ***P<0.001 versus controls). Scale bars = 1 mm in B-D.

NAC administration suppressed LPS-induced angiogenesis on chick CAM and aortic arch explant models

Using the chick CMA model (Fig. 3A), we analyseddifferent combinations of LPS (10 and 100 μg/ml) and NAC (400 and 800 μg/ml) for 2 days. The results showed that both dosages of LPS increase blood vascular densities (10 μg/ml LPS: 27.86±1.35, $p<0.001$; 100 μg/ml LPS: 29.62±3.99, $p<0.001$). The LPS-induced angiogenesis was significantly suppressed by NAC (10 μg/ml LPS & 400 μg/ml NAC: 15.71±1.77 %, $p<0.01$; 100 μg/ml LPS & 400 μg/ml NAC: 24.24±2.66, $p<0.001$; 10 μg/ml LPS & 800 μg/ml NAC: 13.73±1.31%, $p<0.01$; 100 μg/ml LPS & 800 μg/ml NAC: 17.50±1.47, $p<0.001$) (Fig. 3B-I). To confirm the experimental results above, we further employed the *in vitro* explant culture assay of the chick embryo aortic arches as previously described [31], 1μg/ml LPS in culture medium could stimulated the cell extension from the cultured explants of chick aortic arches. Again the LPS-stimulated cell extension from aortic arch explants was significantly regressed by addition of 1000 μg/ml NAC (1 μg/ml LPS: 421.4±3.293, $p<0.001$; 1 μg/ml LPS & 1000 μg/ml NAC: 359.2±25.98, $p<0.001$) (Fig. 3J-M), indicating that NAC administration indeed suppress LPS-induced angiogenesis.

NAC administration suppressed LPS-enhanced adhesion of endothelial cells and angiogenesis-related gene expressions on chick CAM

To investigate whether or not the integrity of blood vessels was altered in presence/absence of LPS and/or NAC, we carried out the Evans blue perfusion experiments in chick

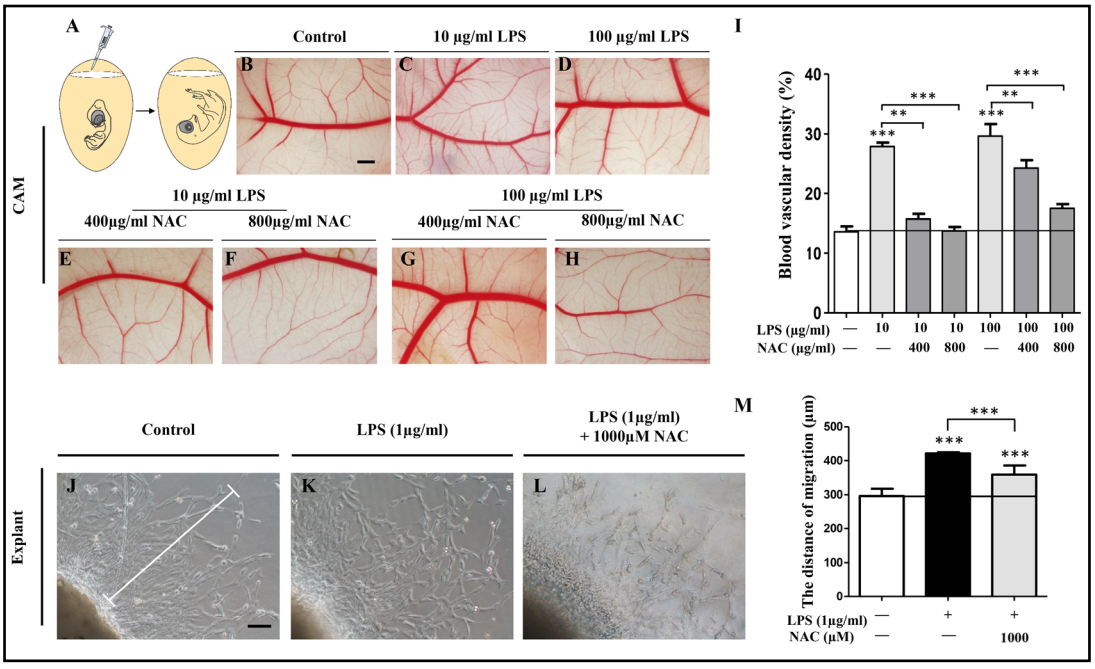

Fig. 3. Assessment of angiogenesis on chick CAM and *in vitro* explant culture in absence/presence of LPS and NAC. A: The sketches illustrate the same way to treat embryos as did in Fig. 2A. B-H: The representative bright-field image of angiogenesis on chick CAM exposed to simple saline (Control, B), 10 LPS (C), 100 LPS (D), 10 LPS + 400 NAC (E), 10 LPS + 800 NAC (F), 100 LPS + 400 NAC (G), and 100 LPS + 800 NAC (H) respectively. I: Bar chart showing the comparison of blood vascular densities on the CAM among control, 10 LPS, 100 LPS, 10 LPS + 400 NAC, 10 LPS + NAC, 100 LPS + 400 NAC, and 100 LPS + 800 NAC groups (n>3 CAM in each group). J-L: The representative bright-field images of the aortic arch explants from 13-day chick embryos were taken at *in vitro* 3-day incubation in presence of sample saline (Control, J), 1 LPS or 1 LPS + 1000 NAC groups. M: Bar chart showing the comparison of cell emigratory distances from the *in vitro*-cultured aortic arch explants among control, 1 LPS and 1 LPS + 1000 NAC groups (n>3 aortic arch explants in each group). Scale bars = 1 mm in B-H and 50 μm in J-L.

CAM model. The amount of Evans blue accumulated in the tissues is inversely related to the integrity of blood vessels. (Fig. 4B).

Furthermore, we determined gene expressions of tight junction and adherens junction molecules using quantitative PCR. Both 10 and 100 μg/ml LPS significantly increased gene expressions of tight junction molecules including Claudin-1, Claudin-5, Claudin-12, ZO1, ZO2 in chick CAM. Increased expression of these genes was blunted by NAC in a dose-dependent manner (except for Claudin-12 and ZO-2 in the 10 μg/ml LPS + 400 μg/ml NAC groups) (Fig. 4C-G). Both dosages of LPS administration also significantly increased the expressions of VE-cadherin, β-catenin, Plakoglobin, Vinculin and Par3; the addition of 400 or 800 μg/ml NAC suppressed the LPS-enhanced expressions of Plakoglobin and Vinculin (Fig. 4H-L). 10 and 100 μg/ml LPS significantly increased gene expressions of Ang2, VEGFR3 and FGF2 on chick CAM. Addition of 400 or 800 μg/ml NAC suppressed LPS-enhanced expressions of these genes (Fig. 5F, H, I). On the contrary, both 10 and 100 μg/ml LPS inhibited the expressions of Ang1 and VEGFR2 on chick CAM, and the expressions of Ang1 and VEGFR2 gradually rose by the addition of 400 or 800 μg/ml NAC (Fig. 5G, J).

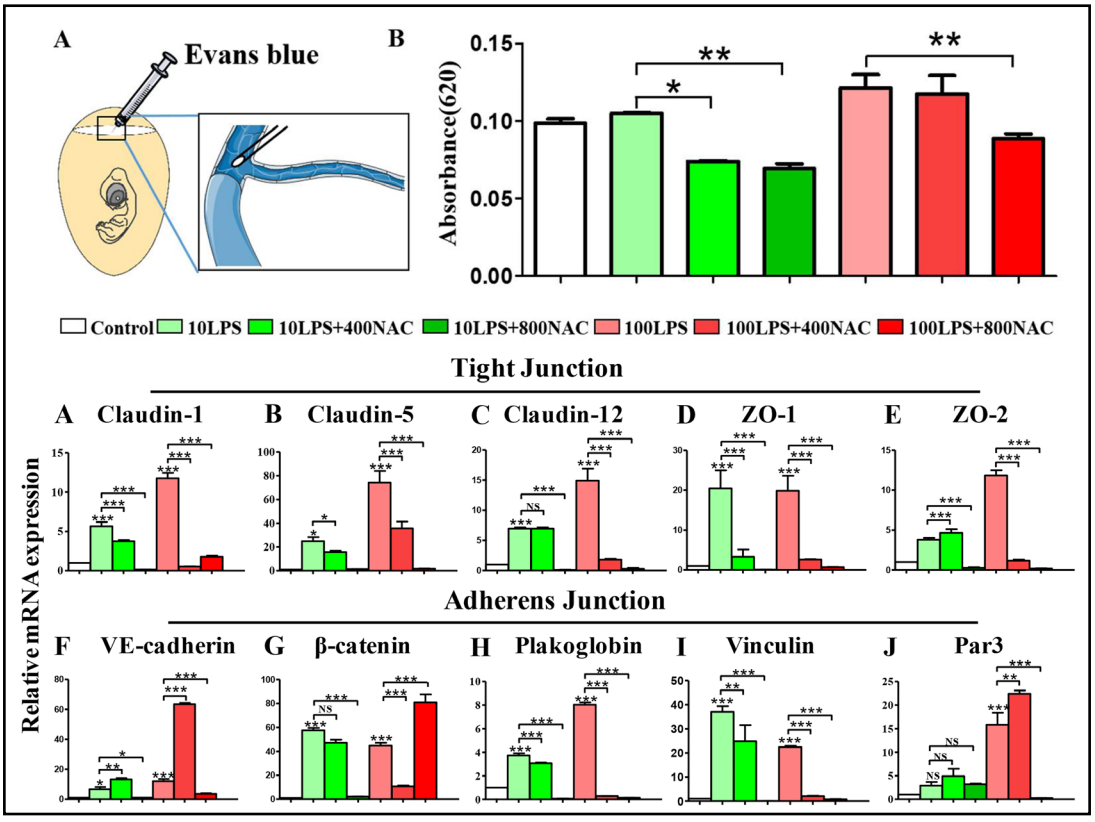

Fig. 4. Assessment of vascular integrity and gene expression of genes involved in tight-junction adhesion molecules, modulation of subcellular distribution of junctions, cell polarity proteins, junction formation and epithelial polarization in chick CAM in absence/presence of LPS and NAC. A: Sketches illustrates how Evans blue was injected into the blood vessel. B: Bar graphs showing blood vessel integrity. The amount of Evans blue accumulated in the tissues is inversely related to the integrity of blood vessels. C-L: Quantitative PCR data showing gene expressions of Claudin-1 (C), Claudin-5 (D), Claudin-12 (E), ZO-1 (F), ZO-2 (G), VE-Cadherin (H), β-catenin (I), Plakoglobin (J), Vinculin (K) and Par3 (L) in control, 10 μg/ml LPS, 10 μg/ml LPS + 400 μg/ml NAC, 10 μg/ml LPS + 800 μg/ml NAC, 100 μg/ml LPS, 100 μg/ml LPS + 400 μg/ml NAC, and 100 μg/ml LPS + 800 μg/ml NAC groups. Each of these experiments was replicated at least three times. (*P<0.05, **P<0.01, ***P<0.001 versus controls).

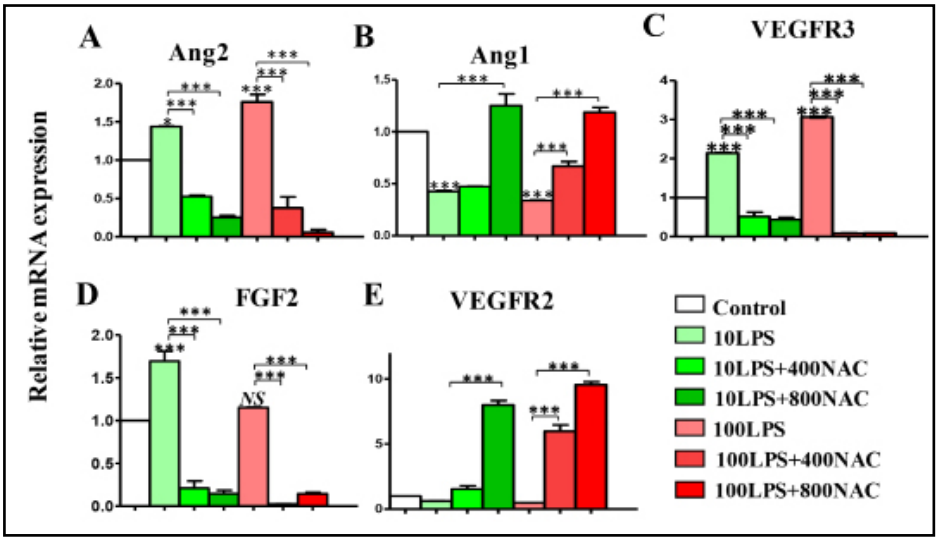

Fig. 5. Assessment of the angiogenesis-related gene expressions in chick CAM in absence/presence of LPS and NAC. A-E: Quantitative PCR data showing the gene expressions of Ang2 (A), Ang1 (B), VEGFR3 (C), FGF2 (D), and VEGFR2 (E)in control, 10 μg/ml LPS, 10 μg/ml LPS + 400 μg/ml NAC, 10 μg/ml LPS + 800 μg/ml NAC, 100 μg/ml LPS, 100 μg/ml LPS + 400 μg/ml NAC, and 100 μg/ml LPS + 800 μg/ml NAC groups. Each of these experiments was replicated at least three times. (*P<0.05, **P<0.01, ***P<0.001 versus controls).

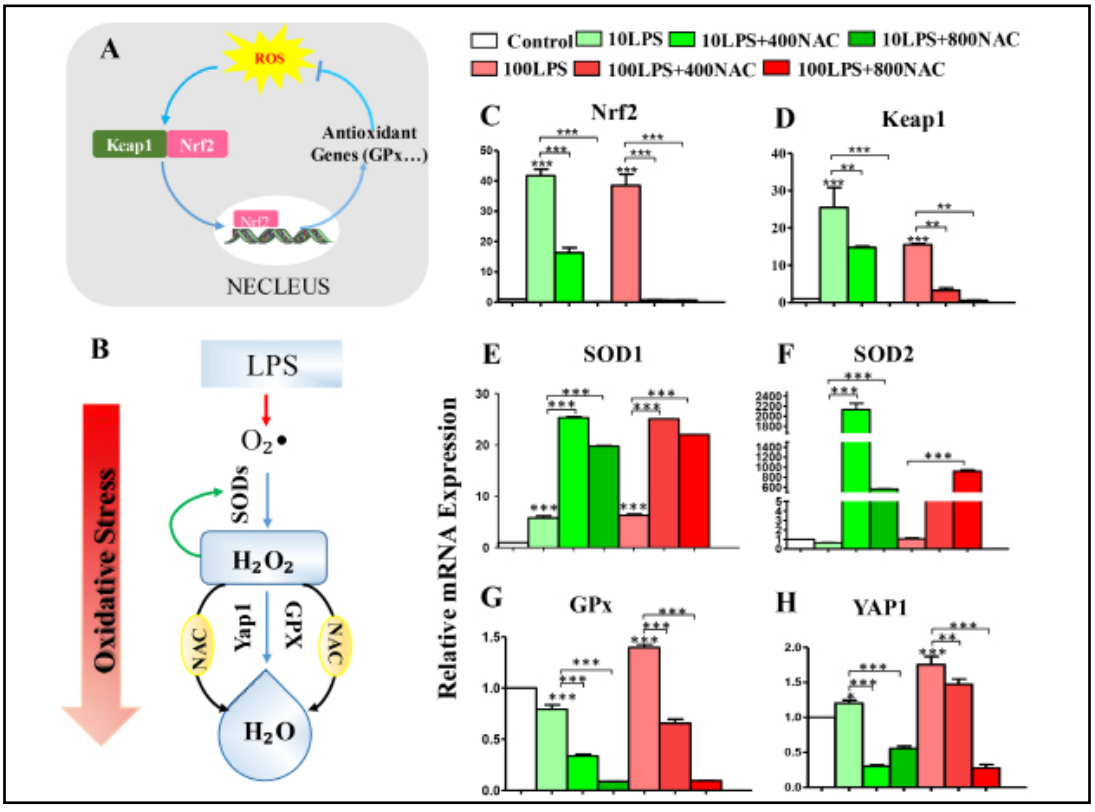

Fig. 6. Assessment of the oxidative stress-related gene expressions in chick CAM in absence/presence of LPS and NAC. A: Sketches illustrating key genes involved in oxidative stress regulation. B :Potential effects of LPS on genes involved in oxidative stress. C-H: Gene expressions of Nrf2 (C), Keap1 (D), SOD1 (E), SOD2 (F), GPx (G), and YAP1 (H) in control, 10 μg/ml LPS, 10 μg/ml LPS + 400 μg/ml NAC, 10 μg/ml LPS + 800 μg/ml NAC, 100 μg/ml LPS, 100 μg/ml LPS + 400 μg/ml NAC, and 100 μg/ml LPS + 800 μg/ml NAC. Each of these experiments was replicated at least three times. (*P<0.05, **P<0.01, ***P<0.001 versus controls).

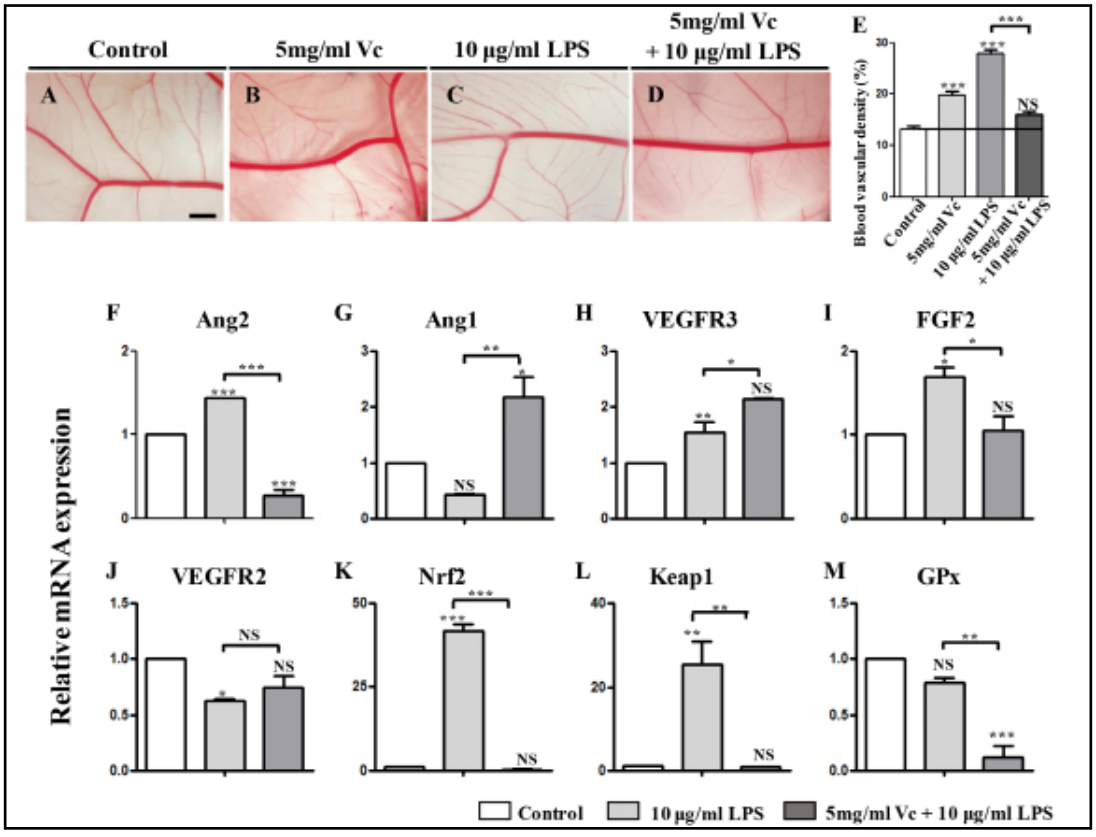

Fig. 7. Assessment of Vitamin C (Vc) effects on angiogenesis in presence/absence of LPS. A-D: Representative bright-field images of angiogenesis on chick CAM exposed to saline (Control, A), 5 mg/ml Vc (B), 10 μg/ml LPS (B), 10 μg/ml LPS + 5 mg/ml Vc (D) , respectively. E: Bar chart showing the comparison of blood vascular densities on the CAM in control, 5 mg/ml Vc, 10 μg/ml LPS, 10 μg/ml LPS + 5 mg/ml Vc (n>3 CAMs in each group). F-M: Quantitative PCR data show the gene expressions of Ang2 (F), Ang1 (G), VEGFR3 (H), FGF2 (I), and VEGFR2 (J), Nrf2 (K), Keap1 (L), GPx (M) in control embryos, embryos treated with 5 mg/ml Vc, 10 μg/ml LPS, 10 μg/ml LPS + 5 mg/ml Vc, respectively (Each of these experiments was replicated at least three times). *P<0.05, **P<0.01, ***P<0.001. Scale bars = 1 mm in A-C.

Oxidative stress is closely related to NAC-suppressed angiogenesis induced by LPS on chick CAM

It is well known that external environment factors often interferes with embryonic angiogenesis through excessive ROS production [34].Using quantitative PCR, we determined the expressions of oxidative stress-related genes (Fig. 6A-B) on chick CAM in absence/presence of LPS or/and NAC. The results showed that both of 10 and 100 μg/ml LPS significantly increased the expressions of Nrf2, Keap1, SOD1, SOD2, GPx and YAP1 genes in chick CAM (Figs. 6C-H); addition of 400 or 800 μg/ml NAC suppressed the LPS-enhanced expressions of GPx, YAP1, Nrf2, , and Keap1genes (Fig. 6E, F, G, H). Addition of 400 or 800 μg/ml NAC to the test system even raised the expressions of SOD1 and SOD2 genes.

Besides, we also investigated the effects of another antioxidant – Vitamin C (Vc) - on angiogenesis in this model, and the result showed that Vitamin C (5 mg/ml) significantly affected the angiogenesis on CAM (Fig. 7B) as well as the blood vascular densities of the vascular nets on chick CAM (5 mg/ml Vc: 19.81±0.63, p<0.001; Fig. 7E). The LPS-induced angiogenesis was significantly suppressedby addition of 5 mg/ml Vc. (10 μg/ml LPS: 27.86±1.35, p<0.001; 10 μg/ml LPS + 5 mg/ml Vc: 16.63±0.64, p<0.001) (Fig. 7E).

The expressions of key genes on chick CAM were assessed using quantitative PCR following the exposure of 5 mg/ml Vc (Fig. 7F-J). 10 μg/ml LPS significantly increased

the gene expressions of Ang2, VEGFR3 and FGF2 on chick CAM. Addition of 5 mg/ml Vc suppressed the LPS-enhanced the expressions of these genes (Fig. 7F, H, I). 10 μg/ml LPS inhibited the expressions of Ang1 and VEGFR2 on chick CAM, and the expressions of Ang1 and VEGFR2 gradually rose by the addition of 5 mg/ml Vc (Fig. 7G, J).

We also determined the expressions of oxidative stress-related genes (Fig. 7K-M) on chick CAM in absence/presence of LPS or/and Vitamin C. The results showed that 10 μg/ml LPS significantly increased the expressions of Nrf2, Keap1 and GPx genes on chick CAM.

Discussion

The chick embryo model has been extensively used to investigate the birth defects in various organ systems [35-40]. In the current study, we used chick embryos and chick chorioallantoic membrane (CAM) as an *in vivo* angiogenesis model to analyze the effects of N-acetylcysteine (NAC) on LPS-induced pathological angiogenesis. The chick gastrula embryos and CAM are an excellent model for studying angiogenesis because they are easily accessible for experimental manipulations and angiogenesis could be observed and analyzed easily as previously described [41, 42]. The chick CAM normally provides the developing embryo with nutrients. The highly vascularization of CAM makes it an excellent model for studying the formation of blood vessels in presence of any compound of interest. Moreover, the results produced are highly reproducible, because NAC concentrations as well as other factors influencing embryonic growth can be strictly controlled in EC culture medium (see Fig. 1).

Our study demonstrated that NAC on its own did not affect angiogenesis in the chick embryo model, but regulates genes expression of enzymes involved in oxidative stress (Fig. 2). Angiogenesis could be induced by administration of lipopolysaccharide (LPS) in CAM. We suggest that this effect is mediated via the LDS receptor being detectable on chicken CAM (unpublished data). LPS substantially induces angiogenesis in chicken CAM, this process, however, could be abolished by NAC treatment (Fig. 3). Using chicken embryo aortic arch assay, we also obtainedsimilar results showing that NAC administration normalized the LPS-enhanced cell extension from the cultured explants of chick embryo aortic arches (Fig. 3). These data are in agreement with previous reports showing that LPS induces endothelial sprouting and angiogenesis via stimulating the release of growth factors or cytokines [43].

The LPS-stimulated angiogenesis was accompanied by an upregulation of genes (Fig. 4) involved in tight-junction adhesion molecules, but also modulation of subcellular distribution of junction and cell polarity proteins, resulting in junction formation and epithelial polarization [44]. These gene expressions were raised following the LPS exposure and most of them dropped by the addition of NAC (Fig. 4), which shows the same patternas seen on blood vessel density in presence of LPS/NAC, suggesting an antagonism of NAC on inflammation-induced pathological angiogenesis. FGF-2 and VEGF are key inducers of angiogenesis [45] and HIF2 (hypoxia-inducible transcription factor 2) plays an important role in blood vessel remodeling [46]. The followed experiments about the LPS/NAC-altered expressions of VEGFR2-3 and FGF2, as well as Ang1 and Ang2, the key genes on maintaining vascular endothelium integrity [47], likewise support the restoring effect of NAC on aberrant angiogenesis (Fig. 5).

To elucidate the underlying mechanism of NAC's restoring effect on LPS-induced pathological angiogenesis, we focused onthe antioxidative potential of NAC. We observed that LPS-enhanced the expressions of Nrf2 and Keap1, the key antioxidative genes. NAC normalized expression of these genes. Moreover, the expressions of key redox enzyme genes such as SOD, GPx and YAP1 showed likewise a trend towards normal expression after NAC treatment (Fig. 6). We found that Vitamin C (a well-known antioxidant drug) could effectively inhibited LPS-induced angiogenesis as well (Fig. 7). These findings suggest that the NAC as antioxidant is able to restore pathological angiogenesis at least partially by modulation genes involved in oxidative stress.

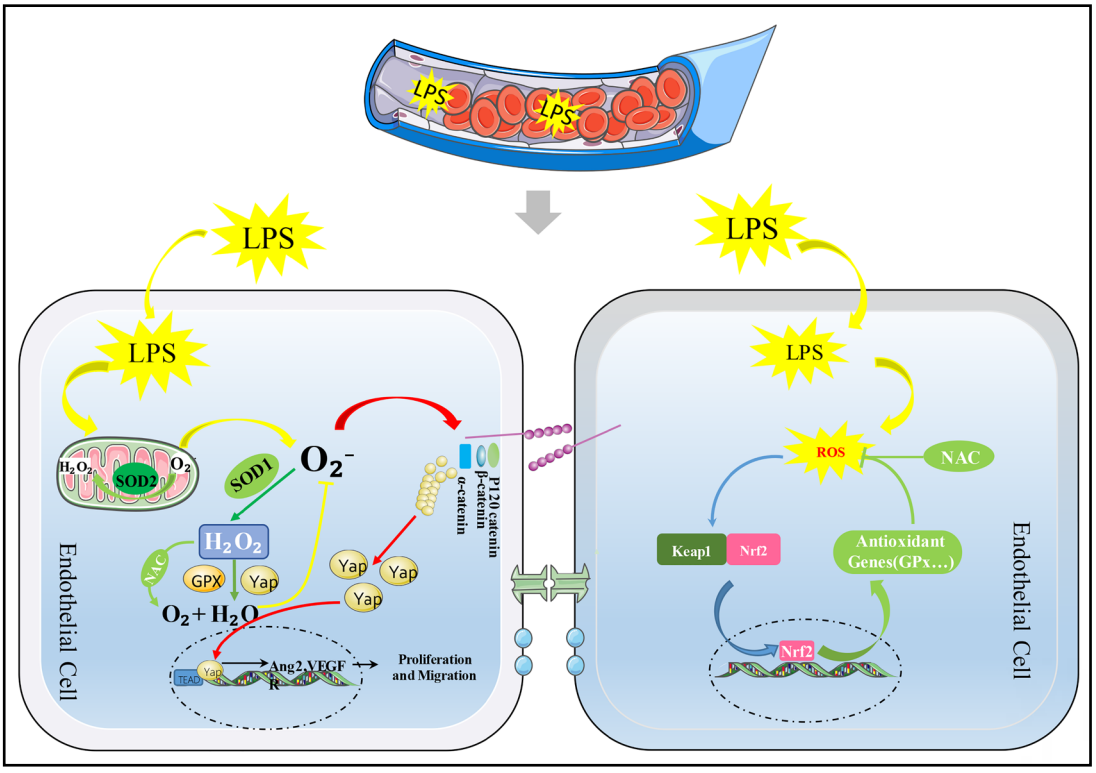

Fig. 8. Hypothetic model illustrating how NAC blunts LPS induced angiogenesis.

It is a clear study limitation that we only analyzed NAC as antioxidant. Thus is is unclear so far weather or not the observed NAC effects are compound specific or a class effect of antioxidants in general.

Conclusion

In summary (Fig. 8), we established a LPS-induced angiogenesis model in chick CAM. NAC administration could effectively inhibit LPS-induced angiogenesis and restore the integrity of endothelium on chick CAM. LPS exposure caused an increased expression of genes involved in oxidative stress in chick CAM, and NAC administration could abolish this effect. We recently demonstrated in the same model that combined ETA/ETB receptor blocade is likewise an effective pharmaceutical approach to inhibit LPS-induced angiogenesis [48]. It would be of interest to see wheater an combination of both strategies potentiate the inhibition on LPS-induced angiogenesis.

Our current studies suggest that NAC might act as a potential drug for the treatment of pathological angiogenesis in clinical practice as well, however, more experiments in models closer to clinical situation are clearly needed.

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Disclosure Statement

The authors declare that there are no competing financial interests.

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