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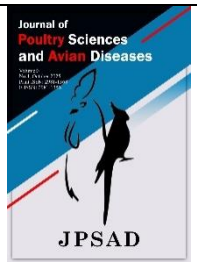

Investigation of the Effects of Lipopolysaccharide on Vascular Development using the Chick Embryo Extraembryonic Membrane Model

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A B S T R A C T

The proper functioning of all body tissues depends on an effective and well-structured vascular system. Consequently, the formation of blood vessels is essential for the development of any tissue. Angiogenesis, the process by which new blood vessels develop from pre-existing ones, plays a fundamental role in maintaining health and is implicated in the pathogenesis of numerous diseases. Lipopolysaccharide (LPS) is a crucial structural component of the cell wall of Gram-negative bacteria, possessing certain pathogenic properties and being regarded as a bacterial toxin. Fourteen fertilized Ross 308 eggs, with an average weight of 51 ± 3 grams, were randomly divided into two equal groups of seven. In the first group (experiment), LPS was injected at a dose of 100 mg/kg body weight into the shell membrane, and in the second group (control), the same amount of PBS was injected. LPS was inoculated at 24, 48, and 72 hours after the start of incubation. Twenty-four hours after the last inoculation, the eggs were longitudinally opened to access the membranes. Then, the shell membrane was removed, and the evolution of the vascular network was evaluated by various image analysis software. The assessment revealed a significant reduction in vessel area, vessel length, number of vessel branches, percentage of new vessel formation areas, and vascular complexity in the experimental group compared to the control group ($p < 0.05$). Based on the obtained results, it was determined that the composition of LPS can have an inhibitory effect on the growth and development of the extraembryonic vascular network of chickens.

Keywords: *Angiogenesis, Chick embryo, Lipopolysaccharide, Shell membrane, Vascular*

1 Introduction

During incubation, the extraembryonic vascular network plays a crucial role in chicken embryo growth, organ development, and overall embryonic health. Proper vascular formation supports normal hatching and reduces embryo mortality (1). Blood vessel development involves two key processes: vasculogenesis and

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angiogenesis. Vasculogenesis begins early in embryogenesis, where epiblast stem cells differentiate into hemangioblasts, precursors of both hematopoietic cells and endothelial cells (ECs). These hemangioblasts further develop into angioblasts, which cluster to form blood islands, the foundation of the primary vascular system (2). This early network, composed of immature EC-lined tubular structures, later evolves into capillaries, veins, and arteries, guided by genetic programming (3).

Angiogenesis is a multistage process of blood vessel formation, beginning with capillary sprouting and followed by vessel maturation. Endothelial cells in differentiated tissues typically remain quiescent but can rapidly respond to stimuli such as hypoxia by forming new vessels to restore oxygen and nutrient supply (4). This process involves the migration, proliferation, and differentiation of ECs, which are regulated by various internal and external factors (5). Depending on physiological conditions, angiogenesis may be either promoted or inhibited. Key promoters include vascular endothelial growth factor (VEGF), Fibroblast growth factor (FGF), Tumor necrosis factor-α (TNF-α), and angiogenin, while inhibitors such as angiostatin and angiogenin-2 suppress vessel formation (6, 7).

Physiological angiogenesis refers to the regulated growth of capillaries that occurs during the healing of wounds, menstrual periods, placental growth, embryo implantation, and ovulation. On the other hand, pathological angiogenesis refers to the uncontrolled proliferation of capillaries by endothelial cells, which is associated with various diseases, including atherosclerosis, diabetic retinopathy, tumors, psoriasis, hemangioma, scleroderma, rheumatoid arthritis, and endometriosis (8-10).

Various models, including in vitro, ex vivo, and in vivo, have been developed to study angiogenesis and its regulatory factors, each with specific advantages and limitations. In vivo models include the corneal micropocket, chick embryo chorioallantoic membrane (CAM), rodent mesenteric sponge/matrix implant, disc assay, Matrigel plug assay, and zebrafish embryo (11). The extraembryonic membranes of birds, especially the chicken CAM, are widely used due to their accessibility, simplicity, and visibility of angiogenesis without additional nutrients or culture media, making them ideal for detailed and rapid study of this process (12, 13).

The historical emergence of lipopolysaccharide (LPS) research dates back to the late 19th century when a toxic substance was identified in putrid fluids, later termed "endotoxin" to describe a heat-stable toxic component found in *Vibrio cholerae* (14, 15). Over time, advances in immunology revealed the key role of LPS in triggering host immune responses, highlighting its importance in bacterial pathogenicity and immune system activation (16). LPS is a major glycolipid component of the outer membrane (OM) of Gram-negative bacteria, such as *Escherichia coli*, where it constitutes about 50% of the outer membrane's weight and covers approximately 75% of the bacterial surface (17). The outer membrane, along with the cytoplasmic membrane and periplasmic space containing the peptidoglycan cell wall, forms a protective barrier against environmental threats (18). LPS acts as a potent endotoxin that triggers acute inflammatory responses and physiological changes, potentially causing fetal pathogenicity. While various chemical compounds have been studied for their effects on embryonic development and vascular formation in poultry, the impact of LPS on embryonic vascular system development remains poorly understood. Evolutionarily, organisms have developed innate and adaptive immune systems to detect and respond to external factors. The innate immune system, the oldest defense mechanism, rapidly recognizes conserved microbial patterns such as LPS through pattern recognition receptors, initiating early immune responses (19). LPS is a powerful activator of innate immunity across diverse eukaryotic species, from insects to humans, and also influences adaptive immune responses (20). Figure 1 shows the structure of the cell envelope of Gram-negative bacteria and its components (21).

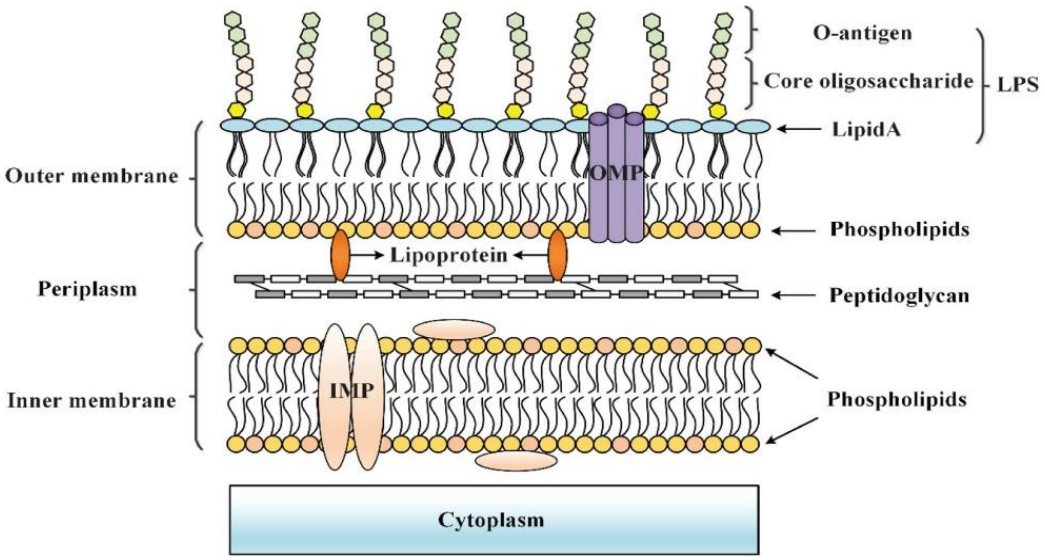

Figure 1. Cell envelope structure of Gram-negative bacteria. The outer membrane (OM) is separated from the inner membrane (IM) by an aqueous periplasmic space, which houses the peptidoglycan cell wall and acts as a protective barrier in bacterial-environment interactions.

LPS is a high-molecular-weight glycolipid responsible for septic shock and endotoxemic reactions during septicemia. It consists of three main components: a hydrophobic lipid A, an oligosaccharide core, and a polysaccharide O antigen (22). Lipid A anchors LPS to the bacterial outer membrane through hydrophobic interactions (23). The oligosaccharide core comprises residues such as Kdo, heptoses, and hexoses, which often have phosphate groups attached. The O antigen is composed of long polysaccharide chains with repeating sugar units (e.g., galactose, mannose, rhamnose) linked by glycosidic bonds, exhibiting significant structural variability among species and strains. This polymorphism, particularly in the O antigen, results in variations in biological properties and antigenic diversity (24-27). Most of the details regarding the structure of LPS are provided in Figure 2 (28).

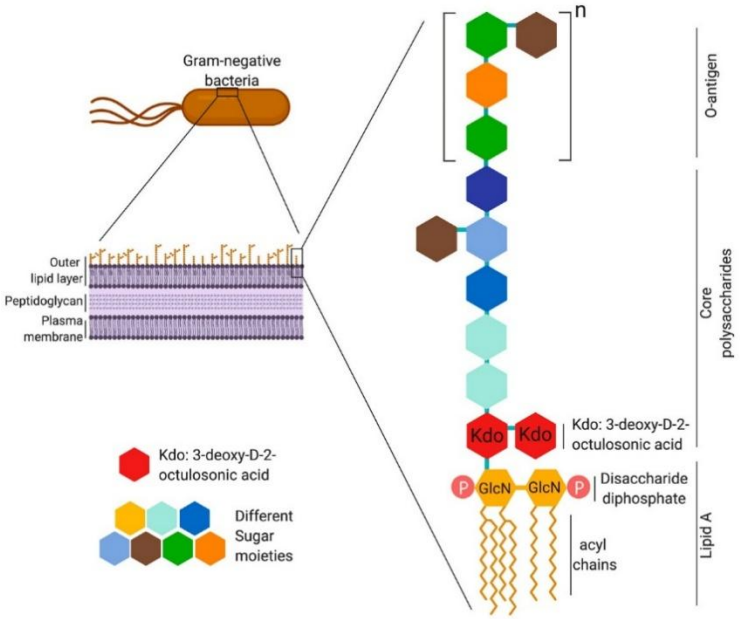

Figure 2. Structural features of lipopolysaccharide from Gram-negative bacteria.

Blood vessels originating outside the embryo enhance blood flow within the extraembryonic membrane and are classified into omphalomesenteric (vitelline) vessels, which transport blood between the embryo and yolk sac, and chorioallantoic vessels, which facilitate gas exchange with the chorioallantois (29). According to the Hamilton Hamburger staging, blood vessel formation begins around 29–33 hours post-incubation, with capillary networks

forming, followed by the onset of blood circulation and the development of more complex vascular channels by 45–49 hours. By 51–56 hours, the chorioallantois is fully enclosed by the sinus terminalis (30). Omphalomesenteric vessels continue to branch and supply blood toward the head and yolk sac throughout incubation, with hemodynamics documented at 65-69 hours post-incubation (31). After hatching, the yolk sac retracts into the body cavity, and omphalomesenteric vessels regress following yolk consumption. The developing embryo is shown in Figure 3, along with a general view of blood vessels (32).

Accordingly, the objective of this study was to evaluate the impact of lipopolysaccharide injection into fertilized chicken eggs on the formation and development of the embryonic vascular system, with particular attention to changes in vascular patterning, density, and overall angiogenic activity.

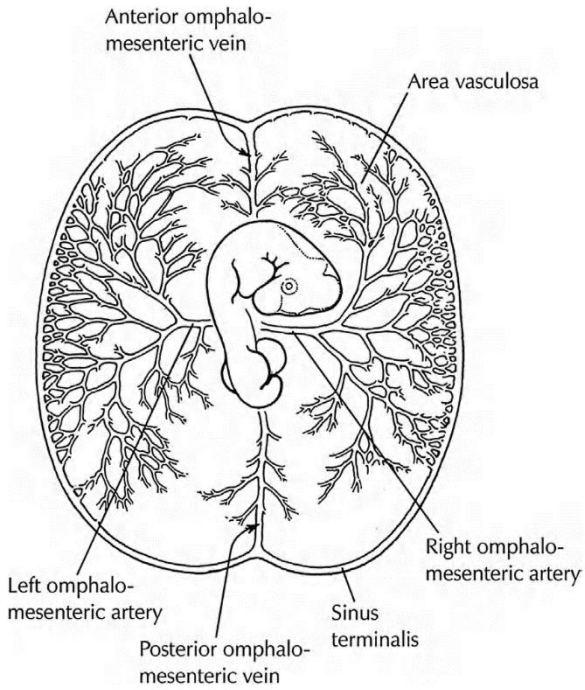

Figure 3. Vitelline blood circulation in the vascular zone on day four of incubation. Vitelline vessels become omphalomesenteric vessels in the embryo.

2 Materials and Methods

2.1 Eggs & Drugs

Fertile chicken eggs (Ross 308 strain) with an average egg weight of 51±3 grams, all of which were produced at the same time, were purchased from the Mahan chicken production complex, a breeder farm with standard conditions of breeding. Lipopolysaccharide from *Escherichia coli O157*, Pure Grade, supplied by LPS Biosciences, was obtained, with each stock containing 100 mg of LPS in lyophilized powdered form. The LPS vial was opened in sterile conditions and then reconstituted with 2 mL of sterile phosphate-buffered saline (PBS; Karmania Pars Gene) to achieve a working concentration of 50 µg/µL. Every 100-microliter portion of the working solution has 5 mg of LPS.

2.2 Experimental protocol

Fertilized eggs were incubated at the Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, using a Belderchi Damavand Co. PLC-DQSH incubator under conditions of 37.7°C and 60% relative humidity, with the eggs positioned vertically, wide end facing upward. On the first day of incubation, the eggs were rotated six times a day at a 90-degree angle, and then the rotation ceased to stabilize embryonic positioning. Each egg was disinfected by swabbing the surface with sterile gauze saturated with 70% ethanol, and 4 milliliters of albumen was very gently pipetted out from the pointed end of the eggs by using a 5-milliliter syringe without damaging the embryo or surrounding membranes to inhibit the embryo from attaching itself to the eggshell (33). Finally, the puncture

sites created by the needle were sealed with melted paraffin to prevent the entry of external pathogens.

Fourteen fertilized eggs were randomly allocated into two equal groups, each containing seven eggs. To conduct the first inoculation, on the first day of incubation, the eggs were removed from the incubator, and a small hole was made at the wide end of the egg, ensuring that the inner membrane remained intact. A volume of 100 microliters of the lipopolysaccharide composition, with a dose of 100 mg/kg egg weight, was inoculated on the shell membrane. The dose was selected based on preliminary pilot experiments aimed at determining a concentration sufficient to induce a measurable effect on angiogenesis in embryonated chicken eggs. Melted paraffin was used to seal the hole, and the eggs were placed back in the incubator. The control group eggs were administered only PBS solution in an equivalent volume. This procedure was carried out at 48-hour and 72-hour intervals, resulting in a total of three inoculations per egg. After 24 hours following the last inoculation, the eggs were removed from the incubator, and the eggshells were longitudinally opened to access the membranes. Then, the shell membrane was removed. The extent of angiogenesis surrounding the embryo was assessed.

2.3 Angiogenesis evaluation

The vascular plexus within the extraembryonic membrane was assessed to determine its response to the induced LPS. This assessment includes measurements of vessel area, vessel length, number of vessel branches, percentage of new vessel formation, and vascular complexity. Digital photographs of the expansion site of the left omphalomesenteric vascular network were captured using a Sony A7C digital camera with a resolution of 4000 × 3000 pixels. The images, each with a uniform cross-sectional area of 110 mm², were analyzed on a computer using image analysis software such as Digimizer® (Version 6.4), which was used for precise geometric measurements of the vessels (e.g., length, diameter, and area); ImageJ® (Version 1.52), which facilitated image enhancement, thresholding, and vascular structure analysis; and MATLAB® (MathWorks Matlab R2021b), which was employed for advanced image processing and statistical evaluation of the extracted data. The effect of the lipopolysaccharide composition on the expansion process of the vascular network was subsequently analyzed based on these images. The average values of the evaluated parameters across all areas of the captured images were calculated for each group.

Vascular complexity was assessed using the fractal dimension (*D*) derived from the box-counting method (34). The *D* value, which encompasses both vessel branching and vessel tortuosity, was determined by measuring the slope of the resulting plot, indicating vascular complexity (35).

The fractional area capillary is the percentage of the tissue region that comprises the formed capillaries, which helps to understand the extent of tissue containing vasculature in the studied area. For the method of estimating the density of capillaries, only areas with the capillary plexus were selected for evaluation of the quantity (36). The surface area with red pixels, which represents the blood, was calculated. The total length of all the blood vessels in a given area of body tissue, or the total length of a body's vascular system, was measured for a blood vessel measurement. Like veins, arteries, arterioles, and capillaries, they comprise the entire vascular system, making this measurement a comprehensive evaluation of the blood vessel network in a region. The branching index is a crucial evaluative measure in vascular studies, as it provides insight into the development of vascularization (37). New blood vessels were formed 24 hours after the final injection, as seen in Figure 4.

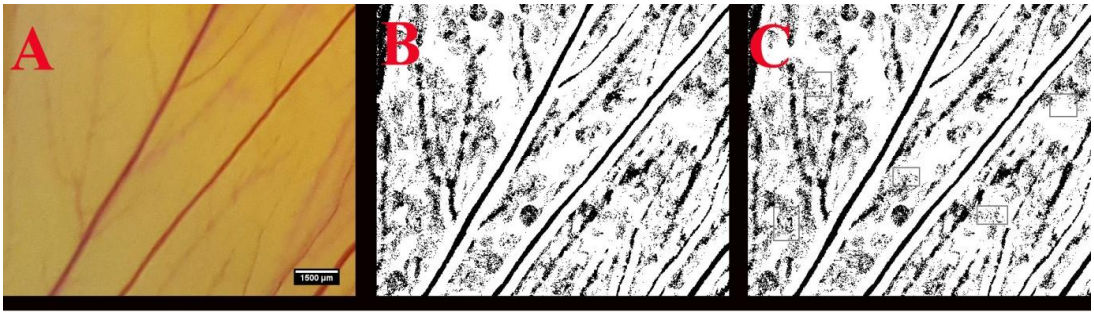

Figure 4. Day 4 of embryonic development. Formation of new vessels in the developing embryo's membrane. (A) Selection of the same cross-section of the embryo membrane. (B) Conversion of the image to binary form. (C) Selection of areas containing the formation of new vessels (highlighted by five rectangles).

2.4 Statistical analysis

Statistical analysis was performed using SPSS version 27. An independent samples T-test was used to compare vascular parameters between the control and LPS-treated groups, including total vessel length, number of vascular branches, capillary density, new vessel formation area, and vascular complexity. A *p*-value of less than 0.05 was statistically considered significant. Results are presented as mean ± SD values, and differences between groups were interpreted in terms of statistical significance where applicable.

3 Results

3.1 Visual and Morphometric Assessment of Vascular Network

A visual comparison of the vascular images revealed a marked reduction in angiogenesis and vasculogenesis in the LPS-treated group compared to the control group. Even to the naked eye, the vascular network in the LPS group appeared less dense and less branched, indicating a substantial impairment in vessel formation. In contrast, the control group exhibited a well-developed and intricate vascular plexus with numerous branching vessels and higher capillary density (Figure 5). Table 1 presents the results of the quantification and morphometric evaluation of the vascular plexus, highlighting a significant decrease in total vessel length, the number of vascular branches, capillary density, and areas of new vascular formation in the LPS-treated group compared to the control group ($p<0.05$).

3.2 Fractal Analysis of Vascular Complexity

Fractal analysis revealed a significant reduction in vascular complexity in the LPS-treated group, as evidenced by a lower fractal dimension (D = 0.85) compared to the control group (D = 1.73). A higher D value corresponds to a more intricate vascular network characterized by increased branching and tortuosity. Thus, the lower D value observed in the LPS group reflects impaired angiogenesis and vascular remodeling (Figure 6).

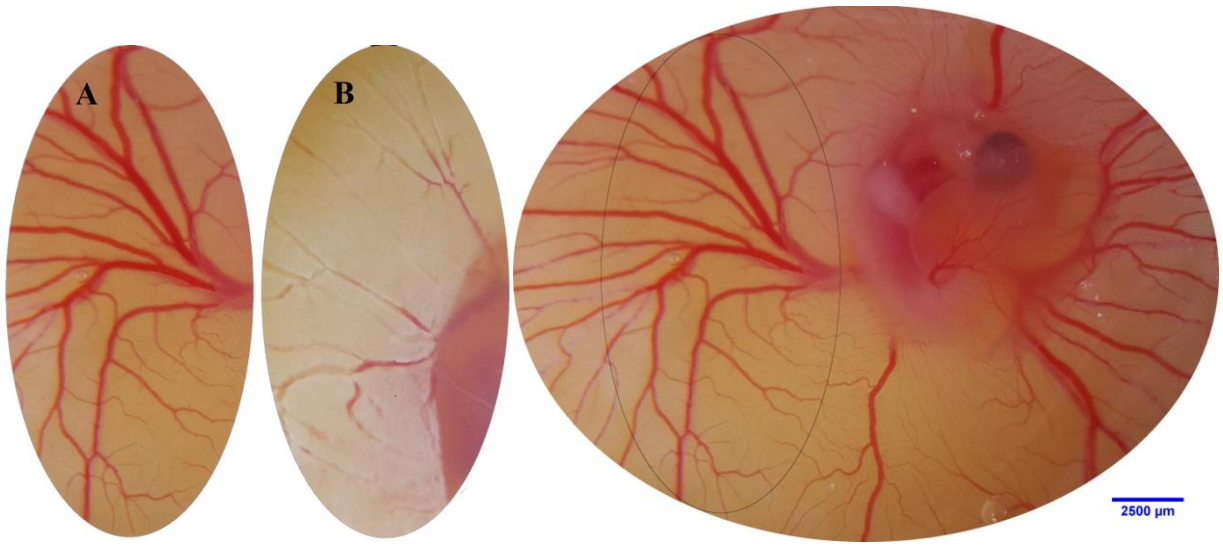

Figure 5. Image of day 4 of embryonic development. Embryonic membrane in the process of development and the pattern of vascular expansion. The image represents a cross-sectional area, measuring 110 mm², taken from the membrane of the developing embryo on the fourth day of the embryonic incubation period. (A) Control group. (B) LPS group

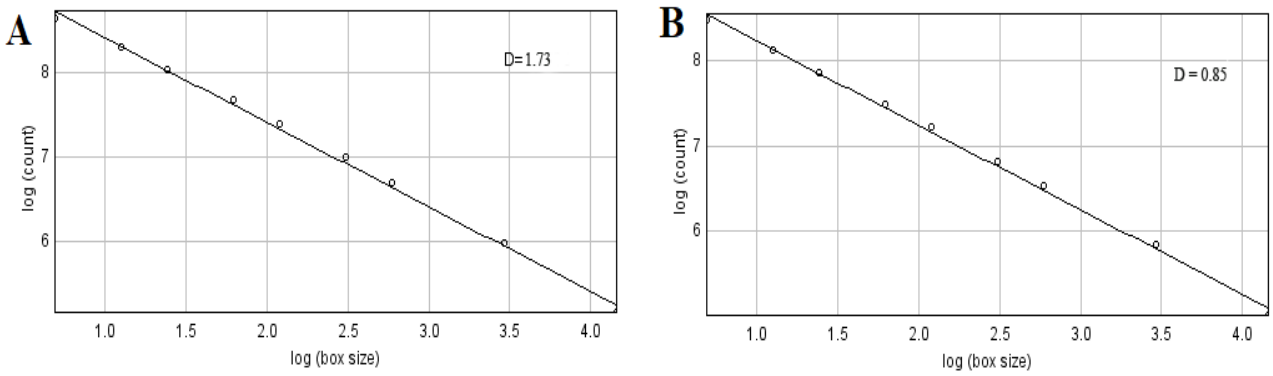

Figure 6. Impact of LPS on vascular complexity. The LPS-treated group (B) exhibited a decline in vascular complexity compared to the control group (A). This complexity was assessed using the fractal dimension (D) obtained via the box-counting technique. The D value, derived from the slope, reflects vascular complexity by incorporating both branching patterns and vessel tortuosity without differentiating between them.

4 Discussion

This research focused on the angiogenic effects of lipopolysaccharide utilizing the chick embryo extraembryonic membrane model. As reported, the group that received LPS treatment showed considerably decreased vascular parameters, including vessel number, length, and tortuosity, compared to the control group. This data suggests that LPS has an antagonistic effect on angiogenesis, most likely through the modulation of pro-angiogenic and anti-angiogenic signaling pathways. More compelling, previous studies have had mixed results about the effects of LPS on angiogenesis. Some studies have shown strong evidence supporting pro-angiogenetic activity (38, 39), whereas others, including our own, have demonstrated strong LPS suppression (40, 41). The apparent dual effect of LPS can be attributed to several factors, including the experimental model employed, the quantity of LPS used, the duration of exposure, and the specific inflammatory mediators involved. Some studies have indicated that LPS can enhance angiogenesis due to its ability to upregulate the proliferation of VEGF and endothelial cells (42). With that said, the reduction in vascular complexity we noted in our study suggests that LPS may have initiated anti-angiogenic mechanisms through oxidative stress, apoptosis, necrosis, and even endothelial migration inhibition (43). Differences in inflammation patterns may rationalize these facts. The aforementioned suggests that lower doses of LPS encourage a mild inflammatory response-driven angiogenesis. In contrast, higher doses, such as the 100 mg/kg dose of egg weight in this experiment, tend to lead to chronic inflammation, endothelial damage, and vascular atrophy. Simultaneously, the ratio of pro-angiogenic and anti-angiogenic factors, such as VEGF and thrombospondins, may differ depending on certain biological conditions and the specific traits of the species in question.

Regarding the test's hypothesis, this could also result from the increased amount of reactive oxygen species (ROS) caused by LPS treatment (44). Stimulation of TLR4 (Toll-like receptor 4) activated both NADPH oxidase and ROS production (45). Increased concentrations of ROS can result in the fragmentation of endothelial cells, lipids, and DNA, leading to apoptosis, necrosis, oxidative stress, and an imbalance between pro- and anti-angiogenic factors, ultimately culminating in cell death. In addition, oxidative stress causes an imbalance in the control of pro- and anti-angiogenic factors (46); for example, it downregulates pro-angiogenic factors, such as VEGF, and upregulates anti-angiogenic factors, including thrombospondin-1 and angiostatin. This oxidative damage may, in part, account for the reduced vascular complexity observed in our study.

The methodology employed in this study, which included high-resolution imaging and quantitative analysis using Digimizer, ImageJ, and MATLAB, enabled an objective assessment of vascular alterations. Nonetheless, molecular corroboration of LPS action on specific pathways is lacking, which limits the power of our findings. Subsequent studies may address protein and gene expression analysis of angiogenic and apoptotic factors, including BAX and BCL-2 genes, as tools to support these hypotheses (47). Even with these limitations, the findings remain in agreement with other studies, which show that some contexts of LPS exhibit anti-angiogenic properties. Angiogenesis plays a crucial role in many physiological and pathological situations, which

warrants additional studies to understand better the context-dependent action of LPS and the molecular mechanisms that explain its contradictory impacts on vasculature. In addition, LPS knowledge may contribute to an understanding of inflammatory diseases in which dysfunction of blood vessels plays a critical role and, hence, may identify therapeutic targets. Understanding the effects of lipopolysaccharide on angiogenesis will open up new avenues for the development of therapeutic strategies, including the blockade of angiogenesis in cancer or ischemic cardiovascular diseases, as well as the stimulation of angiogenesis for purposes such as wound healing.

5 Conclusion

This research was conducted as a basic investigation into the angiogenic properties of lipopolysaccharides using the chicken extraembryonic membrane model as one of the tools. This study demonstrated that LPS disrupts embryonic development by inhibiting angiogenesis, as evidenced by reductions in total vessel length, the number of vascular branches, capillary density, and areas of new vascular formation.

Table 1. Quantification and morphometric evaluation of vascular plexus in LPS-treated and control groups

Quantification and morphometric parameters*				
Groups	Total vessel's length (mm)	Number of vascular branches	Capillary density (%)	Areas of new vascular formation (%)
LPS group	128.7[a]	15[a]	10.19[a]	17.3[a]
Control group	152.13[b]	37[b]	15.82[b]	24.1[b]

* Values are Mean. The heterogeneous letters in each column indicate significantly different (p<0.05).

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Conflict of Interest

The authors declare no competing interests.

Author Contributions

H.B. and H.T. conceptualized the research project, performed data analysis, provided intellectual guidance, developed the study design, and authored the manuscript. M.S. served as the secondary supervisor, facilitated data acquisition, and provided methodological support. All authors reviewed and approved the final version of the manuscript.

Data Availability Statement

Data are available from the first author upon reasonable request.

Ethical Considerations

This research was conducted in full compliance with the European ethical standards for the treatment of animals used in experimental studies. All procedures involving animals were carried out by the principles outlined in Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes.

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