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Ex-Ovo Evaluation of Sevoflurane Exposure on Chick Embryo Development: Investigating Angiogenesis Effects

Sevofluran Maruziyetinin Civciv Embriyo Gelişimi Üzerindeki Ex-Ovo Değerlendirilmesi: Anjiyogenez Etkilerinin Araştırılması

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ABSTRACT

Objective: Angiogenesis, defined as the formation of new blood vessels from pre-existing ones, plays a vital role in both physiological and pathological conditions. Understanding agents that can influence angiogenesis is crucial in managing diseases where angiogenesis is dysregulated. This study focuses on sevoflurane, a commonly used inhalation anesthetic, whose effects on angiogenesis and embryonic development are not well understood. Our objective was to assess the impact of sevoflurane exposure on angiogenesis using the ex-ovo chick chorioallantoic membrane model.

Methods: In this model, fertilized chicken eggs were exposed to sevoflurane at concentrations of 2% and 4%, for varying durations of 1, 2, and 4 hours. The embryos were then divided into control and experimental groups for quantitative angiogenesis assessment using Image J software, followed by statistical analysis with one-way analysis of variance.

Results: The results indicate that sevoflurane exposure has a dose-dependent positive effect on angiogenesis, with significant increases in vascular density observed in embryos exposed to both concentrations compared to the control group. Additionally, the length of exposure was found to further enhance these angiogenic effects.

Conclusion: Despite the dose and duration-dependent impact of sevoflurane on angiogenesis, the existing literature presents mixed findings, highlighting the need for additional research to elucidate sevoflurane's role in angiogenesis. This is particularly important for understanding its implications in various medical conditions, such as cancer, wound healing, and fetal development. Future investigations into sevoflurane's effects on placental angiogenesis could also provide valuable insights into its potential consequences on intrauterine growth.

Keywords: Angiogenesis, sevoflurane, chicken embryo, model organisms, ex-ovo

ÖZ

Amaç: Önceden var olan damarlardan yeni kan damarlarının oluşması olarak tanımlanan anjiyogenez hem fizyolojik hem de patolojik durumlarda hayati bir rol oynar. Anjiyogenezi etkileyebilecek ajanları anlamak, anjiyogenezin düzensiz olduğu hastalıkların tedavisinde çok önemlidir. Bu çalışma, yaygın olarak kullanılan bir inhalasyon anesteziği olan ve anjiyogenez ve embriyonik gelişim üzerindeki etkileri tam olarak anlaşılamayan sevofluran üzerine odaklanmaktadır. Amacımız, ex-ovo civciv korioallantoik membran modelini kullanarak sevofluran maruziyetinin anjiyogenez üzerindeki etkisini değerlendirmektir.

Yöntem: Bu modelde döllenmiş tavuk yumurtaları 1, 2 ve 4 saat gibi değişen sürelerde %2 ve %4 konsantrasyonlarında sevoflurana maruz bırakıldı. Embriyolar daha sonra Image J yazılımı kullanılarak kantitatif anjiyogenez değerlendirmesi için kontrol ve deney gruplarına ayrıldı, ardından tek yönlü varyans analizi ile istatistiksel analiz yapıldı.

Bulgular: Sonuçlar, sevoflurana maruz kalmanın anjiyogenez üzerinde doza bağlı pozitif bir etkiye sahip olduğunu, kontrol grubuyla karşılaştırıldığında her iki konsantrasyona da maruz kalan embriyolarda damar yoğunluğunda önemli artışların gözlemlendiğini gösterdik. Ek olarak, çalışmamızda maruz kalma süresinin bu anjiyojenik etkileri daha da arttırdığı bulunmuştur.

Sonuç: Sevofluranın anjiyogenez üzerindeki doza ve süreye bağlı etkisine rağmen, mevcut literatür karışık bulgular sunmakta ve sevofluranın anjiyogenezdeki rolünü aydınlatmak için ek araştırmalara ihtiyaç olduğunu vurgulamaktadır. Bu özellikler kanser, yara iyileşmesi ve fetal gelişim gibi çeşitli tıbbi durumlardaki etkilerini anlamak için önemlidir. Sevofluran'ın plasental anjiyogenez üzerindeki etkilerine ilişkin gelecekteki araştırmalar, intrauterin büyüme üzerindeki potansiyel sonuçlarına ilişkin değerli bilgiler de sağlayabilir.

Anahtar sözcükler: Anjiyogenez, sevofluran, civciv embriyo, model organizma, ex-ovo

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INTRODUCTION

The human body develops its vascular system through two distinct physiological processes: vasculogenesis and angiogenesis (1). Angiogenesis is defined as the formation of new blood vessels from pre-existing ones (2). This process can be categorized into two types: physiological and pathological angiogenesis. Physiological angiogenesis is a critical step in embryonic development, inflammation, and wound healing. On the other hand, pathological angiogenesis is primarily associated with tumor metastasis. Studying the angiogenesis process, including agents that either promote or inhibit angiogenesis, is crucial for understanding and managing these pathological and physiological phenomena.

The vascular endothelial system is pivotal in the human vascular system, playing a key role not only in vessel formation but also in processes like vascular tone regulation and inflammation (3). Current research focuses on agents with angiogenic or anti-angiogenic properties to develop novel treatments for various diseases linked to pathological angiogenesis, such as cardiovascular ailments stemming from vascular endothelial dysfunction, tumor metastases, rheumatoid arthritis, retinopathy of prematurity, and psoriasis. We should note that it is significant to recognize that using some anti-angiogenic agents, though promising in inhibiting pathological angiogenesis, could lead to adverse effects in several physiological processes, notably fetal development, and wound healing. Furthermore, there is a concern regarding the operating room environment, as some of these agents might diffuse into the air, potentially posing risks to operating room staff due to chronic exposure to inhaled anesthetic gases.

Sevoflurane, a widely used inhalation anesthetic, is recognized for its safety and efficacy in modern anesthesia practices. It is employed for induction and maintenance of general anesthesia across various patient populations, including pediatric and preterm newborns. Its popularity stems from its non-irritating nature to the respiratory tract, ability to rapidly achieve desired anesthesia depth, and facilitation of quick recovery (4). To evaluate exposure to toxic substances and inhalation agents in gas or aerosol form, the literature describes two experimental models: the intratracheal drip and the inhalation chamber methods (5). While the intratracheal drip method allows for controlled dosing of agents, it does not adequately replicate physiological exposure. Conversely, the inhalation chamber method closely simulates the physiological pathway of exposure but requires significant quantities of the agent and specialized equipment.

Among the various models used to study angiogenesis, the chick chorioallantoic membrane (CAM) model stands out due to its simplicity, reproducibility, and capability for quantitative assessment, making it a popular choice in research (2). The CAM is an extra-embryonic membrane characterized by a dense network of capillaries, suitable for study in both in-ovo and ex-ovo formats (6). In the in-ovo model, experiments are conducted within the egg, with observations made through a window created in the eggshell (7). While this model typically results in higher embryo survival, visualizing the CAM can be challenging. Conversely, the ex-ovo model involves removing the eggshell entirely and placing the embryo in a controlled environment for continued development outside the shell. Although this approach offers easier access to the embryo and CAM, it generally has a lower survival rate than the in-ovo method. While in-ovo inhalation exposure models are documented in the literature, the ex-ovo inhalation exposure model has not yet been established (5,8). In our study, we aim to explore the effects of sevoflurane, a common agent in modern anesthesia, on angiogenesis using an ex-ovo chick CAM model, applying a method that is pioneering in this field.

MATERIAL and METHODS

This study was conducted on chicken embryos and an ethics committee decision is not required for these studies and conducted in accordance with the principles of the Declaration of Helsinki at Health Sciences University, Gülhane Training and Research Hospital, Department of Medical Biochemistry, Vascular Biology Laboratory.

Implementing the Ex-Ovo CAM Model

In our study, fertilized chicken eggs for use in the ex-ovo CAM model were obtained from Atak-S chickens produced at the National Poultry Institute (Ankara, Türkiye). All embryos were monitored at constant temperature and humidity (37°C, 85–90% relative humidity) throughout the experiment.

To perform the ex-ovo CAM assay as described in previous studies, eggs were initially cleaned with a dry disposable wipe to remove contaminants like feces, mud, and feathers, ensuring the shell remained intact (6). These cleaned eggs were then placed horizontally in a CIMUKA 40080 serial number incubator, rotated on a moving tray every two hours, and incubated for 72 hours. During this time, constant temperature and humidity levels in the incubator were meticulously maintained. After the 72-hour incubation, eggs were examined with a light source to locate the embryo, then marked with a pencil. In a sterile environment and wearing sterile gloves, the eggs were carefully broken at the bottom, keeping the marked point upwards. The embryos were delicately transferred into 100 mL disposable, sterile weighing boats, ensuring no harm came to them. These eggs, now in weighing boats, were covered with sterile glass and returned to the incubator for an additional three days of incubation. Any embryos lost during this period were promptly removed to avoid contamination. The surviving embryos, after six days of

incubation, were divided into three groups: the control group (C), receiving 50% air-oxygen insufflation at 2 L min^{-1} fresh gas flow; 2% concentration group , exposed to 2% concentration of sevoflurane in a 50% air-oxygen mixture; and 4% concentration group, exposed to 4% concentration of sevoflurane in a 50% air-oxygen mixture. To further investigate the impact of exposure duration on angiogenesis, 2% and 4% concentration groups were subdivided into six, based on exposure times: 1h, 2h and 4h (1, 2, and 4 hours respectively, Figure 1). According to a study by Jiang et al., the air cell in the CAM model functions as a naturally occurring inhalation chamber. Consequently, introducing gas into the air cell simulates early-life inhalation exposure. Although no specific concentration recommendations exist for CAM assays, concentrations of 2% and 4% were selected for our study to approximate real-life exposure scenarios (5). Each group of eggs was placed in distinct incubators equipped with anesthesia circuit hose connections for agent inflation, featuring both inlet and outlet connections.

The CAM models were palced in a sealed chamber equipped with both inflow and outflow connectors. The internal atmosphere of the chamber was regulated to maintain a constant humidity of 80% and a temperature of 37°C. The chamber's entrance port was linked to the inspiratory breathing system of an anesthetic machine (Aisys CS2, Datex Ohmeda, Inc., USA). The fresh gas flow rate was set at 2 L min^{-1}, and bacterial filters were also installed at the circuit's inlet connections. Before initiating sevoflurane inhalation, photographs of all groups were taken at hour 0 as a baseline reference. Sevoflurane (Sevorane®; AbbVie S.r.l., Compoverde di Aprilla (LT), Italy) was administered using a sevoflurane vaporizer (Aladin2, Datex Ohmeda, Inc., USA) attached to the anesthesia machine. The groups were exposed to the pre-determined concentration (2% and 4% concentration) and duration (1, 2, and 4 hours) of the inhalation agent. The chamber's outlet port connected to the machine's expiratory breathing system. A gas analyzer on the anesthetic machine continuously monitored the concentrations of O_2 and sevoflurane delivered. Imaging was then conducted 24-, 48-, and 72-hours post-exposure to monitor changes. These images were systematically archived for subsequent statistical analysis.

The quantitative analysis of ex-ovo embryo examinations was obtained using Image J software (National Institutes of Health, Bethesda, MD, USA). The mean of the control group imaging was taken as 100%, and the imaging was standardized to a percentage of this value.

Statistical Analysis

Data were reported as mean±standard deviation (SD). GraphPad Prism software was used to analyze the data. A two-way ANOVA test was used to assess the differences between the control and treatment groups. $p<0.05$ was determined as a statistical significance.

RESULTS

Our study's primary objective was to investigate the influence of dosage and exposure duration on the effects of sevoflurane on angiogenesis. We discovered that sevoflurane positively affects angiogenesis in a time and dose-dependent manner. Upon analyzing changes in vascular density, a significant

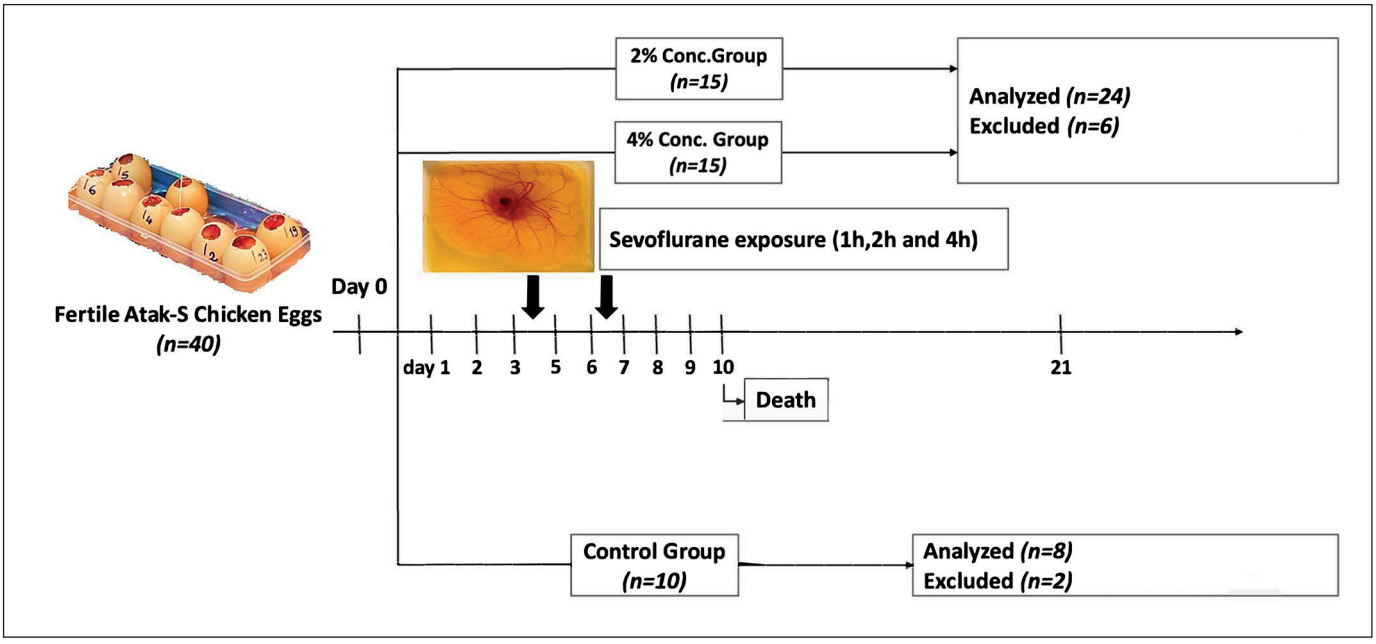

Figure 1: Flow diagram of the study.

increase was noted in the embryos exposed to 2% and 4% concentrations of sevoflurane compared to those in the control group. These findings are visually represented in Figures 2 and 3.

Quantitative analyses were conducted using the Image J program, followed by statistical evaluations. The images captured at 24th hours demonstrated no significant differences in all groups after treatment with 2% and 4% sevoflurane ($p>0.05$). Imaging at the 48th and 72nd hours revealed that embryos exposed to a 2% concentration of sevoflurane for 1 hour and 2 hours exhibited a significant increase in angiogenesis compared to the control group ($p<0.05$ for 48th hours; $p<0.01$ for 72nd hours). Further analysis on the impact of exposure duration showed that within the group treated with 2% sevoflurane, those exposed for 4h demonstrated a notably higher increase in angiogenesis at both the 48th and 72nd hours compared to the control group ($p<0.01$ for 48th and 72nd hours). This indicates that administering sevoflurane at a 2% concentration positively influences angiogenesis, with the effect being dependent on the duration of exposure (Figure 4). In the group exposed to a 4% concentration of sevoflurane for 2h and 4h, our findings suggest that sevoflurane enhances angiogenesis in all subgroups relative to the control group ($p<0.05$ and $p<0.01$ for 48th and 72nd hours), mirroring the results seen in the 2% sevoflurane group. However, the increase in the duration of sevoflurane treatment partially impacted the induction of angiogenesis. Unexpectedly, the most pronounced positive effects on angiogenesis with 4% sevoflurane were observed in the images captured at 72nd hours when the exposure duration to sevoflurane was limited to one hour ($p<0.01$ vs control group; Figure 4).

DISCUSSION

Angiogenesis, the process of forming new blood vessels from existing vascular structures, plays a crucial role in tumor growth, invasion, and metastasis. Recent advancements in cancer treatment have shown promise with the use of anti-angiogenic agents aimed at inhibiting angiogenesis, thereby potentially halting tumor growth and metastasis (9). Mao et al., in their synthesis of various studies, highlighted those volatile anesthetic agents, including sevoflurane, may activate tumoral processes through diverse mechanisms (10). In their review, Tavare et al. reported that various volatile anesthetics could increase the transcription of genes modulating

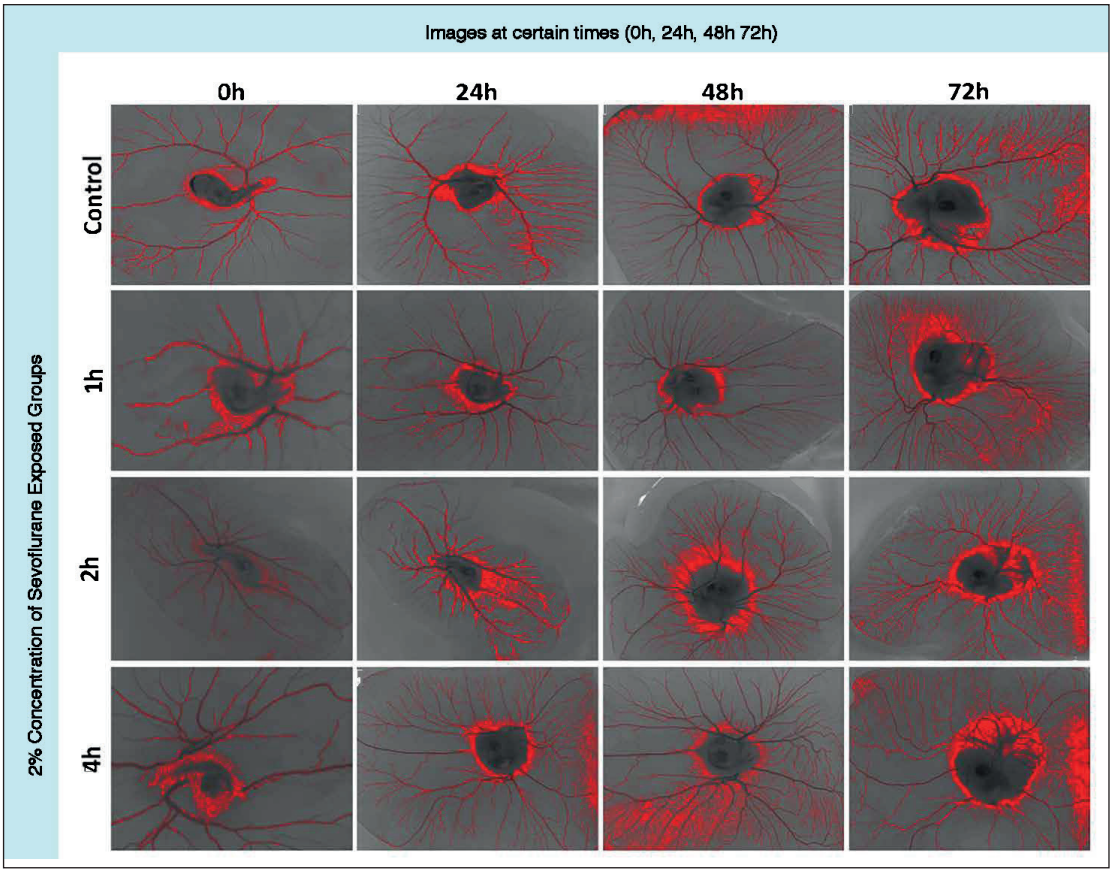

Figure 2: Chicken Ex-Ovo CAM Analysis of Vascular Density in 2% Concentration Group. Change in vascular density over time (0h, 24h, 48h, 72h).

angiogenesis, primarily by up-regulating hypoxia-inducible factor-1a (11). Consistent with this, various animal experiments and in vitro studies utilizing volatile anesthetics have been linked to an increased metastatic potential (12–14). However, the specific effects of sevoflurane on cancer cells and its metastasis potential remain unclear, with existing literature presenting conflicting results regarding its potential impact on angiogenesis.

Angiogenesis initiation and regulation involve a multitude of factors and their inhibitors (1). A crucial balance between angiogenic and anti-angiogenic factors dictates this process. Key angiogenic factors include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietins (1). Various in vitro and in vivo studies have explored the effects of sevoflurane on VEGF, yielding contradictory results (3,15).

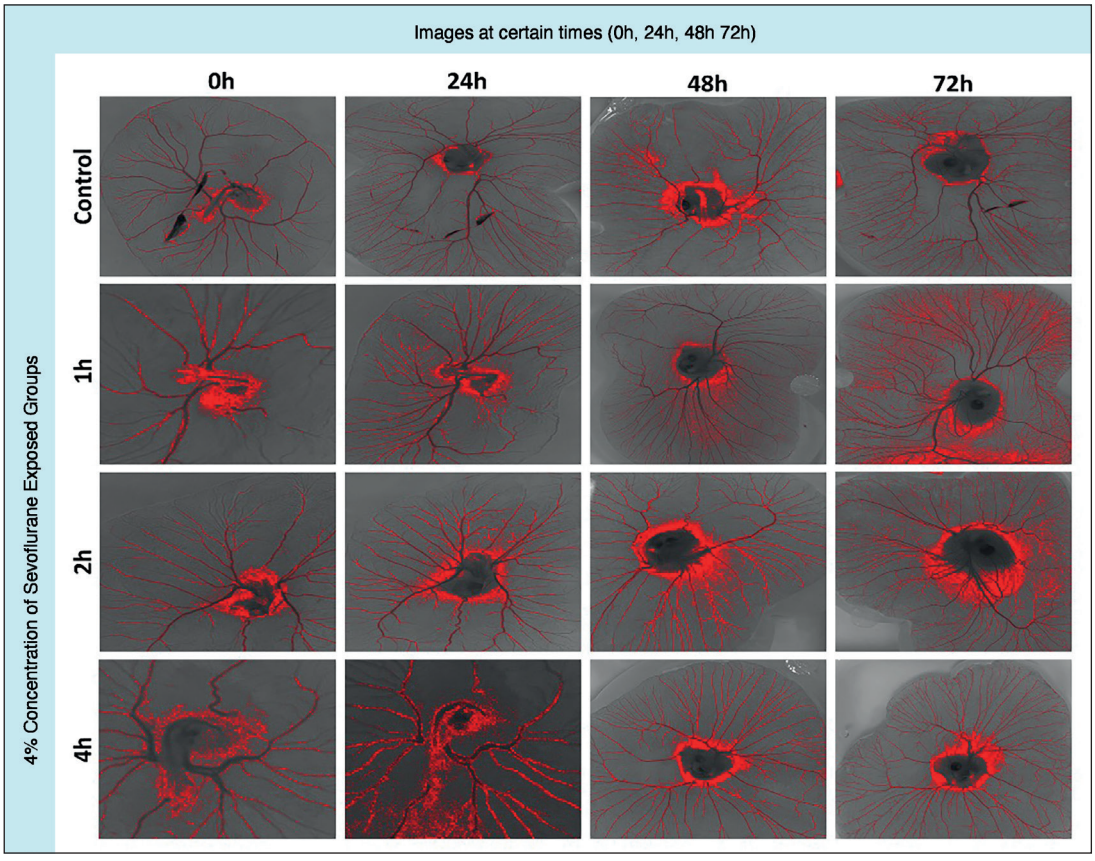

Figure 3: Chicken Ex-Ovo CAM Analysis of Vascular Density in 4% Concentration Group.
Change in vascular density over time (0h, 24h, 48h, 72h).

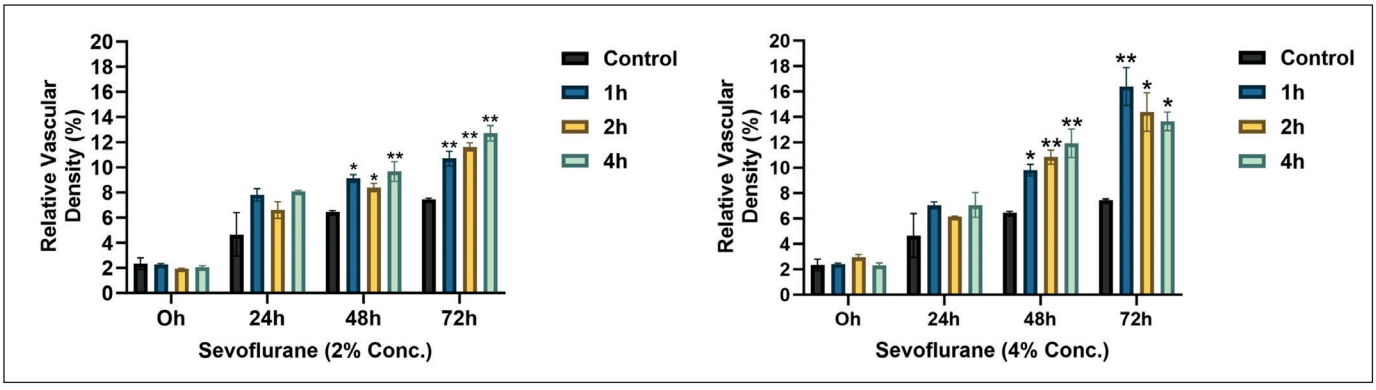

Figure 4: Effect of 2% and 4% Sevoflurane Concentrations on Vascular Density in Ex-Ovo Chicken CAM Assay.
Each group was presented on left and right sides, respectively. For each group, control group and subgroups of 1h, 2h and 4h were also shown with certain color (* p-values < 0.05 and ** p-values < 0.01 and *** p-values < 0.001 vs the control group).

Our experiment, using the chick CAM model, demonstrated that sevoflurane positively influences angiogenesis in a dose-dependent manner. Complementing our findings, Wang et al.'s study on human umbilical vein endothelial cells (HUVEC) revealed that VEGF receptor expression levels increased in a concentration-dependent manner following 48 hours of sevoflurane exposure compared to controls (3). Similarly, Yang et al. reported angiogenic effects in their HUVEC study, citing upregulation of VEGF expression in mesenchymal stem cells incubated with sevoflurane (16). Inconsistent with these findings, Kim et al., in a mouse model of oxygen-associated retinopathy, observed that VEGF expression was suppressed, and retinal angiogenesis inhibited in mice exposed to sevoflurane (4). Furthermore, in their study, Wang et al. noted that sevoflurane inhibited angiogenesis by suppressing Rac1 and Ras signals induced by VEGF and FGF (15).

Angiogenesis also plays a pivotal role in another crucial physiological process: wound healing (17). Cha et al. investigated sevoflurane's impact on wound healing in a rat model. Their study revealed an increase in wound sizes and a significant reduction in regional blood flow and FGF expression in rats exposed to sevoflurane compared to the control group (18). Similarly, Lee et al. conducted an experiment using a comparable rat model and concluded that sevoflurane exposure might adversely affect the wound-healing process by decreasing the expression of FGF and other growth factors in a process-dependent manner (19). However, it is crucial to recognize that the wound-healing process is complex, involving various interrelated mechanisms. While angiogenesis is a significant factor in this process, other multifactorial aspects influence its functioning. Therefore, further research is necessary to comprehensively evaluate sevoflurane's effects on all stages of wound healing.

Providing an adequate substrate for normal fetal growth is crucial during intrauterine development. The formation of new blood vessels is essential to meet the increasing demands of the continuously growing fetus throughout pregnancy. The human placenta is replete with angiogenic factors. Disruption of angiogenesis during intrauterine development may lead to intrauterine growth retardation and various related pathologies (20). While many studies have examined the effects of sevoflurane exposure at different stages of pregnancy, research on its impact on placental angiogenesis remains scarce (21,22). A multicenter cohort study investigating long-term fetal exposure to volatile anesthetic agents assessed 15,317 children born to 9,433 nursing mothers exposed to anesthetic gases during pregnancy. This study found that these children had a higher risk of congenital anomalies compared to children of mothers who were not exposed to such gases (23). However, it is important to note that fetal development is influenced by many different factors. Therefore, additional research is needed to elucidate the relationship between sevoflurane exposure, placental angiogenesis, and intrauterine growth retardation.

CONCLUSION

In conclusion, our study delves into the complex interplay between sevoflurane exposure and angiogenesis, utilizing an ex-ovo CAM model. The findings suggest a dose-dependent positive effect of sevoflurane on angiogenesis, evidenced by increased vascular density at both 2% and 4% concentrations. Additionally, the study reveals that prolonged exposure to sevoflurane further amplifies these angiogenic effects. While existing literature provides valuable insights, it also presents conflicting views. Therefore, further research is essential to fully unravel the intricate role of sevoflurane in the process of angiogenesis.

AUTHOR CONTRIBUTIONS

Conception or design of the work: NOY, OFK
Data collection: NOY, OFK
Data analysis and interpretation: NOY, OFK
Drafting the article: NOY, OFK
Critical revision of the article: NOY
The author (NOY, OFK) reviewed the results and approved the final version of the manuscript.

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