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Current Applied Science and Technology Vol. 24 No. 4

Research article

Alternating Current-Electric Field Inducing Chorio Allantoic Membrane (CAM) Angiogenesis through Exogenous Growth Factor Intervention

Endah Sri Palupi[1,2], Bambang Retnoaji[3], Pudji Astuti[4], Firman Alamsyah[5,6], Warsito Purwo Taruno[6] and Rarastoeti Pratiwi[7]*

[1]Postgraduate Program, Faculty of Biology, Universitas Gadjah Mada, DI Yogyakarta, 55281, Indonesia
[2]Animal Structure and Development Laboratory, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, 53122, Indonesia
[3]Animal Structure and Development Laboratory, Faculty of Biology, Universitas Gadjah Mada, DI Yogyakarta, 55281, Indonesia
[4]Department of Physiology, Faculty of Veterinary, Universitas Gadjah Mada, DI Yogyakarta, 55281, Indonesia
[5]Faculty of Science and Technology, Universitas Al Azhar Indonesia, Jakarta, Indonesia
[6]Center for Medical Physics and Cancer Research, Ctech Labs Edwar Technology, Tangerang, Banten, 15143, Indonesia
[7]Biochemistry Laboratory, Faculty of Biology, Universitas Gadjah Mada, DI Yogyakarta, 55281, Indonesia

Curr. Appl. Sci. Technol. 2024, Vol. 24 (No. 4), e0258584; https://doi.org/10.55003/cast.2024.258584

Received: 10 April 2023, Revised: 14 November 2023, Accepted: 12 January 2024, Published: 20 March 2024

Abstract

Keywords

AC-EF;
angiogenesis;
bFGF;
CAM;
vascularization;
VEGFA

Angiogenesis is widely used in various therapies by promoting or inhibiting the formation of new blood vessels. The use of Alternating Current-Electric Fields (AC-EF) in Electro-Capacitive Cancer Therapy (ECCT) showed its potential as an anti-cancer device, and is characterized by its anti-proliferative and pro-apoptotic effects. However, the role of AC-EF in angiogenesis remains unclear. To investigate the effects of AC-EF on CAM angiogenesis, we used the *ex ovo* culture method of chorioallantoic membrane (CAM). A basic fibroblast growth factor (bFGF) dose of 30 ng/µL was administered as an exogenous growth factor. The ECCT device, generating AC-EF of 150 kHz and 18 Vpp, was exposed to the CAMs. Subsequently, the 24 CAMs of chick embryo were divided into four groups. Two groups were non-bFGF-induced CAM, while the other two were bFGF-induced CAM, and each group was exposed either with or without AC-EF. The vascularization was evaluated through macroscopic observation, while vascular endothelial growth factor A

*Corresponding author: Tel: +62 (274) 580839 Fax: +62 (274) 580839
E-mail: rarastp@ugm.ac.id

(VEGFA) gene expression was measured using qPCR. The data were statistically analyzed using ANOVA with GraphPad Prism 9.5. The results showed that an AC-EF exposure had no effects on normal CAM angiogenesis (P>0.05). Moreover, VEGFA gene expression did not show significant upregulation (P>0.05) in the bFGF-induced CAM with or without AC-EF exposure. Interestingly, the number of new blood vessels was significantly higher (P<0.05) in the bFGF-induced with AC-EF exposure than in the non-bFGF-induced group. In conclusion, AC-EF of ECCT did not affect normal angiogenesis. AC-EF may trigger CAM angiogenesis with bFGF induction. This observation suggested that AC-EF of intermediate frequency could enhance angiogenesis by administration of external growth factors, offering a potential avenue for addressing obstructive vascular conditions.

1. Introduction

Angiogenesis is the new blood vessel development from a pre-existing one, and is a process vital for the transportation of nutrients and gases. This mechanism holds significance in both normal physiological functioning and pathological conditions [1]. In several diseases, such as cancer, angiogenesis promotes and supports cancer growth and metastasis [2, 3]. However, in instances of inadequate angiogenesis, such as coronary artery disease, atherosclerosis, and nephropathy, the emergence of new blood vessels can positively impact patients [1, 4]. This intricate balance has spurred the emergence of potential therapeutic avenues known as angiogenesis-based therapies [5]. Over the last few decades, these therapies have been widely involved in the medical field, including promoting or inhibiting angiogenesis [6, 7].

Principally, angiogenesis is a complex cascade mechanism involving growth factor production, signal transduction, membrane basal degradation, proliferation and migration of endothelial cells, and stabilization of new blood vessels [1, 8]. Among these intricacies, the role of vascular endothelial growth factor A (VEGFA) stands out as an important factor in angiogenesis [9], and this growth factor always becomes a parameter in angiogenesis studies.

Angiogenesis studies were mostly conducted in relation to certain diseases in order to explore and discern potential agents that either promoted or hindered the process, depending on the disease in question. Angiogenic inhibitors were applied in cancer diseases to inhibit cancer growth and metastasis. Recently, a noteworthy and promising development emerged in the form of Alternating Current-Electric Field (AC-EF) devices for Electro Capacitive Cancer Therapy (ECCT). Previous studies proved that AC-EF with intermediate frequency, when used for ECCT, demonstrated anti-tumor capacity due to its anti-proliferative and pro-apoptotic effects [10, 11]. However, its effect on angiogenesis is still unclear.

In a previous investigation, limited data showed that AC-EF of intermediate frequency and low intensity inhibited angiogenesis. Tumor Treating Field device was used in the treatment of glioblastoma (GBM) [12], produced an anti-angiogenic effect by downregulating VEGF, HIF1α, MMP2, and MMP9 in a glioblastoma cell culture model [13]. Another AC-EF device, picosecond Pulsed electric Fields (psPEF), may block angiogenesis in a cervical cancer xenograft model by downregulating VEGF, HIF1α, and HIF2α [14]. In addition, this device has downregulated VEGF and HIF1α when using HeLa cell culture [15]. However, the influence of intermediate frequency generated by ECCT on angiogenesis remains unexplored. Moreover, there is a dearth of information concerning the safety implications of using AC-EF of intermediate frequency in normal angiogenesis.

Considering the essential role of angiogenesis in both normal and pathological conditions, it is important to investigate intermediate frequency AC-EF effects on angiogenesis. In this study, CAM of chick embryo was used as an angiogenesis model to observe the intermediate frequency

AC-EF impact on angiogenesis. The CAM is the most relevant model for angiogenesis investigation [16-18]. It requires simple and easy treatment and is of low cost [19]. Furthermore, compared with the *in ovo* method, the *ex ovo* culture CAM method offers several benefits, such as specifically enhancing accessibility for observation and documentation [20]. The *ex ovo* CAM method provides a larger CAM experimental area and can increase biological replication without adding CAM replication [19]. In addition, CAM may provide representative and reliable data for intermediate frequency AC-EF effects on normal angiogenesis. The *ex ovo* CAM method makes AC-EF exposure applicable since the eggshell may be a barrier for the AC-EF exposure. Therefore, by using the *ex ovo* CAM model, this study aimed to investigate and observe the effect of 150 kHz frequency and 18 Vpp intensity of AC-EF generated by an ECCT on the CAM angiogenesis of chick embryos.

2. Materials and Methods

2.1 Animals and ethics

This study was conducted at the Laboratory of Animal Structure and Development, Faculty of Biology, Universitas Gadjah Mada (UGM). The experimental protocol adhered to ethical clearance legalized by the Ethics Committee of Integrated Research and Testing Laboratory (LPPT) UGM with an ethical clearance number 00008/04/LPPT/VI/2021.

For experimental design, fertile egg quantities were determined using established procedures by Federer [21]. A total of twenty-four fertile eggs of chick embryos were collected from a hatchery in Perusahaan Unggas dan Perakitan Mesin Tetas HTN, Yogyakarta. The eggs were incubated in an incubator with a temperature of 38.5°C and humidity of 60-70%. The *ex ovo* culture was performed on an embryo aged 72 hours of incubation.

2.2 Experimental design

Twenty-four fertile eggs were divided into four groups, with six replications of each. The experimental design in this study consisted of 4 groups as follows:

1. NINT : control group (non-bFGF-induced and non-therapy AC-EF-exposed CAM)
2. NIT : non-bFGF-induced with therapy AC-EF-exposed CAM
3. INT : bFGF-induced and non-therapy AC-EF-exposed CAM
4. IT : bFGF-induced with therapy AC-EF-exposed CAM

2.3 bFGF preparation

Ectopic angiogenesis within CAM was induced by administering a bFGF dose of 30 ng/µL to each egg. The bFGF solution was prepared following procedures of Oktavia *et al*. [22]. The bFGF (Sigma Aldrich; cat no. F3685) was diluted with Tris-HCl buffer (10 mmol/L, pH 7.5) and prepared as a 1 µg/µL concentrated stock solution. bFGF of 30 ng/µL was made by diluting 1 µL bFGF stock solution with 32.33 µL Tris-HCl buffer.

2.4 The *ex ovo* culture preparation

Fertile eggs that had been incubated for 72 h were cultured using the *ex ovo* method. Each eggshell was sterilized with 70% ethyl alcohol and marked for embryo position. Subsequently, the egg was drilled to remove the eggshell, and then the whole embryo, including all components inside the eggshell, was smoothly and gently transferred into a sterile glass bowl. The *ex ovo* culture CAM

was supplied with antibiotics and antimycotics of 10000 U/mL (Gibco™; cat no. 15140122). All procedures of the *ex ovo* culture were conducted in Laminar Air Flow (Biobase). The incubation in the *ex ovo* culture was carried out for four days (up to 7 days of incubation age) before treatment. Daily checking was performed to ensure the *ex ovo* culture was safe from contaminants, and contaminated or dead embryos had to be removed and discarded from the incubator as soon as possible to avoid contamination.

2.5 Alternating current-electric fields exposure on the chorioallantoic membrane of chick embryo

Each treatment was performed meticulously adhered to the previously outlined experimental design. Ectopic angiogenesis was induced with 30 ng/µL concentrated bFGF implanted in 0.5 mm diameter paper disc, while Tris-HCl buffer was provided in paper discs for non-bFGF-induced CAM. 150 kHz and 18 Vpp AC-EF exposure was generated from an ECCT. The ECCT cage was designed by Ctech Labs Edwar Technology.

The angiogenesis assay was carried out for three days, starting from the 7th day of incubation (the 4th day of the *ex ovo* culture). Following the experimental design, both NIT and IT groups were exposed to an intermediate frequency of AC-EF. The AC-EF exposure was conducted for three days (7th up to 10th-day incubation) with 10 h/day exposure with a 2 h rest period in the middle (06.00-11.00 a.m. and 01.00-06.00 p.m. for AC-EF exposure). After the treatments, each CAM was harvested to collect the data.

2.6 Observation and data collection

On the 10th day of incubation, the process of observation and data collection was carried out. The *ex ovo* culture CAM was observed by counting the number of new blood vessels as an angiogenesis response to the treatments. Macroscopic observation was conducted using photographs, and the radial patterns of blood vessels were counted to analyze the angiogenesis response. Immediately, the CAM area implanted on a paper disc was taken, rinsed with PBS, and stored in RNAlater® (Invitrogen; cat. no. AM7024) at -20°C for total RNA extraction.

2.7 Quantitative PCR (qPCR)

The assessment of VEGFA mRNA expression involved the utilization of quantitative PCR (qPCR) as the methodology. The process commenced with total RNA extraction, achieved using a Direct-zol™ RNA purification kit (Zymo Research; cat. no. R2071). The next step was cDNA synthesis using the iScript™ cDNA Synthesis kit (Bio-Rad; cat. no. 1708891), and the RNA concentration in the cDNA template was 500 ng/µL. The qPCR reaction was carried out using the SensiFAST SYBR No-ROX kit (Bioline; cat no. BIO-98005). The GAPDH primers were designed using the Primer-Blast designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer optimization was carried out before qPCR. Primers were shown in Table 1.

Table 1. Primer sequences for the qPCR method

Gene	Primers
GAPDH (NM_204305.2)	F: 5'TCTCTGGCAAAGTCCAAGTG3' R: 5'TCACAAGTTTCCCGTTCTCA3'
VEGFA [23]	F: 5'CAATTGAGACCCTGGTGGAC3' R: 5'TCTCATCAGAGGCACACAGG3'

All qPCR reactions were performed using the CFX96 Touch Real-Time PCR Bio-Rad Detection System (Bio-Rad Laboratories, Hercules, CA, USA). These reactions were done following specific thermal cycling conditions, including pre-denaturation at 95°C for 2 min; denaturation at 95°C for 5 s; annealing at 58.9°C for 15 s; elongation from 65°C to 95°C for 5 s; and increment of 0.5°C. Quantification Cycle (Cq) data were used to analyze the relative gene expression using the Livak method [24].

2.8 Data analysis

The number of new blood vessels and VEGFA gene expression were analyzed statistically using one-way ANOVA ($\alpha = 0.05$) with a significant level of P-value < 0.05. In contrast, the angiogenesis response in percentage data was analyzed using a Kruskal-Wallis non-parametric test. All the statistical analysis and graphs were performed and created by GraphPad Prism software ver.9.5 for Windows [11].

3. Results and Discussion

In this study, an ECCT delivered an intermediate frequency of alternating current electric fields (AC-EF) with 150 kHz frequency and 18 Vpp voltage on CAM angiogenesis. The use of the *ex ovo* culture provided direct access to the CAM surface for AC-EF exposure. Notably, this was the first study that investigated the effect of intermediate frequency AC-EF exposure on CAM angiogenesis. Previous studies using ECCT proved that it inhibited tumor growth, but there was no information about its effect on angiogenesis. A similar characteristically device, TTFields, proved to inhibit angiogenesis in GBM cell culture by downregulating HIF1α, VEGFA, MMP2, and MMP9 [13]. However, there was no data on normal angiogenesis [13], which can be represented using the CAM angiogenesis model.

The e*x ovo* culture CAM method provides a clear and reliable visualization of treatment and angiogenesis. Figure 1A shows the visualization of the new blood vessel development among treatment groups. The bFGF-induced groups (INT and IT) showed massive amount of angiogenesis compared to the non-bFGF-induced groups (NINT and NIT, Figure 1A). The number of new blood vessels per implant in the IT group showed that exposure to intermediate-frequency AC-EF promoted excessive angiogenesis significantly (P<0.05) (Figure 1B). Meanwhile, exposure to AC-EF without bFGF induction did not promote angiogenesis (NIT group).

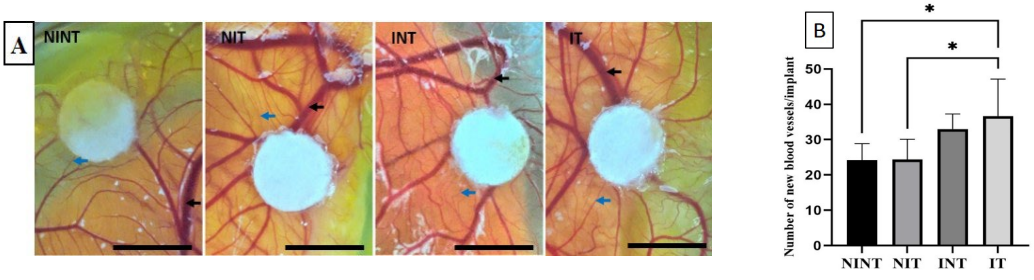

Figure 1. A. Angiogenesis of chorioallantoic membrane of the chick embryo. B. The number of new blood vessels/implants. ANOVA, *<0.05. NINT= non-bFGF-induced CAM, non-AC-EF exposure; NIT= non-bFGF-induced CAM with AC-EF exposure; INT= bFGF-induced CAM, non-AC-EF exposure; IT= bFGF-induced CAM with AC-EF exposure. Non-bFGF-induced CAM = paper disc+buffer Tris-HCl; bFGF-induced CAM = paper disc+bFGF 30 ng/µL. The error bar shows a standard deviation of six replications. Black arrow = main blood vessel; blue arrow = new blood vessel; Scale bar = 5 mm.

The angiogenesis response of the CAM of each treatment is shown in Table 2. The angiogenesis response was calculated and compared to the control group's mean (NINT group). The bFGF-induced CAM with AC-EF exposure (IT group) gave the highest angiogenesis response, which was consistent with the number of new blood vessels (Figure 1B).

Table 2. Angiogenesis response on CAM with and without bFGF-induction and AC-EF exposure

Treatment Group	∑ New Blood Vessels	Angiogenesis Response (%)
NINT	24.17±4.71[a]	0[a]
NIT	24.33±5.79[a]	19.54±10.74[a]
INT	33±4.29[ab]	36.55±17.75[b]
IT	36.67±10.48[b]	51.95±43.04[b]

Note: The values display mean±SD from six replications. The different superscript letter in the same column is significantly different ($P<0.05$). Kruskal-Wallis, $\alpha<0.05$. NINT= non-bFGF-induced CAM, non-AC-EF exposure; NIT= non-bFGF-induced CAM with AC-EF exposure; INT= bFGF-induced CAM, non-AC-EF exposure; IT= bFGF-induced CAM with AC-EF exposure. Non-bFGF-induced CAM = paper disc + buffer Tris-HCl; bFGF-induced CAM = paper disc + bFGF 30 ng/µL.

Figure 2C shows the relative gene expression levels of VEGFA connected with CAM angiogenesis. The results showed that intermediate-frequency AC-EF exposure had no significant impacts on normal CAM angiogenesis (non-bFGF-induced CAM with AC-EF exposure/NIT group) ($P>0.05$). However, VEGFA gene expression had been upregulated insignificantly in bFGF-induced CAM with intermediate frequency AC-EF exposure (IT group) (1.46-fold change).

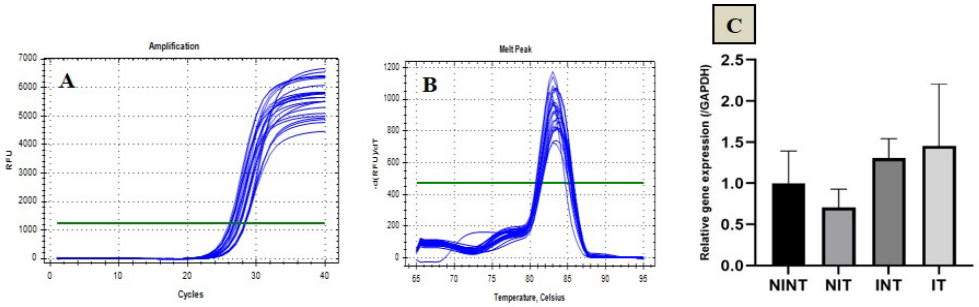

Figure 2. Relative gene expression of VEGFA on CAM angiogenesis of the chick embryo among treatment groups. (A) Amplification chart; (B) Melt peak chart. (C) Relative gene expression. The internal control of relative gene expression is GAPDH. ANOVA, $\alpha<0.05$. NINT= non-bFGF-induced CAM, non-AC-EF exposure; NIT= non-bFGF-induced CAM with AC-EF exposure; INT= bFGF-induced CAM, non-AC-EF exposure; IT= bFGF-induced CAM with AC-EF exposure. Non-bFGF-induced CAM = paper disc+buffer Tris-HCl; bFGF-induced CAM = paper disc+bFGF 30 ng/µL.

In this study, intermediate frequency AC-EF impact on CAM angiogenesis of chick embryos was observed and examined. These findings indicate that exposure to intermediate-frequency AC-EF did not yield any discernible effects on normal CAM angiogenesis, as observed in the NIT group. Furthermore, in the bFGF-induced CAM with (IT group) and without (INT group)

AC-EF exposure treatments, there was an insignificant upregulation of VEGFA gene expression compared to the control (NINT group). Consequently, the number of new blood vessels increased (Figure 1B).

Angiogenesis is a complex process regulated by growth factors such as bFGF and VEGF. These factors bind to tyrosine kinase receptors, initiating endothelial cell proliferation, migration, and differentiation, leading to angiogenesis [25]. Ultimately, the growth factors promote angiogenesis. The results showed that bFGF played a role as an angiogenic growth factor. Consequently, bFGF emerges as a viable candidate for controlling angiogenesis. Given this, it becomes unequivocal that within the context of bFGF-induced CAM (INT group), there is indeed a notable rise in the quantification of newly formed blood vessels.

Interestingly, the number of new blood vessels in the IT group was the highest. This suggests that an intermediate-frequency AC-EF exposure may promote angiogenesis only in bFGF-induced CAM and has no impact on normal angiogenesis (non-bFGF-induced CAM groups) (Figure 1B). This intriguing phenomenon emphasizes that angiogenesis in bFGF-induced CAM with AC-EF exposure is guided by a dual interplay of growth factors, with VEGFA acting as an endogenous factor and bFGF as an exogenous factor. This implies that intermediate-frequency AC-EF exposure may promote excessive CAM angiogenesis through bFGF and VEGFA regulation. This complex interplay suggests that intermediate-frequency AC-EF might exert its influence on the angiogenesis mechanism through distinct pathways, potentially involving multiple growth factors.

A previous study using intermediate frequency AC-EF of 150 kHz and 18 Vpp downregulated IL18 in rat breast tumors [11]. IL18 plays a role in excessive angiogenesis [26]. However, no further information related to AC-EFs effects on angiogenesis existed. In addition, an AC-EF generated from a TTFields device of 0.9 V/cm and 150 kHz was proven to inhibit angiogenesis in GBM cell culture by downregulating VEGFA expression [13]. This result stands in contrast to this study's results, which indicate that AC-EF induces angiogenesis in bFGF-induced CAM. The different results may be influenced by the distinct study method, either *in vitro* or *in vivo*. Furthermore, we hypothesize that the IT group angiogenesis was regulated by AC-EF exposure and bFGF induction since the new blood vessels were formed in bFGF-induced CAM only. However, this hypothesis requires a more in-depth investigation.

Based on the review by Guo *et al*. [27], VEGFR2 plays a critical role in breast tumor angiogenesis. This significance is underscored by its central interaction with VEGFA, the principal growth factor. This is supported by the review result that there were possibilities of VEGF-independent angiogenic factors and VEGFR2-dependent tumor angiogenesis [28]. Therefore, it was logical that the number of new blood vessels significantly increased in the IT group, although VEGFA gene expression was similar among groups.

In contrast to the use of AC-EF, a range of studies exploring the effects of direct current electric fields (DC-EF) showed the potential of DC-EF to promote angiogenesis [29-31]. However, it must be underlined that the data were obtained using different electric sources and research methods, and without information about the electric field effects on normal angiogenesis using the CAM model. However, it may be noted that VEGF receptors play a vital role in that mechanism [29, 32].

Angiogenesis is a crucial mechanism during normal physiology and pathological conditions [1]. Better understanding of the angiogenesis mechanism, and in particular how it is influenced by intermediate frequency AC-EF, should be able to support angiogenesis-based therapies for various diseases that feature the promotion or inhibition of angiogenesis. Such therapies can be used to treat a tumor or vascular disease. The result of this study proves that AC-EF, generated by ECCT, did not affect normal angiogenesis. It becomes a valuable result since ECCT is a potential invention to treat tumors, but it is still debatable.

Future investigation is necessary to answer the hypothesis that exists. Using this reason, an ECCT device, resulting in an AC-EF, should facilitate the *ex ovo* culture to maximize the repetition.

This study had a small sample due to the low survival of the *ex ovo* culture CAM method and tricky procedures using the AC-EF device. Therefore, a potential solution is the development of specialized devices for the *ex ovo* culture methods to reduce contamination risks and increase the survival of the embryos. In addition, growth factor receptors should be investigated to complete the gap.

3. Conclusions

In conclusion, intermediate frequency AC-EF used in ECCT did not affect normal CAM angiogenesis. However, the results showed that the AC-EF induced new blood vessel development significantly on bFGF-induced CAM, although VEGFA gene expression was upregulated insignificantly. This suggested that intermediate frequency of AC-EF was safe for normal angiogenesis. The bFGF and VEGFA may play a role during bFGF-induced with AC-EF-exposed CAM. Therefore, an intermediate frequency of AC-EF may potentially become a solution for obstructive vascular disease.

4. Acknowledgements

This study was supported by the Indonesian Endowment Fund for Education (LPDP) through Beasiswa Unggulan Dosen Indonesia-Dalam Negeri 2019 (BUDI-DN 2019 scholarship). The authors are grateful to Daniel Saputra Wahyudi for his assistance in the molecular assay of this research.

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