

Folic acid and zinc inhibit angiogenesis in chicken chorioallantoic membrane model via angiogenic factor genes

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ABSTRACT

Aims: Angiogenesis occurs only in pathologic conditions such as wound healing and tumorogenesis. Folic acid is a form of a vitamin B. Zinc as an essential mineral plays an important role in tissue repair. Angiogenesis is an important process in wound healing and tissue repair. Despite of this, angiogenic role of folic acid/zinc is not clear on human body. Therefore, the possible angiogenic role of folic acid/zinc had been analyzed on an "in vivo" model in this manuscript. The gene expression alterations of angiogenic factors had been found in this panel.

Methods: The possible roles of Folic acid and Zinc on angiogenesis were evaluated by using an "in vivo" model (chick embryo chorioallantoic membrane model). Total RNA isolation and c-DNA synthesis was performed from vascular structures. Gene expression alterations were analyzed on FGF1, FGF2, VEGFA, VEGFR1, VEGFR2, and NRP1 genes by using c-DNAs as a template in real-time polymerase chain reactions. The results in folic acid/zinc treated groups were correlated with the results in untreated groups for finding the gene expression alterations.

Results: Folic acid and zinc in one by one and combined uses inhibited angiogenesis in certain concentrations. Folic acid inhibited the expressions of VEGFA, VEGFR1 and NRP1 genes. Zinc inhibited the expression of only FGF2 gene in our panel.

Conclusions: In our study, antiangiogenic roles of folic acid and zinc were proven. Folic acid used VEGF-A, VEGFR1 and NRP1 genes in the inhibition of angiogenesis. FGF2 gene was used by zinc in its inhibitory affect on the same panel.

Introduction

Chorioallantoic membrane (CAM) model offers clear advantages to study and manipulate vascular functions with visibility, accessibility, and rapid developmental growth advantages. Many pro- and anti-angiogenic agents (growth factors, hormones, natural molecules, anticancer agents, gases, pro-angiogenic molecules, antibiotics, antibodies and synthetic small molecules) have been tested by quantifying the morphological responses of the CAM vasculature (1). Despite of these findings, the roles of some dietary supplements such as Folic acid (FA) and Zinc (Zn) are not fully clear in angiogenesis.

FA as a vitamin (folate) is an essential molecule take place in various bodily functions. Human body needs FA in DNA synthesis, DNA repair, DNA methylation as well as to act as a cofactor in certain biological reactions. FA is especially important in aiding rapid cell division and growth, such as in infancy and pregnancy. Children and adults both require FA to produce healthy red blood cells and prevent anemia (2, 3). In a recent publication, the suppressor role of FA was explained in tumor growth. FA inhibits cancer cell proliferation using FRα/c-SRC/

ERK1/2/NFKB/TP53 pathway (4). Zn is an essential element used in daily vitamin drugs (5). It is believed to possess antioxidant properties, which may protect skin aging. Zn helps the reactions in wound healing process after an injury. It enhances the body's immune response (5, 6).

Angiogenesis is a normal process during body growth in pregnancy. In adults, it occurs only in pathologic conditions such as wounds, ischemic injury, varicose vein development, tumor formation and cancer metastasis (7). Antiangiogenic agents can be used in tumoral conditions. Angiogenic factors including fibroblast growth factor-FGF and vascular endothelial growth factor-VEGF have important roles in inhibition or induction of angiogenesis (8).

We aimed to analyze the possible roles of FA and Zn on angiogenesis in vivo culture condition. CAM study revealed that FA and Zn decreased the vessel formation on CAM model. FGF1 (OMIM: 131220), FGF2 (OMIM: 134920), Vascular Endothelial Growth Factor A/VGFA/VEGF (OMIM: 192240), VEGF receptor1/VEGFR1/FLT1 (OMIM: 165070), VEGF receptor2/VEGFR2/KDR (OMIM: 191306) and Neuropilin 1/NRP1 (OMIM:

602069) genes were analyzed on the same panel by using Real Time Polymerase Chain Reaction (RT PCR). Our study revealed that FA inhibited VEGFA, VEGFR1 and NRP1 expressions. Zn inhibited only FGF2 expression. So, FA and Zn have antiangiogenic role via these angiogenic factors.

Methods

Preparation of Fa and Zn Solutions: FA was obtained from I.E.Ulagay AS company. ZnCl₂ was supplied from Sigma-Aldrich Company (Cat No: 96468). FA was diluted in physiologic serum (SF) in 0.1 µg concentration (9). ZnCl₂ was diluted in SF in 1 mmol concentration (10).

Atak-S Type Fertilized Chicken Eggs Preparation: Atak-S type fertilized chicken eggs were obtained from the Chicken Production Farm of the Turkish Ministry of Food, Agriculture and Livestock. We performed this project with the ethical decision of Animal Experiments Local Ethics Committee- Gülhane Military Medical Academy (March 8, 2013-2013/4). Three hundred fertilized chicken eggs were selected and incubated in a CO₂ incubator (Heraeus Hanau, Germany) for six days (at 37°C heat, 85-90% humidity). Six days later, the egg shells were cleaned with an antiseptic solution, and the eggs were opened carefully. All the eggs were observed and photographed to check the angiogenesis / neovascularization (11).

Application of Solutions on Fertilized chick CAM: On the 6th day, six different groups (three groups for FA, three groups for Zn) were formed including at least nine to eleven live fertilized eggs for the study (Table 1-2). In FA study, one group for control and two groups for FA treatment were separated. In control group, SF (50 µl) was used. In FA treated groups, we applied 1x10-6 µg/ 50 µl FA in one group and 5x10-5 µg/50 µl FA in another group. In Zn study, one group for control and two groups for Zn treatment were separated. FA treatment procedure was applied similarly in this Zn treated groups (1x10-6 mmol/ 50 µl Zn and 5x10-5 mmol/ 50 µl Zn). The eggs were placed back into the incubator after being covered with parafilm (to prevent the moisture loss and keep the sterilization of the CAM in the eggs).

After 24 hours (7th day) and 48 hours (8th day), the eggs were observed and photographed to check the changes in vascularization. The results obtained in the 6th day were compared with the results obtained in the 7th and 8th days on CAM analysis. In scoring of angiogenesis, Knighton et al.'s methodology was used (11). As known, 24-hour observation (in 7th day) was enough to score the vessels. 48-hour observation (in 8th day) was made for the detection of the embryos for viability due to literature (12).

In Knighton et al.'s scoring methodology, CAM blood vessels near the treatment points were recorded after 24-hour by two different observers. This vascular response was graded as 0, 1+, 2+. As a total differentiation scores of angiogenesis (TDSA), 0 means no change in vessel formation. 1+, and 2+ reflects an increased density and length of vessels converging toward the treatment point (11). In antiangiogenic condition, we noted the inhibitory effect due to the decrease of vessel formation (in density and in length of vessels) (Table 1-2) (13).

RNA Isolation and cDNA Synthesis: All vascular structures were collected from chorioallantoic membrane under stereo microscope (Olympus zoom stereo microscope-ZSX2) in control and 24-hour treated (in 7th day) and 48-hour treated (in 8th day) groups. Total RNAs were obtained from the CAM vascular

Table 1. The total differentiation scores of angiogenesis (TDSA) in control and FA treated groups

	FA Study groups		
	Control	1µl FA	50µL FA
In 24th hour	0*	0*	Inhibition
In 48th hour	0*	0*	Inhibition
TDSA (0) represents no decrease and increase in angiogenesis			

Table 2. The TDSA in control and Zn treated groups

	Zn Study groups		
	Control	1µl Zn	50µL Zn
In 24th hour	0*	Inhibition	Inhibition
In 48th hour	0*	Inhibition	Inhibition
*TDSA (0) represents no decrease and increase in angiogenesis			

structures in 24-hour and 48-hour later FA / Zn treated groups by using RNA isolation kit (NucleoSpin RNA II, Macherey-Nagel). RNAs were converted to c-DNAs by using the cDNA synthesis kit (RevertAid cDNA Synthesis Kit, Fermentas).

Real-time Polimerase Chain Reaction (RT-PCR): The expression patterns of six selected angiogenic factors (FGF1, FGF2, VEGFA, VEGFR1, VEGFR2, and NRP1) were found in RT-PCR analyses. Beta Aktin was used as an internal control in each reaction. All the forward and reverse primers were designed from Primer Bank (<https://pga.mgh.harvard.edu/primerbank/>). Each RT-PCR reaction was performed in 20 µl [10 µl 2 X SYBR (Applied Biosystems), 5 µl c DNA, 1 µl primer, 3 µl d H₂O] in reaction tubes in Roche Applied Science: LightCycler® 480 System. For optimum results, RT-PCR reactions were performed six times for each gene in each condition. The gene expression levels were found in FGF1, FGF2, VEGFA, VEGFR1, VEGFR2, and NRP1 genes in control and FA/Zn treated groups. FA/Zn treated group results were compared with control group results. Mean values were obtained in all groups.

Statistical analyses

The mean values of RT-PCR results were obtained by dividing from each of RT-PCR reaction result. Student's t test (one sample t test) was used for two-group comparisons by using SPSS programme. Student t tests results (p values) represent two group comparisons among the control and the FA/Zn treated groups (Table 1-2).

Results

Results for FA/Zn in CAM model: In 24-hour and 48-hour FA/ Zn treated groups, the TDSA were found due to Knighton et al.'s methodology (Table 1-2) (11). The decrement in density and length of vessels converging toward the treatment point represented the inhibitory effect of FA/Zn on CAM model. No change TDSA was found in control group and in 1x10-6 µg/ 50 µl FA treated groups in 24-hour (7th day) and 48-hour (8th day) treatment. FA decreased the angiogenesis obviously (weakened in main vessels and decreased in capillary vessels) in 5x10-5 µg/ 50µl FA treatment in 24-hour (7th day) and 48-hour

(8th day) groups (Table).

Gene expression results for FA treated groups: In 1x10-6 µg/ 1µl FA treatment, FGF1, FGF2, VEGFA, VEGFR1, VEGFR2, and NRP1 gene expressions were found as similar as in control group in 24-hour and 48-hour groups. In 5x10-5 µg/ 50µL FA treated group, FA inhibited the expressions of VEGFA, VEGFR1 and NRP1 genes two to three times in 24-hour and 48-hour groups. All the results were statistically significant due to control ($p < 0.05$). The gene expressions of FGF1, FGF2, VEGFR2 in 24-hour and 48-hour groups were found as similar as in control groups.

Results for Zn in CAM model: In control group, no change was observed in TDSA result in 24-hour and 48-hour studies. Zn inhibited angiogenesis both in 1x10-6 mmol/ 50 µl and 5x10-5 mmol/ 50µl Zn treated groups in 24-hour and 48-hour observations (Table).

Gene expression results for Zn treated groups: In 1µl Zn treatment, FGF2 gene expression was found decreased approximately two times in 24-hour group. Zn inhibited the expression of same gene approximately two and half times in 48-hour group. In 5x10-5 mmol/ 50µl Zn treatment, Zn inhibited the expression of same gene approximately four times in 24-hour and 48-hour groups. All the results were statistically significant due to control ($p < 0.05$). In FGF1, VEGFA, VEGFR1, VEGFR2, and NRP1 gene analysis, no differences was observed due to control results.

Discussion

Angiogenesis is a process of new vessel formation. Nowadays in medicine it is important to encourage angiogenesis especially for ischemic diseases; instead to reduce angiogenesis for cancer treatment. Inhibiting angiogenesis causes retardation of development and metastasis of malign tumors (Antiangiogenic therapy) (14, 15). CAM model of chick embryos is mostly used as an in vivo working model for angiogenesis (12, 16). This model is used because of its high sensitivity (11). Up to now, no literature was found about the angiogenic and antiangiogenic effect of FA and Zn on CAM with gene expression findings. In our study, the possible effects of FA and Zn on angiogenesis were analyzed on CAM model. FA inhibited the angiogenesis in high concentration (5x10-5 µg/ 50µL FA) in 24-hour and 48-hour observations. Zn also inhibited angiogenesis in low (1x10-6 mmol/ 50 µl Zn) and high concentrations (5x10-5 mmol/ 50µl Zn) in 24-hour and 48-hour observations. The inhibition of angiogenesis is important in angiogenesis-dependent human diseases, such as; inflammatory diseases and cancers (17). There may be a use of FA and Zn in such diseases. On the other hand, there are growing data and a continuing controversy over the effect of FA supplementation on cancer risk. Qin et al. conducted a meta-analysis based on up-to-date published relevant randomized trials to further examine on this issue. Due to these findings, FA supplementation has no significant effect on total cancer incidence, colorectal cancer, prostate cancer, lung cancer, breast cancer or hematological malignancy, but reduces the risk of melanoma (18). In Norway, meta-analysis of ten randomize control trials with mainly elderly men with cardiovascular disease showed a borderline significant increase in incidence of cancer in the FA group compared to controls. When analyzing site-specific cancers, prostate cancer was the only cancer type where increased risk was shown for FA supplements (19). FA likely plays a dual role in prostate carcinogenesis. Epidemiological surveys show that FA can pre-

vent prostate cancer, but fortified FA may increase the risk of the malignancy (20). Weissenborn et al. noticed that the association between FA and cancer is rather complex. FA intake in the range of the dietary condition is associated with a reduced risk for cancer in healthy populations, whereas high intakes of FA might result in an increased risk for cancer incidence or progression in persons with precancerous lesions and under certain conditions (21). As known, our data contains only the effect of FA on angiogenesis. Tumor progression depends on many different factors (gene alteration, heredity, chemical and physical carcinogens etc.) (22). FA applications in cancer treatment may be useful after clarifying the exact role of FA supplementation in cancer progression.

FGF1 and FGF2 proteins belong to FGF family has stimulatory roles in angiogenesis, wound healing, embryonic development and various endocrine signaling pathways as growth factors (23). VEGF is a specific mitogen for vascular endothelial cells (24). The expressions of VEGF genes are potentiated in response to hypoxia, by activated oncogenes (15, 25). In vivo VEGF induces angiogenesis, permeabilization of blood vessels, and plays a central role in the regulation of vasculogenesis (26). As known, VEGFA protein specifically acts on endothelial cells and has various effects, including mediating increased vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis (27). VEGFR genes encode receptors for VEGF gene products. There are three main subtypes of VEGFR, numbered 1, 2 and 3. VEGFR 1-2 genes mainly involved in vasculogenesis and angiogenesis as receptors of VEGF proteins (28). NRP1 gene encodes one of two neuropilins. It contains specific protein domains which allow them to participate in several different types of signaling pathways that control cell migration. Neuropilins bind many ligands and various types of co-receptors; they affect cell survival, migration, and attraction. Some of the ligands and co-receptors bound by neuropilins are vascular endothelial growth factor (VEGF) and semaphorin family members. Neuropilins bind many ligands and various types of co-receptors; they affect cell survival, migration, and attraction (29). So, the mechanisms of antiangiogenic effects of FA and Zn were tried to analyze in our study by finding the gene expression profiles of FGF1, FGF2, VEGF-A, VEGFR1, VEGFR2, and NRP1 genes on vascularization of CAM model. FA inhibited the vascularization of CAM model. In vascular tissue obtained from CAM, FA decreased VEGFA, VEGFR1, NRP1 gene expressions. So, FA inhibits the chick CAM vascularization via VEGFA, VEGFR1, NRP1 genes.

Zn also inhibited angiogenesis on CAM model in 24-hour and 48-hour groups. In our experiments, only the FGF2 gene was found as inhibited by Zn. These results represent the role of Zn on FGF2 gene in angiogenic process. So, Zn may be useful in the treatment of inflammatory diseases and cancers which have benefit in diminished angiogenesis.

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