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Anti-angiogenic role of Ankaferd on chick chorioallontoic membrane model

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SUMMARY

Angiogenesis is needed for tumor growth and metastasis. For this reason, it represents an exciting target for cancer treatment. Some anti-angiogenic drugs may be useful to prevent angiogenesis during cancer treatment. The aim of this study is to investigate if Ankaferd (standardized herbal extract obtained from five different plants namely Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpina officinarum, and Urtica dioica) have an effect on angiogenesis in chick embryo chorioallontoic membrane. Ankaferd was applied at 1, 5, 20, 50% concentrations to the fertilized eggs on the 6th day. On the 7th and 8th days, all the fertilized eggs were opened and vessels were examined. No significant changes were observed in 1% Ankaferd group and control group. Significant inhibition in angiogenesis was observed in 5, 20, 50% Ankaferd groups. In chick embryo chorioallontoic membrane assay, the anti-angiogenic effect of Ankaferd was shown experimentally for the first time in the literature. The antiangiogenic affect of Ancafert was analyzed on vascular endothelial growth factors (VEGF-A,VEGF-C), and hypoxia-inducible factors (HIF1-A and HIF3-A) genes which have possible roles in angiogenesis on human mesenchimal stem cells and human umbilical vein endothelial cells. Ankaferd treatment increased VEGF-A gene expression levels in HUVECs. VEGF-A gene expression levels remained unchanced in hMSCs with %5 Ankaferd treatment. HIF1-A gene expression levels decreased in HUVECs, whereas increased in hMSCs with 5% Ankaferd treatment. The gene expression levels of HIF3-A increased both in HUVECs and hMSCs. In chick embryo chorioallontoic membrane assay, the anti-angiogenic effect of Ankaferd was shown experimentally for the first time in the literature. These findings may represents the potential uses of Ankaferd in cancer treatment as an anti-angiogenic agent. Due to gene expression analyses resuts in our study, Ankaferd affects the angiogenesis in anti-angiogenic procedure on VEGF and HIF genes.

Key Words: *Ankaferd, chick choriallantoic membrane, angiogenesis, vascular endothelial growth factor, hypoxia-inducible factors, human umbilical vein endothelial cells.*

Introduction

Angiogenesis means the development of new blood vessels out of existing ones, due to triger factors such as ischemic diseases, hypoxia, injuries and tumour growth with the secretion of some angiogenic factors (e.g. vascular endothelial growth factor-VEGF, epidermal growth factor-EGF and Hypoxia-inducible factor-HIF) (1-3). These factors activate the endothelial cells by binding to the receptors. So that angiogenesis occurs with the formation of the vascular tubes (4). In order to prevent or decrease angiogenesis, some anti-angiogenic agents have been used in the treatment of cancer (5). Ankaferd (Ankaferd Blood Stopper, İstanbul, TURKEY) as a hemostatic agent, has been provided for the control of some bleeding diseases. It is the first licenced medical Turkish product which includes Urtica dioica, Vitis vinifera, Glycrrhiza glabra, Alpinia officinarum and Thymus vulgaris. Although its hemostatic properties are well known, the molecular mechanisms affected in this process are not fully clarified. The role of Ankaferd on angiogenesis is not known (6). Chick CAM (Chorio-allantioc membrane) model is an usefull easy model in observation the role of therapotic drug and/or extract (such as Ankaferd) in literature (7).

Angiogenesis is mostly studied by using HUVECs which represent the cell migration and the tube formation components of angiogenesis in-vivo (8,9). hMSCs use generally in cell therapy and regenerative medicine because of their multilinaege potentials. hMSCs can differantiate to mesenchymal lineages like osteogenic, chondrogenic, adipogenic (10). Also they can differantiate to other lineages like neurogenic or myogenic (11). Recent reports have suggested that extracellular vesicles released by MSCs have angiogenesis-promoting activity (12).

In our study, the role of Ankaferd in angiogegenesis was analzed in vivo CAM assay.). In order to find out the role of some genes participate in angiogenesis procedure (VEGF-A, VEGF-C, HIF1-A and HIF3-A), the expression patterns were analysed in different concentrations of Ankaferd on human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (h MSCs).

Material and method

Preparation of Ankaferd Solutions: We supplied Ankaferd from the producer company (Ankaferd Ilaç Kozmetik A.S., İstanbul, TURKEY). We used Physiological Saline Solution (PSS) for dilutions in control group and study groups. The five groups were formed including at least eigth live fertilized eggs for Ankaferd study. The Ankaferd solutions were prepared as

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different concentrations (1%, 5%, 20% and 50%) (250 µl for each egg). For control group, 250 µl PSS was added only (13).

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 with the ethical decision of Gülhane Military Academy Animal Experiments Local Ethics Committee (GATA Etik-2013-52). One hundered fifty eggs were selected and kept hold in an incubator for six days (at 37°C heat, 85-90% humidity). Six days later, the egg shells were first cleaned with an antiseptic solution, and the eggs were opened. The opening of the eggs was performed on the sixth day, since vascularization in chick embryos begins on the 5th and 6th days (7). By carefully removing the egg shells with tweezers, the CAM was explored.

Application of Solutions on Fertilized chick CAM: Five groups were selected (control, 1%, 5%, 20% and 50%) from fertilized chick eggs. In each group we tried to find living embrios. Each group contained among 8-11 fertilized eggs (Table 1). Different concentrations of Ankaferd (1%, 5%, 20% and 50%) were applied on these living fertilized chick eggs seperately. In control group, only PSS was applied. All the eggs were observed and photographed to check the vascularization with Sony Digital Camera. The eggs were placed back into the incubator after being covered with parafilm (to prevent the moisture loss and keep the sterilisation of the CAM in the eggs).

Table I. The differantiation scores of angiogenesis in control and Ankafert treatment groups

Groups	n	TDSA	p Values
Control	8	0	p > 0.05
%1 Ankaferd	11	3 (-)	p > 0.05
%5 Ankaferd	9	18 (-)	p < 0.05
%20 Ankaferd	8	16 (-)	p < 0.05
%50 Ankaferd	8	17 (-)	p < 0.05

n: Number of evaluated eggs
TDSA: Total Differantiation Score of Angiogenesis
(-): Represents the decrease in angiogenesis

After 24 and 48 hours, the eggs were observed and photographed to check the changes in vascularization. The 7th and 8th days vascularization of the CAM analyses results were compaired with the 6th day vascularization of the CAM analyses results. Knighton et al.'s scoring methodology was used in the assessments of vascularization in our study (7). In this scoring protocol, CAM blood vessels observed after 24 hours by two different observers. As known, 24 hours observation was enough to score the vessels. 48 hours observation was made for the detection of the embrios for viability due to literature (14).

In Knighton et al.'s scoring methodology, CAM blood vessels near the treatment points were recorded after 24 hours by two different observers. This vascular response was graded as 0, 1+, 2+. As a score, 0 means no change in vessel formation. 1+, and 2+ reflects an increased density and length of vessels converging toward the treatment point (14). This protocol explains the angiogenesis condition in CAM experiments well. For anti-angiogenic condition, we expanded a new evaluation system. In this expanded new system, the decrease of vessel formation repesented numerically as 1- and 2-, whereas 1- reflects a milder form of decrement than 2- in density and in length of vessels.

Cell Culture: HUVECs and hMSCs were used for finding the anti-angiogenic mechanisms in Ankaferd treatment. HUVECs were from ATCC (ATCC® CRL-1730™). hMSCs were derived from bone marrow by primer cell culture in Gülhane Military Medical Academy, Health Science Institute, Cancer Research Center. Primer culture procedure was applied in having hMSCs as described previously (15). The cell lines were cultured in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (BiochromAG, Germany), 1% penicillin/streptomycin (Biological Industries, Israel) at 37 º C', 5% CO2 in an incubator (Heraeus-Hanau, Germany).

XTT Cell Proliferation Assay: The cytotoxic effects of Ankaferd were analyzed by using the protocol of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl)]-2H-tetrazolium hydroxide) assay kit on HUVECs and hMSCs. Ankafert solutions in different concentrations (1%, 5%, 20% and 50%) were applied on cell cultures. The results were obtained by using "ELISA reader" after 24 hours. Due to the XTT assay results, Ankaferd solutions were non-toxic for 1% and 5% concentrations respectively, but 20% and 50% concantrations were above the lethal dose. They were toxic to the cells (16). So, in our study we use 1% and 5% concantrations in the cell cultures.

The application of Ankaferd solutions in cell culture: Three different cell culture groups were chosen for each cell line (one group for control, other group for 1% Ankaferd treatment and another group for 5% Ankaferd treatment). In two grops, 1% and 5% Ankaferd solutions were applied in 2 ml DMEM. In control group, nothing was applied. We performed RNA isolation from cell cultures following the applications of Ankaferd (due to the results of CAM and XTT analyses). The cells were harvested with trypsin and washed with PBS (Sigma-Aldrich) seperatally. RNA isolation procedure was applied in all groups.

Trypan blue exclusion test was done to assess cell viability with trypan blue solution. The cell viability was examined under an inverted microscope (40X magnification). The cells were identified as degenerated (blue-stained) and survived (unstained) (Sigma-Aldrich T8154 Trypan Blue solution) (17). In all well plates, the surviving ratios of the cells were found over 87%.

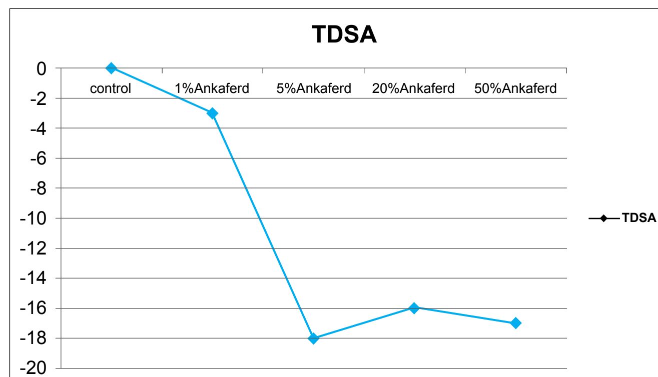
RNA Isolation and cDNA Synthesis: Total RNAs were obtained from the cells in each group by using RNA isolation kit (NucleoSpin RNA II, Macherey-Nagel). Cell viability tests were applied before RNA isolations in all culture groups. RNAs were converted to cDNAs by using the cDNA synthesis kit (RevertAid cDNA Synthesis Kit, Fermentas).

Real-time Polimerase Chain Reaction (RT-PCR): The expression patterns of four selected angiogenic factors (VEGF-A, VEGF-C, HIF1-A and HIF3-A) were analyzed (18-20). Forward and reverse primers were designed from Primer Bank for VEGF-A, VEGF-C, HIF1-A, HIF3-A and GAPDH genes. RT-PCR analyses were performed for finding the effect of Ankaferd on the gene expressions of selected genes. GAPDH

gene was used as internal control in each reaction. Each RT-PCR reaction was performed in 20 µl [10 µl 2 X SYBR, 5 µl c DNA, 1 µl primer, 3 µl d H2O] (Roche Applied Science: LightCycler ® 480 System). SYBR Green PCR master mix was obtained from Applied Biosystems. RT-PCRs were applied for 6 times for each gene seperately. The gene expression levels of VEGF-A, VEGF-C, HIF1-A and HIF3-A in HUVECs and hMSCs were found. The results were compared with control group results. Mean values were obtained in all groups.

Statistical Analyses: 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 1% Ankaferd, 5% Ankaferd, 20% Ankaferd and 50% Ankaferd groups in CAM model (Table I). Student t tests results (p values) represent two group comparisons among the control, the 1% Ankaferd and the 5% Ankaferd groups in gene expression analyses in HUVEC and hMSC cell cultures (Table III).

Table II. In control and Ankaferd treated groups, Total Differantiation Score of Angiogenesis-TDSA results in eggs



Results

Results for CAM model: In 5%, 20% and 50% Ankaferd groups, the differantiation scores of angiogenesis were found due to our new expanded Knighton et al.'s system in each group

(Table I). As seen (-) values represents the decreased density and length of vessels converging toward the treatment point (Figure 1-5). Due to (-) results in CAM analyses, total differantiation scores of angiogenesis (TDSA) were obtained (Table I). No change in the TDSA result was found in control group (Table 1). Ankaferd decreased the TDSA results in %1 and %5 Ankafert groups in a dose dependent manner (Table II). The TDSA result of %1 group was obtained as 3 (-). In 5% Ankaferd solution, the TDSA result was obtained as 18 (-).

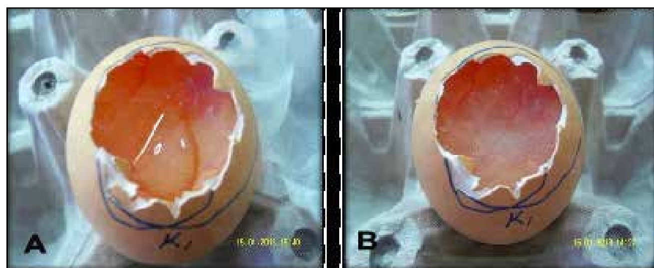


Figure 1. Control group: A.Before serum physiologic treatment B.24 hours later after serum physiologic treatment, No change in angiogenesis

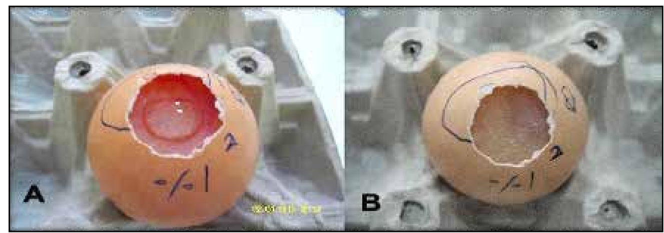


Figure 2. %1 Ankaferd treated group: A. Before Ankaferd treatment B.24 hours later after Ankaferd treatment, No change in angiogenesis

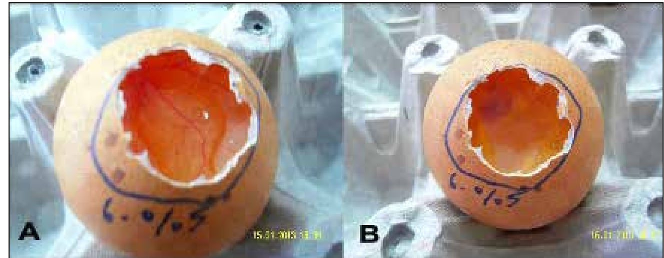


Figure 3. %5 Ankaferd treated group: A. Before Ankaferd treatment B.24 hours later after Ankaferd treatment, Decrease in angiogenesis.

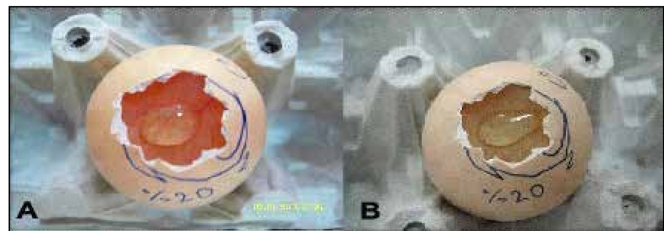


Figure 4. %20 Ankaferd treated group, **A.**Before Ankaferd treatment **B.**24 hours later after Ankaferd treatment, Decrease in angiogenesis

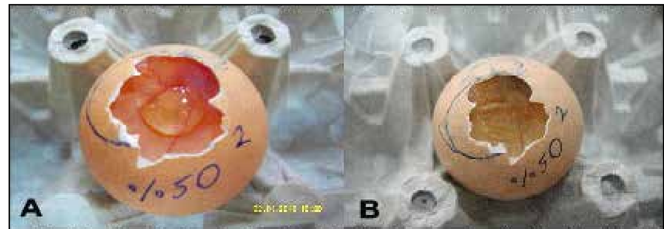


Figure 5. %50 Ankaferd treated group, **A.**Before Ankaferd treatment **B.**24 hours later after Ankaferd treatment, Decrease in angiogenesis

The TDSA results in 20% and 50% Ankaferd solutions were found nearly close to the TDSA result of 5% Ankaferd group. In 20% Ankaferd group, the TDSA result was obtained as 16 (-). In 50% Ankaferd group, the TDSA result was obtained as 17 (-) (Table I and II).

Statitically, no significant difference was found in the TDSA results of control and 1% Ankaferd group ($p>0.05$). Statistically significant difference was observed in the TDSA results of 5%, 20% and 50% Ankaferd groups ($p<0.05$) (Table I). Also, figures 1-5 represented that Ankaferd in 5%, 20% and 50% concantrations had an anti-angiogenic effect in angiogenesis (weakened in main vessels and decreased in capillary vessels) (Figures 1-5). The statistical results in the TDSA values supported the observations in our CAM analyses.

Results for gene expressions in cell cultures: Different concentrations of Ankaferd (%1-%5) were applied in HUVEC and hMSC cells due to XTT analyses results. The expression levels of VEGF-A, VEGF-C, HIF1-A and HIF3-A genes were

presented in Table III. In HUVECs, there was no significant statistical difference for VEGF-A, VEGF-C and HIF3-A genes in 1% Ankaferd group (p0.05). Statistically significant difference was observed in HIF1-A gene expression level in the same group (p0.05) (Table III). In 5% Ankaferd group, no significant difference was found in VEGF-C gene expression level ($p>0.05$). In the same group, there were significant statistical difference in VEGF-A, HIF1-A and HIF3-A gene expression levels due to the control group ($p<0.05$) (Table III). The gene expression level of VEGF-C in 5% Ankaferd group was found nearly similar to the control group in HUVEC study. %5 Ankafed application decreased HIF1-A gene expression almost ten times in the same group. VEGF-A gene expression level increased nearly five times according to the control group. Interestingly, the expression levels of HIF3-A gene highly increased in 5% Ankaferd group (Table III).

In hMSCs, there was no statistically significant difference for VEGF-A, VEGF-C, HIF1-A and HIF3-A genes in 1% and 5% Ankaferd groups ($p> 0.05$) (Table III). The gene expression levels of VEGF-A and VEGF-C in 5% Ankaferd group were found nearly similar to the control group in hMSC study. %5 Ankafed application increased HIF1-A and HIF3-A gene expressions almost two and three times respectively (Table III).

As seen, %5 Ankaferd treatment increased VEGF-A gene expression levels in HUVECs. VEGF-A gene expression levels remained unchanced in hMSCs with %5 Ankaferd treatment. In the same series, VEGF-C gene expression levels were found nearly similar due to control levels in both HUVECs and hMSCs. HIF1-A gene expression levels decreased in HUVECs, whereas increased in hMSCs with 5% Ankaferd treatment. The gene expression levels of HIF3-A increased both in HUVECs and hMSCs (Table III).

Discussion and conclusions

Angiogenesis is a process of new vessel formation. Nowadays in medicine it is important to encourage angiogenesis especially for iscemic diseases; instead to reduce angiogenesis for cancer treatment (anti-angiogenesis). Inhibiting angiogenesis causes retardation of development and metastasis of malign tumours (18,19). CAM model of chick embrios is mostly used as an in-vivo working model for angiogenesis (14,20,21). This model is used because of its high sensitivity (7). Ankaferd is a traditional extract generally uses for hemostasis (6). Up to now, no study has been observed about anti-angiogenic effect of Ankaferd except Turhan et al' s manuscript on gastric and rectum cancers topically in literature (22). Also some studies about anti-angiogenic effect of Vitis vinifera and Glycrrhiza glabra (the ingreditents of Ankaferd) was publicated in literature (23-26).

Table III. The gene expression levels of selected genes in Ankaferd treated groups and control groups in HUVEC and hMSCcells.

Cells	Genes	Ankafert Treated Groups				
		Control	%1	P values	%5	P values
HUVEC	VEGFA	0.42±0.36	1.25±1.32	0.168	2.06±1.12	0.006
	VEGFC	1.20±0.45	0.97±0.87	0.621	1.24±0.21	0.877
	HIF1A	12.74±3.13	2.77±0.12	0.001	1.21±1.41	0.000
	HIF3A	0.003±0.003	0.02±0.01	0.116	0.51±0.83	0.007
hMSC	VEGFA	1.49±0.71	1.04±0.79	0.332	1.49±1.00	1.000
	VEGFC	1.60±0.34	1.01±1.25	0.475	1.65±0.92	0.934
	HIF1A	1.38±0.60	0.80±0.35	0.070	2.30±1.02	0.052
	HIF3A	0.67±0.52	0.23±0.19	0.085	1.71±1.79	0.207

As known, the hemostatic effects of Ankaferd were caused by the total effect of the ingreditents. If one of the ingreditents of Ankaferd was absent in uses, no hemostatic effect was observed (6). In our study, the possible effects of Ankaferd on angiogenesis were analyzed on CAM model. So, the inhibition of angiogenesis was observed with Ankaferd in certain concentrations. Ankaferd can be used in cancer treatment by inhibiting tumour growth and invasion as an extract. There are several studies about the extract uses in medicine in literature such as "Voacangine". It was presented with anti-angiogenic effects on tumour cells by Kim et al. As a natural extract like Ankaferd (27). No side effect of Ankaferd was presented in clinical usage (28-31). Our findings represented that high dosage of Ankaferd has anti-angiogenic affect on angiogenesis. So, local uses may be usefull in anti-tumor treatment to be avare of possible side effects. In angiogenesis, VEGF is a specific mitogen for vascular endothelial cells (32). The expression of VEGF gene is potentiated in response to hypoxia, by activated oncogenes (33,34). In vivo VEGF induces angiogenesis, permeabilization of blood vessels, and plays a central role in the regulation of vasculogenesis (35). As known, VEGF-A 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 (18). VEGF-C participates in angiogenesis, but especially lymphangiogenesis and endothelial cell growth and survival. It can also affect the 6 permeability of blood vessels (36). HIFs are nuclear transcriptional factors that regulate the transcription of genes that mediate cellular and tissue homeostatic responses to altered oxygenation. HIF genes are sensitive in changes in hypoxic conditions especially in cellular environment (37). The products of HIF target genes function in hematopoiesis, angiogenesis, resistance to oxidative stress, cell proliferation, survival and apoptosis, extracellular matrix homeostasis, tumorigenesis and metastasis (38). As a nuclear transcriptional factor, HIF1-A gene is mostly expressed in low oxygen status such as hipoxia or tumors (39). HIF3-A as a DNA binding protein inhibits the hypoxia-inducible gene expressions in condition of low oxgene levels (40). So, the mechanisms of anti-angiogenic effect of Ankaferd were tried to analyse in our study by finding the gene expression profiles of VEGF-A,VEGF-C,

HIF1-A and HIF3-A genes on HUVEC and hMSC cultures. Endothelial cells cultured from human umbilical vein (from cord of newborns), as called HUVEC, is a model for angiogenesis procedure. These cells represent the cell migration and the tube formation components of angiogenesis in cultures (8,9). hMSCs are used in cellular therapy and regenerative medicine because of their multilineage potentials (42). Recent reports have suggested that extracellular vesicles released by MSCs have angiogenesis promoting activity (12). So, HUVECs were used as an in-vitro model for exploring the possible effect of Ankaferd on endotelial cells. hMSCs were used for the property of angiogenesis promoting activity, having possible effect on endotelial cells which have roles on angiogenesis.

In our study, in certain concantrations of Ankaferd, VEGF-A and HIF3-A gene expression levels were increased and HIF1-A gene expression levels were decreased in HUVECs (Table III). No change could be observed in VEGF-C gene expression levels. Increased VEGF-A gene expression level means increased angiogenesis, vasculogenesis and endothelial cell growth. Due to our results, increased HIF3-A gene expression may play the major role in antiangiogenic effect of Ankaferd on vascular endotelial cells. Highly increased HIF3-A, found in our study probably inhibits HIFs which may cause anti-angiogenic effect on HUVECs. So, decresed HIF1-A levels observed in our experiment with anti-angiogenic effect of Ankaferd supported the occurance of Ankaferd's anti-angiogenic effect via HIF3-A gene in HUVECs. This is the first finding which represents the possible molecular mechanism affected in Ankaferd treatment. This may be the result of an hypoxic condition, created in Ankaferd treatment in HUVECs. As known, the overexpression of HIF3-A selectively decreases the hypoxia mediated expression of VEGF-A (43). Certain doses of Ankaferd were increased HIF1-A and HIF3-A gene expression levels in hMSCs. Over-expression of HIF3-A and HIF1-A genes in our experiment may be the result of an hypoxic condition, created in Ankaferd treatment in hMSCs. Despite of this, no changing in the expression of VEGF-A and VEGF-C was observed in the same series. This finding may represent us that the anti-angiogenesis observed in our experiment in our study blongs to the mechanism in HUVECs not in hMSCs. According to our results, Ankaferd has a high anti-angiogenic activity. This finding represent that it may use as an orally used anti-angiogenic extract in cancer.

Aknowlegements

This study was presented by Mert Ruşen Gülşen and Nur Sena Uzunay at 44th TUBITAK High School Students' Project Contest in Ankara, in May 20-24, 2013 and awarded with the first prize. Also it was presented at 25th Europian Union Contest for Young Scientists in Prag, in September 20-25, 2013.

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