Research Article

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Assessment of the synergic effect of Avastin and VEGF siRNA on inhibition of the cell proliferation, invasion and angiogenic factor expression of human breast cancer cells

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Running Title: Combination of VEGF siRNA and Avastin

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Abstract

Background: Vascular endothelial growth factor (VEGF) is a key factor in angiogenesis, tumor growth and metastasis. Various small molecules and several humanized monoclonal antibodies against VEGF have been developed for inhibition of angiogenesis in different cancers. The aim of this work was to investigate the effect of combination of VEGF siRNA and Avastin on breast cancer MCF-7 cell line behavior.

Methods: The cells were treated by Avastin and/or VEGF siRNA and their combination. The cell survival and cell proliferation were assayed by cell counting, trypan blue and MTT tests. The cell migration was assayed by scratching test. VEGF expression was assayed by RT and real-time PCR and ELISA methods.

Results: The cell death and viability were decreased following treatment by Avastin (50% cell death at 100 µg/ml). Cell death with VEGF siRNA transfection was lower than Avastin, however, it was significant. This result in VEGF siRNA+Avastin (100 µg/ml) treatment was greater compared to treatment with each of these compounds alone (47%). Scratching results also showed the synergic effect of VEGF siRNA and Avastin (57% decrease). Real-time PCR results showed that Avastin at concentrations of ≥50 µg/ml led to 2.5 to 7.5-fold decrease in VEGF expression levels. Also, treatment with VEGF siRNA led to 15.5-fold decrease in VEGF expression. Finally, VEGF expression following VEGF siRNA+Avastin treatment led to a significant 47.5-fold decrease in VEGF expression.

Conclusion: Combination of VEGF siRNA and Avastin have a more significant impact on the inhibition of cell growth and migration and it can probably be used as an effective therapeutic approach.

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Keywords:
Breast cancer, Avastin, siRNA, MCF-7, Vascular endothelial growth factor.

Introduction

One of the most important health problems among women is breast cancer. Different methods and protocols such as radiotherapy, chemotherapy and biotherapy have been used for treatment of breast cancer[1,2]. One of the most reliable strategies for inhibition of tumor cell growth and proliferation is blocking of angiogenesis by angiogenesis inhibitors [3]. Angiogenesis is stimulated and triggered by various angiogenic factors such as cell adhesion molecules(Integrins), prostaglandins, Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF) [4]. Among these agents VEGF play the most important role in angiogenesis, cell proliferation, differentiation permeability, vascular tone, the production of vaso-active molecules, tumor growth and metastasis [5]. Most of normal and cancerous cells express and produce VEGF and its receptor FLK-1. In breast cancer VEGF was expressed and secreted by cancerous cells and surrounding fibroblasts which resulted in the formation of intercellular new micro vessels[6]. Therefore, one of the most important target for regulation and inhibition of angiogenesis as a therapeutic manner is VEGF. Several VEGF inhibitors such as a humanized anti VEGF-A monoclonal antibody (Bevacizumab or Avastin), various small molecules, VEGF receptors signal transducers, VEGF receptor chimeric protein and VEGF siRNA have been developed as anticancer agents in last two decades [7-9]. According to The literature reviews and published data, blocking of angiogenesis by some small chemical inhibitors indicated that, these agents might have very limited effect on cancerous cells growth [10-12]. Experiments whit neutralizing antibodies and other inhibitors demonstrated that blockade of the VEGF pathway is not completely sufficient to suppress angiogenesis associated with breast cancer tumors growth too and these agents have some side effects [13.14]. The use Avastin have some limitation from the cost, economic aspect, side effects and so on. For overcoming to these limitation and increasing the efficiency , combination of Avastin by other agents and new type of agents and technology such as gene silencing methods is an attractive subject in the field of cancer research in recent years.

Many reported data have shown that, gene silencing agents such as small interferences RNA(siRNAi) have therapeutic potential when used alone or in combination with other agents [15,16]. However, siRNA –based method relies on successful delivery to the target and in addition, siRNA has been widely explored for use in combination therapy too[17,18]. The use of siRNA in combination with other anti-cancer therapeutics has been shown to improve the sensitivity of breast cancer towards a therapeutic modality, or by working in an additive or synergistic fashion [19,20]. The use of siRNA technology for cancer treatment have some limitation and challenges such as chemical modification, tumour penetration, endosomal escape, target selection and off-target effects. which discussed in detail by Wang et al.[21]. For overcoming to these challenges, interests and researchs on using siRNA by other agents is an attractive subject in the field of cancer research in recent years. Additionaly, researchs on the finding the molecular mechanism and their biological actions are necessary and attractive subject. Because of some limitation and disadvantage of each agent, novell combination approaches to inhibit these processes by probably synergistic effect are under investigation. The aim of this study was to evaluate the potency and synergism of specific VEGF siRNA to suppress human VEGF expression in combination by Avastin on the human breast cancer MCF-7 cells growth, proliferation, migration, VEGF expression and secretion

Material and methods

Cell culture and treatments by Avastan:

Human breast cancer cell line (MCF-7) was provided from American Type Culture Collection (ATCC, Rockville, MD, USA) and were cultured in DMEM medium (Gibson, Paisley, U.K.) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin-streptomycin (100 U-100 µg/ml) ((Gibco, Paisley, U.K.). The cells were trypsinized and sub cultured twice a week in fresh medium. In logarithmic phase of growth, the cells were seeded in every wells of 6 well plates (250 ×103 cells/well) and/or 2.0 ×104 cells/well in 96 flat bottom micro wells (Nunc, Denmark). The cells were incubated over night for complete adherence. Then the cells were treated by 12.5-500 µg/ml of Avastin (Bevacizumab) (Roche, Hafteman-La, Basel, Switzerland). The cells were incubated in DMED medium, supplemented by 10% of FBS for 96 hr at 37°C, 5% of CO2. After 96 hr, the cells pellet and supernatants were separately collected and the total cell number, viability, MTT cell proliferation assays, VEGF expression and secretion were done as below.

SIRNAs transfection

The MCF-7 cells were cultured for 24 hours and transfected by 3double-stranded RNA and oligo targeting VEGF which were purchased from Santa Cruz (Santa Cruz Biotechnology Inc, Bergheimer, Heidelberg, Germany). Oligoes which used as scramble and VEGF siRNA have sequences as:

Scramble siRNA sense: 5/ -GCG GAG AGG CUU AGG UGU

A[TT]-3/.

Antisense: 5/-UAC ACC UAA GCC UCU CCG C[TT]-3/.

hVEGF-siRNA, sense strand RNA, 5/-GGA GUA CCC UGA UGA GAU C[TT]-3/.

hVEGF antisense strand RNA, 5/-GAU CUC AUC AGG GUA CUC C[TT]-3/.

The cells (with 60% of confluency) were incubated in 800 µL of antibiotic and serum free DMEM in six-well plate per well. 10 µL of 3 siRNAs stock (10µmol) and 10 µL of an X-term-GENE (Roche) transfection reagent were diluted to 100 µL antibiotic and serum free DMEM and then incubated separately at room temperature for 5 min. The two mixtures were combined and incubated at room temperature for 30 min. The siRNAs, X-term mixtures were added drop wise to cells in well. The cells were incubated for 6 hours. Then 1000 µL of 20% FBS DMEM were added to cells, after 72 hours' cell detached and analyzed for cell proliferation, viability, qRT-PCR and ELISA assay as described below. To analyze the percent of transfection of cells, the experiment was performed by control siRNA conjugate with FITC (Invitrogen) and the percent of FITC positive cells were analyzed by flow cytometry according to the manufactures recommendations.

For combination study the cells were treated by 25 µg/ml of Avastin and VEGF siRNA.

Cell proliferation and growth

Cell number counting and viability (Trypan blue dye exclusion) were enumerated using a Neobar hemocytometer.

MTT assay: After 96 hr of incubation, 10µl of freshly prepared (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, U.S.A.) solution (5 mg/ml in PBS) was added to each microwell and were incubated for 4hr. Then, 50µl of MTT lysis solution (20% Sodium Dodcyl Sulphate W/V and 50% Dimethy Formamide V/V) was added to each well and incubated overnight. Absorbance was read at 580 nm using a Titretek multiscan ELISA reader (Labsystems Multiskan, Roden, Netherlands).

Semi-quantitative detection of VEGF mRNAs by RT-PCR

Avastin and/or VEGF siRNA treated cells were collected and total RNA was extracted by using the Tripur isolation reagent (Roche, Mannheim, Germany), according to the manufacture's protocol. RNA yield and purity were quantitated by measuring optical density (OD260/280) using a Nanodrop (Beckman Coulter Inc. CA, USA). First-strand cDNA was synthesized from 1 µg of total RNA using Maurine Maloney Leukemia virus (M-MLV, Fermentas) and reverse transcriptase (Fermentas Gmbh, Leon-Rot, Germany) with oligo-dT primer (Fermentas Gmbh, Leon-Rot, Germany), according to the manufacturer's instructions. The VEGF and GAPDH cDNA were amplified by the following primers:

hVEGF forward: 5/-CCA TGA ACT TTC TGC TGT CTT-3/,

and reverse: 5/-ATC GCA TCA GGG GCA CAC AG-3/;

GAPDH forward: 5/-AAT CCC ATC ACC ATC TTC CA-3/

and reverse: 50/GTC ATC ATA TTT GGC AGG TT-3/.

The expected sizes of the RT-PCR product were 121 bp for GAPDH and 250 bp for VEGF. The thermal cycling conditions for amplification of those fragments were as follows: 94 °C for 10 min., followed by 35 cycles at 94 °C for 50s; 54 °C for 30s; 72 °C for 60s. This was followed by re-extension at 72 °C for 10 min. The PCR products were separated on a 2% agarose gel (using 0.5× TBE buffer) and visualized by ethidium bromide staining. For quantitation each band of gel was scanned by image analysis program (ImageJ. Exe). The expression of the target genes was quantified and then normalized by an endogenous reference housekeeping gene (GAPDH) relative to the calibrator (untreated cells). Relative intensity of each gene mRNA and fold of change referred to untreated control cells.

Real-Time PCR quantitative detection of the VEGF mRNAs:

Quantitative real-time PCR was carried out on cDNAs prepared for conventional RT-PCR. We used the ABI PRISMTM 7700 Sequence Detector System (PE Applied Biosystems) and the fluorescent dye SYBR® Green and the amount of PCR products was determined based on the fluorescence produced during the extension step of each cycle in a closed tube. To normalize the amount of total RNA present in each reaction, we used GAPDH as housekeeping gene an internal control. The threshold cycle (Ct) value for each sample was proportional to the log of the initial amount of input cDNA. Relative expression levels of VEGF in each treatment group were derived from normalizing the Ct value of genes against that of an endogenous reference and a calibrator, where by GAPDH was used as a normalizer against untreated cells as a calibrator. Quantitative data were analyzed and relative quantification of VEGF mRNAs were derived by the 2-ΔΔCT methods.

Quantitative ELIZA for VEGF

The amount of secreted VEGF in response to Avastin and/or VEGF siRNA by cells were measured in supernatants by using commercial Calbiochem human VEGF ELISA kits. (Calbiochem-Merck-Bioscience, Darmstadt, Germany) according to the procedure manual of the kits. The method is a "sandwich" enzyme immunoassay employing monoclonal and polyclonal antibodies. Quantitation is achieved by construction of a

standard curve using known concentrations of VEGF calibrators of the kit.

Scratching assay

Cell migration and quantification was done using the wound healing assay as described by Bobadilla and co workers [22]. Briefly, equal numbers of cells (5×105) were plated and kept overnight in 6-well plates. After that the monolayer of cells was carefully wounded by manual scratching with a micro-pipette tip head. Exactly, the cells were treated with different concentration of Rosuvastatin and/or Avastin. Treated cells were cultured in complete growth medium and incubated for 96 hr. The wounded regions were photographed at different times interval (0-96hr) using a phase contrast microscope (Nikon). The photographs of the cells were analyzed using a Cell science software program. The distance between the two sides of scratch layers of images taken from migration of all concentrations at specified times was determined by the Cell science software.

Statistical analysis

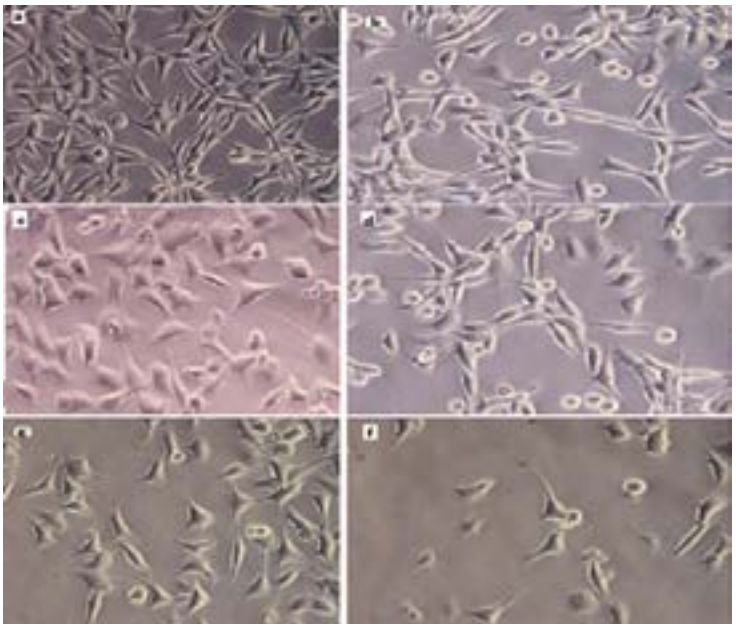
Each experiment was minimally performed three times for all data, each carried out in duplicated sequences. Data were analyzed using a One-Way Analysis of variance (ANOVA) Values were given as the mean ± Standard Deviation (SD) and analytical variables were compared by using the students' t-Test. By convention, a α-level of $p<0.05$ was considered to be statistically significant

Results

Cell proliferation

Initially, the morphology and behavior of cells which treated with different concentrations of Avastin and/or VEGF siRNA and/or combination of them were observed under an invert microscope after 72 hrs. (Figures 1) Avastin and VEGF siRNA treatment did not affect MCF-7 cells morphology and behavior. Increasing of Avastin concentration slightly decreased the cell density, confluency and adherence (Fig 1b-d)> In presence of VEGF siRNA the cell density and confluence decreased moderately (Fig 1E). In combination of Avastin and VEGF siRNA the cells density and confluency were strongly decreased in comparison to untreated cells (Fig 1F)

Figure 1. The effects of Avastin and VEGF siRNA on the morphology, cell proliferation and growth of MCF-7 cells. The cells were treated by Avastin and/or VEGF siRNA and incubated 72 hr and images were photographed by microscope. a: Untreated control cells after 72 hr incubation. b, c and d: The cells treated by25, 50 and 100µg/ml of Avastin respectively. The cells density and proliferation were slightly suppressed by Avastin. e: The cells treated by VEGF siRNA. The cells density and proliferation were moderately suppressed. f: The cells treated by combination of 100µg/ml of Avastin and VEGF siRNA. The cells density and proliferation were strongly suppressed. (Magnification: ×400)



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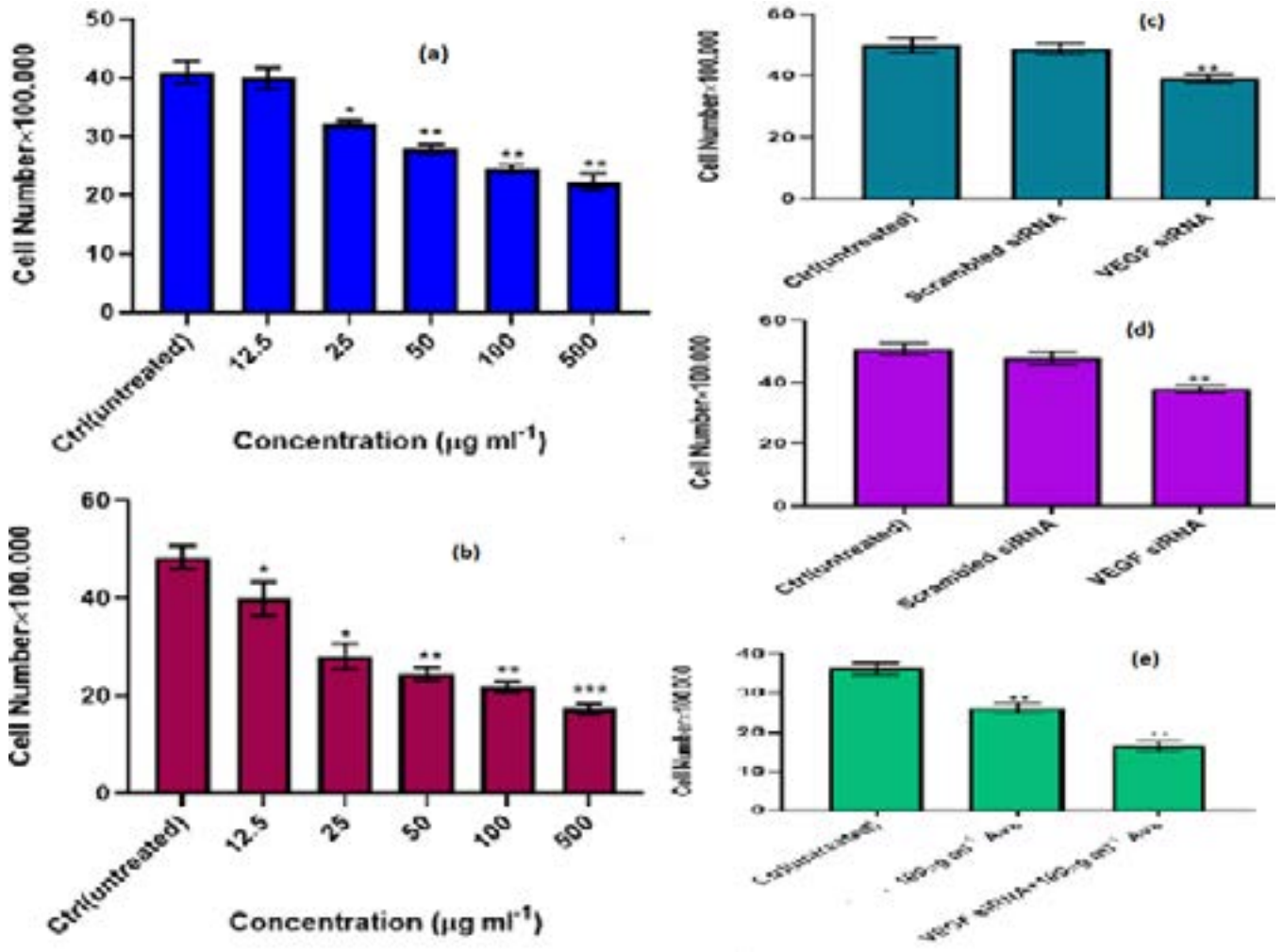
These cells were trypsinized and collected after 72 hr. The cells, viability and growth were assayed by using trypan blue, neobar cell counting and MTT assays. The result of cell counting was shown in figure 2. Avastin inhibited cell growth potential and cell proliferation parameters from 20-50% in the presence of Avastin (25-100 µg/ml) incubation in comparison of untreated control cells after 48-72 hr ($p<0.05$) (Figure 2a and b). The results indicate that, VEGF siRNA inhibit cell proliferation of MCF-7 cells about 15-20% in comparison to control cells after 48-72 hr. (Fig 2c and d). Combination of Avastin and VEGF siRNA decreased cell numbers in comparison by alone treatment (Figure 2E). The results indicate that, combination of 100µg/ml of Avastin by VEGF siRNA inhibited the cell growth and cell numbers about 60% in comparison to untreated cells and about 40% in comparison to alone treatments ($p< 0.01$) (Fig 2 E)

Figure 2. The effect of Avastin and VEGF siRNA on the cell growth and proliferation according cell count. For cell proliferation assay, the control and treated cells were collected after treatment and 48 - 72 hrs incubation. Total cell number was determined using a hemo-cytometer. Cell count after treatment by Avastin for 48 (a) , 72 hr(b) , VEGF siRNA 48 hr(c) and 72 hr (d). e indicate treatment of cells in combination for 48 hr. (* $p<0.1$, ** $p<0.05$ and *** $p<0.01$). All experiments were repeated 3 times in duplicates. The results are mean ± 1.0 SD for three separate experiments.



The cells viability curves of MCF-7 cells in response to Avastin and VEGF siRNA was shown in figure 3. Results indicated that in lower concentration of Avastin (12.5 µg/ml) the viable cells are more than 90% after 48 hr (Fig 3a) and after 72 hr about 20% of the cells were died (Fig 3b). The cells viability was decreased from 75% to 51% in presence of 25-100 µM of Avastin for 48 hr and 50-25% for 72 hr incubation. Treatment of cells by VEGF siRNA have not significant cytotoxic effect on cell viability (Fig

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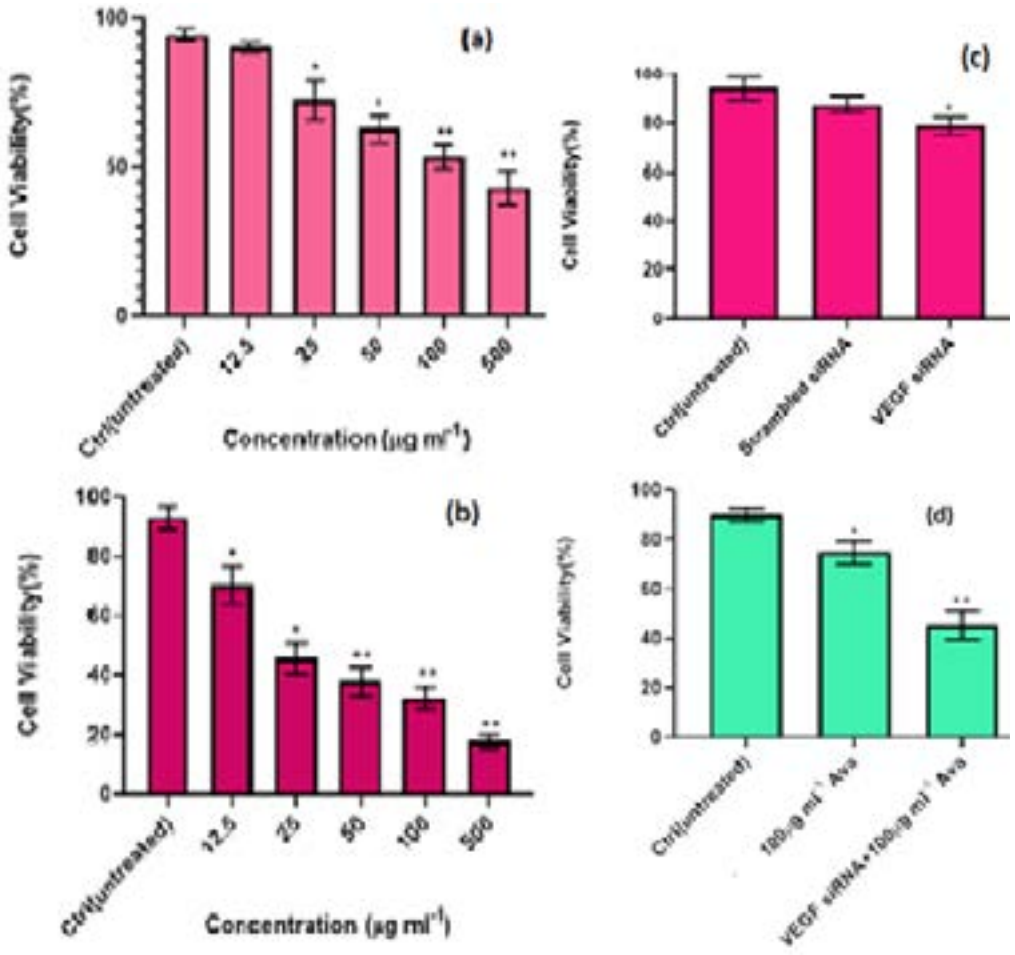
3c). Combination of 100 µg/ml of Avastin by VEGF siRNA resulted in about 15% lowering of viability after 48 hr incubation in comparison by alone treatments ($p<0.05$) (Fig 3d)

Figure 3. The effect of Avastin and VEGF siRNA on the cell viability by trypan blue test. For viability assay, the control and treated cells were collected after treatment and 48 - 72 hrs incubation and trypan blue dye exclusion by viable cells was determined and counted using a hemo-cytometer. % of viable cells after treatment by Avastin for 48 (a) , 72 hr(b) , VEGF siRNA 72 hr (c) and combination treatment for 72 hr(d). (* $p<.0.05$, ** $p<0.01$). All experiments were repeated 3 times in duplicates. The results are mean ± 1.0 SD for three separate experiments



In MTT assay, the absorbance values of 4 independent tests were averaged and growth curves were constructed (Figure 4). The result indicated that, Avastin inhibited cell growth potential and cell proliferation parameters from 25-55% in the presence of Avastin (25-500 µg/ml) incubation in comparison of untreated control cells after 48hr ($p<0.05$) (Figure 4a) and 40-70% after 72 hr $p<0.01$) Figure 4b). The results indicate that, VEGF siRNA inhibit cell proliferation of MCF-7 cells about 20% in comparison to control cells after 48 hr (Fig 4c). Combination of Avastin and VEGF siRNA decreased MTT absorbance in comparison by alone treatment (Figure 4c). The results indicate that, combination of 100µg/ml of Avastin by VEGF siRNA inhibited the cell growth and cell proliferation about 25-30% in comparison to untreated cells and about 30-40% in comparison to alone treatments ($p< 0.01$) (Fig 4c)

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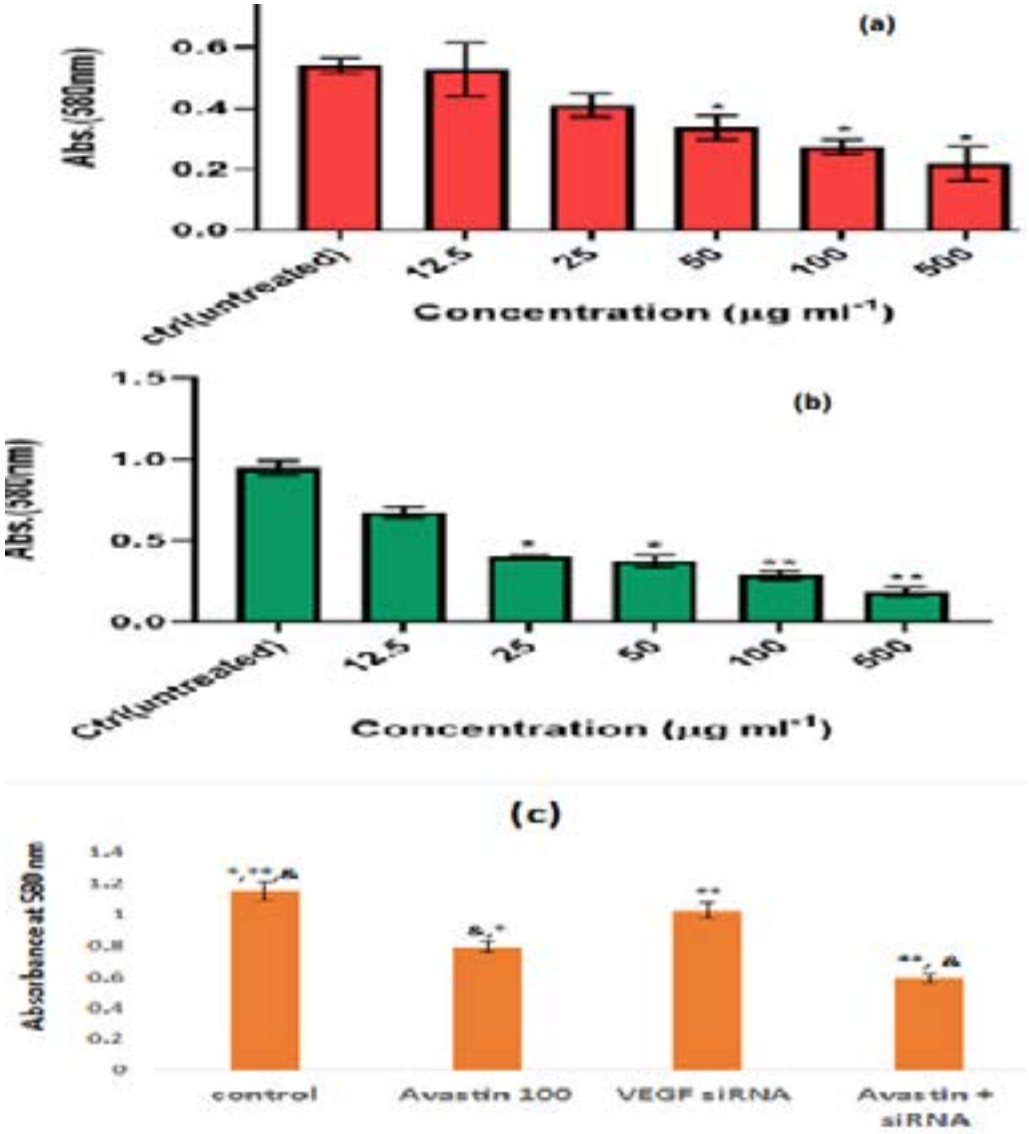
Figure 4. The effect of Avastin and VEGF siRNA on the cell growth and proliferation according MTT assay. The control and treated cells were assayed by MTT after treatment and 48 - 72 hrs incubation as described in materials and methods. Measured absorbance at 580 nm after treatment by Avastin for 48 (a) , 72 hr(b) , VEGF siRNA and combination after 72 hr (c).. (* p<0.05 , ** p<0.01 and & p<0.005). All experiments were repeated 3 times in duplicates. The results are mean ± 1.0 SD for three separate experiments.



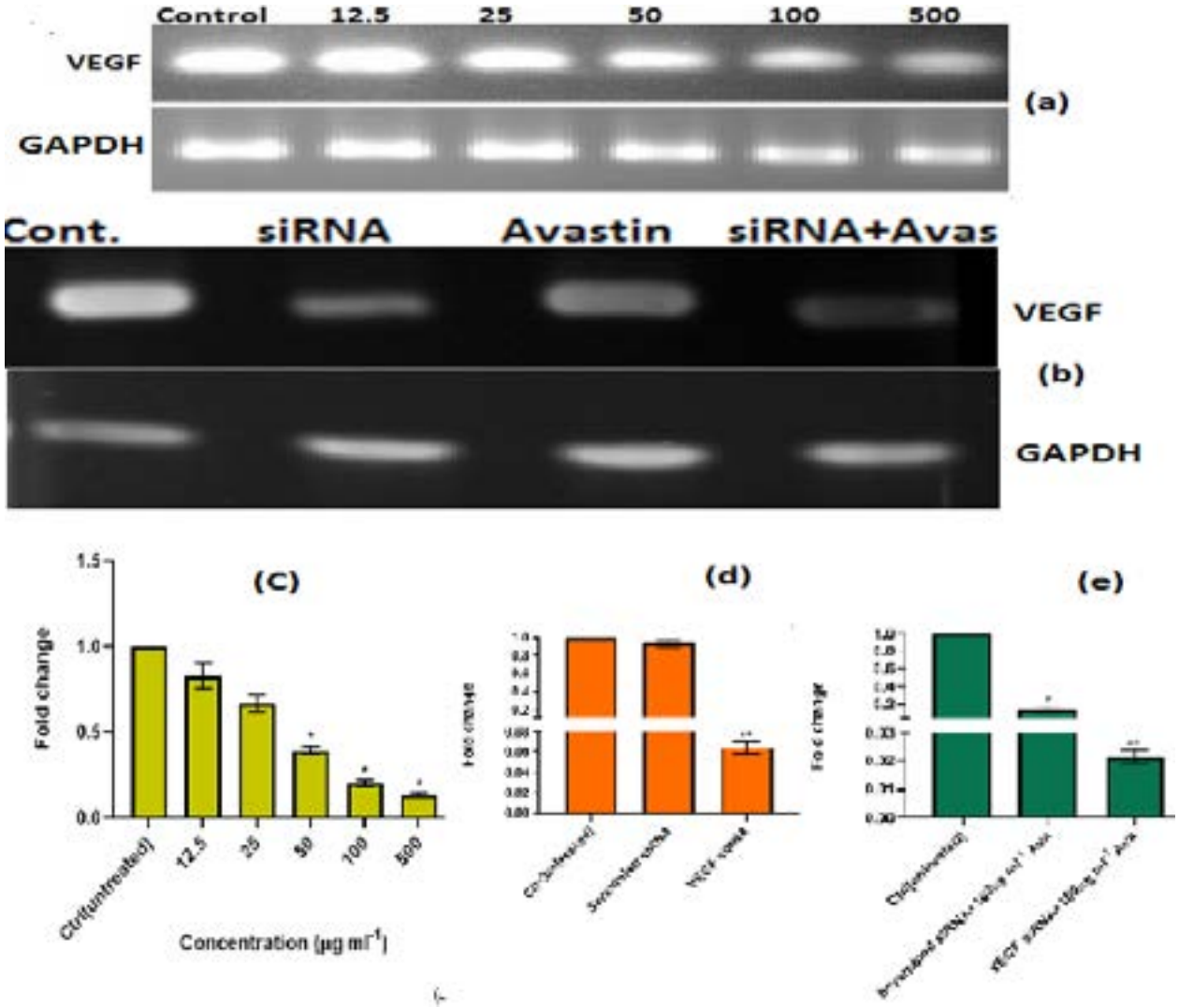
Effect of VEGF siRNA and Avastin on the expression of VEGF:

To examine the level of VEGF mRNA expression by MCF-7 cells in response to VEGF siRNA and Avastin; RT-PCR were performed. The treated cells were collected and total RNAs were extracted and the quality of the nucleic acids was measured by Nano-drop. After that, RT-PCR, and consequently Real-time PCR, were employed by the designed specific primers as described in materials and methods to analyze VEGF gene expression. The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining (Figure 5). The intensity of gels indicates that the expression of VEGF in response to Avastin was decreased in a dose dependent manner (Fig 5a). VEGF siRNA inhibited the VEGF expression and combination treatment was sharply inhibited the expression. For quantitation each band of gel was scanned by image analysis program (ImageJ. Exe) and by Real time PCR too. Quantitative real-time PCR was carried out on cDNAs prepared for conventional RT-PCR. Relative intensity of each gene mRNA and fold of change referred to untreated control cells were shown in figure 5 c, d and e. The results

presented in figure 5c show that, treatment of MCF-7 cells by Avastin inhibited VEGF expression about 20 to 80% in presence of 12.5to 500 µg/ml of Avastin in a linear dose dependent manner($p<0.05$). VEGF expression was decreased from about 98% in presence of VEGF siRNA in comparison by untreated cells and scramble siRNA treated cells (Figure 5d) ($p<0.01$). In combination of 100µg/ml of Avastin by VEGF siRNA the expression was shapely inhibited in comparison to untreated control cells and alone treatment (Fig 5e) ($p<0.001$).

VEGF content in supernatants were quantitavely measured by using VEGF ELISA test too. The VGEF content of samples were calculated from the standard curve of calibrators of the kit. Results show that in presence of Avastin (25-500 µg/ml) and VEGF siRNA and their combination the VEGF content was decreased from > 95% to complete inhibition in secretion respectively in comparison to untreated cells (Data was not shown).

Figure 5. Changes in VEGF genes expression during treatment of MCF-7 cells by Avastin and/or VEGF siRNA. MCF-7 cells were incubated with Avastin (up to 100µg/ml) and/or VEGF siRNA for 72 h as described in "Materials and Methods". Then, total RNA was extracted from untreated and treated cells and amplified by RT-PCR for gene expression. a) The agarose gel electrophoresis pattern of PCR products from Avastin treated cells. b) The agarose gel electrophoresis pattern of PCR products from VEGF siRNA and combination of them by 100µg/ml of Avastin. Total RNA was extracted from untreated and treated cells and amplified by Real-Time RT-PCR for gene expression too. The Real Time PCR results were quantified and normalized against untreated control cells and GAPDH as an internal housekeeping gene control. c , d and e indicate for response to Avastin, VEGF siRNA and combination of them respectively. * indicated 50 and 100µg/ml of Avastin in comparison to untreated control cells($p<0.01$. ** indicated VEGF siRNA and combination of them by Avastin in comparison to untreated control cells($p<0.001$). All experiments were repeated 3 times in duplicates. The results are mean ± 1.0 SD for three separate experiments.



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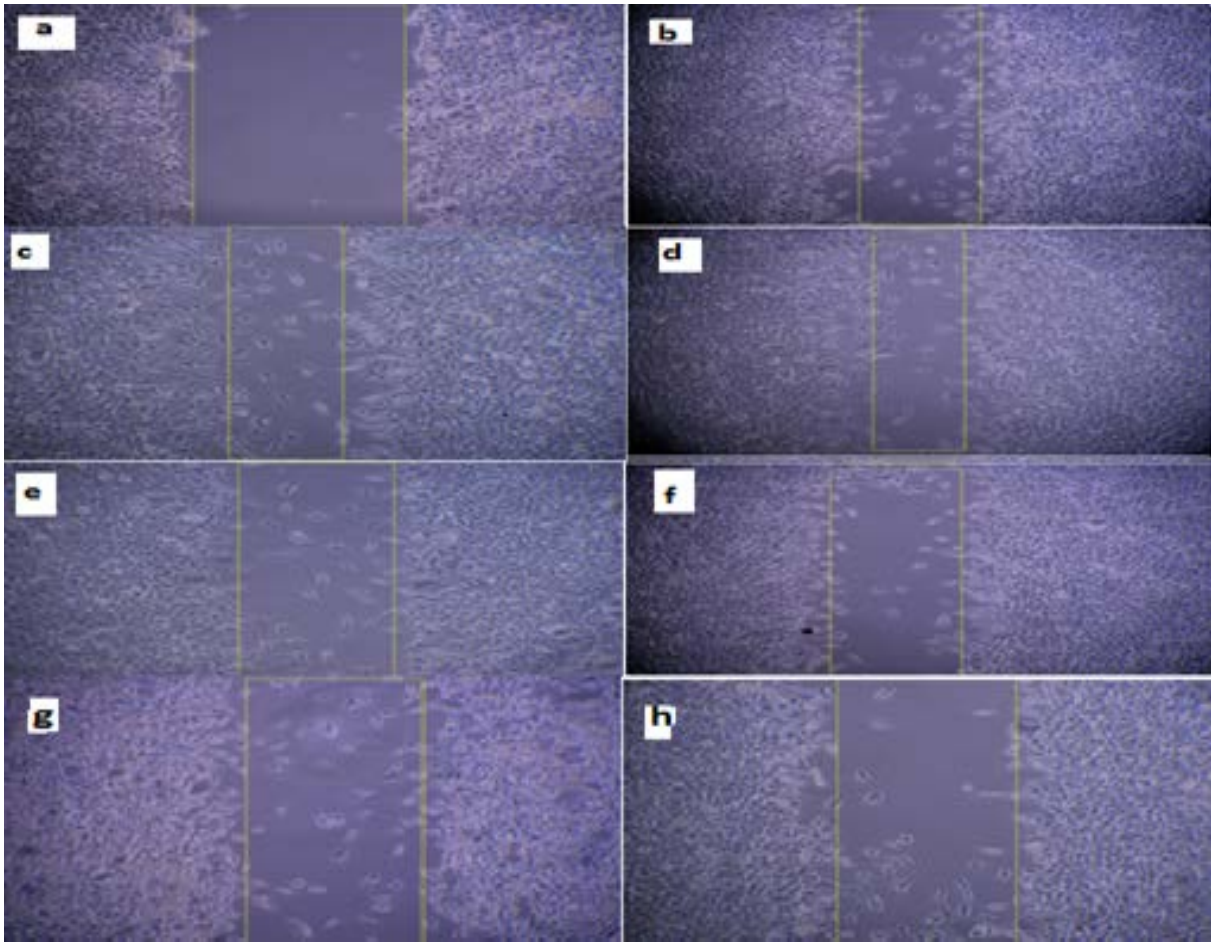
The effects of Avastin and VEGF siRNA on migration of MCF-7 cells in scratching method

The effect of Avastin and/or VEGF siRNA on proliferation and migration of MCF-7 cells were done by scratching assay as described in the Materials and Methods. The wounded regions were photographed after 96hr by using a phase contrast microscope (Figure 6A). The distance between the two sides of scratch layers of images taken from migration of all concentrations at specified times was determined by the Cell science software. The average migration distance in each photograph was calculated. The percentage difference for each concentration was calculated and compared to untreated control and alone treated cells. The calculated data were presented as a graph generated (Figure 6B). The cell migration was inhibited about 20-40% in presence of 12.5- 100 µg/ml of Avastin ($p<0.05$) and about 45% in presence of VEGF siRNA ($p<0.01$) in comparison to untreated cells. In combination of 100µg/ml of Avastin by VEGF siRNA the migration was inhibited about 55% ($p<0.01$) in comparison to untreated cells and alone treatment ($p<0.01$).

Figure 6. The effects of Avastin and/or VEGF siRNA and their combination on the growth, proliferation, migration and healing of MCF-7 cells according scratching wound healing assay after 72 hr as described in materials and methods. The MCF-7 cells were grown in RPMI medium in the absence or presence of those agent. The cells were assessed for migration by the scratching method. The distance of migration and relative surface coverage area were calculated by the program which is described in methods. A) The morphology and photographs of the cells at 72 hr. a) The scratched region at time zero, b) Untreated control cells, c , d , e and f the cells treated by 12.5, 25, 50 and 100 µg/ml of Avastin respectively. g and h indicate for VEGF siRNA and its combination by 100 µg/ml of Avastin respectively (magnification: ×100). B) The distance of migration and relative surface coverage area were calculated and quantified by the program which is described in methods., * indicate 50-500 µg/ml of Avastin in comparison to untreated cells and alone treatment ml of Avastin (*, $p<0.05$). ** indicate VEGF siRNa and its combination by 100 µg/ml of Avastin in comparison to untreated cells and alone treatment (*, $p<0.01$). All experiments were repeated 3 times in duplicates. The results are mean ± 1.0 SD for three separate experiments.



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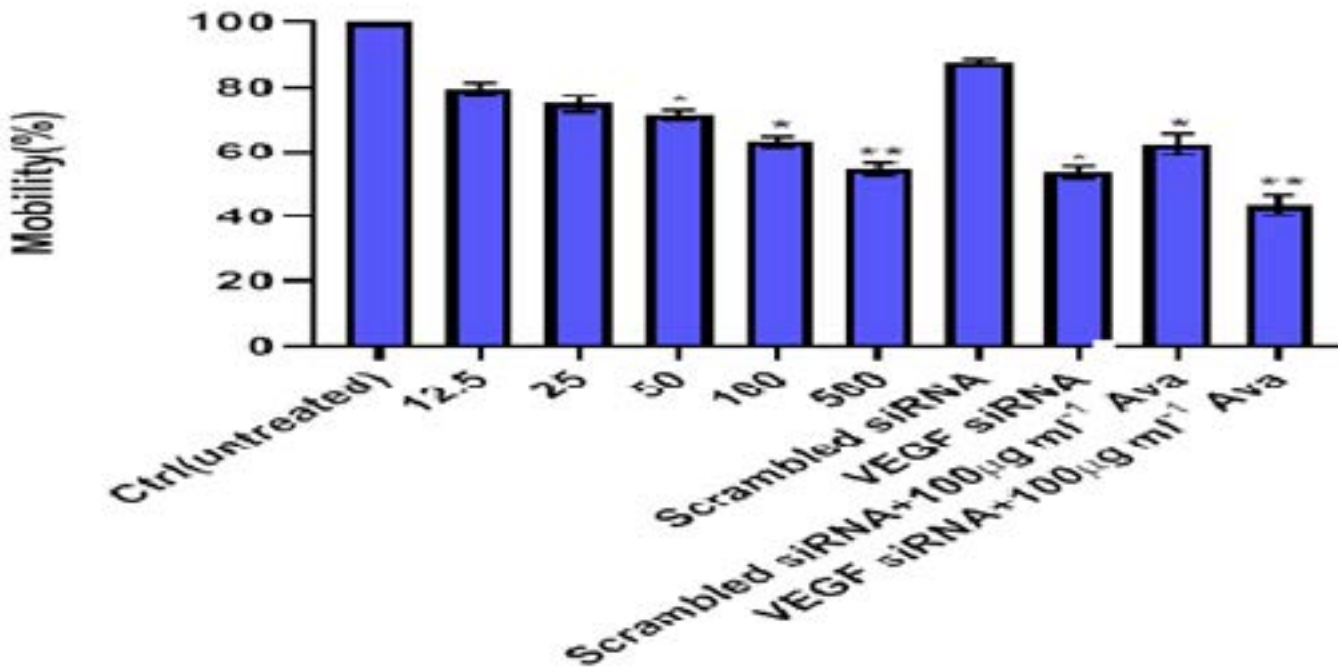

Discussion

Vascular Endothelial Growth Factors (VEGFs) is one of cytokine which expresses by many type of normal and cancerous cells in tissue and tumors. VEGFs and VEGF receptors mediate a plethora of biological processes in angiogenesis, cell proliferation, differentiation permeability, vascular tone, the production of vaso-active molecules, tumor growth and metastasis. Various small molecules and some type of humanized monoclonal antibodies have been developed as anticancer. The use of siRNA in combination with other anti-cancer therapeutics has been shown to improve outcomes by either increasing the sensitivity of cancer towards a therapeutic modality, or by working in an additive or synergistic fashion. Several studies on the combined treatment of angiogenic inhibitors and chemotherapy such as avastin combined with paclitaxel and carboplatin in the treatment of non-small cell lung cancer [23], concomitant delivery of avastin with doxorubicin for the treatment of breast cancer [24] and the simultaneous treatment of VEGF siRNA and doxorubicin have focused on the treatment of liver carcinoma [25].

In general, new strategies are needed to increase the effectiveness of current chemotherapy drugs Therefore, the aim of this research was to investigate the synergistic effect of Avastin and VEGF siRNA gene silencing on the growth, proliferation, migration and VEGF expression of MCF-7 cells. Our results from the cell proliferation aspect showed high cytotoxic effect of avastin after 72 hours of treatment. For this reason, a 48-hour treatment with Avastin was considered the ideal treatment time for subsequent tests. A significant increase was observed in cell death following treatment with avastin at 50, 100 and 500 µg / ml. The rate of cell death in the treatment with VEGF siRNA also increased significantly, which was much lower than the treatment with different concentrations of avastin. However, this increase in cell death was greater in the simultaneous treatment of cells with VEGF siRNA and avastin at a concentration of 100 µg / ml than in the treatment with either of these compounds alone treatment.

These results indicate that this efficient system, use of VEGF siRNA and Avastin, can reduce the dose of the drug and consequently reduce the side effects of avastin. Because of the high economic cost of Avastin, it is also more economical.

The results were confirmed by trypan blue dye exclusion test and cell counting. Thus, the percentage of cell viability as well as the total number of cells were significantly decreased after 72 hours of Avastin treatment, which resulted to the toxicity of this drug when the cells were exposed for a long time. Also, the percentage of survival and the total number of cells decreased in both VEGF siRNA treatments alone and avastin alone, and this decrease in VEGF siRNA transfected cells treated with avastin alone compared to the two groups alone. It was significantly more. As with the MTT results, these results demonstrate the syn-

ergistic effect of the combined use of VEGF siRNA and avastin on overcoming the drug resistance of cells.

In the mechanism of VEGF action in tumor cells, it has been shown that VEGF induce the apoptotic genes and regulate the balance of Bcl2/Bax genes. The Bcl-2/Bax family includes to both anti-apoptotic and pro-apoptotic members of pro-to-oncogenes, which have different effects and mechanism of action in cancerous cells and on mitochondria. Bax can increase the release of cytochrome c from the cytosol into the cytochrome. This leads to inhibition of cytochrome c release, which is involved in the induction of apoptotic effects. VEGF siRNA has been shown to reduce the expression of the anti-apoptotic protein Bcl-2 and has no effect on the Bax apoptotic protein in MCF-7 cells. Thus, it has been suggested that inhibition of VEGF by siRNA by disturbing the balance between members of the Bcl-2 family may lead to increased cellular apoptosis. The expression ratio of anti-apoptotic and pre-apoptotic proteins, such as Bcl-2 / Bax, is essential for the induction of apoptosis, and this ratio determines the cell's susceptibility to apoptosis. A change in the Bcl-2 / Bax ratio stimulates the release of cytochrome c from the mitochondria to the cytosol. It has been shown that this ratio decreases significantly following treatment with VEGF siRNA in MCF-7 cells, and a decrease in this ratio may be the mechanism by which MCF-7 cell apoptosis is induced. In fact, VEGF siRNA has been shown to reduce VEGF expression, followed by a decrease in Bcl-2 / Bax ratio, cytochrome c release, and caspase-3 activation in the lower extremities, leading to cellular apoptosis [26].

Combination treatments strategy relies on the induction and regulation of multiple treatments entities to exploit additive or synergistic effects and enhance the efficiency of cells reactions. Combination chemotherapy refers to the grouping of multiple chemotherapeutic agents that use different mechanisms to treat cancer. The combination strategy not only enhances therapeutic efficiency, but also reduces the risk of severe side effects caused by cytotoxicity of individual drugs [27,28].

Conclusion

Considering the results of the current study, it could be stated that dual treatment with VEGF siRNA and Avastin have a more significant impact on the inhibition of cell growth and migration, and considering the high cost of Avastin and efficacy of siRNA in reducing the gene expression of VEGF, combination treatment of cells with lower effective concentrations, can be assessed as a more efficient approach in in vivo studies and in case these results are replicable, it can be used as an effective therapeutic approach.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Ethical approval: The study protocol was approved by the Research Ethics Committee at National institute of Genetic Engineering and Biotechnology (Tehran-Iran).

Author contributions: AD designed research, wrote the paper, conducted review and editing; BG preformed the experimental work and analysis. All authors read and approved their specific contributions.

Data availability statement: The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials

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