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L-arginine/5-fluorouracil combination treatment approaches cells selectively: Rescuing endothelial cells while killing MDA-MB-468 breast cancer cells

Mozhgan Jahani[a,b], Mehri Azadbakht[b,**], Hassan Rasouli[a], Reza Yarani[c,a], Davood Rezazadeh[a], Nader Salari[d], Kamran Mansouri[a,*]

[a] Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran
[b] Department of Biology, Faculty of Basic Sciences, Razi University, Kermanshah, Iran
[c] Copenhagen Diabetes Research Center, Department of Pediatrics, Herlev and Gentofte Hospitals, University of Copenhagen, Denmark
[d] Department of Biostatistics and Epidemiology, School of Public Health, Kermanshah University of Medical Sciences, Kermanshah, Iran

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ABSTRACT

Reducing the adverse effects of chemotherapy on normal cells such as endothelial cells is a determinant factor of treatment success especially in pregnant women. In this regard, modulatory effect of L-arginine on various cancers is still a controversial topic in cancer therapy. So, this study aimed to compare the effect of L-arginine treatment alone and in combination with 5-fluorouracil (5-FU) on the survival and angiogenesis of primary human umbilical vein endothelial cells (HUVECs) and the breast cancer cell line of MDA-MB-468.

Combinations of L-arginine and 5-FU increased cell survival in HUVECs but induced cell death in MDA-MB-468 cells. Nitric oxide assay showed an increase of this molecule in both cell lines. Assessments of metabolic changes as well as molecular docking indicated a decrease in glycolytic activity of cancer cells but not normal cells. Angiogenesis induction in HUVECs was confirmed through *VEGF* and *MMP-2,9* up-regulated gene expressions. However, a down-regulation of the above-mentioned genes expression was observed in MDA-MB-468. Furthermore, an *in vivo* increased angiogenesis and decreased embryo toxicity was observed in combination treatment. Altogether, these findings clearly suggest that L-arginine inhibits cell death induced by 5-FU in HUVECs through attenuating the adverse effects of 5-FU, while it does not do so in breast cancer cells.

1. Introduction

Angiogenesis is the formation of new blood vessels from previous ones. This phenomenon is vital to many physiological and pathological processes such as embryonic development and tumor growth, respectively (Bhat and Singh, 2008; Bielenberg and Zetter, 2015; Karamysheva, 2008). Regarding cancer treatment, chemotherapy is one of the most widely used therapy choices (Kateb et al., 2011a). However, a decisive factor in treatment success is reducing the chemotherapy side effects on the normal cells including endothelial cells (Moding et al., 2013; Saini et al., 2012).

Endothelial dysfunction and the subsequent vascular toxicity following chemotherapy result in a non-compensatory damage in vascular formation and function. Hypertension, thrombosis, and atherogenesis

Abbreviations: AC-COA, Acetyl coenzyme A; Arg, arginine; Asuc, argininosuccinate; CAM, chick chorioallantoic membrane; Cit, citrate; Citr, citrulline; CPT1, carnitine palmitoyl transferase 1; DCF, 2',7'– dichlorofluorescin; DCFDA, 2',7'– dichlorofluorescein diacetate; DMEM, Dulbecco's Modified Eagle's Medium; EGFR, epidermal growth factor receptor; FCS, Fetal Calf Serum; F6P, fructose 6- phosphate; F1,6P, fructose 1,6-bisphosphate; 5-FU, 5-fluorouracil; GA3P, glyceraldehyde3-phosphate; G6P, glucose 6- phosphate; Glut, glucose transporter; HIF, hypoxia-inducible factor; HK, hexokinase; HUVEC, human umbilical vein endothelial cell; LAT, lateral amino acid transporter; LDH, lactate dehydrogenase; MMP2,9, matrix metalloproteinases 2,9; mTOR, mechanistic target of rapamycin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; OAA, oxaloacetate; Orn, ornithine; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; PEP, phosphoenolpyruvate; 3 PG, 3-phosphoglycerate; PHD2, prolyl hydroxylase domain-containing protein 2; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species; Suc, succinate; TCA, tricarboxylic acid; VEGF, vascular endothelial growth factor

* Corresponding author. Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, 67147-55687, Iran.
** Corresponding author. Department of Biology, Faculty of Basic Sciences, Razi University, Kermanshah, 67149-67346, Iran.
E-mail addresses: azadbakhtm_tmu@yahoo.com (M. Azadbakht), kmansouri@kums.ac.ir (K. Mansouri).

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may also occur under this condition (Curigliano et al., 2016; Focaccetti et al., 2015). Accordingly, chemotherapy effects on the embryonic development should be considered more seriously during cancer therapy of pregnant women.

Cancer incidence during pregnancy is rare with an estimation of about one case in 1000 pregnancies. Breast cancer is diagnosed in about 1–2 per 10.000 pregnancies and the incidence may rise as women delay childbearing. Cancer therapy is also challenging during pregnancy; as depending on the stage of the pregnancy, chemotherapy unfavorably impacts the development and health conditions of the fetus if it passes through the placenta (Ngu and Ngan, 2016). In the first trimester of pregnancy, during which developing processes and organogenesis occur, the fetus is most sensitive to harmful effects of chemotherapeutic agents (Abdalla et al., 2017; Ngu and Ngan, 2016). 5-FU, as an antimetabolite chemotherapeutic drug, has been shown to inhibit angiogenesis by affecting the viability and proliferation of endothelial cells (Focaccetti et al., 2015; Zhang et al., 2010a). So, during fetal development in pregnancy, embryonic angiogenesis could be negatively affected following 5-FU treatment. Hence, it must be used with extra caution and care in pregnant patients due to its teratogenic effects (Dekrem et al., 2013).

L-arginine on the other hand, is a vital precursor for the synthesis of important molecules including polyamines, creatine, proline, ornithine and nitric oxide (NO). Many metabolic pathways important for reproduction, growth, and health are regulated by L-arginine (Wu et al., 2009). This amino acid is commonly prescribed during pregnancy to protect both maternal and fetal health (Chen et al., 2016). NO, as one of its important derivatives, plays a crucial role in normal vessel function (Tousoulis et al., 2012). Nitric oxide has a main function in placental growth and angiogenesis through increasing uterine and placental-fetal blood flow (Krause et al., 2011). Moreover, this molecule is a signal transducer and cellular messenger in homeostasis and host defense mechanisms (Sobrevia et al., 2015). Depending on its concentration, NO is a key modulator of cell proliferation, cell cycle arrest, and apoptosis. Several studies have shown that a relatively low concentration of NO could lead to cell proliferation and anti-apoptotic responses. On the other hand, at high concentrations, it could result in cell cycle arrest, mitochondrial respiration, and finally, apoptosis (He et al., 2014; Napoli et al., 2013; Vander Heiden, 2011). Thus, the positive effect of the L-arginine and its derivative, NO, in cancer treatment is plausible. So that, increasing cell death of the breast cancer cell lines using L-arginine has been indicated by previous study (Jahani et al., 2017). However, its impacts on reducing adverse effect of the chemotherapy drugs on normal cells have not been determined until now.

Designing cancer treatments based on the differences between cancerous cells and normal cells could be of great use since chemotherapy negatively affects both. One of these basic differences is observed in their varying metabolic pathways (DeBerardinis and Chandel, 2016). Therefore, antitumor therapies have been developed based on this metabolic varieties between normal and cancerous cells (Vernieri et al., 2016). Most cancer cells are dependent on glycolysis instead of oxidative phosphorylation (OXPHOS) in order to meet their energy demands. Thus, suppressing glycolytic activity in tumor cells could be effective in cancer therapy (DeBerardinis and Chandel, 2016). However, there is still limited information on the L-arginine (NO) effect on the cell metabolism and its modulatory effect on various cancers has remained a controversial issue. So, herein, we investigated angiogenic and metabolic responses of HUVECs as normal cells and MDA-MB-468 as a triple negative breast cancer (TNBC) cell (TNBC tends to be more aggressive and metastatic than other breast cancer cell) to L-arginine alone and in combination with 5-FU.

2. Materials and methods

Cell culture reagents including Dulbecco's Modified Eagle's Medium (DMEM), Fetal Calf Serum (FCS), and trypsin–EDTA were purchased from Invitrogen (Life Technologies, Gaithersburg, MD, USA). Flasks and other cell culture dishes were from Orange Scientific (Braine l'Alleud, Belgium). 5-FU and L-arginine were purchased from Sigma-Aldrich (Taufkirchen, Germany). Real-time PCR and cDNA synthesis kits were obtained from $EURE_X$ ($EURE_X$ co, Poland); all other materials were obtained from Sigma-Aldrich (Taufkirchen, Germany) and Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.1. Cells and culture conditions

Primary human umbilical vein endothelial cells (HUVECs) were isolated as described previously (Baudin et al., 2007), and a human breast cancer cell line (MDA-MB-468), as the model cancer cells, was purchased from Pasteur Institute, Iran. The cells were grown at 37 °C in a humidified atmosphere of 5% CO2 in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin antibiotics. Cells were periodically analyzed for contamination with a mycoplasma detection kit and kept in culture for less than four months. In some experiments (such as NO assay, HK, glucose, lactate and ROS assessment) data normalization was done on the basis of the supernatant or cell lysate total protein determined by Bradford method.

2.2. Cell viability assay

HUVECs and MDA-MB-468 cells were seeded in a 96-well tissue culture plate with a cell density of 2×10^3 cells/well. Then, 5-FU and L-arginine were added separately to the cells at the concentrations of 15, 30, 45, 60, 90, and 190 μM and 1, 2, 4, 8, 16, and 32 mM, respectively, according to the previous study (Focaccetti et al., 2015; Jahani et al., 2017). Doses of L-arginine and 5-FU were chosen based on cell viability. The two highest doses of 5-FU that caused the highest toxicity and three doses of L-arginine that increased the viability of HUVECs but not MDA-MB-468 were chosen. Finally, in the combination treatment, 4, 8 and 16 mM of L-arginine and 90 μM of 5-FU were used, both separately and as a mixture for further experiments. All concentrations were tested in triplicate, and results were gathered from three sets of independent experiments. After incubation for 48 h (37 °C and 5% CO_2), 20 μl of 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) solution (5 mg/ml in PBS) was added to each well and cells were re-incubated for 4 h at 37 °C. To dissolve formazan crystals formed, MTT-containing supernatants were removed and replaced by 100 μl of dimethyl sulfoxide (DMSO), and plates were kept at room temperature for 30 min. Lastly, the absorbance was read at 570 nm with the background subtraction of 630 nm using a plate reader (Space fax 2100, Awareness, USA).

2.3. NO assay

From each sample, 400 μl of supernatant was deproteinized using 6 mg of zinc sulphate. The samples were centrifuged at 4 °C and 12000 g for 12 min, and then 100 μl of each sample was transferred into each well of the microplate. Then, 100 μl of vanadium chloride, 50 μl of sulfanilamide, and 50 μl of N-(1-naphthyl) ethylene diamine hydrochloride were added to the wells. The microplate was then incubated for 15 min at 37 °C and the optical density (OD) was recorded at 540 nm with background subtraction of 630 nm using a microplate reader (Space fax 2100, Awareness, USA).

2.4. Glycolytic activity assay

2.4.1. Hexokinase activity assay

Hexokinase (HK) is the first and the rate limiting enzyme in glycolysis pathway (Hay, 2016). Hexokinase activity assay was performed using a specific assay kit (Greiner, Germany) in accordance with the manufacturer's protocol. Briefly, cells were seeded at the density of

5×10^4 cells/well in a 24-well tissue culture plate. After 24 h post drug treatment, cell lysate was used for enzyme activity assay. HK activity was measured spectrophotometrically through NAD^+ reduction to NADH as a colored product with intense absorbance at 340 nm.

2.4.2. Glucose uptake and lactate production assays

The phenomenon of high glucose uptake and lactate accumulation by cancer cells, even under normoxic conditions (Warburg Effect), is a well-known common characteristic of these cells (Hirschhaeuser et al., 2011). Accordingly, cells were seeded in 24-well tissue culture plates with a cell density of 5×10^4 cells/well and after 24 h, the medium was removed and the glucose and lactate concentrations were measured by specific colorimetric kits (Greiner, Germany) according to the manufacturer's instruction. All experiments were conducted in triplicate.

2.4.3. Determination of intracellular ROS

Intracellular ROS level was assessed using 2',7'– dichlorofluorescein diacetate (DCFDA). DCFDA is a flourogenic dye deacetylated by cellular esterases to a non-fluorescent compound. Oxidization of this compound by ROS converts it into a highly fluorescent compound, 2', 7' –dichlorofluorescein (DCF), resulting in a green fluorescence. The level of ROS was determined by measuring the intensity of the emitted fluorescence (Shokoohinia et al., 2015). The cells were washed with PBS buffer (pH 7.4) 24 h after being treated with both drugs. Thereafter, cells were incubated with 20 μl DCFDA at 37 °C for 30 min. Then, cells were lysed with Triton X-100 and fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 528 nm using a fluorescence microplate reader (BioTek, H1M, USA).

2.5. Three-dimensional angiogenesis assay

Three-dimensional angiogenesis assay was performed as described by Mansouri et el (Mansouri et al., 2015). In this experiment, passages 3–5 of HUVECs were used. Cytodex 3 microcarrier beads (Amersham Pharmacia Biotech) were prepared according to the manufacturer's instructions. HUVECs were mixed with the cytodex 3 microcarrier beads by mildly shaking the cell suspension and microcarriers every 20 min in DMEM supplemented with 10% FCS for 4 h at 37 °C and 5% CO_2. Thereafter, the cell suspension with microcarriers was transferred to a 24-well tissue culture plate and incubated for 12–16 h under the same conditions. On the following day, the cell-incorporated beads were mixed with a collagen solution (collagen type I, 10X DMEM, 23 mg/ml $NaHCO_3$ and FCS with a ratio of 7.5:1:1:0.5, respectively) on ice, and 50 μl of the solution was added to each well of the 96-well plate and allowed to rigidify in the incubator for 20 min at 37 °C and 5% CO_2. Afterwards, 250 μl of DMEM with different concentrations of L-arginine and 5-FU was added to each well. The effects of both L-arginine and 5-FU on angiogenesis and sprout formation were investigated using Angio Sys 2.0 Image Analysis Software (TCS Cell works, Buckingham, UK), which presents the mean number of sprouts in 20 beads for each treatment. Herein, we reported the percentage of sprout formation after two days of treatment compared to the control (non treatment: NT).

2.6. Chick embryo chorioallantoic membrane (CAM) assay

The effects of L-arginine and 5-FU on angiogenesis were assessed *in vivo* using the CAM assay. The fertilized eggs used in this experiment were stored in a humidified incubator at 37 °C. After incubation for three days, the chorioallantoic membrane was detached from the yolk sac using an 18-gauge hypodermic needle to draw 0.5–1 ml of albumin through a small puncture drilled at the narrow end of the eggs. A small window was created on the shell on the 7th day of chick embryo development under sterile conditions. Thereafter, different concentrations of L-arginine and 5-FU were placed on top of the egg vessels using filter discs containing 10 μl of each drug. After re-incubation of eggs in the incubator for 48 h, the rate of angiogenesis following the L-arginine and 5-FU treatment was investigated macroscopically around the discs. The images were recorded with a loop microscope (SMZ745T; Nikon, Tokyo, Japan). All images were processed in Adobe Photoshop 6.0. The SPIP software (version 6.2.5) was used to measure the 3D surface roughness parameter along with all related parameters to evaluate the angiogenic effect of L-arginine alone and in combination with 5-FU. Blood vessel densities and the angiogenic activity of the samples were determined using the following formula: Angiogenic activity (%) = [(CAM area of control – CAM area of sample)/(CAM area of control)] × 100. For the evaluation of L-arginine effect on reducing 5-FU-induced embryo toxicity, embryos in different drug-treated groups were weighed on the 21stday of development and compared with control group. All assessments were repeated three times and each treated group was comprised of nine eggs.

2.7. Real-time PCR

Total RNA extraction was performed by RNAX-Plus (Cinaclon, Iran) (after 18 h of incubating HUVECs and MDA-MB-468 treated with L-arginine and 5-FU at 37 °C, 5% CO_2 and 80% humidity). cDNA synthesis from RNAs was done using $EURE_X$ kit according to its instruction. *VEGF* and *MMP-2,*9 mRNA levels were assessed with SYBR Green I and amplified with the Rotor Gene 6000 system (Corbett Research, Australia) by real time PCR. Beta-actin (β-actin) was used as a reference gene for normalization.

2.8. Molecular docking

2.8.1. Receptor and ligand preparation

The crystal structure of human hexokinase II (PDB ID: 1V4S (Kamata et al., 2004)) was obtained from protein data bank (http://www.rcsb. org/pdb). Also, the 3D structure of L-arginine (PubChem CID: 6322) was downloaded from PubChem database (https://pubchem.ncbi.nlm.nih.gov/). In order to get the most stable geometry, these structures (receptors and ligands) were optimized by employing the Becke three-parameter Lee-Yang-Parr (B3LYP) hybrid density functional theory at the 6–31 G** basis set using quantum chemistry software Gaussian 03 W. Finally, all water molecules and unknown atoms were removed from receptors and the output results were saved in pdb format.

2.8.2. Docking procedure

Molecular docking calculations (blind docking) were performed by Auto Dock 4.2 program package using the Auto Dock empirical free energy function and the Lamarckian genetic algorithm with local search. Primarily, water molecules were removed from initial structure of both receptors and ligands and then missing hydrogens and Gasteiger charges were added to the system during the preparation of the receptor input file. AutoDock Tools was used for the preparation of coordinate files of ligands and proteins (PDBQT). Afterwards, pre-calculation of grid maps was performed using Auto Grid. The docking calculation was done by locating a grid map with $60 \times 60 \times 60$ Å^3 points and a grid-point spacing of 0.375 Å which was centered on the receptor. The number of independent docking runs performed for each docking simulation was set to 200 with 25,000,000 energy evaluations for each run. The default values of the program were used for other docking parameters.

2.9. Statistical analysis

All statistical analysis was done using SPSS (SPSS software, version 24.0) and graphs were plotted using Graph Pad Prism 5 software (Graph Pad Software, version 5.0, Inc., La Jolla, CA, USA). The statistical significance of experimental data was determined using analysis of variance (ANOVA) followed by the Tukey's test.A P-value < 0.05 was considered to be statistically significant. Each point or column

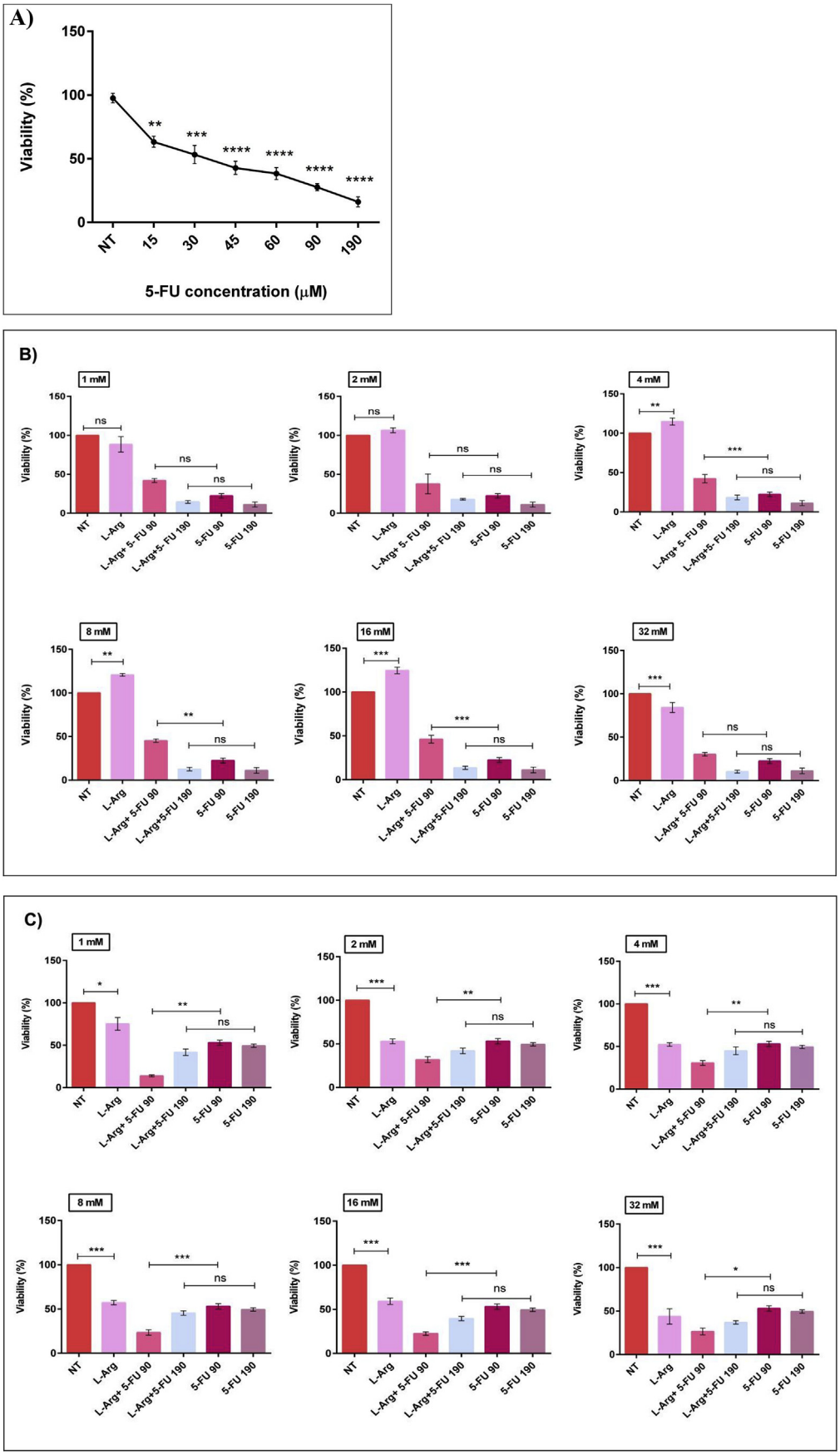

Fig. 1. (A) Effects of different concentrations of 5-fluorouracil on HUVECs viability 48 h after treatment. (B), (C) Effects of L-arginine and 5-fluorouracil on HUVECs and MDA-MB-468 viability, respectively, at 1 (a), 2 (b), 4 (c), 8 (d), 16 (e), and 32 (f) mM of L-arginine (L-Arg) alone and in combination with 90 and 190 μM concentrations of 5-fluorouracil after 48 h (L-Arg + 5-FU 90, 5-FU 190).Data are presented as mean ± SD from 3 to 5 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: nonsignificant.

represents the mean ± SD. (n = 3–5); p < 0.05 (*), p < 0.01 (**), p < 0.001(***), p < 0.0001(****).

3. Results

3.1. The effect of L-arginine and 5-FU on the viability of HUVECs

Cell viability was assessed using MTT assay. The results showed a dose-dependent decrease in the viability of HUVECs after treatment with 5-FU. The maximum cell death occurred at concentrations of 90 and 190 μM (Fig. 1A). Therefore, these concentrations were selected as the optimal doses for combination treatment (5-FU and L-arginine). As it is shown in Fig. 1B, L-arginine alone and in combination with 90 μM of 5-FU could increase HUVECs viability, while it showed no such effect at 190 μM of 5-FU. Other concentrations of 4 (c), 8 (d) and 16 (e) mM of L-arginine resulted in a significant increase in HUVECs survival; however, no significant increase was observed in cell viability at 1 (a) and 2 (b) mM of this amino acid. Nonetheless, the highest concentration of L-arginine (32 (f) mM) led to HUVECs death. Therefore, 4, 8, and 16 mM of L-arginine and 90 μM of 5-FU were selected for our further tests.

3.2. The effect of L-arginine and 5-FU on the viability of MDA-MB-468

In contrast to HUVECs, the results of MTT assay for MDA-MB-468 cell line showed an decrease cell viability compared to the controls at all L-arginine concentrations. Treatment with a combination of L-arginine (in all its concentrations) and 5-FU resulted in a significant reduction of cell survival and cell viability in comparison to 5-FU treatment alone (Fig. 1C).

3.3. The effect of L-arginine alone and in combination with 5-FU on NO production

5-FU reduced the production of NO from HUVECs, while different concentrations of L-arginine significantly increased NO secretion in a dose-dependent manner compared to the control. The combination treatment resulted in a significant increase in NO production in contrast to 5-FU treatment alone (Fig. 2A). Furthermore, NO production was increased significantly by using L-arginine in MDA-MB-468 in all tested concentrations. Using 5-FU resulted in no significant increase in NO production from MDA-MB-468, whereas the NO concentration was noticeably increased in treatment with L-arginine/5-FU combination except for 4 mM of this amino acid (Fig. 2B).

3.4. The effect of L-arginine alone and in combination with 5-FU on the glycolytic activity of cells

3.4.1. Hexokinase activity

HK activity in the HUVECs was decreased in the low concentrations of L-arginine. This is while increasing the concentration of L-arginine increased HK enzyme activity to the controls level. In contrast, enzyme activity in MDA-MB-468 was significantly decreased in both low and high concentrations of L-arginine compared to the controls (Fig. 3A).

3.4.2. Glucose uptake and lactate level

Cell supernatants were used to check the glucose uptake and lactate production in HUVECs and MDA-MB-468 cells under L-arginine and 5-FU treatment. The results showed a decrease in both glucose uptake (indicated by the amount of glucose in the cell supernatant) and lactate production in MDA-MB-468. L-arginine of all concentrations, either alone or in combination with 5-FU, significantly decreased the glucose uptake in MDA-MB-468 compared to both the control and 5-FU treated cells, suggesting its inhibitory role in cancer glycolysis. In contrast to MDA-MB-468, the changes in glucose concentration were not significant in HUVECs except at 8 and 16 mM of L-arginine treatment alone and 16 mM of this amino acid in combination with 5-FU. Furthermore, lactate level in HUVECs was significantly decreased only when cells were treated with 8 and 16 mM of L-arginine combined with 5-FU (Figs. 3B and 4A).

3.4.3. ROS production within the cells

The level of ROS production by HUVECs and MDA-MB-468 was determined using DCFDA. Except for 32 mM of L-arginine, the amount of ROS produced by HUVECs was insignificantly increased in all concentrations compared to the controls. Furthermore, combination treatment resulted in a significant decrease of ROS level in endothelial cells in contrast to 5-FU treatment. Regarding the ROS production in MDA-MB-468, results indicated a great increase in ROS levels after administration of L-arginine alone and in combination with 5-FU (except for L-Arg 4 and L-Arg 8 mM + 5-FU) compared to the controls and 5-FU treatment alone, respectively (Fig. 4B).

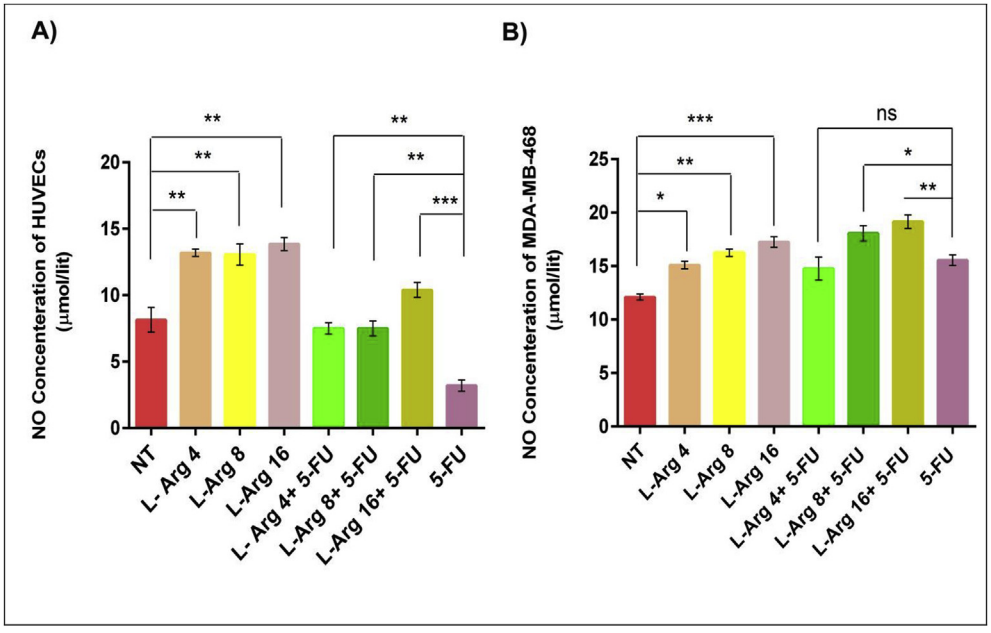

Fig. 2. L-arginine effects on HUVECs and MDA-MB-468 NO production. (A) Increase of nitric oxide production in HUVECs and (B) MDA-MB-468 in the presence of various L-arginine concentrations alone (L-Arg 4, L-Arg 8 and L-Arg 16 are denoted as 4, 8, and 16 mM concentrations of L-arginine, respectively) and in combination with 90 μM of 5-fluorouracil was assessed 24 h after treatment (L-Arg 4, L-Arg 8, and L-Arg 16 + 5-FU). Data are presented as mean ± SD from 3 to 5 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ns: nonsignificant.

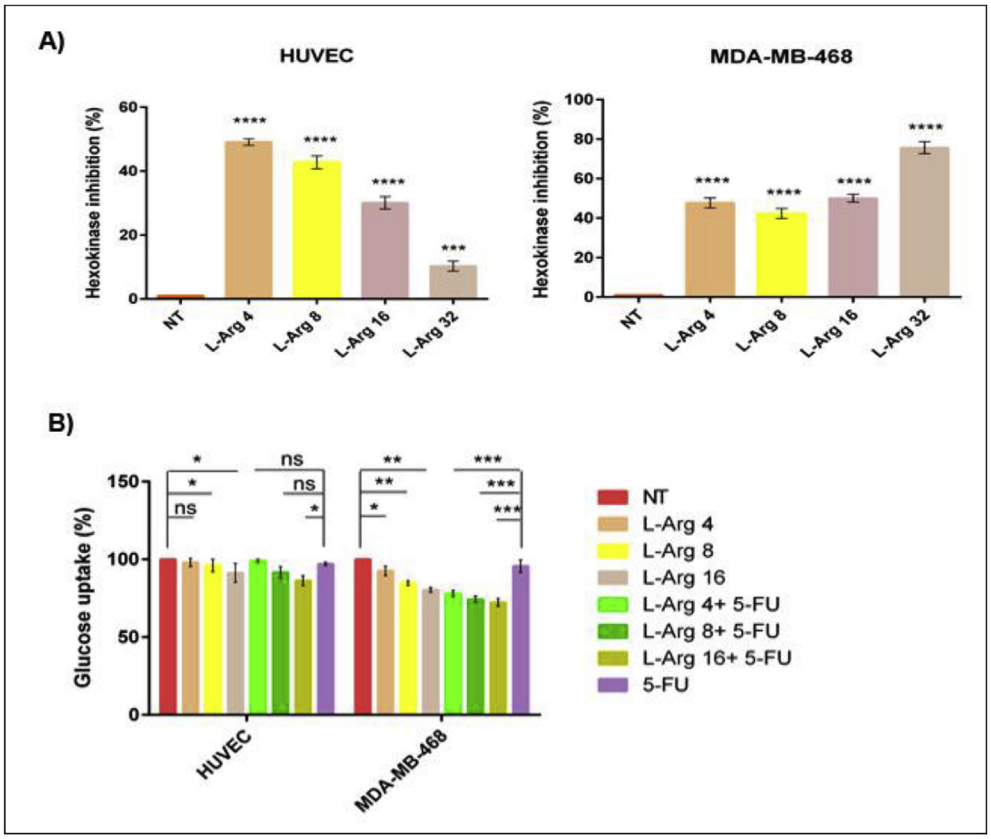

Fig. 3. L-arginine effects on the hexokinase (HK) activity and glucose uptake in HUVECs and MDA-MB-468. (A) After 24 h drug treatment cell lysate was used for HK activity assay in different concentrations of L-arginine alone (L-Arg 4, L-Arg 8 and L-Arg 16 are denoted as 4, 8, and 16 mM concentrations of L-arginine, respectively) and in combination with 90 μM of 5-fluorouracil (L-Arg 4, L-Arg 8, and L-Arg 16 + 5-FU). (B) Glucose uptake was assessed using cell supernatant. Cells were seeded in 24-well tissue culture plates with a cell density of 5×10^4 cells/well. 24 h after treatment, the medium was removed and the glucose uptake was measured by specific colorimetric kits. Data are presented as mean ± SD from 3 to 5 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: nonsignificant.

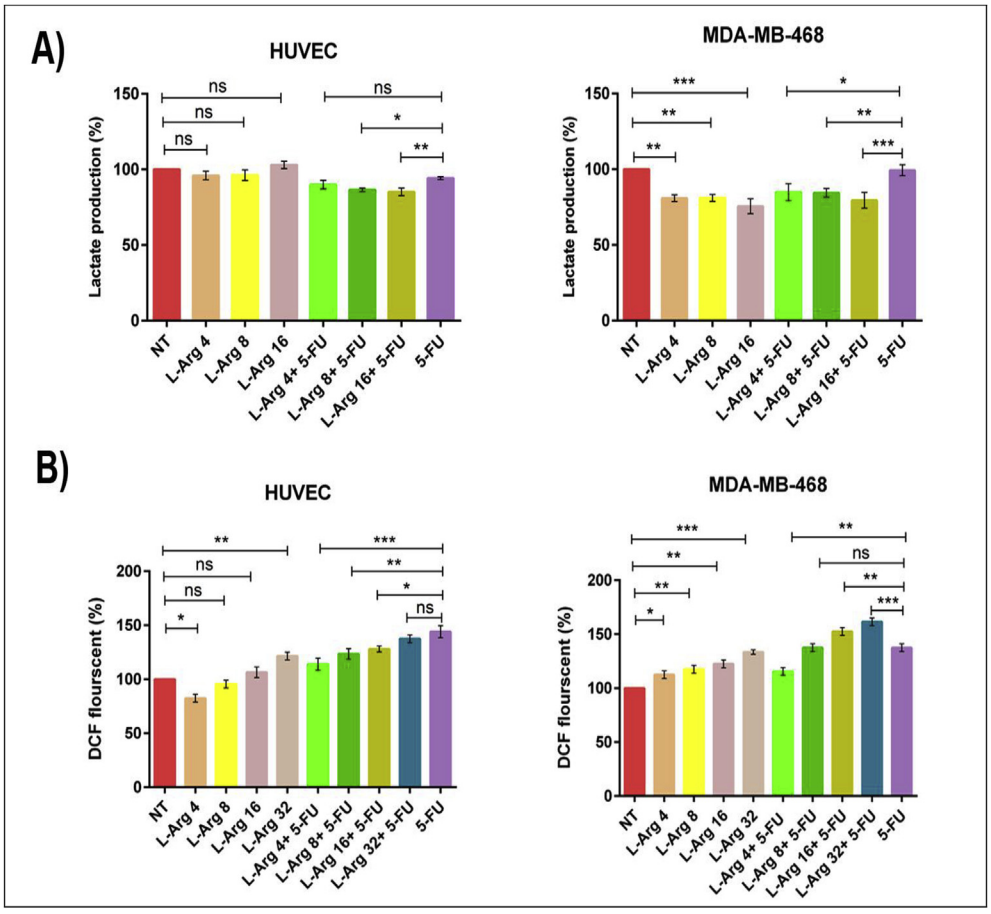

Fig. 4. L-arginine effects on the (A) lactate production and (B) ROS level in HUVECs and MDA-MB-468. Effects of L-arginine alone (L-Arg 4, L-Arg 8 and L-Arg 16 are denoted as 4, 8, and 16 mM concentrations of L-arginine, respectively) and in combination with 90 μM of 5-fluorouracil (L-Arg 4, L-Arg 8, L-Arg 16 + 5-FU) on the lactate production and ROS level was assessed using cells supernatant and DCFDA respectively 24 h after treatment. Data are presented as mean ± SD from 3 to 5 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ns: nonsignificant.

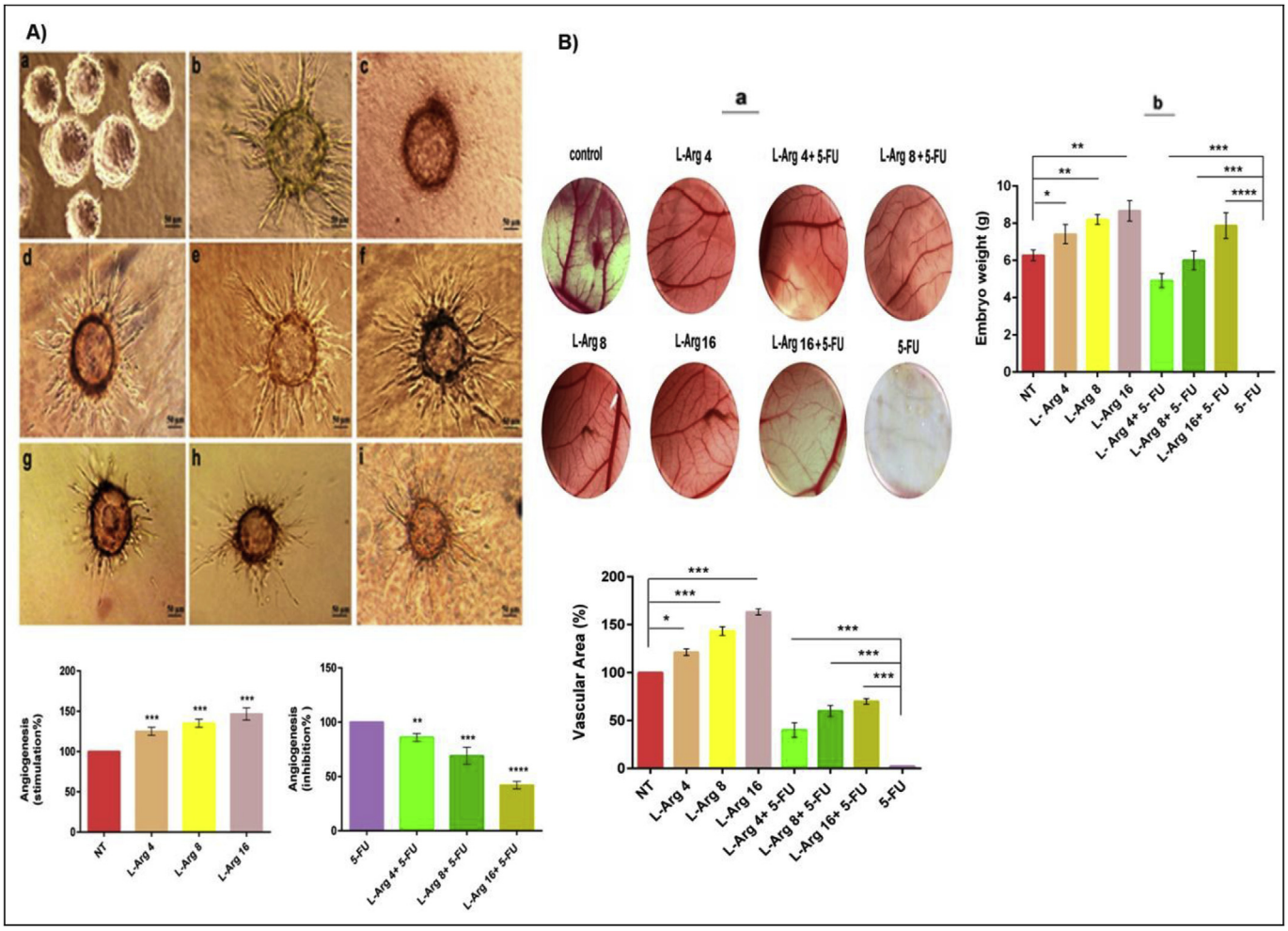

Fig. 5. Effects of different concentrations of L-arginine on *in vitro* and *in vivo* angiogenesis as well as embryonic weight. (A) Angiogenesis of HUVECs mixed with cytodex 3 microcarrier beads (a) was increased by L-arginine alone (L-Arg 4 (d), L-Arg 8 (e), and L-Arg 16 (f), are denoted as 4 8, and 16 mM concentrations of L-arginine, respectively) compared with control (NT) (b). Furthermore, increased angiogenesis occurred in combination treatments of L-arginine with 90 μM of 5-fluorouracil (L-Arg 4 (g), L-Arg 8 (h), L-Arg 16 (i) + 5-FU (c)) as compared to 5-FU. (B) In the *in vivo* angiogenesis assay (48 h after drugs treatment), L-arginine caused angiogenesis promotion at all concentrations (L-Arg 4, L-Arg 8 and L-Arg 16 are denoted as 4, 8, and 16 mM concentrations of L-arginine, respectively) compared to the control (NT) group. 5-fluorouracil (90 μM) inhibited sprout formation, and, in combination with L-arginine (L-Arg 4, L-Arg 8, L-Arg 16 + 5-FU), micro vessel density increased as compared to this chemotherapy medication (a). Furthermore, the embryonic growth and embryonic weight were improved in comparison to the control (NT) by increasing the concentration of L-arginine alone in the range of 4–16 mM, and when L-arginine is combined with 5-fluorouracil as compared to the control (NT) and 5-fluorouracil treatments respectively (b). Data are presented as mean ± SD from 3 to 5 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: nonsignificant.

3.5. The effect of L-arginine treatment alone and in combination with 5-FU on angiogenesis in a three-dimensional in vitro model

The angiogenic activity of HUVECs was increased by L-arginine in a dose-dependent manner compared to the control (b); whereas it was completely inhibited by 5-FU (c). Therefore, treatment with L-arginine at the concentrations of 4 (d), 8 (e), and 16 (f) mM increased angiogenesis by 20, 25, and 35%, respectively in comparison to the control. Combination treatment resulted in an increased angiogenesis (as indicated by an increase in sprout formation) compared to 5-FU. The number of formed sprouts was also increased by 11, 23, and 31.5% at 4 (g), 8 (h), and 16 (i) mM of L-arginine concentrations, respectively (Fig. 5A).

3.6. The effect of L-arginine treatment alone and in combination with 5-FU on the angiogenesis and embryo weight in a chick chorioallantoic membrane (CAM) in vivo model

In this assay, endothelial sprout formation was assessed after nine days, and the angiogenic responses were evaluated both in test and control groups. Obtained results suggest that L-arginine has an increasing, concentration-dependent effect on *in vivo* angiogenesis in the CAM model (25, 43 and 63% increase in 4, 8 and 16 mM of L-arginine alone, respectively). 5-FU showed a significant inhibitory effect on the sprout branching. However, as it is shown in Fig. 5B (a) for combination treatments, the endothelial sprouts were formed and their numbers increased by 30, 40, and 60% upon increasing L-arginine concentrations to 16 mM compared to the 5-FU treatment alone. The embryo weight results showed that L-arginine can fully inhibit the 5-FU embryo toxicity which results in a higher weight of the embryos treated with L-arginine alone or in combination with 5-FU compared to that of controls and embryos treated with only 5-FU (Fig. 5B (b)).

3.7. The effect of L-arginine alone and in combination with 5-FU on VEGF, MMP-2, 9 mRNA levels in HUVECs

Compared to the control, significant increases were observed in the expression of *VEGF* and *MMP-2* when HUVECs were treated with L-

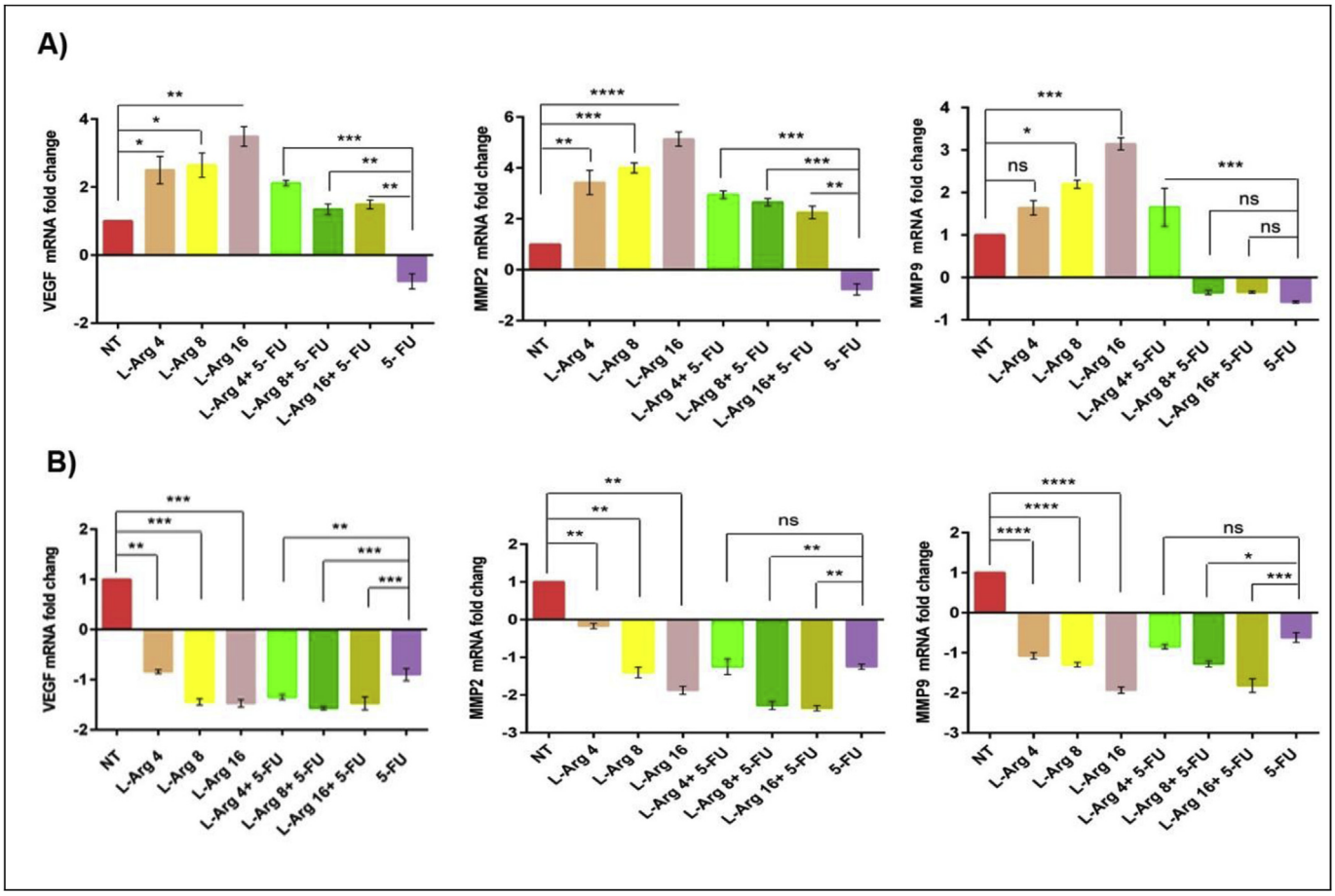

Fig. 6. Vascular endothelial growth factor, matrix metalloproteinase-2,9 mRNA levels in HUVECs and MDA-MB-468 was assessed 24 h after treatment using real time PCR. (A) vascular endothelial growth factor, matrix metalloproteinase-2,9 mRNA levels in the HUVECs increased in the presence of different concentrations of L-arginine alone (L-Arg 4, L-Arg 8 and L-Arg 16, are denoted as 4, 8, and 16 mM concentrations of L-arginine respectively) and in combination with 90 μM of 5-fluorouracil (L-Arg 4, L-Arg 8, and L-Arg 16 + 5-FU) compared to 5-FU alone. (B) However, the same genes showed down-regulation in MDA-MB-468 upon treatment with both L-arginine and 5-fluorouracil alone and in combination. Fold change was normalized against Beta-actin and was compared to control (NT). Data are presented as mean ± SD from 3 to 5 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: nonsignificant.

arginine alone. In contrast, 5-FU treated cells showed a down-regulation in *VEGF* and *MMP-2* expression. On the other hand, the combination of L-arginine with 5-FU significantly up-regulated *VEGF* and *MMP-2* compared to 5-FU treatment alone. On the mRNA level, a non-significant increase in *MMP-9* expression was observed when cells were treated with 4 mM of L-arginine. Except for 4 mM of L-arginine combined with 5-FU, no significant increase was observed in the mRNA level of *MMP-9* in any other combination concentrations compared to 5-FU treatment alone (Fig. 6A).

3.8. The effect of L-arginine alone and in combination with 5-FU on VEGF, MMP-2,9 mRNA levels in MDA-MB-468

*VEGF, MMP-2,*9 mRNA levels decreased when MDA-MB-468 cells were treated with L-arginine alone in comparison to the control. Furthermore, the results of mRNA expression for these genes from combination treatment showed a significant decrease compared to 5-FU treatment alone. However, *MMP-2,9* expression levels did not show any notable change in MDA-MB-468 cells treated with 4 mM of L-arginine combined with 5-FU; while 5-FU treatment alone decreased their expression (Fig. 6B).

3.9. Molecular docking results

To evaluate the direct inhibitory effect of L-arginine on glycolysis enzymes, molecular docking analysis was performed. Studying the molecular docking of small molecules with selected receptors may facilitate the search for novel leading compounds suitable for developing drug studies. The HK is an important enzyme in the glycolytic pathway. In the first step of glycolysis, HK facilitates phosphorylation of glucose into glucose-6-phosphate.The active site residues for HK are Asp205, Lys169, Asn204, Glu256, and Thr168 (Kamata et al., 2004). These residues are located in the deep cleft at the interface between the two lobes of the molecule. This active site is capable of binding two ligands: glucose and glucose-6-phosphate (Kamata et al., 2004). Our molecular docking results showed a high affinity of L-arginine for binding to the active site of the HK protein (Fig. 7).

In addition to Lys169 and Asp205 residues located in the active site of HK, it was displayed that Asp78, Gly81, Ser151, and Asp 409 are also interacting with L-arginine significantly. This result clearly suggests that the interaction between L-arginine and the aforementioned residues in the HK active site may impair the interaction between this protein and glucose. Therefore, it could be concluded that L-arginine inhibits the enzyme's activity. Our experimental data confirmed the direct effect of L-arginine on glycolysis through the inhibition of HK active site which, in turn, results in a significant decrease in cancer cell glycolysis. In this study, the docking of L-arginine with other enzymes involved in the glycolytic pathway was also modeled. It was showed that this ligand can only significantly interact with HK (Table 1). Since HK is a critical enzyme in glycolysis, it is safe to say that L-arginine has

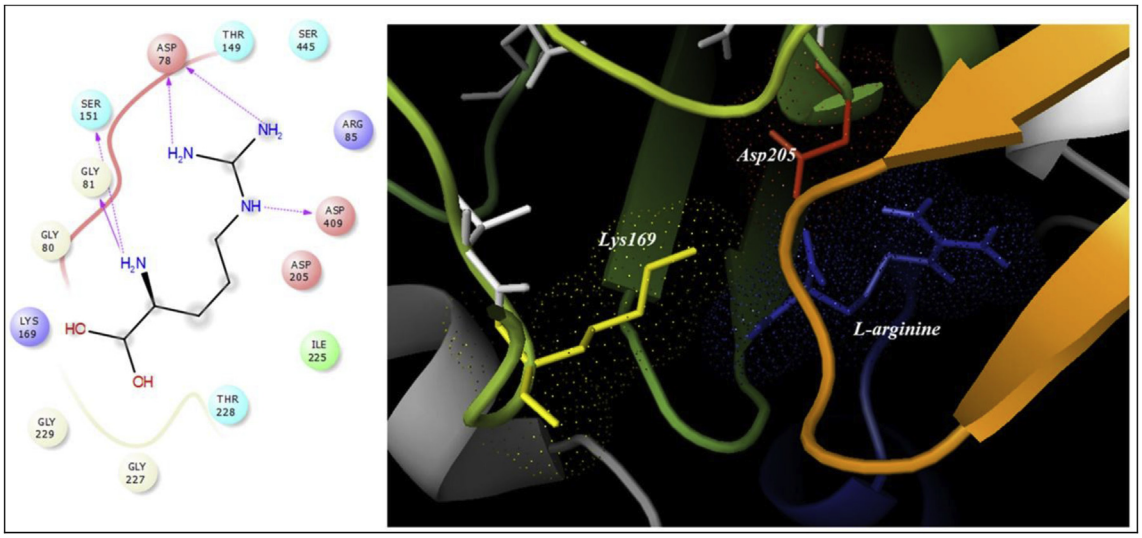

Fig. 7. A schematic illustration of the interaction between L-arginine and the active site of the hexokinase enzyme. The docking score for this interaction was −74.567 kcaL/mol. This figure was generated using the PyMOl software.

Table 1
Calculated binding affinity energies for docking L-arginine into various glycolysis enzymes.

Enzyme	Binding affinity energy (kcal/mol)
Hexokinase	−74.567
Phosphoglucose isomerase	−44.561
Phosphofructokinase	−56.234
Glyceraldehyde 3-phosphate dehydrogenase	−54.989

the obvious potential to inhibit HK and consequently prevent cancer cell proliferation.

4. Discussion

Reducing chemotherapy side effects and accordingly improving the patient's life has been a long time goal in cancer treatment (Saini et al., 2012). Affecting normal cells as well as cancerous ones and chemoresistance are considered the main complications faced during chemotherapy which is the most common type of cancer treatment (Housman et al., 2014).

Upon chemotherapy, endothelial cells, as the key components of angiogenesis (Franses et al., 2013; Karamysheva, 2008), are damaged and could cause cardiovascular disorders. Therefore, cardiotoxicity and endothelial dysfunction are observed in most cancer patients undergoing chemotherapy. This, in turn, results in a compromised quality of life and increased mortality rate due to cardiac problems (Curigliano et al., 2016). Furthermore, endothelial dysfunction can result in chemoresistance in cancer cells; as healthy endothelial cells can increase vascular integrity and decrease their leaky state as well as inducing chemo-sensitivity in cancer cells (Franses et al., 2013). More importantly, side effects of anticancer drugs on pregnant patients must be considered more carefully since saving mother's life with chemotherapy parallels threatening the life of the growing embryo (Abdalla et al., 2017). The use of chemotherapy drugs such as antimetabolites is known to inhibit the proliferation of highly proliferating cell types (Woods and Turchi, 2013); as 5-FU inhibits embryogenesis to varying degrees based on the time of exposure during pregnancy (Dekrem et al., 2013; Yamaguchi et al., 2009). The cardiovascular system and vascular network are among the first systems that develop during the early stages of embryonic development (Bruneau, 2008). In this case, the adverse effects of 5-FU on endothelial cells (anti-proliferative and anti-migratory) have been frequently demonstrated by different studies (Focaccetti et al., 2015; Zhang et al., 2010b). Therefore, the possibility of maldevelopment of the vascular system in the embryo following the use of 5-FU is fairly high. Accordingly, finding and employing safe treatment options for decreasing chemotherapy side effects on normal cells while increasing their antitumor activity could be of great value in cancer treatment, especially during pregnancy (Esposito et al., 2016).

In this study, we showed the effects of L-arginine, a drug often used for cardiac improvement and pregnant women health, on HUVECs, assigned as the normal cells, and MDA-MB-468, as a breast cancer cell line. Interestingly, L-arginine combined with 5-FU enhanced its therapeutic efficacy while simultaneously protected normal cells and, therefore, possibly the fetus from the side effects of this chemotherapy drug.

In cancer therapy, modulatory effect of L-arginine on various cancers has remained a controversial issue (Jahani et al., 2018). L-arginine impact on the cells is greatly linked to the type of metabolism path they adopt. Previous studies investigating the effects of L-arginine in cancer therapy present conflicting results. While some studies confirm that L-arginine enhances tumor growth (Feun et al., 2008; Patil et al., 2016), others introduce this amino acid as an appropriate candidate for cancer treatment (Cao et al., 2016; Yin et al., 2007). It has been shown that in addition to reducing cancer cell viability, L-arginine and its metabolites can evoke antitumor immune response by directly influencing T cells survival and their metabolic fitness (Geiger et al., 2016; Jahani et al., 2017). Moreover, some other studies suggest that L-arginine deprivation in cancer cells could be a good strategy in cancer treatment (Delage et al., 2010; Feun et al., 2008). However, according to Matthew Fletcher's study, L-arginine deprivation in cancer therapy inhibits antitumor responses of T cells by inducing myeloid-derived suppressor cells (MDSC) (Fletcher et al., 2015). Furthermore, L-arginine deprivation using enzymes such as arginine deiminase (ADI) may not be applicable in cancer therapy during pregnancy, as some of the important L-arginine anabolic enzymes have low expressions in the embryo (Köhler et al., 2008). In addition, it has been demonstrated that ADI inhibits capillary-like tube formation in HUVECs and may result in angiogenesis prevention in the embryo (Zhuo et al., 2011). Hence, L-arginine is considered an essential amino acid in embryo development.

As our MTT assay results demonstrated, treatment with 5-FU decreased cell viability in HUVECs as well as in MDA-MB-468 cells. Nevertheless, using L-arginine alone and in combination with 5-FU resulted in an increased cell viability along with cell proliferation in HUVECs and also an increase in cell death in MDA-MB-468. Our

findings showed that in combination therapy, L-arginine decreased the cytotoxic effects of 5-FU on HUVECs and simultaneously enhanced the cytotoxic effects of 5-FU on MDA-MB-468 cells.

L-arginine can exert different effects on cell metabolism through its different metabolites. NO and polyamines are two of the most important L-arginine derivatives generated by nitric oxide synthase (NOS) and arginase, respectively (Wu et al., 2009). NOS activity and NO production are very important characteristic processes in an endothelial cell. Moreover, arginase activity is associated with not only cancer progression but also endothelial dysfunction and results in the changes of fetoplacental endothelial function (Ivanenkov and Chufarova, 2014; Steppan et al., 2013).

Since arginase has a lower affinity for L-arginine compared to NOS, L-arginine of high concentrations is more likely to act as a substrate for NOS rather than arginase and therefore in such conditions, NO concentration will increase (Steppan et al., 2013). According to our results from NO concentration assay, 5-FU decreased NO production from endothelial cells while we observed a non-significant increase in the production of NO from 5-FU-treated MDA-MB-468 cells. On the other hand, treatment with different concentrations of L-arginine was associated with increased NO secretion in a dose-dependent manner compared to the control. Related results also showed that combination therapy significantly increased NO production in both cell lines compared to the 5-FU treatment.

According to previous studies, L-arginine)NO) can have a dual effect on cell viability and proliferation depending on its concentration (He et al., 2014; Napoli et al., 2013).

It has been shown that through NO production, L-arginine along with 5-FU inhibits liver tumor development in nude mice (Yin et al., 2007). A possible mechanism for the cell death induction by NO is that this molecule could affect the redox state of the cell via mitochondrial pathway. NO competes with oxygen (O_2 at low concentrations) for binding to cytochrome C oxidase in the electron-transport chain which results in cell death by subsequent ROS production in the mitochondria and the release of pro-apoptotic proteins (Sarti et al., 2012) (Fig. 8).

Increased sensitivity to oxidative stress in cancer cells could be a result of many phenomena, such as mitochondrial dysfunction, aberrant cell metabolism, and dysregulation of antioxidant enzymes (impaired antioxidant systems). However, normal cells are protected from oxidative damages owing to their intact antioxidant systems (DeBerardinis and Chandel, 2016). In the present study, results from ROS assay in HUVECs and MDA-MB-468 showed that the amount of these species reached the same level as the control in HUVECs after introducing L-arginine in the treatment. However, in contrast to HUVECs, ROS levels increased in the MDA-MB-468 compared to the control. In addition, combination treatment with both drugs decreased the ROS levels in the endothelial cells whereas it increased these levels in cancer cells in comparison to the 5-FU treatment alone. These results were consistent with those of ours from the MTT assay.

Different rate of cell proliferation and energy demands in normal cells and cancerous ones results in a significant difference between their energy metabolism (DeBerardinis and Chandel, 2016) (Fig. 8). In particular, cancer cells generally switch to aerobic glycolysis pathway rather than using glucose in the oxidative phosphorylation pathway, which can be a good target for cancer treatment (DuHadaway and Prendergast, 2016; Lehuédé et al., 2016). Therefore, inhibition of cancer cells metabolism through targeting the glycolytic pathway and its key enzymes is considered to be a promising strategy in cancer therapy. Vander Heidenet al. showed that metabolic pathway switch from glycolysis to OXPHOS induced by L-arginine administration can prevent Warburg effect (Vander Heiden et al., 2009). It has been suggested that the up-regulation of the serine biosynthesis is the mechanism underlying this metabolic change; although the main reason remains elusive (Possemato et al., 2011). Our docking analysis revealed that L-arginine can directly interact with glycolysis enzymes such as HK, the first rate limiting glycolytic enzyme in this pathway. Interaction of L-arginine with HK, in turn, inhibits enzymatic activity of HK which leads to altered glucose uptake and lactate production by the cells. Our data on HK enzymatic activity at high L-arginine-concentrations indicated a decreased activity of this enzyme in MDA-MB-468 cells whereas in HUVECs, the enzymatic activity returns to the control level. Furthermore, our results demonstrated a decreased glucose uptake and lactate production by cancer cells when L-arginine was used alone and in combination treatments compared to the control and 5-FU treated cells. These results support the hypothesis we developed from the docking analysis and is in line with Roger Geiger's study stating that increased L-arginine concentrations inhibits aerobic glycolysis in activated T cells (Geiger et al., 2016). However, it is noteworthy to mention that glycolysis pathway is the preferential pathway in some types of normal cells such as endothelial cells (especially in a high-rate proliferative state) (Polet and Feron, 2013). Nevertheless, according to glycolytic assay results in HUVECs, glycolytic activity changes were not significant in endothelial cells, except in some treatment groups in lactate and glucose assays. So, the important question here is how glycolysis inhibition results in killing cancer cells but not normal ones and what might happen to endothelial cells in a tumor microenvironment. A possible explanation is that after glycolysis inhibition by L-arginine, cancer cells can employ another pathway to provide their energy demands. Activation of TCA cycle, using glutaminolysis mechanism, and consuming other carbohydrates rather than glucose, are the common pathways among these cells (Altman et al., 2016; DeBerardinis et al., 2008). However, because of the elevated ROS production in the cells and the following adverse effects on the activity of the enzymes, including those in TCA cycle, the rate of energy production in these pathways may be disrupted after L-arginine administration (Schieber and Chandel, 2014). Furthermore, it is demonstrated that fructose and galactose, as the common substitute carbohydrates for glucose, cannot change the energy metabolism through glycolysis in cancer cells (Liu et al., 2010; Reitzer et al., 1979). Regarding the endothelial cells, it has been demonstrated that except under pathologic conditions (switching mostly to glycolytic pathway), TCA cycle could be used along with glycolysis under a physiologic condition (Polet and Feron, 2013). So, energy demands in endothelial cells (in physiologic state) could be met through glucose to fructose conversion along with the mechanisms mentioned earlier. Thereafter, fructose phosphorylation occurs by fructokinase activity that has a higher affinity for fructose than glucose. The phosphorylated fructose then enters the glycolysis pathway (Li et al., 2016; Liu et al., 2010).

Regarding the tumor endothelial cells response, a previous study has revealed that normal endothelial cells are different from tumor endothelial cells in both their behavior and gene profile. So, they may respond differently to drugs inhibiting a critical metabolic pathway of theirs. Tumor endothelial cells are genetically abnormal and their viability and activity could be affected by cytokines and growth factors (such as VEGF, bFGF, etc.) secreted by tumor cells or other stromal cells. Hypoxia could also be of the same effect on these cells (Kyoko et al., 2007). From our results, the decreased expression of angiogenic factors and increased NO production from cancer cells could decrease both angiogenic activity and viability of tumor endothelial cells. To achieve a more comprehensive understanding of this tumor endothelial cells response and its precise mechanism, more investigations are required.

GAPDH is another key glycolytic enzyme highly expressed in cancer cells. Inhibition of this enzyme results in decreased ATP availability, which in turn may induce cell death (Tang et al., 2012). Susanne Mohr et al. showed that NO derived from L-arginine or other NO donors cause GAPDH modification through its S-nitrosylation. This modification causes nicotinamide adenine dinucleotide (NADH) to be preferably used as a substrate for the enzyme rather than NAD+, which could inhibit the enzyme activity and glycolysis (Mohr et al., 1996). However, it is demonstrated that GAPDH modification by NO is reversible and can be prevented under normal physiological conditions and redox state

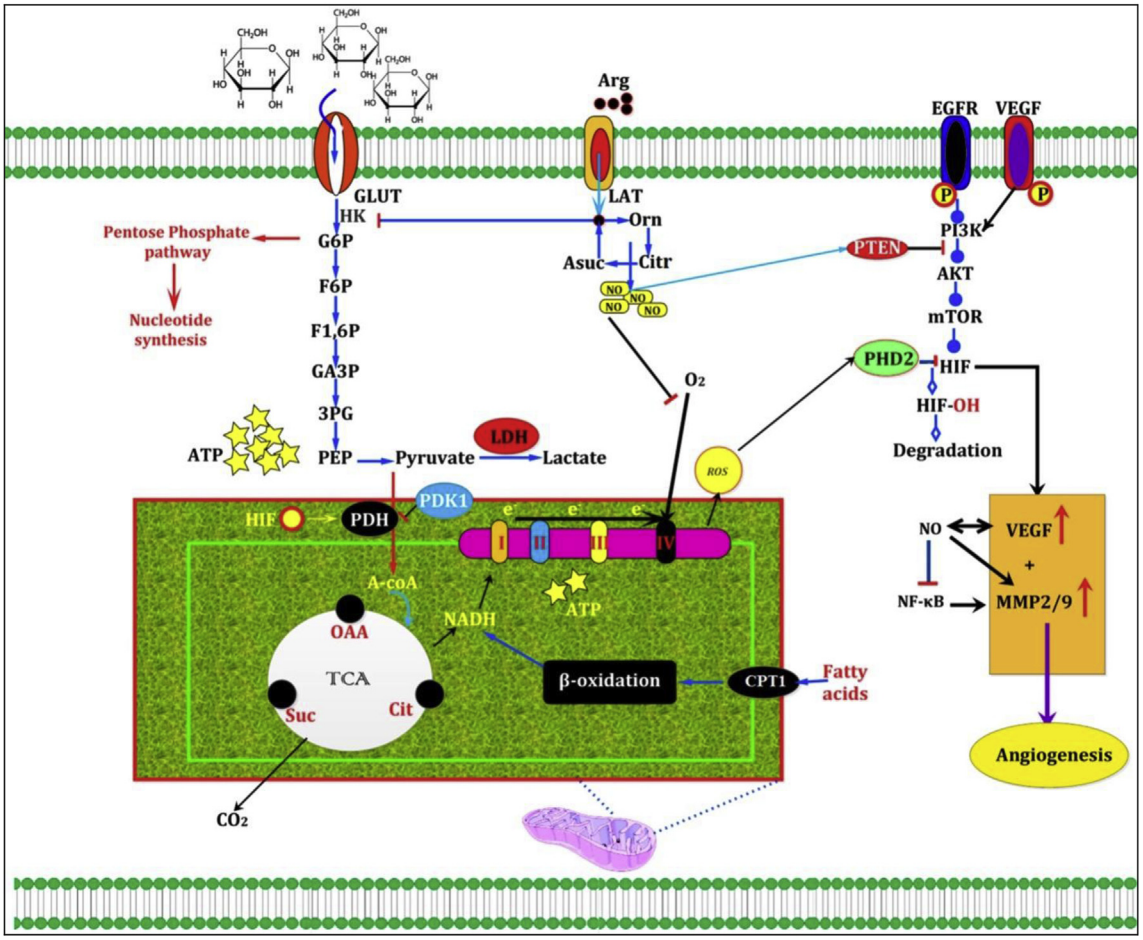

Fig. 8. Probable mechanism for the anticancer activity of L-arginine. L-arginine and its derivative (NO) can inhibit the metabolic pathway and angiogenesis of cancer cells and trigger the apoptotic pathway.

(Padgett and Whorton, 1997). Interestingly, L-arginine inhibits both glycolysis pathway, and ATP production by OXPHOS pathway under hypoxia. As a result, increased production of ROS mediated by NO in mitochondria induces death in cancer cells but not normal cells due to the existence of protective mechanisms in these cells (Fig. 8).

Developing an intact and complete functional vascular network is a vital embryonic process (Bruneau, 2008). Angiogenesis, as an important process in embryo development as well as cancer progression, is dependent on cell proliferation, extracellular matrix degradation, and cell migration (Karamysheva, 2008). Therefore, any interference in these processes could and will result in their inhibition. According to the Zhang et al.'s investigation, 5-FU can inhibit cell proliferation and migration (Zhang et al., 2010b). Furthermore, all DNA synthesis inhibitors like 5-FU can repress the growth of an embryo and lead to early embryonic death which might be due to the non-specific disorder in DNA synthesis caused by these agents (Tanaka et al., 1986). This evidence clearly shows the adverse effects of 5-FU on angiogenesis and embryo survival.

Based on our study, L-arginine treatment induced angiogenic activity of endothelial cells but decreased the expression of proangiogenic factors in cancer cells. According to our qPCR results (on HUVECs), L-arginine alone and in combination with 5-FU increased *VEGF* and *MMP-2,9* expressions compared to control and 5-FU treated cells. However, a down-regulation of these genes was observed in MDA-MB-468 cells treated with both drugs, alone and in combination. Although the expressions of *VEGF* and *MMP-2,9* in MDA-MB-468 cells were decreased, in HUVECs, all the changes in the expression of the mentioned genes were in favor of angiogenesis.

Now, the main question remaining is how L-arginine can decrease the proangiogenic factors expression in cancer cells but not normal cells (HUVECs). This paradox could probably be linked to the L-arginine's derivative, NO.

Compared to our findings, there are some contradictions in the literature regarding the role of NO in angiogenesis (MacLauchlan et al., 2011; Pipili-Synetos et al., 1994). Depending on the concentration of NO, the type of cells, and their metabolism, NO can trigger angiogenesis in both normal and cancer cells. High concentrations of NO result in the inhibition of cell proliferation and angiogenesis while low to moderate levels are associated with angiogenesis stimulation (Napoli et al., 2013; Vander Heiden, 2011). Angiogenesis is tightly controlled by maintaining the balance between proangiogenic and antiangiogenic factors (Karamysheva, 2008). VEGF, an important proangiogenic factor, plays a crucial role in the endothelial survival, proliferation, migration and it also interdependently interacts with NO (Karamysheva, 2008; Lee et al., 2015). According to a study by Papapetropoulos et al., NO can induce VEGF expression in endothelial cells as well as HIF-1α expression through activating PI3K-Akt pathway under normoxic conditions. It thereby activates VEGF production in cardiomyocyte cells (Papapetropoulos et al., 1997). Furthermore, Kuwabara et al. demonstrated that VEGF, in turn, enhances NO production in endothelial cells by activating PI3K-Akt pathway and enhancing eNOS expression (Kuwabara et al., 2006). Two of the matrix metalloproteinases, MMP-2 and MMP-9, are other important factors in angiogenesis process by degrading the basement membrane and extracellular matrix as the earliest event in this process (Khalid and Javaid, 2016). NO also affects *MMP*-2 mRNA level which has been affirmed by different studies

(Molinari et al., 2013; Tabruyn and Griffioen, 2008). Moreover, the dual effect of NO have been demonstrated on *MMP-9* expression as well, as it can decrease or increase *MMP*-9 mRNA level in a concentration-dependent manner (Fig. 8) (O'Sullivan et al., 2014).

As mentioned before, VEGF and MMP-2,9 are three important proangiogenic factors assessed in this study and can be regulated by some transcription factors such as PI3K, PTEN and NF-κB (Rodriguez and Huynh-Do, 2011; Tabruyn and Griffioen, 2008). It has been shown that NF-κB signaling pathway, as a hyper-activated pathway in most cancer cells, results in the induction of an angiogenic activity by up-regulating *MMP-2,9* and *VEGF* expressions (Kateb et al., 2011b; Tabruyn and Griffioen, 2008). It has also been demonstrated that NO can induce or inhibit NF-κB activity and its downstream genes in a concentration-dependent manner (Curado, 2011; Katsuyama et al., 1998). Therefore, as can be seen in Fig. 8, NF-κB inhibition by NO in its high concentrations may prevent pro-angiogenic activities of cancer cells. Furthermore, VEGF down-regulation by NO could be due to PTEN activation followed by PI3K-AKT and HIF1-α inhibition in cancer cells (Bonavida and Garban, 2015). According to these studies, NO effect on the proangiogenic factors expression is exerted through many factors and pathways.

Based on the results from the present study, L-arginine not only increased angiogenesis of endothelial cells but also had the potential to modulate 5-FU mediated anti-angiogenic effects on the endothelial cells. Moreover, both *in vitro* and *in vivo* angiogenesis models confirmed the increase in the percentage of capillary and blood vessel formation following L-arginine treatment in comparison to the control group. On the other hand, although angiogenesis by endothelial cells was completely inhibited by 5-FU, the combination treatment was associated with sprout formation. Even the number of sprouts formed by endothelial cells was enhanced upon increasing L-arginine concentration. As confirmed by evaluating the weight of the embryos, L-arginine was able to completely inhibit the toxic effect of 5-FU on the embryos. Furthermore, the inhibitory effect of L-arginine on the breast cancer cell migration and invasion was demonstrated in our previous study (Jahani et al., 2017).

Nevertheless, the relation between our results of angiogenesis, decreased embryo toxicity and cell viability assay may be challengeable, especially in combined treatment *in vivo* angiogenesis assay. Accordingly, *in vivo* angiogenesis results indicated an increased angiogenesis in contrast to the viability rate in the same treated group. To explain these effect of heterogonous nature, it should be noted that *in vivo* angiogenesis is a complex process that in addition to endothelial cells, requires different cells and factors such as fibroblasts, immune system cells as well as growth factors, cytokines, etc. So, this phenomenon is rather regulated by its microenvironment than just endothelial proliferation. To sum up, a thorough investigation is required in order to elucidate the exact mechanism behind these processes.

5. Conclusion

Based on the results obtained to date, L-arginine enhances chemosensitivity of breast cancer cells but does not exert chemotoxicity on normal cells and fetus through NO-dependent regulation of signaling pathways and subsequent effects on different metabolic pathways. Furthermore, these data cast a new light on the metabolic activity of the breast cancer cells. It is foreseeable that L-arginine could target exclusive metabolic pathways in cancers, which may allow this molecule to lay the foundation for developing novel, more effective therapeutic strategies. Nevertheless, these findings need to be corroborated by further investigations and the interpretation of results must also be considered with caution.

Conflicts of interest

There is no conflict of interest to disclose.

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