Toxicology in Vitro 56 (2019) 10–18

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Toxico-pathological effects of meglumine antimoniate on human umbilical vein endothelial cells

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ARTICLE INFO

Keywords:
Meglumine antimoniate
Toxicity
Apoptosis
Angiogenesis
HUVECs

ABSTRACT

Leishmaniasis is one of the most important parasitic diseases after malaria. The standard treatment of leishmaniasis includes pentavalent antimonials (SbV); however, these drugs are associated with serious adverse effects. There have been very few studies pertaining to their side effects and mechanism of action in the fetus. This investigation examines the effects of meglumine antimoniate (MA) on the survival rate, angiogenesis and cellular apoptosis in the human umbilical vein endothelial cells (HUVECs). HUVECs were treated with varying doses of MA (100–800 μg/ml) for 24, 48 and 72 h and the survival rate was studied by colorimetric assay, flow cytometry, immunocytochemistry, migration (scratch) assay and tube formation assay. The results of quantitative real-time PCR (qPCR) studies indicated that the most important genes involved in presenting angiogenesis included VEGF and its receptors (Kdr and Flt-1), NP1 and Hif-1α genes including the anti-apoptotic gene of Bcl2, were significantly reduced compared to the control group ($p < 0.05$). In contrast, the most leading genes involved in the phenomenon of apoptosis were P53, Bax, Bak, Apaf-1 and caspases 3, 8 and 9, which were significantly up regulated compared to the control group ($p < 0.05$).

1. Introduction

Leishmaniasis is considered as the second most important parasitic disease after malaria (Jabali and Kazemi, 2013; WHO, 2016). More than 12 million people in 101 countries around the world suffer from different forms of leishmaniasis with 2 million new cases annually. More than 1 billion people living in urban and rural areas are at particular risk. Pentavalent antimony compound (SbV), such as meglumine antimoniate (MA, Glucantime®) is the standard treatment for leishmaniasis. The United States Food and Drug Administration (FDA) has

Abbreviations: MTT, 3-(4,5-Di methylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide; Hif-1α, hypoxia inducible factor1-alpha; PI3K, (phosphatidylinositol 3-kinase) and Akt/PKB (Protein Kinase B); MA, meglumine antimoniate (Glucantime®); HUVECs, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; Kdr, kinase insert domain receptor; NP1, neuropilin1; Flt-1, FMS related tyrosine kinase1; Bcl2, B-cell lymphoma; BclxL, B-cell lymphoma-extra large; Tp53, tumor protein; Apaf-1, apoptotic peptidase activating factor 1; ICC, immunocytochemistry; CD31, cluster of differentiation 31; PECAM-1, platelet endothelial cell adhesion molecule

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https://doi.org/10.1016/j.tiv.2018.12.018
Received 30 June 2018; Received in revised form 5 December 2018; Accepted 28 December 2018
Available online 30 December 2018

classified this drug in category C (Galvao et al., 2013). Although MA has been used as first-line treatment since the 1940s, its mechanism of action and its toxicity are not fully understood. In some studies, MA has been indicated to cause DNA damage and so can be portrayed as a mutagen (Coelho et al., 2014; Huang et al., 2017; Miranda et al., 2006; Moreira et al., 2017). On the other hand, several studies have confirmed its side effects on various human tissues and organs, particularly on blood, liver, kidney and heart (Galvao et al., 2013). However, limited studies have been performed on the effects of MA in pregnant women and its congenital consequences (He et al., 2016; Khosravi et al., 2018b; Khosravi et al., 2018a; Miranda et al., 2006; Silveira et al., 2003).

Recent studies suggest the possibility of congenital transmission of leishmaniasis from mother to fetus (Berger et al., 2017). We know that normal fetal growth is an important component of a healthy pregnancy and it effectively influences newborn after birth; this process is influenced by several confounding factors. The toxic effects of the drugs on mother and fetus during pregnancy are the most common causes of diseases, which might induce various degrees of pregnancy defects such as abortion and fetal death (Chaiworapongsa et al., 2016). Many women fail to notice their pregnancy in the early stages, particularly before 8 weeks of gestation, which is the main stage in organogenesis. Consequently, such people are exposed to substances or drugs that are likely to be teratogenic and/or toxic and may cause fetal death during the pre-implantation period. If the exposure occurs during the period between the second to eight week of pregnancy, it increases the probability of structural abnormalities (Mifsud and Sebire, 2014). Defect in the angiogenesis of the ovaries may contribute to a variety of disorders including infertility and even abortion (He et al., 2016).

Several studies have shown that Hif-1α factor expresses > 90 genes. High correlation has been reported between Hif-1α and the genes involved in the phenomena of angiogenesis and apoptosis and it plays a central role in these two phenomena (Ziello et al., 2007). The VEGF-A factor is one of the most important angiogenesis regulators that exerts its effects via binding to VEGFR-2 (Kdr) and VEGFR-1 (Flt-1) receptors as well as the exclusive receptor of the isoform Neuropilin1 (NP1). In addition, VEGF induces endothelial cell proliferation and invasion, reduces pathological apoptosis in endothelial cells and stimulates stromal proteolysis (Alleva et al., 2011; Huang et al., 2017; Pandey et al., 2018).

Apoptosis leads to the death of cell through precise and targeted mechanisms which are controlled by various genes. The most important molecules regulating apoptosis are B-cell lymphoma and caspase family proteins (Nagata, 2018; Watson et al., 2017). Caspases are subclassified into initiator and executable and inflammatory types. Caspase-8 is one of the most important initiators, which activates the executive caspases and finally leads to a caspase cascade (Séïde et al., 2016). These events are completed with the release of cytochrome *c* from the mitochondria, which is regulated by Bcl2 family proteins. A group of Bcl2 family members induces the activity of Bax and Bak, which then lead to the induction of mitochondrial membrane permeability and the release of cytochrome *c* from the mitochondria. Upon the release of cytochrome *c*, it binds to the protein Apaf-1 and leads to the activation of executive caspase-9 which cleaves and activates the apoptosis effector caspases-3 and -7. One of the most essential genes implicated in the inhibition of cell growth is Tp53 which plays a key role in inducing apoptosis. This biological process leads to various nuclear-related events such as DNA fragmentation as the main sign of apoptosis (Zhou et al., 2018).

Drugs can activate apoptosis and cause various diseases through direct effects on the mitochondria (Ruiz-Magaña et al., 2016). Any disturbance in the regulation of apoptosis in endothelial cells leads to harmful effects on the development of the cardiovascular system, which can ultimately leads to fetal death (Gutman et al., 2008; Watson et al., 2017). Investigating the effects of medication used during pregnancy faces serious problems such as the difficulty involved in studying this population due to the vulnerability of the fetus. In addition, many physiological changes such as alterations in blood volume and plasma proteins occur during pregnancy, which affect the dosage and distribution of the drugs (Mifsud and Sebire, 2014).

In the present study, we aimed to use human umbilical veins endothelial cells (HUVECs) which are most similar to endothelial cells in vessels, possess good reproductive capacity and respond well to extracellular stimulants (Zhang et al., 2015). Therefore, the purpose of this study is to answer the following questions: Does MA have a toxic effect on HUVECs? Does MA alter the expression of VEGF genes and their receptors that play a major role in the phenomenon of angiogenesis? Does MA alter the expression of apoptotic genes involved in programmed cell death? To answer these questions, using flow cytometry, qPCR, colorimetric assays, immunocytochemistry and migration (scratch) assay, the toxic, apoptotic and antigenic effects of MA on HUVECs were investigated in this study.

2. Materials and methods

2.1. Cell culturing

HUVECs, as non-cancerous cells, were provided by the National Cell Bank of Iran (Pasteur Institute of Iran). These cells were seeded in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA). All media were provided with 100 µg/ml of streptomycin, 100 IU/ml penicillin and 15% fetal bovine serum (FBS) (Gibco, USA). The cells were maintained at 37 °C in 5% CO_2 incubator (Memmert, Germany) and were passaged every 48–72 h.

2.2. Cytotoxicity tests

For the cytotoxicity assay, 5×10^4 cells were seeded into each well of 96-well micro plate and incubated at 37 °C in 5% CO_2. After 24 h, the former culture medium was replaced with new medium containing different concentrations of MA (100, 200, 400 and 800 µg/ml) and again incubated for 24, 48 and 72 h. The wells containing no drug were considered as the negative control. Following the incubation times, 10 µl of MTT solution (5 mg/ml) secured from the German Merck Company was added to every well and incubated at ambient temperature (25 °C) for 3 h. Then, 100 µl isopropanol, as a stop solution, was added to each well. After 1 h, the absorbance was read at a wavelength of 580 nm by an ELISA reader (Bio Tek-ELX 800 Winooski, Vermont, USA). All tests were performed in triplicates. Fifty percent inhibitory concentrations (CC_{50} values) of the drug were calculated using probit analysis in SPSS software.

2.3. Flow cytometry

For detection of apoptosis, an Apoptosis Diagnostic Kit (annexin V-fluorescein isothiocyanate (FITC-PI) was used. The plasma membrane of the apoptotic cell can be structurally altered via exposure of phosphatidylserine on the cell surface (Marino et al., 2017). For this method, 10^6 HUVECs were used and added into micro tubes containing RPMI 1640 with 10% FBS. MA was then added to each tube at different concentrations of 100, 200, 400 and 800 µg/ml. afterward, they were incubated at 24 °C for a standard time of 72 h. After 72 h, the tubes were centrifuged and washed three times with PBS. Then, a binding buffer was added and incubation was carried out for 20 min in the dark at room temperature (25 °C). Finally, annexin V was added into the reaction medium. The results were read by flow cytometers (Becton Dickinson, USA) and analyzed using Cell Quest software. In parallel, untreated tubes were used as blank. All flow cytometry tests were performed in duplicates.

2.4. RNA extraction

HUVECs were cultured in cell culture flasks and MA was incubated at concentrations of 100, 200, 400, and 800 µg/ml. An additional control group was also added and incubated for 72 h and the cells were

Table 1
Primer sequences used for real – time quantitative PCR.

Gene *HUVEC*	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Product size (bp)
Np1	CAGAAAACACCAGGTCGAATCC	CGCGCTGTCGGTGTAAAAA	69
Kdr	CCA GCA AA CA GG GTCTGT	TGTCTGTGTCATCGGAGTGATATCC	87
Flt1	CAGGCCCAGTTTCTGCCATT	TTCCAGCTCAGCGTGGTCGTA	82
Vegf	CTACCTCCACCATGCCAAGT	GCA GTAGCTGCGCTGATAGA	109
Hif-1alpha	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA	124
Tp53	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC	125
Caspase8	AGAGTCTGTGCCCAAATCAAC	GCTGCTTCTCTCTTTGCTGAA	78
Apaf-1	AAG GTG GAG TAC CAC AGA GC	TCC ATG TAT GGT GAC CCA TCC	116
Bak	ATG GTC ACC TTA CCT CTG CAA	TCA TAG CGT CGG TTG ATG TCG	92
Caspase 9	CTTCGTTTCTGCGAACTAACAGG	GCACCACTGGGGTAAGGTTT	75
Caspase 3	CATGGAAGCGAATCAATGGACT	CTGTACCAGACCGAGATGTCA	139
Bax	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT	155
Bcl2	GGTGGGGTCATGTGTGTGG	CGGTTCAGGTACTCAGTCATCC	89
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC	101
Beta Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	250
HPRT	CCTGGCGTCGTGATTAGTGAT	AGACGTTCAGTCCTGTCCATAA	131

trypsinized and centrifuged. Total RNA was extracted using RNA Miniprep kit (BioBasic, Canada) according to the manufacturer's instructions. A nanodrop was used to evaluate the quality of samples (ND-1000, Thermo Scientific Wilmington, DE, USA).

2.5. cDNA synthesis and qPCR

cDNA was synthesized from 500 ng of total RNA using an RT reagent kit (Takara, Clontech) according to its protocol. qPCR was performed by SYBR premix Taq™ (Takara, Clontech) on a real-time PCR cycler (Qiagen, Chatsworth, CA) in a final volume of 10 µl by the following thermal profile: pre-denaturation at 95 °C for 1 min followed by 40 cycles at 94 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s. qPCR was performed by the specific primers as well as by β-Actin, HPRT and GAPDH as the reference genes for data normalization. We performed the experiments based on the MIQE Guidelines. GAPDH gene was demonstrated to be the most superior and stable gene in the experiments as presented in the supplementary data. The selection of the housekeeping genes is also substantiated by numerous reports, which have conducted similar studies (Żyżyńska-Granica and Koziak, 2012) (Table 1). A positive and a negative control were included in each run. All tests were performed in duplicates. Using the $2^{-\Delta\Delta Ct}$ method, the gene expression level was analyzed and in fold change.

2.6. Immunocytochemistry Staining

To study the biological function, staining was performed for CD31 markers. ICC staining of CD31 was performed using standard procedures on a Dako Omnis (ready-to-use; clone JC70A; Dako, Glostrup, Denmark). The expression of CD31 was measured by counting the positively stained cells and considering the mean in 10 high-power fields (20×).

2.7. Migration (scratch) assay

HUVECs were cultured at a density of 5×10^5 cells/dish (35 mm culture dishes) and MA was incubated at concentrations of 100, 200, 400, and 800 µg/ml. Also, an additional control group was added and incubated for 72 h. After ensuring the proper growth of the cells, the cells were separated by scratch assay using 200-ml pipette tip extensively washed with 1.5 ml of PBS and replaced with 2.5 ml of culture medium (Babaee et al., 2018). To remove the cell debris, we washed the culture plates with 1.5 ml of PBS solution and replaced with 2.5 ml of culture medium. We captured the cells in 0 h and incubated the culture plates at 37 °C with 5% CO_2 and checked the cell migration in 72 h. Finally, the images were analyzed using Analysis LS Professional, Version 2.4.

2.8. Tube formation assay

The 96-well plate was coated with 50 µl Matrigel (BD Biosciences, Cat No 356234). A total of 1×10^4 HUVECs was resuspended in 100 µl of conditioned media with 5% FBS (Invitrogen) and seeded on Matrigel-coated wells. MA was incubated at concentrations of 100, 200, 400, and 800 µg/ml. Also, an additional control group was added and incubated for 6 h to allow formation of tube-like structures. Total tube lengths formed were measured and compared from 20× magnification using an inverted microscope (Olymous, Japan). Pictures were analyzed by using ImageJ software (NIH).

2.9. Statistical analysis

Using a probit model in the SPSS version 20 (SPSS Inc., Chicago, IL, USA) CC_{50} was analyzed. The comparisons among the groups were performed through one-way ANOVA and *t*-test. The mean ΔΔct for treatment and control of each gene was compared. A p value of < 0.05 was considered significant. GRAPHPAD PRISM 6 (Graphpad Software Inc., San Diego, CA, USA) was used to construct the graphs.

3. Results

3.1. Effect of MA on growth inhibition

The effect of MA on cell growth at different concentrations of the drug was evaluated. The OD values were measured in comparison with the untreated control at different concentrations (100, 200, 400 and 800 µg/ml) of MA after 24, 48 and 72 h of incubation. The concentrations were selected based on a previous study (Shokri et al., 2012). The inhibitory effect of MA on cell growth was time- and concentration-dependent and was promoted with increasing concentrations compared to the control group.

The calculated CC50 values for MA were 736.82 µg/ml, 480.21 µg/ml and 208.08 µg/ml after 24, 48 and 72 h, respectively. The highest OD value (OD = 1.3 ± 0.07) was observed at the concentration of 100 µg/ml after 24 h of incubation and the least OD value (OD = 0.497 ± 0.04) was obtained at the concentration of 800 µg/ml after 72 h (Fig. 1).

3.2. Effect of MA on the rate of apoptosis

Initially, the effect of MA on the HUVECs was investigated. HUVECs were incubated with varying MA concentrations (100–800 µl/ml) for 72 h. MA decreased the viability of HUVECs in a dose-dependent manner ($p < 0.05$). The apoptotic value was 5.4% at 100 µl/ml of MA and gradually increased to 30.8% at 800 µl/ml, compared with 96%

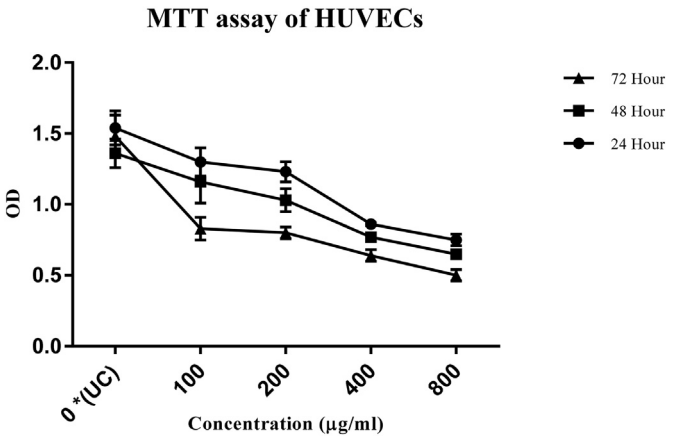

Fig. 1. Inhibition of the growth of HUVECs by MA. Comparison of the overall mean optical density (OD) and the CC_{50} values of MA on the susceptibility of HUVECs by MTT assay. Data are presented as the means ± S.D.; $p < 0.05$. * (untreated control group).

viability for the untreated control group (Fig. 2).

3.3. Effect of MA on apoptotic gene expression

To further investigate, the expression of genes involved in apoptosis, the qPCR was performed. Different concentrations of MA reduced the expression of anti-apoptotic gene Bcl2 and increased the expression of the pro-apoptotic genes of Bak and Bax as well as the expression of caspases-3, -7,-9 and also -8 through P53 in HUVECs. The cells were incubated for an extra 24 h analysis. The qPCR reactions were performed respectively. MA increased the levels of caspase-3 (A), caspase-9 (B), caspase-7 (C), Tp53 (D), Apaf-1 (E), Bax (F) and Bak (G) decreased the expression of Bcl2 (H) by a qPCR test at doses of 100, 200, 400 and 800 µg/ml. The expression levels were normalized to those of GAPDH and were calibrated to controls. As expected in this study, we observed that MA at the highest concentration (800 mg/ml) had the most profound effect on apoptosis of HUVECs. Data are presented as the means ± SD ($p < 0.05$, *) (Fig. 3).

3.4. Effect of MA on angiogenic gene expression

The AKT/PKB signaling pathway is a vital pathway that promotes cell survival. Our results showed that MA suppressed VEGF-A 1 and 2 receptors and VEGF-A ligands were then suppressed in HUVECs. To determine whether MA can interfere with the AKT/PKB pathway, the expression of VEGF-A genes and their receptors VEGFR1 and R2 as well as downstream genes such as Bcl2 and BclxL were evaluated. In comparison with the control, reduced expression of VEGF-A gene and its receptors (VEGFR1, R2) were observed in various drug concentrations. MA reduced the expression levels of VEGF-A 1, 2 and NP1, increased the expression of Hif-1α genes and also reduced expression of VEGF-A in the AKT/PKB pathway induced apoptosis in HUVECs ($p < 0.05$, *) (Fig. 4).

3.5. Effects of MA on ICC results of HUVECs

Results of ICC showed that staining HUVECs with CD31 marker as a platelet endothelial cell adhesion molecule (PECAM-1) with increasing MA concentration, weakened. This indicates the effect of MA on decreasing angiogenesis in HUVECs (Mukherjee, 2016) (Fig. 5).

3.6. Effects of MA on migration potential of HUVECs'

Our findings showed that > 54% of the space between the boundaries of the scratch was repaired after 72 h in control group. Scratch repair in 100, 200 and 400 µg/ml groups was 39.3%, 31.1% and 4.9%, respectively. Additionally, in 800 µg/ml treated groups, not only did no healing occur but also the distance between the edges of the scratch increased in comparison to its initial distance (0 h) (Fig. 6).

3.7. Effects of MA on tube formation of HUVECs'

Our result showed that MA inhibits in vitro angiogenesis, by increasing the concentration of MA, total length tube and total Mesh area significantly reduced compared to the control group (Fig. 7).

4. Discussion

To our knowledge, no broad study has been performed earlier to investigate the cytotoxic, angiogenic and apoptotic effects of MA on HUVECs in an in vitro environment. This unprecedented research sheds light on the involvement of major genes in the phenomena of angiogenesis and apoptosis. The results revealed that there was a significant difference in cell proliferation and cell growth between MA treated HUVECs and the control group. In addition, the inhibitory effects were

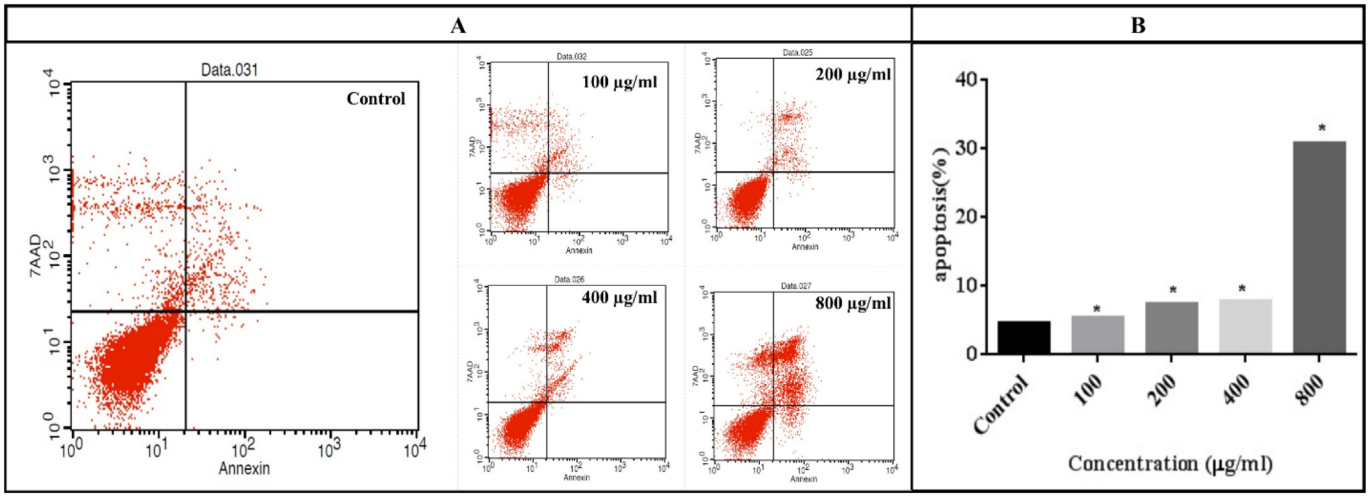

Fig. 2. (A) MA-induced HUVECs apoptosis. The effect of administered at concentration of 100, 200, 400 and 800 µg/ml of MA compared control group was measured on the HUVECs for 72 h in the absence or presence of MA. (B) The percentage was significantly increased in treated groups compared with the control group, data are presented as the means ± SD, ($p < 0.05$, *).

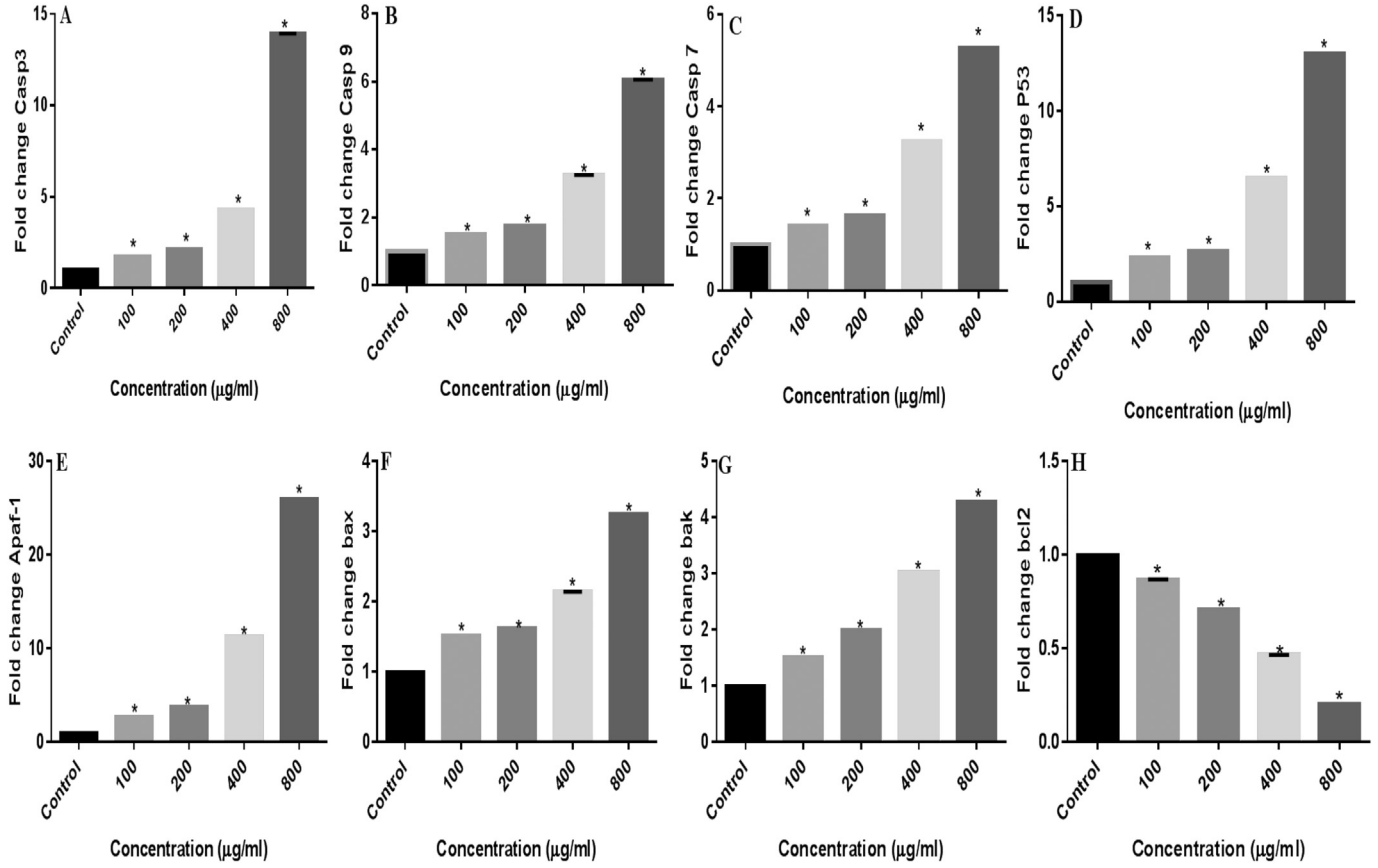

Fig. 3. MA induced the expression of apoptotic mediator genes in the HUVECs. The expression levels of Cas3(A), Cas9(B),Cas7(C),p53(D),Apaf-1(E), Bax (F) and Bak (G) genes were increased and Bcl2(H) gene was decreased in the MA treated HUVECs compared to control. MA was administered at concentration of 100, 200, 400 and 800 µg/mlµg/ml. The expression levels were normalized to those of GAPDH and calibrated to the control (data are presented as fold-change value; error bars show standard error of mean; $*p < 0.05$, One-way ANOVA). Since the standard deviations of the samples were very small, error bars are not clear on the columns.

dependent on dose and time. The varying doses of MA used at different time periods validated that the reduction of survival, induction of apoptosis and inhibition of angiogenesis in HUVECs occurred at the highest level at a concentration of 800 µg/ml and after 72 h of incubation.

The mechanism of action involved in the toxic effect of SbV is not entirely elucidated. However, the stimulation of peroxidase activity in the liver of mice treated with MA is also consistent with the role of trivalent antimony compounds (SbIII) in the mechanism of toxicity associated with this pentavalent antimonial drug (Kato et al., 2014; Liu et al., 2018). It is suggested that SbV acts as a prodrug that transforms into the more toxic SbIII. Indeed, the interference of SbIII with thiol homeostasis increases the generation of reactive oxygen species (ROS), oxidative stress, peroxidase activity and apoptosis (Zhang et al., 2015).

Decreased or increased gene expression for VEGF can contribute to the development of diseases. For example, solid tumor cells, because of insufficient blood supply, grow up to a certain extent, but by increased expression of VEGF-A, they can easily grow and metastasize extensively. In the present study, reviewing VEGF-A and the main genes involved in the angiogenesis phenomenon, using qPCR, demonstrated that MA has the potential to reduce the expression of VEGF-A gene and its main receptors including VEGF-R2 (Kdr), VEGF-R1 (Flt-1) and NP1, also ICC assay and tube formation assay confirmed the result of this study. An important observation in this study is that MA affects tube formation (total tube length and total Mesh area). The balance between the angiogenic and anti-angiogenic factors initiates vascular stability in response of extracellular signals such as ischemia (Aparicio et al., 2005). Furthermore, whole genome sequencing showed that VEGF-A inhibited the expression of a large number of genes involved in apoptosis in cells and multiple cell lines (Parker et al., 2012; Shu et al., 2014).

Moreover, Hif-1α releases VEGF-A and activates other mechanisms, including erythropoiesis and eventually the circulating VEGF-A gene is bound to its receptors on the endothelial cells and leads to angiogenesis through the pathway of tyrosine kinase (Koch and Claesson-Welsh, 2012; Wigerup et al., 2016).

BclxL protein, one of the anti-apoptotic factors of the Bcl2 family, plays an important role in regulation of apoptotic pathway. The heterodimerization of the Bcl2 anti-apoptotic proteins and pro-apoptotic proteins, including Bad, Bak, Bim and Bid, is a key event in regulating apoptosis. Recent studies have shown that the proteins of the Bcl2 family can also interact with other genes, including Tp53, and suppress the transcription of anti-apoptotic genes such as Bcl2 and BclxL (Tiwari, 2012). Interestingly, we observed that MA affected the Akt/PKB, then VEGF-A caused a decrease in the expression of Bcl2 and consequently increased the expressions of “Bak and Bax genes”.

In the intrinsic apoptosis pathway, changes in the permeability of the mitochondrial membrane to cytochrome *c* is relatively determined by pro-apoptotic intermediates (Bax and Bak) (Singh and Kumar, 2016). These pro-apoptotic intermediates lead to the induction of consecutive caspases, including caspase-3, and the result of these actions is the occurrences of definitive events of apoptosis, thus substantiating the data from other studies on the phenomenon of apoptosis. Therefore, the results of this study showed that MA caused a decrease in the expression of anti-apoptotic gene Bcl2 and increased the expression of pro-apoptotic genes of Bax-Bak. Additionally, compared to the control group, Apaf-1, caspase-9 and caspase-3 genes showed increased expression, which caused reduced cell survival. It should be stated that

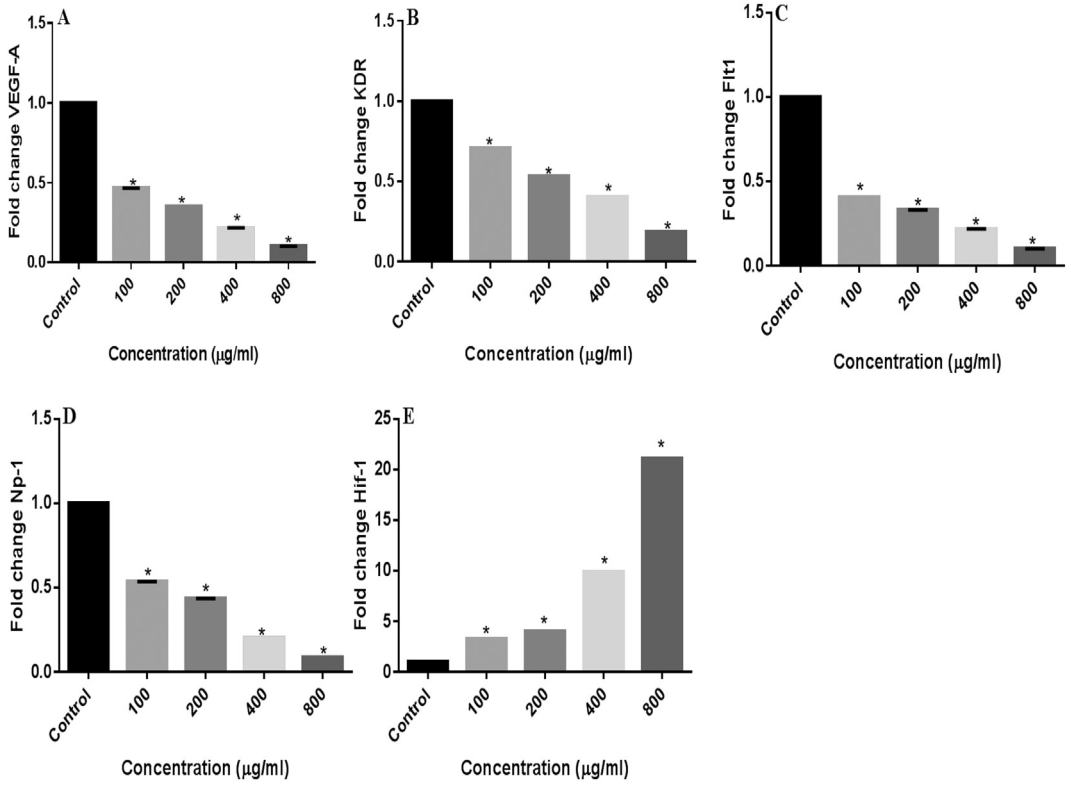

Fig. 4. The expression of active genes involved in angiogenesis (in AKT/PKB pathway) after treatment with MA in HUVECs. MA induced the expression of angiogenesis mediator genes in the HUVECs. The expression levels of VEGF-A (A), Kdr (B), Flt1(C), Np-1(D) and Hif-1 (E) genes were decreased in the MA treated HUVECs compared to control. MA was administered at concentration of 100, 200, 400 and 800 μg/ml. The expression levels were normalized to those of GAPDH and calibrated to the control (data are presented as fold-change value; error bars show standards division (SD); $*p < 0.05$, One-way ANOVA). Since the standard deviations of the samples were very small, error bars are not clear on the columns.

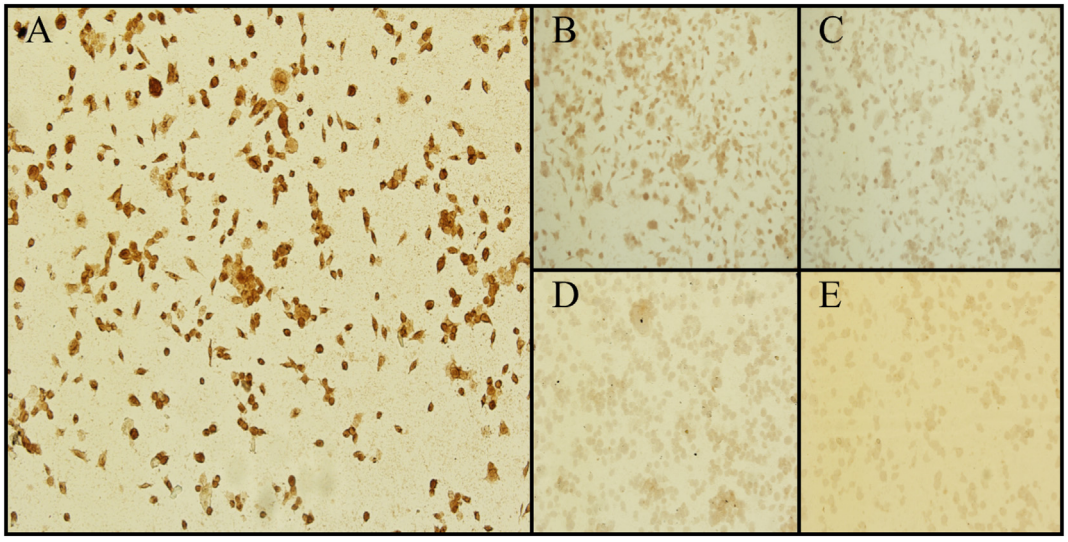

Fig. 5. The effect of MA on ICC in HUVECs. Control (A) staining with CD31 shows strongly positive precursor angioblastic cells with angiogenesis, 100 μg/ml; (B) ICC shows weakly positive cells compared with the control but still precursor angioblastic cells along with decreased angiogenesis are present, 200 μg/ml; (C) ICC shows weakly positive than cells compared with 100 μg/ml but still scattered presence of precursor angioblastic cells without angiogenesis are present, 400 μg/ml; (D) ICC shows very weakly positive precursor angioblastic cells along with sever alteration, 800 μg/ml; (E) ICC shows negative staining.
In Fig. 5A control sample: score 5: 90% of cells are staining with CD31, in Fig. 5B (100 μg/ml): score 4: 55% of cells are staining with CD31, in Fig. 5C (200 μg/ml): score 3:35% of cells are staining with CD31, in Fig. 5D (400μgy/ml): score 2:8% of cells are staining, in Fig. 5E (800 μg/ml): score 0; 0% of cells are staining (Negative).

"MA", given its effects on the proteins of AKT/PKB pathway and VEGF-A along with the activation of caspases-3 and -9 via cytochrome *c*, can contribute to the induction of apoptosis (Bhola and Letai, 2016; Pilatova et al., 2010; Yan et al., 2011; Zhang et al., 2015).

Based on the results of the present study, there was an increase in caspase-8 gene expression, which was significantly distinct from that of the control group. We know that if normal mammalian cells are affected by stress signals such as hypoxia and DNA damage or by some of the

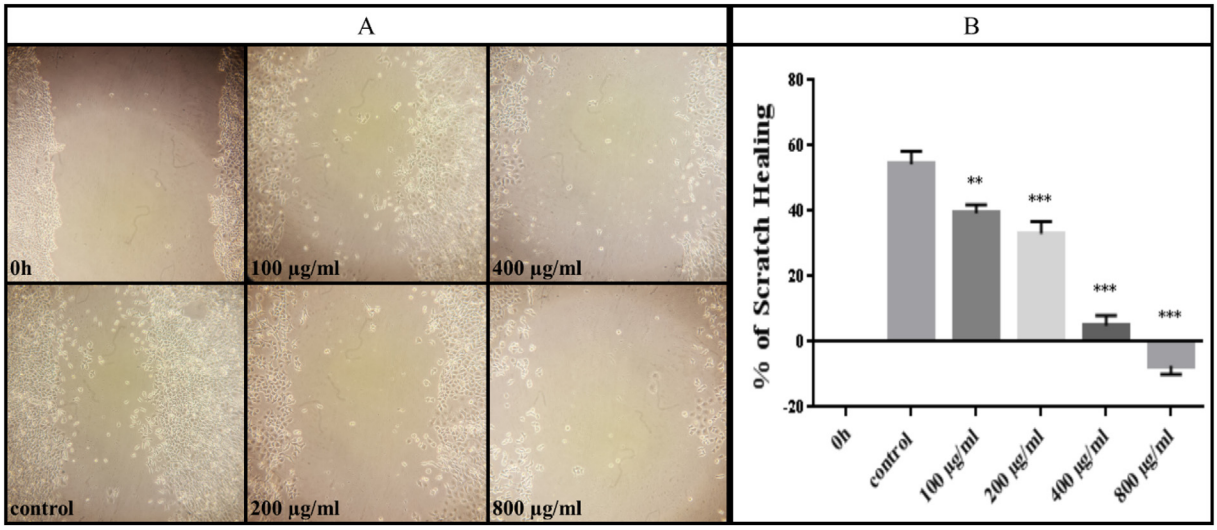

Fig. 6. The effects of MA on migration in HUVECs. The cells were scratched and treated with MA for 72 h. Closure of the scratched area was photographed (A) at 0 h and 72 h in control, 100 μg/ml, 200 μg/ml, 400 μg/ml and 800 μg/ml groups after 72 h. (B) The percentage of scratch renovation was statistically decreased in treated groups compared with the control group. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

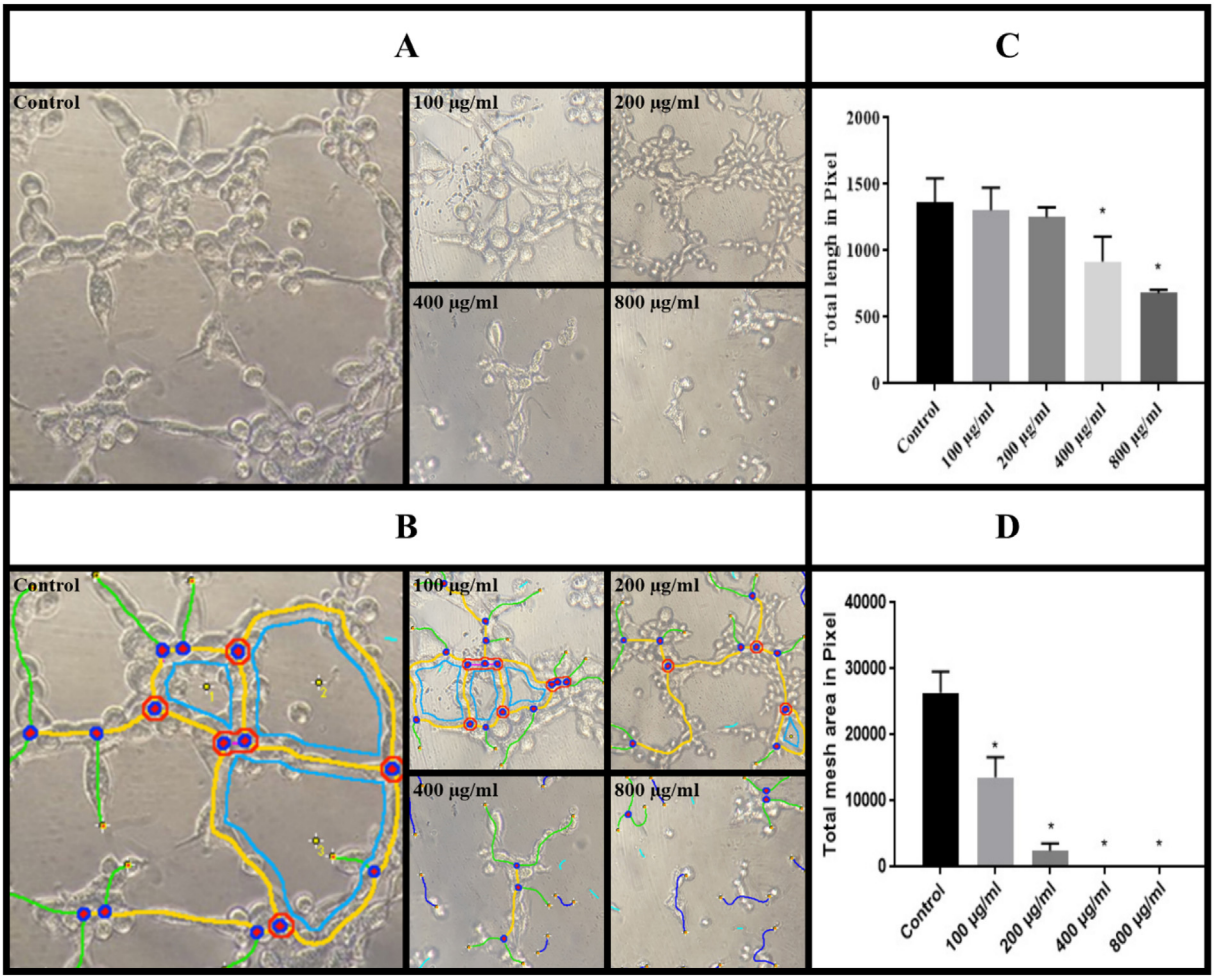

Fig. 7. The effects of MA on tube formation in HUVECs. (A) Representative pictures for angiogenic activity of HUVECs treated with 100 μg/ml, 200 μg/ml, 400 μg/ml and 800 μg/ml of MA, after incubation for 6 h, random microscopic fields were photographed and analyzed. (B) Image J software output (C) Total tube length (pixel), (D) Total Mesh area (pixel), pictures were analyzed from random three fields and were shown as mean ± SD and compared with the control group. (*$p < 0.05$).

drugs, these cells will go through two paths. They either undergo regeneration or apoptosis, which is affected by the Tp53 gene. This gene is one of the most important genes found in human tumors (Coelho et al., 2014). As mentioned before, the Tp53 gene suppresses the transcription of anti-apoptotic genes, and in this study, the effect of MA was shown to be significantly higher than that of the control group. On the other hand, recent studies have shown that MA can act as a potential mutagen by damaging DNA (Coelho et al., 2014; Moreira et al., 2017). Since major human neoplasia can arise from this mechanism, occurrence of tumor lesions in the embryo throughout the pregnancy is not beyond the realms of possibility (Chandrasoma and Taylor, 1991).

One billion people in the world are at risk of leishmaniasis (WHO, 2016) and they live mainly in outlying and rural areas where the main health indicators, including pregnancy, are at low levels. In addition, some studies on abortions in pregnant women treated with MA in these areas reported that many of the women were not aware of their early pregnancy (Mifsud and Sebire, 2014). Infection with different forms of leishmaniasis, especially the visceral form (kala-azar), during the gestation period and the rapid growth of population demand special attention on the issue because only limited information is available in literature (Maciel et al., 2014). Even though the therapeutic protocols in pregnant women with leishmaniasis are being considered in these areas along with the warning that MA cannot be used during pregnancy, the chances of pregnant women receiving this drug are still high. On the other hand, numerous studies have reported that some drugs can disrupt ovarian angiogenesis and cause infertility and abortion (Geva and Jaffe, 2000). Therefore, based on the findings of this study, it is suggested that physicians and health personnel resident in such areas should be more diligent and fastidious about creating awareness among pregnant women and also know the potential risk of the drug and serious fetal consequences. In addition, because of the application of drugs during pregnancy and their association with several barriers, including serious fetal susceptibility, it is recommended to conduct some empirical studies about the effects of MA on the fetus in animal models.

Our study suggested that MA can inhibit cell growth and also induce apoptosis through AKT/PKB patway and VEGF-A. It was indicated that MA could also potentially alter the expression of the targeted genes in the angiogenesis and apoptosis pathways. Furthermore, MA induced apoptosis through the activation of the mitochondrial caspase pathways as well as the activation of “VEGF-A” associated with the AKT pathways and decreased migration capacity of these cells. The present findings have provided new insights into the events leading to the key mechanisms of the genes involved in regulation and development of angiogenesis. Understanding the multiple genetic pathways in a model of healthy cells could provide attractive therapeutic targets for the development of anti-angiogenic drugs. Therefore, the toxico-pathological effects of MA on HUVECs should be considered as a serious clinical implication for the embryo during pregnancy.

Acknowledgements

We would like to thank the personnel of Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran, for their kind assistance in this study. This study was financially supported by Kerman University of Medical Sciences, Kerman, Iran (project no. 94/974/205; ethics no. IR.KMU.REC.1394.525).

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