



## ***In vitro* and *in vivo* modulation of LPS and carrageenan-induced expression of inflammatory genes by amitriptyline**

[Modulación *in vitro* e *in vivo* por amitriptilina de la expresión de genes inflamatorios inducidos por LPS y carragenina]

Laleh Rafiee<sup>1</sup>, Valiollah Hajhashemi<sup>2\*</sup>, Shaghayegh Haghjooy Javanmard<sup>3</sup>

<sup>1</sup>Applied Physiology Research Center, Isfahan Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran.

<sup>2</sup>Department of Pharmacology and Toxicology, Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

<sup>3</sup>Applied Physiology Research Center, Isfahan Cardiovascular Research Institute and Department of Physiology, Isfahan University of Medical Sciences, Isfahan, Iran.

\*E-mail: [vhajhashemi@gmail.com](mailto:vhajhashemi@gmail.com)

### Abstract

**Context:** Amitriptyline, a tricyclic antidepressant is used for the management of psychological disorders and various types of pain. In the previous work, it is founded that amitriptyline inhibited the migration of polymorphonuclear (PMN) into the site of inflammation.

**Aims:** To evaluate the effect of amitriptyline on the expression of some inflammatory mediators such as intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS).

**Methods:** An *in vitro* model system of LPS-stimulated human endothelial cells and U937 macrophages and also *in vivo* model of carrageenan-induced paw edema in rat were used. The expression of inflammatory mediator genes was determined by qRT-Real-time PCR. In endothelial cells, soluble forms of ICAM-1 and VCAM-1 were quantified by ELISA.

**Results:** The expression of ICAM-1, VCAM-1, COX2, iNOS, sICAM-1 and sVCAM-1 significantly decreased by amitriptyline. The finding of this study also confirmed that intraperitoneal (i.p.) injection of amitriptyline inhibited carrageenan-induced inflammation in rat paw edema.

**Conclusions:** The results of the present study provide further evidence for the anti-inflammatory effect of amitriptyline. This effect appears to be mediated by down-regulation of inflammatory genes.

**Keywords:** amitriptyline; cyclooxygenase 2; inducible nitric oxide synthase; inflammation; intercellular adhesion molecule-1; vascular cell adhesion molecule-1.

### Resumen

**Contexto:** La amitriptilina, un antidepresivo tricíclico, se utiliza para el tratamiento de los trastornos psicológicos y diversos tipos de dolor. En un trabajo anterior se demostró el efecto inhibitorio de la amitriptilina sobre la migración de polimorfonucleares (PMN) en el sitio de la inflamación.

**Objetivos:** Evaluar el efecto de la amitriptilina sobre la expresión de algunos mediadores inflamatorios tales como molécula de adhesión intracelular (ICAM-1), molécula de adhesión celular vascular (VCAM-1), ciclooxigenasa 2 (COX2) y óxido nítrico sintasa inducible (iNOS).

**Métodos:** Se utilizó un modelo *in vitro* de células endoteliales humanas estimuladas con LPS y macrófagos U937 y también un modelo *in vivo* de edema de la pata inducido por carragenina en rata. La expresión de genes mediadores inflamatorios se determinó por PCR qRT-Real-time. En las células endoteliales, las formas solubles de ICAM-1 y VCAM-1 se cuantificaron por ELISA.

**Resultados:** La expresión de ICAM-1, VCAM-1, COX2, iNOS, sICAM-1 y sVCAM-1 disminuyó significativamente por la amitriptilina. También se confirmó que la amitriptilina intraperitoneal (i.p.) inhibió la inflamación inducida por carragenina en el edema de la pata de la rata.

**Conclusiones:** Los resultados de este estudio proporcionan evidencia adicional para el efecto antiinflamatorio de la amitriptilina. Este efecto parece estar mediado por la regulación hacia abajo de los genes inflamatorios.

**Palabras Clave:** amitriptilina; ciclooxigenasa 2; inflamación; molécula de adhesión celular vascular-1; molécula de adhesión intracelular-1; óxido nítrico sintasa inducible.

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## INTRODUCTION

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The acute inflammatory reaction is characterized by an increase in vascular permeability and cellular infiltration. This event requires interaction between leukocytes and cell-adhesion molecules (CAMs) that are up regulated on the vascular endothelium (Albelda et al., 1994). Subsequently, it leads to extravasation of fluid, accumulation of leukocytes at the inflammatory site and resulting edema formation. There are a variety of inflammatory mediators such as histamine, bradykinin, serotonin, nitric oxide (NO), and prostaglandins (PG), which are involved in the increased vascular permeability (Posadas et al., 2004).

Amitriptyline is classified as a tricyclic antidepressant with both serotonergic and noradrenergic reuptake inhibitory effects. It is widely used in the management of major depression and different types of pain, including neuropathic pain or migraines (Bansal et al., 2009). In previous studies has been demonstrated that amitriptyline induces a considerable anti-inflammatory activity on carrageenan-evoked paw edema in rats (Gurgel et al., 1999; Hajhashemi et al., 2010). Amitriptyline inhibits the release of pro-inflammatory cytokine such as IL-1 $\beta$  and TNF- $\alpha$  and decreases the migration of polymorphonuclear (PMN) leucocytes into the site of inflammation (Sadeghi et al., 2011). Moreover, it inhibits natural killer cell activity as well as nitric oxide and prostaglandin E<sub>2</sub> production (Yaron et al., 1999). Association between immune activation and depression disorder has been studied extensively. It has been shown that pro-inflammatory cytokines such as INF- $\alpha$  or IL-2 could induce depressive symptoms. Inflammatory cytokines have been demonstrated to increase malfunctioning of noradrenergic and serotonergic neurotransmission (Hashioka et al., 2009). It can be hypothesized that if inflammation plays a causative role in the pathogenesis of major depression, antidepressants may partially act by attenuating pro-inflammatory cytokine production or function. Several *in vitro* and *in vivo* studies have studied the anti-inflammatory effect of antidepressants on central nerves system as well as peripheral inflammations (Taler et al., 2007; Tynan et al., 2012). Despite the definite anti-inflammatory effect of

antidepressants, little is known about the precise mechanisms involved.

This study attempts to investigate further the possible molecular mechanisms involved in anti-inflammatory properties of amitriptyline. Therefore, we evaluated the effect of amitriptyline on the expression of ICAM-1, VCAM-1, iNOS and COX2 using an *in vitro* model of LPS stimulated human U937 macrophage cells and endothelial cells (HUVEC) as well as in carrageenan-induced paw edema in a rat model.

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## MATERIAL AND METHODS

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### Chemicals

Human monocytic cells (U937) and human umbilical vein endothelial cell (HUVEC) were bought from Pasteur Institute (Tehran, Iran). [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), RPMI 1640 cell culture medium, Dulbecco's minimal essential medium (DMEM), trypsin-EDTA and fetal bovine serum (FBS) were purchased from Gibco (USA). ELISA-based kits (96 wells) for sICAM-1 (BMS201) and sVCAM-1 (BMS232) purchased from eBioscience. Phorbol myristate acetate (PMA), lipopolysaccharide (LPS) from *E. coli* 055:B5, dimethyl sulfoxide (DMSO) and dexamethasone were purchased from Sigma-Aldrich (USA) and carrageenan ( $\lambda$ ) were obtained from Fluka Chemical (Switzerland). Amitriptyline hydrochloride was donated by Iran Daru Pharmaceutical Co. (Tehran, Iran), for cells was dissolved in phosphate buffer saline (PBS) and for rat injection in isotonic saline.

### Endothelial cell culture

HUVECs were cultured in Dulbecco's minimal essential medium (DMEM). Fetal bovine serum (10%) was added to DMEM at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. During the growth phase antibiotics, penicillin (100 U/mL) and streptomycin (100  $\mu$ g/mL) were added to the cell culture and removed prior to experimental manipulation. At 70–80% confluency, cells were washed with PBS solution pH 7.4, harvested with 0.025% trypsin – 0.01% EDTA. For the experimental tests

cells were transferred to desired plates. For endothelial cells stimulation, different amount of LPS were added to the cells and the expression of inflammatory mediators (ICAM-1 and VCAM-1) were evaluated, then the best concentration of LPS was chosen (Rafiee et al., 2016b).

For gene expression analysis, cells were pelleted in 6 well plates at a density of  $7 \times 10^5$  cells/well. After 24 h incubation, cells were treated with various amount of amitriptyline from  $10^{-8}$  M to  $10^{-6}$  M. One hour later, proper concentration of LPS (1  $\mu\text{g}/\text{mL}$ ) was added. Control cells and LPS (1  $\mu\text{g}/\text{mL}$ ) alone treated cells were also included. Control cells were incubated in DMEM alone (without LPS or component).

### Human U937 macrophages cell culture

The human monocyte cell line U937 was grown in complete RPMI 1640 medium (Gibco) enriched with 10% FBS and incubated in  $37^\circ\text{C}$  with the humidified atmosphere of 95% air and 5%  $\text{CO}_2$ . During the growth phase antibiotics, penicillin (100 U/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ), were added to the cell culture and removed prior to experimental manipulation.

To differentiate monocytes into adherent macrophages, they were seeded at a density of  $5 \times 10^5$  cells/well and incubated for 48 hours in the presence of PMA at the final concentration of 100 nM into the cellular medium. The cells then were washed and incubated in normal growth medium for additional 24 hours prior to the addition of LPS (1  $\mu\text{g}/\text{mL}$ ). Different concentrations of amitriptyline from  $10^{-8}$  M to  $10^{-6}$  M were added to the medium 1 hour before addition of LPS (1  $\mu\text{g}/\text{mL}$ ).

### Cell viability assay

MTT assay was used to evaluate the toxicity of various concentrations of amitriptyline and LPS on cells.

HUVECs and monocyte-derived macrophages were seeded in 96 well plates at a concentration of  $1 \times 10^4$  cells per well. Cells were incubated for 24 h with various amounts of LPS and different concentrations of amitriptyline from  $10^{-8}$  M to  $10^{-6}$  M at  $37^\circ\text{C}$ . After incubation, the medium was replaced with 100  $\mu\text{L}$  RPMI 1640 phenol red free. Then 10  $\mu\text{L}$  of (12 mM) MTT [3-(4, 5-dimethylthiazol-2-yl)-2,

5-diphenyltetrazolium bromide] (Gibco) stock were added to each well. The ability of the cells to convert MTT shows mitochondrial activity and in consequence cell viability. The cells were incubated for 4 h at  $37^\circ\text{C}$ . Finally the MTT crystals were dissolved by adding 50  $\mu\text{L}$  of dimethyl sulfoxide (DMSO, Sigma-Aldrich) solution and the formazan blue dye was read in a microplate reader (BioTek Instruments, Epoch, USA) at 570 nm (Rafiee et al., 2016b).

### Enzyme-linked immunosorbent assay (ELISA)

For ELISA assay, endothelial cells were seeded in 24-well plate at a density of 40000 cell per well. Different concentration of amitriptyline from  $10^{-8}$  M to  $10^{-6}$  M was added to the medium 1 hour before addition of LPS (1  $\mu\text{g}/\text{mL}$ ). Then cells were incubated for 24 h at  $37^\circ\text{C}$ . Cell culture supernatants were collected after 24 h incubation and frozen at  $-80^\circ\text{C}$  before analysis by ELISA. The quantitative detection of sICAM-1 and sVCAM-1 was performed using a commercially available ELISA kit by Bender MedSystems (eBioscience). The assays were performed according to the protocols set by manufacturers. The concentration of sICAM-1 and sVCAM-1 was expressed as ng/mL. All experiments were repeated at least three times.

### Animals

Male Wistar rats (200–250 g) were obtained from the animal house of the Faculty of Pharmacy, Isfahan University of Medical Sciences, Iran. Animals were held four per cage in a standard polypropylene cages with free access to food and water, under a 12:12 h light/dark cycle. The experiments were performed in agreement with local guidelines for the care of laboratory animals of the Isfahan University of Medical Sciences.

### Carrageenan-induced paw edema

Rats received a subplantar injection of 100  $\mu\text{L}$  of a 1% (w/v) suspension of carrageenan lambda in the right hind paw (Winter et al., 1962). The volume of the paw was measured by a plethysmometer (Ugo Basile, Italy) immediately before and after 4 h of the carrageenan injection. Data were presented as the

increase in paw volume (mL) and compared with control values.

**Experimental design:** Different doses were applied in this study (25 and 50 mg/kg); according to our previous report (Sadeghi et al., 2011). Five groups (n=6 rats in each group) were used. Amitriptyline was given intraperitoneally 30 min before subplantar injection of carrageenan (Rafiee et al., 2016a). The control group received only vehicle. A group of animals pretreated with dexamethasone (1 mg/kg, i.p.) served as the positive control. Paw volumes (mL) were determined prior to carrageenan injection and at the end of the experiment (4 hours later). Then, animals were sacrificed and the inflamed paw tissues (30 mg) were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until they were used for RT-PCR analyses.

### Real-Time PCR

RT-PCR was performed for the detection of the mRNA expressions of VCAM-1, ICAM-1, COX2 and iNOS. According to previous report (Rafiee et al., 2016a), total RNA was isolated from endothelial cells, U937 macrophages and homogenized paw tissues by Gene Jet RNA purification kit (Thermo Scientific, (EU) Lithuania) according to the manufacturer's instructions. The concentration and quality of RNA

preparations were determined by a spectrophotometer (BioTek Instruments, Epoch, USA) and gel electrophoresis. Standardized amounts of RNA were reverse-transcribed to cDNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific, (EU) Lithuania) according to manufacturer's protocol.

The primer sequences for VCAM-1, ICAM-1, COX2, iNOS and housekeeping gene  $\beta$ 2-microglobulin for human cell lines and rat were designed from the sequence list of GeneBank database (National Centre for Biotechnology Information, NCBI) using Beacon Designer eight software and then blasted against GeneBank database sequences. Primer sequences are shown in Table 1.

Real-Time PCR was performed using SYBRGreen (Thermo Scientific, (EU) Lithuania) detection in Corbett machine, Rotorgene 6000 (Australia). Master Mix in each reaction tube included cDNA, dH<sub>2</sub>O, SYBR Green, forward and reverse primer of genes of interest.

The cycling conditions were as follows: initial denaturation at  $95^{\circ}\text{C}$  for 3 minutes and amplification for 45 cycles ( $95^{\circ}\text{C}$  for 12 sec for the denaturation,  $60^{\circ}\text{C}$  for 45 sec for the annealing and extension). The relative amount of gene expression, normalized to the internal control  $\beta$ 2-microglobulin.

**Table 1.** Primers sequences of interested genes in human and rat (Rafiee et al, 2016a).

Genes	Homo sapiens	Rattus norvegicus	
	Sequences (5'-3')	Genes	Sequences (5'-3')
ICAM-1	F: ACGGTGCTGGTGAGGAGAG R: TCGCTGGCAGGACAAAGGT	ICAM-1	F: CCACCATCACTGTGTATTCGTTCC R: ACGGAGCAGCACTACTGAGAG
VCAM-1	F: GCAAGTCTACATATCACCCAAG R: TCACAGAGCCACCTTCTT	VCAM-1	F: CTACATCCACACTGACGCTGAG R: CAGGGAATGAGTAGACCTCCACTT
COX2	F: TGCAGTGAGCGTCAGGAG R: CAAGGATTTGCTGTATGGCTGAG	COX2	F: ATTCTTTGCCAGCACTTCACT R: CCTCTCCACCGATGACCTGATA
iNOS	F: GTCACCTACCACCCGAGATG R: CGCTGGCATTCCGCACAA	iNOS	F: GAGAAGTCCAGCCGCACC R: CAATCCACAACCTCGCTCCAAGA
$\beta$ 2-microglobulin	F: TAGTCGCCGTGCCTACCA R: TGCTGCCTTCTTGGATGT	$\beta$ 2-microglobulin	F: GTTGGTTTTCCGGAAGTGGGC R: GTCGGCATCGTTTATGGTCC

Validation of the reference gene (18S rRNA) and the amplification efficiencies of targets and reference were performed (Livak and Schmittgen, 2001). The fold-change for each sample was analyzed by the  $2^{-\Delta\Delta CT}$  method. The  $2^{-\Delta\Delta CT}$  values obtained from these analyses directly reflect the relative mRNA quantities for the specific gene in response to a particular treatment. Amplification of specific transcripts was further confirmed by obtaining melting curve profiles (Rafiee et al., 2016a). All samples were run in triplicate.

### Statistical analysis

The data are presented as means  $\pm$  SEM. One-way ANOVA (analysis of variance) was performed to define the significance of treatment. Tukey post-hoc test was used to determine the specificity of treatment, to define differences between control and treatment groups. Differences were considered as significant at  $p < 0.05$ . Statistical analysis was performed using the SPSS 19 software.

## RESULTS

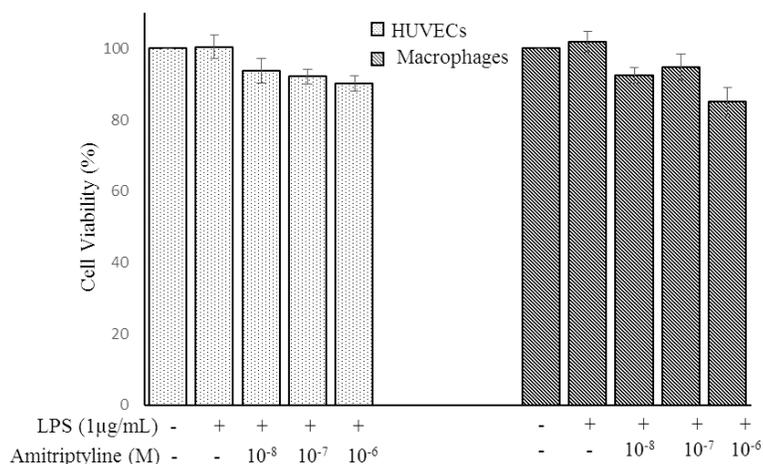
### Cell viability

HUVECs and U937 macrophages were treated with increasing amount of amitriptyline ( $10^{-8}$  M to  $10^{-6}$  M) and LPS ( $1 \mu\text{g/mL}$ ) for 24 h and cell viability was measured using MTT assay. Data obtained from both endothelial cells and U937 macrophages

illustrated that LPS alone and in combination with different concentrations of amitriptyline from  $10^{-8}$  M to  $10^{-6}$  M had no significant effect on cell viability as compared to untreated cells (Fig. 1).

### Amitriptyline inhibits ICAM-1 and VCAM-1 expression in LPS-induced endothelial cells

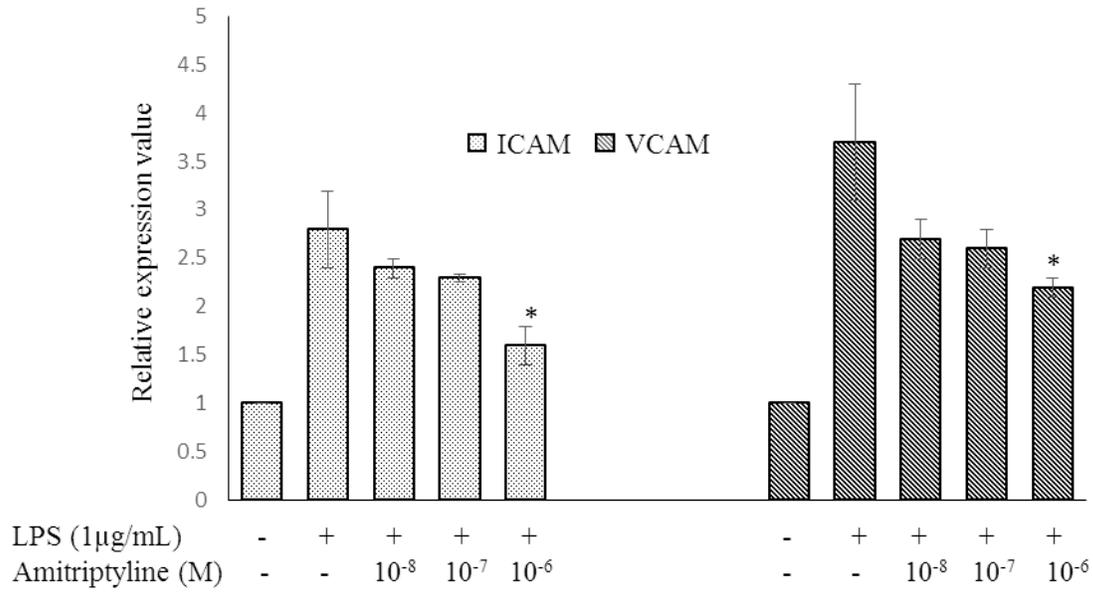
It was investigated whether amitriptyline could inhibit the expression of ICAM-1 and VCAM-1 in LPS-induced endothelial cells. Treatment of HUVECs with amitriptyline decreased the expression of ICAM-1 and VCAM-1 mRNA and the protein levels. Endothelial cells were treated with increasing concentration of LPS and then the mRNA levels of ICAM-1 and VCAM-1 were measured. LPS increased the expression of ICAM-1 and VCAM-1 in a concentration-dependent manner (data not shown) (Rafiee et al., 2016b). One  $\mu\text{g/mL}$  of LPS significantly increased the ICAM-1 and VCAM-1 genes expression with less toxicity. As shown in Fig. 2a, amitriptyline just at the concentration of  $1 \mu\text{M}$  but not  $0.1$  or  $0.01 \mu\text{M}$  significantly decreased the mRNA levels of ICAM-1 ( $p = 0.012$ ) and VCAM-1 ( $p = 0.021$ ) in LPS stimulated endothelial cells in comparison to cells treated only with LPS. As indicated in Fig. 2b, HUVECs after treatment with LPS for 24 h showed enhanced sICAM-1 and sVCAM-1 levels compared with unstimulated cells. When LPS-stimulated HUVECS were incubated with amitriptyline for 24 h, the concentrations of soluble forms of VCAM-1 and ICAM-1 were significantly diminished.



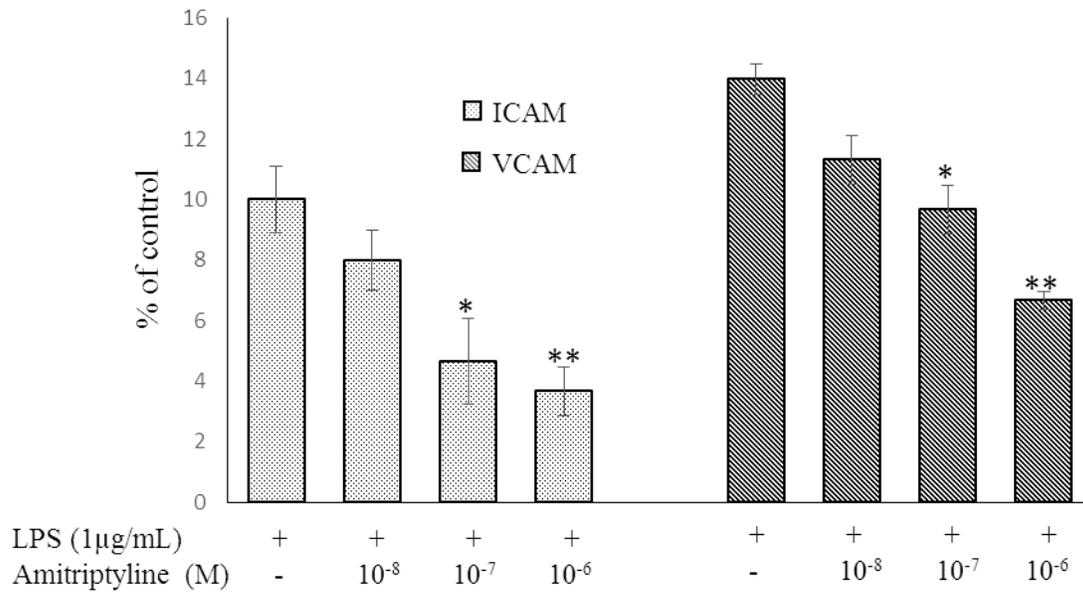
**Figure 1.** Effects of amitriptyline and LPS on cell viability in HUVECS and U937 macrophages.

Values are presented as mean  $\pm$  SEM of three independent experiments.

**A**



**B**



**Figure 2.** Amitriptyline inhibits mRNA (A) and protein (B) expression of ICAM-1 and VCAM-1 in HUVECs.

(A), fold changes relative to control are presented as mean ± SEM.

(B), data are presented relative to control (untreated cells).

Each bar represents the mean ± SEM of triplicate determinations. \*p < 0.05, \*\*p < 0.01 compared with LPS alone treated group.

### **Amitriptyline-mediated inhibition of LPS-induced COX<sub>2</sub> and iNOS expression in U937 macrophages**

It was also evaluated the effect of amitriptyline on the expression of COX<sub>2</sub> and iNOS in U937 macrophages stimulated with LPS. As demonstrated in Fig. 3, amitriptyline alone, at 10<sup>-6</sup> M, significantly decreased COX<sub>2</sub> expression (p = 0.006). However, the expression of iNOS decreased considerably by amitriptyline at the 10<sup>-6</sup> M (p = 0.019) and 10<sup>-7</sup> M (p = 0.033) as compared with LPS alone treated group (Fig. 3).

### **Dose-dependent effect of amitriptyline i.p. injection on carrageenan-induced paw edema**

The *in vivo* anti-inflammatory effect of amitriptyline was performed by carrageenan-induced paw edema test (Hajhashemi et al., 2010). As shown in Fig. 4, i.p. injection of 25 and 50 mg/kg amitriptyline resulted in 33% and 56% reduction in paw edema development, respectively, after the induction of inflammation as compared to the control group. As expected, the reference drug, dexamethasone (1 mg/kg), also caused a significant inhibition (74%) of post-carrageenan edema (Fig. 4).

### **The inhibitory effect of amitriptyline on the expression of ICAM-1 and VCAM-1 on carrageenan-induced paw edema in rats**

The levels of ICAM-1 and VCAM-1 mRNAs in carrageenan-induced paw edema after injection of amitriptyline were measured. As demonstrated in carrageenan injection considerably elevated expression of mentioned pro-inflammatory mediators. Remarkably, pretreatment with amitriptyline significantly reduced the expression of ICAM-1 (p = 0.038) and VCAM-1 (p = 0.034) at 50 mg/kg. Moreover, dexamethasone considerably decreased the expression of ICAM-1 and VCAM-1 in carrageenan-induced paw edema (Fig. 5a).

### **The inhibitory effect of amitriptyline on the expression of COX<sub>2</sub> and iNOS in carrageenan-induced paw edema in rats**

As illustrated in Fig. 5b, amitriptyline at 25 mg/kg (p = 0.049) and 50 mg/kg (p = 0.003) significantly decreased the expression of COX<sub>2</sub>. Simi-

larly, expression of iNOS was reduced at 25 mg/kg (p = 0.033) and 50 mg/kg (p = 0.001) by amitriptyline. As expected, the reference drug, dexamethasone (4 mg/kg), caused a significant reduction in iNOS and COX<sub>2</sub> mRNA levels in carrageenan induced paw edema (Fig. 5b).

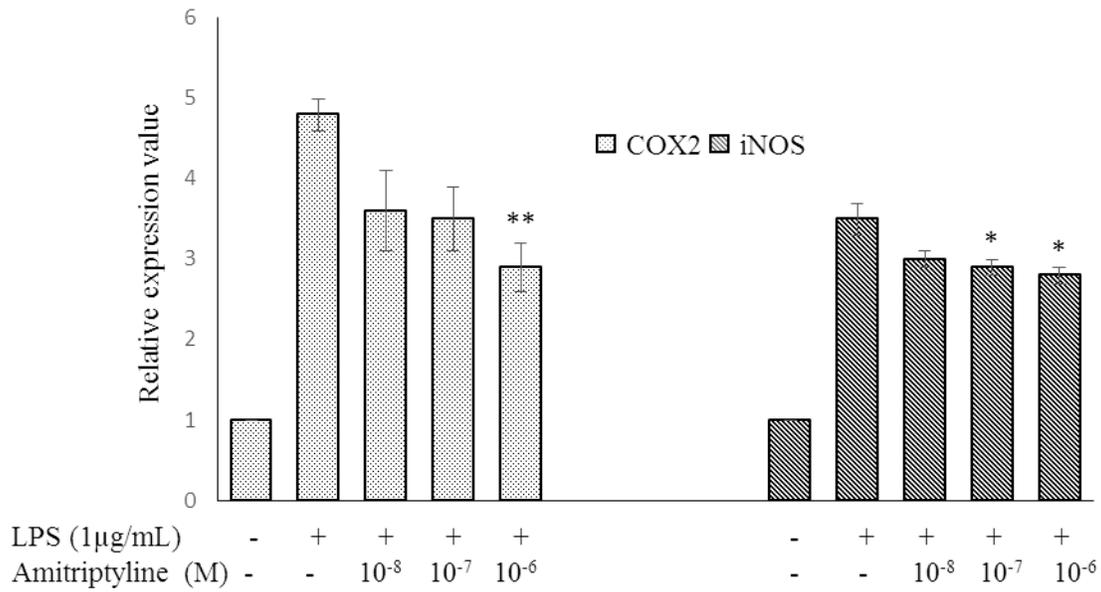
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## **DISCUSSION**

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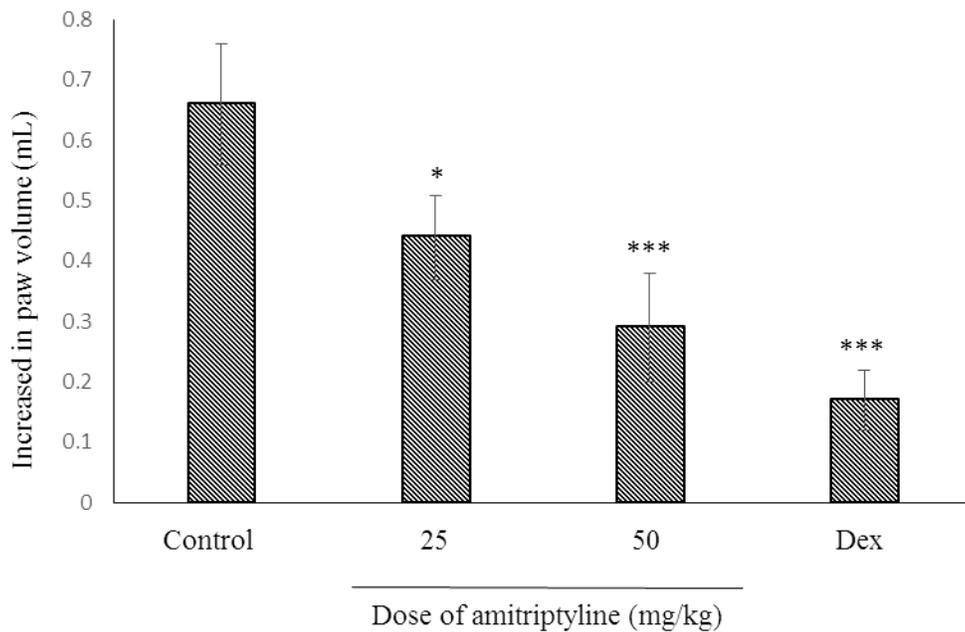
The present study was performed to investigate the potential anti-inflammatory effects of amitriptyline and to clarify the molecular mechanism(s) involved. The findings of this study obviously showed that amitriptyline suppressed the expression of inflammatory mediators both *in vitro* and *in vivo*. In the inflammatory process the main event is the adhesion of circulating monocytes to the endothelial cells (Albelda et al., 1994; Panés et al., 1999). This attachment of monocyte to endothelial cells and transmigration into the intimal spaces is mediated by the adhesive molecules such as VCAM and ICAM (Albelda et al., 1994). As it is shown in results, amitriptyline decreases the expression of ICAM-1 and VCAM-1 in mRNA and protein levels in LPS-stimulated endothelial cells. These results are confirmed by other investigators who have shown that serotonin specific reuptake inhibitors (SSRIs) such as fluvoxamine, fluoxetine and citalopram decreases the TNF-alpha-induced endothelial expression of VCAM-1 and ICAM-1 (Lekakis et al., 2010).

Several reports showed that human peripheral blood mononuclear cells as well as central nervous system possess serotonin transporter, and might be directly affected by antidepressants (Urbina et al., 1999; Fazzino et al., 2008). Moreover, serotonin and noradrenaline are released from lymphocytes and monocytes (Finocchiaro et al., 1988), and can prompt immunomodulatory properties via receptors that are present on immune cells. Therefore, in present study, it was investigated the influence of amitriptyline on monocytes to evaluate if the anti-inflammatory effect of amitriptyline can be mediated by macrophages. The present study showed that amitriptyline also attenuated the expression of iNOS and COX<sub>2</sub> in macrophages. iNOS provokes synthesis of nitric oxide (NO), which is a free radical molecule and could cause cellular damage in inflammatory sites.



**Figure 3.** Effect of amitriptyline on LPS-induced expression of iNOS and COX<sub>2</sub> in U937 macrophages.

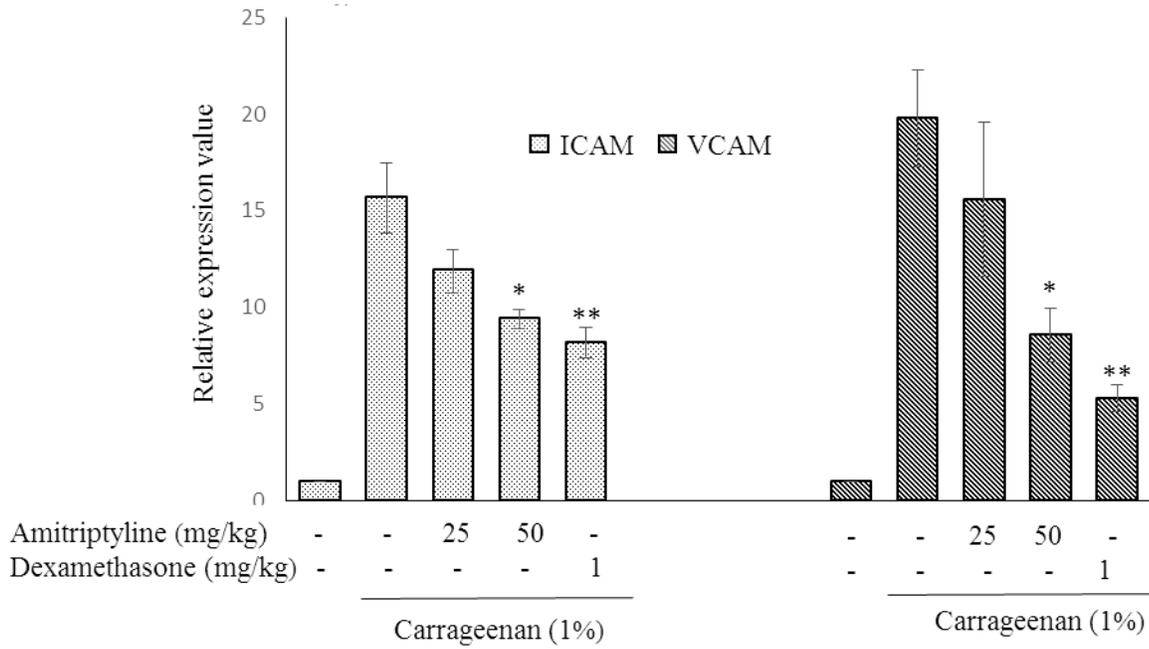
The expression level of control (untreated) was set to 1. Fold changes relative to control are presented. Mean ± SEM values of experiments are shown. \*p < 0.05, \*\*p < 0.01, compared with LPS alone treated group.



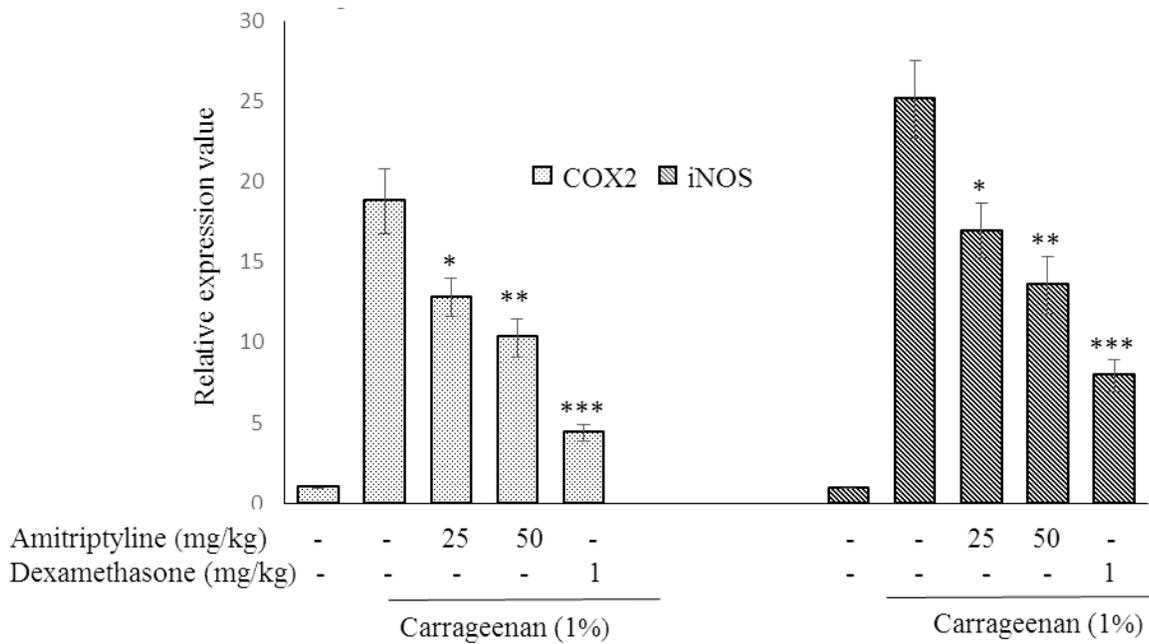
**Figure 4.** Effect of intraperitoneal administration of amitriptyline on carrageenan-induced paw edema in the rat.

The values are represented as mean changes in the paw volume ± SD (n = 6, \*\*\*p < 0.001 compared to control group). Dex: dexamethasone.

A



B



**Figure 5.** Effect of amitriptyline on ICAM-1, VCAM-1 (A), iNOS, and COX<sub>2</sub> (B) gene expression in carrageenan-induced paw edema in rats.

Fold changes relative to control are presented. Mean ± SEM values of experiments are shown. \*p < 0.05, \*\*p < 0.01, \*\*\* p < 0.001 compared to carrageenan only received group.

On the other hand, COX<sub>2</sub> is catalyzing the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) from arachidonic acid (DeWitt, 1991). In line with these results, Taler et al. (2007) reported that the expression of COX<sub>2</sub> decreased with SSRIs (Selective Serotonin Reuptake Inhibitors) on human T lymphocytes. Moreover, amitriptyline and fluoxetine were shown to attenuate the production of pro-inflammatory cytokine-induced PGE<sub>2</sub> and NO by cultured human synovial cells (Yaron et al., 1999). Several other studies also consistently have shown that antidepressants inhibit the NO production (Hashioka et al., 2007; Tynan et al., 2012). In summary, the data indicate that amitriptyline may diminish the inflammatory responses of monocytes; the mechanism could include a cascade of gene expression secondary to effects on the serotonin transporter that is expressed on the surface of monocytes and lymphocytes.

In this study, it has been shown that the expression levels of ICAM-1, VCAM-1, COX<sub>2</sub> and iNOS were reduced in a concentration-dependent manner. Of note, this concentration of amitriptyline is actually within the therapeutic plasma level of this medication (1 μM or below). Many studies used concentrations, which were higher than therapeutic concentrations of antidepressant, and therefore better inhibitory effects were observed (Diamond et al., 2006; Taler et al., 2007). Thus, it is plausible that a higher concentration would potentially inhibit the expression of indicated inflammatory mediators.

The anti-inflammatory effect of antidepressants cannot be obtained just by *in vitro* study, because various additional cell types such as T cells and epithelial cells are involved and may affect the function of amitriptyline. Therefore, we confirmed the anti-inflammatory activity of amitriptyline in an *in vivo* carrageenan-induced paw edema model. The findings showed that i.p. injection of amitriptyline inhibited the development of paw edema over a period of 4 h following carrageenan injection (Hajhashemi et al., 2010). The effect of amitriptyline on ICAM-1 and VCAM-1 gene expression was also evaluated in inflamed paw. Similar to the *in vitro* study, the expression of ICAM-1 and VCAM-1 was attenuated by amitriptyline in this animal model. Based on previous study, amitriptyline elicited a

marked reduction in the infiltration of PMN leucocytes into the carrageenan-treated paws (Sadeghi et al., 2011). Additionally, Gurgel et al. (1999) have illustrated the inhibition of neutrophil migration by heterocyclic antidepressants such as amitriptyline and clomipramine. Collectively, these data suggest that amitriptyline possibly decreases the PMN migration by attenuating some endothelial adhesion molecules expression such as ICAM and VCAM. In agreement with our findings, Achar et al. (2009) demonstrated that amitriptyline significantly reduced the expression of ICAM in a mouse model of unilateral ureteral obstruction. In addition, COX<sub>2</sub> and iNOS expression were determined, and again amitriptyline exerted a significant inhibition of COX<sub>2</sub> and iNOS gene expression at mRNA level.

The exact mechanism by which antidepressants exert their anti-inflammatory effects remains to be elucidated. Several studies have illustrated that many antidepressants increase intracellular concentrations of cAMP through activation of monoamine receptors such as serotonin and noradrenaline receptors. Another *in vitro* study proposed that the anti-inflammatory effects of various antidepressants on microglia are at least partially mediated by the cAMP-dependent protein kinase A (PKA) pathway (Hashioka et al., 2007). In some cell types, it has been shown that cAMP/PKA pathway inhibit the NF-κB activity (Delfino and Walker, 1999), and its activation is identified to induce the expression of iNOS and various pro-inflammatory cytokines in human monocytes. Here endothelial cells and macrophages were stimulated by LPS. Binding of LPS to toll-like receptor 4 (TLR<sub>4</sub>) activates two key signaling pathways, which result in activation of the transcription factor, NF-κB, an important upstream modulator for ICAM, VCAM, COX<sub>2</sub> and iNOS expression (Sharif et al., 2007). Moreover, the carrageenan used in our *in vivo* model can induce innate immune pathways of inflammation mediated by TLR<sub>4</sub> and BCL10. Carrageenan exposure leads to NF-κB activation by both canonical, involving RelA (p65) and p50, and non-canonical, involving RelB and p52 (Borthakur et al., 2012; Abkhezr and Dryer, 2015). Some studies showed that some antidepressants exert their anti-inflammatory effect by inhibition of NF-κB signaling pathway (Koh et al., 2011). Amitriptyline also has been

shown to mediate its anti-inflammatory effect by p38 mitogen-activated protein kinase (Tai et al., 2009). Based on previous studies and present findings, it can be suggested that amitriptyline evokes its suppressive effect on the expression of ICAM-1, VCAM-1, COX2 and iNOS by modulating NF- $\kappa$ B pathway (Achar et al., 2009). To our knowledge, this is the first study to evaluate the anti-inflammatory effect of amitriptyline through the inhibition of ICAM-1, VCAM-1, COX2 and iNOS gene expression in an *in vitro* and *in vivo* model.

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## CONCLUSIONS

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The results of this study provide further evidence for the anti-inflammatory effect of amitriptyline via inhibition of ICAM-1, VCAM-1, COX2 and iNOS gene expression based on *in vivo* and *in vitro* findings. Further studies are needed to evaluate whether these effects are related to neurotransmitters such as norepinephrine and/or serotonin or not.

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## CONFLICT OF INTEREST

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The authors declare no conflict of interest.

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**Author contribution:**

Contribution	Rafiee L	Hajhashemi V	Haghjooy Javanmard S
Concepts or Ideas	X	X	X
Design	X	X	X
Definition of intellectual content	X	X	X
Literature search	X	X	
Experimental studies	X	X	
Data acquisition	X		
Data analysis	X		
Statistical analysis	X		
Manuscript preparation	X	X	
Manuscript editing	X	X	
Manuscript review	X	X	X

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