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A study on the mechanisms involving the anti-inflammatory effect of amitriptyline in carrageenan-induced paw edema in rats

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ABSTRACT

Anti-inflammatory effects of antidepressants have been reported in some studies, but the mechanisms underlying these effects remain unknown. Amitriptyline, a tricyclic antidepressant, is widely used in the management of psychological disorders and various types of pain, including neuropathic pain or fibromyalgia. In our previous work, we found the role of supraspinal mechanisms in the anti-inflammatory effect of amitriptyline. In the line of the indicated study, we sought to evaluate the effects of intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) application of amitriptyline in the carrageenan-induced paw edema in rats in more details. Our findings confirmed that i.p. (40 and 80 mg/kg) and i.c.v. (100 μg/rat) injection of amitriptyline inhibited carrageenan-induced inflammation at different times. We also found that both i.p. and i.c.v. amitriptyline significantly decreased migration of polymorphonuclear (PMN) leucocytes into the site of inflammation, according to pathological evidence and the activity of myeloperoxidase (MPO). Furthermore, i.p. amitriptyline at the applied doses markedly reduced interleukin (IL)-1β and tumor necrosis factor (TNF)-α levels in the paw treated with carrageenan. Our results also showed that i.c.v. amitriptyline noticeably decreased the concentration of IL-1β in the inflamed paws. The TNF-α levels reduced in the i.c.v. group, even though these reductions were not statistically significant. These results confirmed the anti-inflammatory effects of systemic and central amitriptyline in the carrageenan-induced paw edema in rats, and demonstrated that these effects mediated mostly through the inhibition of PMN cells migration and release of IL-1β and TNF-α into the site of inflammation.

1. Introduction

A growing body of evidence suggests that antidepressant drugs are able to produce negative immunoregulatory effects that participate in the pharmacological effects (Janssen et al., 2010; Brustolim et al., 2006; Castanon et al., 2002). In this context, one aspect that has been investigated by some researchers is the anti-inflammatory properties of antidepressants in a number of experimental models of inflammation (Hajhashemi et al., 2010a; Abdel-Salam et al., 2003; Maes et al., 1999; Bianchi et al., 1995). However, the underlying mechanisms in these effects of antidepressant medications remain unclear. In earlier studies, we demonstrated that systemic amitriptyline, as a tricyclic antidepressant, exhibited a considerable anti-inflammatory activity on carrageenan-evoked paw edema in rats (Hajhashemi et al., 2010b). Similar findings about the anti-inflammatory effects of systemic amitriptyline had been reported in other studies (Hajhashemi et al., 2008; Abdel-Salam et al., 2003). We also found that anti-inflammatory effects of amitriptyline were partly mediated through the supraspinal sites (Hajhashemi et al., 2010b).

Carrageenan-induced paw edema is a well-known acute model of inflammation that is broadly used for evaluation of anti-inflammatory activity of different compounds. Carrageenan-induced paw edema divides into two phases: The early phase observed around 1 h is related to the release of histamine, serotonin, bradykinin, and to a less extent prostaglandins, whereas the delayed phase (after 1 h) is attributed to polymorphonuclear (PMN) leucocytes infiltration, and the continuing of the prostaglandin generation (Gilligan et al., 1994; Di Rosa et al., 1971). Release of PMN leucocytes derived reactive oxygen species and free radicals, nitric oxide and pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, and interleukin-1β (IL-1) also involved in the delayed phase of carrageenan-induced inflammation (Halici et al., 2007; Nacife et al., 2004).

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To the best of our knowledge, there are limited evidence about the mechanisms by which anti-inflammatory effects of amitriptyline might be mediated (Hajhashemi et al., 2010b; Martelli et al., 1967). Therefore, the aims of the present study were to (a) evaluate the effect of amitriptyline on the PMN leucocytes infiltration into the site of inflammation, and (b) investigate the role of pro-inflammatory cytokines such as TNF-α and IL-β in the anti-inflammatory effect of amitriptyline.

2. Materials and methods

2.1. 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 housed in standard polypropylene cages, four per cage, under a 12:12 h light/dark cycle with free access to food and water. Following surgical implantation of an i.c.v. cannula, animals were housed one per cage to avoid possible dislocation of the cannula. The experiments were carried out in accordance with local guidelines for the care of laboratory animals of the Isfahan University of Medical Sciences.

2.2. Chemicals

Amitriptyline hydrochloride was donated by Iran Daru Pharmaceutical Co. (Tehran, Iran) and was dissolved in isotonic saline. Carrageenan (lambda) was purchased from Fluka Chemical (Switzerland) and was dissolved in isotonic saline. Indomethacin (Sigma, USA) was suspended in aqueous carboxy methylcellulose (2% w/v). Hexadecyl trimethylammonium bromide (HTAB), aprotinin A, bovine serum albumin, phenylmethylsulfonyl fluoride, benzethonium chloride, ethylenediaminetetraacetic acid (EDTA), and Tween 20 were all purchased from Sigma Chemical Company (St. Louis, MO, USA). TNF-α (R&D Company, USA), and IL-1β (ALPCO, USA) kits were used for measurement of biochemical parameters.

2.3. Surgical procedure

The animals were anesthetized with i.p. injection of a ketamine (50 mg/kg) and xylazine (10 mg/kg) mixture. Then, the animals were placed in a stereotaxic frame (Stoelting, USA), and an i.c.v. cannula was implanted with stereotaxic coordinates: AP, −0.8 mm; L, 1.4 mm; and V, 3.3 mm, according to Paxinos and Watson (Budantsev et al., 1993). The animals were handled daily for five days before the experiments to acclimatize them to manipulation and minimize nonspecific stress responses. Rats with the i.c.v. cannulas were euthanized at the end of the experiments, and their brains were examined to confirm the correct implantation of the cannula.

2.4. Carrageenan-induced paw edema

The rats received a subplantar injection of 100 μl 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 prior to carrageenan injection and then at 1, 2, 3 and 4 h after that. The data were expressed as the variation in the paw volume (ml) and were compared to pre-injection values.

2.5. Experimental design

All doses that applied in the present study were chosen according to our previous studies (Hajhashemi et al., 2010b).

In the first series of experiments, effect of i.p. amitriptyline (40 and 80 mg/kg, n = 6) on paw edema induced by carrageenan was studied. Amitriptyline was given 30 min before subplantar injection of carrageenan. Paw volumes (ml) were determined prior to carrageenan injection, and then again, at 1, 2, 3 and 4 h after carrageenan to determine the difference in paw volume. Control group received only vehicle (i.p.; n = 6). A group of animals that was pretreated with indomethacin (10 mg/kg; n = 6) was used as the positive control. At the end of the experiments, animals were euthanized, and the inflamed paw tissues were collected for cytokine measurements.

In the second series, we used the i.c.v. route to confirm our previous finding about the involvement of supraspinal levels in the anti-inflammatory effect of amitriptyline (Hajhashemi et al., 2010b). The drug was administered smoothly for 1 min through the injection cannula (100 μg/rat, n = 6) 30 min prior to carrageenan in a volume of 10 μl and the paw volumes were recorded according to the indicated method in the previous step. The control group received vehicle (i.c.v.; 10 μl; n = 6). At the end of the experiments, animals were euthanized, and the inflamed paw tissues were collected for cytokine measurements.

In the third series, the objective was to evaluate the effect of amitriptyline on the PMN leucocytes infiltration in the inflamed paw skin. Briefly, rats were randomly divided into 4 groups (8 rats in each group): control (vehicle group), systemic amitriptyline (40 and 80 mg/kg, i.p.), central amitriptyline (100 μg/rat, i.c.v.) and indomethacin (10 mg/kg, i.p.). Half an hour after injection of the indicated treatment, animals received carrageenan and then 4 h after injection of carrageenan, animals were euthanized, and the inflamed paws were collected for pathological and the activity of MPO evaluation.

2.6. Histopathologic examination

Three samples of the carrageenan-treated paws in the third series were removed and fixed by immersion in 10% formaldehyde solution for several days. After that, the fixed tissues were embedded in paraffin and cut into 3–4 μm slices. The slices were mounted on the glass slides and stained with hematoxylin and eosin for light microscopy analysis. The assessment was conducted by a pathologist in a blinded way.

2.7. Determination of MPO activity

MPO activity was measured according to the modified method of Bradley (Bradley et al., 1982). The subcutaneous tissue of carrageenan-injected paws of five samples in the third series was removed and weighted then each sample was finely chopped in 1 ml of 50 mM potassium phosphate buffer containing 0.5% HTAB. The chopped tissue was transferred to a homogenizing tube and the container was rinsed with 2 × 1 ml HTAB in buffer solution. More buffers were added to obtain a concentration equivalent to 5 ml per 0.1 g of paw tissue and homogenized (15,000 rpm) for 4 × 45 s at 1 min intervals. Next, the homogenate was transferred to a sample tube, sonicated in an ice bath for 10 s, then subjected to a sequence of freezing and thawing 3 times, and sonicated again for 10 s. After that, the suspensions were centrifuged at 15,000 rpm for 15 min in 4 °C and then the supernatant decanted for analysis. The MPO activity in supernatants was assessed spectrophotometrically: 0.1 ml of the supernatant was added to 2.9 ml of 50 mM K_3PO_4 buffer (pH = 6.0) containing O-dianisidine dihydrochloride (0.167 mg/ml) and 0.005% hydrogen peroxide. The absorbance of the reaction mixture was measured at 450 nm using a UV–Vis spectrophotometer. MPO activity was expressed in units (U) per gram tissue weight of wet tissue.

2.8. Measurement of the IL-1β and TNF-α levels in the rat paw

Four hours after injection of carrageenan, rats that were assigned for cytokine assays were euthanized by ether and the subcutaneous tissue of carrageenan-injected paws was removed. TNF-α and IL-1β levels in the whole skin of inflamed paws were determined as described

previously (Nacife et al., 2004) by enzyme-linked immunosorbent assay (ELISA). The tissue samples were weighed; snap frozen on liquid nitrogen and stored at −70 °C to be processed for IL-1β and TNF-α determinations. Skin tissue was homogenized in phosphate buffered saline (PBS; pH = 7.4) containing 0.4 M NaCl, 0.05% Tween-20, 0.5% bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, aprotinin A 20 KI, and 10 mM EDTA. The homogenates were centrifuged at 12,000 × *g* for 30 min at 4 °C, and then ELISA was used to measure the levels of IL-1β and TNF-α in the supernatants.

2.9. Statistical analysis

The data are expressed as the means ± S.E.M. The differences between the control and treatment groups were tested by one-way analyses of variance (ANOVA) followed by the Tukey post-hoc test, using SPSS 13.0 software. The probability of $P<0.05$ was considered to show significant differences for all comparisons made.

3. Results

3.1. Effect of i.p. injection of amitriptyline on carrageenan-induced paw edema

As illustrated in Fig. 1, i.p. injection of amitriptyline at doses of 40 and 80 mg/kg significantly inhibited the development of paw edema after the induction of inflammation as compared to the control group. As expected, the reference drug, indomethacin (10 mg/kg), caused a significant inhibition of post-carrageenan edema. As observed, the effect of amitriptyline at the dose of 80 mg/kg on the time course of inflammation was similar to that of indomethacin.

3.2. Effect of i.c.v. injection of amitriptyline on carrageenan-induced paw edema

As shown in Fig. 2, i.c.v. application of amitriptyline (100 μg/rat) produced an inhibitory effect on paw edema formation especially at 3 and 4 h after carrageenan challenge ($P<0.05$) as compared to the control group.

3.3. Pathological examination

As shown in Fig. 3A, there was no sign of inflammation in the paw tissue of normal rats. Specimens from the paw skin of control groups

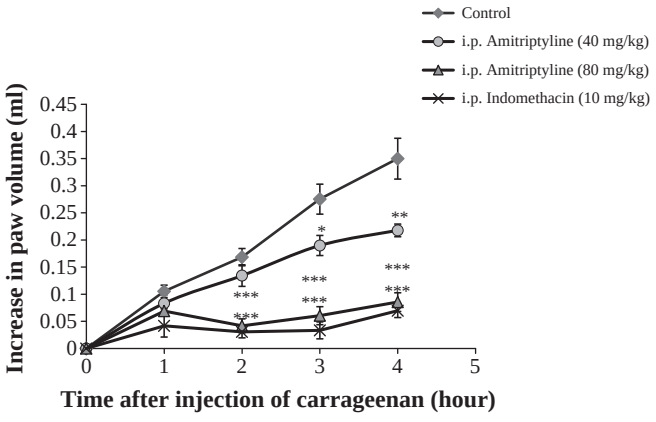

Fig. 1. Effect of i.p. injection of amitriptyline on carrageenan-induced paw edema in rats. Amitriptyline (40 and 80 mg/kg), indomethacin (10 mg/kg) and the vehicle were administered 30 min prior to carrageenan (1%) injection, and the rats were evaluated for paw edema at 1, 2, 3 and 4 h post-carrageenan injection. The values represent the mean variation in the paw volume ± S.E.M. (n = 6, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

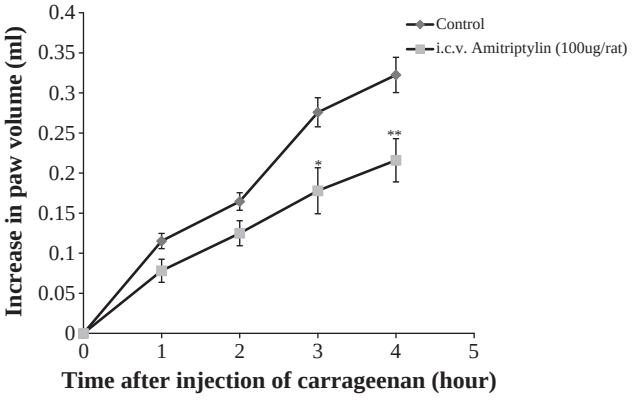

Fig. 2. Effect of i.c.v. injection of amitriptyline on carrageenan-induced paw edema in rats. Amitriptyline or the vehicle was administered 30 min prior to carrageenan (1%) injection, and the rats were evaluated for paw edema at 1, 2, 3, and 4 h post-carrageenan injection. The values represent the mean variation in the paw volume ± S.E.M. (n = 6, $^{*}P<0.05$, $^{**}P<0.01$).

(vehicle-treated rats) showed an acute inflammation in the dermis and epidermis with extensive extravasations, mainly PMN leucocytes along with less lymphocyte. There was also accumulation of PMN around capillaries in dermis.

As observed in Fig. 3(C and D), i.p. injection of amitriptyline at the doses of 40 and 80 mg/kg, noticeably reduced the tissue injuries induced by carrageenan in the paw skin. At the dose of 40 mg/kg, the infiltration of PMN was inhibited and the pattern of infiltration was focal while the pattern of infiltration in control group was diffused. At the dose of 80 mg/kg, not only the PMN infiltration was slight but also congestion was not observed. The paw tissues in this group were more likely a normal tissue than injured tissues.

I.c.v. injection of amitriptyline Fig. 3E, at the dose of 100 μg/rat was also effective in reducing carrageenan-induced injuries. The PMN infiltration and edema were significantly reduced.

3.4. Effect of amitriptyline on the activity of MPO in the paw skin of carrageenan-treated rats

The MPO activity of paw tissue was significantly increased at 4 h after injection of carrageenan. As illustrated in Fig. 4, i.p. treatment of animals with amitriptyline (40 and 80 mg/kg) significantly prevented the increase in the activity of MPO in the paw skin treated with carrageenan ($P<0.05$). The i.c.v. administration of amitriptyline (100 μg/rat) also markedly decreased the activity of MPO in the carrageenan-injected paws, compared to control group ($P<0.001$). Treatment with indomethacin, as a reference drug at a dose of 10 mg/kg, caused a significant reduction in the MPO activity ($P<0.01$).

3.5. Effect of amitriptyline on IL-1β concentration in the rat paws treated with carrageenan

As illustrated in Fig. 5, injection of carrageenan into the rat hind paw induced a marked increase in the hind paw IL-1β concentration 4 h after injection compared with those found after saline injection. The i.p. treatment of rats with amitriptyline (80 and 40 mg/kg) caused a significant reduction of increased IL-1β generation by carrageenan ($P<0.05$). The i.c.v. injection of amitriptyline (100 μg/rat) also displayed a significant inhibitory effect on the production of IL-1β in the carrageenan-injected paws ($P<0.01$). The increase of IL-1β levels was also significantly prevented by indomethacin (10 mg/kg, i.p., $P<0.05$).

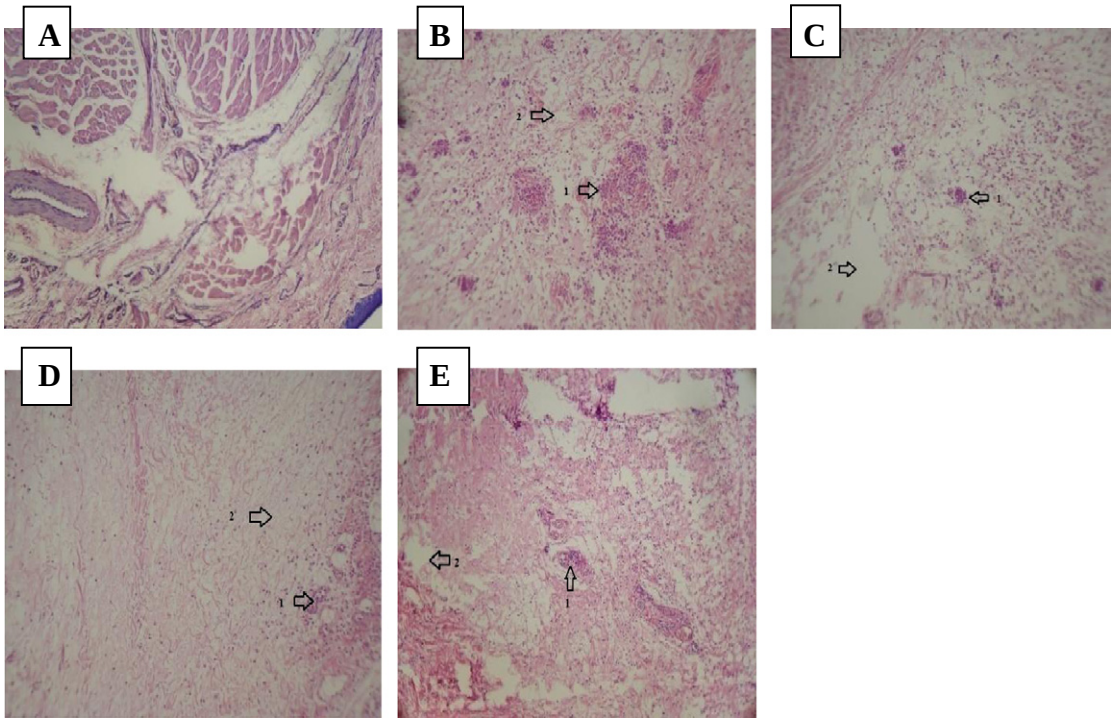

Fig. 3. Histopathologic examination of paw skin of rats treated with amitriptyline, 4 h after subplantar injection of carrageenan. A: Normal rats show the normal appearance of epidermis and dermis without any lesion. B: Carrageenan-injected paw skin in control group (vehicle-treated). Vasodilatation with edema, and migration of leukocytes mainly neutrophils were observed. C: Carrageenan-injected paw skin of rats treated with amitriptyline (40 mg/kg, i.p.). The migration of PMN and edema reduced. D: Carrageenan-injected paw skin of rats treated with amitriptyline (80 mg/kg, i.p.). The appearance of tissues was more similar to the normal tissues, and the infiltration of PMN was noticeable only in some parts. E: Carrageenan-injected paw skin of rats treated with amitriptyline (100 μg/rat, i.c.v.). The injuries induced by carrageenan and the PMN infiltration reduced, and the congestion of capillaries was rare. Sections were stained with hematoxylin and eosin, magnification ×40. 1: Neutrophils. 2: Edema.

3.6. Effect of amitriptyline on TNF-α concentration in the rat paws treated with carrageenan

As illustrated in Fig. 6, injection of carrageenan into the rat hind paw induced an increase in the hind paw TNF-α concentration 4 h after injection compared with those found after saline injection. The i.p. treatment of rats with amitriptyline (80 and 40 mg/kg) caused a significant reduction of TNF-α generation in the carrageenan-injected paws ($P<0.01$). The i.c.v. injection of amitriptyline (100 μg/rat) and indomethacin (10 mg/kg, i.p.) also displayed an inhibitory effect on the production of TNF-α in the carrageenan-injected paw, even though this effect was not statistically significant.

4. Discussion

In the present study, at first, we confirmed our previous findings that i.p. and i.c.v. injection of amitriptyline exhibit a noticeable anti-inflammatory effect in the carrageenan-induced paw edema in rats (Hajhashemi et al., 2010b). In the earlier study, we measured the anti-edematogenic effect of amitriptyline only at 4 h after carrageenan

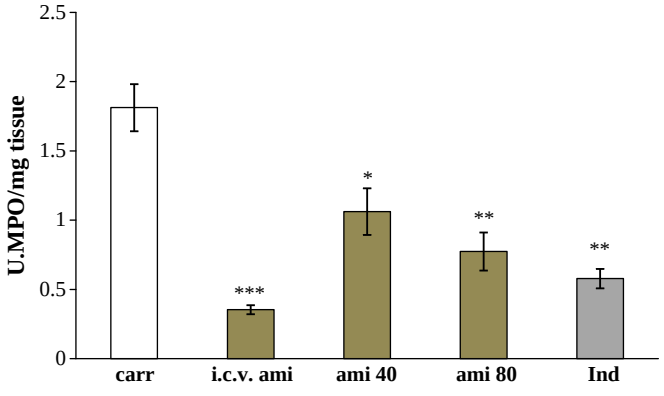

Fig. 4. Effect of amitriptyline on the activity of MPO in the rat paws treated with carrageenan. Animals received i.p. amitriptyline at the indicated doses (40 and 80 mg/kg) or i.c.v. amitriptyline (100 μg/rat) 30 min prior to subplantar injection of carrageenan. The activity of MPO was measured at 4 h after carrageenan injection. Data is expressed as mean ± S.E.M. of five rats ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared to the vehicle-treated group). carr: Carrageenan. ami 40: Amitriptyline 40 mg/kg. ami 80: Amitriptyline 80 mg/kg. i.c.v ami: Intracerebroventricular amitriptyline. Ind: Indomethacin (10 mg/kg).

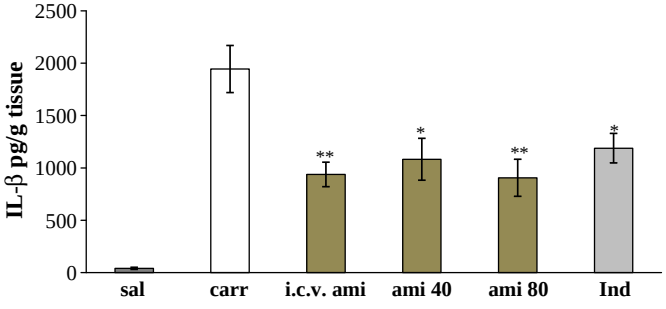

Fig. 5. Effect of amitriptyline on IL-1β levels in the rat paws treated with carrageenan. Rats received indicated doses of amitriptyline (40 and 80 mg/kg, i.p.; 100 μg/rat, i.c.v) 30 min prior to subplantar injection of carrageenan. Four hours after subplantar injection of carrageenan, the paw was homogenized and IL-1β concentration in the supernatant was determined by ELISA. Data is expressed as mean ± S.E.M of five to six rats. ($^{*}P<0.05$, $^{**}P<0.01$, compared to the vehicle-treated group). sal: Saline. carr: Carrageenan. ami 40: Amitriptyline 40 mg/kg. ami 80: Amitriptyline 80 mg/kg. i.c.v ami: Intracerebroventricular amitriptyline. Ind: Indomethacin (10 mg/kg).

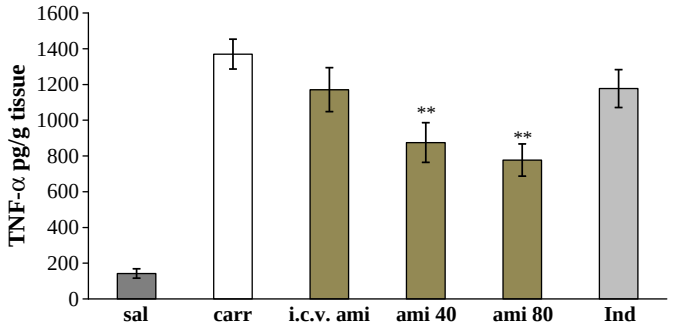

Fig. 6. Effect of amitriptyline on TNF-α levels in the rat paws treated with carrageenan. Rats received indicated doses of amitriptyline (40 and 80 mg/kg, i.p.; 100 μg/rat, i.c.v) 30 min prior to subplantar injection of carrageenan. Four hours after subplantar injection of carrageenan, the paw was homogenized and TNF-α concentration in the supernatant was determined by ELISA. Data is expressed as mean ± S.E.M. of five to six rats. ($^{**}P<0.01$, compared to the vehicle-treated group). sal: Saline. carr: Carrageenan. ami 40: Amitriptyline 40 mg/kg. ami 80: Amitriptyline 80 mg/kg. i.c.v ami: Intracerebroventricular amitriptyline. Ind: Indomethacin (10 mg/kg).

challenge, but in the present experiment, we evaluated the anti-inflammatory activity of amitriptyline at the course of inflammation (1, 2, 3 and 4 h post-carrageenan).Our results showed that amitriptyline was effective in reducing paw swelling in the two phases of inflammation induced by carrageenan.

As mentioned above, carrageenan-induced paw edema is a well-defined model of acute inflammation that a variety of inflammatory mediators participates in its development. It is well characterized that PMN leucocytes infiltration plays an important role in the inflammation induced by carrageenan in hind paw (Gilligan et al., 1994; Di Rosa et al., 1971), therefore one aspect that was investigated in the present study was the interference of amitriptyline with the PMN cells migration. Our results showed that both i.p. and i.c.v. administration of amitriptyline elicited a marked reduction in the infiltration of PMN leucocytes into the carrageenan-treated paws, according to pathological examination and the activity of MPO into the inflamed paw tissues, compared to vehicle-treated group.

Carrageenan injection also provokes the release of some important pro-inflammatory cytokines such as TNFα and IL-1β (Halici et al., 2007; Nacife et al., 2004). Cytokines play a vital part in the generation and/or maintenance of a various types of disease including multiple sclerosis, asthma, arthritis, and inflammatory bowel disease (Codarri et al., 2010; Feldmann and Maini, 2008; Ichinose and Barnes, 2004; Papadakis and Targan, 2000). Of interesting, it has been reported that production of pro-inflammatory cytokines may be implicated in the pathogenesis of major depression (Leonard, 2001; Maes et al., 1995). Therefore, we wanted to determine whether the anti-inflammatory activities of amitriptyline could be related to the inhibition of TNF-α and IL-1β generation in the inflamed site. Our findings demonstrated that i.p. and i.c.v. application of amitriptyline considerably reduced the IL-1β levels into the carrageenan-injected paw tissues. Systemic and central injection of amitriptyline also decreased concentration of TNF-α into the carrageenan-injected paws, although the effect of i.p. injection was only significant. Numerous studies, both *in vitro* and *in vivo* conditions, have evaluated the effect of antidepressants on cytokine levels and functions. For *in vitro* studies, human peripheral blood mononuclear cells or whole blood samples were incubated with different antidepressants and then were stimulated to generate cytokines (Xia et al., 1996). In most cases, *in vitro* studies confirmed that antidepressant drugs noticeably inhibited the stimulated secretion of pro-inflammatory cytokines such as IL-2, IL-1β, and IFN-γ (Castanon et al., 2002). Unlike *in vitro* studies, *in vivo* studies regarding the effects of antidepressant medications on the cytokine concentrations are contradictory. Mikova et al. found that although serum levels of IL-8 and TNF-α increased in depressed patients, treatment with clomipramine, paroxetine, or amitriptyline did not modify the concentrations of these cytokines (Mikova et al., 2001). On the contrary, Sutcigil et al. reported that IL-2 and TNF-α levels in depressed patients diminished after therapy with antidepressants (Sutcigil et al., 2007). Tuglu et al. also found a decline in the increased levels of TNF-α in depressed patients after treatment with Selective Serotonin Reuptake Inhibitors (Tuglu et al., 2003). Interestingly, Hinze-Selch et al. showed that treatment with tricyclic antidepressants, but not Selective Serotonin Reuptake Inhibitors, activated the TNF-α system (Hinze-Selch et al., 2000). Thus, the effect of antidepressants on the cytokine levels remains as an open question. To the best of our knowledge, our results are the first work showing the inhibitory effects of amitriptyline on the PMN leukocyte migration and production of pro-inflammatory cytokines (TNF-α and IL-1β) have an important role in its anti-inflammatory.

It is worth mentioning that there is a strong connection between the inflammatory development and the generation of pain (Marchand et al., 2005). Experimental studies have established that the inhibition of PMN cells migration and pro-inflammatory cytokines lessens the hyperalgesia evoked by different inflammatory stimuli (Ren and Dubner, 2010; Abbadie, 2005). Therefore, it seems possible that the inhibitory effect of amitriptyline on leukocytes migration and TNF-α and IL-1β concentrations, at least partly, participates in its analgesic activity.

In summary, our findings verified our previous studies about the anti-inflammatory properties of i.p. and i.c.v. injection of amitriptyline in the acute model of inflammation (Hajhashemi et al., 2010b). Moreover, we found that the inhibitory effects of amitriptyline on the PMN leucocytes infiltration and the production of pro-inflammatory cytokines (IL-1β and TNF-α) participated in its anti-inflammatory activities. Amitriptyline usually used in the management of fibromyalgia, rheumatoid arthritis and inflammatory bowel disease to combat secondary depression resulting from these inflammatory illnesses (Rahimi et al., 2009; Nishishinya et al., 2008; Bird and Broggini, 2000). Therefore, it is possible that some beneficial effects of amitriptyline in these situations are at least partly mediated through the effects of amitriptyline on the pro-inflammatory mediators.

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