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Roles of Interleukin-1β (Il-1β) and Nitric Oxide (No) in the Anti-Inflammatory Dynamics of Acetylsalicylic Acid Against Carrageenan Induced Paw Oedema in Mice

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Abstract: The study aimed at analyzing the role of each of interleukin-1β (IL-1β) and Nitric Oxide (NO) in development of peripheral acute inflammation in mice in presence/absence of a one hour pre-administered acetylsalicylic acid (ASA). Eighty male mice were utilized. Carrageenan was administered intraplanterly (0.05 ml of 0.5, 1 or 2%, W/V). The examined parameters were paw volume, IL-1β and nitrite levels in plasma and paw infiltrate and the total and differential white blood cells count. Carrageenan administration caused dose-dependent increase tendencies in paw volume, paw infiltrate's IL-1β and plasma nitrite content. Pre-treatment with ASA resulted in augmenting the rise in plasma IL-1β, decreasing IL-1β at the inflammation site and restoring plasma nitrite content to its normal range. The findings indicate that the carrageenan amount is the major determinant of the response to ASA.

Key words: Carrageenan · Interleukine-1β · Acetyl salicylic acid · Inflammation

INTRODUCTION

Acute inflammation is rapid, short-lived and characterized by accumulations of fluid, plasma proteins and leukocytes. At the site of inflammations, the injured vascular endothelial cells and the emigrated leukocytes release a large number of soluble mediators which modulate and maintain the inflammation [1]. The present study is focusing on two of these mediators, namely interleukin-1β (IL-1β) and Nitric Oxide (NO).

Interleukin-1β (IL-1β) is a pleiotropic mediator of the host response to infections and injurious insults. It co-ordinates the activities of other cells and cytokines acts as a costimulant of early innate inflammatory and later specific immune responses [2]. It acts on monocytes and neutrophils, inducing secretion of several cytokines including IL-1 it-self [3, 4]. In addition, IL-1 was reported to prolong the *in vitro* survival of neutrophils, to augment the antigen representing capacity of dendritic cells [5] and to participate in the recruitment of leukocytes to inflammatory sites by inducing the expression of adhesion molecules on vascular endothelium and a number of chemokines that attract neutrophils, eosinophils, macrophages and lymphocytes to the sites of injury [4, 6-8]. IL-1 has been also reported to induce prostaglandins release from fibroblasts [2] and to stimulate apoptosis of pancreatic B cells, presumably through induction of NO [9].

Regarding NO, it was found to possess th pro-and anti-inflammatory actions. Low concentrations of NO produced by constitutive NO synthase (cNOS) inhibit adhesion molecule expression, cytokine and chemokine synthesis and leukocyte adhesion and transmigration [10]. On the other hand, NO produced by the inducible isoform was found to increase carrageenan induced plasma extravasion in rat skin and paw oedema [11]. NO was also found to activate COX-1 in the early phase of carrageenan inflammation and to up-regulate COX-2 expression in the late phase in the skin, resulting in production of PGE2 and PGI2 at the site of inflammation. Thus contributing to exacerbation of the inflammatory process [12]. In addition, the non-selective NOS inhibitor, N-nitro-l-arginine methylester (L-NAME) was found to increase prostacyclin and leukocyte infiltration in carrageenan-soaked sponge implants in rats, which indicate's that th isoforms of NOS may be involved during plasma exudation and leukocyte infiltration into the inflamed tissue [13].

The findings that IL-1β stimulates the expression of iNOS and that NO affects the release of IL-1β [14] point to

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the need for emphasizing the concerns regarding IL-1β and NO while assessing development and treatment of the experimental inflammations.

The anti-inflammatory action of acetylsalicylic acid (ASA) has been generally attributed to direct inhibition of COX-1 and COX-2. Nevertheless, other different mechanisms are likely at work. The anti-inflammatory potential of ASA was reported to take place through inhibiting nuclear factor kappa B (NF-kB) activation in monocytic, lymphocytic and lung epithelial cells by blocking Ikappa-B (IkB) kinase, a key enzyme in NF-kB activation, thereby inhibiting subsequent proinflammatory cytokines expression, like TNF and IL-1 [15-21]. Moreover, aspirin may prevent inflammation by increasing soluble receptor type ∏ (sIL-1R∏) secretion thus; preventing IL-1 from binding target cells [22]. In addition, ASA was found to induce NO release from vascular endothelium [23, 24].

The aim of the present study was to contribute to the understanding of the possible interplay of IL-1β and NO in development of peripheral acute inflammation in presence and in absence of a pre-administered dose of ASA utilizing the mouse paw edema model.

MATERIALS AND METHODS

Animals: The study was performed on adult male Swiss Mice (weighing 23±3g), obtained from the animal house of the National Organization for Drug Control and Research (NODCAR). They were kept under standardized conditions, where diet and water were *ad libitum*.

Drugs and chemicals: Carrageenan lambda (Gelatin, vegetable, Irich Moss, Type IV), vine serum albumin fraction V (BSA) and sulfanilamide were purchased from Sigma-Aldrich Co. Acetylsalicylic acid (ASA) was kindly obtained from El Nasr Pharmaceutical chemicals Co.(Egypt). N-(1-naphthyl)-ethylendiamine dihydrochloride, a laratory reagent, was purchased from the British drug houses LTD (BDH).

Doses preparation and routes of administration: Carrageenan was prepared into three concentrations (0.5, 1 and 2% W/V) using saline as a diluent. It was administered interplanetary to the right paw in a dose volume of 0.05 ml. ASA was prepared as a suspension in distilled water using tween 80 (0.25%). It was orally administered (200 mg kg^{-1}) in a dose volume of 25 ml kg^{-1}.

Experimental design: Three major groups of animals were utilized (90 mice). The first one served for studying the dose-response relationships of carrageenan-induced paw edema, where animals were divided into three subgroups according to the given dose level. The second major group was used for evaluating the anti-inflammatory effect of ASA. It was subdivided into 4 subgroups. They were administered by ASA, 1 h before the administration of carrageenan (0, 0.5, 1 or 2 %). In parallel, the third major group acted as a control by receiving the oral or intraplanter utilized vehicles. Subgroups were always of 10 animals each. Six hours after carrageenan injection, blood was withdrawn from the orbital sinus of each animal into separated different heparinized tubes. These blood samples were utilized for total white blood cells count, differential leucocytic count and for plasma separation. Animals were sacrificed and the paw thicknesses for th contralateral paws were measured using plethysmometer (digital Caliper, U. FA, Germany). The volume of edema was expressed for each animal as the difference between the carrageenan-injected paw and the contralateral one. Paws were then cut. Each of the right hind paws was homogenized in 2 ml saline and centrifuged at 3000 rpm for 10 min after which, exudates (supernatants) were collected. The collected supernatants and plasma samples were then used for assaying IL-1β levels and estimating the nitrite contents.

Determination of IL-1β: IL-1β level was assayed in plasma and paw infiltrate using enzyme-linked immunoadsorbent assay kit in accordance with the manufacturer's recommendations (Biosource International, California, USA), read at 450 using ELISA reader (BioTEK. Instruments Inc., ELx808, USA).

Measurement of plasma and paw infiltrate nitrite content: Nitrite concentration was used as an indication of NO production. The procedure for NO determination was based on the Griess reaction according to Wang and Mazza [25]. One hundred microliters of plasma, paw infiltrate or sodium nitrite standard (7.5-500 μM) was mixed with an equal volume of Griess reagent [a mixture of 0.1% W/V N-(1-naphthyl)-ethylendiamine dihydrochloride reagent) and 1% (W/V) sulfanilamide in 5% (V/V) phosphoric acid, the two parts being mixed together within 1hr. of use]. After 20 min. at room temperature, the light absorbance was measured at 540 nm (Spectrophotometer Heëios α Theromospectonic). The nitrite contents were normalized to the plasma or the infiltrate protein content.

Protein determination: Protein content in each of plasma and paw infiltrate was estimated by the Bradford method [26]. The working dye reagent (Coomassie-brilliant blue G-250) was prepared by diluting the stock solution with distilled water (v/v). Standard curve was performed using several dilutions (0.1-1 μg μl^{-1}) of vine serum albumin fraction V (BSA). Standards and test samples were delivered into 96-well microplate (Grienr lartechni, Kremsmunster, Austria) together with the working dye. The plate was incubated for 15 min and then read at 650 nm using ELISA plate reader (BioTEK. Instruments Inc., ELx808, USA).

Statistical analysis: Data are expressed as mean±SEM. They were analyzed by the one-way analysis of variance (ANOVA), followed by post-hoc Tukey's test for multiple comparisons. The differences were considered significant if the probability was associated with $p<0.05$. Relationships between the given treatments and consequent responses were examined by Pearson's correlation [27].

RESULTS

Effects of carrageenan administration: As seen in Fig. 1, volumes of the inflamed paws have been significantly increased, following all utilized doses of carrageenan. The 1% carrageenan dose did not result in a significant increase in the paw volume as compared to that induced by the 0.5%. On the other hand, that of the 2% induced a very high significant increase, which reached 89 and 63% as higher ($p<0.001$) as compared with that induced by the 0.5 and 1% carrageenan doses, respectively.

The three different ascending doses of carrageenan, elicited a clear dose-dependent rise (with a correlation factor $r = 0.0718$, $p<0.001$) in the paw exudate's IL-1β by 54, 103 and 149%, respectively, as compared to the contralateral saline injected paw (Fig. 2). The statistically significant increase ($p<0.05$) was induced only by the

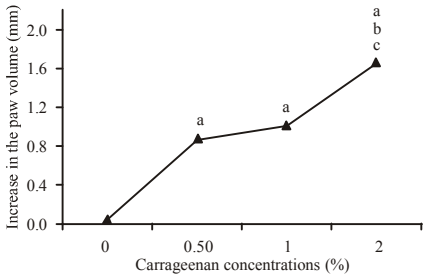

Fig. 1: Effect of administration of carrageenan (0.05 ml of different concentrations) on the paw volume

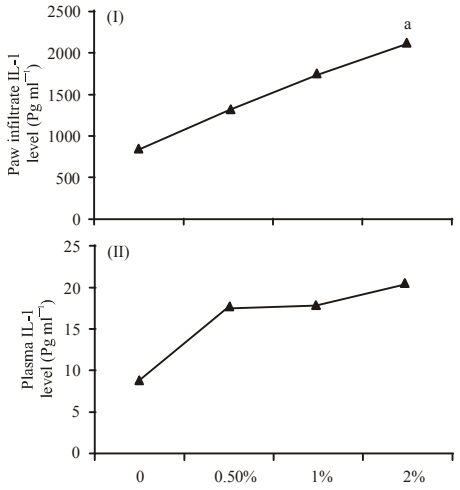

Fig. 2: Effect of administration of carrageenan (0.05ml of different concentrations) on IL-1 levels in the paw infiltrate (I) and in plasma (II)

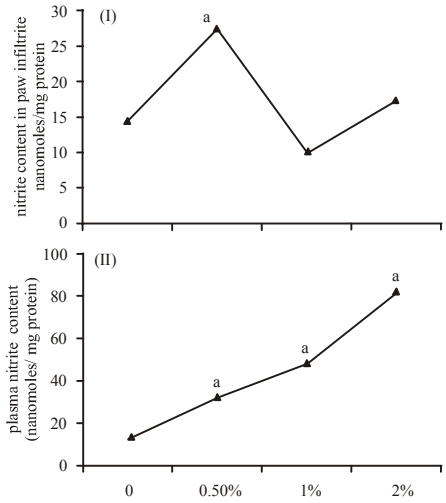

Fig. 3: Effect of administration of carrageenan (0.05ml of different concentrations) on the nitrite contents in the paw infiltrate (I) and in plasma (II)

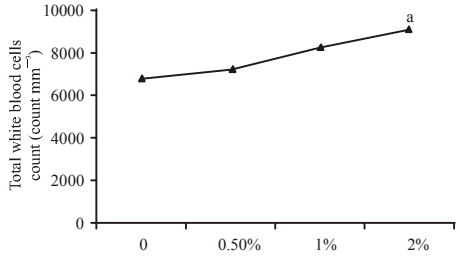

Fig. 4: Effect of administration of carrageenan (0.05ml of different concentrations) on total white blood cells count

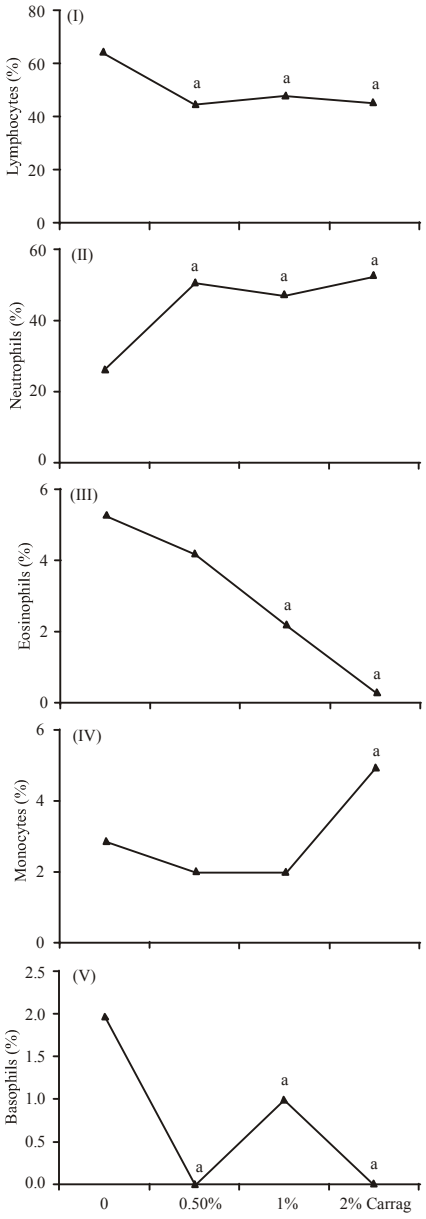

Fig. 5: Effect of different doses of carrageenan on differential blood cells count expressed as percentages of the different types of cells. (I): Lymphocytes (Ð): Neutrophils, (III): Eosinohils, (IV): Monocytes and (V): Basophils

highest used carrageenan dose (2%). At the contrary, the plasma IL-1β levels were not significantly altered by the different used dose levels of carrageenan (Fig. 2).

Administration of carrageenan induced a highly significant ($p<0.001$) dose-dependent increase in the plasma nitrite content (with a correlation factor $r = 0.873$, $p<0.001$). The increase reached 114, 159.5 and 286%,

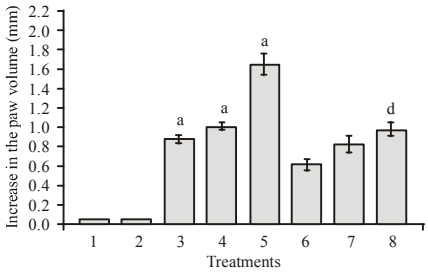

Fig. 6: Effect of preadministration of ASA (200 mg kg^{-1}) on the paw volume changes induced by different doses of carrageenan (0.05 ml of 0.5, 1 or 2% carrageenan)

respectively, as compared to the control group (Fig. 3). The lowest dose of carrageenan (0.5%) was the only one that significantly affected the nitrite content in the paw infiltrate ($p<0.001$), where an increase by 91% took place (Fig. 3).

Concerning the effects of carrageenan on the total and differential blood cells counts (Fig. 4 and 5), only the highest used dose (2%) could induce a significant increase ($p<0.05$) in the total white blood cells count (by 34%) and in the differential monocytes count (by 67%). The three used dose levels of carrageenan exhibited a "ceiling"-like effect on the differential neutrophils count inducing significant increase ($p<0.001$) in their percentages, by 88, 81 and 96%, respectively (Fig. 5). Parallely, the lymphocytes count was significantly decreased by 30, 25 and 31%, respectively (Fig. 5). The decrease in the eosinohils count took place in response to the two highest used doses of carrageenan (1 and 2%) in a dose-dependent manner. Meanwhile, the decrease in the basophils count was dose-independent (Fig. 5).

Ⅱ- Effects of ASA-pretreatment: Administration of 200 mg kg^{-1} of ASA, 1 h before carrageenan injection, induced reduction in the paw volume. This reduction was highly significant ($p<0.001$) and maximum (41%) following administration of the 2% carrageenan as compared to the corresponding carrageenan-saline treated groups (Fig. 6).

Pretreatment with ASA has also significantly reduced the 2% carrageenan-caused rise in IL-1β level in the paw infiltrate by 57%, $p<0.01$ (Fig. 7). Meanwhile, the 2% carrageenan-induced increase in plasma IL-1β level was potantiated by ASA pretreatment.

Administration of ASA without carrageenan challenge resulted in significant increase in the nitrite contents in plasma (by 114%, $p<0.05$) as well as in the paw infiltrate (by 80%, $p<0.001$) as compared to the control

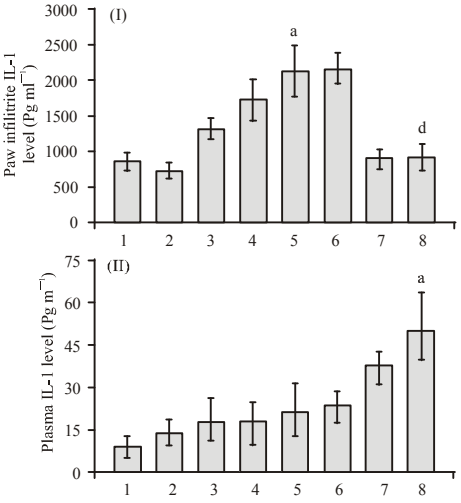

Fig. 7: Effect of preadministration of ASA (200 mg kg^{-1}) on the induced changes in the IL-1 levels in the paw infiltrate (I) and in plasma (Ð) under the influence of different doses of carrageenan (0.05 ml of 0.5, 1 or 2% carrageenan)

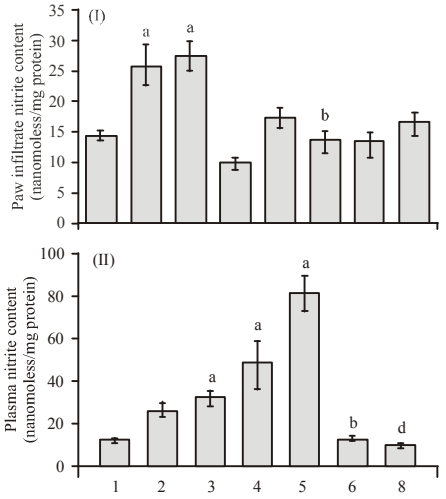

Fig. 8: Effect of preadministration of ASA (200 mg kg^{-1}) on the induced changes in nitrite content in the paw infiltrate (I) and in plasma (Ð) under influence of different doses of carrageenan (0.05 ml of 0.5, 1 or 2% carrageenan)

group (Fig. 8). Pre-administration of ASA resulted in significant lowering of the carrageenan low dose-induced rise in nitrite infiltrate content. On the other hand, ASA administration could prevent the carrageenan-induced rise in the plasma nitrite content and could restore its normal value (Fig. 8).

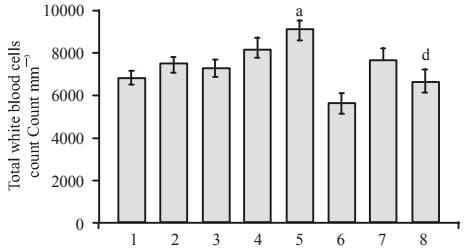

Fig. 9: Effect of preadministration of ASA (200 mg kg^{-1}) on the total white blood cells count changes induced by different doses of carrageenan (0.05 ml of 0.5, 1 or 2% carrageenan)

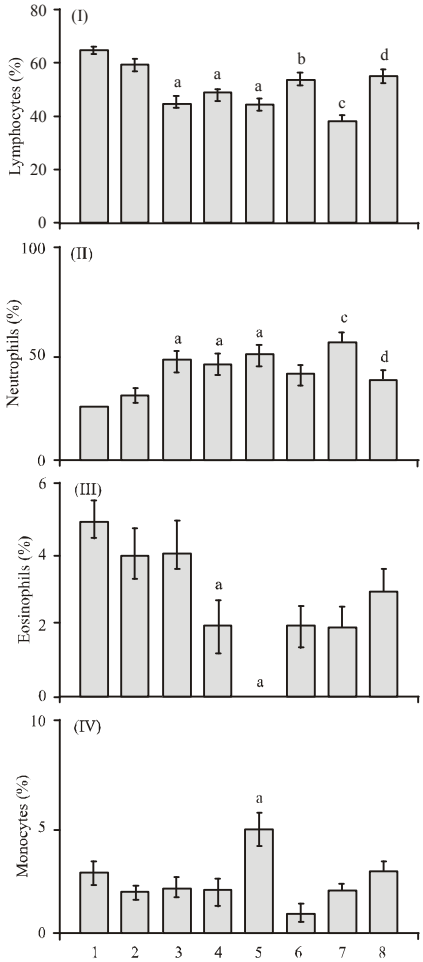

Fig. 10: Effect of preadministration of ASA (200 mg kg^{-1}) on the carrageenan induced changes in the differential blood cells count expressed as percentages of different types of cells (I): lymphocytes (Ð): Neutrophils, (III): Eosinohils, (IV): Monocytes and (V): Basophils. Carrageenan was administered in different doses of (0.5, 1 or 2%)

Pre-treatment with ASA could restore the increase in the total white blood cells count induced by the high dose level of carrageenan (2%) to the control value (Fig. 9). It could modulate the effects of the lowest and highest used carrageenan doses on the differential counts of neutrophils and lymphocytes. On the other hand, pre-administration of ASA, in case of the intermediate used dose level of carrageenan, resulted in reducing the lymphocytes count and increasing that of neutrophils. Moreover, ASA pretreatment could restore the carrageenan-induced decrease in the differential eosinophils count to reach the control value (Fig. 10).

DISCUSSION

The present findings regarding paws parameters came in contrast to those of Henriques *et al*. [28], where edema developed during the first phase was unrelated to the dose of carrageenan. In the present study, the effects induced by 2% carrageenan on paw parameters (paw volume, exudate's IL-1 level and plasma nitrite content) were more pronounced than those induced by the other used two dose levels.

The absence of significant changes in plasma IL-1β following intraplanter administration of carrageenan in the present work coincides with the findings of Pourpak *et al*. [29]. On the other hand, the increase in paw infiltrate IL-1β levels was found to reflect the magnitude of the induced edema as represented by the paw volume, where paw infiltrate IL-1β reached a maximal concentration upon administration of the highest used dose level of carrageenan. Thus, the obtained results in the present study, show positive correlations between each of the significant increase in paw infiltrates IL-1β level and the tendency of increase in that of plasma, the significant increase in plasma nitrite, as well as, the significant increase in the percentage of peripheral neutrophils. All the aforementioned changes were consistent with the incidence of edema induced by different used dose levels of carrageenan in a dose-dependent manner.

The carrageenan induced dose-dependent decrease in the peripheral eosinophils count could be explained by the increase in the endogenous IL-1β level in the paw, whose accumulation there could recruit the local accumulation of eosinophils [8]. On the other hand, the carrageenan exhibited ceiling-like effect on peripheral neutrophils percentages, may be explained by the finding that IL-1β prolongs survival of neutrophils [2]. Moreover, the aforementioned fact together with the observation in the present study that neutrophils constitute aut 50% of the circulating leucocytes and an induction of a relative rise occurred in plasma IL-1β of carrageenan treated mice, may explain the carrageenan resulted rise in the total leucocytes count.

The biological effects of IL-1β are known to be modulated by IL-1 receptor antagonist (IL-1ra) and by the shed soluble forms of IL-1 receptors (sIL-1R), [30-32]. Expression of th type Ⅰ and Ⅱ receptors is tightly regulated to control inflammatory and immune responses. th types are shed by activated neutrophils and monocytes. The solubilized shed receptors were found to bind IL-1 β and to inhibit its functions [33-35]. Soluble IL-1RⅡ is present in normal plasma, serum and synovial inflammatory exudates. These receptors not only function as specific inhibitors for IL-1β, but also block the processing of proIL-1β by inhibiting the propeptide with higher affinity [2]. Previous studies demonstrated that ASA could induce rise in sIL-1R type Ⅱ concentration *in vitro* as well as *in vivo* [22]. In this regard, the present study revealed that, pretreatment with 200 mg kg^{-1} ASA, potantiated the rise in plasma IL-1β levels, the matter which may have resulted in a consequent increase in sIL-1RⅡ levels [22] in plasma as well as in exudates of mice injected with carrageenan. These receptors act as a "decoy" receptor for IL-1β [30-32]. They down regulate the level of extra cellular IL-1β confirming the anti-inflammatory effect of ASA. The exerted anti-inflammatory effects in the present study appeared as decreasing IL-1β levels in the paw and attenuating activation and recruitment of neutrophils and eosinophils, with a consequent decrease in the paw volume. According to Krakaure *et al*. [2], the found high plasma and tissue levels of IL-1ra in patients suffering from various inflammatory diseases were interpreted that; endogenously produced IL-1ra may represent a normal homeostatic response to limit the proinflammatory pathogenic effect of IL-1β.

Thus, in the present study, it may be suggested that, the high plasma IL-1β levels in mice pretreated with ASA and injected with either of the three dose levels of carrageenan, may be present as an outcome of the ASA induced high levels of sIL-1RⅡ as well as the carrageenan induced high level of IL-1ra with the consequence of blocking the processing of inflammation, by inhibiting IL-1β functions. This interpretation may be furtherly supported by the present study found corresponding decrease in IL-1β level in the site of inflammation (paw infiltrate) as well as by the found reduction in the paw volume.

According to the existing knowledge, eNOS is responsible for the nitrite content in the paw. These contents are known not to be changed by inflammatory doses of carrageenan in the early phase [36]. The obtained results showed no change in nitrite content in the 1 and 2% carrageenan-induced inflammation, the matter which goes in accordance with the findings of Posadas *et al*. [36]. On the other hand, the lowest used dose (0.5%) caused a marked increase in nitrite content which is aut 2 folds of the control value and 3 folds that of the higher administered dose of carrageenan (1%). This is difficult to interpret. However, a possible explanation may be that, the used relatively small dose of carrageenan may be associated with activating the iNOS. Thus, it may have led to massive increase in the nitrite content. This possibility may be established on basis of the findings of Connelly *et al*. [14], where the existence of a dual effect of NO on NF-κB, have been observed. This may explain in part-the ability of NO to exert th pro and anti-inflammatory actions. However, this possible explanation needs to be examined through a detailed comparative analysis regarding qualitative and quantitative differences in the inflammatory mediators released by the low (0.5%) and the high (2%) doses.

In the present study, the increase in nitrite content, either of plasma or paw exudates of normal mice treated with ASA, is consistent with the reported findings that ASA induces increase in NO production in leucocytes [37], increases plasma nitrite/nitrate levels [23, 37] and enhances release of NO from vascular endothelium [24]. In addition, unstable angina patients taking ASA were shown to possess a lower systemic inflammatory response and a high eNOS protein expression in their neutrophils [38]. The increased NO contents under influence of ASA may be mediated through its ability to trigger 15-epi-lipoxin A4, as a consequence of acetylation of active COX2 in the endothelial or epithelial cells, thus resulting in eliciting NO synthesis, which is known to negatively regulate leukocyte-endothelium interaction in the microcirculation [23].

The induced decrease in nitrite content in each of plasma and paw exudates following ASA-readministration to carrageenan treated animals may be explained on the basis that addition of NO through an exogenous source (which is ASA in the present study) before carrageenan administration, could cause an inhibition of NF-kB and a consequent inhibition of NOS. In this regard, Connelly *et al*. [14] showed that addition of exogenous source of NO immediately before addition of lipopolysaccharids (LPS) causes a distinct biphasic effect on NF-kB activity, where at low concentration; it induces an enhancement of NF-kB, while at a higher concentration it induces an inhibition of NF-kB.

Finally, from the obtained results concerning the parameters of carrageenan-induced paw edema (paw volume and IL-1β and nitrite levels in the paw exudates and plasma), it seems likely that pretreatment with 200 mg kg^{-1} ASA exhibited a better anti-inflammatory effect in case of utilizing the highest concentration of the inflammatory agent (2% of carrageenan) as compared to the effects of the other two used lower concentrations (0.5% and 1%). A possible explanation may be that the highest used concentration of carrageenan was reflected homeostatically in endogenous production of IL-ra which is known to counteract the proinflammatory effect of IL-1β, the matter which may be subjected to synergism by the ASA possibly induced rise of the sIL-RÐ which is known to inhibit th, the IL-1β and the proIL-1β. Accordingly, it may be possible to consider that the amount of carrageenan is a determinant of the response to ASA.

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