EFFECTS OF AN EXTRACT OF *UNCARIA TOMENTOSA* (UÑA DE GATO) ON TNF-A AND IL-10 PRODUCTION, AND ON LEUKOCYTE MIGRATION IN THE MOUSE AIR POUCH MODEL

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Abstract
The effect of an hydroalcoholic extract of "Cat's claw" or "Uña de Gato" (UG) on the production of tumor necrosis factor-alpha (TNF-α) and interleukine-10 (IL-10) was tested in the BALB/c mouse air pouch model of inflammation. The cytokine levels were measured by ELISA, and the number of leukocytes determined in inflammatory exudates and peripheral blood. Mice inoculated with the irritant agent carrageenan significantly increased their TNF-α production (3,608.0 ± 79.4 pg/ml) as compared to control (61.0 ± 14.1 pg/ml). Oral treatment of inflamed animals with UG caused a dose-dependent lowering of the TNF-α production (48.2% by UG 100, 54.2% by UG 250) ($p< 0.01$) that was even more significant (96.3%, $p < 0.01$) after the simultaneous administration of UG 100 and pentoxifylline. On the other hand, UG 250 stimulated 1.45 times the production of IL-10; stimulation was 3 times higher when UG was supplied with pentoxifylline, pentoxifylline by itself increasing by 5 times the IL-10 level. UG also inhibited the carrageenan-induced leukocyte infiltration to the pouch (66.0% by UG 100, 70% by UG 250) ($p < 0.01$). Blood leukocyte analysis showed significantly lower lymphocyte counts in the carrageenan as compared to the PBS group. Leukocyte extravasations, as measured by the emigration ratio (ER), increased up to 123.2% in the carrageenan-inflamed mice, while in UG-treated mice ER attained 29.9% (UG 100) and 22.3% (UG 250). The present results strongly support the *in vivo* anti-inflammatory action of the hydroalcoholic extract of UG.

Key words
Inflammation, Cytokines, TNF-α, IL-10, *Uncaria tomentosa*, Immunomodulation

Introduction

Uncaria tomentosa Willd DC., Rubiaceae, popularly known as Uña de gato (UG), is a medicinal plant that grows in the Peruvian tropical forest; it is used in the traditional South American medicine because of its claimed therapeutic properties. Particularly, it

has been used as anti-inflammatory, antiviral, antioxidant, and as inmunostimulating and anti-mutagen agent [1]. UG contains diverse compounds including glycosides and cincholic acids, alkaloids of indol and oxindole: pentacyclic oxyndole alkaloids (PAO), or tetracyclic oxyndole alkaloids (TOA) types, triterpenes, tannins, sterols, flavonoids, and different polyphenols [1,2,3,4,5]. POA have been shown to exert anti-inflammatory, immunomodulatory, and apoptotic effects [2,4,6,7,8,9].

It has been reported that extracts of UG play an immunomodulating role through the inhibition of TNF-α synthesis in vitro [10]. In addition, we have shown [8] that a hydroalcoholic extract of UG exert an inhibitory action on NF-κB.

The air pouch model is formed by the daily subcutaneous injection of air in the back of mice for several days [11]. The injection of carrageenan in the pouch produces a local inflammatory reaction characterized by the production of biochemical mediators in the exudates fluid, including PGs, leukotrienes and some cytokines, and the affluence to the pouch of PMNs and macrophages [12, 13].

The present study evaluates the potential beneficial effects of UG, assessing the biological activity of an hydroalcoholic extract of UG in the mouse air pouch model by measuring the levels of the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-10, and its effect on the quality and quantity of leukocytes in the inflammatory exudates and peripheral blood.

Materials and methods

Animals

Male BALB/c mice (6-8 wk old, 25-30 g) were obtained from the Instituto Nacional de Salud (Lima, Peru), and maintained with water and food *ad libitum*. International ethics norms for animals were strictly followed and the study was approved by the Ethic Committee for use of experimental animals of the Universidad Peruana Cayetano Heredia.

Uncaria tomentosa extract

We used a spray-dried hydroalcoholic extract of UG (drug extract ratio 8:1), containing 5.6% total oxindole alkaloids (90.2% POA, 9.8% TOA) determined by HPLC, as reported previously [8]. **(Fig 1)**.

Experimental animal groups

The following experimental animal groups (five mice per group) were established: a) Negative control group: supplied orally with distilled water and injected with 1 ml of PBS in the air pouch; b) Carrageenan-treated group: subdivided in two subgroups: (i) supplied orally with distilled water, and injected with 1 ml of 2% carrageenan in the air pouch; (ii) mice injected with 0.8 ml of 2% carrageenan and 0.2 ml of 3% pentoxy (Sigma Chemicals, USA, 30 mg/ml); c) Mice treated with UG 250: subdivided in two subgroups; (i) supplied orally with 250 mg/kg weight of UG and injected with 2% carrageenan in the air pouch; (ii) mice treated with UG as in (i) and injected with 2%

carrageenan and 3% pentoxy as in group b (ii); d) Mice treated with UG 100: subdivided in two subgroups, treated as in group c, but supplied orally with 100 mg/kg weight of UG; e) Mice treated with 10 mg/kg weight of Indomethacin: Five mice supplied orally with Indomethacin and injected with 1 ml of 2% carrageenan in the air pouch.

The mouse air pouch model

Mice from the different groups were anaesthetized (ketamine/xylazine) and air pouch was induced as describe Edwards et al. (11). Mice received 1 ml/day of distilled water (a, b groups) or UG (c, d groups) through a gastric catheter for 5 days of treatment, and Indomethacin (e group) for 3 days. At day 6, after one hour of having received their respective UG oral dose, the animals were injected in the pouch with 1 ml of PBS (a group) or 1 ml of 2% carrageenan (with or without pentoxy) (b, c, d, e groups) in physiological saline. After four additional hours, mice were sacrificed with an overdose of ketamine/xylazine, and the inflammatory exudates collected from the air pouch with a sterile syringe. The air pouch cavity was rinsed twice with 2 ml of PBS; washings were pooled and centrifuged at 500 x *g* for 10 min at 4 oC to remove leukocytes and the supernatant stored at -20 oC for later cytokine measurements. The cellular pellet was resuspended in PBS to count the total number of leukocytes using the Turk solution. A cardiac puncture was also performed to obtain peripheral blood samples to count the total number of circulating leukocytes; differential leukocyte counting was performed with the Wright solution.

Cytokine determination in the air pouch

TNF-α (5 pg/ml assay limit detection) and IL-10 (23 pg/ml assay limit detection) were measured by commercial ELISA kits from Pharmingen (BD, USA). Cytokine concentration calculations were done according to the manufacturer's instructions.

Emigration ratio of leukocytes

The leukocyte extravasations activity from peripheral blood to the inflammatory site was estimated by ER, which is the ratio of total leukocytes found in the air pouch related to leukocytes in the peripheral blood after the injection of the inflammation agent [12,14].

Statistical analysis

Data were expressed as mean ± SEM and analyzed by one-way ANOVA for multiple comparisons and the Bonferroni test (statistical package SPSS version 10.0). Significance was established at $p < 0.05$.

Results

Cytokine levels in the inflammatory exudates from the air pouch

TNF-α level (expressed in pg/ml) in the exudates after the PBS injection was 61.0 ± 14.1; the carrageenan injection provoked a dramatic local increase of this cytokine (3,608.0 ± 79.4). The TNF-α increment was 74.1% lower (936.0 ± 103.68) ($p < 0.01$) when carrageenan was injected together with its specific inhibitor pentoxy. Mice injected with carrageenan but supplied with the UG extract displayed a significant ($p <0.01$) UG-dose-dependent reduction of the TNF-α levels in the exudates: 1,867.0 (48.3%, by UG 100) and 1,652.0 (54.2%, by UG 250). TNF-α reduction was stronger (96.3%, $p < 0.01$) if

mice were supplied with UG 100 and injected with 3% pentoxy **(Fig. 2)**. IL-10 level (expressed in pg/ml) in the exudates of carrageenan-injected mice was 610.19 ± 29.7, whereas it was higher in mice that received Indomethacin (950.5 ± 249.7)(not show), UG 100 (1,344.2 ± 58.7) and UG 250 (1,499.2 ± 103.7, $p < 0.01$). Mice receiving UG 100 plus pentoxy exhibited 1,667.4 ± 211.8, whereas those that received higher amounts of UG 250 the value was 1,938.4 ± 58.4 ($p < 0.01$). Mice getting only pentoxy had a 427% increment of IL-10 (3,214.7 ± 304.1) **(Fig. 3)**.

Characterization of the cellular infiltrate caused by carrageenan in the air pouch

Results are expressed as total number of leukocytes x 106 cells **(Fig. 4)**. Few cells were found in the exudates of the air pouch when injected with PBS (0.6 ± 0.2); however, when carrageenan was injected a big increment in the number of leukocytes was observed (6.2 ± 0.6). In the carrageenan UG-treated groups, the total leukocytes were about a third of those found in the group injected only with carrageenan, indicating a reduction of leukocyte infiltration towards the site of inflammation of ~ 66% (2.1 ± 0.1 for UG 100) and 70% (1.9 ± 0.2 for UG 250) ($p < 0.01$). Similarly, the cells count of exudates from the carrageenan/pentoxy-treated group decreased ~ 69% (2.1 ± 0.4) and that of the pentoxy/UG 100 -treated group ~ 72% (1.7 ± 0.2) ($p < 0.01$).

Total blood cells count in mice treated with UG

The highest amount of blood leukocytes (expressed in x 103 cells/mm3) was observed in the PBS-injected control group (9.7 ± 0.8) and the lowest in the carrageenan-injected group (5.0 ± 0.3). Total leukocytes in the UG-supplied groups were 7.1 ± 0.4 for 100 mg/kg ($p < 0.05$) and 8.4 ± 0.2 for 250 mg/kg ($p < 0.01$).

In the leukocyte differential profile **(Fig. 5)**, the lymphocytes were found significantly diminished in the carrageenan-injected group (3.6 ± 0.2) as compared to the PBS group (7.7 ± 0.3, $p < 0.01$); in those mice that received the carrageenan injection and were supplied with UG at 100 mg/kg, lymphocytes were 5.9 ± 0.2 ($p < 0.01$) whereas at 250 mg/kg the lymphocytes were 6.6 ± 0.1 ($p < 0.01$).

Changes related to ER are shown in **Table 1**; in the carrageenan/UG-treated mice, ER decreased 75.73% (UG 100) and 81.90 % (UG 250) compared to carrageenan-injected mice.

Discussion

The present results show that the UG extracts was able to cause a dose-dependent lowering of TNF-α levels in the air pouch exudates of animals treated with pro-inflammatory molecules, observing about 50% reduction in TNF-α production when UG was supplied at 100 mg/kg. A significant decrease of cellular infiltrate in the air pouch was seen in parallel. Treatment with pentoxy, a specific inhibitor of TNF-α, produced similar effects to those induced by UG.

The induced expression of TNF-α in the air pouch would in turn induce the expression of COX-2 and consequently, an increment of PGE2 [13]. The inhibitory effect caused by the hydroalcoholic extract of UG on TNF-α would induce also an inhibitory effect on the expression of COX-2, as we have previously shown by in vitro studies [8]. This effect

may be also attributed to the inhibition of NF-kB, as previously been demonstrated [8]. Recently Elgawish et al has demonstrated an hepatoprotective actions with UG extracts through, at least in part, by inhibition of NF-kB [14].

In the present study a synergy for IL-10 production was observed between UG and pentoxy, a fact that can contribute to a beneficial effect in immunotherapy. Apparently, UG extracts were able to shift the cytokine production in carrageenan-inflammed mice toward the anti-inflammatory side of the cytokine response, decreasing TNF-α production and increasing IL-10. In addition, the sustained and elevated production of IL-10 in the systemic circulation might lead to an immunostimulated state.

The local expression of adhesion molecules induced by the production of cytokines like TNF-α is essential for the transmigration of leukocytes from the blood to extravascular inflammatory sites [12]. This is seen in all those in vivo studies where direct injection of TNF-α induces inflammation, whereas the inhibition of its biological activity by different anti-TNF-α treatments reduces inflammation [16,17]. In our present study, mice that received the irritant agent carrageenan showed a higher than normal amount of leukocytes in the pouch exudates and in parallel, a lower number of leukocytes in the blood.

Ding et al. [12] showed that the surface integrins LFA-1 and Mac-1 participate in the firm adhesion of leukocytes to the endothelium and in their extravasations when analyzing the leukocyte emigration ratio (ER). Since UG treatment reduced leukocyte extravasations to the inflamed air pouch, we might infer an inhibitory action of UG on the adhesion and/or integrins molecules resulting in reduced leukocyte interactions with the endothelium, a hypothesis that should be confirmed. By blocking leukocyte infiltration, the UG extract could be acting as inhibitor of the leukocyte extravasations induced by carrageenan in the air pouch, decreasing the leukocyte ER towards this site in a dose-dependent manner. Thus, UG would seem to interfere with the cytokine and adhesion molecules expression in vivo. Consequently, TNF-α inhibition would lead to the inhibition of the adherence and chemotaxis of inflammatory cells, as also suggested in our study by the observed ER reduction.

The inflammation was most efficiently inhibited (~ 96 %) in carrageenan-injected mice that received UG extract and pentoxy, suggesting that these two agents have mechanisms or sites of inhibition, related to their action on TNF-α production, that are different. Krakauer et al. [18] showed that pentoxy inhibited in vitro the production of TNF-α, IL-1α and IFN-α, suggesting that the inhibition occurred at the transcriptional level. Neutrophil functions such as adherence, degranulation and superoxide production induced by the inflammatory cytokines TNF- α and IL-1 are also blocked by pentoxy [19].

Another finding in our study is the action of UG extracts on the number of peripheral blood lymphocytes. UG-treated groups had the tendency to recover the normal number of lymphocytes in peripheral blood in a dose-dependent manner. A similar observation was reported by Sheng et al. [20] who found an increase of lymphocytes in UG-treated rats

injected with doxorubicin to induce leucopoenia. Also, the anti-inflammatory effect has been postulated to explain the hepatoprotector effect demonstrated in the experimental non-alcoholic fatty liver disease (NAFLD) in obese mice [21]. In addition, recently, a publication by Navarro-Hoyos et al [22] show that other phytocompounds like propelargonidin dimers found in leaves of *U. tomentosa* may have important effects.

In conclusion, in the present in vivo studies it has been shown that an hydroalcoholic extract of *U. tomentosa* had a strong anti-inflammatory activity. Finally, our results also suggest that the pentacyclic oxindole alkaloids content of UG or some of the other known UG components, simultaneously concentrated in the extract, play important, unique or synergistic roles in the above described effects.

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FIGURAS

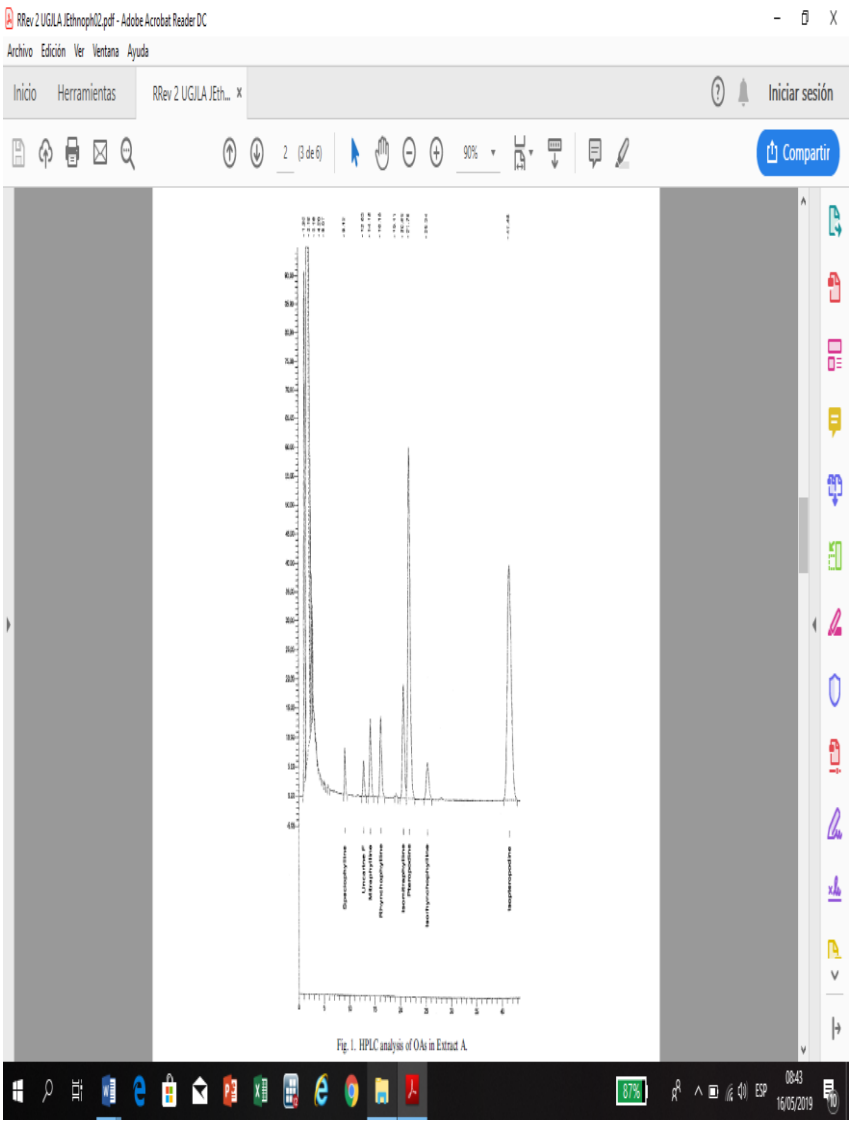

Fig. 1. HPLC analysis of alcoholic extract of UG

HPLC quantification of different oxindole alkaloids determinated in the spray-dried hydroalcoholic extract of UG.

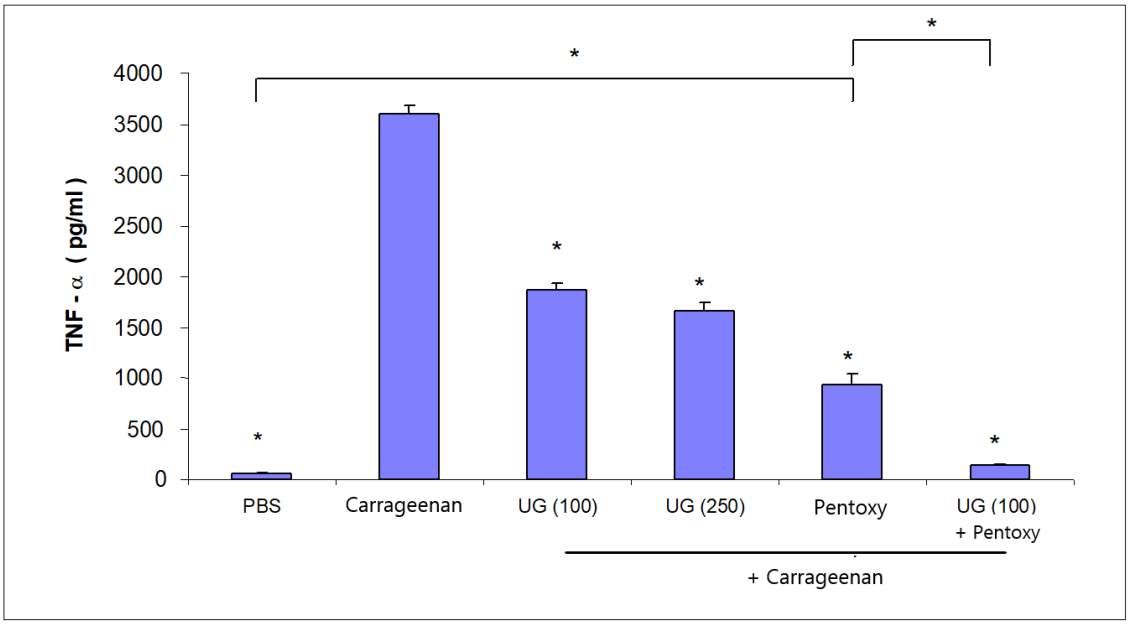

Fig 2. Reduction of TNF-α production by treatment with UG extracts.
Air pouch were induced and animals were treated with carrageenan alone or plus UG-100, UG-250, pentoxy, and UG-100 + pentoxy as described in Material and Methods. Production of TNF-α was evaluated by ELISA from exudates. Each value represents the mean of five animals per group ±SE. *P< 0.01 difference was found between carrageenan-treated group and other groups.

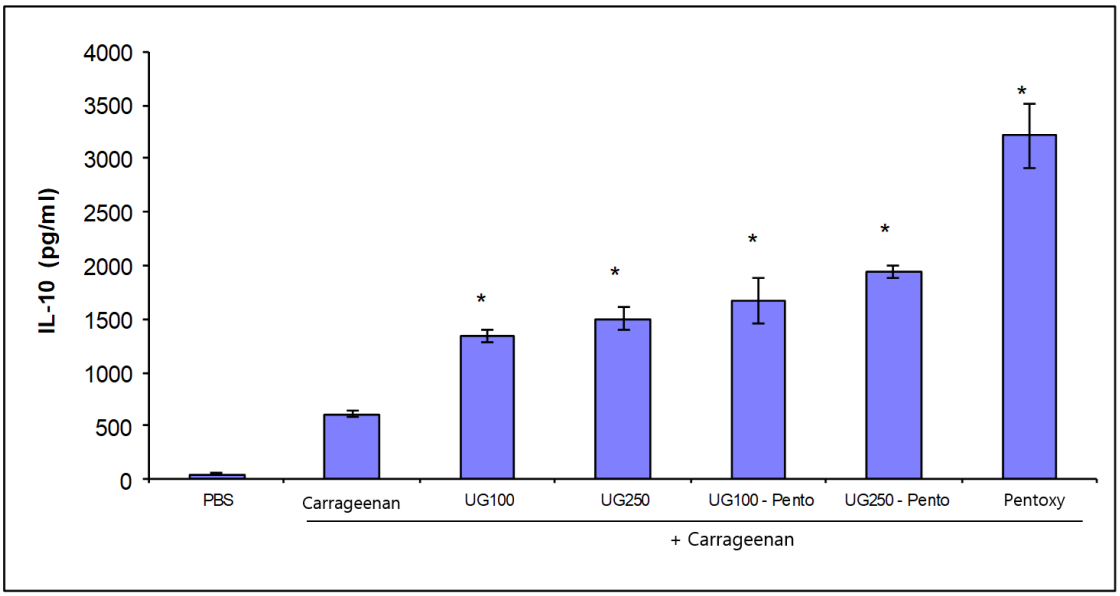

Fig 3. Treatment with UG-extracts augments the production IL-10.
Air pouch were induced and animals were treated with carrageenan alone or plus UG-100, UG-250, pentoxy and UG-100+, or pentoxy as described in Material and Methods. Production of IL-10 was evaluated by ELISA from exudates. Each value represents the mean of five animals per group ±SE. *P< 0.01 difference was found between carrageenan-treated group and other groups.

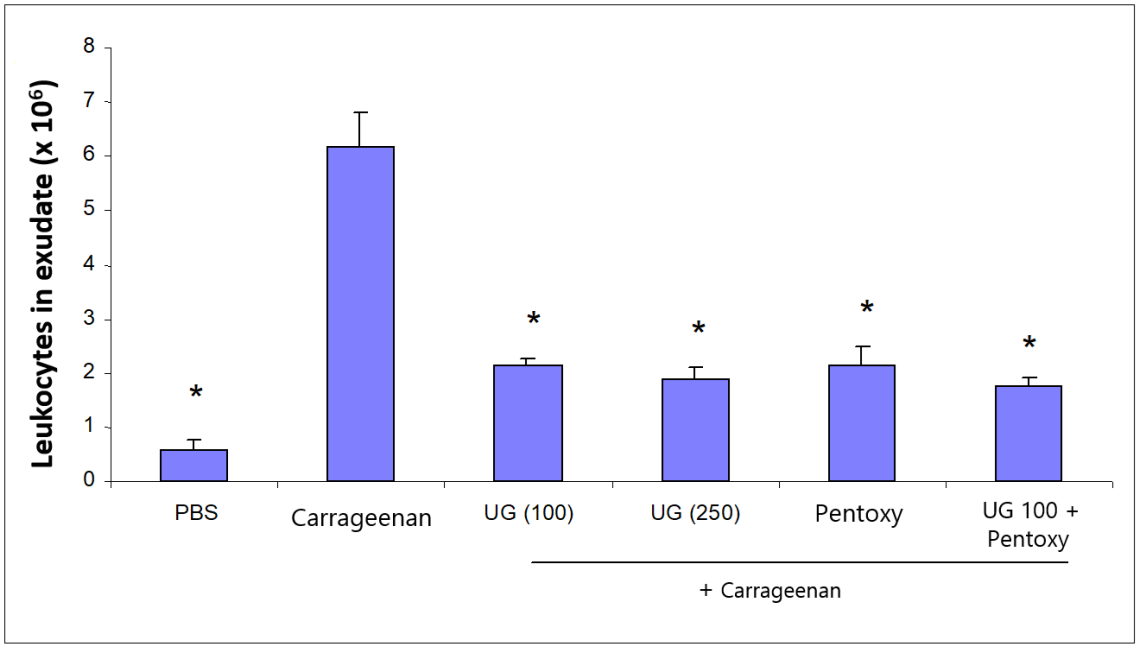

Fig 4. Leukocyte accumulation in the air pouch exudates in response to the carrageenan injection with different treatments.
Data are expressed as Mean SEM, of a total of 5 mice per group. * P < 0.01 *vs* carrageenan group.

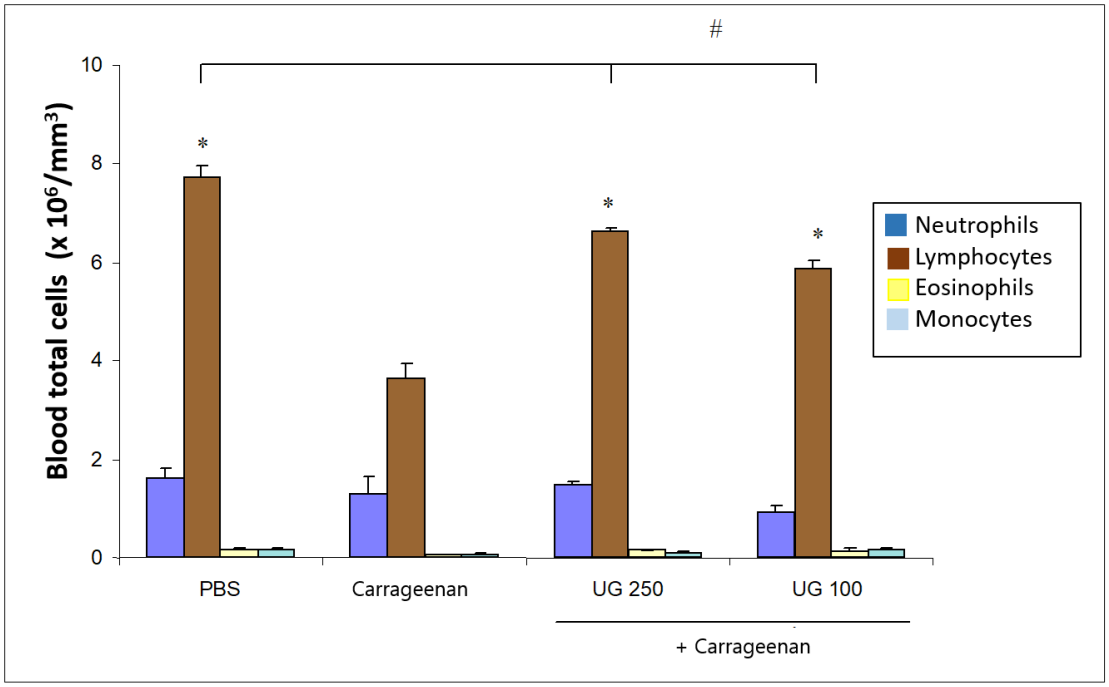

Fig 5. Blood leukocytes in response to the carrageenan injection with different treatments.
Data are expressed as Mean SEM, of a total of 5 mice per group. * $p < 0.01$; $^{\#}$ $p < 0.05$.

Table 1. Emigration ratio (ER) of leukocytes

	Emigration ratio (%)[a]	Change in the emigration ratio [b]
	Leukocytes	Leukocytes
PBS	5.80	0.00
Carragenina	122.52	2010.55
UG (100)	29.98	416.50
UG (250)	22.41	286.06

[a]ER =(Leukocytes in the air pouch / leukocytes in peripheral blood after the carrageenan injection) x 100%. [a]Change in the percent of ER = [(ER of mouse leukocytes corresponding to a given treatment / ER of leukocytes in carrageenan mice) - 1] x 100%.