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ORIGINAL RESEARCH

Arnica montana does not affect mast cell populations in experimentally induced oral ulcers in rats

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BACKGROUND: Studies have shown that Arnica montana shows anti-inflammatory and antioxidant activities. It has been used in traditional medicine for the treatment of several disorders. The aim of this study was to investigate the effect of Arnica montana on mast cells during the wound healing of oral ulcers.
METHOD: An ulcerated lesion was chemically induced on the tongue of 75 male albino rats and, then, treated topically for seven days using saline solution (control), Arnica montana gel or tincture. The animals were killed after 2nd, 7th, 14th, 21th and 42th day of treatment. The tongues were removed and subjected to routine laboratory (0.2% toluidine blue staining). The numbers of mast cell were determined in two regions: superficial and submucosa.
RESULTS: The numbers of mast cells were significantly increased for all groups in the region of the deeper tissue when compared to the superficial region. No statistical difference was observed in mast cell numbers for each group.
CONCLUSION: This study revealed that Arnica montana tincture and gel were unable to change mast cell population during wound healing of oral ulcer of rats. According to these results, the anti-inflammatory effects of Arnica montana were not related to inhibition of mast cell degranulation.

KEYWORDS: Arnica montana, Mast cell, Oral mucosa, Oral ulcer, Inflammation.

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INTRODUCTION

Oral ulcer is the name for the appearance of an open sore inside the mouth caused by a break in the continuity of the mucous membrane or the epithelium on the lips or surrounding the mouth. The types of oral ulcers are diverse, with a multitude of associated causes including: physical or chemical trauma, microorganism infections (virus, bacteria, and fungi), systemic diseases, and some drugs.[1]

This kind of lesion can affect several anatomical regions of the mouth, especially the tongue, the gingiva, and the cheeks.[2-3] The formation of an ulcer occurs when superficial layers of the oral mucosa are lost. Morphologically, oral ulcers appear as circular or irregular lesions. Once formed, the ulcer may be maintained by inflammation and/or secondary infection.[4] Thus, its prevention continues to be of concern for both clinical practitioner and researchers. Treatment of this condition is based on the removal of etiological factors and on the prescription of antimicrobial mouthwash. Several clinical protocols for ulcer treatment have been reported. In addition, several herbal agents have been employed in order to help in the repair of these oral lesions.[3]

Arnica is an herbaceous perennial plant. The subspecies montana is widely distributed, and grows in mountainous areas and thrives in nutrient-poor siliceous meadows and acid soils. Its flowers contain several active ingredients, including sesquiterpene lactones, flavonoids, volatile oil, mucilage, polysaccharides, and tannins. The flowers contain more arnicin than the rhizome, but no tannin. Some components such as Helenalin and

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Dihydrohelenalin produce anti-inflammatory and analgesic effects. Sesquiterpene lactones (SL), which are secondary plant metabolites from the flowerheads of Arnica, exert anti-inflammatory effects mainly by preventing nuclear factor (NF)-kappa B activation because of alkylation of the p65 subunit.[5] Despite its known immunosuppressive action, Arnica has been classified as a plant with strong potency to induce allergic contact dermatitis.[6]

Mast cells are cells of hematopoietic origin that terminally differentiate and become mature in tissues.[7] They contribute to both innate and adaptive immune responses. Mast cells are long-lived secretory cells viewed as sentinels, able to rapidly respond to modifications in their environment.[8-9] Once activated, mast cells have the ability to secrete a wide array of inflammatory mediators. Thus, mast cells can modulate the intensity of organ injury depending upon the pathophysiological context.[10] The aim of this study was to investigate the effect of Arnica montana on mast cells during the wound healing of oral ulcers treated by gel and tincture.

MATERIALS AND METHODS

Ethical Approval

The experimental protocol of the present study was approved by Committee of Ethics in Animal Experimentation at the Pontifícia Universidade Católica do Paraná.

Animals and drugs

Seventy-five male albino rats (Rattus norvegicus) weighing 200-250 g were used in this study. Animals were maintained in individual cages and received standard solid food and water ad libitum. An ulcerated lesion was chemically induced on the surface of the tongue using 40% sodium hydroxide after general anesthesia induced with thiopental sodium® (Cristália, Brazil, 20 mg/Kg). The ulcerated lesion was produced by topical application using a small cotton ball soaked in the chemical solution. The chemical agent was left to act on the edge of the tongue for approximately one minute.

Groups

The animals were divided into three groups:

i) Control: 25 animals that were treated daily by topic applications of saline solution during seven days.
ii) Gel: 25 animals received topical applications of a gel of 30% A. montana during seven days.
iii) Tincture: 25 animals received topical applications of 30% A. montana tincture during seven days.

The animals were treated with an analgesic with peripheral action (no anti-inflammatory effect) to control postoperative pain and facilitate the feeding of animals. The drug used was dipyrone (50 mg/kg) 4 times a day during three days. The mouth ulcers were treated by topical administration with the aid of a cotton pellet soaked in the test substances. The tongues of the animals were examined daily. Any alteration in the normal course of the repair process was recorded. The animals were killed under general anesthesia induced with thiopental sodium® (CRISTÁLIA, Brazil, 20 mg/Kg) after the 2nd (5 animals), 7th (5 animals), 14th (5 animals), 21th (5 animals) and 42th (5 animals) day of treatment. The tongues were removed surgically and fixed in 10% formalin solution. All tissue specimens were processed in the laboratory of Experimental Pathology of Universidade Católica do Paraná. Histological slides were prepared after sectioning at a thickness of 6μm and staining with 0.2% toluidine blue.

Mast Cell counting

The morphology and the location of mast cells were assessed microscopically. Mast cells were counted separately in sections of the ulcerated area using a light microscope OLYMPUS BX50 equipped with an objective PLAN 10X/0,25 and oculars WH10X-H/22 (OLYMPUS, Tokyo, Japan). This microscope was connected to the Color video camera CCD-IRIS (SONY, Tokyo, Japan) that allowed the capture of images in the fields of histology slides. ImagePro Plus software version 4.0.1 (Media Cibernetics, Atlanta, GA, USA) was used to count cells. Mast cells were counted in four counting fields per section at 100x magnification. In order to assess distribution, the histological fields were separated in two areas: Superficial (epithelium/connective tissue interface) and Deep (submucosal region). The number of mast cells was expressed as cells/mm^2 (mean ± SD.).

Statistical analysis

All data were tabulated and statistical tests were performed with SPSS for Windows 13.0 (SPSS Inc., Chicago, Illinois, USA). Tests of normality (Kolgomorov-Smirnoff test) and homogeneity of variances (Levene's Test) were used. Differences between groups were examined using the Games-Howell test. Difference was considered statistically significant when $p < 0.05$.

RESULTS

No significant change was observed during the process of wound repair in the tongues of animals. Large numbers of mast cells were found immediately under the basement membrane in all sections studied. These cells were round or oval and were located especially in certain regions, namely lamina propria, inflammatory infiltrates, perivascular region and in the deep connective tissue. No mast cells were observed in the epithelium.

The mean number of mast cells in the superficial region was greater in animals treated by tincture and gel of Arnica montana at 7th post-treatment days, as shown in Table 1. The lowest density of mast cells was observed in the control group at 14 post-treatment days. However, none of the differences was statistically significant ($p > 0.05$).

Table 1 - Mean numbers of mast cells in the superficial region for groups untreated and treated by *Arnica montana*

Period	Control (Mean ± sd)	Gel (Mean ± sd)	Tincture (Mean ± sd)	p value
	23.0 ± 16.27	20.0 ± 10.81	-	1,0000
2 days	23.0 ± 16.27	-	37.0 ± 23.56	0,9999
	-	20.0 ± 10.81	37.0 ± 23.56	0,9928
	14.62 ± 9.20	32.6 ± 31.87	-	0.9983
7 days	14.62 ± 9.20	-	54.3 ± 36.74	0,7829
	-	32.6 ± 31.87	54.3 ± 36.74	1,0000
	7.66 ± 6.50	16.8 ± 16.68	-	0,9996
14 days	7.66 ± 6.50	-	9.6 ± 8.96	1,0000
	-	16.8 ± 16.68	9.6 ± 8.96	1,0000
	26.75 ± 19.66	15.8 ± 8.07	-	0,994
21 days	26.75 ± 19.66	-	8.75 ± 7.46	0,9496
	-	15.8 ± 8.07	8.75 ± 7.46	0,9967
	18.0 ± 14.53	16.75 ± 8.66	-	1,0000
42 days	18.0 ± 14.53	-	14.25 ± 9.92	1,0000
	-	16.75 ± 8.66	14.25 ± 9.92	1,0000

The numbers of mast cells were significantly increased for all groups in the deeper tissues when compared to the superficial region ($p = 0.003$), as shows the Figure 1. The mean mast cell number in the submucosa was greater in the experimental group when compared to control group at 2, and 21 post-treatment days, as shown in Table 2. As previously noted, the lowest density of mast cells was observed in the control group at 14 post-treatment days. However, none of these differences was statistically significant for this region ($p > 0.05$)

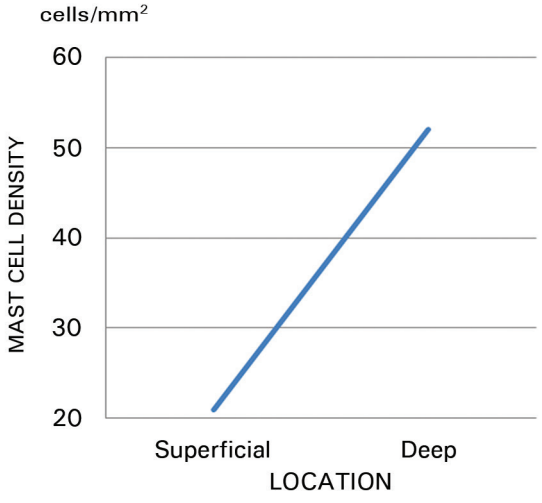

Figure 1 - Superficial and deep average density of mast cells in the region of an ulcer. Averages for control and treated animals, which were not different between them.

DISCUSSION

Evaluation of the biological activities of compounds in Arnica montana and elucidation of the mechanisms of their functions may provide substantial clues for the development of new drug candidates. Some previous studies have demonstrated that Arnica montana exhibits potential anti-inflammatory activity.[11-15] Additionally, other studies have demonstrated the beneficial effects of Arnica montana in wound healing,[16-18] pain relief,[19] antioxidant activity and cytoprotective effect against oxidative damage.[20] However, to our knowledge the mechanism by which Arnica montana acts on the oral wounds is not known.

This study investigated the effect of Arnica montana upon the mast cell population during wound healing of oral ulcers in rats. Mast cells are located in the vicinity microcirculatory vessels involved in vasodilation. They mature under the influence of various cytokines. Human skin and mucosal mast cells play an essential role in various physiological and pathological processes and mediate early hypersensitive reaction and allergic diseases.[21] In the present experiments, the morphology of mast cells was not altered in the presence of Arnica (gel or tincture). Furthermore, no statistical difference was observed in the number of mast cells when the groups were compared. These results demonstrate that Arnica montana compounds were probably unable to inhibit mast cell migration and degranulation.

Table 2 - Number of mast cells at submucosa for groups untreated and treated by Arnica montana

Period	Control (Mean ± SD)	Gel (Mean ± SD)	Tincture (Mean ± SD)	p value
	58.87 ± 31.60	20.0 ± 10.81	-	0,7667
2 days	58.87 ± 31.60	-	53.25 ± 22.94	1,0000
	-	20.0 ± 10.81	53.25 ± 22.94	0,6548
	33.37 ± 12.51	36.8 ± 17.88	-	1,0000
7 days	33.37 ± 12.51	-	60.9 ± 12.26	0,3969
	-	36.8 ± 17.88	60.9 ± 12.26	0,7297
	47.83 ± 25.93	52.8 ± 13.70	-	1,0000
14 days	47.83 ± 25.93	-	60.9 ± 16.70	1,0000
	-	52.8 ± 13.70	60.9 ± 16.70	1,0000
	68.0 ± 31.15	55.0 ± 26.58	-	1,0000
21 days	68.0 ± 31.15	-	66.5 ± 28.80	1,0000
	-	55.0 ± 26.58	66.5 ± 28.80	1,0000
	60.62 ± 24.99	62.87 ± 28.96	-	1,0000
42 days	60.62 ± 24.99	-	50.5 ± 29.52	1,0000
	-	62.87 ± 28.96	50.5 ± 29.52	1,0000

The cytoplasm of the resting mast cell is filled with large granules containing histamine, prostaglandins, and other proinflammatory mediators.[22] The activation and degranulation of mast cells are major contributors to inflammation.[23] This reaction is noticeable when the mean number of mast cells is analyzed in relation to time. In our study, the results showed that the lowest number of mast cells present in the surface region of the wound was recorded after 14 days of treatment. This phase of the repair process coincides with the period of chronic inflammation when collagen fibers are being deposited and angiogenesis is evident. According to Trautmann et al.[24] as the healing process progresses, mast cells migrate to the site of injury, dramatically increasing in numbers.

Most cases of oral ulcers observed in general practice are due to recurrent aphthous ulceration, infection or trauma. However, many of the reports in the literature have not been validated in controlled clinical trials. Ulcers related to trauma usually resolve in about seven days after removal of the cause and with the use of pharmacological agents to minimize the undesirable effects induced by inflammation.[1]

Ganzera et al.[25] demonstrated that sequiterpenes, flavonoids and phenolic acids are among the biologically active ingredients in the flowerheads of the Arnica Montana. Quercetin 3-O-glucuronic acid was the most dominant flavonoid, whereas 3,5-dicaffeoylquinic acid was the major phenolic acid; the total content of flavonoids and phenolic acids varied in the samples from 0.60 to 1.70%, and 1.03 to 2.24%, respectively.

Although the therapeutic use of A montana is established, some side effects have been described.[26] It is classified as a plant with a strong power to induce allergic contact dermatitis.[6] However, no allergic reactions were observed in animals during our clinical follow-up and neither in the histological evaluation of mast cells.

This study also showed that the number of mast cells in the deeper tissues is higher than those in the superficial regions of the ulcer. These findings corroborate the results of Natah et al.[27] who observed similar results when investigating the population of mast cells within traumatic ulcers and recurrent aphthous ulcerations.

The vehicle used for topical applications is a potentially important factor in the pharmacologic effects. Bergamante et al.[28] investigated the ability to dissolve, release, and induce the permeation of helenalin (flavonoid responsible for the anti-inflammatory activity of the Arnica montana extract) in two types of gels and microemulsions. Their study showed that a microemulsion could be a good vehicle to increase the permeation of helenalin. In our study, the greatest numbers of mast cells were observed the groups treated by the tincture of Arnica Montana. In this scenario, the ethanol used in the tincture may have influenced the results. Additionally, the mast cell population was significantly increased in the region of submucosa. This result corroborates the findings of Pumpa et al.[19] and demonstrated that both preparations of Arnica Montana used in this study were unable to act on mast cells in the muscle tissue of the tongue.

The present research represents the first study suggesting that Arnica Montana does not change the mast cell population in oral ulcers during the wound healing. This fact reinforces the concept that the anti-inflammatory effect of Arnica Montana is not related to mast cell inhibition. Thus, others studies are necessary to determine the true mechanism of action of A Montana during the wound healing of oral ulcerations.

ARNICA MONTANA NÃO AFETA POPULAÇÕES DE MASTÓCITOS EM ÚLCERAS ORAIS INDUZIDAS EXPERIMENTALMENTE EM RATOS

OBJETIVO: Sabe-se que a Arnica montana mostra atividade anti-inflamatória e anti-oxidante e tem sido usada em medicina tradicional para o tratamento de vários distúrbios. O objetivo deste estudo foi investigar o efeito da Arnica montana em mastócitos durante a cicatrização de feridas de úlceras orais.

MÉTODO: Uma úlcera foi quimicamente induzida na língua de 75 ratos albinos machos e, em seguida, tratada topicamente durante sete dias, utilizando solução salina (controle), gel ou tintura de Arnica montana. Os animais foram sacrificados após 2, 7, 14, 21 e 42º dia de tratamento. As línguas foram removidas e submetidas a rotina de laboratório (coloração com 0,2% de azul de toluidina). A densidade de mastócitos foi determinada em duas regiões: superficial e submucosa.

RESULTADOS: O número de mastócitos aumentou nitidamente para todos os grupos na região mais profunda do tecido peri-ulceroso, quando comparada à região superficial. Nenhuma diferença estatística foi observada no número de mastócitos entre os grupos.

CONCLUSÃO: Este estudo revelou que a tintura ou o gel de Arnica montana foram incapazes de interferir na população de mastócitos durante a cicatrização da úlcera oral de ratos. De acordo com estes resultados, os efeitos anti-inflamatórios de Arnica montana não foram relacionados à inibição da degranulação dos mastócitos.

UNITERMOS: Arnica montana, mastócitos, mucosa oral, úlcera oral, da inflamação.

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