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Effect of Exogenous Melatonin on Acute and Chronic Inflammatory Process in Rats

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SUMMARY. Melatonin influence (4 mg/kg) was investigated on acute inflammation using rat paw edema and on chronic inflammation through granuloma test and adjuvant arthritis. Melatonin inhibited the edema produced by carrageenan in acute model. However, failed to inhibit the proliferative phase in the granuloma test and the acute and chronic phase in the adjuvant arthritis. These results suggest melatonin shows different activity on the tested inflammatory models at the same doses.

RESUMEN. "Efecto de melatonina exógena en procesos inflamatorios agudos y crónicos en ratas". Se investigó la influencia de melatonina (4 mg/kg) en la inflamación aguda usando el modelo de edema de pata en rata y en la inflamación crónica mediante la prueba del granuloma y artritis inducida por adyuvante. Melatonina inhibió el edema producido por carragenina en el modelo agudo, pero no presentó acción inhibitoria en la fase proliferativa de la prueba del granuloma y en la fase aguda y crónica de la artritis inducida por adyuvante. Estos resultados sugieren que melatonina, a la misma dosis, muestra diferentes comportamientos en los modelos inflamatorios ensayados.

INTRODUCTION

The molecule of melatonin seems to have been conserved throughout evolution. Its presence has been demonstrated in almost all groups of organisms, from plants and protozoa to people. The universal presence of melatonin may be ascribed to its lipophilic nature, which enables it to cross all biological barriers.

There is scientific evidence of melatonin role as therapeutic agent in the treatment of circadian rhythm associated to sleep disorders. It has been well established that this hormone, produced principally by the pineal gland, modulates various immune functions in laboratory rodents [1-3] and counteracts the immunodepression induced by acute stress, drug or corticosteroids on antibody production [4].

Cuzzocrea *et al.* [5] suggest a protective effect for melatonin in carrageenan induced models of local inflammation; part of these anti-inflammatory effects may be related to an inhibition of the expression of the inducible NO synthase, while another part may be related to oxyradical and peroxynitrite scavenging.

Several experiments were performed by the group of Hansson *et al.* [6-8] in the collagen-induced arthritis model. Mice kept in constant darkness developed more severe arthritis than those kept in constant light or in a normal dark/light rhythm (12 h dark -12 h light). Therefore, the authors concluded that exacerbation of arthritis in darkness might be due to higher level of melatonin in these animals.

Cardinali *et al.* [9] showed that in young rats injected with mycobacterial adjuvant, 100 µg melatonin had an inflammation promoting ef-

KEY WORDS: Adjuvant arthritis, Anti-inflammatory activity, Carrageenan-induced rat paw edema, Granuloma test, Melatonin.
PALABRAS CLAVE: Actividad antiinflamatoria, Artritis inducida por adyuvante, Edema de pata de rata inducida por carragenina, Melatonina, Prueba del granuloma.

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fect. Besides, melatonin administration (10 and 100 µg) to old rats restored the inflammatory response in rat hind paws injected with Freund's adjuvant, to levels found in young Sprague-Dawley rats.

Missbach *et al.* [10] identified a class of thiazolidine diones with high potency in suppressing chronic inflammation and joint destruction in the adjuvant arthritis on rat; however, these compound showed to be specific to activate the retinoid Z receptor/retinoid acid receptor-related orphan receptor α (RZR/RoRα) in low (nanomolar) concentrations. Several lines of evidence have shown that melatonin is a natural ligand of RZR/RoR [11,12].

The aim of this study was to examine the effect of exogenous administration of melatonin on acute inflammation using rat paw edema model [13] and on chronic inflammation through granuloma test [14] and adjuvant-induced arthritis [15]. Melatonin dose of 4 mg/kg and pre-treatment time of 15 min in acute model was chosen according to prior assays (unpublished data).

MATERIAL AND METHODS

Experimental animals

Fifty-four adult male Wistar rats (120-150 g) were used in the experiments. They were housed in polypropylene cages under conditions of 24 ºC ± 1ºC and 12 h light-dark cycle with humidity (55 + 5%), maintained with water and food ad libitum.

Materials

Carrageenan (type V), Freund's complete adjuvant, Dexamethasone, Indomethacin and Melatonin were purchased from Sigma Chemical Co, St Louis Mo.

Paw edema by carrageenan

The animals were divided into control, reference and experimental groups of six animals each. Melatonin was dissolved in 100% ethanol and diluted in saline solution to a final concentration of 1% ethanol and it was administered to the experimental animals at 4 mg/kg intraperitoneally. Indomethacin (4 mg/kg) was suspended in the same delivery system and it was given to reference group animals. Control group received only the vehicle. The compounds were administered intraperitoneally at nine o'clock to each group 15 min before carrageenan injection. The edema was induced by subcutaneous injection of 0.1 ml of a 2% suspension of carrageenan in saline solution in the subplantar region of the left hind paw in all the animals.

The paw edema was measured plethysmographically (Ugo Basile 7150, Varese Italy plethysmograph) before injection and at intervals of 1, 3, 5 and 7 h after injection of carrageenan. The edema volume is expressed in each animal as the difference found between the left hind paw volume compared against the right hind one. The inhibition percentage of edema was calculated for each animal group in comparison with control group.

Cotton pellet granuloma formation

Adult male Wistar rats (weight range 120 g) were divided in three groups of five animals. Each animal was anesthetized with ether and a small piece of sterile cotton (50 mg) induced granuloma into the dorsal area. Melatonin was dissolved in 100% ethanol and diluted in saline solution to a final concentration of 1% ethanol and it was administered to the experimental animals at 4 mg/kg intraperitoneally (17 µmoles/kg). Dexamethasone 8 mg/kg (15 µmoles/kg) was dissolved in the same delivery system and it was given to reference group animals. Control group received only the vehicle. The compounds were administered 0.3 ml to each animal. All the injection was administered subcutaneously at nine o'clock in the morning for 6 days. At day 7, the rats were killed by cervical dislocation, granulomas were dissected out and weighed. The increased in the pellet weight was considered as granuloma tissue deposit. The inhibition percentage of inflammation was calculated for each animal group in comparison with the control group.

Arthritis

Experimental arthritis was induced in rats according to the method of adjuvant-carrageenan-induced inflammation (ACII). A total of 21 rats were divided into groups of seven animals each. Experimental group received melatonin (4 mg/kg) dissolved in 100% ethanol and diluted in saline solution to a final concentration of 1% ethanol. Reference group received indometacin (4 mg/kg), dissolved in the same delivery system that melatonin. Control group received only the vehicle. On day 0, all of them received an injection of 0.1 ml Freund's complete adjuvant intradermally at the base of the tail. The compounds were injected intraperitoneally 0.5 ml to each animal at nine o'clock in the morning from 6 to 20 days after adjuvant inoculation. On the

sixth days and one hour after drug administration, a suspension of 0.1 ml of carrageenan (type V; 2% w/v in saline solution) was injected in the subplantar region of the left hind paw of each rat. The degree of pedal edema was determined by measuring both hind paws volume by plethysmography (Ugo Basile). Plethysmographic measurements were made before the adjuvant injection (day 0) and were repeated again 6 days later just before the injection of carrageenan at 3, 5 h (acute phase) and continued from days 7 to 20 (chronic phase) after carrageenan injection. All the measurements was made at nine o'clock in the morning. The edema volume is expressed in each animal as the difference found in the left hind paw compared with the right hind one. The inhibition percentage of edema was calculated for each animal group in comparison with the control group.

Statistical analysis

Data obtained from the pharmacological experiment are expressed as mean ± SD. Differences between the control and the treatments in this experiment were tested for significance using Dunnett's test. A probability of $p<0.05$ was considered significant and a probability of $p<0.01$ was considered very significant.

RESULTS

Effect of Melatonin on inflammation induced by carrageenan

Intraplantar injection of carrageenan in rats led to an increase in paw volume (Table 1).

Time	Control group (vehicle)	Melatonin		Indomethacin	
	Edema volume (ml)	Edema volume (ml)	Inhibition %	Edema volume(ml)	Inhibition %
1 h	0.34 ± 0.07	0.25 ± 0.09	26	0.22 ± 0.11	34
3 h	0.59 ± 0.12	0.19 ± 0.03	68**	0.14 ± 0.05	77**
5h	0.94 ± 0.07	0.27 ± 0.06	71**	0.234 ± 0.11	75**
7h	0.87 ± 0.19	0.43 ± 0.09	50**	0.33 ± 0.15	62**

Table 1. Effect of Melatonin treatment on carrageenan induced paw edema in rats. Six animals were used in each group. Tabular values represent the mean ± SD of difference between injected left paw volume and non-injected right paw volume (edema volume ml), and volume inhibition percent of edema compared with the control group treated only with carrageenan. Dunnett's test, ** $p< 0.01$.

The maximal increase in paw volume was observed at 5 h after carrageenan administration (0.94 ml ± 0.07 ml). Treatment with melatonin (4 mg/kg) reduces significantly at 3, 5, 7 h the inflammatory activity on the experimental group.

The anti-inflammatory activity caused by 4 mg/kg of melatonin was comparable to the reference drug indomethacin and was highly significant $p< 0.01$.

Cotton pellet granuloma formation

The effect of melatonin and dexamethasone on the cotton pellet granuloma is summarized in Table 2. Granuloma formation in animals treated with Melatonin 4 mg/kg was not significantly different from ones in controls. The anti-inflammatory drug Dexamethasone at 8 mg/kg caused a highly significant reduction in granuloma formation ($p<0.01$).

Group	Treatment	Granuloma (mg)	Inhibition (%)
Control	Vehicle	693,5 + 21,0	–
Reference	Dexamethasone	366,9+ 29,7**	47
Experimental	Melatonin	736,2 +19,2	-6

Table 2. Effect of Melatonin treatment on cotton pellet granuloma formation in rats. Five animals were used in each group. Values represent means ± S.D. of granuloma weights and inhibition percent of granuloma formation. Dunnett test ** $p< 0.01$.

Adjuvant arthritis

Table 3 shows the effect of melatonin and indomethacin on the arthritis. Indomethacin decreased the edema produced in acute phase on day 6 at 3 and 5 hours after carrageenan injection, but Melatonin was not active. In the chronic phase (days 7-20) Indomethacin clearly reduced the edema with effect pronounced.

Time	Control Group	Melatonina		Indomethacin	
	Edema volume (ml)	Edema volume(ml)-	Inhibition %	Edema volume (ml)	Inhibition %
3 h (6 days)	0.87 ± 0.24	0.42 ± 0.18	25	0.57 ± 0.12	34*
5 h (6 days)	1.09 ± 0.28	0.78 ± 0.19	12	0.70 ± 0.09	35*
7 days	0.93 ± 0.25	0.59 ± 0.10	36*	0.63 ± 0.10	32*
8 days	1.29 ± 0.24	0.96 ± 0.18	25	0.73 ± 0.11	43*
9days	0.78 ± 0.16	0.67 ± 0.22	14	0.55 ± 0.23	29
10 days	0.82 ± 0.26	0.76 ± 0.18	7	0.42 ± 0.17	48*
11 days	0.84 ± 0.28	0.79 ± 0.21	5	0.63 ± 0.19	24
12 days	1.02 ± 0.27	0.97 ± 0.25	4	0.76 ± 0.23	25
13 days	0.98 ± 0.19	0.95 ± 0.23	3	0.78 ± 0.17	20
14 days	1.14 ± 0.17	0.86 ± 0.21	24	0.74 ± 0.11	35*
15 days	1.09 ± 0.20	0.91 ± 0.19	16	0.69 ± 0.12	36*
16 days	0.99 ± 0.14	0.87 ± 0.21	12	0.70 ± 0.18	29
17 days	1.13 ± 0.27	0.88 ± 0.20	20	0.76 ± 0.10	32*
18 days	1.28 ± 0.14	1.17 ± 0.19	8	0.79 ± 0.11	38*
19 days	1.37 ± 0.29	0.94 ± 0.15	31*	0.83 ± 0.10	39*
20 days	1.53 ± 0.14	1.24 ± 0.11	20	0.93 ± 0.12	39*

Table 3. Effects of Melatonin and Indomethacin treatment on adjuvant carrageenan induced inflammation. Seven animals were used in each group. Tabular values represent the mean ± SD of difference between injected left paw volume and non-injected right paw volume (edema volume ml), and volume inhibition percent of edema compared with the control group. Dunnett test* $p < 0.05$.

Melatonin showed to have a significant inhibition only on 7th and 19th days. These results demonstrated that melatonin did not show anti-inflammatory activity on both acute and chronic phase of arthritis model at 4 mg/kg.

DISCUSSION

It is well known that in acute inflammatory processes, in which vascular permeability increases and leukocyte migration occurs, neutrophil-derived reactive oxygen species including hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{-\bullet}$) and $\bullet OH$ are involved [16].

The cellular and molecular mechanism of the carrageenan-induced inflammation is well characterized [17]. It appears that the early phase of the carrageenan edema is related to the production of histamine, leukotrienes, platelet activating factor and possibly cyclooxygenase products, while the delayed phase of carrageenan-induced inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide and hydroxyl radical, as well as to the release of other neutrophil derived mediators [18-20].

Cotton pellet granuloma bioassay in rats frequently used as an experimental model for the chronic inflammation has three phases after the pellet implantation [21]; in the last phase, there is a cell proliferation and this phase can be prior inhibited, especially by anti-inflammatory steroids.

The etiology of rheumatoid arthritis remains unknown; it has been suggested that cytokines, especially pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), IL-1 and IL-6 play a pivotal role in the pathology of the disease [22].

Melatonin, the pineal hormone, transduces

the effect of photoperiods onto the neuroendocrine system and thus regulates seasonal rhythms including reproduction [23,24]. During the last years, melatonin has been proposed to play an important role on inflammatory process and inmunological system.

Cuzzocrea *et al.* [25] have demonstrated that melatonin (25-50 mg/kg) exerts potent antiinflamatory effects on acute peritonitis; however, inflammatory effect induced by melatonin (1 mg/kg b.w.) was observed for Hansson *et al.* [6,7] in the collagen-induced arthritis model.

Different animals models such as carrageenan paw edema, granuloma test and arthritis adjuvant-induced were employed in our experiment to evaluate the antiinflamatory activity of 4 mg/kg dose of melatonin.

The findings of the present study revealed a protective effect of melatonin on the rat paw edema induced by carrageenan, principally on the delayed phase that response to neutrophil derived free radicals. These results are in concordance with the other authors [5] and should be related to its direct scavenging effects or its ability to regulate lymphocyte migrations.

Neither an anti-inflammatory effect nor inflammatory effect of melatonin at the used doses was observed for us on chronic process. In summary, melatonin showed a different behavior on inflammatory process at the doses used, because it would be acting by a different mechanism. Although our results are insufficient to conclude about melatonin role in chronic inflammatory processes, it is important that research on the different activities of these hormone be continued because in recent years numerous reports have exposed the virtues of using melatonin to treat inflammatory diseases. The popular image of melatonin as a "wonder drug" will lead to increase its use in the future, but more studies are needed to substantiate its potential therapeutic effect.

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