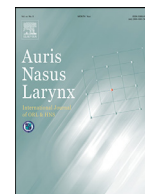




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Original Article

Therapeutic effects of melatonin on an ovalbumin-induced allergic rhinitis model in rats

Asli Cakir Cetin^{a,*}, Mustafa Cenk Ecevit^a, Mukaddes Gumustekin^b,
Cetin Pekcetin^c, Seda Ozbal^c, Hande Efe^d, Pelin Koca^b, Ozge Akcay^b,
Yesim Tuncok^b

^aDepartment of Otorhinolaryngology, Dokuz Eylul University, School of Medicine, Izmir 35340, Turkey

^bDepartment of Pharmacology, Dokuz Eylul University, School of Medicine, Izmir 35340, Turkey

^cDepartment of Histology & Embriology, Dokuz Eylul University, School of Medicine, Izmir 35340, Turkey

^dDepartment of Medical Biology, Dokuz Eylul University, School of Medicine, Izmir 35340, Turkey

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ABSTRACT

Objective: We aimed to investigate the therapeutic effects of melatonin in an experimental AR model.

Methods: Thirty-two Wistar rats were randomised into four groups ($n = 8$ each). The experimental AR model was established in the saline (SF), ethanol, and melatonin groups via intraperitoneal (i.p.) injections and intranasal application of ovalbumin. The SF, ethanol, and melatonin groups received daily i.p. saline, 2% ethanol dissolved in saline, and 10 mg/kg melatonin dissolved in 2% ethanol and saline. The control group received the same amount of i.p. and intranasal saline. Total nasal symptom scores were recorded in all rats on days 1 (baseline), 15, 20, 25, and 30. Serum ovalbumin-specific IgE, IL-13, and melatonin levels were measured on days 1 (baseline), 15, and 30. The nasal mucosa of all rats was scored histopathologically.

Results: The total nasal symptom scores and serum ovalbumin-specific IgE values of the SF, ethanol, and melatonin groups were significantly higher on day 15 than those of the control group. On day 30, the scores and serum ovalbumin-specific IgE values of the melatonin group were similar to those of the control, whereas the SF and ethanol groups had statistically higher scores. The histological scores of the SF and ethanol groups were significantly higher than those of the control and melatonin groups, but no significant difference was found between the melatonin and control groups.

Conclusion: Melatonin reduced total nasal symptom scores and serum ovalbumin-specific IgE levels and improved histological inflammation parameters in the ovalbumin-induced rat experimental AR model.

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1. Introduction

Allergic rhinitis (AR) is a chronic T helper 2 lymphocyte-dominated and IgE-mediated inflammation of the nasal mucosa. The symptoms of AR are allergen-induced sneezing,

* Corresponding author.

E-mail addresses: aslicakir84@hotmail.com, asli.cakir@deu.edu.tr (A. Cakir Cetin).

nasal itching, nasal discharge, and obstruction [1]. AR is a serious disease of public health concern with a prevalence of 10–40%, affecting more than half a billion of people over the world, causing the development of asthma, and complications of asthma control. Treatment remains mostly symptomatic, except for immunotherapy as a disease-modifying treatment [2]. However, immunotherapy is a long-term and challenging method that is not completely free of complications, limitations, and failure [3,4].

Recently, melatonin has been popularised as a promising therapeutic agent in several experimental animal models and clinical studies of *T* helper 2-dominated, IgE-mediated atopic and allergic diseases such as allergic asthma and atopic dermatitis, for its anti-allergic, anti-apoptotic, antioxidant, cytoprotective, and immunomodulatory effects [5,6]. Considering that the pathogenesis of AR resembles that of allergic asthma and that of atopic dermatitis [1], it is unclear whether melatonin plays a role in the treatment of AR.

We sought to investigate the potential therapeutic effects of melatonin in an ovalbumin-induced AR model.

2. Materials and methods

2.1. Compliance with ethical standards

This project was approved by the Ethics Committee of the Research of Laboratory Animals, Dokuz Eylul University (protocol number: 51/2016). All procedures performed in studies involving animals were in accordance with the ethical standards and the guidelines prepared by the Animal Use Committee of the institution.

2.2. Animals and sample size

Thirty-two healthy male Wistar rats (200–220 g and aged 20–22 weeks) bred in the Multidisciplinary Animal Laboratory of Dokuz Eylul University (20–22 °C temperature, 40–60% relative humidity, 12 h light/dark cycle, and free access to pellet food and water) were used. Four rats shared one cage and each cage was 30 × 18 × 24 cm.

2.3. Study design

The animals were randomised into four groups ($n = 8$ each) after baseline symptom scoring. Twenty-four subjects were administered 0.3 mg of ovalbumin (Sigma-Aldrich, St. Louis, MO, USA) and 30 mg aluminium hydroxide dissolved in 1 ml of 0.09% saline intraperitoneally (i.p.) on alternate mornings for 14 days to induce the first sensitisation of AR, while eight subjects (control group, group 1) were given pure saline (1 ml) during this period between 11 AM and 12 AM. For the next 16 days, AR symptoms were induced by applying 10 μ L of 20 mg/mL of ovalbumin dissolved in 0.09% saline in each nostril of the sensitised animals ($n = 24$) with a micropipette, once daily between 10 AM and 12 AM (second sensitisation) [7–9]. The control group (Group 1) was administered the same amount of intranasal 0.09% saline in the same way. During the second sensitisation, the three groups of animals which

developed AR were daily given one of the following one hour before intranasal applications: 1- 1 ml of 0.09% saline i.p. ($n = 8$, Group 2), 2- 2% ethanol (Ethanol, Sigma-Aldrich, Cat No: 32,205 (Sigma-Aldrich Corp., Missouri, USA)) dissolved in 1 ml of 0.09% saline i.p. ($n = 8$, Group 3), 3- 10 mg/kg melatonin-included 2% ethanol dissolved in 1 ml of 0.09% saline [10] (Melatonin, Sigma-Aldrich, Cat No: M5250 (Sigma-Aldrich Corp., Missouri, USA)) ($n = 8$, Group 4) (Fig. 1. Flow Diagram).

2.4. Study place and time schedule

The establishment of the AR model and behavioural tests were performed at the Centre of Laboratory Animal Breeding and Experimental Studies at Dokuz Eylul University between 10.07.2019 and 13.08.2019. Each group's intervention was started on consecutive days. The histological procedures were completed in the Department of Histology and Embryology, Dokuz Eylul University.

2.5. Experimental procedure

2.5.1. Total nasal symptom score

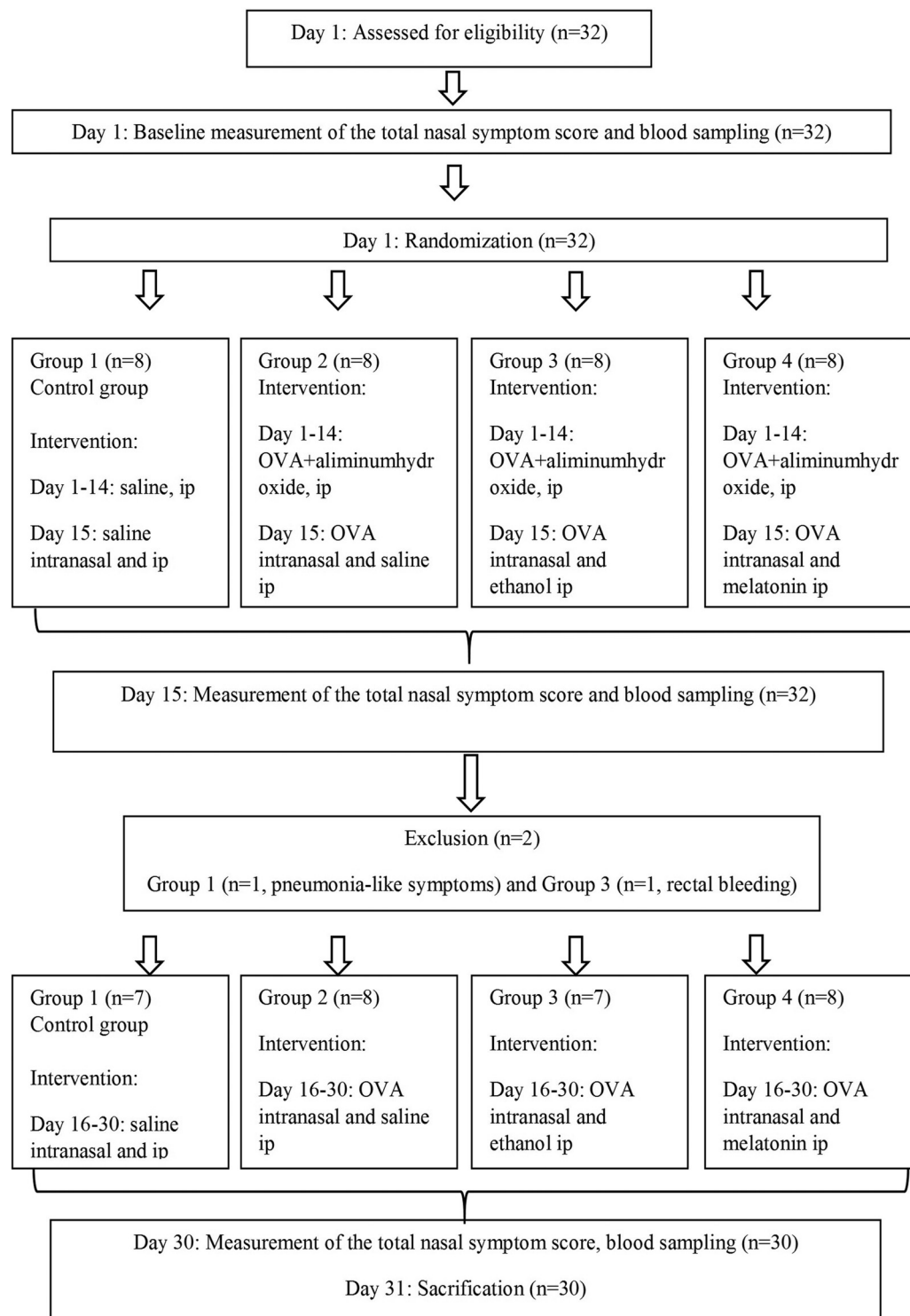
The total nasal symptom score was described as the sum of every single nasal symptom score evaluated on a four-grade scale [7–9]. We monitored nasal itching (scratches, rubbing) and sneezing; and the sum of these two symptoms' scores represented AR when it was four (Table 1) [11,12]. Each animal was separately observed and scored for ten minutes after a ten-minute interval following the intranasal application. The baseline scoring (day 1) was performed initially without any intranasal challenge. The total nasal symptom score was examined on days 1, 15, 20, 25, and 30 between 10 AM and 12 AM.

2.5.2. Blood sampling and biochemical assays

In all rats, 2-ml blood samples were drawn through warm water-dripped tail vein puncture with a 24 gauge needle under diethyl ether inhalation anaesthesia on days 1, 15, and 30, and all were performed after total nasal symptom scoring between 10:10 AM and 12:10 PM. Blood samples were centrifuged for 20 min at 3000 rpm (revolutions per minute), and the sera obtained were stored at -80 °C to be analysed via enzyme-linked immunosorbent assay (ELISA). All samples were measured for ovalbumin-specific IgE ((Rat Ovalbumin-Specific Immunoglobulin E ELISA Kit (OVASigE), My Biosource, Cat No: MBS039847 (My Biosource, Inc., San Diego, USA)) interleukin-13 (IL-13) (Rat IL-13 ELISA Kit, Fine Test, Cat No: ER0034 (Wuhan Fine Biotech Co., Ltd.; Wuhan, Hubei, China)), and melatonin (Rat MT (Melatonin) ELISA Kit, Elabscience, Cat No: E-EL-R0031 (Elabscience Biotechnology Co., Ltd, USA) by ELISA method at the same time, in line with the manufacturer's instructions [8,13,14].

2.5.3. Blinding

All i.p. injections and blood sampling were carried out by the same non-blinded author (A.C.C). To prevent bias,



n: Number of animals, OVA: Ovalbumin, ip:intraperitoneal

Fig. 1. (Flow diagram). experimental design and flow diagram.

the symptom scoring was performed by three blinded authors (P.Ka., O.A., and M.G.). Centrifuging and sera storage were carried out by two other blinded authors (Y.T and C.E.). ELISA tests were performed by another blinded author (H.E.), and sacrifice and histological examination were achieved

by two other blinded authors (S.O. and C.P.). Data recording was double-checked, and the statistical analysis was performed by another colleague who did not contribute as a co-author (P.Ke.).

Table 1. Nasal symptom and histological scores.

Nasal Symptoms	Score			
	0	1	2	3
• Nasal itching	0/min.	1–3/min.	4–6/min.	> 6/min.
• Sneeze	0/min.	1–3/min.	4–9/min.	> 9/min.
Histological Parameters				
• Eosinophil infiltration	No change	Mild	Moderate	Severe
• Inflammatory cell infiltration	No change	Mild	Moderate	Severe
• Vascular congestion	No change	Mild	Moderate	Severe
• Vascular proliferation	No change	Mild	Moderate	Severe
• Goblet cell increase	No change	Mild	Moderate	Severe
• Hypertrophy in chondrocytes	No change	Mild	Moderate	Severe
• Cilia loss	No change	Mild	Moderate	Severe

/min.: per minute.

The total nasal symptom score refers to the sum of the two nasal symptom scores.

The histological score refers to the arithmetical mean of the seven histological parameters.

2.5.4. Histological examination and scoring

On day 31, all animals were sacrificed by cervical dislocation and decapitated. The nasal mucosa of each rat was stripped and decalcified in ethylenediaminetetraacetic acid (EDTA) for two months, after fixation of the head in 10% neutral buffered formalin for three days and 5% trichloroacetic acid for seven days. The tissue blocks were embedded in paraffin. The sections were stained with haematoxylin and eosin (H&E), Alcian blue, and periodic acid Schiff (AB/PAS) to investigate the histology of goblet cells using a DP71 camera (Olympus Optical, Tokyo, Japan) connected to a model BX51 light microscope (Olympus Optical, Tokyo, Japan) at magnifications of $\times 40$ and $\times 100$. Images were processed using the 3D Reconstruct Program 1.1.0.1 (Medical College of Georgia, Georgia Health Sciences University, Augusta, GA, USA) [7]. The arithmetical mean of the seven histological parameters that scored semi-quantitatively represent the histological score [7,8] as shown in Table 1.

2.5.5. Experimental outcomes

The outcomes were the total nasal symptom score, serum ovalbumin-specific IgE, IL-13, melatonin, and histological parameters. Parametric and non-parametric quantitative variables were expressed as mean $\pm$ standard deviation and median (minimum, maximum) in 95% confidence intervals.

2.6. Statistical analysis

The data were statistically analysed using the Statistical Package for Social Sciences (SPSS) 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The distribution pattern was checked using the Shapiro-Wilk test. One-way ANOVA and post-hoc Tukey–Kramer tests were used to compare the parametric quantitative variables between the independent groups. Kruskal–Wallis test and post-hoc Dunn–Bonferonni adjustment were performed to compare non-parametric quantitative variables between the independent groups, whereas the Friedman test and post-hoc Dunn–Bonferonni adjustment were used to assess the difference be-

tween the repeated measures of each group [7,8,14]. The results were presented in a 95% confidence interval. $p < 0.05$ was considered significant.

3. Results

3.1. Animals

Two animals, of which one developed pneumonia-like symptoms in group 1 (control) and the other experienced rectal bleeding in group 3 (ethanol-given), were excluded from the study and thus from the statistical analysis of all outcome measures (total nasal symptom scores, serum ovalbumin-specific Ig-E, IL-13, melatonin, and histological parameters).

Besides, serum samples had to be run three times due to a failure in the serum IL-13 and melatonin ELISA kits, which led to a significant reduction in the number of samples examined in each group. The final sample sizes for IL-13 and melatonin ELISA analyses were 4, 6, 3, and 4 for groups 1–4, respectively (Table 2).

3.2. Results of experimental outcomes

The total nasal symptom scores of the groups on days 1, 15, 20, 25, and 30 and the serum ovalbumin specific IgE values of the groups on days 1, 15, and 30 are presented in Figs. 2 and 3.

The total nasal symptoms and histological scores, the serum ovalbumin-specific IgE, IL-13, and melatonin results of the groups on days 1, 15, and 30, and the significant values of the statistical analyses between groups and repeated measurements are shown in Table 2.

3.3. Total nasal symptom score

The results are expressed as mean values in bars and standard deviation values above each bar. The baseline (day 1) total nasal symptom scores were similar amongst the groups ($p = 0.869$) but significantly different on day 15 ($p = 0.001$),

Table 2. Results of outcome measures of the groups on days 1, 15, and 30, and the significance values of the statistical analyses between groups and measurements.

	Groups		Day 1	Day 15	Day 30	Friedman p
Total Nasal Symptom Score	1.Control	Mean $\pm$ SD	0.29 $\pm$ 0.49	0.29 $\pm$ 0.49	0.57 $\pm$ 0.54	0.156
	n = 7	Median (Min, Max)	0 (0–1)	0 (0–1)	1.00 (0–1)	
	2.Saline n = 8	Mean $\pm$ SD	0.25 $\pm$ 0.46	5.38 $\pm$ 0.92	5.50 $\pm$ 0.76	< 0.001*
		Median (Min, Max)	0 (0–1)	6.00 (4–6)	6.00 (4–6)	
	3.Ethanol	Mean $\pm$ SD	0.17 $\pm$ 0.41	5.50 $\pm$ 0.55	5.83 $\pm$ 0.41	0.001*
	n = 7	Median (Min, Max)	0 (0–1)	5.50 (5–6)	6.00 (4–6)	
	4.Melatonin	Mean $\pm$ SD	0.13 $\pm$ 0.35	5.38 $\pm$ 0.52	1.38 $\pm$ 1.06	< 0.001*
	n = 8	Median (Min, Max)	0 (0–1)	5.00 (5–6)	1.50 (0–3)	
	Kruskal-Wallis p		0.869	0.001*	< 0.001*	
Serum Ovalbumin-specific IgE (ng/ml)	1.Control	Mean $\pm$ SD	0.83 $\pm$ 0.43	0.41 $\pm$ 0.34	0.35 $\pm$ 0.17	0.042*
	n = 7	Median (Min, Max)	1.06 (0.29–1.37)	0.26 (0.13–0.99)	0.35 (0.13–0.57)	
	2.Saline n = 8	Mean $\pm$ SD	0.73 $\pm$ 0.56	16.14 $\pm$ 14.95	14.16 $\pm$ 9.79	0.009*
		Median (Min, Max)	0.48 (0.24–1.74)	11.59 (2.96–41.30)	15.12 (3.64–26.94)	
	3.Ethanol	Mean $\pm$ SD	0.50 $\pm$ 0.02	7.68 $\pm$ 7.47	11.13 $\pm$ 10.56	0.007*
	n = 7	Median (Min, Max)	0.50 (0.48–0.54)	3.06 (2.51–18.75)	3.96 (3.27–25.18)	
	4.Melatonin	Mean $\pm$ SD	0.50 $\pm$ 0.01	9.78 $\pm$ 11.44	3.96 $\pm$ 3.24	0.006*
	n = 8	Median (Min, Max)	0.50 (0.49–0.51)	3.36 (2.07–29.41)	3.11 (1.56–10.20)	
	Kruskal-Wallis p		0.663	0.001*	0.002*	
Serum IL-13 (pg/ml)	1.Control	Mean $\pm$ SD	36.42 $\pm$ 7.19	73.39 $\pm$ 69.50	43.58 $\pm$ 45.55	0.274
	n = 4	Median (Min, Max)	36.25 (26.25–47.75)	51.75 (10.75–195.75)	26.25 (7.75–129.25)	
	2.Saline n = 6	Mean $\pm$ SD	26.08 $\pm$ 17.03	109.13 $\pm$ 82.83	90.83 $\pm$ 68.04	0.311
		Median (Min, Max)	21.00 (10.75–56.25)	116.50 (12.25–220.25)	86.75 (25.25–184.75)	
	3.Ethanol	Mean $\pm$ SD	39.13 $\pm$ 22.82	60.17 $\pm$ 55.67	123.75 $\pm$ 31.11	0.223
	n = 3	Median (Min, Max)	35.00 (16.25–70.25)	37.00 (12.25–154.25)	123.75 (101.75–145.75)	
	4.Melatonin	Mean $\pm$ SD	34.33 $\pm$ 22.38	97.63 $\pm$ 87.57	74.85 $\pm$ 72.80	0.779
	n = 4	Median (Min, Max)	41.25 (0.25–56.25)	68.50 (0.00–219.25)	47.25 (9.75–198.75)	
	Kruskal-Wallis p		0.598	0.699	0.353	
Serum Melatonin (ng/ml)	1.Control	Mean $\pm$ SD	0.80 $\pm$ 0.24	0.77 $\pm$ 0.36	0.90 $\pm$ 0.31	0.223
	n = 4	Median (Min, Max)	0.79 (0.49–1.05)	0.65 (0.37–1.23)	0.73 (0.56–1.33)	
	2.Saline n = 6	Mean $\pm$ SD	0.81 $\pm$ 0.15	0.59 $\pm$ 0.18	0.57 $\pm$ 0.35	0.368
		Median (Min, Max)	0.80 (0.60–1.02)	0.61 (0.30–0.88)	0.58 (0.02–0.97)	
	3.Ethanol	Mean $\pm$ SD	0.85 $\pm$ 0.19	0.48 $\pm$ 0.24	0.59 $\pm$ 0.44	0.115
	n = 3	Median (Min, Max)	0.84 (0.56–1.11)	0.54 (0.16–0.71)	0.56 (0.12–1.32)	
	4.Melatonin	Mean $\pm$ SD	0.76 $\pm$ 0.25	0.46 $\pm$ 0.28	0.41 $\pm$ 0.20	0.223
	n = 4	Median (Min, Max)	0.79 (0.39–1.03)	0.40 (0.15–0.85)	0.38 (0.12–0.72)	
	Kruskal-Wallis p		0.887	0.442	0.083	
Histological Score	1.Control	Mean $\pm$ SD	0.08 $\pm$ 0.07	ANOVA p < 0.001*	Post-hoc p Groups 1–2 Groups 1–3 Groups 1–4 Groups 2–3 Groups 2–4 Groups 3–4	< 0.001** < 0.001** 0.118 0.850 < 0.001** < 0.001**
	n = 7					
	2.Saline n = 8	Mean $\pm$ SD	1.11 $\pm$ 0.27			
	3.Ethanol	Mean $\pm$ SD	1.21 $\pm$ 0.27			
	n = 7					
	4.Melatonin	Mean $\pm$ SD	0.37 $\pm$ 0.28			
	n = 8					

"Post-hoc p" of ANOVA refers Tukey-Kramer test (SPSS version 22.0).

Day 1, 15, and 30 represent the baseline, end of the first sensitization, and final measurements, respectively.

n: Number of animals, SD: Standard Deviation, Min: Minimum, Max: Maximum, ANOVA: Analysis of Variance,.

* p < 0.05,.

** post-hoc p < 0.05,.

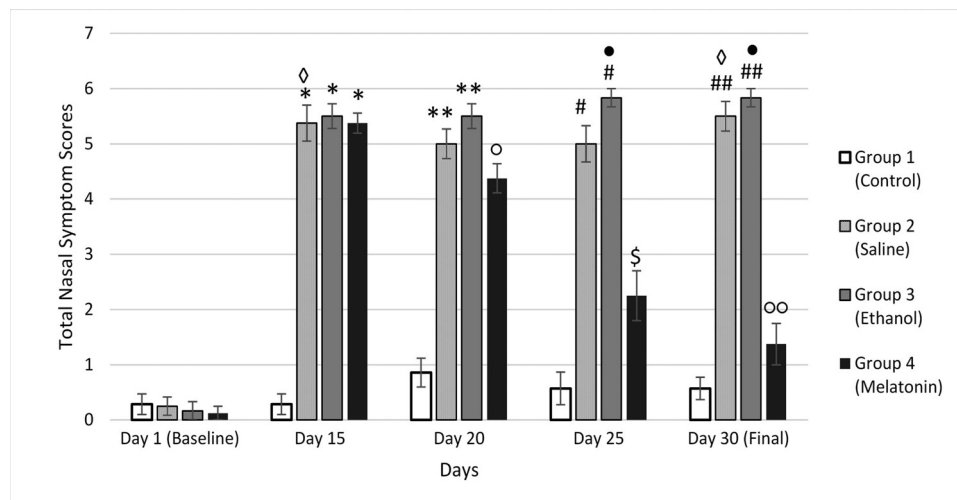


Fig. 2. Total nasal symptom scores of the groups on days 1, 15, 20, 25, and 30 (each symbol refers to a statistically significant post-hoc p value). *: compared to Group 1. **: compared to Group 1. #: compared to Group 1. \$: compared to Group 2. ## compared to both Group 1 and Group 4. ◇: compared to Group 2 on Day 1. • Compared to Group 3 on Day 1. ○ Compared to Group 4 on Day 1. ○○ compared to Group 4 on Day 15.

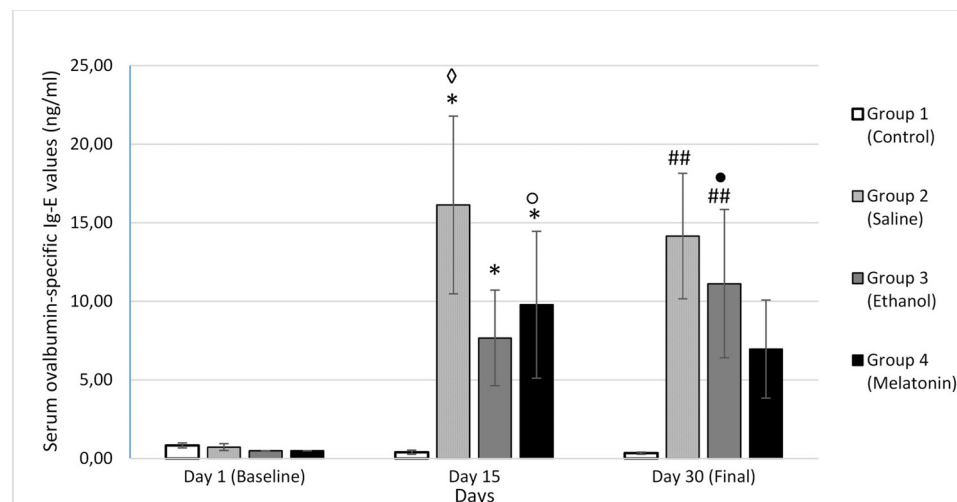


Fig. 3. Serum specific IgE values of the groups on days 1, 15, and 30 (each symbol refers to a statistically significant post-hoc p value). *: compared to Group 1. ## compared to both Group 1 and Group 4. ◇: compared to Group 2 on Day 1. ○ Compared to Group 4 on Day 1. •, compared to Group 3 on Day 1.

20 ($p < 0.001$), 25 ($p < 0.001$), and 30 ($p < 0.001$) (Kruskal–Wallis test).

On day 15, the scores of groups 2, 3, and 4 were ≥ 4 , which confirmed AR. The scores of groups 2, 3, and 4 were significantly higher than those of the control group (post-hoc Dunn–Bonferroni $p = 0.002$, 0.005 , and 0.006 , respectively). The other pairwise post-hoc comparisons on day 15 indicated non-significant results (each $p > 0.05$).

On day 20, the scores of groups 2 and 3 were significantly higher than those of the control group (post-hoc Dunn–Bonferroni $p = 0.003$ and < 0.001 , respectively). The other pairwise post-hoc comparisons on day 20 indicated non-significant results (each $p > 0.05$).

On day 25, the scores of groups 2 and 3 were significantly higher than those of the control group (post-hoc Dunn–Bonferroni $p = 0.002$ and $p < 0.001$, respectively). Group 4 showed significantly lower scores than those of group 2 (post-

hoc Dunn–Bonferroni $p = 0.013$). The other pairwise post-hoc comparisons on day 25 indicated non-significant results (each $p > 0.05$).

On day 30, the scores of groups 2 and 3 were similar (post-hoc Dunn–Bonferroni $p = 1.000$) and significantly higher than those of the control group (post-hoc Dunn–Bonferroni $p = 0.002$ and 0.001 , respectively). Group 4 remained similar to the control group (post-hoc Dunn–Bonferroni $p = 1.000$) but significantly lower than those of groups 2 and 3 (post-hoc Dunn–Bonferroni $p = 0.019$ and 0.012 , respectively).

According to the statistical analysis of the repeated measures of each group, the control group remained similar during the experimental procedure ($p = 0.156$) but groups 2–4 showed significant differences ($p < 0.001$, 0.001 , and < 0.001 , respectively) (Friedman test). Pair-wise comparisons showed significant differences between days 1–15 (post-hoc Dunn–Bonferroni $p = 0.005$) and 1–30 (post-hoc Dunn–

Bonferroni $p = 0.001$) for group 2; days 1–25 (post-hoc Dunn–Bonferroni $p = 0.019$) and 1–30 (post-hoc Dunn–Bonferroni $p = 0.019$) for group 3; and days 1–15 (post-hoc Dunn–Bonferroni $p < 0.001$), 1–20 (post-hoc $p = 0.003$), and 15–30 (post-hoc Dunn–Bonferroni $p = 0.003$) for group 4. The results of the remaining pairwise comparisons were non-significant (each $p > 0.05$). The scores of group 4 followed a decreasing pattern, which was different from the other groups.

3.4. The results of biochemical assays

The results are expressed as mean values in bars and standard deviation values above each bar. The baseline (day 1) serum ovalbumin-specific IgE values were similar among the groups ($p = 0.663$) but significantly different on days 15 ($p = 0.001$) and 30 ($p = 0.002$) (Kruskal–Wallis test).

On day 15, the values of groups 2, 3, and 4 were higher than those of the control group (post-hoc Dunn–Bonferroni $p = 0.001$, 0.046, and 0.041, respectively). The other pairwise post-hoc comparisons on day 15 indicated non-significant results (each $p > 0.05$).

On day 30, the scores of groups 2 and 3 were significantly higher than those of the control group (post-hoc Dunn–Bonferroni $p = 0.002$ and 0.024, respectively), but there was no significant difference between groups 1 and 4 (post-hoc Dunn–Bonferroni $p = 0.170$). The other pairwise post-hoc comparisons on day 30 indicated non-significant results (each $p > 0.05$).

According to the statistical analysis of the repeated ovalbumin-specific Ig-E measurements of each group, the control group remained similar during the experimental procedure (Friedman test $p = 0.042$, post-hoc Dunn–Bonferroni $p > 0.05$ for each comparison), but groups 2–4 showed significant differences (Friedman test $p = 0.009$, 0.007, and 0.006, respectively). For groups 2 and 4, the significant differences were between days 1 and 15 (post-hoc Dunn–Bonferroni $p = 0.012$ and 0.004, respectively). For group 3, the significant differences were between days 1 and 30 (post-hoc Dunn–Bonferroni $p = 0.005$).

3.5. Histological score

The individual histological score of each parameter is presented in Table 3.

The histological structure of the nasal mucosa was normal in group 1 (Figs. 4 and 5), whereas mild to severe inflammatory changes were observed in the majority of the histological parameters, and significantly increased histological scores were observed in groups 2 and 3 (Table 2, Figs. 4,5). A normal histological structure was seen in group 1, the control group. Histological inflammatory changes related to ovalbumin-induced AR are seen in groups 2–4. White stars indicate mononuclear inflammatory cell infiltration, white arrows show eosinophils, and black stars demonstrate vascular proliferation and congestion predominantly in groups 2 and 3, and less frequently in melatonin-administered group 4 (Fig. 4). The normal nasal mucosa was seen in group 1, the

control group. Black arrows indicate the increase in goblet cells predominantly in groups 2 and 3, and less frequently in melatonin-administered group 4 (Fig. 5).

The histological score of group 4 remained similar to the control group, and group 4 demonstrated the most frequent mild histological changes (Table 2, Figs. 4,5).

4. Discussion

In this study, we found that melatonin significantly improved the diagnostic parameters of AR, including the total nasal symptom score, serum ovalbumin-specific IgE, and histological score.

In this study, our total nasal symptom scores indicated that, AR was successfully achieved by ovalbumin in groups 2–4 and melatonin significantly decreased the total nasal symptom score in group 4. AR models in experimental animals were explained by a consensus article in 2019, in which ovalbumin was suggested to be a valid and reliable agent to induce AR [13]. This model is usually an acute model that predominantly manifests histamine-mediated early phase responses and is confirmed by behavioural tests in which some of the nasal and ocular symptoms such as nasal itching, sneezing, nasal discharge, congestion, and lacrimation are scored for 3–10 min after allergen exposure [7–9,11–13]. According to our previous experience in experimental behavioural research [15], it was not feasible to evaluate symptoms other than nasal itching or sneezing. The nasal discharge and lacrimation became invisible because of the rubbing movement in most of the animals, and nasal crackles were almost inaudible due to sneeze attacks.

Elevation of serum ovalbumin-specific IgE level indicated the presence of AR in groups 2–4. Melatonin-administered animals (group 4) demonstrated a reduction in serum ovalbumin-specific IgE levels on day 30, reaching a low level, which did not differ statistically from that in the control group. The most frequently used biochemical method to verify AR is the measurement of ovalbumin-specific IgE in serum by ELISA [8,13,14]. Considering the ovalbumin-induced AR model in rats, many studies [8,14] have detected an increase in serum ovalbumin-specific IgE levels. In our study, unlike groups 2 and 4, no statistically significant difference was found in the pairwise comparison between days 1 and 15 in rats treated with ethanol (group 3). However, as the exposure to the allergen continued, the serum IgE levels continued to increase and finally reached statistical significance on day 30.

In the current study, we did not find a significant difference in serum IL-13 levels between groups. In the experimental AR models, early phase responses are generally thought to be IgE and mast cell-mediated, while the late phase and especially the increased nasal airway resistance observed in late phase are IL-13-mediated rather than eosinophilia-mediated [16]. Some of the previous studies presented elevated IL-13 levels in the experimental models of AR [8,14,17]. The IL-13 result in our study could be explained by two theories: First, the sample size was small due to running ELISA kits three times, and it might have decreased the reliability of our

Table 3. Individual scores of each histological parameter of animals regarding groups.

Animals	Eosinophil infiltration	Inflammatory cell infiltration	Vascular congestion	Vascular proliferation	Goblet cell increase	Hypertrophy in chondrocytes	Cilia loss
Control 1	0	0	0	0	1	0	0
Control 2	0	0	0	0	0	0	1
Control 3	0	0	0	0	0	0	0
Control 4	0	1	0	0	0	0	0
Control 5	0	0	0	0	0	0	1
Control 6	0	0	0	0	0	0	0
Control 7	0	0	0	0	0	0	0
Saline 1	1	1	1	1	1	1	3
Saline 2	1	2	1	1	2	2	1
Saline 3	0	1	2	2	1	1	1
Saline 4	1	1	1	1	2	1	1
Saline 5	0	1	1	1	1	1	1
Saline 6	0	0	1	1	1	1	0
Saline 7	1	1	2	1	1	1	1
Saline 8	1	1	2	2	1	1	1
Ethanol 1	0	1	1	1	2	2	0
Ethanol 2	1	2	1	1	1	1	1
Ethanol 3	1	1	2	2	1	1	0
Ethanol 4	0	1	1	1	2	1	1
Ethanol 5	1	1	1	2	1	1	3
Ethanol 6	2	2	2	1	3	1	1
Ethanol 7	0	1	1	1	1	1	2
Melatonin 1	0	1	1	1	1	1	1
Melatonin 2	0	1	0	0	1	0	0
Melatonin 3	0	0	0	0	1	1	0
Melatonin 4	1	0	1	1	0	0	0
Melatonin 5	1	1	1	0	0	1	1
Melatonin 6	0	0	0	0	0	0	0
Melatonin 7	0	0	1	0	0	0	0
Melatonin 8	0	0	1	1	0	0	0

0: No change 1: Mild change 2: Moderate change 3: Severe change.

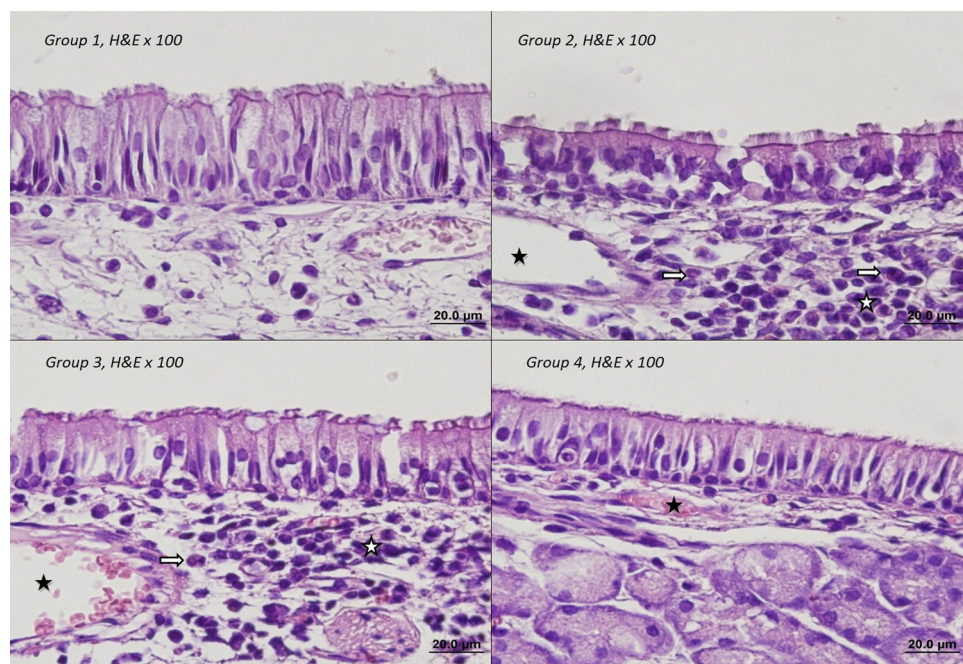


Fig. 4. Light-microscopic view of haematoxylin and eosin stain (H&E) (× 100 optical zoom) in groups 1–4. White stars indicate mononuclear inflammatory cell infiltration, white arrows show eosinophils, and black stars demonstrate vascular proliferation and congestion predominantly in groups 2 and 3, and less frequently in melatonin-administered group 4.

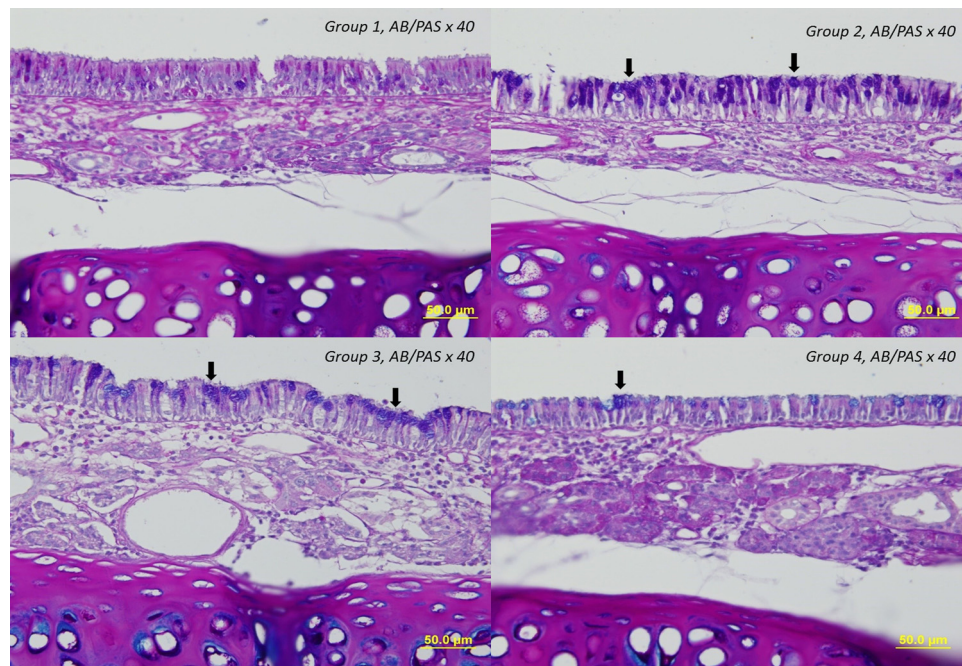


Fig. 5. Light-microscopic view of Alcian blue and periodic acid Schiff (AB/PAS) stain ($\times 40$ optical zoom) in groups 1–4. Black arrows indicate the increase in goblet cells predominantly in groups 2 and 3, and less frequently in melatonin-administered group 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

IL-13 results; second, the late phase of AR could not have developed in our model. As a special case in clinical practise, the late phase may be absent in local AR, and only the early phase may occur [18]. However, the demonstrative histological findings of groups 2 and 3 such as increased chondrocyte hypertrophy which represents tissue remodelling related to AR rules out the second theory.

To assess the association between serum melatonin levels and AR, we measured the serum melatonin level by ELISA. However, our statistical results regarding serum melatonin levels were not significant (Table 2). Low baseline and peak salivary melatonin levels have been found in patients with allergic rhinitis [19]. In our study, melatonin was administered after the first sensitisation phase, as mentioned in experimental allergic asthma models [10] and AR models with different treatment regimens [7,9]. It has been reported that exogenous melatonin may both increase [20] and decrease [21] the amount of endogenous melatonin. In our model, AR seemed to slightly decrease serum melatonin levels on day 15 in groups 2–4, different from the control group, but the result was non-significant. The exogenous melatonin administered to group 4 did not seem to affect the endogenous serum melatonin level, as well. However, our limited sample size should also be considered while interpreting this result.

Histological confirmation of allergic inflammation was also achieved in our model (groups 2 and 3). Furthermore, melatonin was shown to successfully and significantly reduce allergic inflammation in the nasal mucosa (group 4). Since there is no experimental AR model or a clinical study performed with melatonin, in which the histological aspect has been studied, we can only compare our results with an ovalbumin-induced

asthma model [10] which documented the therapeutic effects of melatonin on histological parameters, similar to ours.

Recently, the potential role and mechanism of action of melatonin on atopic and allergic Ig-E-mediated T helper 2 dominated diseases have been detailed [1]. The reduction of both baseline and peak melatonin levels correlated with the exacerbation of allergic asthma and symptom severity [22] whereas decreased serum and bronchoalveolar levels of melatonin were detected in experimental allergic asthma models [6]. Likewise, the nocturnal melatonin peak and serum melatonin levels were observed to be significantly lower in patients with atopic dermatitis than in controls, which is also speculated to be responsible for sleep disturbances [23]. Patients with atopic dermatitis who experienced sleep disturbances also showed more severe symptoms, diminished nocturnal serum melatonin levels, and lower IFN- γ values [5,23]. A recent meta-analysis reported a remarkable association between sleep disorders and AR [24]. This theoretical basis pioneered the use of melatonin in children with atopic dermatitis and sleep disorder [25].

Considering what has been known up to date, it can be speculated that the potential therapeutic effect of melatonin might depend on its immunomodulatory action and its relationship with IFN- γ . As an immunomodulator, melatonin modulates T helper 1, 2, 17 and T regulatory responses by increasing cytokines like IL-2 and IL-12 and thus triggering T helper lymphocytes to transform to T helper 1 that secrete IFN- γ [1,26]. Induced T helper 1 lymphocytes are known to release IFN- γ , which stimulates melatonin production [1,23]. Likewise, low melatonin levels reduce IFN- γ , leading to a decrease in melatonin concentration [5]. As known, T helper 2

dominates inflammation in AR, which manifests as low levels of IFN- γ [26]. Both the baseline and peak salivary melatonin levels [20] and serum IFN- γ concentrations were reported to be low in AR [27]. As IFN- γ emerges after the first sensitisation period of the AR [1,26], we started to apply melatonin immediately after this phase. However, we did not measure serum IFN- γ and other cytokines in the AR pathway. Thus, in our opinion, the therapeutic mechanism of melatonin needs to be further investigated.

4.1. Limitations

We introduce the low sample size of serum IL-13 and melatonin ELISA analyses as a limitation. Additionally, the fact that such cytokines, particularly IFN- γ , IL-4, and IL-5 were not measured due to the financial constraints of our project budget and technical problems, is another limitation, making it difficult to explain the pathway underlying the therapeutic mechanism of melatonin.

5. Conclusion

In conclusion, melatonin is offered as a new therapeutic option in the ovalbumin-induced rat model of AR, owing to its suppressive effects on nasal symptom scores, serum ovalbumin-specific Ig-E levels, and allergic inflammation in histological sections. Nevertheless, we emphasise the necessity of further experimental and clinical studies to support our results and clarify the exact therapeutic mechanisms of melatonin in AR.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

CRediT authorship contribution statement

Asli Cakir Cetin: Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Mustafa Cenk Ecevit:** Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Mukaddes Gumustekin:** Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Cetin Pekcetin:** Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Seda Ozbal:** Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Hande Efe:** Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Pelin Koca:** Conceptualization, Methodology, Data curation, Investigation,

Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Ozge Akcay:** Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Yesim Tuncok:** Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

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Compliance with ethical standards

This project was approved by the Ethics Committee of the Research of Laboratory Animals, Dokuz Eylul University (protocol number: 51/2016). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Availability of data and material

This manuscript is based on the Ph.D. thesis of the corresponding author and will be available online for public access in the website of Turkish Council of Higher Education Thesis center <https://tez.yok.gov.tr/UlusalTezMerkezi/giris.jsp> within six months. Until this process takes place, the datasets generated and analysed during the current study are available from the corresponding author on request of the editorial board or reviewers, but cannot be published or shared.

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