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The Potential Anti-inflammatory Effects of Naringenin on a Histamin Induced Otitis Media with Efussion Rat Model

Histamin ile Oluşturulmuş Efüzyonlu Otitis Media Rat Modelinde Naringeninin Potansiyel Antiinflamatuar Etkileri

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ABSTRACT Objective: The aim of this study was to histopathologically compare the effects of naringenin with methyl-prednisolone (MP) on a histamine induced otitis media with effusion (OME) animal model. **Material and Methods:** Twenty seven male Albino Wistar rats weighing 250-300 g were used in this study. A histamine induced OME model was created by trans-tympanic 0.2 mL injections of 1 mg/mL histamine in both ears in all rats. Three groups of 9 rats were formed by a random selection. Group 1 did not receive any treatment, Group 2 received 1 mg/kg of intra-peritoneal MP treatment for 14 days and Group 3 received 50 mg/kg oral naringenin treatment. All rats were otomicroscopically examined on the 48th hour in order to confirm the development of OME. At the end of the treatment, all rats were otomicroscopically re-examined and later sacrificed. The temporal bulla of all rats was examined with light microscopy and was evaluated in terms of submucosal neutrophil leucocyte count. **Results:** The first otomicroscopic evaluation revealed that 52 ears of 26 rats developed OME (96%). In the second oto-microscopic evaluation, OME was present in 16 ears (88%) in Group 1, 3 ears (19%) in Group 2 and 7 ears (39%) in Group 3. In terms of submucosal neutrophil count, a statistically significant difference was present between Group 1 and Group 2, and 3 ($p<0.05$) however no significant difference was present between Group 2 and 3 ($p=0.21$). **Conclusion:** Naringenin was found as effective as MP in both oto-microscopical examinations and histopathological evaluations in a histamine induced animal model of OME.

Keywords: Otitis media with effusion; naringenin; methyl-prednisolone

ÖZET Amaç: Bu çalışmanın amacı, histamin ile oluşturulmuş deneysel efüzyonlu otitis media (EOM) hayvan modelinde naringenin ve metil-prednizolonun etkilerini histolojik olarak karşılaştırmaktır. **Gereç ve Yöntemler:** Bu çalışmada, ağırlıkları 250-300 g olan 27 adet erkek Albino Wistar ratları kullanıldı. Tüm ratlarda her iki kulağa transtimpanik 0,2 mL 1 mg/mL histamin enjeksiyonu ile EOM modeli oluşturuldu. Rastgele seçimle 9 rattan oluşan 3 grup oluşturuldu. Grup 1'e herhangi bir tedavi uygulanmadı, Grup 2'ye 14 gün 1 mg/kg intraperitoneal metil-prednizolon tedavisi ve Grup 3'e 50 mg/kg oral naringenin tedavisi verildi. EOM gelişimini doğrulamak için tüm sıçanlar 48. saatte otomikroskopik olarak incelendi. Tedavi sonunda tüm sıçanlar otomikroskopik olarak tekrar incelendi ve daha sonra sakrifiye edildi. Tüm sıçanların temporal bullası ışık mikroskobu ile incelendi ve submukozal nötrofil lökosit sayısı açısından değerlendirildi. **Bulgular:** İlk otomikroskopik değerlendirmede, 26 ratın 52 (%96) kulağında EOM geliştiği görüldü. İkinci otomikroskopik değerlendirmede, Grup 1'de 16 kulakta (%88), Grup 2'de 3 kulakta (%19), Grup 3'te 7 kulakta (%39) EOM mevcuttu. Submukozal nötrofil sayısı açısından Grup 1 ile Grup 2 ve 3 arasında istatistiksel olarak anlamlı fark vardı ($p<0,05$), ancak Grup 2 ile 3 arasında anlamlı fark yoktu ($p=0,21$). **Sonuç:** Histamin ile oluşturulan EOM hayvan modelinde hem otomikroskopik incelemelerde hem de histopatolojik değerlendirmelerde naringenin, MP kadar etkili bulunmuştur.

Anahtar Kelimeler: Efüzyonlu otitis media; naringenin; metil-prednizolon

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Otitis media with effusion (OME) is a condition of inflammatory fluid formation in the middle ear without signs or symptoms associated with acute middle ear infection.[1] In the literature, the cohort studies indicate that the OME prevalence is approximately 20%.[2,3] While the incidence of OME is highest in the first year of life, it is stated that every child that reached 3 years of age experiences at least one episode of OME in their lives.[4] Although the underlying mechanism of OME development is not clearly understood, it is thought that middle ear effusions are mostly formed after an acute infection and the post-inflammatory changes of the middle ear mucosa as goblet cell metaplasia and increased inflammatory metabolite secretion contribute to the persistence of fluid accumulation. The eustachian tube disfunction, itself is also considered as a contributing factor in the whole OME process.[5]

Although most of the OME cases resolve without medical or surgical intervention chronic OME do require treatment. According to the widely accepted current guidelines, children with additional developmental issues and effusions persisting more than 3 months are recommended to be treated with surgical procedures as ventilation tubes with or without adenoidectomy.[6,7] Medical treatment agents as antibiotics, decongestants, anti-histamines, local or systemic steroids are not recommended because of their long-term inefficiency or either safety concerns.[6]

The aim of this experimental animal study was to compare the effects of methyl-prednisolone (MP) which has strong anti-inflammatory effects with naringenin, a natural flavonoid molecule that has begun to attract attention due to its anti-inflammatory and anti-oxidant properties.[8] In this respect, the submucosal neutrophil count in the temporal bulla were compared after both treatments with an additional control group. As far as we are concerned, this is the first study investigating the possible efficacy of naringenin on OME animal model.

MATERIAL AND METHODS

The ethical approval was given for this study by Health Sciences University Ankara Training and Research Hospital Local Committee of Animal Experiments (date: March 9, 2022, no: 0070). All procedures in this study were in line with the Helsinki Declaration principles. The study was performed in line with the experimental ethical guidelines and animal protection laws. Twenty seven male Albino Wistar rats weighing 250-300 grams were used in this study. The rats were kept in steel cages with free access to food and water. The study was initiated following sufficient acclimation under stable temperature and moisture with 12 hours light and dark cycles.

All rats were oto-microscopically examined and in order to form OME, they were trans-tympanically injected with 0.2 mL of 1 mg/mL histamine solution (Sigma-Aldrich Chemical Co., Germany) under anesthesia with intramuscular ketamine (Ketalar, Parke-Davis, Türkiye) injections. Trans-tympanic injections were done with a 27-gauge needle. On the 48th hour, all rats were otomicroscopically re-examined to confirm OME formation. Rats in which oto-microscopic findings as air-fluid levels and bubble formations behind the tympanic membrane (TM), retractions, and hyper-vascularization of the TM were detected were accepted to have developed OME. A TM evaluated as OME is presented in Figure 1. In 26 rats, bilateral OME was confirmed while in 1 rat, which was excluded from the study, OME was not detected in either ear.

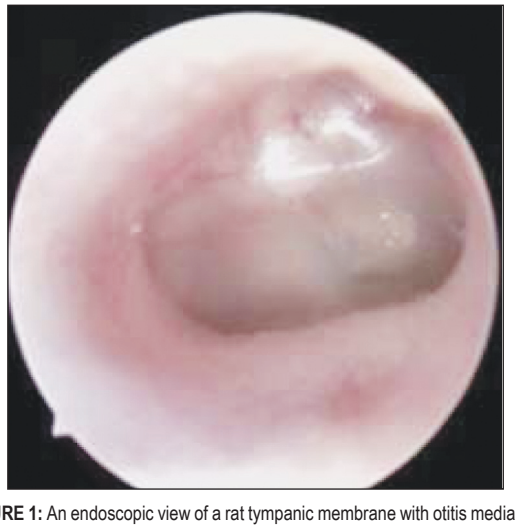

FIGURE 1: An endoscopic view of a rat tympanic membrane with otitis media with effusion.

Three groups were randomly formed. Group 1 (n=9) was the control group and did not receive any treatment, Group 2 (n=8) was the MP treatment group and was administered intra-peritoneal 1 mg/kg MP (Mustafa Nevzat Drug Company, İstanbul, Türkiye) for 14 days, Group 3 was the naringenin treatment group and was administered oral 50 mg/kg naringenin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) for 14 days.

On the 16th day, all rats underwent oto-microscopic examination once more and were than sacrificed with 80 mg/kg intra-peritoneal pentobarbital injections. In the last oto-microscopic examination, the persistence of OME was evaluated. The resolution of air-fluid levels and bubble formations, regression of the hyper-vascularized state of the TM was accepted as recovery. In order to assess histopathologic evaluation, the temporal bulla of all rats was excised and taken out. The preparations were fixed with neutral formalin and were decalcified with ethylenediaminetetraacetic acid for 10 days. Following this procedure, all preparations were embedded in paraffin and tissue blocks (4 μ) were serially sectioned with a microtome. All sections were than stained with hematoxylin and eosin and were blindly evaluated with light microscope by a single pathologist. Histologically, by x100 magnification, the mean submucosal neutrophil counts were obtained from 25 different areas of the temporal bulla mucosa in each preparation and were than compared.

STATISTICAL ANALYSIS

Statistical analyses were done with one-way analysis of variance (ANOVA) test. If difference was detected with ANOVA, as post hoc test, the Tukey statistics were evaluated. The significance level was set at $p<0.05$. While comparing the effusion existence before sacrificing the rats, the chi-square, Fisher's exact-test, and two-proportion Z-test were used. The evaluation of the data was done with www.e-picos.com (MedicReS, USA) New York software and MedCalc (MedCalc Software, Belgium) statistics packet program.

RESULTS

The oto-microscopic examination done in the 2nd day to confirm OME development revealed that 26 rats developed bilateral OME (96%). One rat did not develop OME in either ear and was excluded from the study. The second oto-microscopic examination done before sacrificing the rats revealed that in Group 1, 16 (88%) of total 18 ears (n=9), in Group 2, 3 (19%) of total 16 ears (n=8), and in Group 3, 7 (39%) of total 18 (n=9) OME persisted. In terms of oto-microscopic OME persistence, there was a statistically significant difference between Group 1 and both Group 2 and 3 ($p<0.001$). However, there was no statistically significant difference between Group 2 and Group 3 ($p=0.2$; $p>0.05$) Table 1.

In terms of submucosal neutrophil counts, there was a statistically significant difference between groups ($p<0.05$). The mean submucosal neutrophil count was 77.72±14.02 in Group 1, 10.75±2.91 in Group 2, and 20.83±14.34 in Group 3. Between Group 1 and both Group 2 and 3, there was a statistically significant difference. However, there was no statistically significant difference between Group 2 and Group 3 ($p=0.21$). The statistical analysis of our

TABLE 1: The otomicroscopic comparison of tympanic membranes in terms of effusion existence.

		Control	Methyl-prednisolone	Naringenin	Total	p value*	p value** 1 vs. 2, 1 vs. 3, 2 vs. 3
Non-serous otitis	Count	2	13	11	26	<0.0001	<0.001, 0.002, 0.2
	% within group	11.1%	81.3%	61.1%	50.0%		
Serous otitis	Count	16	3	7	26		
	% within group	88.9%	18.8%	38.9%	50.0%		
Total	Count	18	16	18	52		
	% within group	100.0%	100.0%	100.0%	100.0%		

*p value: Chi-square, Fisher's exact test; **p value: Two-proportion Z-test.

TABLE 2: The statistical evaluation of submucosal neutrophil counts between methyl-prednisolone treatment, naringenin treatment, and control groups.

Submucosal neutrophil counts	Total (n=26) X̄±SD	Control (n=9) X̄±SD	Methyl-prednisolone (n=8) X̄±SD	Naringenin (n=9) X̄±SD	p value*	p value** 1 vs. 2, 1 vs. 3, 2 vs. 3
Right ear	36.38±34.73	72.11±27.36	10.13±2.36	24±22.91	<0.001	<0.001, <0.001, 0.43
Left ear	38.46±35.78	83.33±17.54	11.37±4.24	17.68±13.91	<0.001	<0.001, <0.001, 0.61
Both ears	37.42±32.29	77.72±14.02	10.75±2.91	20.83±14.34	<0.001	<0.001, <0.001, 0.21

*p value: Analysis of variance; **p value: Post hoc Tukey; SD: Standard deviation.

study is summarized in Table 2. Oto-microscopic sections that serve as a model for Group 1 and Group 3 are presented in Figure 2 and Figure 3, respectively.

DISCUSSION

The pathogenesis of OME is currently not thoroughly enlightened with its every aspect. From past to present, many studies had been designed to reveal the underlying complex mechanisms of this pathological process. In the literature, the inflammatory pathways associated with OME is one of the subjects that have been extensively researched. In OME, combined with the decreased drainage function of the middle ear, inflammatory pathways are stated to trigger an accumulation of fluid rich of cytokines, protein, and inflammatory mediators.[9] Thus, research has shown that molecules as prostaglandin, histamine, and immune globulins (Ig) may be found in vast amounts in the effusion fluid.[10,11]

In our study, histamine was chosen to form a OME model due its low morbidity and high rates of success. Although the evaluation of OME in rats is a relatively subjective process, Kaytez et al. have published research done with a rat model in which histamine was used and reported 100% success in creating experimental OME.[12] In another study the success rate was assessed as 90%.[13] In line with the literature, in our study histamine demonstrated a success rate of 96%.

MP is reported to be efficient in terms of resolution of the effusion in the middle ear by several different mechanisms. MP reduces the formation of inflammatory mediators, the viscosity of the effusion and the volume of lymphoid tissues as the adenoid tissue.[13,14] Moreover, by increasing the surfactant production, it eases the resolution of middle ear effusion.[15] Although MP is reported to have these critical properties, the current guidelines do not recommend corticosteroid agents alone or combined with antibiotics because of adverse effects and lack of evidence.[6,16] Instead of systemic use of corticosteroids, nasal steroids were suggested as local steroids do not demonstrate the systemic adverse effects. Research

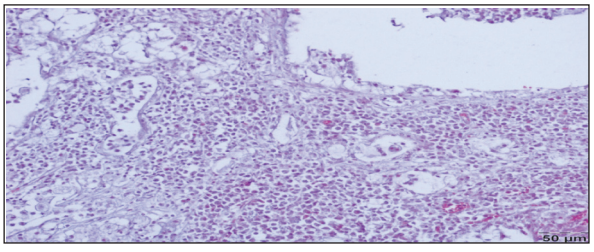

FIGURE 2: A preparation of the control group, severe neutrophil infiltration is observed, hematoxylin and eosin, original magnification x200.

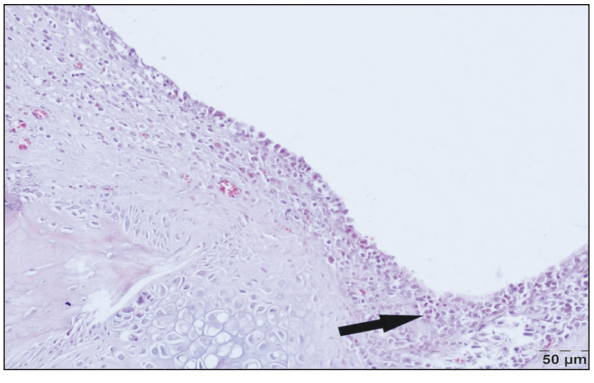

FIGURE 3: A preparation of the naringenin treatment group, mild neutrophil infiltration is observed. The black arrow indicates the submucosal space, hematoxylin and eosin, original magnification x200.

done with nasal corticosteroids have demonstrated controversial results. In the literature, there are studies that found nasal steroid use effective but on the other hand, there are also several more studies that concluded that their use had no significant effect on OME.[17,18] In other words, there is no consensus on the effectiveness of nasal corticosteroid use in OME treatment. Furthermore, the current guideline does not recommend their use.[6]

Although the clinical effect of corticosteroids on OME is still a subject to debate, in experimental animal studies, significant anti-inflammatory effects of corticosteroids are reported.[13,14,19] When the literature was analyzed, it was observed that, in order to manifest a potentially anti-inflammatory molecule's effects, it was compared with the effects of MP.[12,13,20] Therefore, we designed our study by forming a control group, naringenin treatment group, and an additional MP treatment group.

Naringenin is a flavonoid group molecule which is naturally found in tomato, cacao, and citrus fruit. Due to its anti-oxidant and anti-inflammatory properties, many studies have been done with this molecule and these studies revealed promising results.[8,21,22] In brief, many inflammatory pathways have been shown to be affected by naringenin administration.[21,22] Since OME shares similar inflammatory pathways, it was envisioned that naringenin could be effective on middle ear effusions. The dosage of naringenin treatment was determined as 50 mg/kg orally based on the stated previous studies.

Pudrith et al. previously reported that one of the most important indicators of OME was submucosal neutrophil count therefore in line with the literature, in all groups the mean neutrophil counts were obtained and compared.[23] Parallel with other studies done in the literature, MP treatment was found statistically significant when compared to the control group. Apart from the anti-inflammatory effects of MP, also the mineralocorticoid effect may have contributed to this finding.[24] The promising result of our study was that the naringenin treatment group did not only demonstrate significant effects when compared to the control group but also it had similar effects as MP on OME in terms of submucosal neutrophil count. According to the statistical findings, there was no statistically significant difference between the MP treatment group and naringenin treatment group (p=0.21).

There are several factors that may have contributed to this result. Şahin et al. reported that inflammatory pathways were found to be affected after naringenin treatment. In that, it was reported that the pro-inflammatory mediators as interleukin (IL)-4, IL-5, and Ig-E had been decreased with naringenin treatment.[22] In another study, it was published that the expression of tumor necrosis factor-alpha (TNF-a), which is another molecule that is also used to form experimental OME, had been limited by treatment.[21] One other study that emphasized on the anti-inflammatory properties of naringenin was conducted by Jayaraman et al. and according to the results of this study, naringenin had decreased not only TNF-a levels but also reduced the levels of IL-1, IL-6 and COX-2 enzyme.[25]

The hydroxyl group that naringenin contains also gives the molecule an ability of acting as an antioxidant molecule.[26] The antioxidant effect of naringenin is dose-dependently, through the inactivation of free oxygen radicals.[27] The oxidative micro environment during the whole pathological process of OME formation may be limited by the anti-oxidant effects of naringenin. Another pathological process that takes place in OME formation is that the angiogenic alternations following the inflammatory cascade.[28] Naringenin, apart from its anti-oxidant and anti-inflammatory properties, does also have a direct effect in reducing vascular endothelial growth factor expression.[29]

The comparison of naringenin and MP treatment groups revealed that there is no statistically significant difference detected in terms of submucosal neutrophil count. Especially when the possible drastic adverse effects of MP treatment are taken into account although wide scaled further research is needed to completely refer naringenin as a safe molecule, in the current literature, there has not been a single reported adverse effect of naringenin treatment in animal models up to date.[30] While this study contributes precious information to the literature, there were of course some drawbacks as well. In our study, the OME model was formed by trans-

tympanic histamine injections and the effusions that were detected behind the TM were accepted as sterile effusions. Culturing of the effusions were not done. Another weak aspect of this study was that a group in which no medical or surgical intervention was applied was not unfortunately designed. However, comparisons with the untreated group are sufficient to compare with abnormal histopathological data.

CONCLUSION

Managing OME is today still an economic burden in health care systems. A completely safe and effective treatment modality is a subject that research is still ongoing. Currently, surgical treatment options also have their own risks. Naringenin is thought to be an alternative medical agent that can be used in the treatment of OME, if further animal and human studies reveal its effects in terms of efficacy and safety.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Mustafa İbas, Necmi Arslan;* ***Design:*** *Necmi Arslan, Ramazan Öcal;* ***Control/Supervision:*** *Necmi Arslan, Rahmi Kılıç;* ***Data Collection and/or Processing:*** *Songül Dursun, Nihat Yumuşak;* ***Analysis and/or Interpretation:*** *Mustafa İbas, Ramazan Öcal;* ***Literature Review:*** *Mustafa İbas, Songül Dursun;* ***Writing the Article:*** *Mustafa İbas;* ***Critical Review:*** *Necmi Arslan, Rahmi Kılıç;* ***References and Fundings:*** *Mustafa İbas, Songül Dursun;* ***Materials:*** *Nihat Yumuşak.*

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