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Effects of penicillin and montelukast sodium on middle ear mucosa in rats with experimental acute otitis media

Deneysel akut otitis medialı sıçanlarda penisilin ve montelukast sodyumun orta kulak mukozasına etkileri

Seçil Uçar Topalsan[1], Tural Huseynov[1], Sülen Sarıoğlu[2], Bülent Şerbetçioğlu[1]

[1]*Department of Otorhinolaryngology, Medical School, Dokuz Eylül University, İzmir, Turkey*
[2]*Department of Pathology, Medical School, Dokuz Eylül University, İzmir, Turkey*

Abstract

Objective: To develop an experimental acute suppurative otitis media model and compare the responses of rats to penicillin and combinations of leukotriene antagonist with respect to histopathological observations conducted at both early and late phases.

Methods: A total of eighty-three ears from fifty six Wistar rats used in this study. Pneumococcus suspension was injected transtympanically to all the rats. Subjects were classified under four different groups having 14 rats at each. In Group A, intramuscular penicillin G was injected to rats for a period of five days. In Group B, intraperitoneal montelukast was injected for 21 days in addition to penicillin. In Group C, intraperitoneal montelukast and in Group D intraperitoneal isotonic NaCl was injected to rats for 21 days. Cross-sections were semi-quantitatively graded with respect to various inflammatory components.

Results: No significant difference was found between the groups, apart from mucosal vascularization with respect to mucosal and tympanic membrane (TM) parameters at early phases. However, statistically significant differences were found for the improvement of TM thickness with the help of penicillin treatment. Furthermore, considerable deviations were observed for the recuperation of TM and mucosal inflammation for groups where subjects were injected with montekulast as compared to other groups of the study.

Conclusion: The results of this study clearly show that the beneficial effects of the antibiotic (penicillin) as well as leukotriene antagonist (montelukast) is statistically different those of placebo in acute otitis media in rats. When the parameters of inflammation in the rat middle ear were compared with each other, most of these parameters did not show any statistically different beneficial effects in montelukast and penicillin groups.

Key words: Acute otitis media, leukotriene, montelukast, tympanic mucosa.

Özet

Amaç: Deneysel akut supuratif otitis media modeli geliştirilmiş sıçanlarda penisilin ve lökotrien antagonisti kombinasyonuna verilen erken ve geç evre yanıtların histopatolojik açıdan karşılaştırılması amaçlanmıştır.

Yöntem: Çalışmada Wistar tipi 56 sıçanın 83 kulağı incelendi. Bütün sıçanlara transtimpanik yolla pnömokok suspansiyonu enjekte edildi. Örnekler, her biri 14 sıçan içeren dört gruba ayrıldı. Grup A'daki sıçanlara beş günlük bir periyotta intramüsküler penisilin G, Grup B'dekilere penisiline ek olarak, 21 gün boyunca intraperitoneal montelukast enjekte edildi. Yirmi bir gün intraperitoneal tedaviye alınan son iki gruptan Grup C'deki sıçanlara montelukast ve Grup D'dekilere de izotonik NaCl verildi. Örneklerden alınan kesitler çeşitli enflamasyon parametrelerine göre semikantitatif derecelendirildi.

Bulgular: Erken evrede mukoza ve timpanik membran (TM) parametreleri açısından gruplar arasında mukozal vaskülarizasyon dışında anlamlı fark yoktu. Fakat penisilin tedavisi etkisinde TM kalınlığında düzelmede istatistik açıdan anlamlı farklar bulundu. Ayrıca montelukast enjekte edilen gruplar diğerleri ile karşılaştırıldığında TM ve mukoza enflamasyonunda iyileşme açısından dikkate değer farklar gözlendi.

Sonuç: Çalışmanın sonuçları akut otitis medialı sıçanlarda antibiyotiğin (penisilin) ve lökotrien antagonistlerinin (montelukast) iyileştirici etkilerinin plaseboya göre istatistiksel olarak üstün olduğunu açıkça ortaya koymaktadır. Sıçan orta kulağındaki enflamasyon parametreleri kendi aralarında karşılaştırıldığında, parametrelerin çoğunun montelukast ve penisilin gruplarındaki bu etkiler açısından istatistiksel farklılık göstermediği saptandı.

Anahtar sözcükler: Akut otitis media, lökotrien, montelukast, timpanik mukoza.

Correspondence: Bülent Şerbetçioğlu, MD. Dokuz Eylül Üniversitesi, Tıp Fakültesi, Kulak Burun Boğaz Hastalıkları Anabilim Dalı, İzmir, Turkey.
e-mail: serbetcioglu@gmail.com

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Otitis media (OM) is an inflammatory response of the middle ear (ME) caused by multiple factors such as infection and Eustachian tube dysfunction.[1] Acute otitis media (AOM) is one of the common pediatric diseases.[2-4] *Streptococcus pneumonia* and *Haemophilus influenzae* are the most common causative microorganisms for AOM.[5-7] A patient with AOM presents namely two important problems, the infection and the effusion.[4] The infection is usually under control within a few days with successful antibiotic treatment. However, the middle ear effusion lasts for months.[4] In most cases, AOM is a self-limiting disease, and it has been argued that untreated infections might promote the production of antibodies and thus strengthen the immune response and improve resistance in cases of repeated exposure.[8] Furthermore, development of bacterial resistance to common antibiotics is an increasing problem in some parts of the world, which, however, could be limited by strict indications and prescription of narrow-spectrum antibiotics.[8] On the other hand, risk of potentially life-threatening complications clearly justifies the use of antibiotics.[8] Earlier studies of pneumococcal otitis media in the rat demonstrated that the clinical course of pneumoccocal AOM in the rat resembles to those in human beings. Mucosal changes were shown to be present during the acute phase of infection, but these changes were persisting throughout the study period.[7] Mucosal alterations have been suggested to be of importance for the subsequent development of secretory otitis media, and of recurrent episodes of AOM.[5]

The pathogenesis of the effusion in AOM is extraordinarily complex. Viral, bacterial and/or inert respiratory antigens stimulate a complicated interaction between macrophages, mast cells, eosinophils and neutrophils.[9] The cells produce a cascade of many types of inflammatory mediators, which result in damage by causing epithelial shedding, plasma leakage, and edema and mucus formation.[4] Of the numerous inflammatory mediators found in a middle ear effusion (MEE), leukotrienes have been shown to be a major cause of edema, mucus production and Eustachian tube dysfunction.[10-12] LT C4 and LTD4 stimulate mucus production in human airway cells in vitro and have been shown to be a depressive effect on human nasal cilia.[4,13] Cysteinyl leukotrienes are products of arachidonic acid metabolism and are released by various cells that are involved in the inflammatory cascade. It seems clear that arachidonic acid metabolites are important in the pathogenesis of MEE and may even be responsible for the sensorineural hearing loss that is occasionally observed with OME.[4,14]

Montelukast sodium is a selective and orally active leukotriene receptor antagonist. It seemed appropriate to investigate the possibility that montelukast sodium might be beneficial in an experimentally rat model.[4] Even though montelukast has been used for a long time in clinical practice, this drug has rarely been a topic of interest in terms of experimental rat models seeking effectiveness for therapy of AOM.

Materials and Methods

Fifty six female Wistar rats (weight 200-250 g) were used for this study. They were kept under standard laboratory conditions and given food (pellets) and water ad libitum. All ears of the rats examined by otomicroscopy and 83 ears that are free of middle ear infections were included. All animals were anesthetized with a combination of 50 mg/kg ketamine hidrochloride (Ketalar®) and xylazine hydrochloride given intraperitoneally. All procedures were performed under sterile conditions. Pneumonococcal otitis media was induced in each ear by transtympanic inoculation with 0.03 ml of suspension of type 3 Pneumococci (ATCC 49619), at a concentration of 109 CFU /ml).

The animals were initially examined at 48 hours post-inoculation by otomicroscopy and confirmed the presence of otitis media. The animals were randomly divided into four groups:

Group A (*Antibiotic-treated group*): Two days after inoculation, 14 animals (20 ears) were treated with intramuscular penicillin G 160.000 U/kg once daily for five days.

Group B (*Antibiotic and montelukast co-treated group*): Two days after inoculation, 14 animals (23 ears) were treated with Pen G and montelukast Na (10 mg/kg/day) intraperitoneally for 21 days.

Group C (*montelukast treated group*): Two days after inoculation, 14 animals (21 ears) were treated with montelukast Na (10 mg/kg/day) for 21 days.

Group D (*placebo group*): Two days after inoculation, 14 rats (20 ears), were not given any medication, only 2 cc phosphate buffer saline intraperitoneally.

Two rats, one in antibiotics and montelukast co-treatment group, one in infected controls were died, and excluded from the study.

Histologic Preparation

On days 7 and 21 after inoculation, 7 rats randomly selected from each group were otomicroscopically examined

under anesthetization with an intramuscular injection of 50 mg/kg ketamine hydrochloride and 5 mg/kg of xylazine. They were sacrified by intraperitoneal injection of 80 mg/kg pentobarbital. Their temporal bones were removed, fixed in 10% formalin for one day and decalsified with formic acid for 2 days.

Morphologic Examination

Specimens divided into two equal parts were embedded in paraffin following a dehydration procedure and sectioned for light microscopy (0.5 µm). Hematoxylin & eosin and Alcian blue staining was performed for morphologic examination.

The following parameters were scored semiquantitatively by a pathologist blinded to the experimental groups as normal, mild, moderate and severe for increase in tympanic membrane (TM) thickness, vascularization, inflammation as well as middle ear mucosal thickness, mucosal vascularization and mucosal inflammation. Middle ear mucosal secretory metaplasia was graded +1 when transformed cells accounted <%50 cells observed, and +2 when transformed cells accounted >%50 cells observed.

Results

Otomicroscopic Findings

On day 7, in all groups, inflammatory response was as severe as control group, drum vessels were dilated, tympanic membranes were thick and inflammed.

On day 21, in antibiotic treated group, 8 ears of 7 rats, inflammatory response was less severe than infected groups, but more severe than antibiotic-montelukast co-treated and montelukast treated groups.

In antibiotic-montelukast co-treated group, 9 ears of 6 rats, inflammatory response was least severe than infected groups and only antibiotic treated group. Six ears showed no inflammatory sign on their TM and one showed myringosclerosis.

In montelukast treated group; 9 ears of 7 rats, inflammatory response was least severe than infected groups and only antibiotic treated group. Six ears showed no inflammatory sign on their TM, 3 ears shows effusion.

In infected control group; 9 ears of 6 animals, inflammatory response was as severe as on day 7. Tympanic membranes of 6 rats showed effusion, 2 of them showed retraction of TM, and only one was normalized.

Histological Findings

In several areas of the middle ear and bulla, the inflammatory response of the mucosa showed a similar pattern. Semi-quantitative analytical results on the degree of inflammatory response are noted.

On day 7; 43 ears of 28 animals were histopatologically observed for seven parameters: TM thickness, vascularization and inflammation, mucosal thickness, vascularization, inflamation and secretory metaplasia. All groups presented decreased mucosal vascularization when compared with placebo group. But in other parameters, all groups showed similar changes (Tables 1 and 2).

On day 21; 38 ears of 26 animals were histopathologically observed for seven parameters; TM thickness, vascularization and inflammation, mucosal thickness, vascularization, inflamation and secretory metaplasia.

All groups showed significant decrease in TM thickness when compared with control group. Antibiotic-montelukast co-treated group and montelukast group showed significant decrease in mucosal thickness when compared with infected control group. Montelukast group only showed significant

Table 1. For all parameters in the early period of the average scores obtained from four groups.

	Pen	Pen + Mont	Mont	Placebo
TM thickness	1.20±1.22	0.91±1.13	0.86±1.21	1.33±0.86
TM vascularization	0.10±0.31	0.27±0.64	0.43±0.78	0.56±0.52
TM inflammation	1.30±1.16	0.83±1.11	0.86±1.21	1.33±0.86
M thickness	2.00±1.0	1.75±0.86	0.78±1.20	1.78±0.66
M vascularization	0.55±0.52	0.75±0.62	0.67±0.86	1.67±0.50
M inflammation	1.82±0.75	1.58±0.66	1.44±1.04	1.78±0.66
M secretory metaplasia	0.64±0.67	0.92±0.28	0.56±0.52	0.78±0.44

TM: tympanic membrane, M: middle ear mucosa, Pen: penicillin, Mont: montelucast

Table 2. Early period to compare the groups in terms of mucosal vascularization.

Groups	Mucosal vascularization
A-B	0.438
A-C	0.933
A-D	**0.001**
B-C	0.642
B-D	0.004
C-D	0.016

Table 3. Results of the comparisons between groups in the late period (p values).

TM thickness	TM vascularization	TM inflammation	M thickness	M vascularization	M inflammation	M metaplasia
0.003**	0.944	0.054	**0.007****	**0.015***	**0.036***	0.611

TM: tympanic membrane, M: middle ear mucosa, *p<0.05, **p<0.001

decrease in mucosal vascularization when compared with antibiotic treated group and infected control group. Antibiotic-montelukast co-treated group and only montelukast treated group showed significant decrease in mucosal inflammation when compared with infected control group. In all groups, no significant difference is found in TM inflammation, TM vascularization and mucosal secretory metaplasia data. In all groups, chronic inflammatory cells in middle ear and fibrosis of TM were observed, but in infected control group acute inflammatory cells were still observed at submucosal sections (Tables 3-5). Mucosal and TM changes are shown on day 21 in all groups (Fig. 1).

Discussion

As reported previously,[7,16] untreated experimental pneumococcal AOM in rats causes changes in the middle ear mucosa that persist for at least 6 months. The histological changes include a thickened mucosa, an increased number of glands and the occurrence of ciliated cells. Beneficial effects on the mucosal changes were reported in experimental pneumococcal AOM when penicillin V was given prophylactically or used as an early treatment.[8,17,18]

The study results of Cayé-Thomasen et al.[8,17,18] demonstrated that penicillin administration clearly prevented the differentiation of epithelial cells into goblet cells. The increased goblet cell density is conceivably a result of both hyperplasia (involving cell division) and metaplasia (involving cell differentiation). To our knowledge, few middle ear data exist on these aspects, but when the tracheobronchial epithelium is exposed to various noxious stimuli, new and perhaps pre-existing goblet cells proliferate by division, and basal and indeterminate epithelial cells differentiate into goblet cells. The mechanisms leading to goblet cell hyperplasia, an epithelial cell metaplasia are comparatively unknown, although several strong indications exist. Airway infection is accompanied by hypersecretion of mucus from goblet cells. In turn, loss of intracellular mucus to a critical level stimulates goblet cells to enter the cell cycle. Mediators of the inflammatory response to bacterial colonization also play a role, as elucidated by stimulation of goblet cell hyperplasia by neutrophil lysates, inhibition by corticosteroids and NSAIDs and administration of antibiotics during middle ear infection. Specific mechanisms underlying these findings are partly due to products and metabolites of the cyclooxygenase and lipooxygenase pathways of arachidonic acid. These are activated through cell membrane release, induced by noxious and other agents released during inflammation. Prostaglandin E and eicosanoids are examples of such products, which have been shown to induce differentiation of epithelial cells to goblet cells and the release of mucous glycoproteins, possibly through a regulatory mechanism mediated by cyclic adenosine monophosphate. But in our study, goblet cell metaplasia in

Table 4. The groups differed significantly in terms indicating parameters compared to late periods.

Groups	TM thickness	M thickness	M vascularization	M inflammation
A-B	0.620	0.144	0.428	0.308
A-C	0.454	0.097	**0.010**	0.166
A-D	**0.010**	0.361	0.641	0.358
B-C	0.847	0.928	0.081	0.667
B-D	**0.007**	**0.007**	0.507	**0.025**
C-D	**0.002**	**0.002**	**0.005**	**0.009**

TM: tympanic membrane, M: middle ear mucosa

Table 5. Groups for all parameters mean values obtained in the late period.

	Pen	Pen+Mont	Mont	Placebo
TM thickness	1.00±0.00	0.88±0.64	0.82±0.60	2.00±0.70
TM vascularization	0.20±0.44	0.13±0.35	0.09±0.30	0.11±0.33
TM inflammation	0.80±0.44	0.63±1.00	0.45±0.93	1.22±0.66
M thickness	2.00±1.00	1.25±0.88	1.25±0.75	2.44±0.52
M vascularization	1.00±0.57	0.75±0.70	0.25±0.45	0.89±0.33
M inflammation	2.00±1.00	1.50±0.75	1.42±0.76	2.44±0.72
M secretory metaplasia	0.71±0.48	0.63±0.51	0.92±0.51	0.89±0.60

TM: tympanic membrane, M: middle ear mucosa, Pen: penicillin, Mont: montelucast

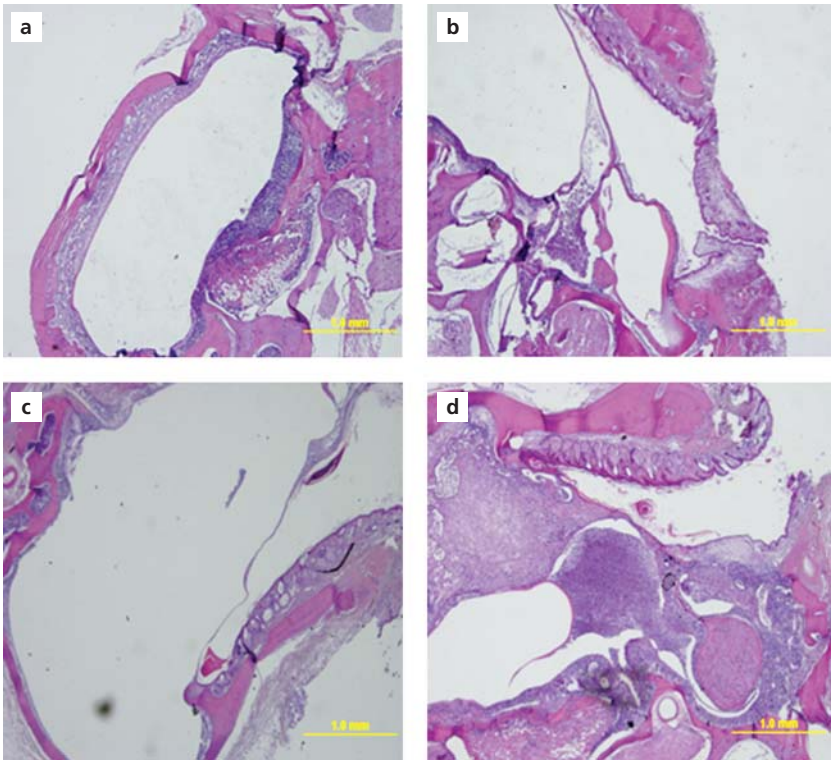

Fig. 1. Microscopic appearances of the middle ears from different groups at 21st day of the experiment, (**a**) Penicillin-treated group: mild inflammation, (**b**) Penicillin and montelukast co-treated group: minimal inflammation, (**c**) Montelukast treated group: almost nearly no inflammation, and (**d**) placebo group: severe inflammation.

mucosa is not statically significant in any group compared with infected controls.

Although clinical trials proving the efficacy of this combination therapy in inflammatory diseases have been reported in the literature, the effect of combination therapy with antibiotics and montelukast in experimental AOM has not been investigated yet. The study results of Cumbs et al.[4] demonstrated that combination treatment with antibiotics and montelukast in clinical treatment showed a significantly beneficial effect on acute otitis media, compared with an antibiotic-treated group.

Cysteinyl leukotrienes (CysLTs) are being increasingly implicated in the aetiology of acute and chronic inflammatory diseases of nonallergic origin, including cardiovascular diseases, autoimmune diseases, and certain malignancies. The spectrum of proinflammatory activities of CysLTs may therefore extend beyond eosinophils, monocytes/macrophages, type 2 helper T (Th2) lymphocytes, and airway smooth muscle cells, to other types of inflammatory cells such as neutrophils.

Montelukast inhibits physiological actions of LTD4 at the CysLT1 receptor without any agonist activity. It also exerts a substantial and apparently direct inhibitory effect on 5- lipoxygenase activity in vitro. By this way, montelukast decreases vascular permeability, inhibits inflammatory cell activation, smooth muscle proliferation, bronchoconstriction and activates mucociliary clearance.

Ganbo et al.[12] showed that in guinea pigs, LTD4 progressively inhibits ciliary activity, while PGE2 promoted it. Leukotriene C4 also induced ciliary inhibition. This may be another way of montelukast decreasing the duration of middle ear effusion after AOM.

This study indicates that it may have a place in the medical management of AOM. Acute otitis media commonly presents with only a partial effusion, and spontaneous resolution of the effusion generally occurs in about 50% of ears at 1 month. A full effusion occurs only about 30% of the time. A full effusion is not only more durable than a partial effusion, it is also more predictive of a clinically important conductive hearing impairment. Unilateral conductive hear-

ing loss is not nearly as bad as a bilateral impairment. It may be that montelukast will prove to be most appropriate and cost effective for a small subset of AOM with bilateral disease and full effusions.

The present study was performed in rats. Thus, it is not possible to generalize these findings to the clinical studies. However, it is obvious that earlier effects of penicillin or montelukast did not differ to those of placebo. This is correlating with the results of the previous studies. Probably this is related to the fact that the accumulating effects of the antibiotic as well as leukotriene antagonist (montelukast sodium) become to be beneficial as late as seven to ten days after the innoculation (inflammation).

However, the results of this study clearly show that the beneficial effects of the antibiotic (penicillin) as well as leukotriene antagonist (montelukast) is statistically different those of placebo in AOM in rats. When the parameters of inflammation in the rat middle ear were compared with each other, most of these parameters did not show any statistically different beneficial effects in montelukast and penicillin groups.

Conflict of Interest: No conflicts declared.

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