ORIGINAL ARTICLE

# Effects of Calcium-rich Diet in Experimentally Induced Otitis Media Subtypes in the Formation of Tympanosclerosis in Rats

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**Objective:** The aim of this study is to evaluate the effect of calcium-rich feed in the development of tympanosclerosis in rats with acute otitis media and otitis media with effusion and to compare the effects of these conditions in the formation process of tympanosclerosis.

**Materials and Methods:** Thirty rats were equally divided into six groups. The first two groups were used as control. The first group was fed calcium-normal feed, while the second group was fed calcium-rich feed. The third group (otitis media with effusion) and fourth group (otitis media with effusion) were calcium-normal and calcium-rich feed, respectively. The fifth (acute otitis media) and sixth groups (acute otitis media) were fed calcium-normal and calcium-rich feed, respectively. Otitis media with effusion was induced with transtympanic injection of histamine dihydrochloride. Acute otitis media was induced with transtympanic injection of suspension of Streptococcus pneumonia. Calcium content of feeding for calcium-normal and calcium-rich feed were 0.67% and 3%, respectively.

**Results:** Total serum calcium values were higher in calcium-rich feed groups than calcium-normal feed groups. However, total serum calcium levels were within normal limits in both groups (2,61±0,07 mmol/lt and 2,54±0.07 mmol/lt, respectively). Comparing the groups of otitis media with effusion, the formation process of tympanosclerosis was higher in the calcium-rich feed group than in the calcium-normal feed group; there was a statistically significant difference. However, here was no statistically significant difference between calcium-rich and calcium-normal feed groups in the acute otitis media and control groups regarding the formation process of tympanosclerosis. There was also no statistically significant difference between the otitis media with effusion and acute otitis media groups.

**Conclusion:** Experimental otitis media with effusion and acute otitis media are effective in the formation of tympanosclerosis. There is no significant difference between the effects of these pathologies in the formation process of tympanosclerosis. Calcium-rich feed is effective in experimental otitis media with effusion in the formation process of tympanosclerosis but not in acute otitis media. Furthermore, calcium-rich feed is not the reason for hypercalcemia.

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## Introduction

Tympanosclerosis (TS) is a sequela of acute otitis media (AOM), otitis media with effusion (OME), and chronic otitis media consisting of calcification of connective tissue in the middle ear and tympanic membrane. The incidence of TS in patients with chronic otitis media varies between 20 and 43%. TS may cause hearing disability, which sometimes is clinically severe. The factors underlying development of TS remain unclear, but it appears that inflammation is a major factor in this condition.[1-3] In cases of inflammatory response, the action of bioactive molecules that interact with the cells of middle ear mucosa plays an important role in the pathogenesis of this condition. Previous studies have demonstrated the role of several cytokines, inflammatory mediators, and growth factors in the inflammation of middle ear mucosa.[4] When the tympanic membrane is solely engaged, defined as myringosclerosis, it seldom

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impairs hearing; however, when the tympanosclerotic plaques are located on the epitympanum, promontory, or inner ear, hearing problems can occur.[1] Histologically, TS is characterized by a progressive fibroblast infiltration causing an increase in collagen fibers that lead to poor formation of cells and blood vessels causing hyalinization, which can progress to calcification.

Physiologically calcium (Ca) levels depend on absorbed and excreted Ca. Persistent hypercalcemia, especially with normal or high serum phosphate levels, can cause ectopic deposition of Ca and phosphate on the walls of blood vessels, heart valves, connective tissue close to joints, tendons, gastric mucosa, cornea, and renal parenchyma. Calcium-rich feed (CRF) may lead to an increase in the formation of TS. This may related with or without hypercalcemia.[5]

The aim of this study is to compare the effects of experimental AOM and OME to formation of TS and to evaluate the effect of CRF on TS development in both pathologies.

## Materials and Methods

Animals used in the present study were housed, supervised, and handled according to the Suleyman Demirel University Medical School Guidelines for Care and Use of Laboratory Animals. The study protocol and animal conditions were approved by Suleyman Demirel University Ethical Committee for Animal Studies (The date/number of Institutional Ethical Committee: 03.03.2009/7). Thirty young, adult male healthy Wistar-Albino rats with no middle ear pathology weighing 325 ± 30g were enrolled in this study. The experimental animals were housed in polypropylene cages under laboratory conditions of 28±2°C temperature with 75% relative humidity and photoperiod of 12 h light/dark cycle.

The rats were divided into six groups. Each group contained five animals. Groups I and II were defined as control. As shown in Table 1, Group I received calcium-normal feed (CNF) with 0.67% Ca concentration ), while Group II received (CRF) with 3%Ca concentration.

In Groups III and IV, both ears of the rats were inoculated with histamine dihydrochloride to induce OME. Histamine solution (Histamine dihydrochloride, Sigma®) was prepared with saline (1 ml histamine contained 1 ml saline). During the OME induction and reassessments, rats were anesthetized with Ketamin (Ketalar®) at 90 mg/kg and Xylazin (Alsazin®) in 10 mg/kg dosages. Histamine was inoculated in both ears using a 27 gauge needle in a concentration of 0.1 ml to induce OME (Table 1, Fig. 1). Groups III and IV were divided and fed with CNF and CRF, respectively (Table 1).

Groups V and VI were divided in the same way. Both ears of all rats in these groups were inoculated using a 27 gauge needle with an approximately 0,1 ml suspension of Streptococcus pneumonia type 3 (Str. Pneumonia type 3, Microbiologics® Inc.) in a concentration of 107 CFU to induce AOM (Table 1, Fig 2). Similarly as described above, Group V was fed with CNF, and Group VI was fed with CRF.

**Table 1.** General characteristics of study groups: OM, Otitis Media; AOM, Acute Otitis Media; OME, Otitis Media With Effusion; Ca, calcium.

| | Rat | Ear quantity in the study | Type of OM | Diet Ca level | Agents used |
|---|---|---|---|---|---|
| Group I | 5 | 10 | Control | 0,67% | None |
| Group II | 5 | 10 | Control | 3% | None |
| Group III | 5 | 10 | OME | 0,67% | Histamine Dihydrochloride |
| Group IV | 5 | 10 | OME | 3% | Histamine Dihydrochloride |
| Group V | 5 | 9 | AOM | 0,67% | S. Pneumonia |
| Group VI | 5 | 10 | AOM | 3% | S. Pneumonia |

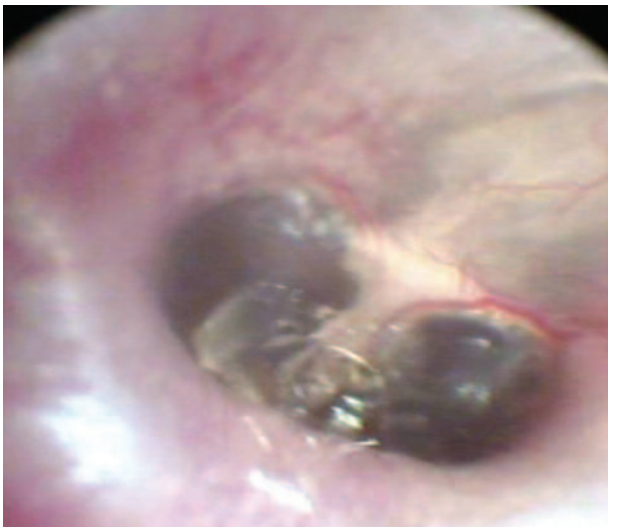

**Figure 1.** Retraction pocket in pars tensa due to transtympanic injection of Histamine dihydrochloride (otoendoscopic view)

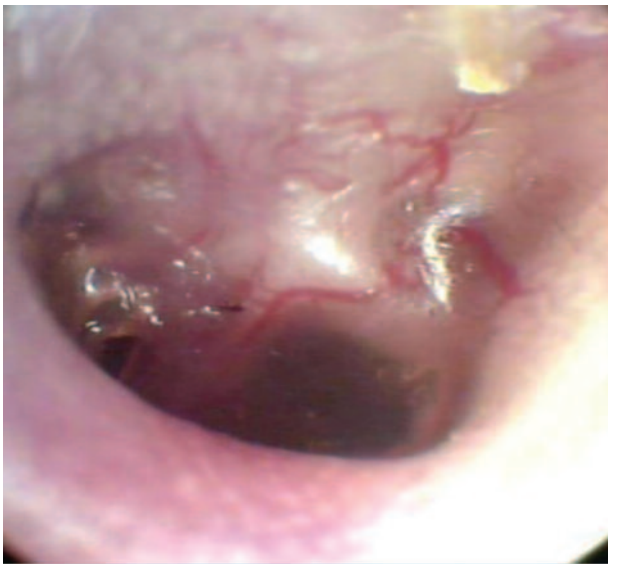

**Figure 2.** Hyperemia and perforation in tympanic membrane due to induced acute otitis media (otoendoscopic view)

*Histopathologic Examination*

After four weeks, the rats were sacrificed; tympanic bullaes were dissected out and fixed in 10% formalin; and blood samples were taken from the vena cava to screen the serum Ca levels. Next, the material was submitted to decalcification in formic acid at 10% for a period of two days and again fixed in buffered 10% formalin. After the decalcification process, the specimens were embedded in paraffin. Cross-sections from pars tensa of TM of 5 μm thickness were made on the axial plane. The first, third, and the fifth sections of the consecutive six sections were stained with Hematoxilen eosine; the rest of the sections were stained with Masson Trichrome (Fig. 3).

The sections were analyzed under light microscopy for histopathologic examination. To facilitate the interpretation of histopathologic findings, the inflammatory processes were classified into five stages, with stage 0 indicating no histological findings, stage 1, predominance of exudate, stage 2, by intense vascular neoformation, stage 3, by fibrotic characteristics, and stage 4, by characteristics of hyalinization with presence of calcifications. Statistical analysis was performed with the nonparametric Mann–Whitney U test. The significance level was set at $p < 0.05$.

## Results

All tympanic membranes and middle ears were examined by otomicroscopic examination 24 hours after the histamine inoculation. Groups III and IV were assessed for the existence of OME. In Groups III and IV, 90% and 100% of the ears, respectively showed OME. After seven days of S. pneumonia inoculation, Groups V and Group VI were assessed for the existence of AOM; 90% and 100%, respectively, were evident with AOM by otomicroscopic examination.

After four weeks, the animals were sacrificed, and histopathologic findings were evaluated. In Group I, there was no histopathologic abnormality except for one ear, which showed submucosal neutrophil infiltration (Fig. 4). In Group II, there were four abnormal histopathologic findings, two of which were submucosal neutrophil infiltration and two of which were fibrotic characteristics. These findings were determined as AOM attacks during the follow-up period.

Considering both groups with OME (Groups III and IV), inflammation was found in advanced stages. In Group III, one ear had normal histological finding; two ears had vascular neoformation (Fig. 5); one ear had fibrotic changes; and the remaining six ears had hyalinization. In Group IV, all ears showed hyalinization with one having calcification.

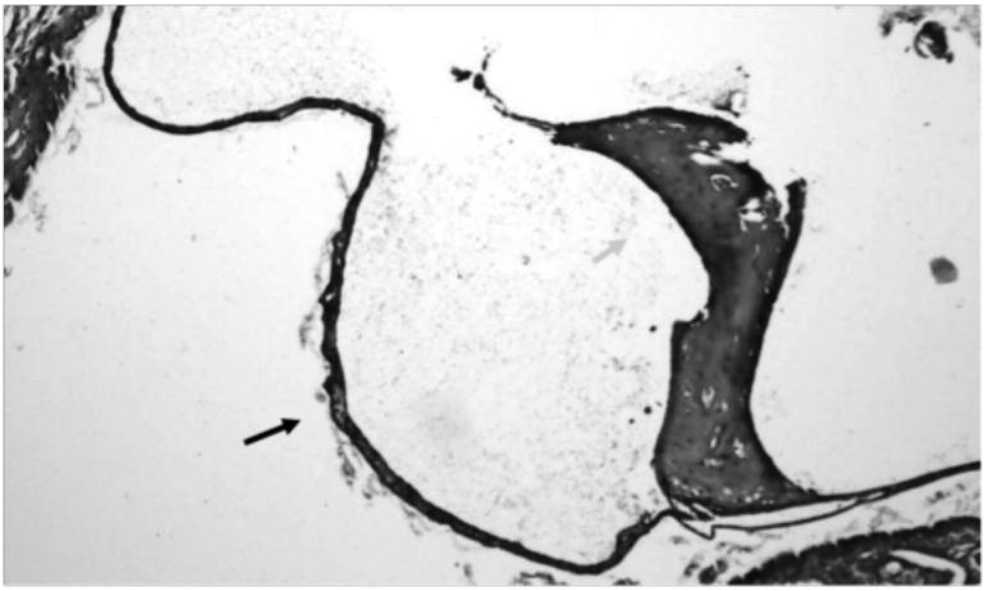

**Figure 3.** Normal tympanic membrane (black arrow) and malleus (yellow arrow) (masson trichrome; original magnification; ×40)

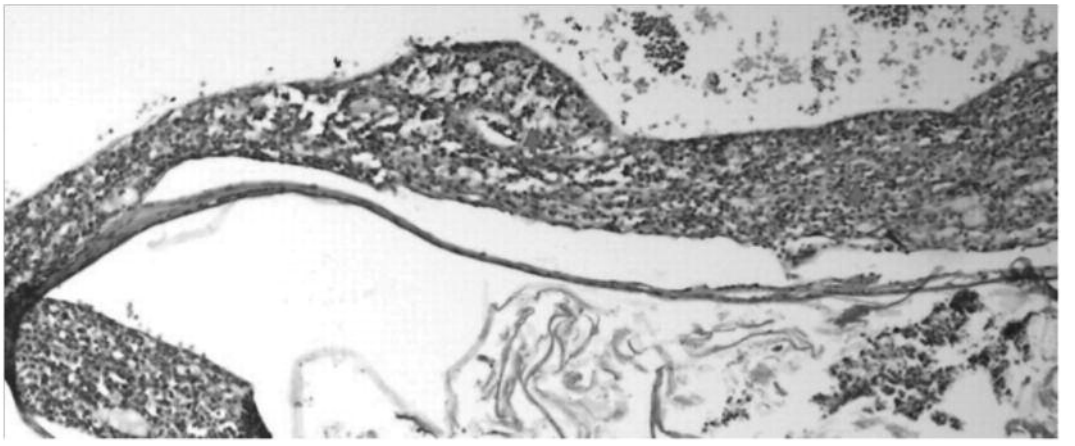

**Figure 4.** Predominance of exudate (red arrow) (hematoxylin eosin; original magnification; ×40)

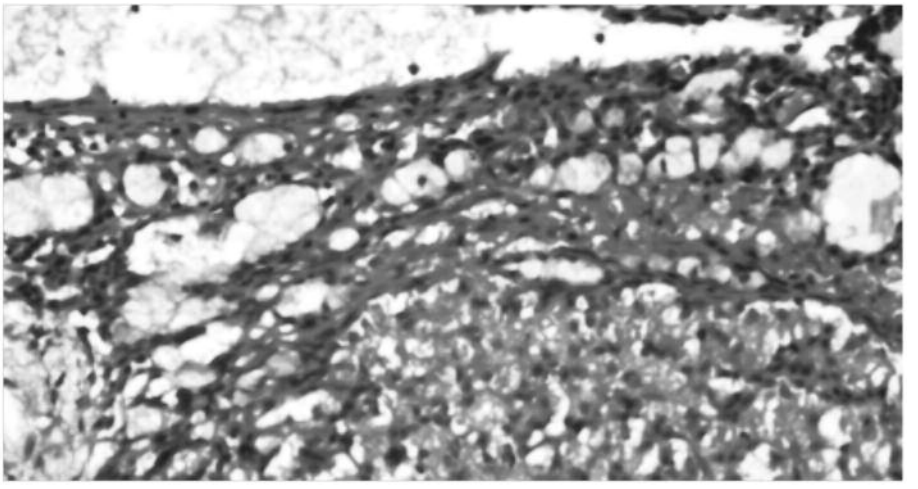

**Figure 5.** Vascular neoformation and retraction of tympanic membrane (masson trichrome; original magnification; ×100)

Similarly, in groups with AOM, inflammation was found in advanced stages. One ear in Group V was excluded from the study due to damage to the tympanic bulla during preparation of specimen. In Group V, one ear had vascular neoformation and eight ears had hyalinization (Fig. 6) with one having calcification. In Group VI, one ear had vascular neoformation, and the rest had hyalinization with no calcification (Table 2).

Comparing the inflammatory stages of the middle ear and TM in Group I with Group II, the statistical difference was not significant (p>0,05). While the statistical difference between OME groups (Groups III and IV) was significant, the difference between both AOM groups (Groups V and VI) was not significant (Table 3).

Comparing the control group with CNF (Group I) with Group III, the statistical difference was significant (p<0,05). Comparing Group I with Group V, the statistical difference was significant (p<0,05). Comparing the Group III with Group V, the statistical difference was significant (p<0,05) (Table 3).

Comparing the control group with CRF (Group II) with Group IV, the statistical difference was again significant (p<0,05). Comparing the Group II with Group VI, there was again a statistically significant difference (p<0,05. Comparing the Group IV with Group VI, there was a statistically significant difference (p<0,05) (Table 3).

Comparing serum Ca and phosphate values of CNF groups and CRF groups, the mean values in CRF groups was higher than in the CNF groups. There was no statistically significant difference between the mean values in both groups, and mean values were within normal limits (Table 4).

## Discussion

The etiopathogenesis of TS development is still unclear. However, it seems likely that inflammation contributes to the development of this condition. TS is characterized by a progressive fibroblast infiltrate, causing an increase in collagen fibers that originate poor formation of cells and blood vessels, suffering hyalinization, which can progress to calcium deposition, histologically. Extracellular vesicle matrix indicate an important role of the in the calcification process of various tissues under normal and pathologic conditions. OME and AOM are known entities in the

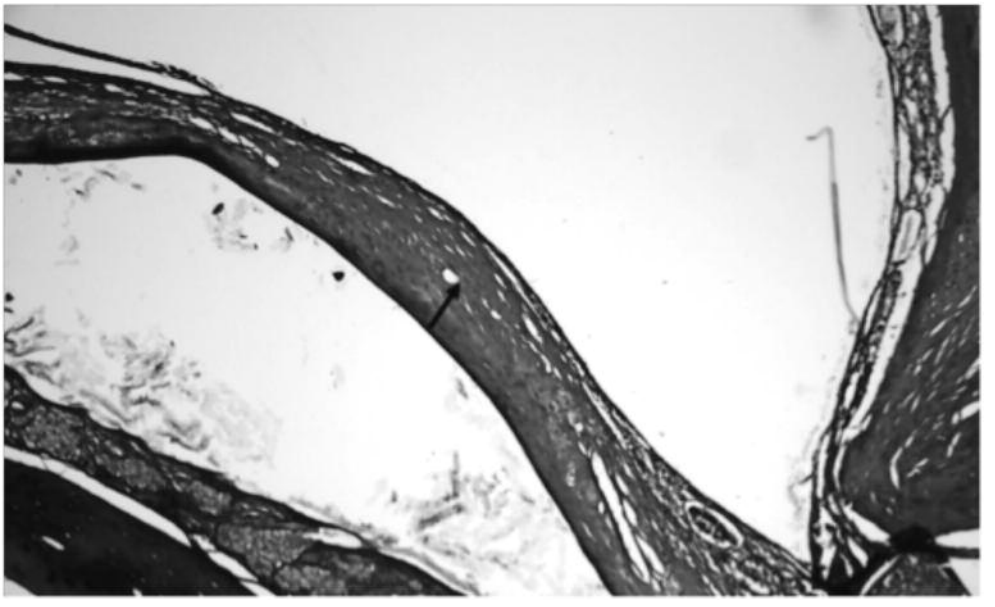

**Figure 6.** Hyalinization (black arrow) (masson trichrome; original magnification ×40)

**Table 2.** Distribution of stages of inflammation (for formation process of tympanosclerosis) in groups

| Stage | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Group I | 9 | 1 | - | - | - |
| Group II | 6 | 2 | - | 2 | - |
| Group III | 1 | - | 2 | 1 | 6 |
| Group IV | - | - | - | - | 10 |
| Group V | - | - | 1 | - | 8 |
| Group VI | - | - | 1 | - | 9 |

**Table 3.** The statistical comparison of groups and p values

| Groups compared | p value |
|---|---|
| Group I – II (CNF Control – CRF Control) | p>0.05 |
| Group III – IV (CNF OME – CRF OME) | p<0.05* |
| Group V – VI (CNF AOM – CRF AOM) | p>0.05 |
| Group I- III (CNF Control – CNF OME) | p<0.05* |
| Group I – V (CNF Control – CNF AOM) | p<0.05* |
| Group III – V (CNF OME – CNF AOM) | p>0.05 |
| Group II – IV (CRF Control – CRF OME) | p<0.05* |
| Group II – VI (CRF Control – CRF AOM) | p<0.05* |
| Group IV – VI (CRF OME – CRF AOM) | p>0.05 |

**Mann Whitney U test (p<0.05 Statistically significant)*

**Table 4.** The mean serum Ca and Phosphate values of groups: Ca, calcium; CNF, Calcium Normal Feed; CRF, Calcium-rich Feed

| | Groups with CNF (Group I, III, V) | Groups with CRF (Group II, IV, VI) |
|---|---|---|
| Ca | 2.54±0.07 mmol/lt | 2.61±0.07 mmol/lt |
| Phosphate | 2.3 ±0.26 mmol/lt | 2.5 ±0.35 mmol/lt |

formation process of TS.[3,5,6] AOM is the bacterial infection that affects the mucosa of the middle ear and mastoid air cell system. OME is an inflammation of the middle ear accompanied by an accumulation of fluid in the middle ear space without signs or symptoms of acute infection.[7,8]

Numerous studies involving pharmacologic stimulation, microbiologic stimulation, immunologic stimulation and cold stimulation to the external ear have been used for the experimental induction of OME.[9-12] We used transtympanic injection of histamine to induce OME [7,13,14] and suspension of Streptococcus pneumonia type 3 to induce AOM.[5]

Previous studies have shown that, in general, a few weeks are sufficient to assess the formation process of sclerosis.[5,15-17] Selcuk et al.[15] reported that clinically

experimental sclerosis is observed at the end of the fourth week by otomicroscopic examination; these results correlate with the histopathologic findings. We sacrificed the rats after four weeks of follow up.

Numerous studies have used a decalcification process to assess to the formation of sclerosis.[5,16,17] Decalcification was necessary in our analysis, because we aimed to assess not only the tympanic membrane but also the tympanic bulla in the animals, which would not be possible without decalcification.

In a normal serum level, Ca is closely regulated with normal total calcium of 2.25 to 2.68 mmol/L (9-10.2 mg/dl). The protein bound fraction of Ca represents 30 to 55%; diffusible ionic complexes of Ca comprise approximately 10 to 15%; and approximately 50% is freely ionized. Ionized Ca accounts for the biologically active form of serum Ca, and normal range of serum level is 1.1 to 1.4 mmol/L. In our study, the mean Ca value of the CRF groups was higher than for the CNF groups. However, both mean Ca values were within normal range (see Table 4). Carvalho et al.[5] accepted feed with 3% Ca concentration as Ca supplementation or experimental hypercalcemia, but there were no data about Ca levels of rats. Kahonen et al.[18] accepted feed with 3% Ca concentration is not cause to experimental hypercalcemia in rats. We agree that CRF is not the reason for hypercalcemia in rats.

In the present study, CRF was not more effective than CNF in AOM and control groups in the formation of TS. Carvalho et al. [5] assert that CRF may lead to an increase in the formation of TS in AOM. However, the results of their study were not statistically significant. According to the results of both studies, CRF is ineffective in the formation of TS in AOM.

To the best of our knowledge, there is no previous study comparing experimental AOM and OME in the formation of TS. We found that AOM was not more effective than OME in the formation process of TS or vice versa.

CRF was more effective in OME than CNF in the formation of TS. CRF may lead to an increase or acceleration in the formation of TS without causing hypercalcemia in OME.

## Conclusion

Experimental OME and AOM are effective in the formation of TS. There is no significant difference between the effects of these pathologies with respect to the formation process of TS. CRF is effective in experimental OME in the formation of TS but not in AOM. Besides, CRF is not the reason of hypercalcemia.

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