Biochemical Determination of Tissue Ossification Markers in Experimentally-induced Myringosclerosis

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Abstract: Objectives: To explore myringosclerotic tissue levels of two bone modeling markers: osteopontin (OPN) and osteoprotegerin (OPG) in experimentally-induced, histologically-confirmed myringosclerosis (MS). **Materials & Methods:** The right middle ear of 24 normal healthy growing male Wister rats was inoculated, via transtympanic access, by Streptococcus pneumoniae type 3, after a period of 8 weeks the tympanic membrane (TM) was examined Otomicroscopically for transparency and graded as normal TM, mild or marked opacification. Then, tympanic bullae were removed and a part of myringosclerotic plaque was excised for ELISA estimation of tissue extract levels of OPN and OPG and the remainder of the TM was stained with hematoxylin-eosin for light microscopic grading of TM inflammation according to extent of calcification into 5 grades. **Results**: 20 ears developed otoscopically defined myringosclerotic changes, 14 ears showed mild and 6 had marked opacification that was localized in 4 ears and diffuse in 2 ears. Histological examination reported inflammation of grade III in 7, grade IV in 13 and grade V in 4 specimens. Mean estimated tissue extract level of OPN and OPG in studied animals were significantly higher compared to control animals with a positive significant correlation between histological grading and tissue-extract levels of both OPG and OPN and a positive significant correlation between otoscopic grading and tissue-extract levels of OPG, but the correlation was non-significant with OPN **Conclusion:** Increased myringosclerotic tissue extract levels of both bone modeling markers indicated their possible role in initiation and/or progression of sclerotic changes in TM after chronic suppurative otitis media.

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1. Introduction

Tympanosclerosis involves the hyalinization and calcification of the collagen layer in certain areas of the tympanic membrane. The process is most often seen in the TM but may also involve other sites in the middle ear. The incidence of tympanosclerosis was found to be 35.6% of patients with chronic suppurative otitis media, but 77.8% of these patients had dry ear and the majority of them had hearing loss of the conductive type, [(1)].

The pathogenesis of TS is still unclear and various studies tried to explore the etiopathogenesis of MS especially that occurred in conjunction with or secondary to middle ear aeration using ventilation tube. The relationship between oxygen-derived free radicals and occurrence of MS has been proven in experimental models, and it was also shown that the formation of MS after experimental myringotomy could be reduced by application of various free radical scavengers, [(2)]. Kazikdas et al., [(3)] reported that the formation of MS after experimental myringotomy can be diminished by intramuscular alpha-tocopherol injections and Uneri et al., [(4)] reported similar results after topical application of vitamin E in human subjects.

Osteopontin (OPN) is a phosphorylated glycoprotein that is initially identified in osteoblasts as a mineralization-modulatory matrix protein and is constitutively expressed in mineralized tissues and in epithelial surfaces, [(5)]. In calcified tissues OPN plays an important regulatory role in bone mineralization and tissue remodeling, through the control of bone cell adhesion, osteoclast activation and matrix mineralization, [(6)]. OPN has been studied as a multifunctional protein that is upregulated in a variety of acute and chronic inflammatory conditions, such as wound healing, fibrosis, autoimmune disease, and atherosclerosis, [(7)].

Osteopontin plays an important role in the inflammatory response, in which it stimulates macrophage and T-lymphocyte migration and activation, [(8)]. In the latter cells, OPN polarizes the early Th1 cytokine response and inhibits Th2 cytokine expression, [(9)]. During inflammation, OPN is also expressed by cells of both innate and adaptive immunity, such as activated T lymphocytes, macrophages and resident fibroblasts, [(10)]. Moreover, OPN has been found to play an important role in neoplastic disorders and in the control of blood vessel neoformation (angiogenesis) via the stimulation of endothelial cells proliferation and progression, [(11)].

Osteoprotegerin (OPG) is an extracellular regulator of osteoclast differentiation and activation, [12]. OPG is synthesized as a propeptide (401 amino acids for the human, mouse, and rat forms), of which the signal peptide (21 amino acids) is cleaved, thus generating the 380 amino acids mature peptide, [13]. In contrast to all other TNFR superfamily members, OPG lacks transmembrane and cytoplasmic domains and is secreted as a soluble protein. Moreover, OPG mRNA has wide tissue distribution not restricted to bone or immune tissues and high levels of OPG mRNA have been detected in lung, heart, kidney, liver, stomach, intestine, skin, brain, spinal cord, thyroid gland, and bone, [14]. In addition, high OPG mRNA levels have also been detected in endothelial cells, aortic smooth muscle cells, fibroblastic cells, ovarian and breast cancer cell lines, and monocytic dendritic and B lymphocytic cell lines, [15].

As the calcification process and the sclerotic plaques of the drum mimics features of bone tissue, this study was designed to explore myringosclerotic tissue levels of two bone modeling markers: osteopontin (OPN) and osteoprotegerin (OPG) in experimentally-induced, histologically- confirmed myringosclerosis.

2. Materials and Methods

After approval of the study protocol from the Local Ethical Committee with regards to rules for dealing with experimental animals; the study comprised 30 normal healthy growing male Wister rats, weighing 200-400 gm. Rats were kept under standard conditions, temperature 20^{o}C, humidity 60% and 12-hs day/night cycle, and maintained on standard diet and free water supply till the start of study regimens. The animals were divided into 2 groups, each in a separate cage: Control group included 6 rats and Study group included 24 rats.

The rats were anesthetized with chloral hydrate at 10% by intra-peritoneal injections after which they were placed over the surgical bench, left lateral position, so that the examiner could explore the right ear. Under otomicroscopy, using surgical microscope brand D.F. Vasconcelos, model MC-M31, the middle ear of each animal was inoculated, via transtympanic access, by 0.1ml of solution containing 10^7 colony-forming units (CFU) of Streptococcus pneumoniae type 3, after which the animals were sent back to their respective cages. All animals were injected in right ear only. After elapse of a period of 8 weeks control and study rats were sacrificed with a lethal dose of chloral hydrate and the TM was examined Otomicroscopically for transparency and graded as normal TM (TM appeared thin and transparent), mild opacification (diffuse or isolated mild loss of transparency) and marked opacification (diffuse or isolated marked TM thickness with total loss of transparency), [16]. Then, tympanic bullae were removed and after decalcification of specimens in nitric acid at 7.5%, a part of myringosclerotic plaque was excised for biochemical assay and the remainder of the TM together with the external auditory canal was fixed in formaldehyde at 10%. In the post-fixation process, bullae were placed in a hot oven for 40 minutes in alcohol and 20 minutes in alcohol-xylol and later they were placed in xylol outside the hot oven, and then they were dehydrated, mounted in paraffin bloc (50 minutes). The material was sectioned at axial sections of 4mm thickness, with intervals of 50mm, encompassing the whole TM, and stained with hematoxylin-eosin and then examined under light microscopy, [17].

Microscopically, TM inflammation was graded into 5 grades: Grade 1: exudation, characterized by marked polymorphonuclear infiltrate; Grade 2: granulation, characterized by marked neovascularization and presence of elements of the macrophage mononuclear system (fibroblasts, lymphocytes, macrophages); Grade 3: fibrosis, characterized by major proliferation of fibroblasts and formation of collagen fibers, reduction of vascularization; Grade 4: hyalinization, characterized by reduction in number of fibroblasts, which are replaced by collagen fibers, which fuse forming plaques and Grade 5: calcification, characterized by calcium and phosphorus deposits, giving the collagen matrix an aspect similar to that of cartilage or bone tissue, [18].

Immediately after removal of the sclerotic plaque; specimens were homogenized in 10 mg/ml ice-cold phosphate-buffered saline to get a concentration of 10% (W/V). Homogenization was performed for 1 minute with the aid of a motor driven homogenizer at 5000 rpm on an ice background, [19]. The homogenate was then centrifuged to get the supernatant for Enzyme-Linked Immunosorbent Assay (ELISA) estimation of tissue extract levels of Osteoprotegerin using the RayBio® Human Osteoprotegerin ELISA, [13] and Osteopontin using the R&D Systems, Inc., [20].

Statistical analysis

Obtained data were presented as mean±SD and ranges. Results were analyzed using Wilcoxon Z-test. Possible relationships were investigated using Pearson linear regression. Statistical analysis was conducted using the SPSS (Version 10, 2002) for Windows statistical package. P value <0.05 was considered statistically significant.

3. Results:

At the end of the study period, 20 of the study animals developed otoscopically defined myringosclerotic changes. The TM of 4 ears (16.7%) appeared normal, while 14 ears (58.3%) showed mild opacification. The remaining 6 ears (25%) developed marked opacification that was localized in 4 ears (Fig. 1) and diffuse in 2 ears, (Fig. 2).

Seven (29.1%) specimens showed grade-3 changes with hyalinization, reduced number of fibroblasts and appearance of collagen fibers, (Fig. 3). Thirteen (54.2%) specimens showed grade 4 changes with fibrosis, proliferation of fibroblasts and formation of collagen fibers associated with reduction of vascularization, (Fig. 4). Four specimens (16.7%) showed grade-5 changes with calcification giving the collagen matrix an aspect similar to that of cartilage or bone tissue, (Fig. 5). All studied samples showed microscopic evidence of sclerosis varied between fibroblastic proliferation and evident calcification. On contrary, clinical evaluation failed to detect sclerotic changes in 4 ears with sensitivity of 83.3%.

Mean estimated tissue extract level of OPG in studied animals (127±12.5; range: 103.9-165.4 ng/ml) was significantly ($p<0.05$) higher compared to that estimated in control animals, (71.9±7.6; range: 62.3-81.4 ng/ml). Also, mean estimated tissue extract level of OPN in studied animals (204.3±45.5; range: 135-265 ng/ml) was significantly ($p<0.05$) higher compared to levels estimated in control animals, (342.5±110.9; range: 194-654 ng/ml), (Fig. 6).

Moreover, there was a positive significant correlation between tissue-extract levels of OPG and otomicroscopic grading, ($r=0.430$, $p=0.036$) and histological findings, ($r=0.581$, $p=0.003$), (Fig. 8). Also, there was a positive significant correlation between tissue-extract levels of OPN and histological grading, ($r=0.445$, $p=0.029$), while the correlation was non-significant with otomicroscopic grading, ($r=0.314$, $p>0.05$), (Fig. 9).

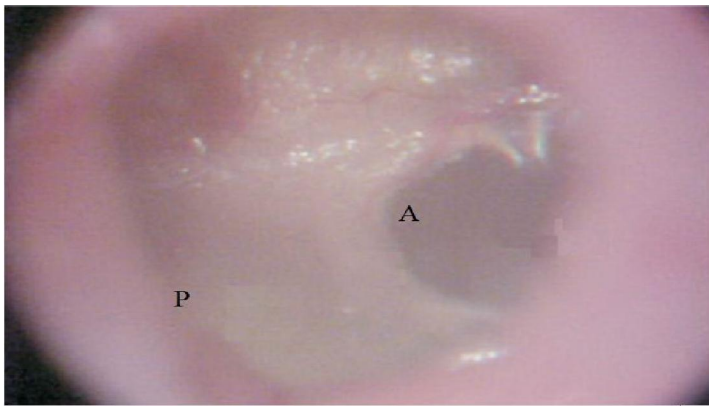

Figure 1: Otomicroscopic examination of one affected ear showing marked opacification of the posterior portion (P) of TM with normal transparency of the anterior portion (A) of TM

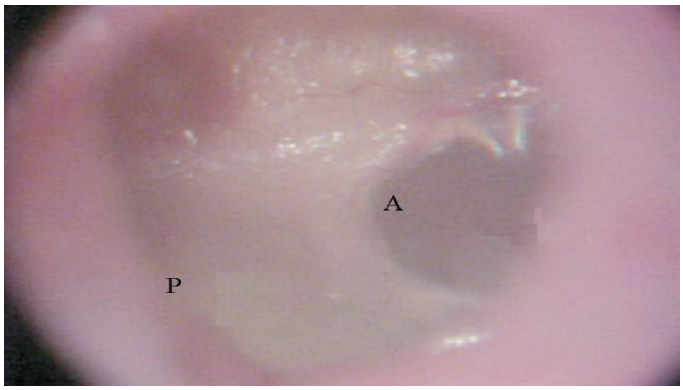

Figure 2: Otomicroscopic examination of one affected ear showing marked diffuse opacification of the TM (O)

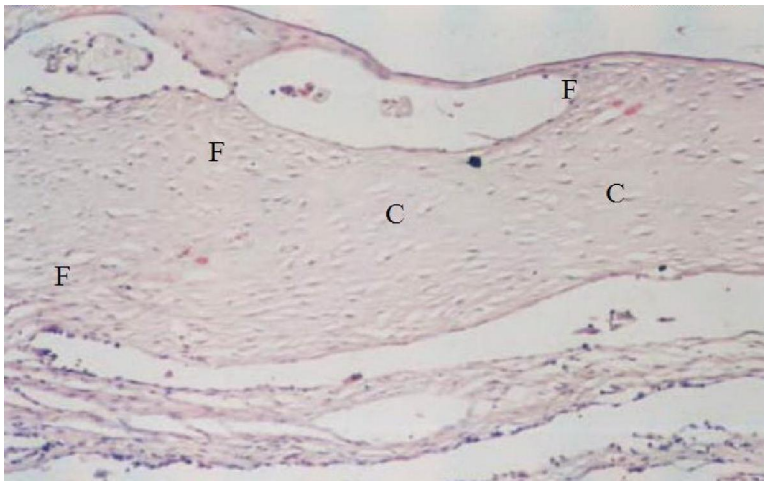

Figure 3: TM showed scattered fibroblasts (F) with areas of hyalinized collagen (HC), (Hx &E, x100).

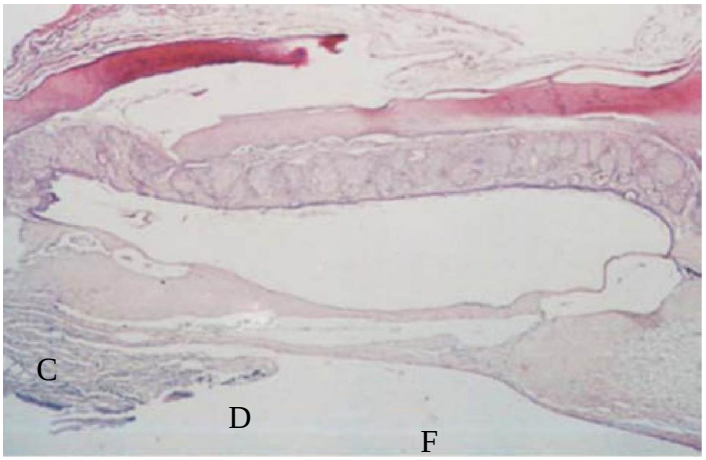

Figure 4: TM showed collagen (C) deposition with fibrosis (F) and areas of hyalinized degeneration (D), (Hx &E, x10).

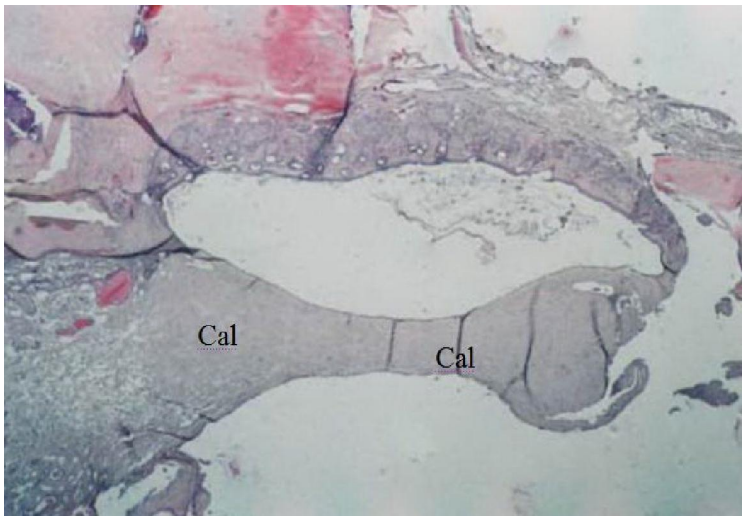

Figure 5: TM showed calcification (Cal), (Hx &E, x10).

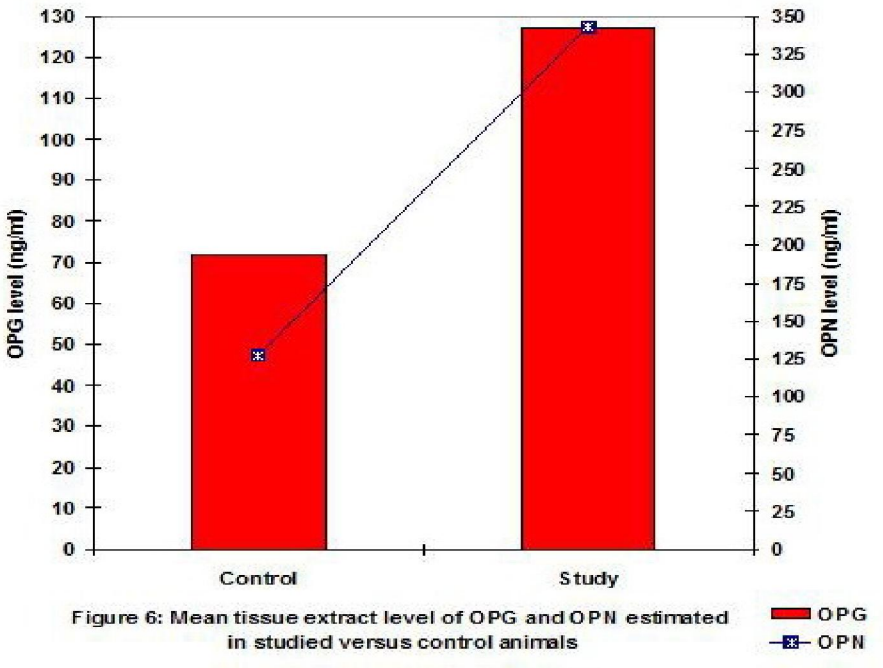

Figure 6: Mean tissue extract level of OPG and OPN estimated in studied versus control animals

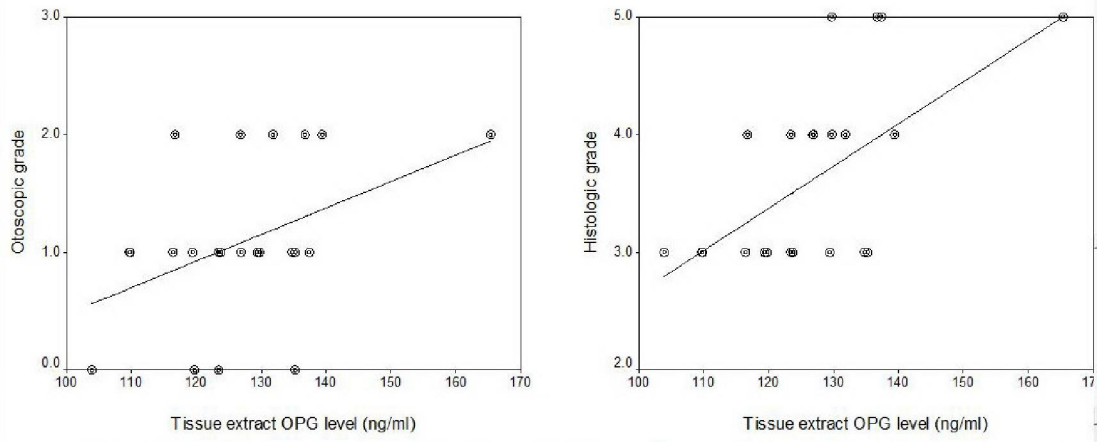

Figure 7: Correlation between otoscopic and histological grading and tissue extract levels of OPG

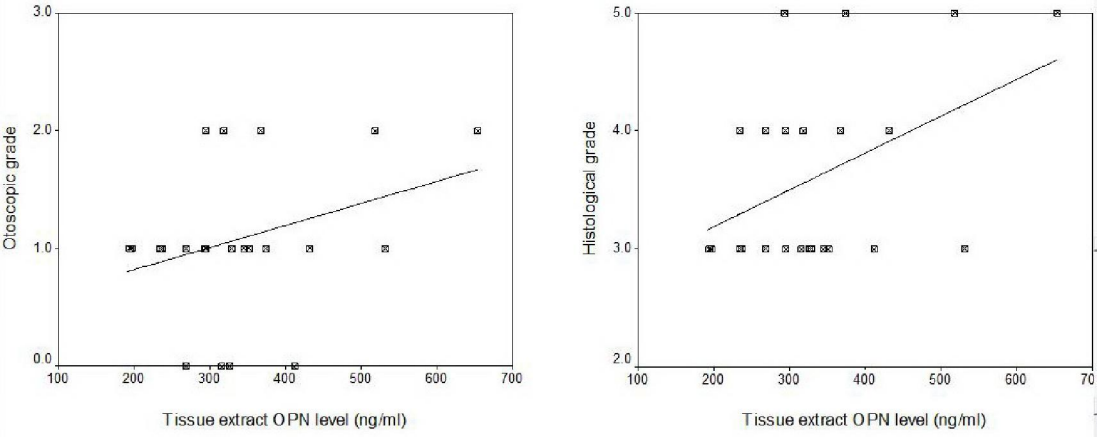

Figure 8: Correlation between otoscopic and histological grading and tissue extract levels of OPN

4. Discussion

Myringosclerosis was induced inoculation of the middle ear of studied animals, via transtympanic access, by 0.1ml of solution containing 10^7 CFU of Streptococcus pneumoniae type 3 and animals were sacrificed 8 weeks after inoculation. Such induction protocol goes in line with de Carvalho et al., [21] who induced myringosclerosis in rats and obtained plaques after 7 weeks of inoculation with Streptococcus pneumoniae and the choice of type of bacterial species was dependent on the previous data of Raustyte et al., [22] who found Streptococcus pneumoniae type 3 provokes a severe clinical course of acute otitis media that healed with scarring and myringosclerosis formation in the tympanic membrane and clinically visible myringosclerosis develops after middle ear infection caused by Streptococcus pneumoniae type 3, but not in cases caused by non-typeable Haemophilus influenzae.

Otoscopic examination missed 4 ears appeared to have normal TM but histological examination of excised drum specimens determined the presence of sclerotic changes of grade 3 in these four drums with sensitivity of 83.3%; a figure consistent with Santos et al., [23] who reported that from the comparison of the otomicroscopic data in relation to the histological findings, otomicroscopy showed sensitivity of 80% and specificity of 75% for diagnosis of myringosclerosis especially in mild cases

The reported histological findings coincided with that previously reported in studies concerned with tissue changes in myringosclerosis; Kazikdas et al., [3] reported that under light microscopy, extensive sclerotic lesions were found in the tympanic membranes; these sclerotic deposits were located in the lamina propria and myringosclerosis occurred predominantly adjacent to the handle of the malleus, but also near the annular region. Also, Stenfeldt et al., [24] found that during infection, the collagen layer was thickened and stained strongly for type II collagen and collagen types I and III were found in the edematous connective tissue around the main collagen layer and around dilated blood vessels and 3-months after perforation or infection, all 3 collagens were present in the lamina propria of the tympanic membrane with extensive amounts were present in the scar tissue.

Mean estimated tissue extract levels of OPG and OPN in studied animals were significantly higher compared to that estimated in control animals with a positive significant correlation with both otoscopic and histological findings. These findings agreed with Makiishi-Shimobayashi et al., who immunohistochemically evaluated tissue expression of OPN in experimental tympanosclerosis, [25] and at the calcification sites of cholesteatoma in humans, [26] and reported that in hyalinized tissues with macroscopic calcification and fibrous tissues with microscopic calcification, OPN was found in the calcification sites and in inflammatory tissues with microscopic calcification, OPN was also found in the calcifying foci, and many OPN mRNA-expressing cells, determined by in situ hybridization, located around their foci and concluded that these results suggest that OPN secreted by exudate macrophages might be an important regulator in the calcification of tympanosclerosis and the pathological calcification that occurs in association with cholesteatoma. Also, Grases et al. [27] found all rats subjected to calcinosis induction showed OPN 8 days post-induction, and was clearly associated with calcified areas.

Raustyte et al., [28] using a rat model of acute otitis media (AOM) determined the expression of OPN and OPG by immunohistochemistry and detected calcium depositions accumulated in the cytoplasm of macrophages and dispersed in the connective tissue layers of the pars flaccida and tensa, but late accumulation occurred in the lamina propria of pars tensa, OPN and OPG expression was found early in inflammatory cells including especially macrophages and late in pars tensa fibrocytes, but OPG expression was also located late to the inner basal membrane of pars flaccida.

These findings spotlight on the role played by inflammatory cells in pathogenesis of TM either through precipitation in tissues and subsequent release of fibrosis-inducing cytokines or through the release of bone-remodeling cytokines and supported that previously reported by Lui et al. [29] who found all the examined sclerotic plaques were surrounded with macrophages and bone morphogenetic protein 2 positive cells and were synchronously expressing CD68 positive and BMP2 positive.

It could be concluded that increased myringosclerotic tissue extract levels of both bone modeling markers indicated their possible role in initiation and progression of histologically documented sclerotic changes in tympanic membrane after chronic suppurative otitis media.

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