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Original Article

Histopathological Evaluation of the Effect of Bromelain on Myringosclerosis in Rats

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BACKGROUND: Myringosclerosis is frequently detected after otitis media with effusion treatment via ventilation tube insertion. The study was performed to investigate the effect of bromelain as an antioxidant and anti-inflammatory on myringotomized rats.

METHODS: The myringotomy operations of 15 Wistar Albino rats were performed under an otomicroscope. Three groups were formed by randomly separating these animals. The day after unilateral myringotomy, the control group was injected with 0.9% NaCl (saline) intraperitoneally (i.p.). The rats of the second group were injected 15 mg/kg/day of bromelain i.p. The third group received 30 mg/kg/day of bromelain i.p. On the 22nd day, the myringotomized ears of the animals were investigated via otomicroscope to determine myringosclerosis. After euthanasia of the animals, tympanic membrane (TM) thickness and inflammation of middle ear mucosa were investigated histopathologically.

RESULTS: The control group had higher myringosclerosis scores than the bromelain curative groups ($P = .048$). The median TM thickness of the control group (64.75 µm) was higher than the bromelain 15 mg/kg group (34.95 µm) ($P = .009$) and the bromelain 30 mg/kg group (27.84 µm) ($P = .008$). The inflammation scores were higher in the control group compared to the bromelain curative groups ($P = .039$). There were no statistically significant differences according to myringosclerosis scores ($P = .117$), TM thickness ($P = .079$), and inflammation scores ($P = .490$) between the bromelain low and high dose groups.

CONCLUSION: Bromelain treatment prevented the formation of myringosclerosis, reduced TM thickness, and supressed inflammation in myringotomized rats.

KEYWORDS: Bromelain, myringosclerosis, myringotomy, rat

INTRODUCTION

Myringosclerosis is an irreversibly developing eardrum lesion characterized by dystrophic calcification and hyalinization.[1] On otoscopic examination, it is usually seen as white chalky patches in the anterior and posterior lower regions of the eardrum. Histopathologically, the lamina propria has an increase in collagen fibers because of extracellular calcium deposition, hyaline degeneration, and progressive fibroblast infiltration.[2] Myringosclerosis impairs the quality of life as it can cause hearing loss in the patients.[3] The factors during the development of myringosclerosis include ventilation tube placement, middle ear infection, trauma, genetic predisposition, and increased generation of oxygen-derived free radicals.[4] However, the occurrence of myringosclerosis can be prevented by using antioxidant agents that can neutralize the effects of oxygen-derived free radicals.[5,6]

Bromelain is composed of thiol-endopeptidase, ribonucleases, phosphatases, peroxidases, glycosidases, cellulases, glycoproteins, and carbohydrates from the stem/fruit of *Ananas comosus* (pineapple).[7] During inflammation, bromelain increases the activities of superoxide dismutase (SOD) and glutathione (GSH).[8] Bromelain also reduces tissue malondialdehyde (MDA) and myeloperoxidase (MPO) levels.[7] This research was performed to evaluate the effect of bromelain on the development of myringosclerosis using otomicroscopic and histopathological examination in rats.

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MATERIAL AND METHODS

Animals

The G Power Analysis (3.1.9.4 version) for Windows was used for sample size determination, which was based on the 2-tailed hypothesis. The minimum number required for investigation was determined as n = 15 when the alpha error is 0.05, the power of the test is 0.8 (80%), and effect size is 0.96 (the fibrinolytic activity of bromelain).[9] The experimental study was approved by the Ondokuz Mayıs University Animal Experiments Local Ethics Committee with the approval number: OMU-HADYEK 2022/02 and date: 24.02.2022. Fifteen Wistar-Albino male rats (200-250 g) were obtained from Ondokuz Mayıs University Experimental Animal Research Center, Samsun, Türkiye. They were in an environment with sufficient water and food for 21 days at 22 ± 2°Cand 60 ± 5% humidity.

Experimental Design

Ketamine HCl (50 mg per kg, i.p.) (Ketalar®, Eczacıbası Parke-Davis) and Xylazine HCl (10 mg per kg, i.p.) (Alfazyne®, Alfasan International B.V.) were used for the anesthetic induction of rats. The animals were placed on a heating blanket to maintain their body temperature at 35°C during anesthesia, and the rectal temperature was also monitored. The otoscopic inspection of the external auditory canal and TM of the rats was conducted. Animals with perforation of the TM, external/middle ear infection, and TM opacification were excluded from the study. The rats participating in the study were separated into 3 groups, and in all groups, the myringotomy was done on the left eardrums with a myringotomy knife under the otomicroscope. The day after myringotomy, the control group (n = 5) was injected i.p. with an equal volume of saline matching the total substance given to the bromelain group in 1 day for 20 days. The second group (n = 5) was injected i.p. with 15 mg/kg/day bromelain (Sigma-Aldrich®) for 20 days. The third group (n = 5) received i.p. 30 mg/kg/day bromelain for 20 days. Saline was used to dissolve bromelain.

Otomicroscopic Examination

On the 22nd day, the left ears of all rats were evaluated under an otomicroscope and defined as normal, mild, moderate, or severe in terms of myringosclerosis and recorded (Table 1) (Figure 1,2,3,4). The scoring of the myringosclerosis was as follows: no sclerotic lesion (normal), a sclerotic lesion next to the malleus arm (mild), a sclerotic lesion next to the malleus arm and in the upper anterior of the pars tensa (moderate), and sclerotic lesions from the malleus along to the annulus (severe).[3,10,11] The rats were euthanized under anesthesia after the determination.

Histopathological Examination

The left temporal bones of the animals were taken. After 3 days of fixation in 4% formol, the tissues were decalcified via a solution of formic acid-sodium for 14 days. Sections from the TM and middle ear were achieved. Then the tissue follow-up stages were performed. The sections were embedded in paraffin blocks, which were then cut into 4-5 µm thick sections. The slices were stained with hematoxylin-eosin (H&E) and evaluated under a light microscope, and also photographed via a photomicroscope. The thickness of the TM was determined in 10 regions, and then the average data of the regions were recorded for each animal. The inflammation score in the middle ear was defined as normal, mild, moderate, and severe (Table 2).[11]

Statistical Analysis

The outputs of the study were analyzed with SPSS version 22.0 (IBM SPSS Corp.; Armonk, NY, USA) package program. The Kolmogorov–Smirnov test was used to decide if the data were normally distributed. The data were expressed as mean ± standard deviation (min-max), median, frequency (n), and percentage (%). The Chi-square test was used for comparing the categorical variables. Kruskal–Wallis analysis of variance was applied to compare 3 groups when the normality of the data was not verified, and Mann–Whitney *U* test with Bonferroni correction was used for post-hoc comparisons when necessary. *P*-values of $P < .05$ were considered statistically significant (the statistical significance value for the Mann–Whitney *U* test with Bonferroni correction was accepted as $P < .017$).

MAIN POINTS

- It has been found that 15 mg/kg and 30 mg/kg intraperitoneally (i.p.) doses of bromelain were effective in diminishing the myringosclerosis score compared to the control group via otomicroscopic examinations in myringotomized rats.
- It has been determined that bromelain 15 mg/kg and 30 mg/kg i.p. doses diminished tympanic membrane (TM) thickness and inflammation scores of myringotomized rats compared to the control group when histopathological examinations were performed.
- There were no statistically significant differences found according to myringosclerosis score, TM thickness, and inflammation score between the bromelain 15 mg/kg and 30 mg/kg groups.

RESULTS

According to the otomicroscopic determination in all rats, the myringotomy perforation located on the left eardrum was closed by the 22nd day (Figures 1,2,3,4). The myringosclerosis scores of the control group were significantly higher than those of the bromelain 15 mg/kg and the bromelain 30 mg/kg curative groups ($P = .048$). There were no statistical difference according to myringosclerosis scores between the bromelain 15 mg/kg and the bromelain 30 mg/kg groups ($P = .117$) (Table 1).

Table 1. Scores of Myringosclerosis by Otomicroscopic Examination

	Control (n)	Bromelain 15 mg/kg (n)	Bromelain 30 mg/kg (n)	Total (n)
Normal	0	2	5	7
Mild	3	2	0	5
Moderate	1	1	0	2
Severe	1	0	0	1
Total	5	5	5	15

Table 2. Histopathologically Assessment of Inflammation

Normal	No Inflammation
Mild	Inflammatory cells in the perivascular area
Moderate	Inflammatory cells in the perivascular and subepithelial area
Severe	Diffuse inflammatory cells

Table 3. Tympanic membrane Thickness (µm) of the Groups

	n	Mean ± Std. Deviation	Median	Min.	Max.	*P*
Kontrol[1]	5	62.72 ± 6.07	64.75	52.51	67.82	.004**
Bromelain 15 mg/kg[2]	5	36.60 ± 7.51	34.95	27.83	45.89	1-2 = .009* 1-3 = .008*
Bromelain 30 mg/kg[3]	5	25.33 ± 7.40	27.84	17.25	34.17	2-3 = .079*

*Mann–Whitney *U* test with Bonferroni correction, **Kruskal–Wallis test.

The median TM thickness was statistically higher in the control group (64.75 µm) than in the bromelain 15 mg/kg group (34.95 µm) and the bromelain 30 mg/kg group (27.84 µm) (*P* = .009 and *P* = .008 respectively) (Figure 5). There was no statistical difference according to the median TM thickness between the bromelain 15 mg/kg and 30 mg/kg groups (p = .079) (Table 3).

The control group had statistically higher inflammation scores than the bromelain 15 mg/kg and the bromelain 30 mg/kg groups (*P* = .039) (Figure 5). There were no statistical differences in inflammation scores between the bromelain 15 mg/kg and the bromelain 30 mg/kg groups (*P* = .490). Mild and moderate inflammation scores were higher in the control group, while normal and mild inflammation scores were higher in the bromelain 15 mg/kg and 30 mg/kg groups (*P* = .042, *P* = .026 respectively) (Tables 2and 4).

DISCUSSION

Myringosclerosis is a common complication after myringotomy.[12,13] The study, which was created for evaluating a myringosclerosis model in rats, demonstrated extensive sclerotic deposits in the lamina propria of the TM in the myringotomy group.[14] The formation of free oxygen radicals has a key role in the occurrence of myringosclerosis after myringotomy.[14,15] The free oxygen radicals cause irreversible damage in the tissue because of fibrosis and hyaline degeneration in the tissue repair sites of the wound.[16,17] Several studies demonstrated that antioxidants are effective in reducing or preventing myringosclerosis.[3,5,18,19] In a study evaluating the effect of systemic L-carnitine on myringotomized Sprague–Dawley rats, it was shown that L-carnitine, as an effective antioxidant, prevented the development of myringosclerosis in rats.[20] Song et al[2] found that the caffeic acid phenethyl ester i.p. was effective in the prophylactic treatment of myringosclerosis in Sprague–Dawley rats. In another study, decreased myringosclerosis was documented in myringotomized Sprague–Dawley rats treated with systemic selenium.[21] The experimental study of Dogan et al[15] also showed that systemic treatment of N-nitro L-arginine methyl ester diminished fibroblastic proliferation and the occurrence of myringosclerosis in myringotomized Wistar-Albino rats. It has also been documented that systemic ascorbic acid was effective in reducing the TM thickness of the myringotomized Wistar-Albino rats.[19]

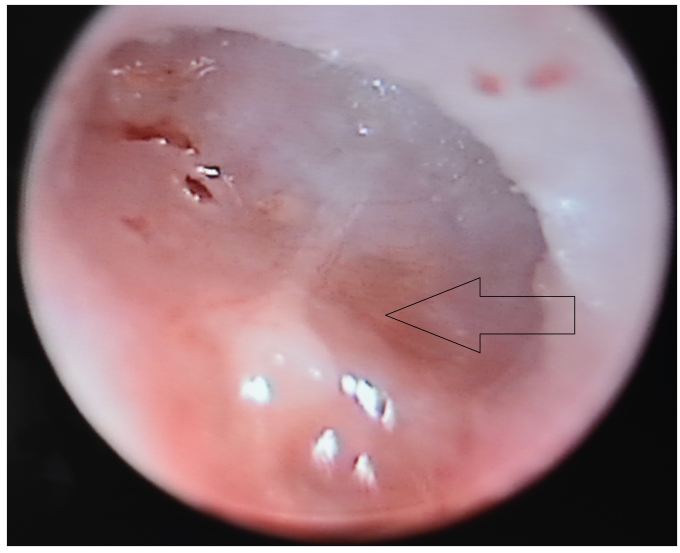

Figure 2. Mild myringosclerosis.

The enhancement of free oxygen radicals during inflammation due to otitis media is increased with ventilation tube insertion.[5,15] Thus, the ventilation tube insertion treatment also increases the possibility of myringosclerosis formation.[17] Kokten et al[5] reported that antioxidant

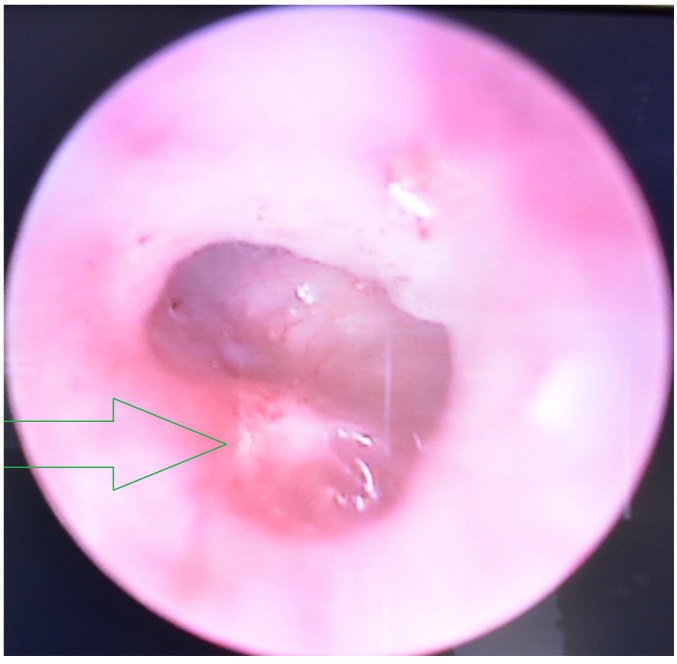

Figure 1. Normal TM via otomicroscopic examination, malleus (green arrow).

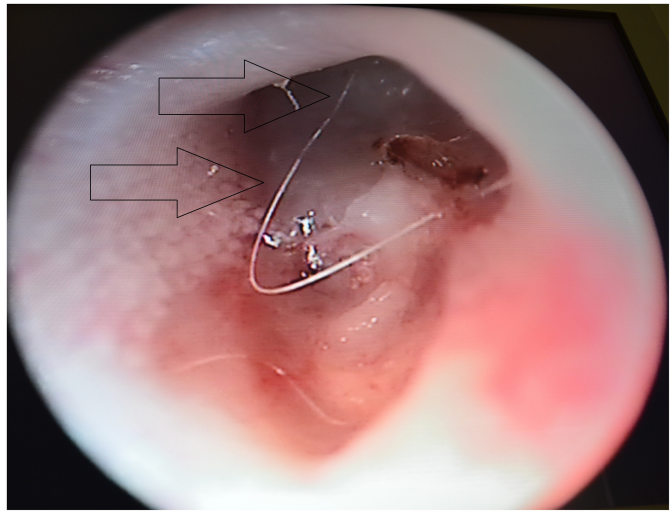

Figure 3. Moderate myringosclerosis.

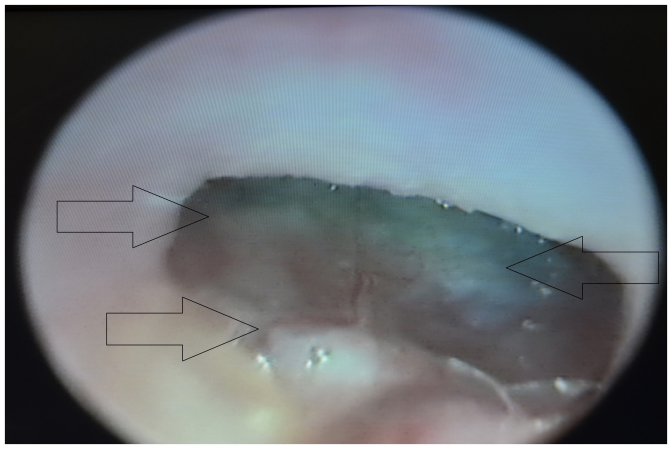

Figure 4. Severe myringosclerosis.

black cumin seed oil was effective in preventing experimentally induced myringosclerosis in Guinea pigs, this effect was a result of suppression of the inflammation and fibroblastic activity in the lamina propria. In another study, in relation to low inflammation scores and low cellular infiltration, the expression of vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) were reduced in myringotomized Guinea pigs treated with N-acetyl cysteine. Thus, decreased myringosclerosis was documented in these animals.[18] Gunes et al[19] also demonstrated that the edematous lamina propria resolution and prevention of myringosclerosis were achieved with the use of antioxidant ascorbic acid in myringotomized Wistar-Albino rats.

Bromelain has been suggested to have anti-inflammatory effects which may be useful in reducing inflammation associated with injuries or surgical procedures. Several different experimental and clinical studies have demonstrated the anti-inflammatory and antioxidant effects of bromelain.[7,8,22,23] Consistently in this current study, but probably for the first time in the literature, bromelain was effective in preventing inflammation and myringosclerosis after myringotomy. Although, in a recent study, it has predicted that bromelain has limited antioxidative and also anti-inflammatory activities,[24] bromelain provides anti-inflammatory and antioxidant properties by reducing tissue MDA and MPO levels and elevating GSH, SOD.[7,25] In this manner, bromelain has a dose-dependent effect against oxidative stress, inflammation, and tissue damage.[25] Bromelain reduces lipid peroxidation of the membrane by activating SOD and GSH nuclear transcription factors, NrF-1 and NrF-2. These factors bind to the DNA area which stimulates the expression of genes of the antioxidant enzymes.[8] Bromelain also inhibits cyclooxygenase, thereby modulating prostaglandins and inflammation, thromboxane and coagulation. Furthermore, bromelain hydrolyzes bradykinin.[26] Although, there were no statistically significant differences found according to myringosclerosis score, TM thickness, and inflammation score between the bromelain low and high dose groups, the myringosclerosis score, the TM thickness, and the inflammation score of the bromelain 30 mg/kg curative group were lower than those of the

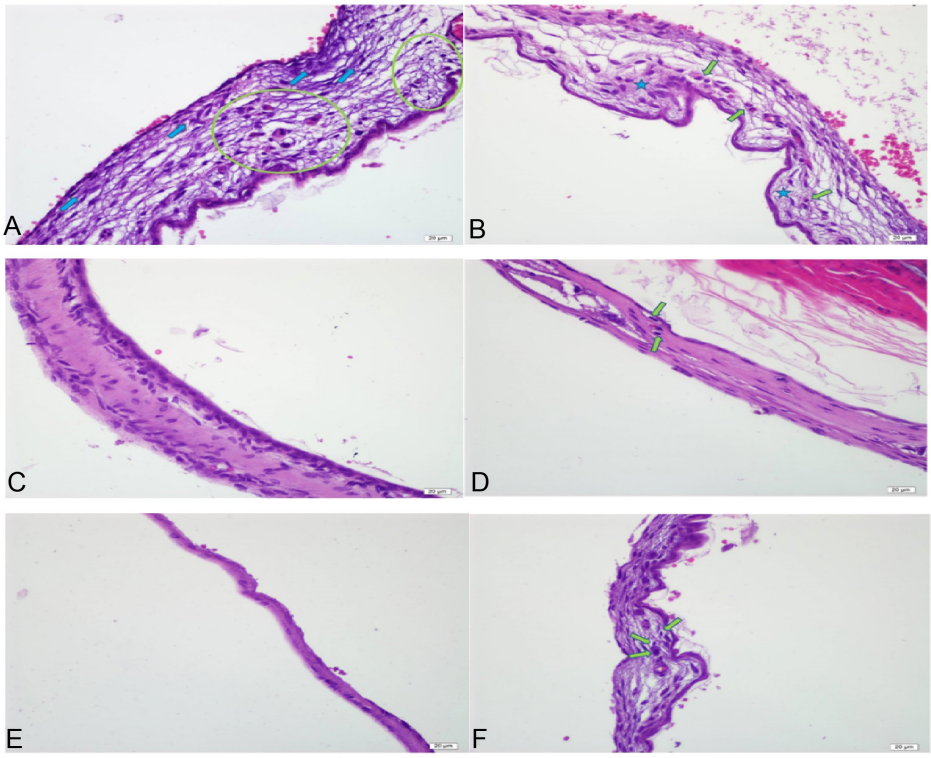

Figure 5. A. Control group. Tympanic membrane with moderate inflammation; inflammation in the perivascular and subepithelial area (green circle) and fibroblastic proliferation (blue arrow). **B.** Control group. TM with mild inflammation; inflammation in the perivascular area (green arrow) and fibroblastic proliferation (blue star). **C.** Bromelain 15 mg/kg group. Tympanic membrane with fibroblasts and absence of inflammation. **D.** Bromelain 15 mg/kg group. Tympanic membrane with fibroblasts and mild inflammatory cell infiltration (green arrow). **E.** Bromelain 30 mg/kg group. Minimally thickened TM with the absence of inflammation. **F.** Bromelain 30 mg/kg group. Tympanic membrane with mild inflammatory cell infiltration (green arrow) (H&E; ×400).

Table 4. Inflammation Scores of the Groups

	Control (n)	Bromelain 15 mg/kg (n)	Bromelain 30 mg/kg (n)	Total (n)
Normal	0	3	4	7
Mild	2	2	1	5
Moderate	3	0	0	3
Severe	0	0	0	0
Total	5	5	5	15

bromelain 15 mg/kg curative group. Bromelain has immunomodulatory and fibrinolytic activity[23] but because of the different bromelain doses, the effect of bromelain on inflammation and also inflammation markers including IFNγ, IL-2, IL-5, IL-6, IL-8, IL-10, IL-13, PGE-2, CRP, and fibrinogen varies.[27]

Limitations

The tympanometric measurements, which would have been useful for supporting the results, were not carried out before and after the procedure. However, the otomicroscopic and histopathological examinations were sufficient for achieving the contributions to the literature.

CONCLUSIONS

The myringosclerosis score, the TM thickness, and the inflammation score were reduced in both bromelain curative (15 mg/kg and 30 mg/kg) groups in myringotomized rats.

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: This study was approved by the Ethics Committee of Ondokuz Mayıs University (approval no.: OMU–HADYEK 2022/02, date: February 24, 2022).

Peer-review: Externally peer reviewed.

Author Contributions: Concept – M.D.A., D.O.; Design – M.D.A., D.O.; Supervision – M.D.A.; Materials – M.D.A., D.O., A.E., S.S.K.; Data Collection and/or Processing – M.D.A., D.O., A.E., S.S.K.; Analysis and/or Interpretation – M.D.A., D.O., A.E., S.S.K.; Literature Search –M.D.A.; Writing – M.D.A.; Critical Review – M.D.A.

Declaration of Interests: The authors have no conflict of interest to declare.

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