ORIGINAL ARTICLE

Protective Effect of Pyrrolidine Dithiocarbamate on Myringosclerosis

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Objective: Myringotomy is most often used to treat recurrent acute otitis media and chronic effusion otitis media. The most common sequela of myringotomy is myringosclerosis. It has recently been shown that the development of myringosclerosis after myringotomy occurs concomitantly with an increased concentration of reactive oxygen species in the middle-ear cavity and an inflammatory reaction in the tympanic membrane. To assess the effect of pyrrolidine dithiocarbamate on acute inflammation due to myringotomy.

Materials and Methods: This is prospective randomised study. Thirty Sprague–Dawley rats were divided into three groups. Group one constituted controls. Group two underwent myringotomy. Group three underwent myringotomy and also received 100 mg/kg pyrrolidine dithiocarbamate intraperiteonaly two days after surgery. Following sacrifice 48 hours after myringotomy, the animals' right ears were used to determine the concentration of reactive oxygen species, using the chemiluminescence method; left ears were used for histopathological study.

Results: Reactive oxygen species levels were significantly decreased in group three compared with group two ($p < 0.001$). The density of inflammatory cells in group three was significantly less than that in group two ($p < 0.05$). Lamina propria thickness and vessel density were also significantly decreased in group three compared with group two ($p < 0.05$).

Conclusion: Our results indicate that intraperitoneal pyrrolidine dithiocarbamate decreases reactive oxygen species concentration and acute inflammation in the tympanic membrane after myringotomy. Systemic pyrrolidine dithiocarbamate administration might have a significant protective effect after miringotomy.

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Introduction

Myringotomy is most often used to treat recurrent acute otitis media and chronic otitis media with effusion.[1] The most common sequela of myringotomy is myringosclerosis.[2] It has recently been shown that the development of myringosclerosis after myringotomy occurs concomitantly with an increased concentration of reactive oxygen species in the middle-ear cavity and an inflammatory reaction in the tympanic membrane.[3,4] Free radicals effect the lipids, proteins, carbonhydrates and DNA of the cell and cause tissue damage.

The protective mechanisms against reactive oxygen species and inflammation comprise enzymatic and nonenzymatic free radical scavengers.[5] Previous reports have shown that, following experimental myringotomy, the development of acute inflammation and myringosclerosis can be reduced by the topical application of various free radical scavengers such as vitamin E, ascorbic acid, L-carnitine and N-acetylcysteine. [6-9]

Pyrrolidine dithiocarbamate (PDTC) is a metal chelator composed of low molecular weight thiol particles. PDTC has many biological activities including balancing redox status, chelation of heavy

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metals and enzyme inhibition.[10-12] PDTC is well known as a nuclear factor-κB (NF-κB) inhibitor.[13] PDTC has also been used for treatment of hepatic and cerebral ischemia, spinal cord injuries, and neonatal asphyxia.[14-18]

The first aim of the current study was to assess the level of reactive oxygen species in the tympanic membrane of rats following myringotomy, using the chemiluminescence method. The second aim was to assess the protective effect of PDTCon rat tympanic membrane following myringotomy, including the assessment of any tympanic membrane histopathological changes.

Materials and Methods

Experimental design

The study was approved by the animal ethics committee of the Istanbul University Medical Faculty (no:24/2.25.2010). Thirty healthy Sprague–Dawley rats (weight, 250–300 g) were used. All animals had been kept in a 14-hour light/10-hour dark cycle with free access to food and water.

The animals were anaesthetised with 50 mg/kg ketamine hydrochloride intraperitoneally. They were then examined and assessed otoscopically for evidence of ear disease. Any animal showing signs of ear disease was excluded from the study.

The animals were randomly assigned to three groups of 10 animals each. In groups two and three, myringotomy was performed in the upper posterior quadrant of the tympanic membrane in both ears, with a sterile myringotomy lancet and aural speculum, under otomicroscopy (S1, 300 mm lens; Carl Zeiss, Oberkochen, Germany) and using a sterile technique.

The group one rats constituted the control group, and did not receive myringotomy or any other treatment. The group two rats received no pre- or post-myringotomy treatment. However, the group three rats received 100 mg/day PDTC (Sigma-Aldrich Chemical Corp, MO, USA) via intraperitoneally for one day pre-myringotomy and two days postmyringotomy.

Forty-eight hours after myringotomy, all animals were sacrificed via injection with a lethal dose of ketamine hydrochloride intraperitoneally. The tempintraperitoneal bones were harvested and the tympanic bullae cracked with scisserbest oksijen radikalis. Under a dissecting microscope, the middle-ear mucosa and tympanic membrane were peeled off the underlying bone. The right tympanic membrane of each animal was used for luminolenhanced chemiluminescence measurements, while the left tympanic membrane was used for histopathological study.

Chemiluminescence

The tympanic membranes were washed with ice-cold saline solution and analysed immediately. After 10 minutes, the specimens were assessed for reactive oxygen species, using luminol chemiluminescence as described previously.[6] Chemiluminescence was measured at room temperature using a Mini Lumat LB 9506 luminometer (EG & G Berthold, Germany) in the presence of 0.2 mmol/l luminol containing phosphate-buffered saline 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid buffer (0.5 mol/l phosphate-buffered saline containing 20 mmol/l 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid). Counts were obtained at 5-second intervals over a period of 5 minutes. Results were recorded using relative light area per mg of tissue (rlu/mg) as the unit of measurement, expressed as the area under the curve for the 5-minute counting period.

Tissue specimens were drained and weighed at the end of each assessment. The average specimen weight was approximately 1–2 mg.

Histopathology

For histopathological study, tempintraperitoneal bones were fixed in 10 per cent formalin for four days. Specimens were then decalcified in 10 per cent formaldehyde for five days. Specimens were subsequently washed for 3 hours to remove acidity.

A tracking process was then performed for 13 hours using an automatic tissue tracking machine. After this processing, the specimens were embedded in paraffin,

sectioned to a thickness of 3 µm with a microtome, and stained with haematoxylin and eosin. The sections were evaluated by a blinded pathologist using a light microscope (Olympus Bx-50, Olympus Optical, Hamburg, Germany).

On light microscopic examination, the inflammatory cell density, lamina propria thickness and tympanic membrane vessel density were evaluated semiquantitatively using the following grading system: 0= absent, 1= slightly increased, 2=moderately increased and 3= severely increased.

Statistical analysis

The NCSS 2007 and PASS 2008 statistical software programs (Kaysville, Utah, USA) were used for statistical analysis. Relevance of data to the standard distribution was determined by the Kolmogorov–Smirnov test. The significance of differences between experimental groups was analysed using the one-way analysis of variance test and the Tukey HSD (Honestly Significant Differences) test. The chi-square test was used to analyse qualitative data. Differences were considered significant when the probability was $p < 0.05$.

Results

Chemiluminescence

Free radical levels of the tympanic membrane increased following myringotomy and this increase was documented with luminol chemiluminescence method. Tissue luminol chemiluminescence levels of Group 1 was 31.37 ±3.64 rlu/mg whereas levels of Group 2 was 68.39±11.84 rlu/mg and the difference was very significant ($p <0.001$)(Table 1).

Free radical levels of the group PDTC administred in addition to myringotomy were 30.75±4.53 rlu/mg adn these levels were significantly differently to myringotomized group ($p <0.001$) (Table 1). Levels of group 3 were lower compared to levels of group 2 ($p<0.05$). There was a significant increase in ROS levels of Group 2 compared to group 1 ($p<0.05$). Luminol chemiluminscence levels of Group 3 were similar to group 1 and no statistically significant difference was documented (Figure 1).

Table 1. Luminol-amplified Chemiluminescence values. Data represent relative light units per mg tissue. No =number; SD= standard deviation

LUMINOL	AMPLIFIED CHEMILUMINESCENCE VALUES		
Rat	**Group 1**	**Group 2**	**Group 3**
1		81.7	
2	35.8	68.1	
3	30.1	80.3	
4		75.4	
5	34.7	58.0	
6	30.0	68.9	
7	31.1	72.0	
8	27.7	75.6	
9	32.1	61.4	
10	24.1	42.5	
Mean±SD	31.37 ±3.6468.9±11.84		30.5±4.53

Data represent relative light units per mg tissue.
number; SD standard deviation

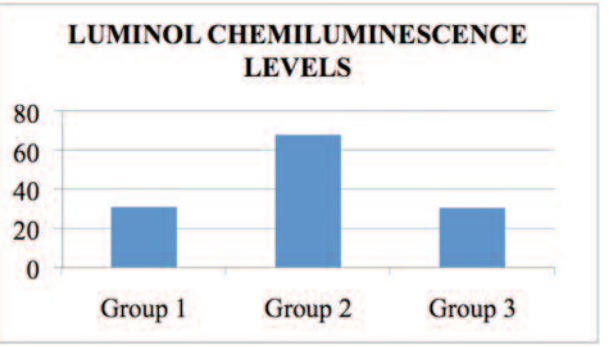

Figure 1. Comparison of luminol chemiluminescence (representing reactive oxygen species concentration) for the three groups (mean ± std)

Histopathology

In the tympanic membrane specimens from group one animals, the observed structure of the tympanic membrane was normal, with an inner mucosal layer and a thin lamina propria, without inflammatory cells or angioneogenesis (Figure 2).

Group 2 myringotomized rats (PDTC was not administred) had moderately increase in inflammatory cells (especially neutrophils), vascular thickness of the tympanic membrane increased and thickness of lamina propria has increased (Figures 3 and 4).

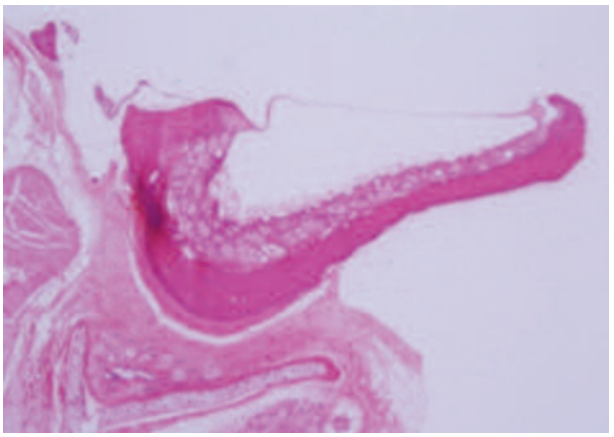

Figure 2. Increase in inflammatory cells, increased vessel thickness in tympanic membrane and increased thickness of lamina propria. No perforation (H&E, 4X) (Group 1)

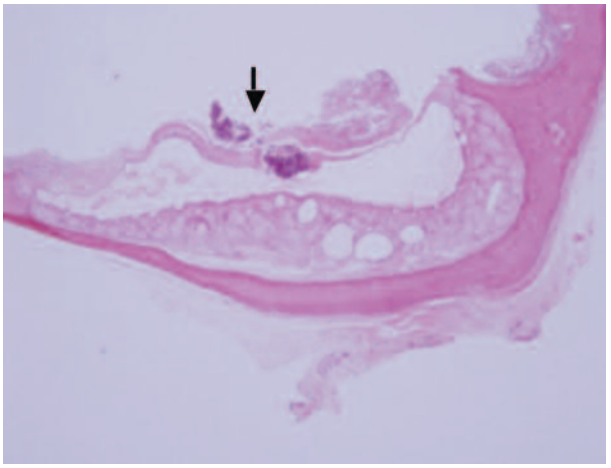

Figure 3. Moderate increase in inflammatory cells, vascular thickness of the tympanic membrane increased and thickness of lamina propria has increased (H&E, 4X) (Group 2)

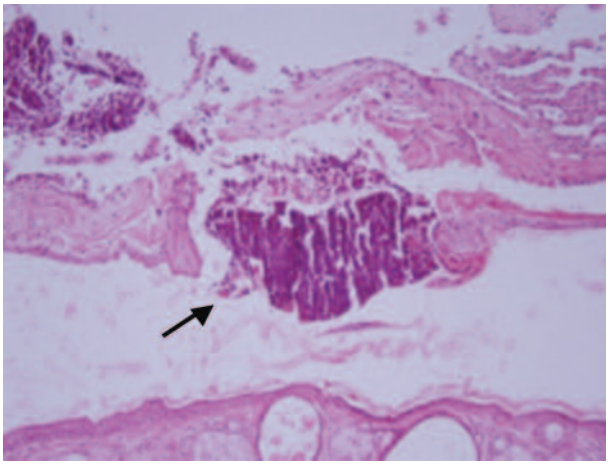

Figure 4. Severe increase in inflammatory cell infiltration, neutrophils (H&E, 20X) (Group 2)

Group 3 (PDTC administred group) increase in number of inflammatory cells, increased thickness of tympanic membrane vascularization and lamina propria was either minor (Figures 6 and 7) or absent (Figure 5).

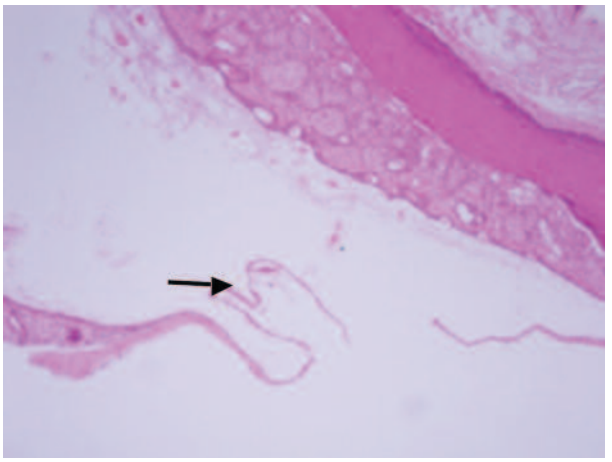

Figure 5. The number of inflammatory cells, vascularization of the tympanic membrane and thickness of the lamina propria are normal. Tympanic membrane is perforated. (H&E, 20X) (Group 3)

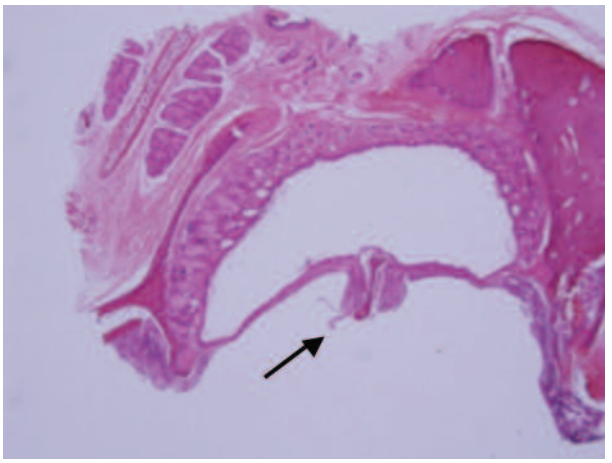

Figure 6. The number of inflammatory cells, vascular thickness of tympanic membrane and lamina propria has slightly increased (H&E, 4X) (Group 3)

Microscopic evaluation of Group 2 revealed increased histopathological changes which were significantly increased compared to group 1 ($p < 0.05$). Histopathological changes of Group 3 were significantly lower compared to group 2 ($p<0.05$).

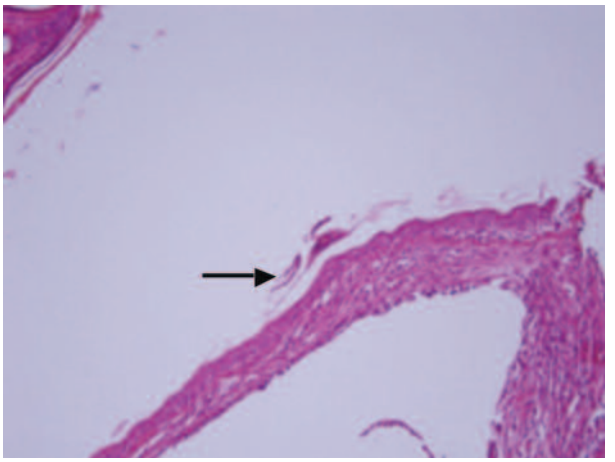

Figure 7. Number of inflammatory cells, vascular thickness of tympanic membrane and lamina propria has slightly increased (H&E, 20X) (Group 3)

Increase in number of inflammatory cells of group 3 was statistically higher compared to group 1 ($p<0.05$) but no difference was documented in terms of thickness of tympanic membrane and lamina propria. Inflammatory cell density was either normal or slightly increased in group 3, whereas group 2 had significantly increased cell density ($p<0.001$). Lamina propria thickness was either normal or slightly increased in group 3, whereas group had moderate to severe increase which was statistically significant ($p<0.001$). Histopathological changes observed in the groups are further detailed in Table 2.

Inflammatory cell infiltration, thickness of the lamina propria, and vascular proliferation of the tympanic membrane were evaluated with luminol chemiluminescence and nonparametric correlation analysis was performed. There was a negative correlation between the vascular proliferation of tympanic membrane and luminol chemiluminescence levels.

Discussion

Our study findings indicated that intraperitoneal administration of PDTC before and after myringotomy resulted in a reduction in reactive oxygen species levels and acute inflammation in the tympanic membranes of myringotomised rats, compared with the tympanic membranes of myringotomised rats not thus treated. To the best of our knowledge, the current study represents the first published report evaluating the effectiveness of PDTC in reducing acute tympanic membrane inflammation following myringotomy.

Table 2. Graded histopathological changes. Grade 1 =normal; 2 = slightly increased; 3 = moderately increased; 4 =severely increased. Grp = group; infl = inflammatory; LP = lamina propria; TM = tympanic membrane

Grade	Grp 2 (n %)	Grp 3 (n %)
Infl cell density		
1	0(00)	4(40)
2	0(00)	3(30)
3	2(20)	1(10)
4	8(80)	2(20)
LP thickness		
1	0(00)	6(60)
2	1(10)	2(20)
3	7(70)	1(10)
4	2(20)	1(10)
TM vessel density		
1	0(00)	7(70)
2	0(00)	2(20)
3	8(80)	1(10)
4	2(20)	0(00)

Grade 1 = normal; 2= slightly increased; 3= moderately increased; 4= severely increased.
Grp= group; infl= inflammatory; LP= lamina propria; TM= tympanic membrane

The oxygen concentration in the middle-ear cavity is approximately 5.5–12.1 per cent, much lower than that of ambient air.[19] Myringotomy permits passage of ambient air into the middle-ear cavity, resulting in relative hyperoxia.[20] This hyperoxia increases the formation of reactive oxygen species in mitochondria and endoplasmic reticulum. A previous study showed that myringotomy is associated with increased levels of reactive oxygen species.[5] Increased reactive oxygen species and impaired antioxidant defence mechanisms have been postulated to be causative factors in inflammatory disease.[21] Increased production of reactive oxygen species may also be the first stage in the accumulation and aggregation of calcium and phosphorus, forming sclerotic deposits and eventually causing myringosclerosis.[4]

Free radicals have a very limited life span, therefore it is a challenge to measure their levels with accuracy.

Matson et al. showed myringosclerosis occurs at 9th hour and inflammatory process peaks at twenty-fourth hour following myringotomy.[1] Therefore free radical levels were measured accordingly, 24 hours after myringotomy. Luminol chemluminescence is an accurate method for measuring detecting levels of H2O2, HOCL-I and OH which are byproducts of oxidative metabolism.

Numerous studies have been published about utilization of antioxidant enzymes and elements for attenuating oxidative damage in myringotomized tympanic membranes. Vitamin E, ascorbic acid, L-carnitine, and N-acetylsistein have attenuating roles in the process of myringosclerosis.[6-9]

Pyrrolidine dithiocarbamate is a potent antioxidant and NFkB inhibitor. Its antioxidant effects work by means of changing redox status, metal chelation, enzyme inhibition, its antitoxic effect on free radicals and blocking effects of proinflammatory cytokines.[10-13] Furthermore, PDTC exacerbates gene expression of antioxidant enzymes such as Superoxide dismutase and glutathione peroxidase.[22,23] Our current study proves PDTC reduces levels of free radicals via chemliuminescence method. Our results in group 3 were significantly lower compared to group 2. Furthermore results of group 3 were almost equal to results of group 1. This proves the antioxidant effect of PDTC.

Kahya et al.[24] has shown the antioxidant effect of pomegranate extract on myringosclerosis and found chemiluminescence levels of the pomegranate extract administred group had 50 % higher results compared to the control group whereas chemiluminescece levels of the PDTC administred group in our study was equal to the control group. This shows PDTC has even higher antioxidant properties compared to pomegranate extract which has been shown to have higher antioxidant effect than vitamin C, E, coenzyme Q-10, α-lipoic acid, blueberry, cranberry, black mulberry, orange and grape.[25]

Üneri et al. measured 50 % higher chemiluminescence levels compared to control group.[26] Our study revealed similar results between PDTC administred and control group. PDTC, evaluated by chemiluminescence method, is the most effective way of attentuating free radicals in myringosclerosis. Free radical formation is the key factor in physiopathology of myringosclerosis, therefore PDTC might be the most effective way to prevent myringosclerosis.

Following tympanic membrane perforation, the wound healing process starts immediately, with the proliferation and migration of inflammatory cells.[27] Inflammatory cells are thought to be involved in the tissue formation and remodelling phases, in addition to their known role in cleaning the area around the wound. Schiff et al. believe that myringosclerosis may be triggered by exposure of damaged collagen to an intense inflammatory cell infiltrate.[28] During myringosclerosis, angioneogenesis occurs along the handle of the malleus and the annulus region to enable increased blood flow, which increases myringosclerotic plaque formation.[29] Ilknur et al. shows that bioflavonoids decreased angiogenesis and inflammation fort his reason prevent of experimental myringosclerosis.[30] In the current study, we observed decreased inflammatory cell density, lamina propria thickness and tympanic membrane vascular density in myringotomised rats treated with PDTC, compared with myringotomised rats not thus treated. These findings suggest that PDTC may reduce acute tympanic membrane inflammation, and may also decrease the formation of myringosclerosis, following myringotomy.

Histopathological evaluation of myringosclerosis revealed increase in collagen fibers, hyalen degeneration in lamina propria and extracellular calcium accumulation.[5] PDTC has been shown to decrease collagen fiber accumulation in tissues.[31] Our findings confirm these results; thickness of lamina propria and vascular proliferation of the tympanic membrane was significantly lower in PDTC administred group. PDTC is a potent inhibitor of NF-κB.13 NFkB has a key role in regulating inflammatory process of the vascular tissue, by means regulating interaction between the endothelium and circulating leukocytes.[32,33] Our current findings are parallel with

these results; inflammatory cell density of PDTC administred group was significantly lower. Proinflammatory cytokines act as the key factor on regulation of the inflammatory response in middle ear and production of free radicals.[34] PDTC has been shown to inhibit proinflammatory genes in previously published studies.[16] Lower number of inflammatory cells in PDTC administred group can be interpreted as the result of PDTC's inhibiting effect on proinflammatory cytokines.

Conclusion

In conclusion, current study states the predominating effect of PDTC in preventing myringosclerosis compared to other experimentally used agents. Furthermore PDTC minimalizes the results of acute inflammation and it can be used for preventing myringosclerosis by means of attenuating numerous pathophysiological mechanisms.

Our findings indicate that PDTC may be useful in this clinical setting. These materials may help reduce the complications of myringotomy. However, further studies on indications and dosages are needed before clinical application becomes possible.

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