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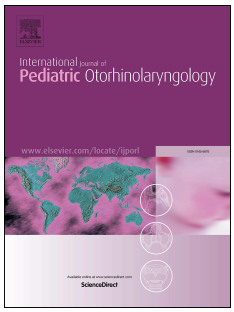


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# The Effect Of *Nigella sativa* Oil On Prevention Of Myringosclerosis In A Guinea Pig Model

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**ABSTRACT:**

**OBJECTIVES:** In this study, our aim was to identify the possible effects of *Nigella sativa* L. (NS) [blackcumin] seed oil on the prevention of experimentally induced myringosclerosis (MS).

**MATERIALS AND METHODS:** Fifteen Guinea pigs were used and they were divided into three groups. Tympanic membranes (TM) of all animals were perforated and then group I was treated with saline soaked gel foams as a control group, group II was treated with 0.5 ml NS oil soaked gel foams at 0, 24 and 48 hours and group III was treated with 5 ml NS oil orally at 0, 24, 48, 72 and 120 hours. After 15 days, all animals were euthanized. Tympanic membranes were evaluated macroscopically and histopathologically.

**RESULTS:** Groups I showed extensive myringosclerosis in contrast to those of Groups II and III which had significantly less changes ($p < 0.05$). The fibrosis and inflammation in the lamina propria of the tympanic membranes of Groups I was found to be significantly more pronounced ($p < 0.05$). The tympanic membranes were found to be significantly thinner in Groups II and III when compared with Groups I ($p < 0.05$).

**CONCLUSIONS:** The results of this study suggested that topical or oral administration of NS oil supressed the inflammation and fibroblastic activity in the lamina propria of the myringotomized TMs of the Guinea pigs. For providing further evidence to use plant extracts as antioxidant and antiinflammatory therapy after myringotomy or ventilation tube insertion, further clinical studies with larger population will be essential.

**Keywords:** myringosclerosis, Nigella sativa oil, antiinflammatory, Guinea pig

## 1. INTRODUCTION:

Myringosclerosis (MS) is an irreversible degenerative pathology affecting tympanic membrane (TM) and synchronous dystrophic calcification occurs in the fibrous layer [1]. Generally, MS is caused by myringotomy, ventilation tube insertion, trauma, chemical agents, chronic middle ear effusion and infection, and autoimmunity [2]. It is histopathologically characterized by hyaline degeneration, calcification, and rise in collagen fiber in the lamina propria (LP) of the TM [3]. In addition, as calcium and phosphorus are accumulated on this structure, crystallization and sclerosis will occur [2].

In paediatric population, otitis media with effusion is a common disease and often necessitates a surgical intervention like myringotomy or ventilation tube insertion which leads to a common sequel, myringosclerosis (MS). The exact pathogenesis of MS is not known very well. In previous studies, the course of MS has been investigated by making a TM injury with trauma or paracentesis [4]. These studies have shown that traumatic perforations or formation of oxygen derived free radicals because of hyperoxidative conditions may be the primary reason in the formation of MS [4,5]. Therefore, MS may be reduced or prevented by inhibiting the harmful effects of oxygen derived free radicals and administration of anti-inflammatory or antioxidant agents. Starting from this point of view, for minimizing the formation of MS after experimental paracentesis, several forms of free radical scavengers have been used in previous reports in the literature [3,4,6-10].

*Nigella sativa* L. (NS) is a plant from the family Ranunculaceae [11]. It is also known as black seed or black cumin in English [12]. The seeds using for medicinal purpose are the most important parts of this plant. They contain fixed oil, volatile oil, alkaloids, proteins, and saponins [11]. Primarily Thymoquinone (TQ) and other active constituents of NS have recently been reported to possess several potential therapeutic properties; antimicrobial,

analgesic, antidiabetic, anticancer, anti-inflammatory, antioxidant and immunomodulator [11]. And also it has been evaluated in clinical trials with various health conditions such as, hyperlipidemia, diabetes mellitus, hypertension, asthma, allergy, cough, bronchitis, fever, headache, infertility, rheumatoid arthritis and gastrointestinal diseases [13].

In this study, the purpose was to investigate the possible preventive effect of oral and topical forms of NS oil on the development of MS in the myringotomized TMs of the Guinea pigs using histopathology and otomiscroscopy techniques.

## 2. MATERIALS AND METHODS:

### *2.1. Animal maintenance and experimental material:*

This study was approved by the Animal Experiments Ethic Committee of Yeditepe University (YUDHEK) (prot.no:01.11.2016-505). Fourteen Guinea pigs, male, weighing 400-600 g, at age 16-18 weeks, bred in Yeditepe University Animal Experiments Laboratories were used in this study. The animals were kept in ordinary cages and can access freely to food and water with artifical lighting from 8:00 a.m to 8:00 p.m. at a relatively humidity of 50±10% and a temperature of 23±3 ˚C. Before and after the surgery, if any signs of external and middle ear infection in the animals was detected, those were excluded from the study.

NS oil was administered topically and orally in two groups of animals. Cold pressed NS oil [Black Cumin Seed Oil- *Nigella sativa* Oleum preparation] was provided by Zade Vital Natural Supplements, Konya, Turkey. The composition of the Nigella sativa seed oil, which was used in the present study was determined by GC-MS technique and found to be as follows: Linoleic acid (57.537%), oleic acid (23.851%), palmitic acid (11.75%), stearic acid (3.086%), cis-11,14-eicosapentaenoic acid (2.413%), gondoic acid (0.33%), linolenic acid (0.247%), arachidic acid (0.194%), palmitoleic acid (0.171%), myristic acid (0.139%), erusic acid (0.085%), heptadecanoic acid (0.064%), cis-10-heptadecanoic acid (0.044%), behenic acid (0.030%), cis-13,16-docosahexaenoic acid (0.037%), lignoceric acid (0.023%).

### *2.2. Experiment designing and surgical procedure:*

All animals were randomly divided into three groups and two groups include five and one group includes four male Guinea pigs. Animals were anesthetized with intramuscular injection of xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (50 mg/kg). A sterile perforation with a 0,5 mm pick through an ear speculum was performed in the superior posterior quadrant of the TMs in bilateral ears. In group I, a gelfoam particle soaked with

saline solution was applied on the perforation of both TMs of animals as a control group. Animals in group II had treated topically with a gelfoam particle soaked with NS oil immediately after myringotomy, and 0.5 ml NS oil was dropped into the ear in the 24$^{th}$ and 48$^{th}$ hours [3,4,6]. In group III, 5 ml NS oil solution were given orally five times; immediately after myringotomy, in the 24$^{th}$ h (day-1), 48$^{th}$ h (day-2), 72$^{nd}$ h( day-3), and 120$^{th}$ h (day-5). We administered high doses compared to previous human studies because minimal toxic effect of NS oil have been reported previously [11]. We researched the preventive effect of NS oil via using consecutive high doses to obtain optimum systemic effect in a short time. Dose ranging studies were not performed because this was a preliminary study to show the preventive effect of NS oil. The dose ranging study is also needed as a secondary issue.

*2.3. Otomicroscopic Examination:*

Otomicroscopic examination was performed on the 15th day of the experiment after all animals were euthanized with carbon dioxide. The author who scored the degree of tympanic membrane inflammation was blinded to the treatment group of the animals being examined. The extent of MS in the pars tensa of the TMs was evaluated semiquantitatively as follows: (0) if no visible MS; (+) if there was white halo around umbo; (++) if there was white halo around umbo and white line beside the handle of the malleus and along the annulus; and (+++) if there was a confluent whitish deposit forming a horseshoe pattern [14].

*2.4. Histopathological Evaluation:*

After the otomicroscopic evaluations were completed, tympanic bulla was opened by microdissection and TM, its surrounding bony annulus, and cochlea were removed together. The specimens were fixed in 10% buffered formaldehyde solution overnight and then decalcified with ethylenediamine tetra-acetic acid for 10 days. All specimens were cut into two pieces from the same region and at the same angle through a line bisecting the

perforation. Both pieces were embedded in parafine and sectioned in 5 µm thickness. Four serial sections were taken from each parafine block (two were stained with Masson's Trichrome (Bio-optica, Milan, Italy) and two were stained with Hematoxylin & Eosin staining.) to evaluate the inflammation, fibrosis, and thickness of the TMs in each animal. For examining the collagen fibers and sclerotic changes in the LP of TMs, Masson's Trichrome staining was used. Sclerotic areas were stained with Masson's Trichrom staining and appeared as blue areas rather than white. Also, calcification was irregular bordered areas that appears as dark purplish pink color in H&E staining. For measuring the thickness of the TM, the healed perforation area of the TM (i.e. the area where inflammation occurs) observed in the sections was evaluated.

Evaluation of all specimens was done by a pathologist blinded to treatment types given to each group. Histopathologically, all the micro measurements were done quantitatively on the digital image output that was taken by the Olympus DP73 camera by using the software program of the same digital microscope and the intensity of the fibrosis degree, inflammation, and the thickness of the TMs were evaluated using a digital light microscope Olympus BX53. Then the fibrosis degree and the sclerotic changes in the LP were scored as: (0) for no fibrosis; (1) for mild fibrosis; (2) for moderate fibrosis and (3) for marked fibrosis and sclerosis and the intensity of the inflammation was scored as: (0) for no inflammation; (1) for mild inflammation; (2) for moderate inflammation; and (3) for marked inflammation, intense exudation and granulation [4].

*2.5. Statistical Analysis:*

Statistical analyses were performed using SPSS v.21 (SPSS, Inc. Chicago, IL, USA) and a value of $p< 0,05$ was accepted as significant. Mean thickness of the TMs were compared with

Mann Whitney U test and otomicroscopic MS scores and inflammation in the LP in each group were compared with Pearson Chi-Square test.

## 3. RESULTS:

### *3.1. Otomicroscopic Examination:*

All TM perforations of the animals were healed and closed on the 15$^{th}$day of the experiment. More myringosclerotic plaques were seen in control group (group I), but the TMs of the animals of group II (treated with NS oil topically) and group III (treated with NS oil orally) showed significantly less or no sclerotic plaques (Figure 1a-d).

The results of MS degree evaluated under otomicroscopy are seen in Table-1 and Figure 2. MS degree of TMs was '0' or '+' in 30% of TMs in group I (control), 80% in group II (topical) and 100% in group III(oral), but '++' and '+++' in %70 of TMs in group I, 20% in group II and 0% in group III and the results were significantly less pronounced in group II and III ($p=0.034$, $p<0.05$).

### *3.2. Histopathological Examination:*

In group I, 6 (60%) TMs had moderate, 1 (10%) TMs had marked and 3 (30%) had mild inflammation. But in group II, 1 (10%) TMs had moderate, 5 (50%) had mild and 4 (40%) had no inflammation and in group III, 5 (50%) TMs had none, 3 (30%) had mild and 0 (0%) had moderate or marked inflammation (Figure 3a-d) (Table-2). As a summary, in group I, inflammation in the LP of the TMs were found to be significantly more severe than group II and III (Figure 4) ($p=0.015$, $p<0.05$).

Sclerotic deposits and fibrosis degree were histopathologically less extensive in TMs of group II and III. Nine of 10 (90%) TMs in group II and 8 of 8 (100%) TMs in group III had none or mild fibrosis. On the other hand, in group I 2 of 10 (20%) TMs had marked and 6 (60%) had moderate fibroblastic proliferation (Table-3) (Figures 5, 6, 7) ($p<0.001$).

The thickness of TMs at the myringotomy site in group II and III were noted thinner than group I. The mean thickness of the TMs of group I were 52±32.24 µm; in group II 21±18.37 µm and in group III 12.50±8.45 µm (Figure 8). According to the Mann Whitney U test, significant differences were observed between group I and II ($p<0.05$) and between group I and III ($p<0.05$). There was no significant difference between group II and III according to the mean thickness of TM ($p=0.873$).

## 4. DISCUSSION:

In this study, it was shown that the topical and oral application of NS oil reduced fibrosis, suppressed inflammation, and prevented the MS development. Previously, several studies revealing the antiinflamatory and antioxidant effects of NS oil were reported [15,16], but to our knowledge, this is the first study to evaluate the use of NS oil for prevention the development of MS.

MS occurs in the middle ear and usually includes the TM. After surgical intervention for exudative otitis media; such as myringotomy and ventilation tube insertion, the commonest sequel is MS [3,4]. Although the morphology of MS has been described, the exact pathogenesis and etiology is not known very well [3]. It is characterized with calcification and synchronous hyalinization in the LP of TM [3-5]. Some hypothesis like mechanical injury, immunologic sensitivity, reactive oxygen species, and metabolic disturbances have been proposed for elucidating the etiopathogenesis of MS [3,5,17,18]. Tissue injury and exaggerated inflammatory response in the TM is one of the most underlined factors contributing the formation of MS [4]. Paracentesis causes a damage to the TM and an inflammatory reaction which leads to tissue damage was initiated by wound healing. After tissue injury, MS is created with calcification, hyalinization, and irregular collagen synthesis in the LP of TM [4]. It was previously reported that after myringotomy, sclerotic changes develops within 9 hours and intense inflammatory response were established in the pars flaccida within 12-24 hours [19].

Recently, in several studies it was shown that MS is caused by the oxygen derived free radicals made in the middle ear cavity under hyperoxic conditions [5,20,21]. These free radicals may be induced from both the hyperoxic environment and inflammatory reaction generated from inflammatory cells around sclerotic plaques and the inflammation provoked

by TM injury [3]. During arachidonic acid metabolism and phagocytosis, superoxide radicals are produced and also may contribute to formation of MS [22]. In a study from Mattson et al. [21], it was reported that rats exposed to different concentrations of oxygen levels were evaluated and after paracentesis, MS lesions of the TMs increased with higher oxygen concentrations. Normally, in a healthy individual the oxygen concentration of middle ear cavity is lower than ambient air and approximately 5-10%. But, after myringotomy, ambient air (in which oxygen concentration is 21%) can enter into the middle ear cavity and produce relative hyperoxic conditions.

A wide variety of free radical scavengers like L-carnitine, ascorbic acid, zinc aspartate, N-acetylcysteine, selenium, *Ginkgo biloba*, vitamin C and E, Caffeic acid phenyl ester (CAPE), N-nitro L-arginine metyl ester ($_{L}$-NAME), melatonin, *Hypericum perforatum*, and enoxaparin sodium have been proposed to use for preventing the development of MS in TM in several studies [1,2,6-10,11,20,21,23-27]. Free radical scavengers process via suppressing the generation of active oxygen species and free radicals, scavenging them, and repairing damage. Several antioxidants have been identified as preventing MS previously. The antioxidant enzymes, elements, and exctracts were applied to the myringotomized area of animals and the results indicate that the treatment with free radical scavengers and antioxidants reduced or inhibited the development of MS. Free radicals have been shown to stimulate apoptosis and antioxidants can offer protection, as has been shown in various cell types.

In terms of disadvantage of use of free radical scavengers, although free radicals are considered prooxidants, antioxidants can also have prooxidant attitude. Vitamin C is considered a potent antioxidant, but it can also become a prooxidant. This will happen when it combines with copper and iron, reducing Cu3+ to Cu2+ (or Fe3+ to Fe2+), which in turn reduces hydrogen peroxide to hydroxyl radicals [28]. One of the useful and powerful

antioxidant is α-Tocopherol but in high concentrations it can become a prooxidant because of its antioxidant mechanism. If there is not enough ascorbic acid for its regeneration than it will remain in this highly reactive state and promote the autoxidation of linoleic acid [29]. Although not much evidence is found, it is proposed that carotenoids can also react as prooxidant especially through autoxidation in the presence of high concentrations of oxygen-forming hydroxyl radicals. Even flavonoids can act as prooxidants, although each one responds differently to the environment in which it is inserted [30].

*Nigella sativa* L., commonly known as black cumin or black seed, is belonging to Ranunculaceae family and used in herbal medicine all over the world. It is widely cultivated in Mediterranean countries, Middle East, Western Asia and Eastern Europe [11]. It has been commonly used as a natural remedy against a broad range of illnesses and conditions such as asthma, diabetes, hypertension, cough, bronchitis, inflammation, eczema, headache, dizziness, influenza and fever [12]. Its seeds or oil are used as diuretic, lactagogue, carminative and vermifuge. NS seeds contain proteins, alkaloids, saponins, fixed oil and volatile oil [11]. The fixed oil contains mainly unsaturated fatty acids that include the unusual C20:2 arachidic acid and eicosadienoic acids [15]. The main components of volatile oil are mainly thymoquinone (13,7%), ρ-cymene (37,3%), carvacrol (11,77%), carvone (0.9%), thymol (0.33%), t-anethole, longifoline and 4-terpineol [13,16]. As the antioxidant effect of NS, it has been shown in a study from Houghton et al. that both tymoquinone and as well as fixed oil of NS inhibit non-enzymatic lipid peroxidation in liposomes [15]. It was also reported that components of NS (including carvacol, 4-terpineol, t-anethole and thymoquionone) had characteristics of free radical scavenger [16]. In some other in vitro tests, these compounds were found to have antioxidant features but not prooxidant [11]. Many human dieases and illnesses occur as a cause of generation of free radicals. For this reason, the antioxidant action of NS that was proven previously may explain its usefulness in folk medicine. It was used against carbon

tetrachloride ($CCl_4$) hepatotoxicity, liver fibrosis and cirrhosis, and liver damage because of *Schistosoma mansoni* infection [11]. The possible mechanism of anti-inflammatory effects of NS has been studied in previous studies. Thymoquinone, by inhibiting both cyclooxygenase and lipooxygenase, had found to be a potent inhibitor of eicosanoid generation that were leucotrienes $B_4$ and thromboxane $B_2$ [15]. It was also reported that the fixed oil of NS had both anti-eicosanoid and antioxidant effects greater than thymoquinone, the main active constituent [15]. In a study from Al-Ghamdi, carrageenan-induced paw oedema of rats was used as a model of inflammation and aqueous extract of NS was found to have significant anti-inflammatory action [31]. These findings make some confidence to the folk medicinal usage of NS plant as an anti-inflammatory and antioxidant substance.

About safety concerns of NS, the extract of seeds and its components appear to have a minimal toxicity level [11]. El Daly reported that the intraperitoneal administration of NS extract at 50 mg/kg to rats for 5 days did not cause any significant effect to the hepatic and renal function [32]. Also Khanna et al. used oral seed oil as 10 mL/kg in rats and mice and they did not observe any mortality or toxicity during 48 hours [33]. Likewise, Zaoui et al. administered 10 mL/kg NS fixed oil orally to rats for 12 weeks and they did not report any mortality or alterations in the hepatic enzymes level [34]. Badary et al. found the $LD_{50}$ value of thymoquinone as 2.4 g/kg and reported that administration of high doses like 2 g/kg or more might cause difficulty in respiration and hypoactivity [35]. They also mentioned that high doses could affect glutathione levels in liver, heart and kidney and damaged these organs. In that study, concentrations up to 0.03% of thymoquinone was included to the drinking water of mice for 90 days and it was reported that except for prominent decrease in fasting plasma glucose concentration, there were no sign of toxicity during the experiment [35]. In literature, there are two case reports stating allergic contact dermatitis and maculopapular eczema after using pure oil of NS topically, although it was presented for

marketing to help in acne, eczema, inflammation and disorders of skin dysfunction. These cases were treated with topical steroids [11]. As reported in references [32-34], oral usage of 10 mL/kg even 2 g/kg NS oil did not cause any serious toxic effect to the rats and mice, therefore we administered 5 ml/kg NS oil orally to the animals to avoid the toxic effects.

In this study, the results suggested that topical and oral administration of NS oil suppressed the inflammation and fibroblastic activity in the LP of the myringotomized TMs of the Guinea pigs by its antiinflammatory and antioxidant components. Also it was found that TMs of the topically or orally treated groups were significantly thinner than the control group. These results also suggest that NS oil reduces the formation of MS by its anti-inflammatory and antifibrotic effects.

## 5. CONCLUSION:

According to our knowledge, there have been no studies on the preventive effect of NS oil on myringosclerosis development; for this reason, this study represents the only study to interpret the association between NS oil and myringosclerosis development. The results of the study were similar to results of the previous myringosclerosis studies. The present study showed that NS oil treatment inhibited myringosclerosis development in TM of guinea pigs and suggest that in the future, this data could be a value for developing approaches to prevent myringosclerosis after myringotomy or ventilation tube application in human patients. At the same time, further studies are required for NS oil to be introduced into the clinical practice.

**Conflict of interest:** None

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**FIGURE LEGENDS:**

**Fig. 1 a-d.** Otomicroscopic views of the TM of different groups from lateral to medial on last day of the experiment. (a) Otomicroscopic view from group I and MS degree was (+++). Asterisk shows dense sclerotic plaques near umbo. (b) View from group I, MS degree was (++). Asterix shows the sclerotic plaques around the umbo, beside the handle of the malleus and along the annulus. (c) View of the TM from group II and MS degree was (+). Asterisks show white sclerotic plaques near umbo and the healed myringotomy site. (d) Otomicroscopic view of TM from group III, MS degree was (0).

**Fig. 2.** Statistical and graphical analysis of otomicroscopic grading of MS in the TMs is shown.

**Fig. 3 a-d.** Light microscopic views of TMs of Guinea pigs (H&E staining, all four photos are at original magnification 40x and arrows are indicating the TMs in the section) (a) Light microscopic photo of pars tensa from a Guinea pig treated with topical NS oil (Group I). There is a prevelant inflammation in the LP and a thicker TM (Inflammation degree- (3) ) (b) Light microscopic photo of a TM from group I (control group) again. The inflammation degree is evident (Inflammation degree (2) ) (c) Light microscopic photo of a Guinea pig from group II, mild inflammation in the LP and more thinner TM from other groups (Inflammation degree-(1) ). (d) Light microscopic photo of pars tensa from a Guinea pig treated with oral NS oil. There is no evidence of inflammation in the TM (Inflammation degree-(0) )

**Fig. 4.** Graphic of the inflammation degree in the LP of the TMs (results of H&E staining).

**Fig. 5.** Graphic of the fibrosis degree in the LP of the TMs (results of Mason-trikrom staining).

**Fig. 6 a-d.** Light microscopic views of tympanic membrane of Guinea pigs ( Masson-Trikrom staining, all four photos are at original magnification 40x and arrows are indicating the TMs in the section) (a) Light microscopic photo of a Guinea pig from group I (control group), showing increased fibroblastic activity in the TM and more thicker LP and TM from other groups (fibrosis degree-(3) ). (b) Light microscopic photo of a TM from group I again. Sclerosis is more evident and the TM is comparatively thicker (fibrosis degree (2) ) (c) Light microscopic photo of pars tensa from a Guinea pig treated with topical NS oil (Group II). There is a mild fibroblastic activity in the TM (fibrosis degree- (1) ). (d) Light microscopic photo of pars tensa from a Guinea pig treated with oral NS oil. There is no evidence of sclerosis in the TM (fibrosis degree-(0) ).

**Fig.7.** Inflammation and sclerotic areas in the tympanic membrane ((a) H&E and (b) Masson's trichrome staining, 100x magnification).

**Fig. 8.** This graphic shows the mean thickness of the TMs (micrometer) and statistical analysis between groups.

**Table-1** Otomicroscopic degrees of TMs of Giunea pigs

| | ***0*** | + | ++ | +++ |
|---|---|---|---|---|
| *Control (Saline)* | 0 | 3 | 6 | 1 |
| *NS oil (Topical)* | 5 | 3 | 2 | 0 |
| *NS oil (Oral)* | 5 | 3 | 0 | 0 |

NS: Nigella sativa TM: tympanic membrane

**Table-2** Inflammation degrees of TMs of Giunea pigs

| | ***None*** | ***Mild*** | ***Moderate*** | ***Marked*** |
|---|---|---|---|---|
| *Control(Saline)* | 0 | 3 | 6 | 1 |
| *NS oil (Topical)* | 4 | 5 | 1 | 0 |
| *NS oil (Oral)* | 5 | 3 | 0 | 0 |

NS: Nigella sativa TM: tympanic membrane

**Table-3** Fibrosis degrees of TMs of Giunea pigs

| | ***None*** | ***Mild*** | ***Moderate*** | ***Marked*** |
|---|---|---|---|---|
| *Control(Saline)* | 0 | 2 | 6 | 2 |
| *NS oil (Topical)* | 4 | 5 | 1 | 0 |
| *NS oil (Oral)* | 8 | 0 | 0 | 0 |

NS: Nigella sativa TM: tympanic membrane