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Does the use of antibiotic spacer disrupt induced membrane function?

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ABSTRACT

Treatment of large segmental bone defects has been a challenging and long process for both physicians and patients. At present, the induced membrane technique is one of the reconstruction techniques commonly utilized in treating large segmental bone defects. It consists of a two-step procedure. In the first one, after bone debridement, the defect is filled with bone cement. The aim at this stage is to support and protect the defective area with cement. A membrane is formed around the area where cement was inserted 4-6 weeks after the first surgical stage. This membrane secretes vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), as shown in the earliest studies. In the second step, the bone cement is removed, and the defect is filled with cancellous bone autograft. In the first stage, antibiotics can be added to the applied bone cement, depending on the infection. Still, the histological and micromolecular effects of the added antibiotic on the membrane are unknown.This study investigates the molecular and histological effects of antibiotics addition into bone cement on the induced membrane.In this study, conducted on 27 male New Zealand rabbits, the 2 cm long defects of a bone were created in the rabbit femurs. Three groups were formed by placing antibiotic-free, gentamicin, and vancomycin-containing cement in the defect area.These groups were followed for six weeks, and the membrane formed at the end of 6 weeks was examined histologically. As a result of this study, it found that the membrane quality markers (Von Willebrand factor (vwf), Interleukin 6-8 (IL), Transforming growth factor beta (TGF-β), Vascular endothelial growth factor (VEGF) were significantly higher in the antibiotic-free bone cement group. Our study has shown that antibiotics added to the cement have negative effects on the membrane. Based on the results we obtained, it would be a better choice to use antibiotic-free cement in aseptic nonunions. However, more data is needed to understand the effects of these changes on the cement on the membrane.

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Introduction

Repairing bone abnormalities is a challenging procedure for both surgeons and patients. Bone repair occurs following operations performed to treat bone infections, to remove tumors, and to address fractures and nonunion. However, such bone repair approaches as autografting or conventional cancellous bone grafting are not suitable for the treatment of critical size defects. These defects are basically flaws with a diameter that is 2–2.5 times larger than the problem area. Given that nonvascular bone grafts are conventionally resorbed, the suitable therapy often involves either vascularized bone grafts or a distraction osteogenesis procedure. These approaches, however, are not without challenges [1].

The induced membrane technique was first described in 1986. This technique has been the topic in over 300 articles [2] and is now regarded as a gold-standard therapeutic approach. The first two phases in this procedure are the debridement and filling of a defective region with bone cement (i.e., polymethylmethacrylate or PMMA) and the establishment of bone stability. Over time, osteogenic and angiogenic membranes grow around the PMMA as a response to the presence of a foreign body (i.e., PMMA). After 4–8 weeks, a second stage surgery is conducted [3]. Following the removal of the PMMA, the cavity created in the membrane is filled with bone transplant [4].

Many studies on membrane qualities, fixation techniques, scheduling of second stage surgery, and graft materials to be

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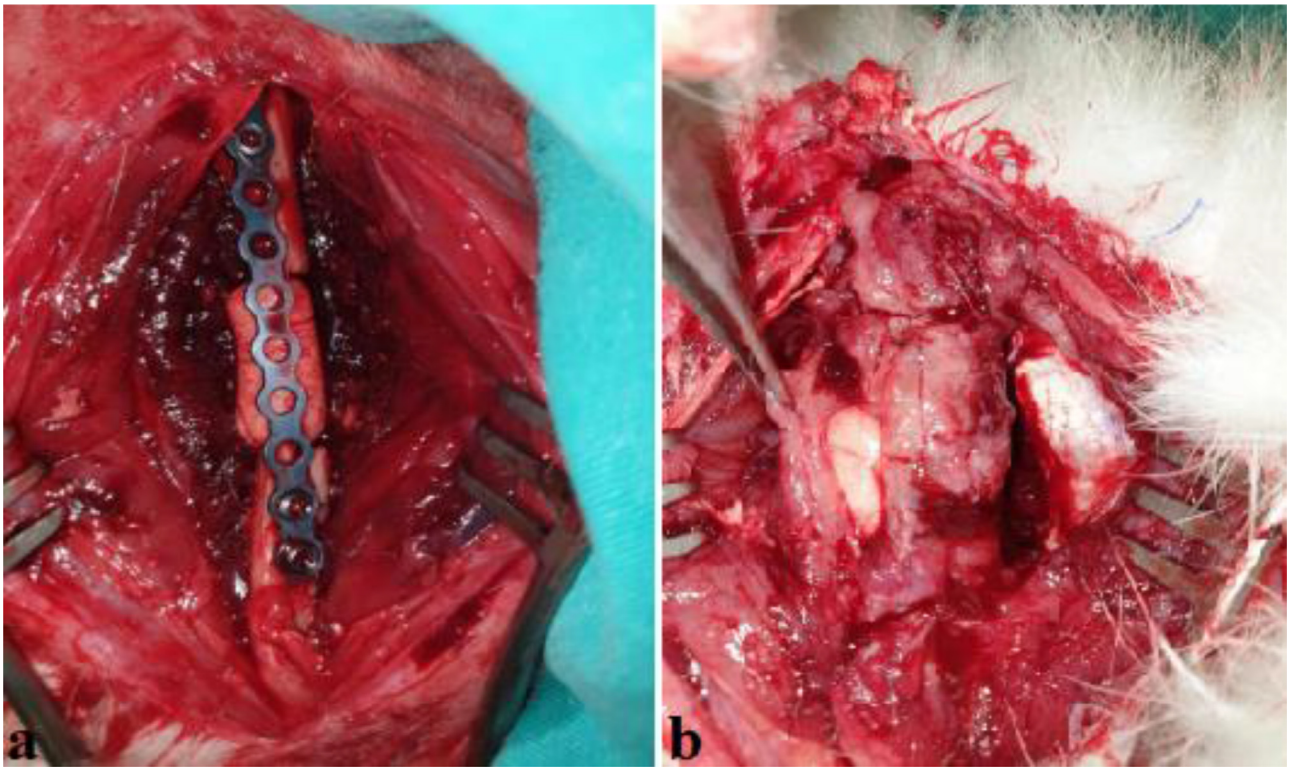


Fig. 1. a) The bone defect was filled with PMMA; b) appearance of the induced membrane at 6 weeks.

used are being conducted. Note that membrane characteristics vary depending on the kind of PMMA used. For instance, antibiotic-free PMMA was used in Masquelet's technique [5]. According to Masquelet, an induced membrane lacks the ability to maintain an area free from unwanted elements; moreover, appropriate debridement is even more crucial, and medications alter membrane shape [5]. In clinical research, however, the use of PMMA in combination with antibiotics had demonstrated positive effects. Heat-stable antibiotics, such as clindamycin, gentamicin, and vancomycin, are actually used in therapeutic applications in combination with PMMA [6]. The current work aims to demonstrate the immunohistochemical characteristics of an induced membrane generated following the application of PMMA without the antibiotics, such as gentamicin and vancomycin.

Materials and method

This research was conducted between April and May in 2020; a research permit was granted by the Eskişehir Osmangazi University Experimental Animals Ethics Committee on January 25, 2018, with a file registration number 639. Our research involved three groups: the antibiotic-free cement (control) group, the vancomycin group, and the gentamicin group, wherein each group contained 9 male New Zealand rabbits. Anesthesia consisting of 44 mg/kg ketamine HCl and 5 mg/kg xylazine was delivered intramuscularly to the rabbits, followed by the administration of antibacterial prophylaxis (20 mg/kg cefazolin). During the surgery, periosteal tissue was excised above the femoral diaphysis after reaching the femur; a tissue was obtained between the biceps femoris and the tensor fascia lata. After a 2 cm bone defect was created, fixation was performed by securing the proximal and distal ends of a 2 mm-thick titanium alloy 10-hole microplate by using two locking screws. Biofix 1 cement-synimed antibiotic-free mixture containing 40 g PMMA and 2 g vancomycin and 40 g PMMA and 2 g gentamicin were prepared. The defective regions in the three groups were filled with the appropriate PMMA preparation (Fig. 1a), and then the skin incision was closed. The rabbits were monitored in clear cages at 25–35 °C for 12 h during daytime and 12 h at night in naturally vantilated and light intensity-regulated rooms (40 lux per cage). The rabbits were fed ad libitum with Esyem rabbit food and were provided with tap water. Six weeks later, the rabbits were re-operated while under ketamine 44 mg/kg and xylazine 5 mg/kg anesthesia. The generated defect region was accessed by creating a new incision over the incision scar. Samples were obtained from the biological membranes that formed around the cement. (Fig. 1b)Once the procedure was completed, the rabbits were sacrificed under anesthesia by cervical dislocation.

Histological materials and methods

Histological procedures

The obtained membrane tissues were immediately rinsed with 0.9% NaCl solution, cleaned from blood, and fixed in 10% formaldehyde. After 24 h, the tissues were rinsed with running tap water and passed through an ethanol series (70%, 80%, 96%, and 100%). After being rendered lightly permeable using xylene, they were passed through a combination of xylene and liquid paraffin and then through three different paraffin solutions before being prepared into paraffin blocks. For general histological investigation, 5 µm-thick serial slices were cut from the paraffin blocks using a microtome, and then the slices were stained with H-E.

General histopathlogical procedures

The histological examination performed by an experienced and independent obserwer, who was blinded to the groups. Examined under a 40× objective, five different locations in each tissue segment were scored; thus, each rabbit received a score. A score of 0, 1, 2, or 3 was assigned when no, few, moderate, or high amounts

of edema, large cells, and small-diameter blood vessels were observed, respectively.

Immunohistochemical procedures

4 µm-thick sections were cut from the paraffin blocks, transferred to slides, and placed in an oven at 60 °C for 1 h. Subsequently, the sections were deparaffinized by using xylol for 3 × 5 min. The slides were passed through a series of alcohol with decreasing concentrations (100%, 96%, 80%, and 70%) before being run through distilled water for 1 min. To unmask the antigen, a citrate buffer diluted 1/10 (p:6) (AP-9003–999; Thermo Scientific) was used in combination with the PT Module (A80400012 Lab Vision). The slides and coverslip were placed in an immunohistochemistry stainer and placed in rack slots; subsequently, they were washed with PBS for 5 min. Endogenous peroxidase activity was inhibited after 10 min of activation with 3% hydrogen peroxide (TA-125-HP ThermoScientific). The tissues were washed with PBS and blocked for 5 min with a blocking solution (TA-125-PBQ ThermoScientific). The tissues were incubated with antibodies against von Willebrand factor (vWF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), bone morphogenic protein 2 (BMP-2), interleukin 6 (IL-6), and interleukin 8 (IL-8). The preparations were rinsed with PBS for 5 min the following day after being incubated at +4 °C overnight and then stained with AEC Chromogen (TA-125- HA Thermo Scientific). Background staining was performed for 30 s with hematoxylin (HHS32 Sigma). Any excess hematoxylin was removed with distilled water. The slides were allowed to dry naturally and then sealed using a water-based sealing solution (TA-125-UG Thermo Scientific).

Immunohistochemical examination

Examined under the microscope, the sections were scored 0–3 based on the location of the applied antibody in the tissue (surface, deep, or both), on the staining intensity (light, moderate, or strong), and on the quantity of the staining (low, medium, large) (45). The results obtained from the membranes were statistically compared. (The Kruskal–Wallis test was used to compare the score variables obtained from the histological evaluation. The score variables that were significant in the test were subjected to pairwise comparison using Dunn's test. Descriptive statistics (median, Q1, and Q3) were used for group evaluation. IBM SPSS Statistics 21.0 was used in the analysis. A p value of <0.05 indicated statistical significance) (Table 1).

Table 1
Summary of statistical data.

Variables	Groups	Median(Q1-Q3)	Kruskal Wallis	*p* value
H-E	Control	3 (2-3)	11.432	<0.001
	Gentamicin	(1-1)		
	Vancomycin	0 (0-0)		
BMP2	Control	2 (2-2)	2.727	0.256
	Gentamicin	2 (1-2)		
	Vancomycin	1 (1-2)		
VEGF	Control	2 (2-2)	23.209	<0.001
	Gentamicin	1 (1-1)		
	Vancomycin	0 (0-0)		
VWF	Control	2 (2-2)	16.832	<0.001
	Gentamicin	2 (2-2)		
	Vancomycin	1 (1-1)		
TGF-β	Control	2 (2-2)	9.026	0.011
	Gentamicin	2 (2-2)		
	Vancomycin	1 (1-2)		
IL6	Control	3 (3-3)	7.143	0.028
	Gentamicin	2 (2-3)		
	Vancomycin	2 (2-3)		
IL8	Control	2 (2-2)	2.009	0.339
	Gentamicin	2 (2-2)		
	Vancomycin	2 (2-2)		

Results

The control and antibiotic (vancomycin and gentamicin) groups were compared. The Kruskal–Wallis test results showed that the histological findings, the H-E staining, and the VEGF, WVF, TGF-, and IL6 levels were statistically higher ($p = 0.05$) in the control group than in the two other groups. The BMP-2 and IL-8 levels had high histological scores, but they did not significantly differ between the groups. No evidence of infection was observed in the groups. The groups were then compared in pairs using the Dunn's test for the factors that were significant. The general histological and immunohistochemical findings are graphically shown in Fig. 2.

General histological findings (H-E)

Fig. 3 show the representative microscopic images of the examined membranes. Membrane induction was more apparent in the control group than in the two other groups. A large number of multinucleated giant cells and the breadth of the edematous patches were noticeable in the control group. In the gentamicin group, giant cells and edematous regions were less prevalent, whereas they were nearly absent in the vancomycin group. Blood vessels were seen in various regions in all groups. The mean induction scores of the control, gentamicin, and vancomycin groups were 2.71, 0.89, and 0.22, respectively. Each of the antibiotic groups significantly differed from the control group ($p = 0.20$ for gentamicin group and $p < 0.001$ for the vancomycin group), whereas no significant difference was observed between the gentamicin and vancomycin groups ($p = 0.323$).

Immunohistochemical findings

vWF

Fig. 4 shows the representative microscopic images of the examined membranes. In the control group, the vWF expression was prevalent and intense, which was practically comparable to that in the gentamicin group; the expression in the vancomycin group was drastically reduced. The mean expression scores for the control, gentamicin, and vancomycin groups were 2.22, 2.00, and 1.00, respectively, which were significantly different ($p < 0.001$) from one another. While the difference between the control group and the gentamicin group was not significant ($p = 1.00$), the difference ($p < 0.001$) between the control and vancomycin groups was significant. The gentamicin and vancomycin groups also significantly differed ($p = 0.005$).

VEGF

Fig. 5 shows the representative microscopic images of the examined membranes. VEGF expression was prevalent and intense in the control group, whereas it dropped in the gentamicin group and was significantly low in the vancomycin group. The mean expression scores in the control, gentamicin, and vancomycin groups were 2.11, 1.11, and 0.11, respectively, which significantly differed ($p < 0.001$) from one another. The gentamicin group had higher VEGF scores than the vancomycin group ($p = 0.048$).

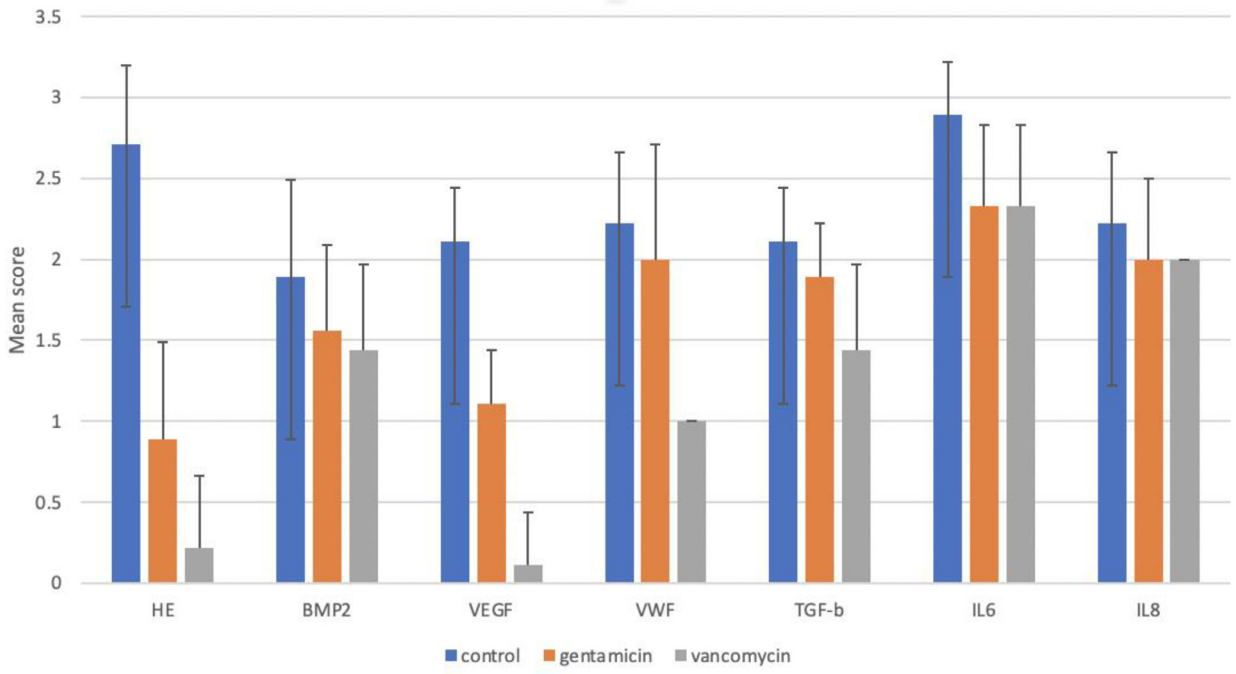


Fig. 2. Graphical representation of the mean score and standard deviation of the examined parameters in the three groups. The sections are examined under a microscope, and the distribution of the applied antibody in the tissue (on the surface, in the deep or both), the intensity of the staining (light, medium, strong) and the amount (low, medium, high) are between 0 and 3 rated.

TGF-β

Fig. 6 shows the representative microscopic images of the examined membranes. TGF-β expression in the membrane tissue was more prevalent and stronger in the control group, whereas it was lower in the gentamicin group and fell significantly in the vancomycin group. The mean expression scores in the control, gentamicin, and vancomycin groups were 2.11, 1.89, and 1.44, respectively, which significantly differed ($p = 0.011$) from one another. While the difference between the vancomycin and the control groups was significant ($p = 0.10$), the differences between the gentamicin and vancomycin groups and between the gentamicin and control groups were not significant ($p = 0.130$ for the gentamicin–vancomycin groups; $p = 1.00$ for the gentamicin–control group).

BMP-2

Fig. 7 shows the representative microscopic images of the examined membranes. BMP-2 expression in the membrane tissue was more prevalent and intense in the control group, somewhat reduced in the gentamicin group, and marginally decreased in the vancomycin group. The average expression scores for the control, gentamicin, and vancomycin groups were 1.89, 1.56, and 1.44, respectively, which did not significantly differ ($p = 0.256$) from one another.

IL-6

Fig. 8 shows the representative microscopic images of the examined membranes. IL-6 expression in the membrane tissues was more prevalent and intense in the control group, whereas a minor drop was observed in the gentamicin and vancomycin groups, which showed comparable expression levels. The mean expression scores of the control, gentamicin, and vancomycin groups were 2.89, 2.33, and 2.33, respectively.

The Kruskal–Wallis test results indicated that at least one group differs from the others in terms of IL-6 expression; however, the Dunn's test was unable to identify the group pair that significant differed. This is because of the scoring factors. The groups were not homogenous, but this could not be confirmed given that scores were used as a metric.

IL-8

Fig. 9 shows the representative microscopic images of the examined membranes. Although IL-8 expression was somewhat more intense in the control group, comparable expression levels were observed in the three groups. The mean expression scores of the control, gentamicin, and vancomycin groups were 2.22, 2.00, and 2.00, respectively. The difference between the antibiotic and control groups was not statistically significant ($p = 0.366$).

Discussion

Fracture healing relies heavily on the vascularity of the surrounding soft tissue. By following the steps Masquelet outlined, a pseudosynovial membrane was successfully created; this membrane was claimed to prevent the grafts implanted in the faulty location from resorbing, and it promotes corticalization and provides vascularity. This membrane was formed as a response to the presence of a foreign body (i.e., PMMA) [7]. Evidence suggests that this membrane is responsible for the secretion of osteoinductive factors, such as BMP-2, VEGF, and TGF-β [8]. The created barrier also prevents the protrusion of soft tissues into the defective area. This induced membrane approach has already achieved a healing percentage of higher than 90% [7]. Given that distraction osteogenesis is a slow process and prone to problems and that vascularized bone transfer is technically challenging ([9,10]), the induced membrane approach has received tremendous attention and has been the topic of several investigations.

The time of evaluation, the kind of antibiotics utilized, and the spacer material all play a role in determining the final membrane quality following stabilization. To be clear, Masquelet did not combine PMMA with any antibiotics [11], as the quality of membranes may be altered by antibiotics [6]. Nevertheless, PMMA infused with antibiotics has been used successfully as a spacer in clinical prac-

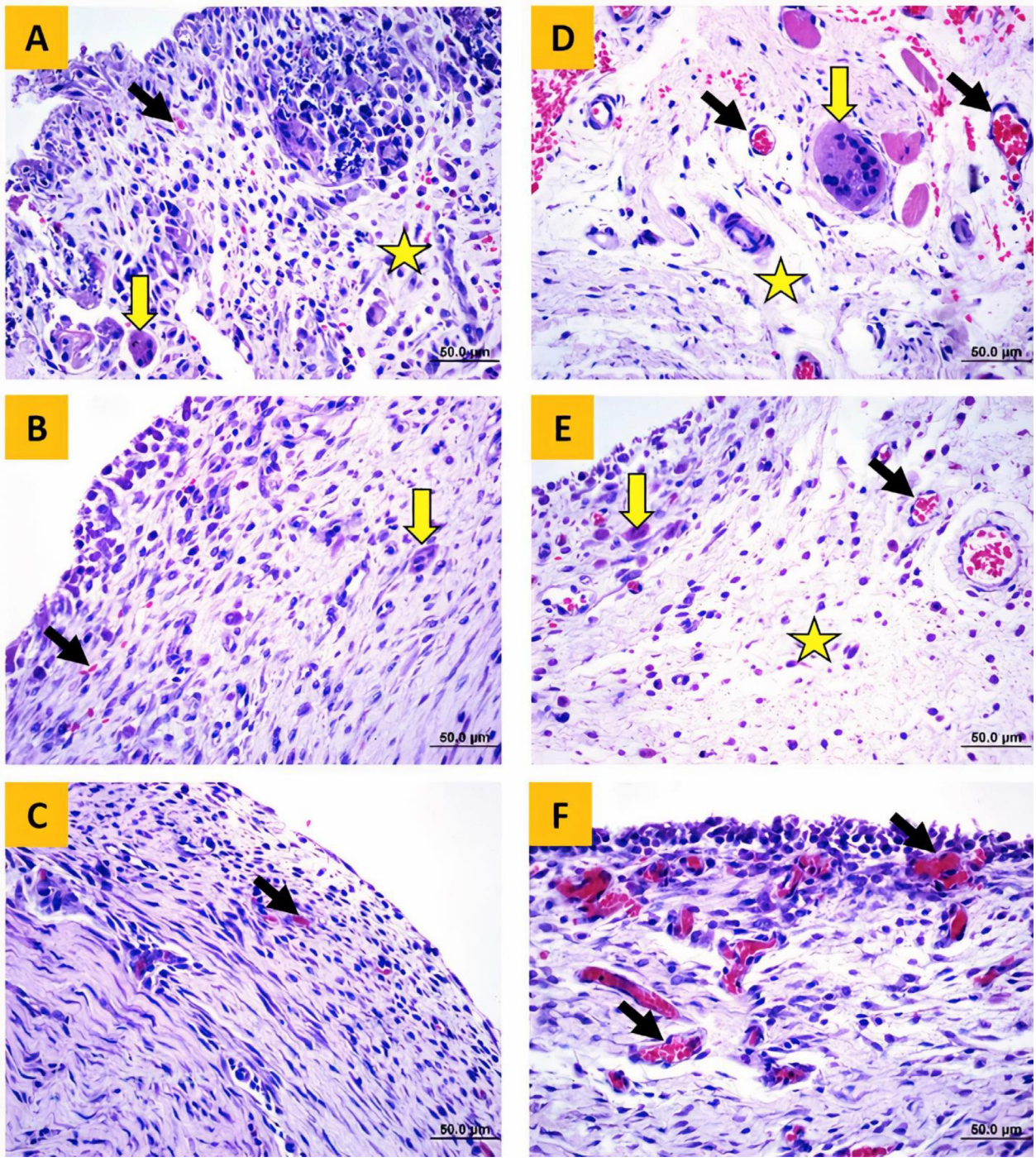


Fig. 3. Histological images of the examined membrane tissues and the cement-facing side is oriented upwards. A: The control group displays an irregular membrane surface, a large edematous area (star), one giant cell (yellow arrow), and a small capillary (black arrow). B: The gentamicin group had a more regular membrane surface, had fewer giant cells (yellow arrow), and contained capillaries (black arrow). C: The vancomycin group had a regular membrane surface, was devoid of giant cells, and contained capillaries (black arrows). D: The control group had a large edematous area (star), one giant cell (yellow arrow), and small capillaries (black arrows). E: The gentamicin group still had an edematous area (star), fewer giant cells (yellow arrow), and capillaries (black arrow). F: The vancomycin group was free from giant cells but still had capillaries (black arrows). Scale bars: 50 µm (The tissues were stained with H-E).

tice. A meta-analysis has shown that an antibiotic cement has decreased the need for grafting procedures [12].

PMMA containing antibiotics, such as vancomycin and gentamicin, are commonly used in revision arthroplasty and osteomyelitis [13]. However, disagreements exist on how much antibiotics should be added to PMMA. Primary arthroplasty cases often involve the use of low-dose preventive antibiotic PMMA (0.5 g in 40 g PMMA), whereas high-dose antibiotic PMMA may be used safely to treat infections [14]. However, the properties of PMMA with antibiotics desired to be obtained with the induced membrane technique are quite different; neither low doses used in arthroplasty surgery to preserve the biomechanical properties of PMMA nor high doses used in the treatment of osteomyelitis are needed when deciding on the amount of antibiotic.

The vascular structure and osteogenic potential formed by the induced membrane form the basis of the technique. Antibiotics

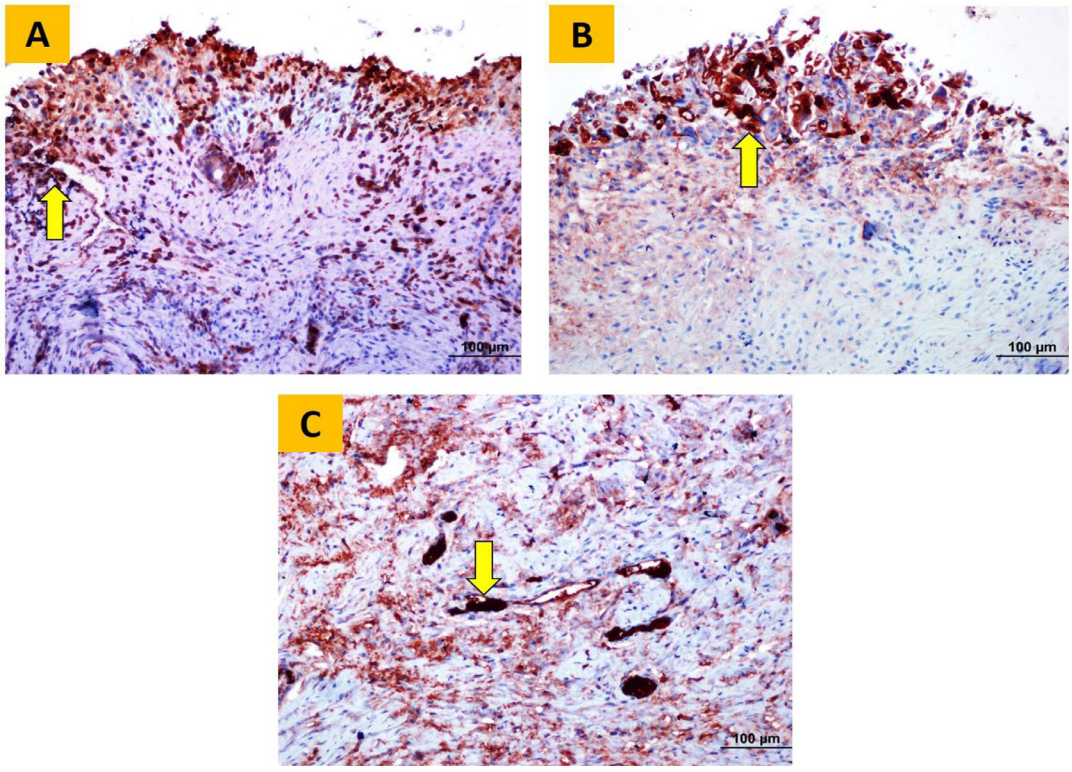


Fig. 4. Representative images showing the vWF expression in the examined membrane tissues and the cement-facing side is oriented upwards. A: Positive and intense staining (yellow arrow) was observed in the control group. B: The gentamicin group had a slightly decreased (yellow arrow) vWF expression. C: vWF expression was intense only in the blood vessels (yellow arrow) in the vancomycin group. Scale bars: 100 µm.

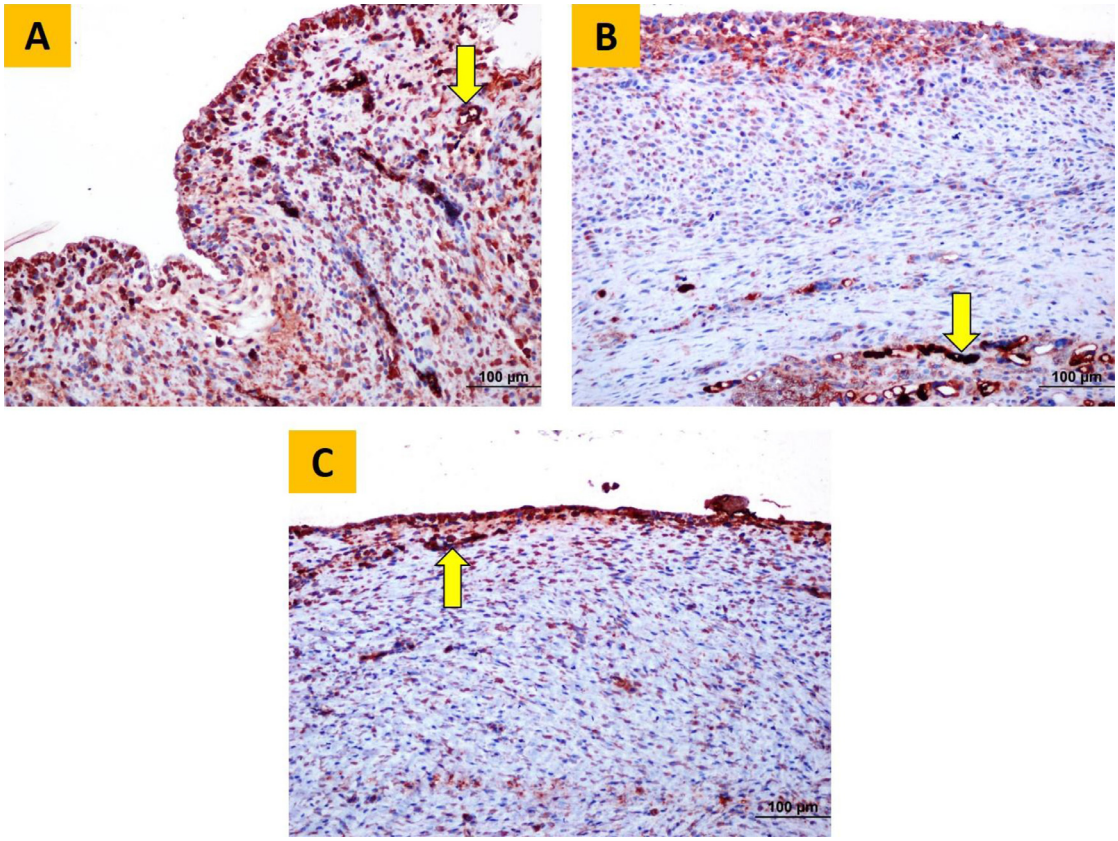


Fig. 5. Representative images showing the VEGF expression in the examined membrane tissues and the cement-facing side is oriented upwards. A: The control group showed a positive and intense staining (yellow arrow). B: The gentamicin group showed a slightly reduced VEGF expression on the membrane surface but a strong expression in the blood vessels (yellow arrow). C: In the vancomycin group, VEGF expression decreased in the membrane, but it was intense in the blood vessels (yellow arrow). Scale bars: 100 µm.

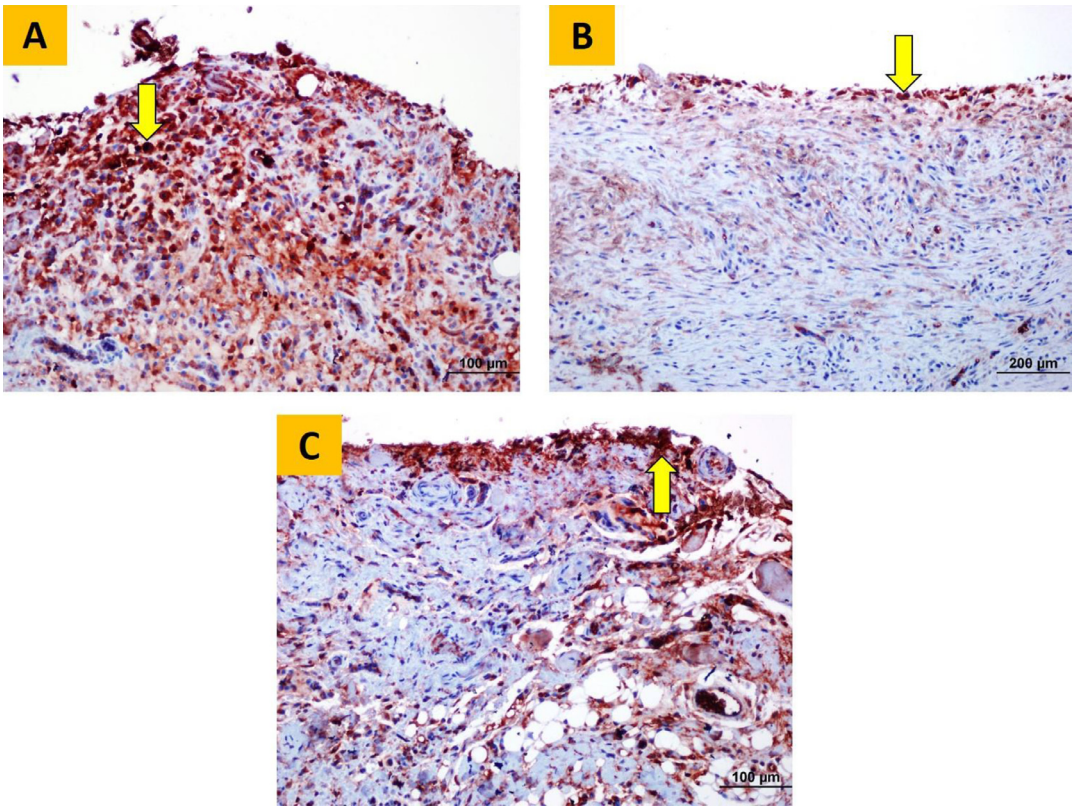


Fig. 6. TGF-β expression in the examined membrane tissues and the cement-facing side is oriented upwards. A: Positive and intense staining (yellow arrow) was observed in the control group. B: Expression decreased (yellow arrow) in the gentamicin group. C: The expression also decreased (yellow arrow) in the vancomycin group. Scale bars: 100 µm.

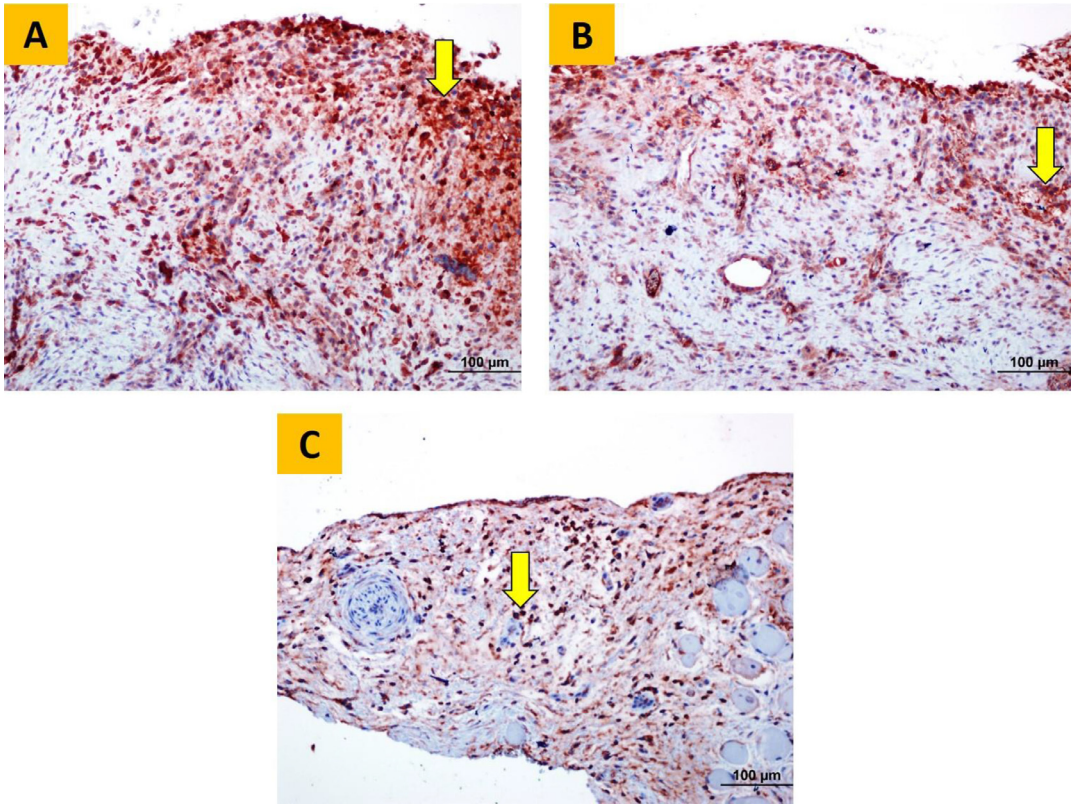


Fig. 7. Representative images showing the BMP-2 expression in the examined membrane tissues and the cement-facing side is oriented upwards. A: Positive and intense staining (yellow arrow) was observed in the control group. B: Expression was slightly decreased (yellow arrow) in the gentamicin group. C: Expression decreased (yellow arrow) in the vancomycin group. Scale bars: 100 µm.

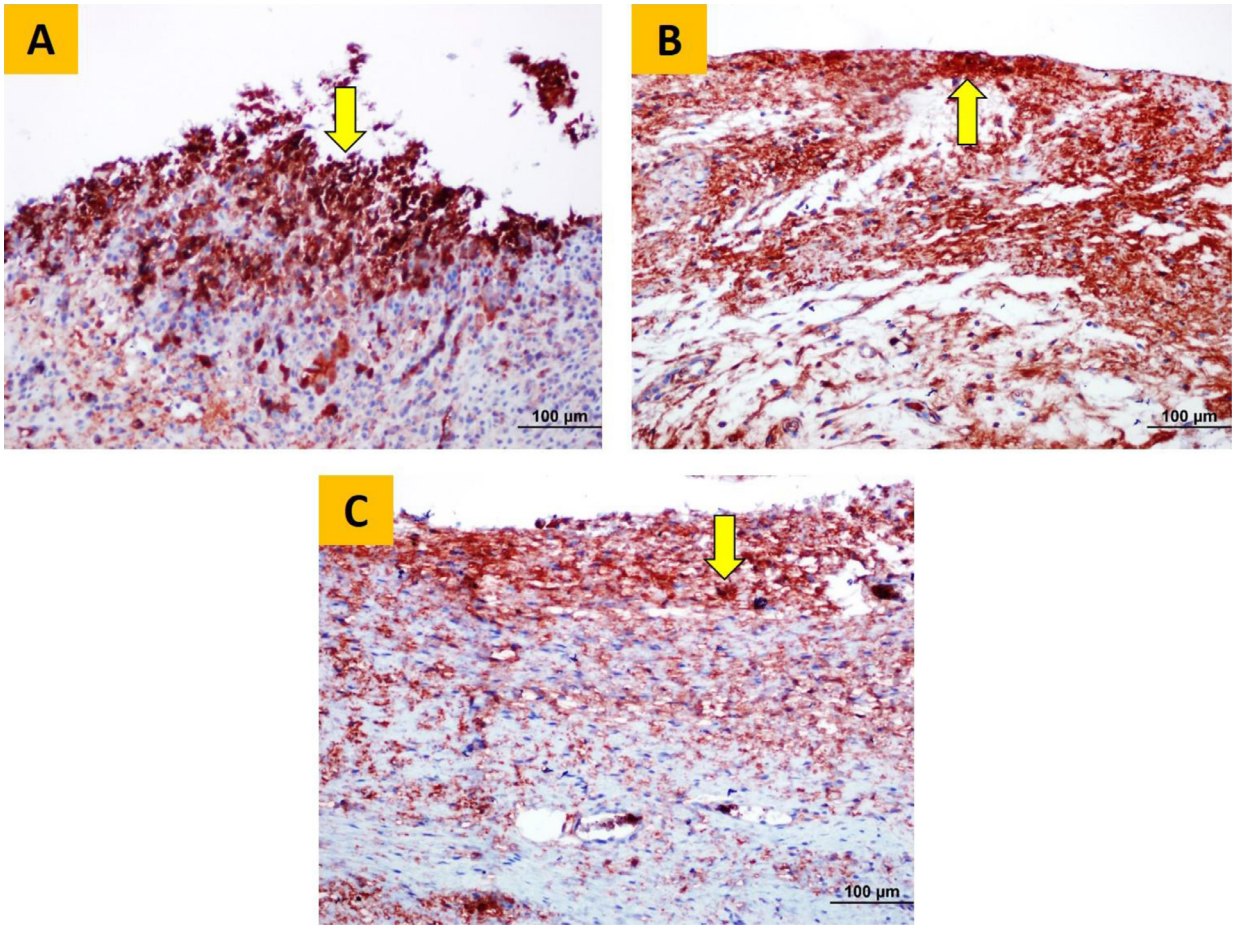


Fig. 8. Representative images showing the IL-6 expression in the examined membrane tissues and the cement-facing side is oriented upwards. A: Positive and intense staining (yellow arrow) was observed in the control group. B: The expression in the gentamicin group was similar to that in the control group, except that the staining intensity decreased and that intense staining was observed in certain places (yellow arrow). C: The expression in the vancomycin group decreased, but intensely stained areas (yellow arrow) were observed. Scale bars: 100 µm.

have a dose-dependent influence on osteogenic activity. High-dose local antibiotics have a detrimental impact on osteoblastic function [15]. High concentrations of clindamycin are known to exert a cytotoxic effect [16]. Vancomycin and gentamicin have been shown to exert no impact on fracture healing [17]. In a rabbit experiment, Jie et al. examined the change in membrane quality as a function of vancomycin dosage. It was noted that 1–4 g of vancomycin had no effect on membrane's osteogenic and angiogenic capabilities [19].

Furthermore, the use of combination antibiotics is currently suggested for the treatment of periprostatic joint infections; however, the phenomenon known as passive opportunism is associated with this mixture, and the use of combination antibiotics in large dosages may also have a detrimental impact on elutions [18]. In vivo and in vitro experiments have already established that low-dose antibiotic PMMA may lead to the development of antibiotic resistance. The use of antibiotics at doses below 2 gs has been met with some resistance, rendering the use of such dosages controversial in light of this information, the use of high-dose antibiotics was avoided in our study involving rabbits, considering the animals' sizes; moreover, mixtures consisting 2 g vancomycin or gentamicin and PMMA were prepared. In addition, the antibiotic concentrations used in our study are comparable to the antibiotic cements used in the market.

Induced membrane formation requires rigid fixing. Masquelet's first system made use of an external fixator. Plate and nail applications provide excellent results in contemporary applications [5]. The use of fixators increases the likelihood of stress fracture in the future [12]. The use of nails is supposed to lessen the quantity of graft necessary, and the nail imparts a load-bearing characteristic [20]. PMMA may be conveniently inserted in the defective region with the use of a plate, and suitable reduction can be accomplished, although there is danger of bending or fractures [21]. In our investigation, we chose to fix with a plate because we only wanted to examine the membrane quality and because it was easier to apply to rabbits.

Pelissier et al. have demonstrated that the best period for the second stage grafting is between 4 and 8 weeks [8]. According to a recent meta-analysis, second stage procedures are generally conducted at 6–8 weeks, and grafting before 8 weeks is advised [22]. Experiments have demonstrated that VEGF expression, BMP-2 release, and membrane thickness increase at 2–6 weeks. Beyond 6 weeks, a membrane's bioactivity starts to decline [23]. Thus, the membrane examination period in our investigation was set at 6 weeks.

A 1 cm defect was generated in the femur of 72 adult rats in a research conducted by Nau et al.; this bone defect was sealed with a plate and cement containing palacos + gentamicin, copal + gentamicin + vancomycin, and copal + gentamicin + clindamycin; after 2, 4, and 6 weeks, the induced membrane that formed around the cement was sampled and evaluated for vascularization, proliferation, and membrane thickness. Membrane thickness increased in the palacos group from 2 weeks to 6 weeks, but it decreased in the copal group. Vascularization increased from 2 weeks to 6

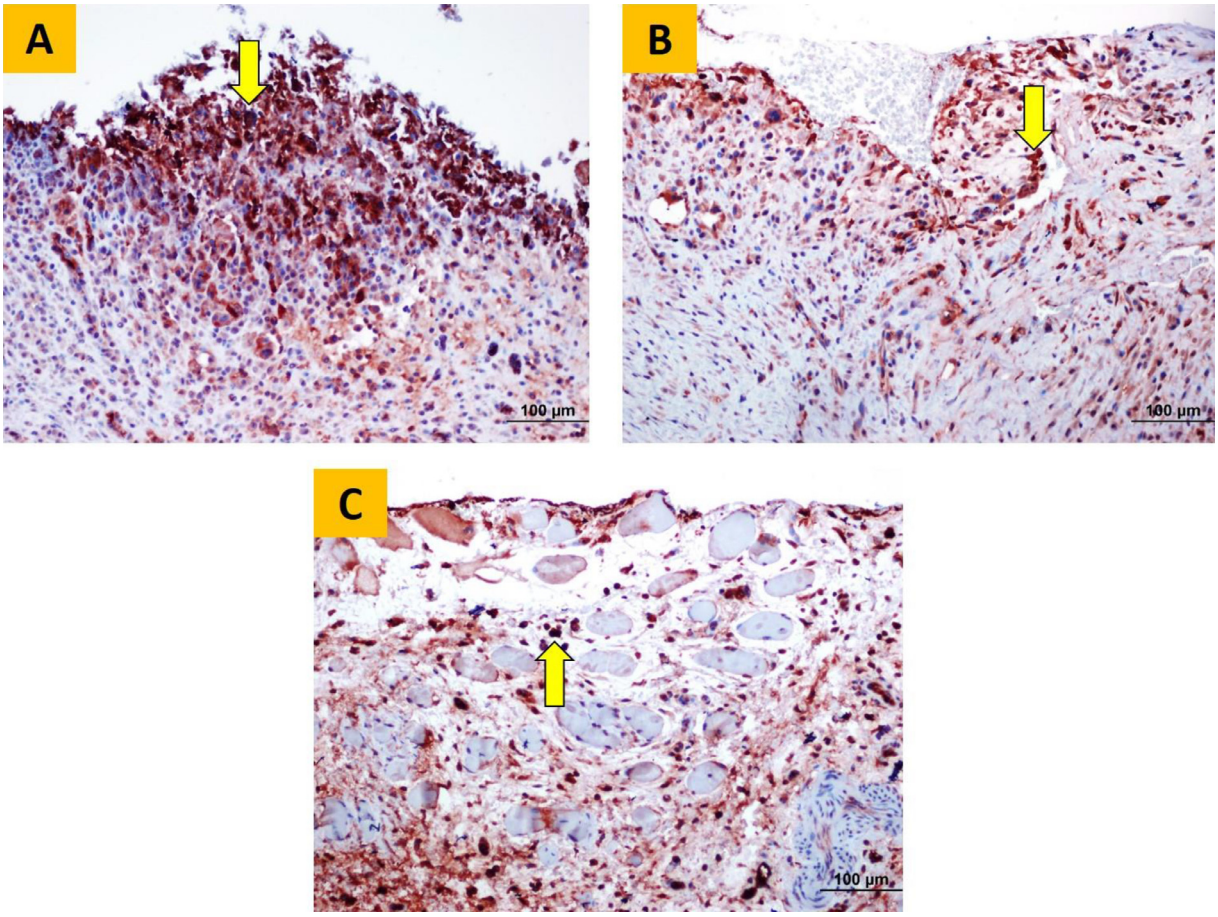


Fig. 9. Representative images showing the IL-8 expression in the examined membrane tissues and the cement-facing side is oriented upwards. A: Positive and intense staining area (yellow arrow) was observed in the control group. B: Similar expression was observed in the gentamicin group, except that the staining intensity decreased and that intense staining was observed in certain places (yellow arrow). C: The expression decreased in the vancomycin group, but intensely stained areas (yellow arrow) were also noted. Scale bars: 100 µm.

weeks in the palacos + gentamicin and copal + gentamicin + vancomycin groups but not in the copal + gentamicin + clindamycin group. Gentamicin was added to all groups in this research [6]. The primary difference of our research is that the PMMA, gentamicin, and vancomycin groups were evaluated independently.

The immunohistological examinations performed after the rabbits were sacrificed at 6 weeks revealed that, with the exception of the BMP2 and IL-8 levels, the values for the membrane quality parameters dramatically decreased in the group treated with antibiotic-containing cement. The reason for this is that the antibiotics used have a cytotoxic effect on the membrane. Compared with the vancomycin group, the gentamicin group had greater VEGF and VWF levels, which are membrane quality indicators. No changes in TGF-β levels were observed in the vancomycin and gentamicin groups. This might be related to the dynamics of the cement–antibiotic release and the peak times in inflammatory cytokine release. More giant cell formation was observed in the control group. More studies are needed to explain the clinical impact of this condition. Our obvious observation as a result of the evaluations is that the antibiotic loaded cements damage the induced membrane structure.

The limitation of our research is that it was conducted within a 6-week period using only two medicines at specific dosages. Various dosages of antibiotics applied over different study periods may be compared so that changes in membrane quality can be assessed. The second part of the investigation where we can observe bone healing can be used to analyze clinical reflection. Aside from the parameters used to assess membrane quality, KI-67 protein may be analyzed as a cell proliferation marker and DDR-2 (didoxin domain receptor-2) as a periosteal marker in osteoblast precursor cells.

Conclusion

Our experiments showed that combining antibiotics with PMMA had a detrimental impact on membranes. Thus, an antibiotic-free cement would be a superior option in aseptic nonunion. More clinical investigations are warranted given that positive clinical findings have been observed from the usage of PMMA infused with antibiotics. Future studies should focus on shortening the time between the materials to be added to the cement and the stages of the technique.

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