ORIGINAL ARTICLE

Histological characteristics of induced membranes in subcutaneous, intramuscular sites and bone defect

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KEYWORDS
Induced membrane; Subcutaneous; Intramuscular; Bone graft

Summary
Background: The induced membrane technique was proposed as a treatment of large segmental bone defects. The influence of the surrounding tissues on its characteristics remains unknown. It is therefore not known which kind of plastic surgery procedure (muscular or faciocutaneous flap) would optimize bone osteointegration within a bone defect reconstructed using the induced-membrane technique.

Hypothesis: We hypothesized that membrane characteristics could be influenced by the soft-tissue environment either subcutaneous or muscular.

Objective: To evaluate the histological characteristics of poly-methylmethacrylate (PMMA) induced membranes in intramuscular, subcutaneous and bony environment (radius defects) at 2 steps: spacer implantation; secondary bone graft and its subsequent osteintegration after spacer removal.

Methods: PMMA-induced membranes were obtained in the three sites of 15 rabbits. Subsequent new bone formation was studied in the same environments in 24 other rabbits. Six weeks after the initial implantation, PMMA spacers were replaced with iliac autografts. Animals were euthanized at 2, 4, and 8 weeks postoperatively. Tissue samples were harvested and

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stained with hematoxylin and eosin. The histological characteristics of the membrane (thickness and microvessel density) and the newly-formed bone (cortical thickness) were quantitatively analyzed.

**Results:** The membranes in the subcutaneous sites developed quicker, were thicker and had the lowest microvessel density \(P<0.01\). The membranes in the intramuscular sites developed later and were thinner \(P<0.01\). The membranes in the osseous defects had the greatest microvessel density \(P<0.01\). After bone grafting, induced membranes became thinner and their microvessel density decreased substantially, but maintained better in osseous site. The newly-formed bone that developed in the radius defects, had the thickest cortices \(P<0.01\).

**Conclusions:** The evolution of membranes induced in the intramuscular and subcutaneous environments was close to that of the bone defect model, although bone formation appeared weaker.

**Level of evidence:** Basic science study III.

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**Introduction**

The induced membrane technique described by Masquelet et al. has emerged as a well-established technique of reconstructing large bone defects of the tibia, femur, humerus, hand, wrist, ulna and mandibular [1–5]. In contrast to the femur and tibia that are covered by muscles, the mandibular and wrist bones are subcutaneous. These anatomic differences may be of importance, because surrounding soft tissues are hypothesized to influence the histological characteristics of membranes [6]. In particular, plastic surgery is often needed in association with this large bone reconstructions [6,7] and it is therefore not known which kind of plastic surgery (muscular or facio-cutaneous flap) would optimize bone osteointegration within a bone defect reconstructed using the induced-membrane technique.

To the best of our knowledge, no study has yet investigated the difference of induced-membrane characteristics in these different environments (intramuscular or subcutaneous). Furthermore, it is not known how much time induced membranes would maintain their histological characteristics and effect on bone healing following bone grafting.

We hypothesized that membrane characteristics would be influenced by the soft-tissue environment subcutaneous or muscular. To test this hypothesis, the histological characteristics of induced-membrane and of the subsequent bone formation in subcutaneous, intramuscular and osseous sites were compared. The protocol was built so as to respond to 3 questions:

- is the model of paravertebral implantation (subcutaneous and intramuscular) realistic, compared to a real bone defect model of the distal radius?
- are the membrane characteristics in the 2 sites comparable (subcutaneous and intramuscular)?
- what are the degradation characteristics of induced membrane in the 3 sites after completion of the bone graft?

**Method**

**Animal**

Thirty-nine New Zealand white rabbits from the experimental animal center of Wenzhou Medical University were operated on (avg wt: 3.0 kg) under general anesthesia using a solution of Chloral Hydrate (Solarbio, China) in semi-sterile conditions.

**Surgical procedure**

In 15 rabbits, PMMA cylinders (4.5 mm in diameter, 15 mm in length) were moulded ex vivo and placed into bilateral subcutaneous, intramuscular sites and radial defects. Two PMMA cylinders were respectively placed in paravertebral muscles and in the subcutaneous tissues of the lumbar area. In the radius defect model serving as a control, a 3-cm longitudinal incision was made along the lower third of the forearm. A 15-mm bone resection was performed in each distal radius, before being filled using a PMMA cylinder. Penicillin 20,000 IU/kg was administered intramuscularly immediately preoperatively and 24, 48 hours postoperatively. The operated limbs were immobilized for 2–8 weeks using splints. Five animals were euthanized and the ten specimens of each site served for the histological examination at 2, 4 and 8 weeks postoperatively, respectively.

Twenty-four other rabbits served for studying the evolution of the membrane after bone graft completion. The animals underwent unilateral implantation of PMMA cylinders in the three sites. The cylinders were then removed at 6 weeks and the voids in the induced membrane were filled in with morcelized bone autografts harvested from the iliac crests. Finally the membranes incisions were sutured using 4-0 prolene. Postoperative anti-inflammatory drugs (Penicillin 20,000 IU/kg) were ordered and a splint immobilization was used. Eight animals were euthanized and the eight specimens of each site served for the histological examination at 2, 4 and 8 weeks postoperatively, respectively.

**Histological examination**

Tissue samples were fixed in 10% formalin. Samples containing bone tissue were additionally demineralized in a 10% formic acid solution. Cross-sections of the membrane and longitudinal sections through the mid-sagittal plane of the bone samples were obtained and embedded in paraffin, stained with hematoxylin and eosin, and examined with a microscope (Olympus, Japan) and photographed...
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(Sony, Tokyo, Japan). There were 10 specimens in the first series and 8 specimens in the second series in each site at each time point. Three measurements were carried out in 3 separate fields of each specimen and averaged. Computed-assisted measurements of the histological parameters (membrane thickness, microvessel density and cortical bone thickness) were obtained using an automated image analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA). Membrane thickness was defined as the distance separating the cement from the adjacent connective tissues. Microvessel density was defined as the number of microvessels per membrane area in microscopic fields (×400). Cortical bone thickness was defined as the distance between the marrow cavity and the membrane. The difference of membrane thickness and microvessel density between the end and the beginning of each time interval were calculated, so as to determine the speed of membrane growth and microvessel proliferation per week.

Statistical analysis

Statistical analysis was performed with SPSS16 software (SPSS Inc., Chicago, IL). The non-parametric Kruskal-Wallis test was used to examine differences in membrane thickness and microvessel density among different sites at each time point. Cortical bone thickness was compared among different sites using one-way analysis of variance. The least-significant difference (LSD) test was used for pairwise comparison. Differences were considered as statistically significant with a P-value of less than 0.05.

Result

Induced membranes in the distal radius were thinner but displayed a greater microvessel density. Substantial differences appeared between the 2 subcutaneous models, the former in the paravertebral region, the latter in the distal radius. Microvessel density was lower and progressed slower in the non-osseous site. Mean membrane thickness was 256.31 ± 92.52 μm, and 178.44 ± 50.94 μm in the subcutaneous and the distal radius, respectively (P < 0.01, Figs. 1 and 2). The mean speed of thickness increase within the first 4 weeks was 50.61 ± 21.85 μm/week, and 24.63 ± 6.45 μm/week respectively (P < 0.01). Membranes showed a maximum microvessel density of 7.03 ± 2.09 × 10⁴/μm², and 32.71 ± 8.10 × 10⁴/μm² in the subcutaneous, and the distal radius respectively. The mean speed of microvessel density proliferation within the first 4 weeks was 1.76 ± 0.52 × 10⁴/μm² week and 7.05 ± 1.35 × 10⁴/μm² week respectively (P < 0.01).

There were also substantial differences between the 2 non-osseous sites. Induced membranes were thicker in
the subcutaneous site and displayed a lower microvessel density. Mean membrane thickness was 256.31 ± 92.52 μm, 55.43 ± 10.41 μm in the subcutaneous, and in the intramuscular site respectively (P < 0.01, Fig. 1). The mean speed of thickness increase within the first 4 weeks was 50.61 ± 21.85 μm/week, 13.85 ± 2.60 μm/week (P < 0.01). Membranes showed a maximum microvessel density of 7.03 ± 2.09 × 10^4/μm^2, 15.32 ± 3.2 × 10^4/μm^2 in the subcutaneous and intramuscular site respectively. The mean speed of microvessel density proliferation within the first 4 weeks was 1.76 ± 0.52 × 10^4/μm^2 week and 3.83 ± 0.80 × 10^4/μm^2 week respectively (P < 0.01).

In the rabbits that received a bone graft within their induced membrane, a significant and constant decrease in membrane thickness and their microvessel density was observed (Figs. 2 and 3). It was quicker in the subcutaneous site and slower in the intramuscular site than in the osseous site. Four-weeks membrane thicknesses were 4.72%, 59.34% and 17.04% of the original membrane thickness in the subcutaneous, intramuscular and osseous site, respectively. The 2-week microvessel densities were 14.90%, 58.10% and 14.29% of their original values in the subcutaneous, intramuscular and osseous site, respectively.

Although their membranes underwent the slower degeneration, bone cortices reabsorbed (Figs. 3 and 4) significantly in the intramuscular site only (P < 0.01). Finally (8 th week), cortical thickness was best preserved in the distal radius.

**Discussion**

The main findings of the present work are that the induced membrane formed quicker in the non-osseous site, but they appeared less active, with a lower microvessel density. It suggests that the subcutaneous paravertebral implantation may underestimate the real osteogenic properties of induced membranes developing in a real bone defect. The same difference was observed between the 2 paravertebral implantations, respectively in the subcutaneous and the intramuscular site. Thicker induced membrane was observed in subcutaneous sites, which may play a mechanical role in preventing soft tissue protrusion [2], subsequently trying to slow down bone resorption. In fact bone resorption was the greatest in the intramuscular site, which had the thinner membrane.

As the membrane in different sites showed different thickness in our study, a possible explanation was warranted: the tissue origin of fibroblasts is a crucial factor for membrane thickness. Fibroblasts are the major components of the induced membrane [8] and induced membrane is a product of soft-tissue reaction to the PWMA spacer [9]. A significant cellular response is activated when implants are placed in the subcutaneous site [10], leading to thicker fibrous membrane. Whereas, in intramuscular environments, only early-appearing myogenic cells differentiate into myofibroblasts [11], leading to a relatively thinner one.

Second, although the paravertebral implantation represents an imperfect model of segmental bone defects, membrane characteristics follow the same evolution over time. These results suggest that a subcutaneous environment would produce less bone resorption than a muscular environment. It shows that the induced membrane technique suits to subcutaneous defects, and looks to justify the use of facio-cutaneous flap associated with the skeleton reconstruction in the treatment of large post-traumatic segmental defects. Previous studies [12–14] reported that no difference in the rate of complications and failures was found between muscular and facio-cutaneous flaps. Regarding to their osteogenic properties, further clinical investigations should be conducted to examine the characteristic of induced membranes covered by muscular or facio-cutaneous flaps.

Third, the membranes in each site exhibited significant degradation after bone graft implantation inside the membrane cavity. Although the membranes in the osseous site displayed a moderate thickness, they had a slow degradation speed, and induced the stronger bone formation. In the contrary, the membrane in non-osseous site did not prevent graft resorption in particular in the intramuscular environment. It is possible that membrane degradation provoked direct bone graft contact with the surrounding soft tissues, thus favoring bone resorption. Clinical
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Figure 3  Photomicrographs of representative sections show the histological changes of membrane degradation and new bone formation in the phase of membrane degradation in intramuscular tissue (a), subcutaneous tissue (b) and radial defects (c) of rabbits. 1 = 2 weeks, 2 = 4 weeks, 3 = 8 weeks (ST, subcutaneous tissue; IM, induced membrane; M, muscle; NB, new bone; BG, bone graft; C, capillary; EO, endochondral ossification. Hematoxylin and eosin staining; original magnification 200×).

Figure 4  Evolution of cortical bone thickness following bone grafting inside the induced membrane in the 3 sites of implantation. The y-axis indicates the thickness of new cortical bone in micrometers (±1SD). The control is represented by the mean thickness of non-operated distal radius in the rabbit.

experience also suggested that morsellized autologous bone graft reabsorbed if placed within a well-vascularised muscu-
lar environment [15,16]. The microvessel density decreased after bone graft, raising the question of the capacity of these membranes to promote durably bone vascularization. Some authors suggested that induced membranes could facilitate vascularization of bone graft [5,8], because they observed no resorption of the within-membrane grafts [6], in the con-
trary to other studies [4,17].

Based on the current study, we showed that the soft-tissue environment influenced the characteristics of the induced-membrane, as well in the formation than in the degradation phase. The subcutaneous paravertebral implantations may not represent an ideal model of the bone defects in the osseous sites. Although their membrane were thicker, and their degradation speed in the subcutaneous site was close to that of the distal radius, they had a lower microves-
sel density, and were finally not able to optimize bone formation as well as the induced membrane of the distal radius. In addition, the phenomenon of membrane degrada-
tion cannot be ignored and its maintenance is challenging.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.
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