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Scaffold implantation in the omentum majus of rabbits for new bone formation

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ABSTRACT

Restoration of the mandible after defects caused by ablative surgery remains challenging. Microvascular free flaps from the scapula, fibula or iliac crest remain the 'gold standard'. A drawback of these methods is donor-side morbidity, availability and the shape of the bone. Former cases have shown that prefabrication of a customized bone flap in the latissimus dorsi muscle may be successful; however, this method is still associated with high donor-side morbidity. Osteogenesis in the omentum majus of rabbits by wrapping the periosteum into it was confirmed recently and is particularly interesting for bone endocultivation.

Twelve adult male New Zealand white rabbits were used. In each, two hydroxyapatite blocks were implanted in the greater omentum with autologous bone or autologous bone + rhBMP-2.

Bone density measurements were performed by CT scans. Fluorochrome labelling was used for new bone formation detection. The animals were sacrificed at week 10, and the specimens were harvested for histological and histomorphometric analysis. In histological and fluorescence microscopic analysis, new bone formation could be found, as well as new blood vessels and connective tissue. No significant differences were found regarding the histological analysis and bone density measurements between the groups.

It could be demonstrated that the omentum majus is a practical way to use one's own body as a bioreactor for prefabrication of tissue-engineered bony constructs. Regarding the influence and exact dose of rhBMP-2, further research is necessary. To establish and improve this method, further large-animal experimental studies are also necessary.

1. Introduction

Presently, the reconstruction of defects in the mandible remains challenging. After trauma, ablative oncological treatment or severe infections, large defects of the continuity of the bone occur. Moreover, radiotherapy disturbs soft-tissue integrity.

The complex anatomy of the mandible consists of a parabola-shaped bone with a centrally located canal with the alveolar nerve and artery. During mastication, the bone is stressed in different ways; therefore, adequate strength is needed. The typical donor regions for autologous microvascular bone flaps are the fibula, scapula, and iliac crest, and they are established in daily use (Warnke et al., 2004). However, there are different drawbacks of these donor regions such that there is a need for adequate bone block culturing (Clavero and Lundgren, 2003; Giannoudis et al., 2005; Nkenke et al., 2002; Summers and Eisenstein, 1989). The aim of ectopic bone block culturing is the reconstruction of bone defects without creating donor site defects (Warnke et al., 2006). Autogenous grafts are the best choice to avoid host-versus-graft reactions and ensure good long-term results. Adequate stability of the transplant can be reached by

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osseointegration of the graft only (Moore et al., 2001). To reach these goals for mandible reconstruction, different in vitro and in vivo trials have been performed. To avoid donor-site morbidity, synthetic bone substitute materials, e.g., hydroxyapatite (HA), tricalcium phosphate (TCP), bioactive silicon, polymers, and composites, have been developed in the last decade (Giannoudis et al., 2005; Van Heest and Swiontkowski, 1999). The key points of these materials are the surface that should induce neoosteogenesis (Cypher and Grossman, 1996; Giannoudis et al., 2005). Osteoinduction can be reached by mesenchymal stem cell activation, which osteoblasts use to differentiate themselves. All synthetic bone substitute materials are osteoconductive only (Bishop and Einhorn, 2007). Bovine hydroxyapatite is well described in the literature as an osteoconductive material with an adequate porosity comparable to that of human bone (Acil et al., 2000; Warnke et al., 2004). Porosity has the advantage of a lead compound for the new bone and vascularization (Janssen et al., 2006). Due to the missing osteoinductivity of hydroxyapatite, there is a need for stimulation by proteins. Since 1988, bone morphogenic proteins (BMPs) have described that stimulation from stem cells to osteoblasts is possible when different trials were performed (Wang et al., 1988; Wozney et al., 1988; Kirker-Head, 2000; Yamaguchi et al., 1996). Implantation of bone substitute material leads to inflammation, which increases neovascularization (Inoda et al., 2007). Multipotent cells become expressed by neoangiogenesis and can be differentiated into bone-forming cells (Katagiri et al., 1994; Yamaguchi et al., 1996). BMPs induce proliferation and differentiation in the cells (Sampath et al., 1992; Yamashita et al., 1996). In some cases, higher osteoinduction was observed by the application of BMPs in muscles (Katagiri et al., 1997; Yoshida et al., 1998). Higher osteoinduction in the muscle is explained by immature cells, myoblasts that could be differentiated into osteoblasts by BMP stimulation (Bosch et al., 2000; Levy et al., 2001). In animal trials, the latissimus dorsi muscle was used for adequate ectopic bone culturing (Okubo et al., 2000; Terheyden et al., 2001a, b). In 2004, a case report documented the reconstruction of a mandible after culturing a bone block with titanium meshes, hydroxyapatite and rhBMP-7 in the latissimus dorsi muscle (Warnke et al., 2004, 2006). However, there were also drawbacks, e.g., the loss of function of the muscle due to nerve damage of the thoracodorsal nerve. Therefore, another area for ectopic bone culturing is needed. The greater omentum showed adequate results in soft-tissue reconstruction in the head and neck (Bayles and Hayden, 2008). Kamei et al. showed osteogenesis in the omentum of the rabbit for the first time in 2010 using the periosteum as the origin for the cells. Bone marrow formation and woven bone were observed (Kamei et al., 2010). This leads to the conclusion that the omentum is an area for ectopic bone formation using hydroxyapatite as a scaffold. Moreover, the omentum consists of fat compartments where the expression of VEGF with angiogenetic effects is increased (Goldsmith et al., 1984; Zhang et al., 1997). In this study, new bone formation was investigated using hydroxyapatite blocks with autologous cancellous bone plus rhBMP_2 after insertion into the greater omentum in rabbits.

2. Material and methods

As experimental animals, twelve New Zealand White Rabbits (Charles River, Sulzfeld, Germany) with an average age of 8 months and a total weight of 3.5–4.0 kg were used. Two groups were created:

1) Hydroxyapatite blocks + autologous bone;
2) Hydroxyapatite blocks + autologous bone + rhBMP-2 (200 μg).

This study was approved by the Ministry of Environment Schleswig–Holstein (No. V 312-72241.121-14 (73-6/11).

2.1. Anaesthesia

A combination of 0.5 ml of Ketamin (Ketamin 10 %; Bremer Pharma GmbH, Warburg, Germany) and 0,25 ml of Xylazin per kilogram bodyweight (Rompun®¬ 2 Pharma %; KVP Pharma + Veterinärprodukte GmbH, Kiel, Deutschland) was injected intraperitoneally for general anaesthesia. If necessary, narcosis could be extended and deepened by administering 1/3 of the maintenance dose. Local anaesthesic (1,2 ml of Citocain, Sopria Citocain, 40 mg/ml of Articain hydrochloride, 10 mg/ml of Epinephrine; Heraeus Kulzer GmbG, Hanau, Germany) was injected at the incision area to minimize pain and bleeding.

For analgesia and prophylaxis of infection, 0,3 ml of Carprofen (Rimadyl, 4 mg/kg bodyweight; Zoetis Deutschland GmbH, Berlin, Germany) and 0,5 ml of Penicillin G (40.000 U/24 h, Grünenthal GmbH, Aachen, Germany) were injected subcutaneously for 5 days postoperatively.

2.2. Operation procedure

The scaffold used for prefabrication was a hydroxyapatite block (Bio-Oss®; Geistlich Pharma, Wollhusen, Switzerland) of 10 × 10 × 20 mm. Two blocks were implanted in the greater omentum of each animal. Before implanting the scaffolds, a central canal was milled into the block to facilitate placing a central blood vessel into the gap to imitate the mandibular canal and improve the blood supply (Fig. 1). Furthermore, the blocks were flushed with NaCl solution before implanting them. First, autologous bone was taken from the iliac crest. After shaving and disinfecting the hip region, the iliac crest was carefully prepared and exposed. Two to three pieces of iliac crest bone were removed with a Luer Tong and milled in a bone mill. Subsequently, the wound was closed in layers. The blocks were soaked with blood of the iliac crest wound and covered with the milled bone. Thereafter, the rabbit was turned over, and an abdominal incision of 10 cm was performed to expose the greater omentum. Two large arterial blood vessels were prepared and put into the milled canals of the blood-soaked and bone-covered hydroxyapatite scaffolds. In one animal group, rhBMP-2 (200 μg) dissolved in 1 ml of NaCl solution was added to the implant before soaking with blood. The omentum majus was mobilized, and both blocks could be wrapped into the omentum and fixed with absorbable suture material (Vicryl 3-0, Ethicon GmbH, Norderstedt, Germany). The abdominal wound was also closed in layers, and the animals were cleaned with NaCl solution. A deposit of 4 ml of NaCl solution was injected subcutaneously for better regeneration.

For the first five days postoperatively, the rabbits were kept separated in single cages. This reduced the stress level and risk of injuries. Thereafter, they were accommodated in groups of 6 animals according to the experimental groups.

2.3. Fluorochrome sequence labelling

For histomorphometric imaging, fluorochrome markers were applied weekly by injecting them intraperitoneally through peripheral venous access (Braunüle®, green: 1,3 × 45 mm, G 18; Fa. B. Braun Melsungen AG, Melsungen, Deutschland) (Table 1).

2.4. Bone density measurement

With a CT scan (SOMATOM Sensation64; Siemens Healthcare GmbH, Erlangen, Germany), the bone density, indicated in Hounsfield Units (HU), was determined in the 2nd, 5th, 8th and 10th

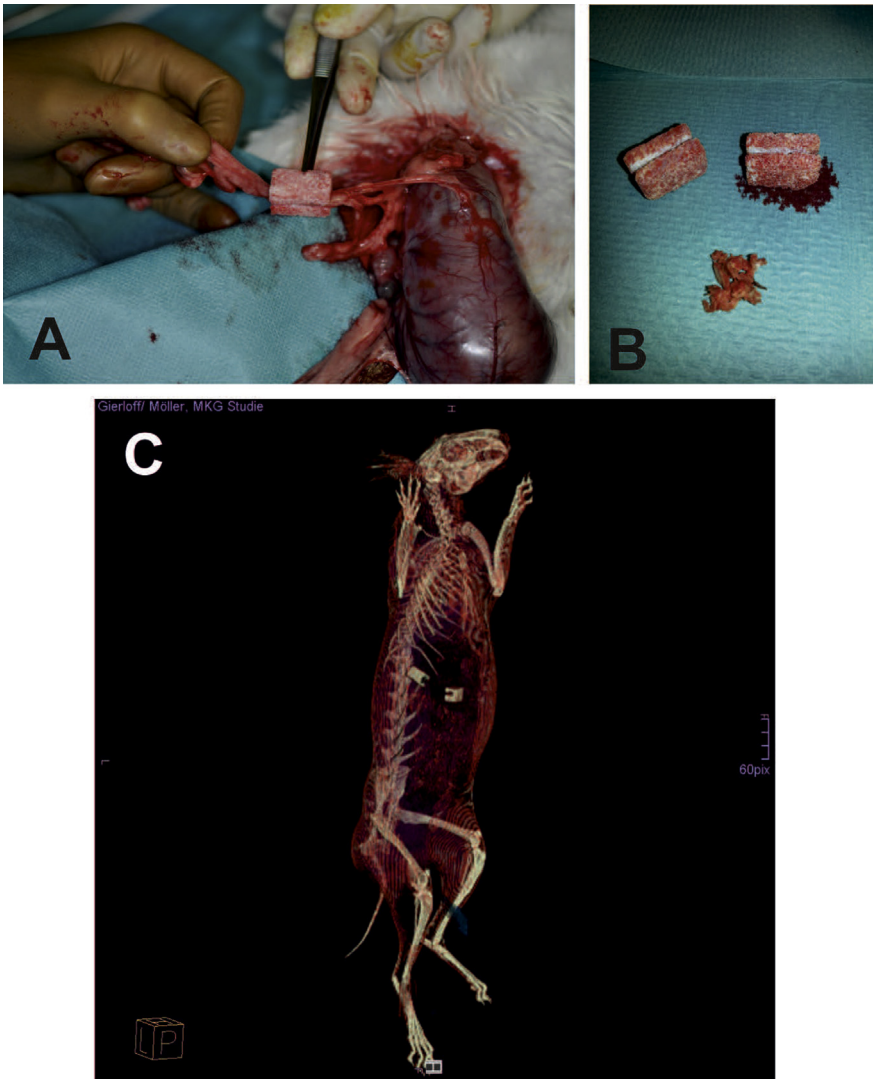

Fig. 1. Surgical procedures. A) Hydroxyapatite block with the blood vessel in the central canal before wrapping into the omentum. B) Hydroxyapatite blocks with the harvested autologous bone from the iliac crest. C) 3D CT data reconstruction with the two inserted blocks.

Table 1
Fluorescence labeling.

Fluorochrome	Dose	Time of labelling
Xylenol orange	6 % in 2 % $NaHCO_3$-solution, 1.5 ml/kg bw (Sigma–Aldrich, Steinheim, D)	2nd and 6th week after implantation
Calcein green	1 % in 2 % $NaHCO_3$-solution, 0.5 ml/kg bw (Sigma–Aldrich, Steinheim, D)	3rd and 7th week after implantation
Alizarin complexone	3 % in 2 % $NaHCO_3$-solution., 0.5 ml/kg bw (Sigma–Aldrich, Steinheim, D)	4th and 8th week after implantation
Calcein blue	0,04 % in 2 % $NaHCO_3$-solution, 1 ml/kg bw (Sigma–Aldrich, Steinheim, D)	5th and 9th week after implantation

weeks postoperatively. Imapx 6 (Agfa HealthCare GmbH, Bonn, Germany) was used to measure the bone density in two different areas in the hydroxyapatite block (two blocks each animal). The first area measured was adjacent to the milled canal and was named “centre”. The second area was located directly next to the first area, in the middle of the block, and was named “top”. To identify the correct point for the measurement, the CT-scan pictures of the blocks had to be turned and shown in a cross-sectional view. Both measured areas had a volume of 2 mm^3.

2.5. Specimen harvesting

Ten weeks postoperatively, all the animals were sacrificed by injecting 5 ml of potassium chloride intracardially. The sacrifice was performed under general anaesthesia. To explant the blocks (two blocks per animal), the same abdominal access was used that was used for implanting.

2.6. Histological sections

After explanting the preparations, every block (two blocks per animal) was split into two parts and fixed in formalin (10 %) for 2 weeks.

After being dehydrated in a PSI-embedding station (Pool of Scientific Instruments, Grünewald GmbH & Co KG, Laudenbach, Germany), the first half was fixed in an embedding medium in a refrigerator at 6 °C and was hardened in a glass vessel at 37 °C. To create a specimen of 40 μm in thickness, all preparations had to be

sawed (Metabo® GmbH, Bochum, Germany), ground (Struers® GmbH, Erkrath, Germany) and bonded to a slide using adhesive film (Technovit®, Exakt Apparatebau GmbH & Co. KG, Norderstedt, Germany). After fixing the preparation to the slide, it was ground and polished to the final thickness of 40 μm (Exakt Apparatebau GmbH & Co. KG, Norderstedt, Germany). Before washing out the fluorochromes and staining the preparations with Toluidine Blue, all the specimens were photographed in a 25× magnification in epifluorescence illumination (Axio Scan.Z1, Carl Zeiss AG, Oberkochen, Germany). Finally, the preparations were immersed in formic acid (0,1 %) for 3 min, cleaned with distilled water, immersed in methanol for 90 min and stained with a Toluidine blue solution for 2 min.

The second half was used for HE staining. The preparations were decalcified with 12,5 % ethylene-diamine-tetra-acetic acid solution (EDTA, pH 7,4) in an ultrasonic bath (Ultraschall-Entkalker USE 33, Medite, Burgdorf, Germany), dehydrated, embedded in paraffin (TES 99, Medite, Burgdorf, Germany) and cut into slices of approximately 5 μm in thickness (Microm International GmbH, Walldorf, Germany). To stain the preparations with HE, they had to be deparaffinized and rehydrated. The specimens were also photographed at 25× magnification.

2.7. Statistical evaluation of computed tomography

The Kolmogorov–Smirnov Test was used to check the normal distribution. To analyse the bone density measurements, the ANOVA-Post hoc-Tukey-Test was used. The average of all measurements was taken as reference values. Several graphs were designed (GraphPad Prism 6, GraphPad Software, Inc., La Jolla, USA). The statistical significance is $p > 0.05$.

3. Results

3.1. Clinic

The operation technique is very practicable. The abdominal wounds and wounds at the iliac crest showed normal wound healing without any infections. The animals were not restricted in eating or moving and showed no signs of pain. After the second CT scan, one animal of each group died, probably as a result of the narcosis. The autopsy showed no signs of intraperitoneal infection, wound infection or any other reason for death caused by the operation. The hydroxyapatite blocks were explanted, and the collected data up to that point were included in the studies.

3.2. Computed tomography

The following data were analysed and compared.

1) Development of the bone density from the 2nd to 10th weeks, both groups separately: No significant increase was found in the bone density during the experimental time period in both groups. In single measurements, there was a significant increase in the bone density in the "centre" area of the AUTOLOGOUS BONE-group (Fig. 2).
2) Development of the bone density in the "centre" and "top" areas from the 2nd to 10th weeks: The measurements showed a higher bone density in the "centre" area; however, the difference is not significant.
3) Comparison of the bone density of the two groups in the 5th and 10th weeks: In single measurements, the bone density of the AUTOLOGOUS BONE-group was significantly higher than that of the AUTOLOGOUS BONE-rhBMP-2-group in the 5th to 10th weeks (Fig. 2). In summary, no significant difference was found between the groups.

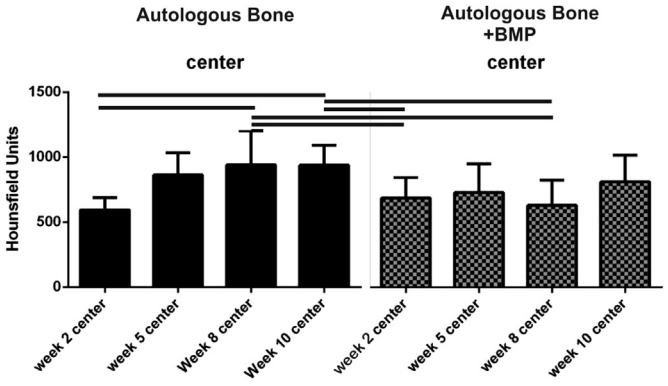

Fig. 2. Bone density of Group 1 and Group 2. Increased bone density was evaluated by CT data in Hounsfield units. However, the increased data are not reflected in the histological data for Group 1. The added rhBMP-2 did not increase the bone density in the CT data or in the histological sections in Group 2. The horizontal bar indicates $p \leq .05$.

3.3. Fluorescence microscopy

Chronological bone apposition during the experimental time is shown in polychromic sequential labelling. The staining bands of Xylenol-orange (2nd and 6th week) and Calcein-green (3rd and 7th week) can be seen in all specimens. The fluorochromes Alizarin and Calcein-blue cannot be seen in any specimen. The green and yellow bands of Xylenol-orange and Calcein-green are located at the margin of the preparations, and no difference was found between the groups. In general, there was no new bone formation; only single findings in some hydroxyapatite edges were observed (Fig. 3).

3.4. Toluidine Blue staining

Toluidine blue can stain even hard embedded preparations and colourize the different tissues in different blue shades. Bony structures appear dark purple and connective tissue appears lighter purple. The hydroxyapatite-free area in the central canal shows much fat and connective tissue, the central blood vessel that was implanted there, and even more vascularization. In some areas, bone apposition and some Haversian canals can be seen. No significant difference was found in bony apposition between the experimental groups.

3.5. Haematoxylin-Eosin staining

HE staining is used to provide an overview of the different tissues. There is connective and fat tissue in the cavities of the hydroxyapatite block. Additionally, there are many blood vessels,

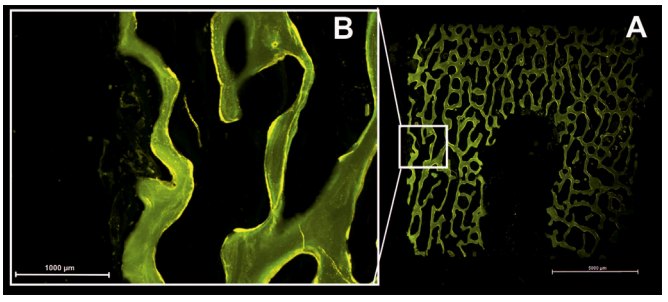

Fig. 3. Fluorescence microscopy. Hydroxyapatite block after fluorescence labelling.

mostly in the centre of the block, near the central canal, as suspected. No bony apposition was detected in the HE-stained specimens, and no difference was noted between the experimental groups (Fig. 4).

4. Discussion

The challenges of mandible reconstruction are felt daily by working with the patients. In part, the patients develop donor-site morbidity manifested as reduced activities, pain, or aesthetic aspects. Many approaches have been investigated in the last decades for bone cultivation and ectopic endocultivation of bone blocks (Beck-Broichsitter et al., 2015; Becker et al., 2012; Terheyden et al., 2001a, b; Warnke et al., 2004, 2006). Different proteins have been identified to improve bone metabolism. This study investigated a new method in the endocultivation of scaffolds. We used the omentum majus in rabbits as an accessible space for scaffolds. The ectopic bone cultivation was tried by autologous cancellous bone rinsed with rhBMP-2 wrapped into the omentum. The omentum majus was described as a region with sufficient space for scaffolds and the opportunity for new bone formation. In a trial with rabbit, bone specimens were wrapped into the omentum, and histological evaluations were performed. Within the first week, woven bone was formed (Kamei et al., 2010). In a case report with an individual treatment attempt after ablative oncological surgery of the jaw, the omentum was used for new bone formation. A titanium mesh cage with hydroxyapatite blocks and rhBMP-2 was inserted into the omentum. The reconstruction of the jaw was performed three months after insertion, and histological evaluations showed new bone formation (Wiltfang et al., 2016). Therefore, the omentum can be helpful in ectopic new bone formation due to adequate surgical access, good vascularization, and no additional bone donor-site morbidity. The CT data showed increasing Hounsfield units with a difference of approximately 300 HU compared with the initial values. There are different reports of increased HU within several weeks (Beck-Broichsitter et al., 2015). However, the increased HU did not reflect new bone formation within the inserted scaffolds. The increased values arose from soft-tissue invasion into the highly porous hydroxyapatite scaffolds. The histological evaluations by H&E and Toluidine blue staining showed much soft tissue with blood vessels and fat. New bone formation occurred in rare areas only; however, this was confirmed by fluorescence labelling.

Several investigations have reported on the subject of critical-size bone defect healing (DeNicolo et al., 2015). Ectopic new bone formation should also be designated as such. In a porcine model, bone marrow-derived stromal cells were compared with dental pulp-derived stromal cells (DPSCs) in defects with 15 × 10 mm. For DPSCs, superior bone healing was identified and explained by faster differentiation into the osteogenic lineage (Jensen et al., 2016). In another trial with rats, ectopic bone formation and significant healing were identified. Moreover, a good neovascularized environment promoted by the DPSCs was described (Petridis et al., 2015).

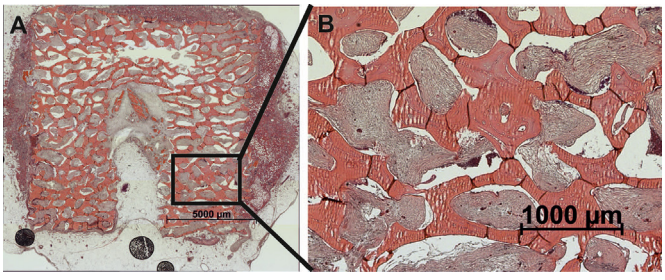

Fig. 4. Histological section with H&E staining. Hydroxyapatite block with soft-tissue invasion.

In the present study, cancellous bone was used; thus, remodelling processes could be too slow to observe intramembranous ossification in this model. A cartilage core needed for endochondral ossification, which is mechanically stimulated with stress, tension, or microdamage, could be an alternative method due to the complex biological stimulation of mesenchymal cells (Kamei et al., 2010).

A recent endocultivation trial in the latissimus dorsi muscle in rats with delayed rhBMP-2 injections showed better results for multi-dose application (Beck-Broichsitter et al., 2015). In all groups of this trial, 200 µg of rhBMP-2 was used but was divided into single doses of 66.6 µg or 100 µg. A higher blood vessel count and a higher bone density were observed in the groups with two single doses of 100 µg in week 1 and week 2 after scaffold insertion. The authors discussed an effective threshold dose of 100 µg due to poorer outcomes in the group with three single doses of 66.6 µg (Beck-Broichsitter et al., 2015). Next, the authors also discussed the endogenous expression of rhBMP-2 after three weeks and the imbalance of BMP-2 inhibitors (Beck-Broichsitter et al., 2015; Kloen et al., 2012; Nakagawa et al., 2001). The threshold dose in rabbits might be higher for the induction of neovascularization or bone formation than that in rats.

Many optimal modalities exist in the application of BMP. However, until now, the best application method has not been described and it is unclear what may have the strongest effect, e.g., dosage, time point of application, coating, or drug. Moreover, the kinetics of BMP are very complex, especially in living bioreactors, and differences exist in the muscle and omentum. In general, an imitation of the physiological processes will be the best method, indicating complete integration of the BMP into the resorbable scaffold. During resorption of the scaffold, the BMP will increase osteogenesis comparable to the remodelling process. Based on the present scaffold materials and manufacturing, this production is not possible today. The present techniques can coat the scaffolds with BMP by covalent bonding (Hettiaratchi et al., 2017; Kim et al., 2016; Wang et al., 2018).

This study possesses different limitations in interpreting the results. Osteoconductive and osteoinductive parts are needed to compare the critical size defects for ectopic new bone formation. The hydroxyapatite scaffolds were used in several trials and can be accepted as an ideal highly porous osteoconductive framework. However, this scaffold is quickly colonized by soft tissue cells that form hard tissue ruins. Therefore, compared with guided bone regeneration in dental surgery, a collagen membrane should be used to keep the soft tissue outside. There are different membranes described with various degradation times (Ghanaati, 2012). The membranes keep supporting the cancellous bone of the scaffold by diffusion until vascularization is completed. rhBMP-2 is one of many proteins that support bone healing or growth. Knowledge of the accurate amount of protein for stimulation is still lacking. Some reports have described the quantities that increase new bone formation; however, due to individual metabolism, there can only be an approximate approach. We used 200 µg for rabbits with a body weight of 4.0 kg and a scaffold of approximately 2.0 cm^3. It is possible that the used amount should be adjusted based on these aspects. Moreover, continuous application by a micropump for several weeks could influence the new bone formation.

Additionally, in fracture healing with two fixed fracture ends, micromovements are necessary as functional loads for stimulation. In the omentum, there is no adequate functional appeal for bone formation. Mechanical stimulation of the scaffolds may increase the bone formation by misleading the cells of the cancellous bone graft. This could be generated by reduced rigidity of the scaffolds in parts.

In our trial, the observation period was stopped after 10 weeks. This observation period was too short for sufficient results within the scaffolds. Fracture healing needs optimally approximately 6–8

weeks, which was not reflected by our set-up. The trial most likely reflects a critical size defect. Therefore, bone formation could be expected after 6 months or more.

5. Conclusion

The omentum majus is a technically adequate space for scaffold endocultivation. Soft-tissue invasion into the scaffolds should be reduced in further investigations with collagen membranes. Moreover, more investigations are needed concerning the threshold dose of rhBMP-2 for new bone formation in rabbits.

Ethics approval

This study was approved by the Ministry of Environment Schleswig–Holstein (No. V 312-72241.121-14 (73-6/11).

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Conflicts of interest

The authors declare that they have no competing interests.

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