Analysis of New Bone Formation Induced by Periosteal Distraction in a Rat Calvarium Model

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ABSTRACT

Background: A controlled, gradual distraction of the periosteum is expected to result in the formation of new bone.

Purpose: This study was designed to estimate the possibility of new bone formation by periosteal distraction in a rat calvarium model.

Material and Methods: Sixteen animals were subjected to a 7-day latency period and distraction rate at 0.4 mm/24 hours for 10 days. Two experimental groups with seven rats each were killed at 10 and 20 days of consolidation period and analyzed by means of microcomputed tomography, histologically and histomorphometry.

Results: In the central regions underneath the disk device, signs of both bone apposition and bone resorption were observed. Peripheral to the disc, new bone was consistently observed. This new bone was up to two and three times thicker than the original bone after a 10- and 20-day consolidation period, respectively. Signs of ongoing woven bone formation indicated that the stimulus for new bone formation was still present. There were no statistically significant differences regarding bone density, bone volume, and total bone height between the two groups.

Conclusion: The periosteal distraction model in the rat calvarium can stimulate the formation of considerable amounts of new bone.

KEY WORDS: bone, distraction osteogenesis, periosteum, rat

INTRODUCTION

Distraction osteogenesis (DO) is a technique that induces the formation of hard and soft tissues by a progressive elongation of the gap created by osteotomy.[1,2] In recent years, DO attracted attention in the craniofacial region, while avoiding the necessity of grafting hard or soft tissues.[3] DO was subsequently introduced as a predictable treatment of vertically deficient alveolar ridges,[4,5] recommended in cases with greater need for bone height.[6] Nevertheless, indications for alveolar DO may be limited with regard to the type and the stage of alveolar ridge resorption.[7] If the osteotomy results in a transport segment consisting only of the cortical bone, reduced blood supply and increased likelihood of secondary resorption may be expected.[3,8] A greater occurrence of mandibular fractures during the consolidation period should also be considered if the residual bone height is less then 10 mm.[9]

Compared with conventional DO, the distraction gap formed by periosteal DO (PDO) is bordered by the original, intact surface of the bone base and by the periosteal (i.e., cambial) layer. A gradual distraction of the periosteum from the original bone surface is expected to result in the formation of new bone so the need for performing an osteotomy and therefore the difficulties associated with conventional DO may be avoided.

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Several experimental studies reported the formation of new bone following PDO; variations, however, existed regarding animal models, site and surgical technique, the rate of augmentation, as well as the length of the consolidation period.[10–14]

The evidence of new bone formation was demonstrated using a disc with screw on the rabbit calvarium, but the instability of the distraction screw frequently caused displacement of the distraction device.[13] On the lateral surface of the rabbit mandible, a distraction mesh was elevated using a screw supported by an external U-shaped body.[10,12,15] Following the activation of 7 mm and consolidation period of 60 days, an average bone gain of 1.4 mm[15] and 2.9 mm[10] was achieved. Incomplete bone formation was attributed to the disengagement of the mesh from the central screw.[10]

New bone formation on the basal bone of the rabbit mandible was found underneath the entire screw-supported titanium mesh.[16] Surrounding soft tissue pressure caused an insufficient elevation of the peripheral part of the titanium mesh and transformation of the mesh into the form of a tent. Approximately twice the original calvarial bone height was achieved, when the mesh plate was elevated at one end by distraction screw in rabbits[17] or using two distraction screws in minipigs.[11] While the previous reports demonstrated the evidence of new bone formation in bigger animals, the purpose of this study is to estimate the possibility of new bone formation by PDO using a rat model.

MATERIALS AND METHODS

Housing and experiments were in accordance with the European communities Council Directive (86/609/EEC) for the care and use of laboratory animals. The animals were housed in a specialized animal facility with an adjusted climate (temperature 22–24 ± 2°C, humidity 30–60 ± 5%, a light/dark cycle of 14:10 hours) with standard rodent diet and water ad libitum. The rats were kept in groups of three prior to surgery and thereafter, single in environment cages. The protocol was approved by the Committee for Animal Research, State of Bern, Switzerland (approval no. 117/07).

Surgery was performed under conditions of general anesthesia induced by subcutaneous injection of ketamine (70 mg/kg) and medetomidine (0.3 mg/kg). After induction, the rats were placed in a prone position on a heating pad to keep body temperature at 37°. Anesthesia was maintained with isoflurane/O_2 (0.5–2%) delivered by a Bain system via face mask. Animal evaluation during surgery was performed by clinical and instrumental monitoring (Datex As3, Datex-Ohmeda Inc., WI, USA) of physiological parameters (T°, Electrocardiography, Respiration, Et CO_2 and ETiso, SpO_2). Perioperative analgesia was provided by the balanced anesthesia protocol thanks to the analgesic properties of ketamine and medetomidine. Before surgical procedure, a local anesthesia was performed with mepivacaine (10 mg/ml) and adrenaline (1:200,000). At awakening, the rats received one dose of meloxicam SC (0.2 mg/kg) and thereafter, the same dose once a day for 4 days post-op.

The operative area was washed and aseptically prepared with iodine solution and sprayed with 70% ethanol. A midsagittal incision was made through the skin and periosteum. The skin was reflected and the periosteal flap carefully elevated from the forehead to expose the calvarial bone on both sides of the midline to the fossa temporalis. The experimental device used was a hemispherical disc of 0.5 mm in height with a disc diameter of 8 mm, which is surrounded by a bordering ring, likewise 0.5 mm in height (Figure 1). The wound was closed in layers, with the distraction screw protruding the skin.

The distraction protocol for devices of both diameters included a latency period of 7 days and a distraction rate at 0.4 mm/24 hours for 10 days. Sixteen animals were divided in two groups with a consolidation periods of 10 (group I) and 20 days (group II), respectively. Euthanasia was performed by CO_2 overdose after placement in an empty Plexiglas box.

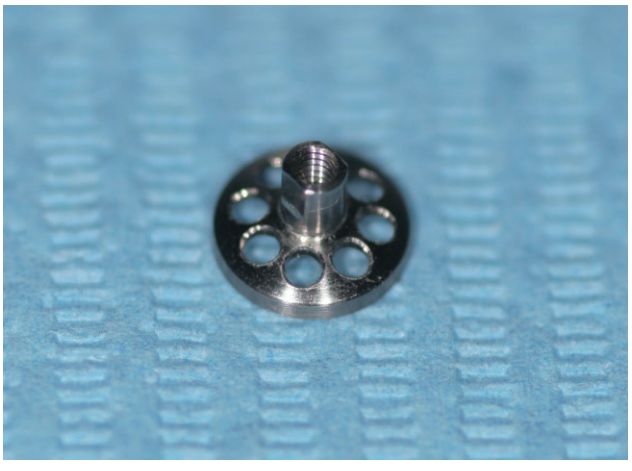

Figure 1 Illustration of disc distraction device.

Micro-CT Analysis

Following sacrifice, the calvariae were block-resected using the oscillating autopsy saw. The distraction site was subjected to radiography (25 kVP for 10 seconds) in two projections.

All scans were made by µCT 40 (Scanco Medical AG, Brüttisellen, Switzerland), a desktop cone beam scanner with the following parameters: the X-ray source (E) was set at 70 kVp with 114 µA at high resolution (1,000 projections/180°), which showed an image matrix of 2,048 x 2,048 pixels. The diameter of the sample holder was 30.7 mm, which allowed an increment (resolution) of 15 µm (=voxel size). Integration time was set at 3 seconds. The µCT slices (700) were reconstructed perpendicular to the saggital axis of the calvarium. At the lateral side to the disc for each animal, the region was selected manually. The evaluation of the reconstructed two-dimensional images was made with a 3D Segmentation of Volume of Interest (Scanco Medical AG), gauss sigma at 0.8, and gauss support at 1. Bone mineral density (mg HA/mm^3), bone volume, and total bone height were determined.

Comparisons using paired *t*-test were made to ascertain differences of bone mineral density, bone volume, and bone height between the two groups. An independent *t*-test was used to compare differences between the groups. The statistical analysis was processed using SPSS for Windows (release 17.0, standard version; SPSS®, Chicago, IL, USA).

Histologic and Histomorphometric Analysis

Prior to histologic preparation, the recovered segments were immediately immersed in a solution of 4% buffered paraformaldehyde combined with 1% $CaCl_2$ for at least 48hours at ambient temperature. The specimens were processed for the production of undecalcified ground sections as described by Schenk and colleagues.[18] Briefly, the samples were rinsed in running tap water, dehydrated in ascending concentrations of ethanol and embedded in methylmethacrylate. Ten tissue slices cut in the axis of the distraction device into approximately 400 µm-thick ground section using a slow-speed diamond saw (Varicut® VC-50, Leco, Munich, Germany). After mounting the sections onto acrylic glass slabs, they were ground and polished to a final thickness of about 100 µm (Knuth-Rotor-3, Struers, Rodovre/Copenhagen, Denmark) and surface-stained with basic fuchsin and toluidine blue/McNeal. Digital photography was performed using a ProgRes® C5 digital camera (Jenoptik Laser, Optik, Systeme GmbH, Jena, Germany) connected to a Zeiss Axioplan microscope (Carl Zeiss, Göttingen, Germany).

All available ground sections were used for histomorphometric measurements of the total bone thickness at various regions. Measurements were made underneath and peripheral to the distraction device. Two excluded animals were counted as dropouts.

RESULTS

Qualitative Histological Analysis

Two rats were excluded from the study because of postoperative death in one animal and exposure and loss of the device during activation in the other animal. The remaining 14 rats were killed at two time points with seven animals per group. Healing was uneventful and all 14 animals showed a normal behavior throughout the experimental period. There were no histological signs of further exposure of the distraction devices and no infections observed. All observations were similar for all sites for a given consolidation period. At the entry site of the screw into the skin, a minimal and locally confined inflammatory reaction was observed. Regarding new bone formation, findings in the µ-CT images corresponded to the observations made in the histological sections. Inspection of all ground sections revealed that it was necessary to make a distinction between two regions, within the distraction device and peripheral to the distraction device.

Group I (10 Days Consolidation Period)

At the periphery of the distraction device, a thick layer of new bone was observed on top of the old bone of the calvaria (Figure 2A). At some sites, the thickness of the new bone layer exceeded that of the old calvarial bone (Figure 2B). For all the samples, the thickness of the new bone decreased with increasing distance from the distraction device. The new bone was composed of cavities of immature bone marrow and woven bone reinforced by parallel-fibered bone. Osteoid and osteoblasts were present at various sites within the newly formed bone. The surface contour of the newly formed bone was even and covered by a distinct periosteal layer. New bone and old bone, composed of a tabula externa and a tabula

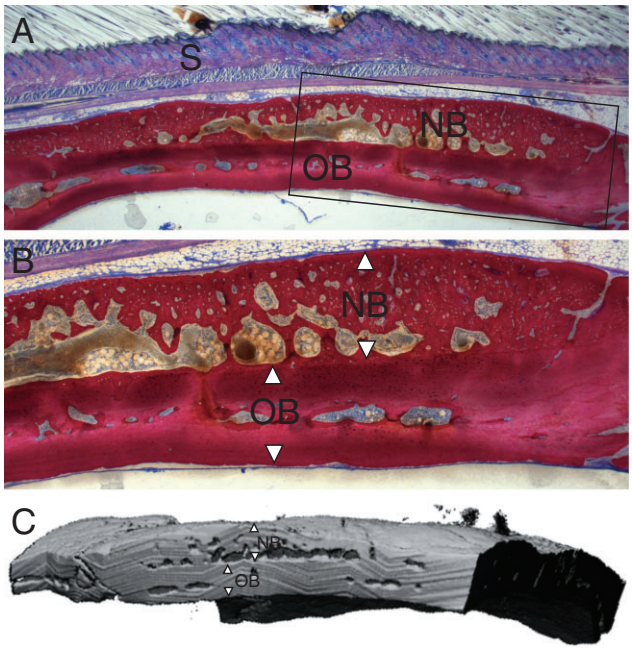

Figure 2 Transversal histological section *A*, *B*, and μ-CT *C* of the calvarium at the periphery of the distraction device after a consolidation period of 10 days. The rectangle in *A* is enlarged in *B*. *A*, Overview showing the old bone (OB) of the calvarium and the new bone (NB), which formed between the OB and the skin (S) of the skull. *B*, The OB of the calvarium consists of the tabula interna, the tabula externa, and an intervening mature bone marrow. The new bone is thicker than the OB layer and consists of immature bone and large bone marrow cavities. *C*, The μ-CT image illustrates a thick layer of newly formed bone on top of the old calvarial bone.

interna with intervening small marrow cavities, were recognizable histologically (Figure 2B) and in the μ-CT image (Figure 2C).

In the region covered by the disk of the distraction device, both bone apposition and bone resorption were observed (Figure 3A). Bone resorption occurred at sites were the screw or the tilted disk of the distraction device touched the bone surface. Numerous Howship's lacunae and osteoclasts were present at these sites. At sites not affected by bone resorption, a thickening of bone was observed (Figure 3, A–C). Similar to at peripheral sites, there were sites where the thickness of the new bone layer exceeded that of the old calvarial bone (Figure 3B). The new bone was composed of woven bone reinforced by parallel-fibered bone. Osteoblasts and osteoid were seen. A distinct periosteal layer was not discernible in the center of the distraction device, but the distracted space between bone and the disk was occupied by a highly vascularized loose connective tissue (Figure 3A). Furthermore, remnants of the coagulum and granulation tissue were observed toward the bone surface.

Group II (20 Days Consolidation Period)

At peripheral sites, the same observations as for the 10-day consolidation period were made for the 20-day consolidation group with the following exceptions (Figure 4). The maximum thickness of the newly formed bone at peripheral sites exceeded by far that observed after a 10-day consolidation group (compare Figure 4, B and C with Figure 2B). Fine orbicular structures of woven bone indicative of ongoing bone apposition were observed at the leading edge of bone

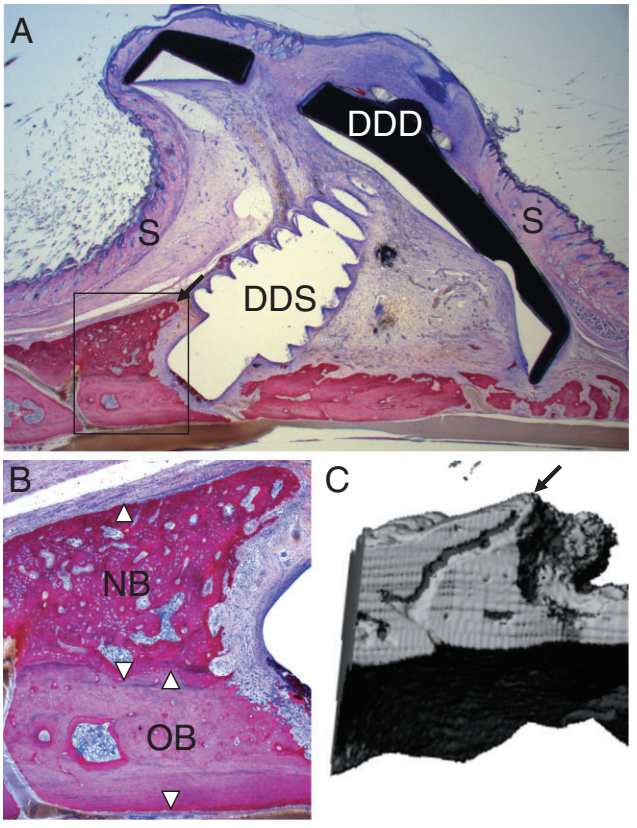

Figure 3 Transversal histological section *A*, *B*, and μ-CT *C* of the calvarium illustrating the central region of the distraction device after a consolidation period of 10 days. The rectangle in *A* is enlarged in *B*. *A*, Overview showing the empty space where the screw of the distraction device (DDS) was located and the disc of the distraction device (DDD), which is completely covered by the skin (S) of the skull. Bone resorption is evident at sites where the screw and the disc came in contact with the bone surface. *B*, Right adjacent to one resorption site, new bone (NB) formation is evident on top of the old bone (OB). The maximum thickness of the newly formed bone is greater than that of the old calvarial bone layer. *C*, The μ-CT image illustrates the new bone lateral to the screw of the distraction device and matches the histological observations (arrows in *C* and *A*).

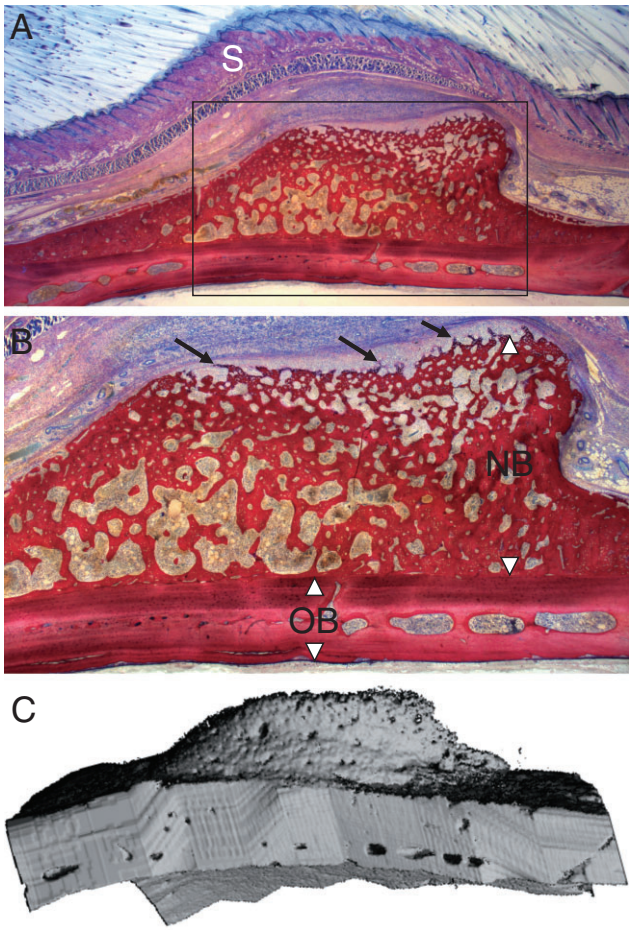

Figure 4 Transversal histological section *A*, *B*, and µ-CT *C* of the calvarium at the periphery of the distraction device after a consolidation period of 20 days. The rectangle in *A* is enlarged in *B*. *A*, Overview showing a massive thickening of the calvarium by newly formed bone. *B*, The new bone (NB) was up to three times thicker than the original thickness of the old bone (OB) of the calvarium. The new bone was more mature toward the interface to the old bone. Toward the skin (S), a fine trabecular network of woven bone (*arrows*) was indicative of ongoing bone formation. *C*, The µ-CT image matches the histological observations and shows vast new bone formation lateral to the distraction device.

formation facing the soft connective tissue, whereas the new bone appeared more mature toward the old bone (Figure 4B).

Similar to after the 10-consolidation period, new bone formation was observed also in the central region underneath the distraction device (Figure 5). Bone resorption sites were still recognizable at sites where the distraction device touched the bone surface and massive bone formation occurred next to the resorption sites (Figure 5A). The newly formed bone was composed of bone marrow cavities and woven bone reinforced by parallel-fibered bone (Figure 5B). A periosteal layer was lacking and the soft tissue between the new bone and the disk of the distraction device was highly vascularized. The total bone thickness as measured in the ground section (Figure 5B) precisely matches that obtained by micro-CT (Figure 5C).

Histometric and Statistical Analysis

Bone mineral density, bone volume, and maximal total bone height, as determined by µ-CT, are shown in

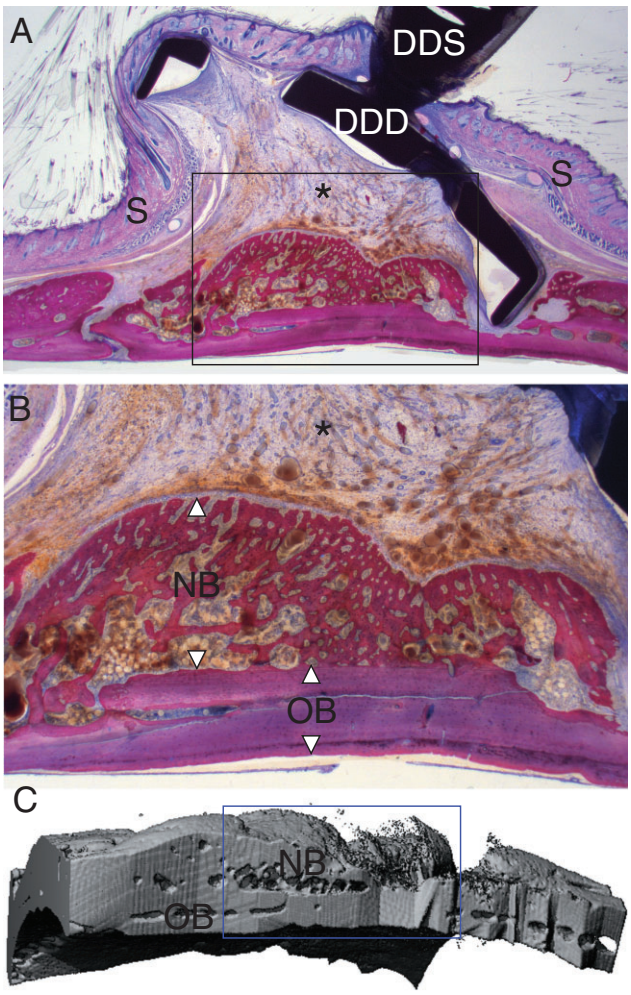

Figure 5 Transversal histological section *A*, *B*, and µ-CT *C* of the calvarium illustrating the central region of the distraction device covered by the skin (S) of the skull after a consolidation period of 20 days. The rectangle in *A* is enlarged in *B*. *A*, Overview showing the disk (DDD) and screw (DDS) of the distraction device. Bone resorption is evident where the disk touches the bone surface. In the space underneath the disk between the resorption site and the calvarial suture, new bone formation is evident. *B*, The thickness of the new bone (NB) is almost twice as much as that of the old bone (OB). The soft tissue adjacent to the new bone (*) is highly vascularized. *C*, The outlined area in the µ-CT image corresponds to *B* and illustrates the region of new bone formation underneath the distraction device. The massive increase in bone thickness corresponds to the observations made in the histological sections.

TABLE 1 Values of Bone Mineral Density, Bone Volume, and Maximal Bone Height for Both Groups of Animals as Measured on the μCT (All Values Are Expressed as Means ± SD)

Groups	Bone Mineral Density (mg HA/mm^3)	Bone Volume (mm^3)	Maximal Bone Height (mm)
I	105.3422 ± 11.93	862.1634 ± 18.37	2.07 ± 0.62
II	112.3682 ± 17.83	860.0991 ± 10.05	2.13 ± 0.46

Table 1. There were no statistically significant differences ($p > 0.05$) between the two groups. When determined histomorphometrically, the mean total bone thickness was 1.68 mm internal and 2.04 mm external to the distraction device for the 10-day consolidation period. The corresponding values were 1.82 mm and 2.16 mm for the 20-day consolidation period. In one animal, the maximum total bone thickness external to the distraction device was 2.80 mm. The mean thickness of the pristine calvaria for all animals was 0.72 mm. Thus, the total bone thickness amounted to approximately three times the thickness of the pristine calvaria, in one animal even up to four times.

DISCUSSION

The PDO technique is in line with the basic principle of tissue engineering by inducing the endogenous formation of hard and soft tissues. The application of PDO might be in cases of advanced ridge resorption in an attempt to avoid the drawbacks and limitations of standardized treatment modalities. Using different locations and distraction devices, the amount of newly formed bone following PDO was achieved to a various extent.[11,12,14] The results of the present study confirmed the augmentation of bone at the site of its apposition to the existing bone surface. Modifications of devices were suggested to prevent the tension of soft tissues,[10,16] but an increased rigidity of device might also harbor a risk of wound dehiscence, with plate exposure and subsequent site infection.[13] One of the 16 animals experienced such a problems in the present study. Calvarial bone has been demonstrated as an adequate model for skull and facial bone repair with a relatively simple surgical access.[19,20] Although displacement of the device occurred frequently, no further evidence of infection was encountered in the present study. The good head vascularization allowed a rapid tissue healing process with very low infection risk in the present model. Different outcomes regarding new bone formation were, however, achieved underneath and peripheral to the distraction disc. The bone in the central sections underneath the device, which was deprived from the periosteum, showed signs of both resorption and apposition. Bone resorption was due to overcompression exerted by the screw and tilted disc of the distraction device. In contrast, new bone formation without any signs of bone resorption was observed peripheral to the disc, thus at a site where a periosteum was present quite close to the surface of the original calvarial bone. The new, flat bone peripheral to the disc device was covered with a distinct periosteum layer showing signs of ongoing bone apposition only.

Bone can be formed in a place where it has not existed before, beyond the genetically determined "skeletal envelope."[21,22] Nakajima and colleagues[20] estimated the apposition of new bone on the parietal bone of rats using the polytetrafluorethylene disc with the thickness of 1 mm placed between the bone and periosteum. Twelve weeks after disc placement, appositional bone occupied an average of 50% of the inner space. This percentage corresponds to the previous findings of Yamada and colleagues[22] following 12 weeks of placement of the perforated, 4-mm high titanium cap on the rabbit's calvarium. These findings correspond to the central sections underneath the disc device in the present study where the periosteum was incised and placed outside the actual distraction space. The presence of a prominent coagulum bordered by a granulation tissue likely had delayed new bone formation since it is known that the coagulum needs to be resorbed before new bone formation can occur.[23] The tilting of the disc device may have affected the continuation of periosteum, which is most relevant for its function during distraction.[24] Depending on the model, periosteum ruptures at strains between 40% and 50%.[25,26] A more stable device is thus considered for future studies on PDO in rats.

The periosteum in the present study was elevated from the midline laterally and sutured over the perforated device; it is to be expected that the vascularization of periosteum remains preserved mainly at the periphery.[27] The periosteum of adult animals, which has been immediately elevated did not seem to contribute to the supraosteal bone formation.[28,29] The injury of the periosteum may be attributed to the surgical trauma caused by the incision, elevation, and the placement of device. A barrier membrane used for guided bone regeneration thus has to be sufficiently occlusive to prevent invasion of fibrous connective tissue into the area where bone formation is wanted.[28,30]

The contact between periosteum and bone seems to be essential for the osteogenic capacity of the periosteum.[27,29,31] If unstimulated (under physiological conditions), the expression of bone matrix molecules in the periosteal cells is barely detectable.[32] Undermining of periosteum during surgical procedure and its immediate reposition was demonstrated to stimulate new bone formation in mature bone.[33–35] In comparison with the periosteum of rats' tibia, undifferentiated mesenchymal cells in the calvarial periosteum may have the potential to differentiate only into osteoblasts.[36] In contrast to previous findings on the deprived periosteum,[32] a considerable amount of newly formed bone was achieved in the present study.

The periosteum appears to be the most crucial structure for successful bone regeneration during conventional DO.[37] The functional level of the periosteum returns to normal already during the activation period.[38] The tensile strain in vitro induced expression of runt-related transcription factor 2.[39] The distraction rate of 0.5 mm daily produced excellent regenerate tissue 28 days after conventional DO in the rat mandible.[40] Approximately the same distraction rate was applied in all animals in the present study, but only initial signs of lamellar bone formation were observed at 20 days of consolidation period. Besides the tilting of the distraction disc, the strain distribution outside the disc is hard to predict.[24] The periosteal strains might be of less importance regarding osteoblast differentiation than the accidental rupture because the periosteum can locally grow. In the present model, we were not able to detect major differences regarding new bone formation and maturation between the two groups of animals. The consolidation period longer than 20 days might be expected to affect the amount and quality of newly formed bone since the presence of osteoid and new blood vessels could be seen even 6 weeks after reposition of the periosteum on the calvaria of rats.[41]

Rabbits, dogs, and miniature pigs were used to analyze the procedure of PDO. The present experimental study confirmed the possibility of new bone formation by gradual distraction of the periosteum from the calvarial bone surface in rats. The mean total bone thickness was three times as thick as the pristine calvaria for both consolidation periods. In one animal, the maximum total bone thickness after the 20-day consolidation period was even four times thicker than the pristine bone. This extraordinary gain in bone thickness indicates the powerful potential of PDO in the rat calvaria model. Further mechanism exploration of PDO deserves to be clarified. The precise contribution of the periosteum and the old bone to de novo bone formation remains to be elucidated in future studies on the rat calvarium model of PDO.

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