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Tissue reactions to subperiosteal onlays of demineralized xenogenous dentin blocks in rats

Arne Mordenfeld[1,2,3], Mats Hallman[1,2], Sven Lindskog[4]

[1]Department of Oral & Maxillofacial Surgery, Public Health Service, Gävle; [2]Center for Research and Development, Uppsala University/Gävleborg County Council, Gävleborg; [3]Department of Biomaterials/Handicap Research, Institute for Surgical Sciences, Göteborg University, Göteborg; [4]Department of Pathology and Cytology, Karolinska University Hospital, Stockholm, Sweden

Correspondence to: Dr Arne Mordenfeld, Department of Oral & Maxillofacial Surgery, Public Health Service, Gävle Hospital, S-801 87 Gävle, Sweden
Tel.: +46 26 155646
Fax: +46 26 155347
e-mails: arne.mordenfeld@lg.se; christine_berthold@yahoo.de

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Abstract – *Objectives:* This study was undertaken to examine the influence of partial demineralization of xenogenous dentin on bone formation in an osteoconductive environment. *Materials and methods:* Sixty dentin blocks, 2–3 mm thick and 4 mm in diameter, were prepared from developing teeth of young pigs. Forty blocks were demineralized in 24% ethylenediaminetetraacetic acid (pH 7.0) for 1, 2, 6 or 12 h. Forty adult rats divided into eight groups with five rats in each group were used. A sagittal midcranial incision was made from the occipital to the frontal region. Through a subperiostal dissection, a pocket was created on each side of the skull. One demineralized block was placed on one side, and a non-demineralized block was placed on the contralateral side, or the pocket was left empty as controls. Thus, eight experimental groups with five rats in each were formed. *Results:* Resorption increased significantly with increasing degree of demineralization while bone formation increased significantly with increasing degree of demineralization, provided inflammation was compensated for. This suggests an important role for inflammation or infection control during the healing period of osteogenic implants to optimize osseous integration in an osteoconductive environment. *Conclusion:* Partial demineralization of xenogenous dentin blocks may provide a method for optimizing the integration of dentin onlays in an osteoconductive environment, thus stabilizing the implant and slowing down replacement resorption.

Introduction

Bone defects in the jaws caused by trauma, tumours, infection or loss of teeth are often reconstructed with autogenous bone grafts. Because of its osteogeneic properties and immunological compatibility, autogenous bone is considered the gold standard (1). However, donor site morbidity and limitations in the amount of available bone have encouraged research to find an acceptable bone substitute. The optimal bone substitute should possess both osteoinductive and osteoconductive properties leading to rapid osseointegration and probably more predictable results without inducing unwanted side effects such as excess inflammation-induced resorption. Studies have shown that demineralized bone and demineralized dentin are osteoinductive, most likely due to bone morphogenetic proteins (BMP) as part of their organic matrix (2–5). However, particulated demineralized dentin or hydroxyapatite ceramics applied as bone onlays are frequently encapsulated in fibrous tissue, which has been suggested to be as a result of instability of the graft and/or ruptures in the periosteum (6–10). In a more sheltered environment such as implanted in abdominal muscle pouches, dentin demineralized for 24 h or more in ethylenediaminetetraacetic acid (EDTA) or hydrochloric acid induce formation of new bone (11, 12). However, allogeneic dentin blocks demineralized in 24% EDTA at pH 7.0 for 3 min and subsequently implanted in the palatal connective tissue in rats appear not to induce any bone formation up to 4 weeks postimplantation (13). Hence, there is reason to believe that for dentin, both the demineralization procedure and the implant environment play important roles in osteoinduction and osteoconduction.

This study was undertaken to examine the influence of partial demineralization of xenogenous dentin on bone formation in an osteoconductive environment.

Materials and methods

The study was approved by the Local Ethics Committee on Animal Experiments, Stockholm, Sweden. Sixty dentin blocks, 2–3 mm thick and 4 mm in diameter, were prepared with a trephine bur from unerupted developing pig teeth from which the enamel/enamel matrix had been removed. During the entire procedure, the specimens were continuously irrigated with water. Forty of the dentin blocks were demineralized in 24% EDTA (pH 7.0) for 1, 2, 6 or 12 h, respectively, in room temperature. After thorough rinsing in tap water, the blocks were stored for one to 2 months in a freezer at −18°C before implantation. Another 20 dentin blocks

were prepared but not demineralized and also stored in a freezer before used as one of the controls.

Forty adult rats with a body weight ranging from 340 to 400 g were used. The rats were divided into eight groups with five rats in each group. Anaesthesia was provided by intramuscular injection of fentanyl citrate (0.4 ml equivalent to 0.126 mg kg^{-1} body weight, Hypnorm™; Janssen Pharmaceutica, Beerse, Belgium).

A sagittal, midcranial incision was made from the occipital to the frontal region. Through subperiosteal dissection, a pocket was created on each side of the midline. One demineralized dentin block was placed on one side, and on the other side, a non-demineralized block was placed or the pocket was left empty. Thus, eight experimental groups with five rats in each were formed as follows:

Group I Non-demineralized dentin block on the left side and dentin block demineralized for 1 h on the right side.

Group II Non-demineralized dentin block on the left side and dentin block demineralized for 2 h on the right side.

Group III Non-demineralized dentin block on the left side and dentin block demineralized for 6 h on the right side.

Group IV Non-demineralized dentin block on the left side and dentin block demineralized for 12 h on the right side.

Group V Only subperiosteal dissection on the left side and dentin block demineralized for 1 h on the right side.

Group VI Only subperiosteal dissection on the left side and dentin block demineralized for 2 h on the right side.

Group VII Only subperiosteal dissection on the left side and dentin block demineralized for 6 h on the right side.

Group VIII Only subperiosteal dissection on the left side and dentin block demineralized for 12 h on the right side.

After placement of the xenogenous dentin onlays, the wounds were closed with interrupted non-resorbable sutures. All rats were sacrificed after 4 weeks, and the cranial bone was carefully excised and separated in the midline. The implantation area was dissected free and fixed in 4% neutral buffered formalin. Subsequently, radiographs of the specimens were taken for orientation of any possible hard tissue formation. The specimens were then demineralized in neutral buffered 24% EDTA, dehydrated and embedded in paraffin. Serial sections of 7 μm thick were cut transversally through the midpart of the xenogenous dentin onlays, stained with haematoxylin and eosin and analysed in a light microscope.

All xenogenous dentin onlays and recipient sites were evaluated semiquantitatively for degree of inflammation, encapsulation, resorption and bone formation and given the scores indicated in brackets as follows:

Inflammation none (0), slight (1), moderate (2) and intense (3).

Encapsulation (% of the implant surface) < 50% (0) and > 50% (1).

Resorption (% of the implant surface) < 50% (0) and > 50% (1).

Bone formation (% of the implant surface) 0% (0), 0–33% (1), 33–67% (2) and 67–100% (3).

Outcome variables were either binary or ordinal categorical.

Statistical analyses

First, for each of the four outcome variables (inflammation, encapsulation, resorption and bone formation), the Wilcoxon signed ranks test was performed to determine the presence of differences between control (non-demineralized dentin) and demineralization regardless of time (1, 2, 6 or 12 h of demineralization). The Bonferroni–Holm method was used to compensate for possible mass significance. Adjusted *P*-values equal to or smaller than 0.10 were considered statistically significant.

Secondly, for each of the four outcome variables (inflammation, encapsulation, resorption and bone formation), a Kruskal–Wallis test was performed to determine the presence of differences between the time of demineralization; 1, 2, 6 or 12 h of demineralization. The Bonferroni–Holm method was again used to compensate for possible mass significance. Adjusted *P*-values equal to or smaller than 0.10 were considered statistically significant.

If the tests showed any statistical significant differences, the next step was to perform pairwise comparisons between 1, 2, 6 and 12 h of demineralization, respectively. The Wilcoxon rank sum test was used for the pairwise comparisons. No compensation for mass significance was used as the presence of differences between the times of demineralization was already established by the Kruskal–Wallis test.

All calculations were performed using SAS 9.2, and the result data were processed within the SAS/Windows environment.

Results

The soft tissue in all 40 rats healed uneventfully, and no macroscopic signs of infection were observed. In one rat, the xenogenous dentin onlay was dislocated and found in the soft tissue. In another rat, the histological evaluation revealed abscesses with a completely resorbed dentin block. Both cases were in control sites (non-demineralized) and were excluded from the analyses. In the control group where subperiosteal dissection only was performed, no inflammation was present and bone formation was negligible. Demineralized dentin presented with significantly more resorption than non-demineralized dentin. The results from the statistical analyses to determine the presence of differences between the time of demineralization: 1, 2, 6 and 12 h of demineralization, are presented in Table 1.

The results from the pairwise comparisons between 1 and 2 h, 6 and 12 h of demineralization, respectively, are presented in Table 2.

Seven of 58 sites with dentin blocks presented moderate to intense inflammation. They were found in the groups with dentin blocks demineralized for 2 h

($N = 2$), 6 h ($N = 2$) and 12 h ($N = 3$) (Fig. 1), respectively. This was considered a surgical complication rather than a reaction to the dentin blocks as such reactions were not seen in the non-demineralized control group even though the xenogenous dentin onlays had not been sterilized. Hence, we formed subgroups excluding sites with moderate to intense inflammation (Tables 1 and 3).

All non-demineralized dentin blocks and dentin blocks demineralized for 1 h were encapsulated to more than 50% of the dentin onlay surface; however, no significant differences was observed between the demineralization groups with or without exclusion of the moderate and intense inflammation sites, with respect to encapsulation.

Resorption increased with increasing time of demineralization without exclusion of the moderate and intense inflammation sites and was significantly higher after 6 h of demineralization or longer. Resorption lacunas were often identified and were sometimes occupied by multinucleated giant cells surrounded by means of osteoblasts (Fig. 2). In the non-demineralized dentin group, there were no signs of resorption.

Inflammation was not significantly higher in any of the groups with or without exclusion of the moderate and intense inflammation sites. Bone formation did not significantly differ between the groups; however, when the moderate and intense inflammation sites were excluded, there was significantly more bone formation in both the 6- and 12-h demineralization groups.

Bone formation towards the periosteum was negligible except in the 12-h demineralization group where bone formation sometimes was prominent (Fig. 3). Bone formation was often found at the short ends of the xenogenous dentin onlays where the space probably initially had been filled with a coagulum, which was subsequently replaced by immature bone (Fig. 4). In some cases, direct bone formation was seen on the dentin onlay surface facing, but without direct contact to the surface of the calvarium. In the non-demineralized dentin group, no signs were observed of bone formation.

Discussion

Autogenous bone is considered the gold standard in bone grafting. However, graft resorption is unpredictable, and the degree of resorption of onlay grafts varies widely in the literature (14–16). There is a need for a both osteoinductive and osteoconductive bone substitute that will be replaced by newly formed bone with a minimum of graft resorption.

The experimental procedure in this study was designed to examine the effect of partial demineralization of xenogenous dentin implants on bone formation in an osteoconductive environment in an attempt to control osseous integration. In a clinical situation, early partial osseous integration may serve to stabilize an onlay graft, and a central non-demineralized part of the xenogenous dentin onlay may slow down replacement of the graft by bone and subsequently maintain the graft volume over time. Significant osteogenic properties primarily resting in its content of BMP have previously been demonstrated for demineralized dentin (17, 18) and to a lesser degree or none for non-demineralized dentin (19) especially, if it is not sterilized (20). According to Bang and Urist (11), resorption of non-demineralized dentin starts about 8–12 weeks later than totally demineralized dentin when implanted in rat abdominal muscle. In addition, Yeomans and Urist (21) demonstrated that non-decalcified dentin also induced scanty bone formation but only after a latent period of 8–12 weeks in rabbit muscle, while others demonstrated no new bone with non-demineralized dentin grafts in the middle ear of rat (19). Hence, the graft environment appears to play an important role for any significant expression of the osteogenic properties of dentin as recently also demonstrated by Andersson (22) for different intrabony sites.

In the present study, the potential expression of osteogenic properties of partially demineralized dentin was probably enhanced by transplantation subperiostally in an osteoconductive environment. Hence, no attempt was made to differentiate between osteoinduction and osteoconduction. After subperiosteal dissection (an osteoconductive environment), the dentin blocks were placed as onlays on the rat skull bone without any preparation of the recipient site. Bone marrow exposure might have resulted in explosive bone formation, swifter graft incorporation and earlier onset of remodelling (23), while perforation at the recipient site might have induced migration of the bone marrow into the graft (24). This could explain the integration of non-demineralized dentin blocks showed by other researchers (22, 25, 26). However, bone marrow exposure would have interfered with the interpretations of osteoinductive and osteoconductive properties of the dentin blocks themselves.

The purpose of the demineralization was not a total removal of mineral from the dentin blocks but rather to

Table 1. Results from the Kruskal–Wallis tests to determine the presence of differences between the time of demineralization with (included) or without (excluded) inclusion of the sites presenting moderate or intense inflammation

	Included			Excluded		
Variable	*N*	*P*-value	Adjusted *P*-value	*N*	*P*-value	Adjusted *P*-value
Bone formation	40	0.082	0.246	33	0.008	0.031*
Inflammation	40	0.113	0.246	33	0.259	0.352
Resorption	40	0.022	0.089*	33	0.044	0.132
Encapsulation	40	0.463	0.463	33	0.176	0.352

The Bonferroni–Holm method was used to compensate for possible mass significance. Adjusted *P*-values equal to or smaller than 0.10 were considered statistically significant (*).

Table 2. All demineralized dentin blocks are included. All controls (non-demineralized dentin blocks) are excluded

Treatment	*N*	Bone formation Median (min–max)	Inflammation Median (min–max)	Resorption Median (min–max)	*P*	Encapsulation Median (min–max)
1 h EDTA	10	0 (0–1)	0 (0–1)	0 (0–1)	–	1 (1–1)
2 h EDTA	10	0 (0–2)	1 (0–3)	0 (0–1)	0.131	1 (0–1)
6 h EDTA	10	1 (0–2)	0 (0–3)	1 (0–1)	0.008	1 (0–1)
12 h EDTA	10	1 (0–3)	1 (0–3)	1 (0–1)	0.008	1 (0–1)

Median values, minimum and maximum (min–max) from semiquantitative histological evaluation of the experimental materials are presented as described in the Materials and methods and *P*-values (*P*) from pairwise comparisons between 1, 2, 6 and 12 h of demineralization, respectively with respect to resorption (see Table 1). The Wilcoxon rank sum test was used for pairwise comparisons.

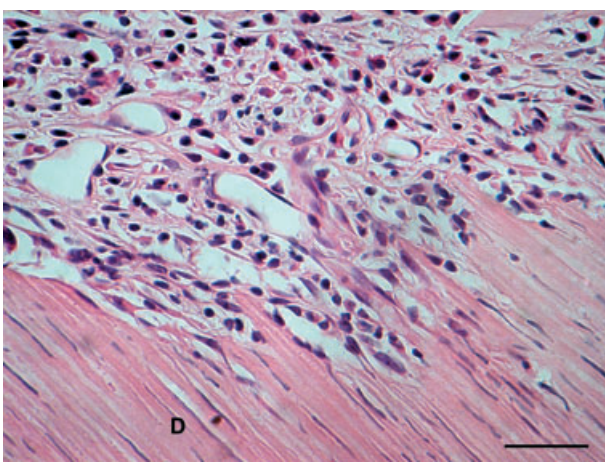

Fig. 1. Intense inflammation around a dentin (D) onlay demineralized for 12 h. Bar = 100 μm.

modify the surface facing the surrounding tissue (periosteum and bone surface) known to be able to induce both bone formation and resorption. Hence, the blocks were exposed to EDTA for relatively short periods. EDTA at neutral pH was chosen as it has been shown to selectively remove mineral from a dentin surface preserving integrity of the collagenous matrix, unlikely the effect of etchants operating at low pH (27). Furthermore, the morphogenetic pattern of dentin is more stable than that of bone matrix in EDTA (12). Urist (12) also claimed that bone morphogenetic properties are lost if the matrix is exposed to chemical solvents that denature or otherwise derange the three-dimensional framework of the fibrous proteins. EDTA however seems to preserve the integrity of intercellular structures (28). The authors also found that the calcium extraction from the bone was very rapid in the beginning, and they suggested that EDTA had to diffuse into the bone tissue after the amounts of calcium in the superficial layer were reduced. In fact, Birkedal-Hansen (29) found that the decalcification in EDTA of elephant's ivory dentin differs in the rate of penetration between plain sheet, where demineralization depth was directly proportional to the square root of the time and cylinders in which the rate of penetration decreases initially and later increases again.

In the present study, bone formation increased significantly with increasing degree of demineralization, provided inflammation was compensated for. This suggests an important role for inflammation or infection control during the healing period of xenogenous dentin onlays to optimize osseous integration in an osteoconductive environment. Hence, to isolate the effect of partial demineralization of the xenogenous dentin onlays, the few sites (7 of 58) with moderate and intense inflammation were excluded in a subgroup as it was considered to be a complication to the surgical procedure and lack of proper sterilization of the dentin blocks rather than a reaction to the dentin surfaces *per se*. Antibiotic treatment and proper sterilization would probably have decreased the number of inflammation sites. By excluding these sites, the analyses of other variables, which were the main purpose of the study, could be refined. In the subgroup where moderate and intense inflammation had been excluded, significantly more bone formation was observed after a demineralization period of 6 h or more. Hence, this suggests that moderate or intense inflammation probably has a negative effect on bone formation, at least in the early healing phase. This is in agreement with the results presented by Machado et al. (20). Sterilization of the dentine blocks in ethylene oxide could have been an alternative to solve this problem

Table 3. Dentin blocks at experimental sites presenting with moderate and intense inflammation are excluded

Treatment	*N*	Bone formation Median (min–max)	*P*	Inflammation Median (min–max)	Resorption Median (min–max)	Encapsulation Median (min–max)
1 h EDTA	10	0 (0–1)	–	0 (0–1)	0 (0–1)	1 (1–1)
2 h EDTA	8	0 (0–2)	0.955	0.5 (0–1)	0 (0–1)	1 (0–1)
6 h EDTA	8	1 (0–2)	0.011	0 (0–1)	1 (0–1)	1 (0–1)
12 h EDTA	7	1 (0–3)	0.016	1 (0–1)	1 (0–1)	1 (0–1)

Median values, minimum and maximum (min–max) from semiquantitative histological evaluation of the experimental materials as described in the Materials and methods, and *P*-values (*P*) from pairwise comparisons between 1, 2, 6 and 12 h of demineralization with respect to bone formation (see Table 1). The Wilcoxon rank sum test was used for the pairwise comparisons.

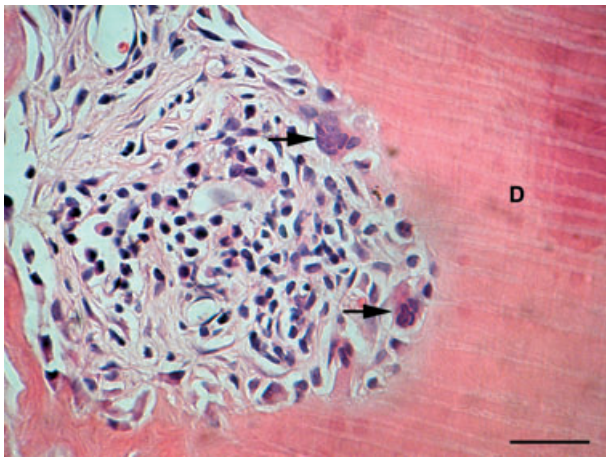

Fig. 2. Resorption lacunae with multinucleated resorbing cells (arrows) surrounded by mononucleated osteoblasts-like cells on the surface of a dentin (D) onlay demineralized for 6 h. Bar = 100 μm.

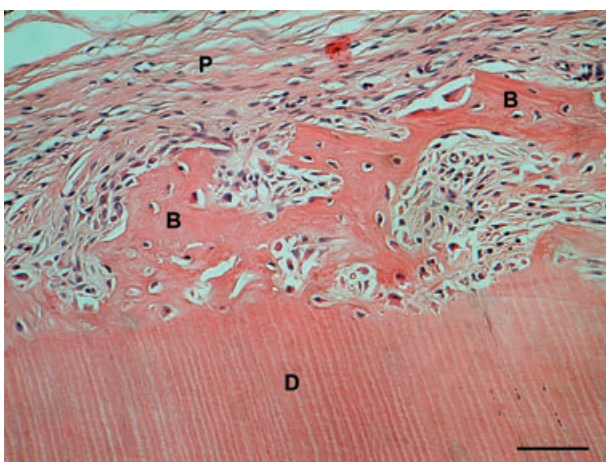

Fig. 3. Bone formation (B) towards the periosteum (P) on a dentin (D) onlay demineralized for 12 h. Bar = 200 μm.

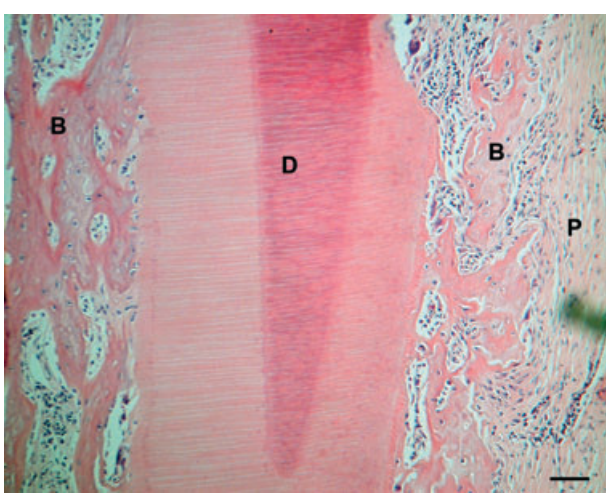

Fig. 4. Prominent bone formation (B) surrounding a dentin (D) onlay demineralized for 6 h. Bar = 200 μm.

because the process does not seem to reduce the inductive potential in demineralized bone (30). However, this procedure would not have eliminated contamination during the surgical procedure.

Increase in bone formation was seen with demineralization of 6 h or more in sites with no or negligible inflammation (Table 2). Similarly, resorption increased with demineralization of 6 h or more (Table 3), although not coupled directly with bone formation in the same statistical data set. This indicates that inflammation most likely contributed to increase resorption (31), while absence of inflammation seemed to have a positive effect on bone formation (32). However, it may also indicate that resorption is a prerequisite for bone formation in accordance with evidence of physiological coupling between resorption and bone formation (33), although resorbing cells find a more highly mineralized surface attractive to cell colonization (34) while bone induction requires that more of the matrix is exposed (35).

The results of the present study may have clinical implications suggesting that dentin onlays may be customized for optimal osseous integration in an osteoconductive environment. From a clinical point of view, rapid bone anchorage of onlays is crucial for stability and further replacement by bone. Partial demineralization of xenogenous dentin implants may thus provide a method for optimizing integration of dentin onlays in an osteoconductive environment, which may serve to stabilize the graft, slowing down replacement resorption and preserve as much as possible of the volume of the graft.

Conclusions

Bone formation in an osteoconductive environment appears to increase significantly with increasing degree of demineralization in grafted xenogenous dentin onlays, provided inflammation is controlled for. This suggests an important role for infection control during the healing period of dentin onlays. Thus, partial demineralization of xenogenous dentin blocks may provide a clinical method for optimizing integration of onlays in an osteoconductive environment.

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