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Bone sialoprotein-induced reparative dentinogenesis in the pulp of rat's molar

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Abstract Bone sialoprotein (BSP), an osteogenic protein (OP), mixed with a carrier, was implanted in the pulp of rat first upper molars (OP group). Cavities were prepared with dental burs and pulp perforation was carried out by pressure with the tip of a steel probe. After 8, 14, and 30 days, the rats were killed and the pulps of the OP group were compared with (1) a sham group (S group), (2) a group where the carrier was implanted alone (C group), and (3) capping with calcium hydroxide (Ca group). After 8 days, a few inflammatory cells were seen, mostly located at the pulp surface near the perforation. In the Ca group, a dentin bridge started to form, in contrast to the other groups. After 15 days, globular structures were seen in the pulps of the S and C groups. A reparative osteodentin bridge isolated the pulp from the cavity in the Ca group. Variable reactions were seen in the OP group, with some evidence of cell and matrix alignments or plugs of osteodentin in continuity with an inner layer of reparative dentin. After 30 days, irregular osteodentin formation was observed in the pulps of the S and C groups, with a tendency for globular structures to merge, but with interglobular spaces filled by pulp remnants. In the Ca group, osteodentin was observed in the mesial part of the pulp chamber. In the BSP-implanted group, the osteogenic protein stimulated the formation of a homogeneous dentin-like deposit occupying most of the mesial part of the pulp. Apparently, BSP stimulates the differentiation of cells which secrete an organized extracellular matrix more efficiently than any other capping material used so far. Altogether, the results reported here support that bone sialoprotein displays novel bioactive properties and is capable of stimulating in 1 month's time the development of a thick reparative dentinal tissue in the pulp, occluding the perforation and filling the mesial third of the pulp chamber.

Key words Reparative dentin · Pulp · Biomineralization · Bone sialoprotein · Calcium hydroxide

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Introduction

It has been well-documented for a long time that intramuscularly implanted HCl-demineralized bone matrix (DBM) induces the formation of bone [32]. The DBM contains one or more so-called bone morphogenetic proteins (BMP), most of which are members of the TGFβ family. This group of molecules induces the proliferation and differentiation of undifferentiated mesenchymal cells firstly into chondrocytes, which produce a cartilage matrix, which then is destroyed and replaced by bone. This endochondral ossification occurs in subcutaneous or intramuscular uncalcified tissues [32, 38]. In cranial induced defects ≥8 mm in diameter which do not heal spontaneously, the same sequence of cellular and extracellular events occurred, despite the expected membranous mineralization [17, 38]. Using DBM, Wang et al. [34] reported that only small clusters of cartilage were formed in cranial defect models, and the initial cellular response was an immediate proliferation of mesenchymal cells which differentiated into osteoblasts, synthesizing a bone matrix which rapidly mineralized. Osteopontin and bone sialoprotein were identified in the new bone formed in the defect [35]. The small amount of cartilage was resorbed and replaced by bone after 9–10 days. The original bone matrix implants and the small amount of cartilage synthesized during repair are resorbed and replaced by new bone. Although the exact biological mechanisms

need still further clarification, it is clear that the implantation of DBM stimulates the expression of two phosphorylated proteins (osteopontin and bone sialoprotein) involved in biological functions and in the mineralization processes. The implants also seem to play a role in the recruitment of mesenchymal cells, which underwent further differentiation into osteoblasts. Wang et al. [35, 36] have shown that bone sialoprotein (BSP), originally termed bone sialoprotein II, is implicated in bone formation in surgically developed calvarial defects of critical size, in contrast to osteopontin (OPN), a molecule which has also been termed bone sialoprotein I, which did not induce bone formation at days 10, 21, or 30 under the same experimental conditions. It is now well-recognized that OPN is also found in nonmineralized tissue and expressed by many types of cells. In contrast, BSP is mostly found in mineralizing tissues such as bone, cartilage, dentin, and cementum. At this step in their work, Wang et al. [35, 36] concluded that BSP was a major component of DBM and, in conjunction with bone morphogenetic proteins, has a bone-inductive capacity.

The reasons why BSP displays an osteoblastic activity are not elucidated, but it was shown that, after implantation of purified BSP with carrier in critical cranial defects, direct bone formation occurred without significant endochondral activity [36, 37]. BSP has a M_r of 57 kDa, of which almost half is carbohydrate, and a protein core of 33.6 kDa. BSP has a variable content of sialic acid ranging from 13% in bovine adult bone to 5% in rat bone. It is a phosphorylated protein with tyrosine sulphatation and RGD cell attachment sequence [4]. BSP is characterized by the presence of several stretches of polyglutamic acid involved in binding to HAP, and the molecule has the potential to mediate the initial formation of hydroxyapatite crystals [8]. In addition, BSP enhances the fibrillogenesis of collagen [5]. Chen et al. [2, 3] have shown that BSP mRNA is expressed in odontoblasts of the incisor during dentinogenesis and that polyclonal antibodies raised against BSP react positively with epitopes located in odontoblast cell bodies and processes and in peritubular dentin. Ameloblasts, as well as pulp cells, do not express BSP messengers, nor do they react with the anti-BSP antibody. Because BSP bears all these interesting biological properties, it was interesting to evaluate its properties in the induction of reparative dentin after surgical exposure. In the past few years, much evidence has been produced that dentin matrix components, which stimulate odontoblasts and the formation of reactionary dentin [23], are also involved in the proliferation and differentiation of osteoprogenitor cells [24]. According to the terminology used by Lesot et al. [11], odontoblasts elaborate primary and secondary dentin in nonpathological conditions. In response to caries, odontoblasts secrete reactionary dentin. Reparative dentinogenesis implicates the differentiation of pulp cells which elaborate an initial osteodentin layer, sometimes followed by the formation of a true orthodentin. In this context, the stimulation of reparative dentin formation is clinically relevant.

The present report is in line with what has been done previously using a recombinant human osteogenic protein-1 (OP-1 or BMP-7), except that we did not use a growth factor but rather a matrix protein specifically found in mineralized tissues. Sampath et al. [21] were able to induce new bone formation in vivo with OP-1. Reparative osteodentin-like formation was observed in amputated pulps capped with crude allogenic BMP in dogs [12, 14, 15]. In the dental pulp of monkey, OP-1 was shown to induce reparative osteodentin formation after direct capping [19, 20]. The biological mechanisms of such events are clarified by the fact that BMP and BMP receptor genes have been identified in human, rat, and bovine embryonic and adult pulps [7, 25–27]. However, OP-1 was shown to produce a porous osteodentin. Therefore, the stability of the reparative tissue in case of the failure of filling and bacterial colonization is questionable. In addition, it is still not known whether the mechanism of formation of reparative tissue and its mineralization will be limited and stopped shortly after it has been used or whether it has a long-term potential to pursue its action, also retaining the potential of mineralizing the soft tissues surrounding the tooth. In this context, BMP combined with collagen matrix as a carrier is capable of inducing the transformation and mineralization of soft tissues. In contrast, BSP induces osteogenesis only in bony sites. Therefore, important differences between OP-1 and BSP appear in bone healing. Since nothing is known about the action of BSP on the dental pulp, it was consequently interesting to evaluate its effects in reparative dentinogenesis after direct capping of the pulp in an in vivo animal model.

Materials and methods

Thirty-six Sprague Dawley rats aged 6–7 weeks were used for this investigation. Each animal was anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg). Electrosurgery of the gingival tissue was carried out with a Servotom (Satelec, France) to prepare an access to the mesial aspect of the right and left upper first molars. The next steps: preparation of cavities, implantation in the pulp, and filling with glass-ionomer cement was done using a microscope (Epidiascope) at ×16 magnification. Half-moon class V-like cavities were then prepared in 1–2 seconds in the cervical third of the mesial aspect of the first upper molars with a high speed contra-angle working at 120 000 rpm. Round tungsten carbide burs (size 0.6 mm., 0.05 ISO, Maillefer, France) were used cooled with copious sterile water to flush the cutting area adequately. The burs were changed after every fourth cavity. Two teeth per rat were prepared. Pulp perforation was accomplished by pressure with the tip of a steel probe.

The upper molars were divided into four groups of 18 teeth each: In the sham group of teeth (S), the cavity was prepared but filled only with Fuji IX, a light-curing glass ionomer cement (GIC) (GC Corporation, Tokyo, Japan).

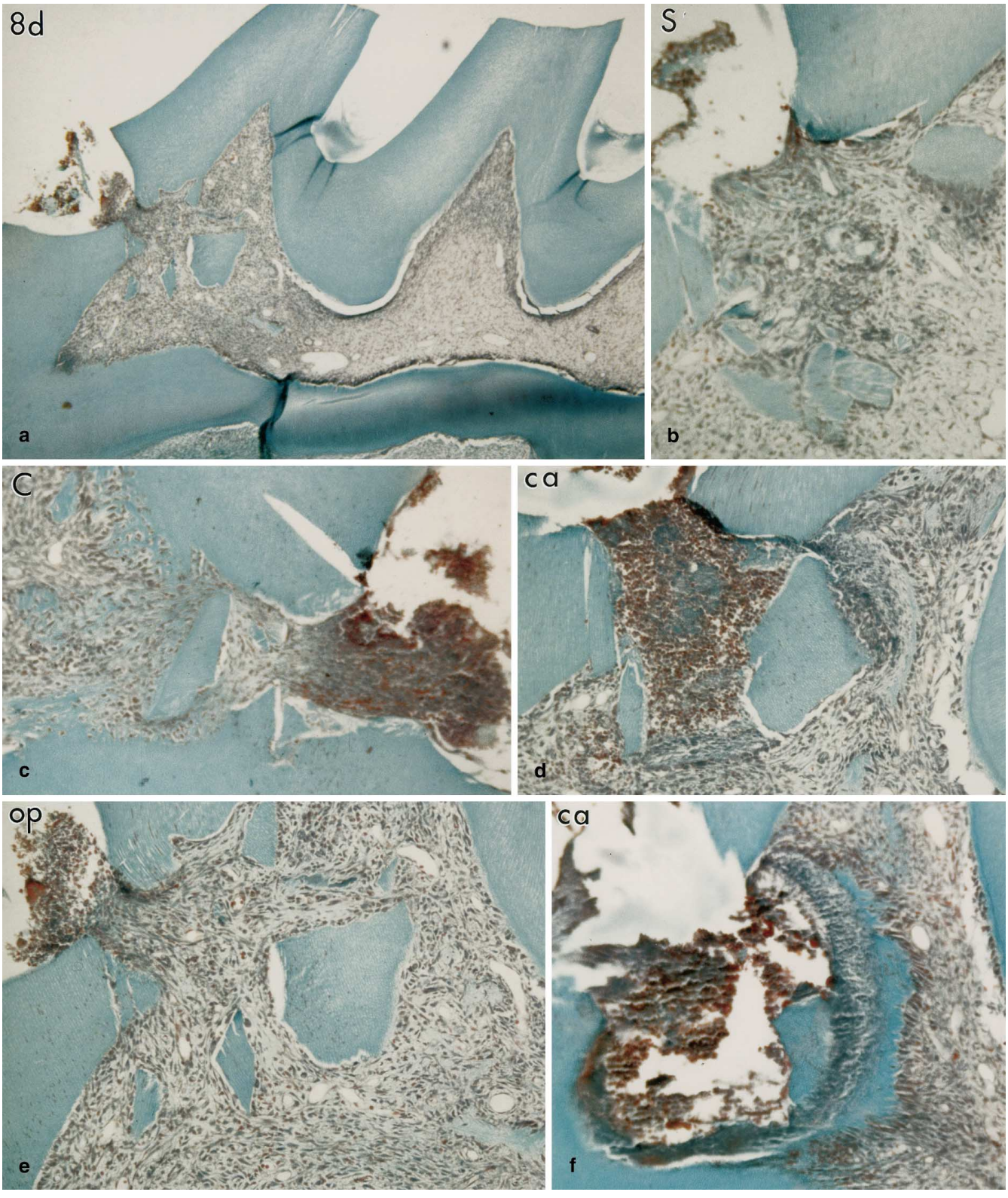

Fig. 1a–f **a** Visualization of the cavity prepared in the first maxillary molar. Sham group. Some glass ionomer remnants are seen in the cavity. Projections of dentin debris into the mesial part of the pulp, ×10. **b** Sham at day 8. Bottom of the cavity and pulp perforation are seen at the upper left. Dentin debris were pushed inside the pulp during the preparation. The inflammatory reaction is moderate, ×120. **c** Compared with **b**, a more severe inflammation was induced by the carrier at day 8, ×120. **d,f** Calcium hydroxide at day 8. Beginning of a reparative bridge formation, ×120. **e** Osteogenic protein at day 8. No inflammation is detected, ×120

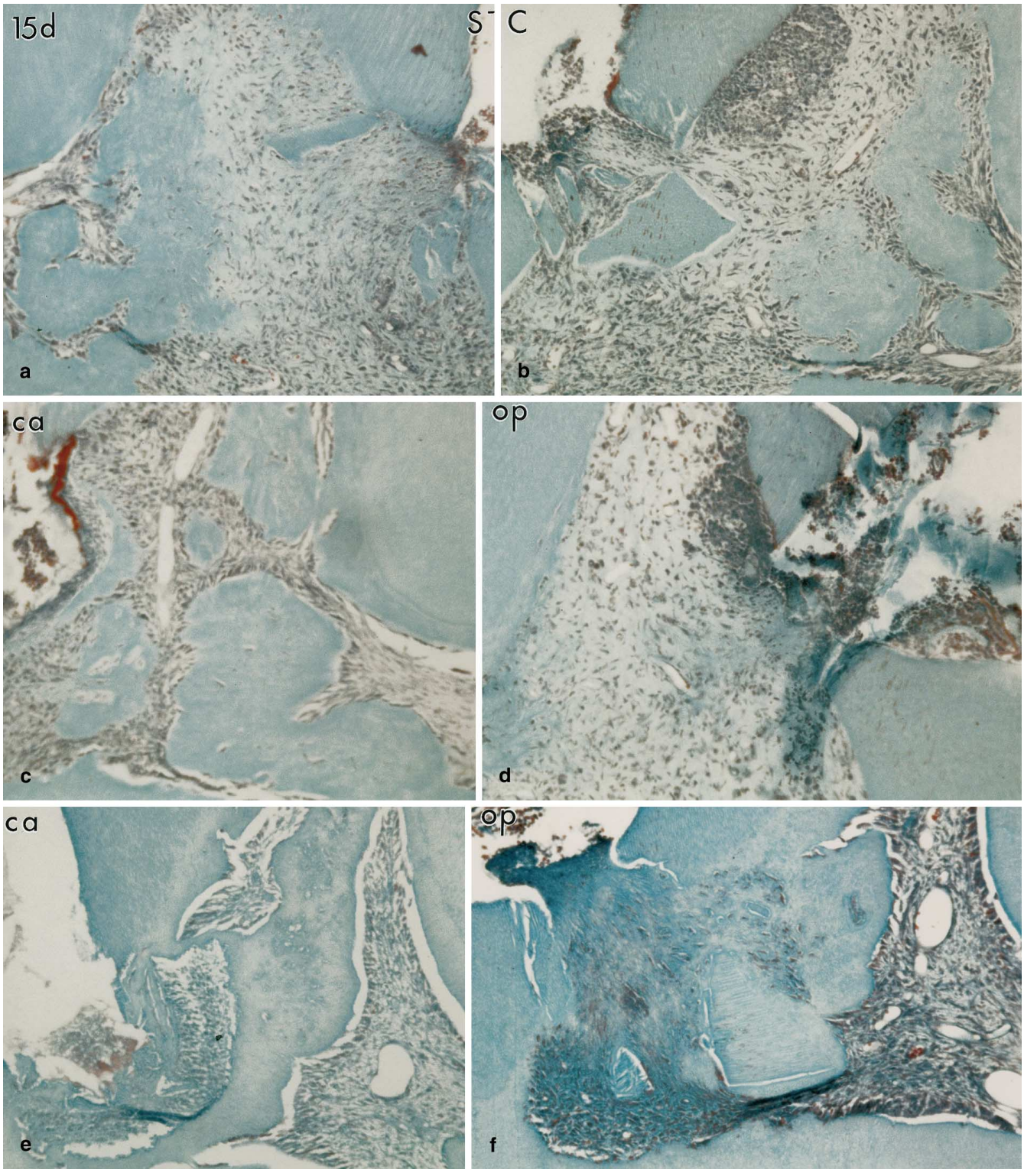

Fig. 2a–f **a** After 15 days, in sham (*S*) reparative globular structures are seen in the pulp near the junction between the mesial and central parts, but not near the perforation, ×120. **b** 15 days after implantation, the carrier (*C*) still induced an inflammatory reaction. Globular calcospheritic structures are seen in the pulp far from the perforation, ×120. **c,e** After 15 days, a thick reparative dentin bridge is observed in the Ca group, ×120. **d,f** Two different aspects of BSP induced reaction (OP group). In **d**, inflammatory cells are seen in the upper superficial part of the pulp. In the lower parts, pulp cells are not more numerous than in the sham group. In contrast in **f**, osteodentin and dentin formation are clearly seen around dentin debris, ×120

In the carrier (gelatin) group (C), the carrier alone was implanted in the pulp and the cavity filled with GIC. In the osteogenic protein (OP) group, BSP together with the carrier was implanted and protected with the GIC. BSP was purified by one of the authors (E. Salih) in his laboratory, as previously described [35–37]. A total of 30 μg BSP mixed with 5 mg gelatin were used for the 18 teeth of this group. The effects of BSP on the formation of reparative dentin or osteodentin in dental pulp were compared with a calcium hydroxide group (Ca). The rats were randomly treated and the right-side treatment differed from that of the left, so that after 8 days, 15 days, and 30 days respectively, six rats per group were killed by perfusion of the fixative solution (10% neutral formaline) through the heart (Table 1). The upper molar groups were dissected out from the maxillary after 5 to 6 min. Block sections including the three molars, bone, and gingiva were immersed in the fixative and kept at 4°C for 24 h. They were demineralized either with sodium formiate or with 4.13% EDTA and embedded in Paraplast. Serial sections of 7 μm were stained with either Masson's trichrome or H&E. A few slides were also stained with the Brown and Brenn method for visualizing bacteria in tissue sections [1]. The evaluation criteria used for histological investigation were: inflammation (mild, moderate, severe), vasodilatation of the capillaries, formation of reparative osteodentin and orthodentin, presence of calcospherites, and presence or absence of odontoblasts.

Results

After 8 days, moderate inflammation was seen in the S group (Fig. 1a). Unidentified inflammatory cells were grouped near the perforation area. Dentin debris was pushed by the probe inside the pulp. The inflammatory reaction seen around such fragments was weak (Fig. 1b). In the C group, the carrier alone induced a more severe inflammatory reaction, mostly detectable near the wounded surface of the pulp. In deeper zones, the tissue was apparently normal (Fig. 1c). In the Ca group, the capping induced an inflammatory reaction at the surface of the pulp. At the border between implanted material and pulp, continuous or discontinuous fibrous structures began to form, underlined by an odontoblast-like palisade (Figs. 1d,f). More fibroblast-like cells were observed in the OP group near the perforation area. It is not possible at the present stage to establish any actual discrimination between inflammatory cells and cells which are recruited and differentiate into reparative dentin-forming cells. The rest of the pulp was apparently normal, despite the presence of dentin fragments pushed into the pulp during the preparation (Fig. 1e, Table 1)

After 15 days, there was already some tendency in the S group to self-repair some distance away from the wound, mostly in the cervical junction between the pulp chamber and root canal (Fig. 2a). Inflammation was still visible in some areas in the C group, and heterogeneous globular structures were seen in the pulp but not near the perforation (Fig. 2b). In the Ca group, chips of osteodentin forming bridges of variable thickness were detectable in many molars together with calcospheritic structures located some distance away in the pulp (Fig. 2c,e). In the OP group, alignments of cells and organized matrix were visible, but still no evidence of reparative dentin or osteodentin (Fig. 2d). This material seems to be involved in the initial formation of a premineralized tissue, filling the mesial part of the pulp chamber. In a few cases, new deposits were seen beneath a plug of osteodentin around the fragments of dentin pushed into the pulp during preparation of the tooth. Such constructions were seen in the mesial part of the pulp but not in inner locations (Fig. 2f, Table 1).

After 30 days, pulps in the S group were not healed, despite the spontaneous formation of calcospherites or globules of mineralized structures, which displayed some tendency to merge and filled the mesial pulp horn. Inclusion of cells inside these structures was still visible, suggesting that, at best, osteodentin had formed (Fig. 3a,b). The same was seen in the carrier group: a mixture of large areas of globular structures which were probably mineralized before light microscopy processing and cell-rich areas of pulp remnants (Fig. 4a). In very

Table 1 Materials used in the four groups (six first upper molars/period-of-time/group) and summary of the results

Sham (S): cavity + GIC + dentin debris	Carrier (C): implant of gelatin + dentin debris + GIC	Calcium hydroxide (Ca) +dentin debris + GIC	Osteogenic protein (OP): BSP + carrier + dentin debris + GIC
8 days: moderate inflammation	8 days: more severe inflammation	8 days: beginning of the formation of a reparative bridge (osteodentin)	8 days: recruitment of undifferentiated cells
15 days: tendency for self-repair at some distance from the wound	15 days: inflammation and formation of globular heterogeneous structures distance away from the open wound	15 days: osteodentin bridge of variable thickness + calcospherites; closure of the wound	15 days: from the organization of cells and matrix to the closure of the wound and initial mineralization
30 days: calcospherites and heterogeneous mineralized structures of the osteodentin type; tendency to merge in the mesial horn + pulp remnants	30 days: very similar to sham; many pulp remnants	30 days: thick osteodentin formation filling partially the mesial horn + pulp remnants	30 days: initial formation of a layer of osteodentin, followed by the formation of thick atubular (mineralized) structure filling the mesial horn

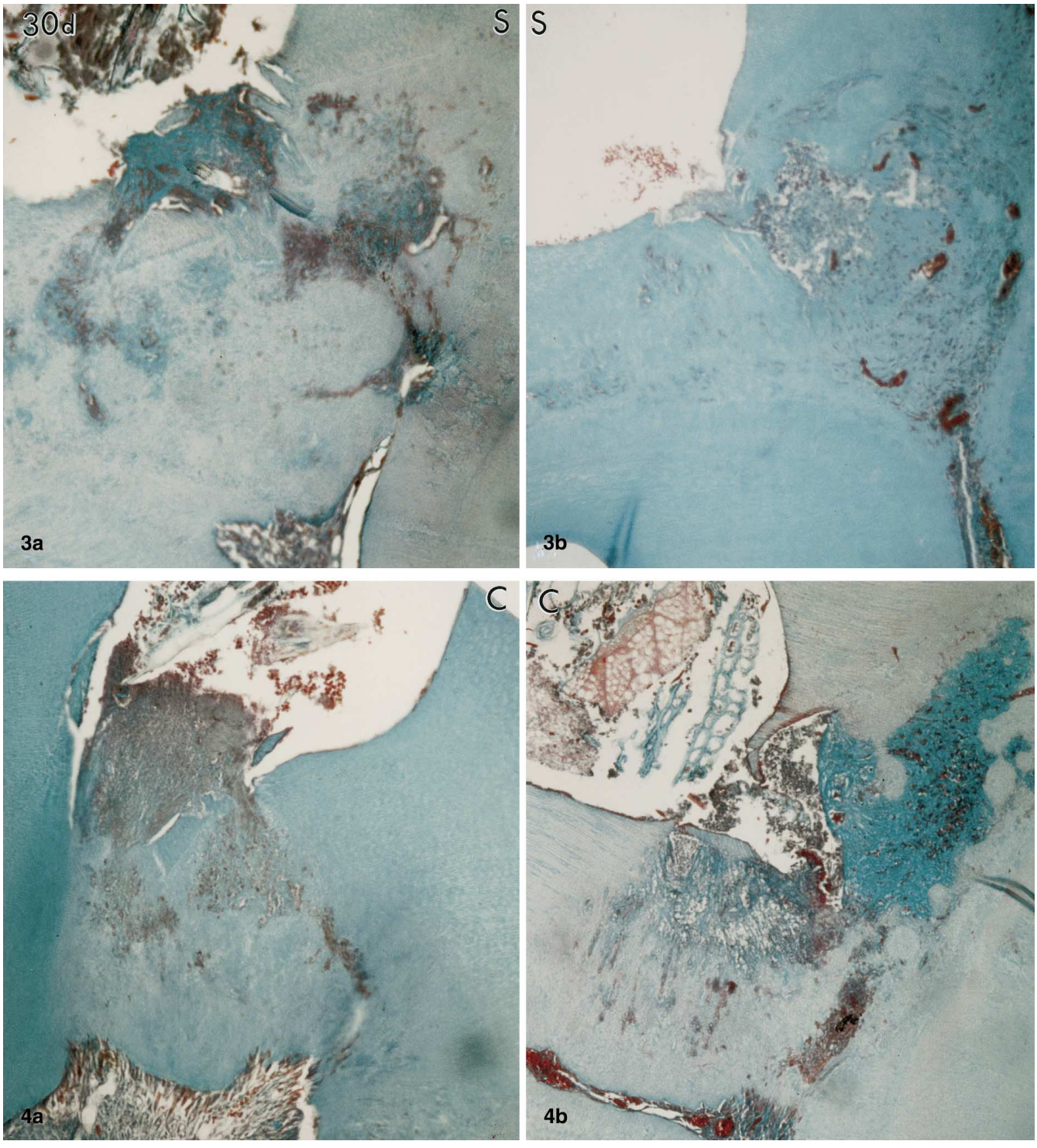

Fig. 3a,b Sham day 30. Osteodentin formation, globular structures, inclusions of cell debris and empty spaces containing pulp remnants are seen. The tendency for self-repair is enhanced by the presence of dentin and predentin debris which are inducers of reparative dentin formation, ×120

Fig. 4a,b Carrier group after 30 days. Formation of reparative dentin occurred also spontaneously, or under the influence of dentin debris (a). The situation is worst in another tooth where osteodentin, pulp remnants and heterogeneous filling of the pulp are seen (**b**), ×120

few cases, a thick layer of what appeared to be orthodentin partially filled the mesial part of the pulp chamber beneath an osteodentin-type plug. It was also clear that dentin and predentin debris may be involved in the formation of reparative dentin/osteodentin (Fig. 4b). At day 30 in the Ca group, the mesial part of the pulp chamber was filled with a mixture of cells and mineralized tissue of the osteodentin type (Figs. 5a,b). In contrast, in the OP group, the mesial third of the pulp was totally filled

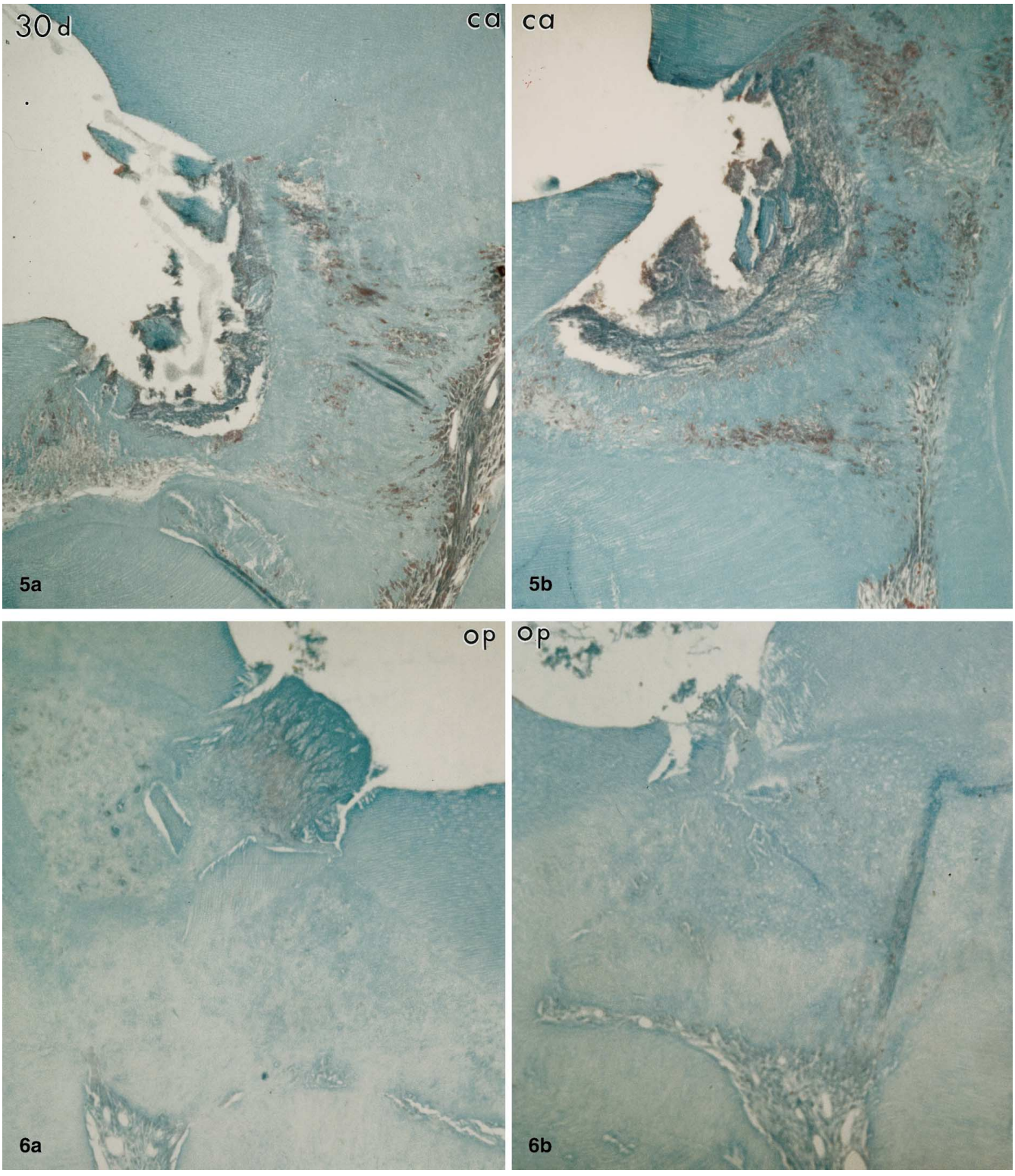

Fig. 5a,b Calcium hydroxide-treated pulps after 30 days. Osteodentin was formed massively. Pulp remnants are surrounded by reparative dentin, ×120

Fig. 6a,b Implantation of the osteogenic protein induces the formation after 30 days of an homogeneous reparative dentin, filling the mesial part of the pulp, ×120

by reparative tissue which was more homogeneous than in the Ca group (Figs. 6a,b). Initially, the reparative tissue that formed appeared to be of the osteodentin type, but shortly after this first deposit, a thick homogeneous atubular structure filled the pulp or at least its major part, leaving a thin pulp horn containing what seemed to be normal tissue (Table 1).

Discussion

Our aim was to characterize the effects of a bone sialoprotein implant in the pulp of rat's molars. Because there is a well-known tendency of self-repair in the rat tooth, controls were carried out on what we called a sham group but was seen in fact to contain dentin and predentin debris. These fragments are actually osteoprogenitor cells and reparative osteodentin inducers [24, 30, 31]. The effect of the carrier alone (gelatin) was also evaluated. The results obtained were compared with calcium hydroxide-capped pulps. Although the rat is a difficult model and therapeutical effects are difficult to identify, the comparison between the four groups allows some conclusions or at least to observe a tendency. It was confirmed that sound dentin and predentin debris pushed into the pulp during perforation and/or implantation are capable of inducing the formation of osteodentin 30 days after preparation. Direct implantation of the carrier induced a temporary inflammation. However, the reparative processes were not impaired after 30 days. In both cases, osteodentin was formed some distance away from the perforation. This was also largely the case with calcium hydroxide and, in addition, a layer of reparative osteodentin tended to fill the perforation. This layer increased in thickness and, at some stage orthodentin seemed to be formed, with odontoblast-like cells organized in a palisadic structure as has been reported previously with calcium hydroxide or calcium-β-glycerophosphate or α-tricalcium phosphate [9, 10, 13, 22, 31]. After direct implantation of BSP, the formation of a reparative mineralized tissue was slower at the beginning than with calcium hydroxide but enhanced at later stages, homogeneously filling nearly all the mesial third of the pulp chamber after 1 month whereas, with calcium hydroxide, pulp remnants were still present within or near osteodentin formation. The sections provide evidence of efficient cell recruitment and cell and matrix organization in the narrow pulp space. Due to the demineralization, a prerequisite for light microscope histological studies, mineralization of the reparative tissue cannot be appreciated, but the density of the structures formed is apparently much superior to what resulted from calcium hydroxide implantation, the actual reference in pulp capping. Characterization of the inflammatory cells (PMNs, macrophages, T- or B-cells) and identification of the recruited cells and the matrix components implicated in the reaction are now under investigation using immunocytochemical and immunohistochemical methods.

This is not the first time that rat molar was used to investigate the bioactive properties of a molecule or biomaterial [6, 16, 18, 28, 29, 33]. The technique used here has some specificities. Firstly, the gingival papilla was removed without bleeding by electrosurgery. This allows the preparation of cavities not related to the tip of pulp horn but drilled in an area related to the middle or lower third of the mesial wall of pulp chamber. The remaining dental tissues possess better mechanical resistance to occlusal pressures, which tend to dislodge the glass ionomer filling. In fact, a few fillings were lost, especially in the 30-day groups. As a result, bacterial colonization could occur. As the Brown and Brenn method [1] was used in only a few sections and not systematically, it is, however, difficult to conclude in these slides why no bacterial contamination was detected in the pulp. In any case, there were apparently no abscesses leading to pulp degeneration.

Secondly, pulp effraction was not carried out with the bur but by pushing the thin layer of dentin isolating the pulp from the cavity with a probe. This avoided stretching of the pulp tissue around the bur and uncontrolled pulp damages. In addition, the projection of unaltered dentin and predentin debris inside the pulp may contribute to spontaneous repair of the lesion [30, 31]. This implies that what we called sham is not a pure control of the effects of cavity preparation but that an endogenous biomaterial was active in that group and may be responsible for the tissue repair detected in the absence of calcium hydroxide or osteogenic protein implantation. This points out the crucial question of what is due to self-repair processes and what results from the BSP treatment. The comparison between pulps where dentin and predentin debris were implanted incidentally, pulps which were in contact with the carrier alone, and those where BSP was implanted aims to answer this question. Up to now, calcium hydroxide has been the gold standard in dentistry, so that a comparison between this material and BSP was mandatory. With a sufficient number of rats per group, clearcut conclusions might be expected. In fact, there is also uncertainty on how and how much of the protein was implanted, depending on the size of the implant. Since 30 μg BSP combined with 5 mg gelatin was used in the 18 molars of the OP group, it may be assumed that about 1.6 μg of BMP was implanted per tooth. However, there is some doubt about the homogeneity of the pellets implanted and the size of the contact area between the implant and pulp. The only answer to this series of questions was the intragroup homogeneity of the results.

It is interesting to note that BSP has bone-inductive capacity, in contrast to osteopontin, another phosphorylated matrix protein which lacks such a capacity [36, 37]. To the best of our knowledge, this is the first time that such a novel bioactive property showing stimulation of reparative dentin formation was reported, opening new perspectives for future therapy. BSP is expressed by odontoblasts and also found in peritubular dentin but not in pulp cells or pulp extracellular matrix [2, 3]. Pulp is an ambiguous tissue because it, on the one hand, does not mineralize spontaneously but on the other hand retains the ability to mineralize gradually with aging and, in case of trauma, to form reparative dentin, diffuse mineralizations, calcospheritic structures, or pulp stones. The recruitment of osteogenic cells, the interaction with collagen fibrillation, and the potential role of glutamic acid-rich sequences in the nucleation of hydroxyapatite seem to characterize the major biological properties of BSP. These biological properties might

explain why BSP is a molecule restricted to the mineralized matrices of bone, dentin, calcifying cartilage, and cementum and consequently may play a role in the formation of reparative dentin after implantation into the pulp.

To conclude, the data presented here provide insights on: (1) the possibility of using BSP, a biologically active molecule, to stimulate the formation of a thick reparative dentin layer which may act as a barrier against the cytotoxic effects of restorative biomaterials and (2) future possibilities to reduce or even suppress endodontic therapy. At the moment, it is not known whether the mineralization processes observed here 1 month after implantation cease at some time or if all the pulp in the crown and roots are gradually filled. This will be investigated in the near future.

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