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Osteogenic proteins (bone sialoprotein and bone morphogenetic protein-7) and dental pulp mineralization

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Bone sialoprotein (BSP) cross-linked to collagen/gelatin was implanted in the pulp of rat's upper molars. Comparison was carried out with a sham group (non implanted), with a group of rats receiving the carrier alone, and a group of molars where the perforated pulps were capped with calcium hydroxide. The cavities were occluded with a glass-ionomer cement (GIC). After 8, 14 and 30 days respectively the rats were killed by intracardiac perfusion of the fixative and processed for light microscopy. Dentin and predentin debris pushed into the pulp during the preparation enhanced self-repair processes, with large pulp remnants. The carrier alone induced slight inflammation, and calcium hydroxide the formation of a reparative dentin bridge. BSP stimulated the recruitment of cells which produced an homogeneous atubular dentin-like structure, filling after one month the mesial third of the crown pulp. Osteogenic protein (OP-1) used in the same experimental conditions induced the formation of osteodentin in the coronal pulp and the radicular part of the pulp was totally filled by a mineralized material. The differences reported here suggest two possible different therapeutic approaches with the two osteogenic proteins, BSP inducing pulp mineralization in the crown part, and OP-1 occluding the root part of the pulp.

1. Introduction

Since long calcium hydroxide has been used in dentistry as an unique capping material having the capacity to induce the formation of a mineralized dentin bridge. This barrier is the only appropriate response in case of incidental wound of dental pulp. The rationale of such procedure is that calcium hydroxide acting as an alkaline agent burns superficially the pulp, and induce a scar which favor odontoblast-like cells differentiation. Under this scar, recruited mesenchymal or STEM cells differentiate and elaborate a newly formed dentin-like layer which increase in thickness with time and mineralize more or less homogeneously [1, 2]. Depending either on the capacity of true odontoblasts to stimulate the formation of reactionary dentin, or on the reactivation of replacement cells of the odontoblast lineage, namely the so-called Höhl cells, calcium hydroxide stimulates the formation of reparative dentin and therefore has a long-standing history in the everyday practice of dentistry.

More recently, following Urist and Reddi's pioneering works, the discovery that bone morphogenetic proteins (BMPs) or osteogenic proteins (OP) form mineralized bone-like nodules in ectopic sites has opened new prospects in tissue engineering [3–5]. In the past few years, and because their potential usefulness in the treatment of various organs including the dental pulp, new tools were suggested, which are now studied extensively. In this context, Rutherford *et al*. [6] used BMP7, also named OP-1, a member of the TGFβ family, in order to induce the mineralization of the radicular pulp. OP-1 is a growth factor shown to be efficient after direct implantation in monkey's pulp, inducing the transformation of pulp into osteodentin [6, 7]. Other BMPs have been also used [8–10], as well as extracellular matrix proteins, all these approaches aiming to induce reparative dentinogenesis or pulp mineralization [11–14].

In this line, we have shown recently that BSP-cross-linked to collagen/gelatin and implanted in the pulp of

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rat's maxillary molars has the capacity to induce within one month the formation of large mineralization areas [15]. BSP is an extracellular matrix protein, found in bone and dentin, [16–18] but apparently not present in the dental pulp. This is diverging with the fact that BMP genes and receptors are actually expressed in human dental pulp cells [19, 20]. BSP on the other hand has been suggested to have various biological functions: (1) modulate *in vitro* mineralization, (2) is a nucleator of hydroxyapatite [21, 22], (3) mediate attachment of cells through its RGD sequence [23], (4) has strong affinity to collagen fibrils [24], (5) is strongly expressed during the repair of calvarial defects [25], and in this context BSP was suggested to have functions other than these stated above and (6) is capable of inducing the differentiation of osteoblast-like cells from undifferentiated mesenchymal cells [26]. Implanted in subcutaneous sites, the new bone formation is significantly less than in calvarial defects [27, 28]. This constitutes another strong difference with BMPs, known to induce mineralization in any ectopic site [29]. In order to get some insights on the biological mechanisms which control the development of such mineralizing processes, we therefore decided to compare the effects of BSP and OP-1 in an *in vivo* upper rat molar model.

The rat molar provide a good model for such investigations. However, strong pitfalls have to be taken into consideration before any final conclusion may be drawn. Firstly, due to the exceptional resistance of the rat, spontaneous healing can occur, a phenomenon which may interfere with the results obtained in this experimental situation. The effects of cavity preparation have to be taken into account, since it is impossible to disconnect the preparation with the fact that during pulp perforation with the tip of a probe, fragments of dentin and predentin are pushed into the pulp. This is the only method to avoid pulp damages, the tissue being rolled up around the bur and subsequently pulled out. It is now well established that these fragments induce pulp mineralization [11–14]. Secondly, the carrier might also have some specific effects, which have to be clearly identified. Finally, because the clinical reference is calcium hydroxide, a comparison is also needed on the specific effects of this biomaterial. Using this series of controls, the respective effects of BSP and OP-1 on dental pulp were evaluated.

2. Materials and methods

Forty-eight Sprague Dawley rats aged six to seven 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 two upper first molars, right and left. The surgery, and the next steps, preparation of cavities, implantation in the pulp and filling with glass-ionomer cement (GIC), was done using a microscope (Epidiascope) at × 16 magnification. Half-moon class V-like cavities were then prepared in 1–2 s in the cervical third of the mesial aspect of 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 adequately flush the cutting area. The burs were changed after every fourth cavity. Two teeth were prepared for rat. Pulp perforation was accomplished by pressure with the tip of a steel probe.

For the BSP part of this investigation, the upper molars were divided into four groups, each of 18 teeth (total number of teeth, 72). In the Sham group of teeth (S), the cavity was prepared but filled only with Fuji IX, a light cured GIC (GC Corporation. Tokyo, Japan). In the carrier (gelatin) group (C), the carrier alone was implanted in the pulp, and the cavity filled with GIC. In the osteogenic protein group, BSP together with the carrier were implanted and protected with the GIC. BSP was purified by one of us (E. Salih) in his laboratory, as previously described [30]. Thirty microgram BSP covalently cross-linked to with 5 mg gelatin were used for the 18 teeth of this group (between 0.25 and 0.30 μg/pulp). The effects of BSP on the formation of reparative dentin or osteodentin in dental pulp were compared with a calcium hydroxide group (Ca). Rats were randomly treated, and the right side treatment differed from 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.

For the OP-1 groups of rats, polyglycol was used as a carrier, two groups of 6 molars were prepared after implantation for 8 and 30 days with the carrier alone. Two other groups of molars were implanted with 2.5 μg recombinant human OP-1/mg polyglycol. One pellet was used per pulp, and the rats killed after 8 or 30 days. They were perfused in the same way.

The upper molar groups were dissected out from the maxillary after 5–6 min. Block sections including the three molars, bone and gingiva, were immersed in the fixative, kept at 4 °C for 24 h. They were demineralized either with sodium formiate or with 4.13% EDTA, and embedded in Paraplast. Seven micrometer serial sections were stained either with Masson's trichrome or with hematoxylin-eosin.

3. Results

3.1. BSP experiment

3.1.1. Eight days

Fig. 1 is an example of the preparation of the cavity in the cervical third of the mesial aspect of the first upper molar. The reaction to the carrier after eight days is limited to the mesial third of the pulp. Fig. 2 shows the weak reaction in a sham preparation (S). Fragments of dentin and predentin which is associated were pushed by the probe during the perforation. The inflammatory reaction was weak. A more severe reaction was induced by the carrier (gelatin/collagen) (Fig. 3). After one week the beginning of the formation of a thin bridge was seen. Odontoblast-like cells are seen in the inner border of the bridge (Fig. 4). In contrast, BSP did not induce any similar reaction. Compared with Fig. 2, numerous cells which have not yet been identified were observed within the pulp (Fig. 5).

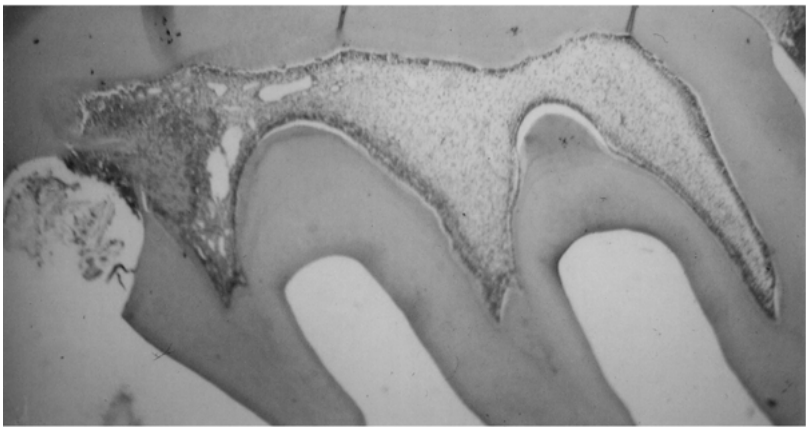

Figure 1 Carrier 8 days A cavity is prepared in the mesial aspect of the first maxillar molar. Hematoxylin-eosin × 120.

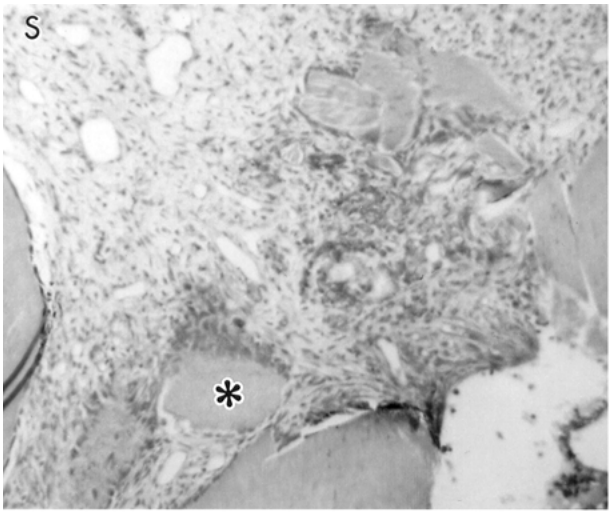

Figure 2 Sham (S) 8 days. Dentin debris (asterisk) were pushed in the pulp during the preparation of the molar. Inflammatory cells are seen at the surface of the perforation. Trichrome × 240.

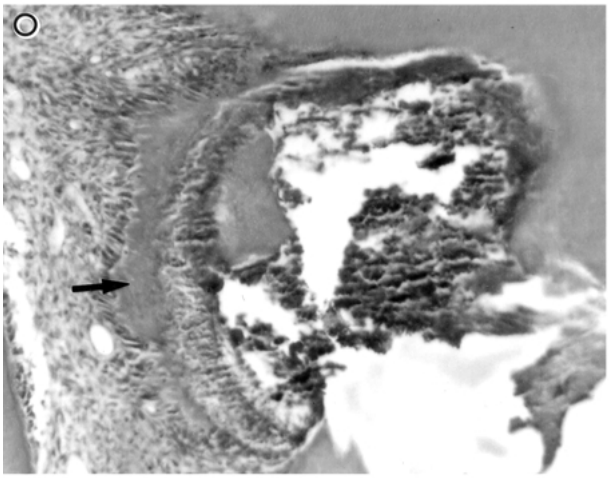

Figure 4 Calcium hydroxide (O) 8 days. A reparative dentin bridge begins to be formed (arrow). Trichrome × 240.

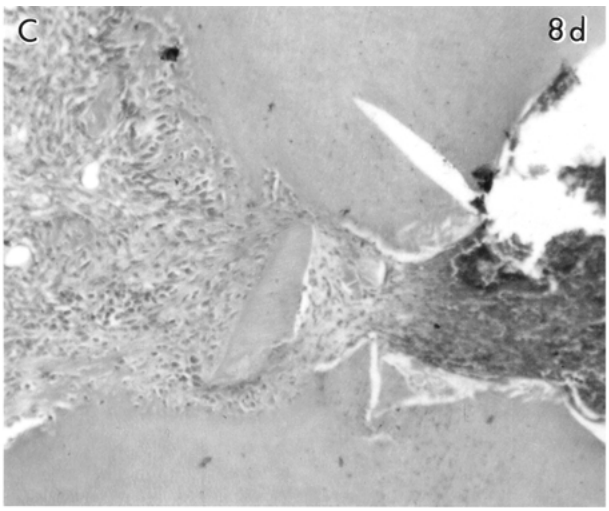

Figure 3 Carrier (C) 8 days. Mild inflammation. Trichrome × 240.

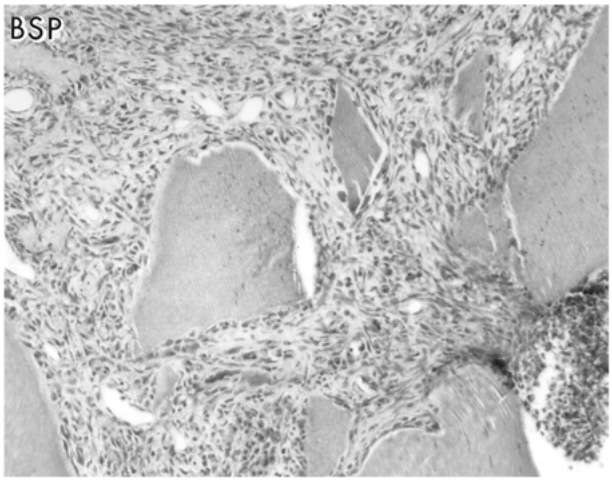

Figure 5 BSP/Gelatin 8 days. Inflammation is moderate. Trichrome × 240.

3.1.2. Two weeks

A tendency to spontaneous repair was observed in the sham group after two weeks, but the calcospheritic structures were not located near the pulp perforation, in the outer part, but more centrally, near the junction between the mesial and the central parts of the pulp chamber (Fig. 6). Inflammatory processes persisted after 14 days in the carrier's group (Fig. 7). The dentin bridge formed in the presence of calcium hydroxide displayed increased thickness. The mesial part of the pulp exhibited normal appearance (Fig. 8). In the BSP group, the outer part of the perforation was filled with osteodentin, but immediately beneath a dentin-like structure where no tubules were detected starts to fill the mesial part of the pulp. Around dentin fragments and debris, newly formed reparative tissue was detected (Fig. 9).

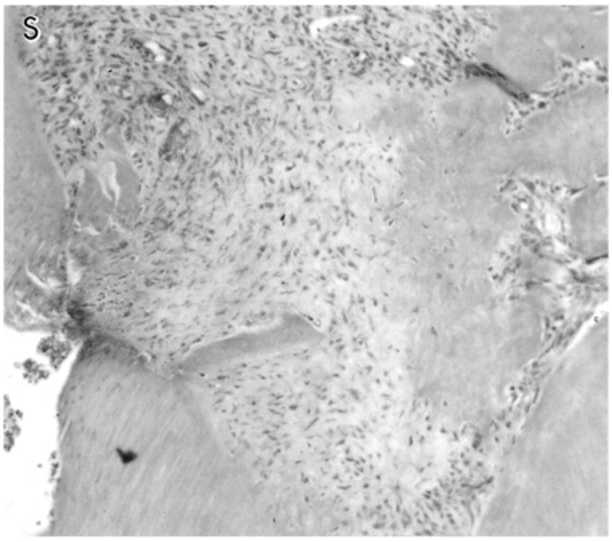

Figure 6 Sham (S) 2 weeks. Self-repair processes have already started in the opposite part of the pulp, some distance away from the perforation. Trichrome × 240.

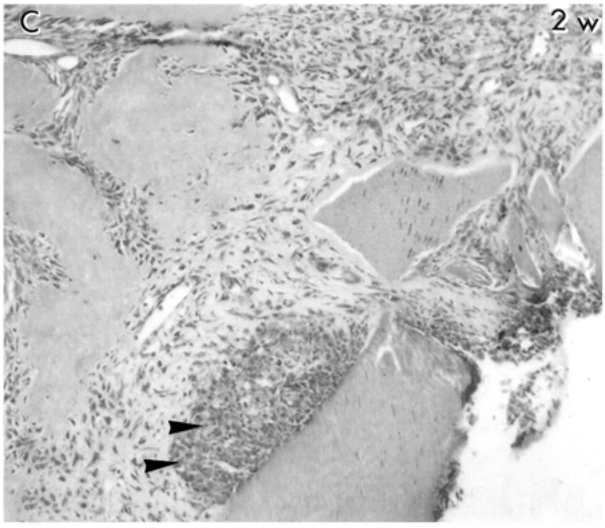

Figure 7 Carrier (C) 2 weeks. Inflammatory cells are still present and grouped (arrowheads) in the pulp. Trichrome × 240.

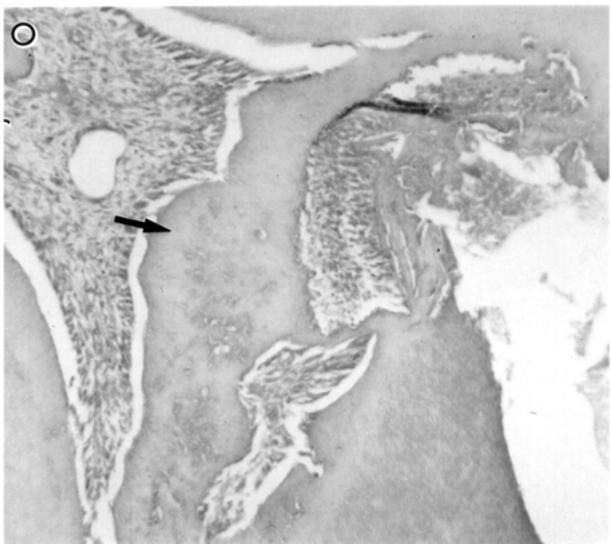

Figure 8 Calcium hydroxide (O) 2 weeks. The reparative dentin bridge is thicker (arrow). Trichrome × 240.

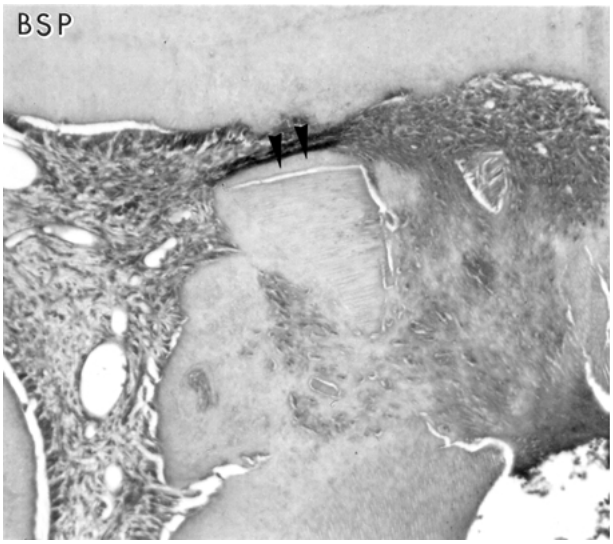

Figure 9 BSP 2 weeks. Mineralization of the pulp is expanding from the surface of the perforation toward deeper parts of the pulp. Mineral has been deposited along dentin fragments pushed during the preparation of the molar (arrowheads). Trichrome × 240.

3.1.3. Thirty days

Despite some spontaneous tendency for self-repair, calcospherites did not fill the mesial part of the pulp, and in many areas, unmineralized tissue containing many cells was observed (Fig. 10). The same was also true for the carrier's group (Fig. 11). After one month the dentin bridge induced by calcium hydroxide was thicker. However, most of the tissue was of the osteodentin type, with large areas of pulp remnants (Fig. 12). In the BSP group, after one month the mesial part of the pulp was filled with an homogeneous dentin-like material. Dentin debris were hardly identified, totally surrounded by new areas of mineralization. In most cases, no remnant of the pulp was detected in the mesial part of the pulp, whereas the rest of the pulp looked normal (Fig. 13).

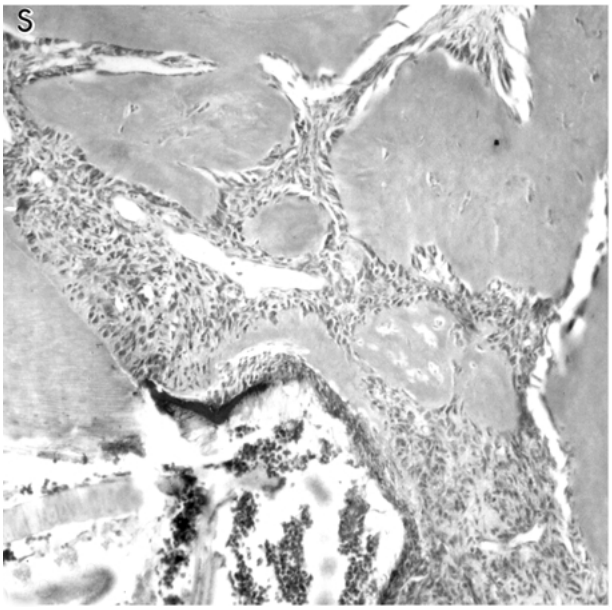

Figure 10 Sham (S) 30 days. Calcospherites are seen in the pulp. Spontaneous repair does not induce homogenous mineralization of the pulp, as seen from pulp remnants. Trichrome × 240.

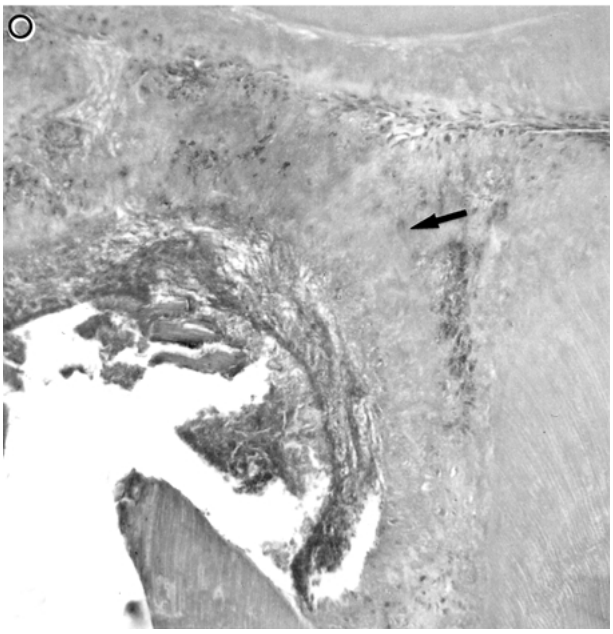

Figure 12 Calcium hydroxide (O) 30 days. Despite the formation of a thick reparative dentin bridge (arrow), many pulp areas remain unfilled by mineralized material. Trichrome × 240.

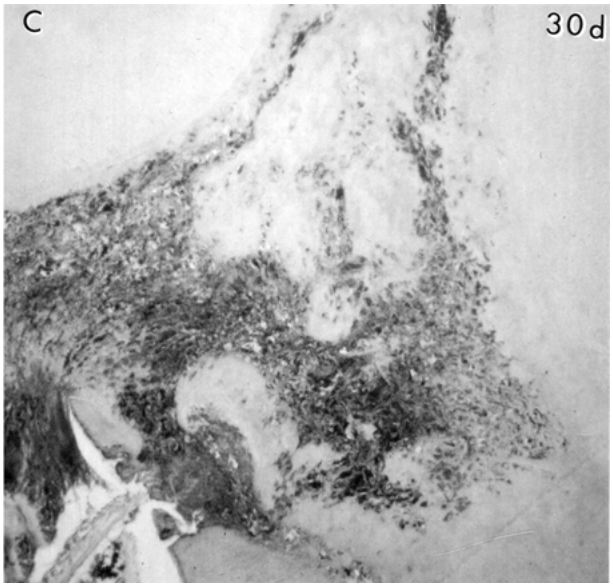

Figure 11 Carrier (C) 30 days. Heterogeneous mineralization is seen in the pulp, with many area containing pulp remnants. Hematoxylin-eosin × 240.

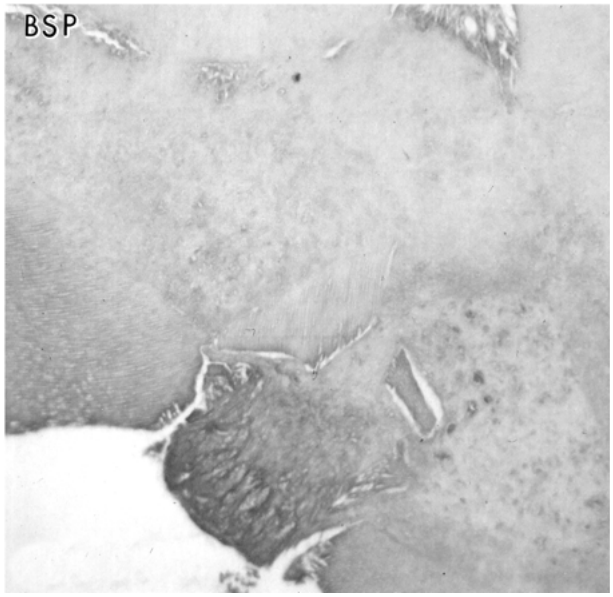

Figure 13 BSM 30 days. An homogeneous material fills the mesial third of the coronal pulp chamber. Trichrome × 240.

3.2. OP-1 experiment

After eight days or 30 days, the carrier (polyglycol) induced no more than a weak inflammatory reaction. In the eight days molars implanted with OP-1, many cells of unknown origin were recruited in the mesial part (Fig. 14). After 30 days, osteodentin was seen instead of pulp tissue. Some pulp remnants persisted (Fig. 15). In addition, an induced mineralization was seen in the mesial root. A calciotraumatic-like line was seen at the limit between the root dentin present before implantation and the homogeneous biological filling induced by the BMP in the radicular pulp (Fig. 16).

4. Discussion

The present data confirm that implantations of BSP or OP-1 in the pulp of a rat's molar induce the formation of reparative dentinogenesis. The two molecules stimulate the recruitment of new cells, which differentiate into osteogenic or dentinogenic cells. The nature and origin of such cells in the dental pulp need still further clarification. They might be mesenchymal cells, STEM cells, fibroblasts, pericytes or endothelial cells, as it was speculated after direct capping with calcium hydroxide [31]. It is clear that odontoblasts which are post-mitotic cells cannot participate in such processes. There is a high

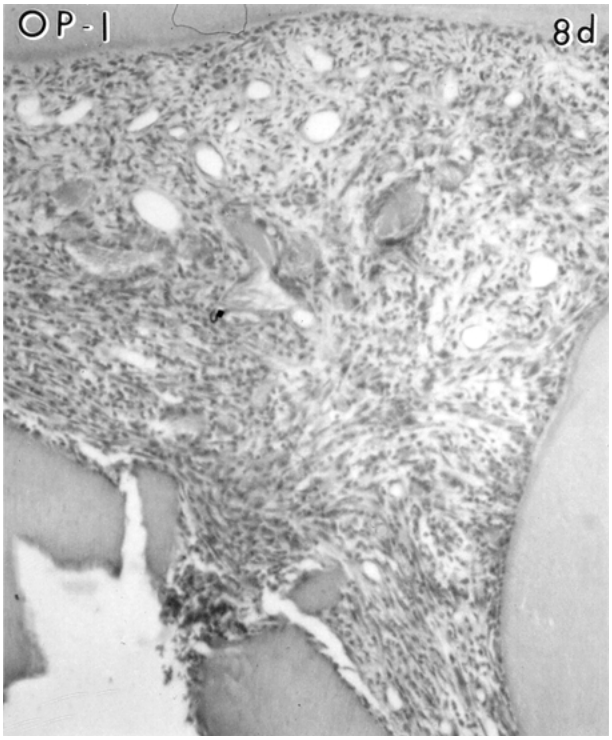

Figure 14 OP-1 8 days. An inflammatory response is seen. Hematoxylin – eosine × 240.

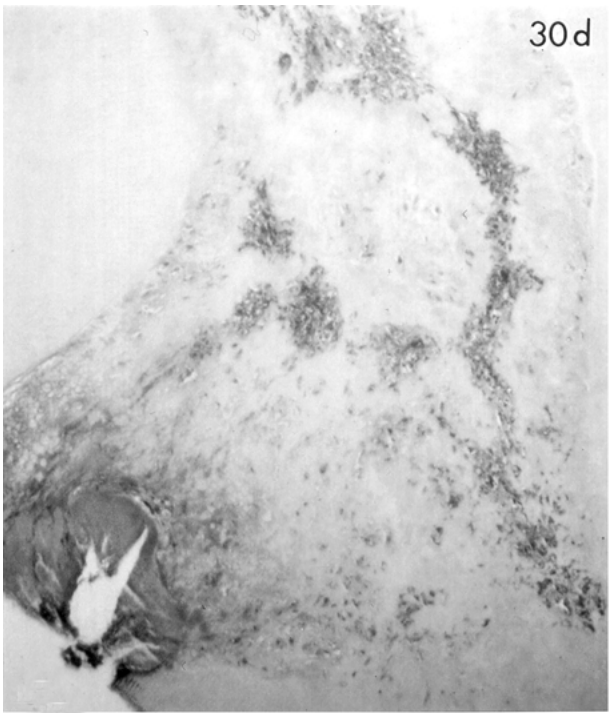

Figure 15 OP-1 30 days. Reparative dentin of the osteodentin type is formed and fills the mesial aprt of the coronal pulp. Some pulp remnants are still present. Trichrome × 240.

probability that odontoblasts and Höhl cells are involved in the elaboration of reactionary dentin but not reparative dentin (see Lesot *et al*. [32] for terminology). Once differentiated, osteoblast-like or odontoblast-like cells produce eventually an extracellular matrix which mineralize. In a pilot study using undemineralized sections we have confirmed with a scanning electron microscope microprobe that the material which fills the pulp after such treatments has a high calcium and phosphorus content, and therefore is mineralized. Depending on the stimuli, bone-like structure (osteodentin) or atubular dentin-like structures were formed. In this context, the present data provide evidence that pulp has a high potential to mineralize. It has been established that colonies of cells emerging early from pulp explants mineralize after 30 days of culture [33]. The actual phenotype of these cells has not yet been characterized. Altogether, this supports that BSP acting mostly in bonny sites and to a smaller extent in ectopic areas [29] exert strong effect *in vivo* in dental pulp. BSP stimulate calcification in osteoblast-like cells [34]. It is, however, important to note that in osteogenesis induced by BMP, there is a time dependent expression of BSP, and therefore some evidence of a cascade where BSP plays an important role [35]. Hence, the two molecules have an overlapping activity. In any case, using BSP or OP-1 the induced mineralization area was larger and more homogeneous than could be seen with calcium hydroxide, the gold standard in operative dentistry.

It is noteworthy that BSP induce the formation of an atubular dentin-like structure in the crown part of the pulp, whereas a porous and inhomogeneous osteodentin resulted from OP-1 implantation. This was also the case during reparative dentin formation in monkey by OP-1 [6]. More important is the fact that OP-1 stimulate the formation of reparative dentin in the radicular part of the pulp, which was not apparently the case with BSP, at least for the experimental period of 30 days. This specific reaction suggests that two different strategies might be used, each of the OP having its own target. Direct pulp capping with BSP leads to the formation of a thick reparative dentin-like structure filling the mesial third of the pulp located in the crown. With respect to the clinical application of this finding, this would prevent diffusion of cytotoxic free monomers from resins and resin-enriched GIC, and the subsequent alteration of the remaining part of the pulp. Alternatively, OP-1 might be used as a powerful substitute to root canal treatment. Such biologic tool may prevent the potential failures of endodontic treatments. BMPs other than BMP-7 may have similar mechanisms and therefore may produce the same effects. They have now to be evaluated in this context.

This also suggests that substantial differences exist between the crown and the radicular part of the pulp. The crown part is the result from the development of an embryonic pulp, originally a mesenchymal tissue enriched in neural crest-derived cells. This part, surrounded by an enamel organ, is clearly influenced by the diffusion of enamel proteins, namely amelogenin, toward odontoblasts and pulp cells [36–39]. The radicular part is formed during the formation of the root and linked to the tooth eruption. Even if the cells have the same embryological origin, a point which is still open for discussion, epithelial Hertwig root sheath cells (EHRS) do not synthesize and consequently cannot secrete amelogenin. In contrast, ameloblastin and bone and cement matrix molecules are expressed by EHRS cells, now recognized to be subjected to epithelio-connective interconversion. [40]. Hence, the cell/matrix

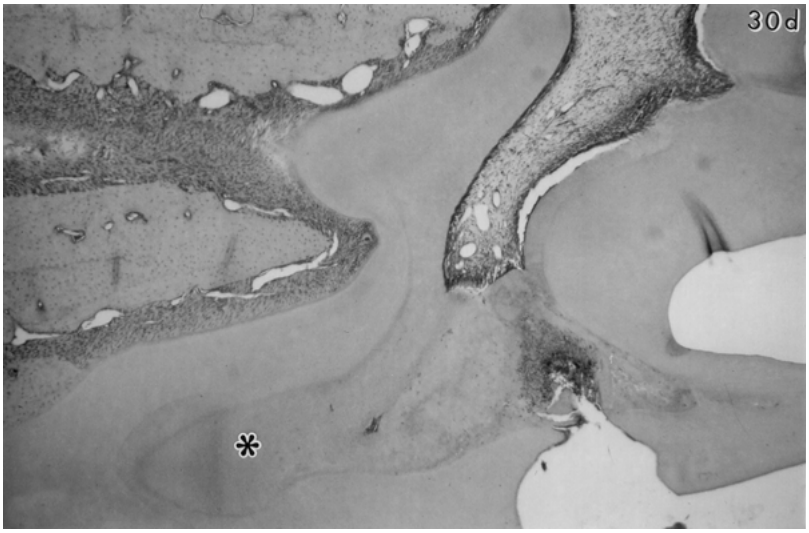

Figure 16 OP-1 30 days. Mineralization of the root part of the pulp (asterisk). Hematoxylin-eosin × 120.

relationships are totally different. In addition, during tooth development, endothelial cells and axons penetrate the embryonic pulp papilla and constitute a specific coronal organization. Later, a second group of vascular cells and neurons associate to form another network in the root, which bears its own specificity. Such differences may contribute to the differences reported here, recognized for the first time, to the best of our knowledge.

Finally, it is interesting also to note that the rat's molar provide a valuable biological model. The results obtained with OP-1 are very similar to what was observed in monkey [6, 7]. The comparison between the sham and carrier groups and experimental groups allows to identify what is under the control of self-repair processes, even enhanced by the projection of dentin and predentin fragments in the pulp, which certainly contribute to the healing or to the heterogeneous mineralization of the tissue. Up to now, this model has been used mostly for the evaluation of dental biomaterials [41–45], but is clearly suitable for study of the effects of different types of OP.

Acknowledgments

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