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In vivo reactions to particulate rhenanite and particulate hydroxylapatite after implantation in tooth sockets

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To gain more information on the *in vivo* behaviour of rhenanite ($CaNaPO_4$), particles of this material and of hydroxylapatite were mixed with gelatin or saline and inserted in tooth sockets of beagle dogs for periods of 3 and 6 months. Rhenanite appeared to transform into an apatite containing carbonate, sodium and magnesium. Resorption of both calcium phosphate particles was not observed histologically and was not shown histomorphometrically. Calcium phosphates were not found in lymph node tissue. The presence of particles in the sockets gave rise to new bone formation. Histomorphometry demonstrated that statistically significant more bone deposition occurred on rhenanite particles than on hydroxylapatite particles. Also gelatin, meant as a spacer, contributed to new bone formation.

1. Introduction

In previous studies on calcium phosphate resorption and transformation [1, 2], divergent reactions to rhenanite ($CaNaPO_4$) were observed when implanted in the forehead of domestic pigs (*Sus scrofa*) for periods of 6 weeks and 3 months. Most rhenanite (Rh) implants were surrounded by a wide soft tissue periimplant containing a large number of osteoclast-like cells strongly resorbing the implants. In contrast two Rh implants revealed intimate contact with bone after some initial resorption at the surface had taken place. This resorption had obviously stopped as no resorptive cells were found. Electron probe X-ray microanalysis (EPXMA) showed that the chemical composition of the two Rh implants had changed, and according to X-ray diffraction (XRD) the crystal structure had turned into an apatite. To gain more information on the *in vivo* behaviour of this still rather unknown Rh as a biomaterial, another *in vivo* experiment was carried out in which the reactions to Rh were compared with the reactions to hydroxylapatite (HA). Changes in the chemical composition and crystal structure of Rh were again evaluated.

2. Materials and methods

Blocks of Rh were formed by making mixtures of $NaHCO_3$ (Merck 6329) and $CaHPO_4$ (Baker 0080) and pressing in a cylindrical mould. Sintering was carried out at 1300 °C for 16 h under a stream of air, followed by slow cooling to room temperature. This resulted in the formation of about 20 vol % microporous Rh, pore size 4.4 ± 2.2 μm, measured by electron microscope during EPXMA. HA was formed by making a slurry of $Ca_5(PO_4)_3$, H_2O and 30% H_2O_2 which was heated overnight at 100 °C and sintered at 1300 °C. The resulting HA contained 50 vol % pores. Pore size of HA was not measured as no EPXMA was performed on HA. The blocks of Rh and HA were crushed and sieved to obtain 0.5–0.7-mm sized particles. To create sufficient space for bone growth between the particles, 40 % w/w Rh or HA particles were mixed with gelatin.

Gelatin was prepared by dissolving 1.00 g calf skin 225 Bloom gelatin (Aldrich) in 20 ml of water and sterilized by passing the solution through a millipore filter (pore diameter 0.2 μm). Stability at body temperature was achieved by a cross-linking procedure [3] comprising storage in 5% DIC (1.6 di-isocyanatohexane 98%, Aurich Chemie, Germany) in 100% ethyl alcohol for 24 h. Washing out of the DIC was done with alcohols of decreasing percentage over 72 h, followed by washing with sterile physiological saline. A pH value of 7 was established by storage in 0.1 $mol\,l^{-1}$ phosphate buffer for 48 h. Biocompatibility was demonstrated by the *in vitro* growth of human fibroblasts on samples of the stabilized gelatin in a medium as used by Wijnbergen-Buijen van Weelderen *et al.* [4].

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TABLE I Distribution of the type of implant materials over the extraction sockets per animal and per experimental period: gel = gelatin, sal = saline

Dog number	Experiment period (months)	Sockets left:		Sockets right:	
		2 molar	2 premolar	2 premolar	2 molar
1	3	Rh + gel	Rh + gel	Rh + gel	Rh + gel
2	3	HA + gel	HA + gel	HA + gel	HA + gel
3	3	Rh + gel	Rh + sal	HA + sal	HA + gel
4	6	Rh + gel	Rh + gel	Rh + gel	Rh + gel
5	6	HA + gel	HA + gel	HA + gel	HA + gel
6	6	Rh + gel	Rh + sal	HA + sal	HA + gel

The fresh extraction sockets of the fourth premolars and the first molars at both sides of the lower jaws of six beagle dogs served as implantation sites. The dogs were divided into two groups of three dogs, with experimental periods of 3 and 6 months. As the *in vivo* reactions to Rh were still unpredictable and these reactions might influence the reactions to HA, the implant material containing Rh was kept separated from the material containing HA. In each group therefore, all eight extraction sockets in one dog were filled with Rh in gelatin and in another dog with HA in gelatin. To be able to compare the reactions with the two different implant materials in one dog, in each group the two molar sockets at one side of the third dog were filled with Rh in gelatin, and with HA in gelatin in the two molar sockets at the other side.

In this third dog the effectiveness of the spacing between the particles by the gelatin was studied comparatively by filling the two premolar sockets at one side of the lower jaw with Rh particles mixed with a drop of saline, and at the other side with HA particles mixed with saline. No unimplanted control sockets were kept. The distribution of the different materials is given in Table I.

The sockets were filled with the sterile materials to a level maximally 2 mm below the crest to facilitate bone growth over the implants. The extraction wounds were completely closed by mobilizing and suturing a mucosal flap. X-rays of the implant sites were taken immediately after the operation and every month thereafter. Intra-oral inspections were done at the same time and more frequently during the healing period. At the time of sacrifice perfusion fixation was carried out using physiological saline followed by neutral 4% formaldehyde solution. Blocks of tissue containing the implants were embedded in PMMA, sawn to 50-μm-thick undecalcified sections which were Giemsa stained. During histological evaluation by light microscopy the differences in rate of bone growth between the particles, bone deposition on the particles and resorption of the particles were of special interest.

To support the histological evaluation on bone deposition on the particles, histomorphometry was performed on sections of a 6-month animal (dog number 6, Table I). This animal contained all types of implant material and also the only socket where the Rh particles in saline appeared to be unclotted and allowing tissue growth between the particles. Of each implant one representative section was drawn using an inverted microscope. The particles on the drawings were traced and measured with a computerized tablet digitizer (Hitachi, model HDG 1111B), which discriminated the distance over which the particles had either bone or soft tissue contact. Simultaneously the surface areas of the individual particles were calculated. To study any reduction in particle size upon implantation, socket-size holes were prepared in self-curing PMMA and filled with Rh and HA particles in gelatin or saline. Sections were prepared, followed by similar measurements of the surface areas. These areas were statistically compared with the surface areas upon implantation.

The following statistical tests were performed two-sidedly:

1. the Mann and Whitney U-test (Wilcoxon's two-sample test);
2. a combination test based on the combination of Mann and Whitney statistics for a number of pairs of experimental groups.

Reproducibility of the technique of tracing and measuring was checked by repeating the tracings and measurements of the first 40 particles at the end of the series, using the Wilcoxon ranked sign test.

Possible changes in the chemical composition and the crystal structure of Rh were studied by quantitatively and qualitatively analysing particles in histological sections at 3 and 6 months by EPXMA (Camebax MB-1, operating at 20 kV and 3 nA beam current, measuring time 2 min), XRD (Philips X-ray diffracto-meter, CuK_α radiation and Ni filter) and infrared spectroscopy (IR) in KBr tablets (Perkin Elmer type 457). X-ray spectra were also recorded of surrounding bone and unimplanted Rh. As in the previous study no changes were found by XRD in the crystal structure of the applied HA; no XRD, IR and EPXMA were done on HA.

Submandibular lymph nodes were excised and undecalcified sections were examined for the presence of calcium phosphates by light microscopy, unstained, and after alizarin staining, XRD and EPXMA.

3. Results

3.1. Intra-oral inspections and X-ray photographs

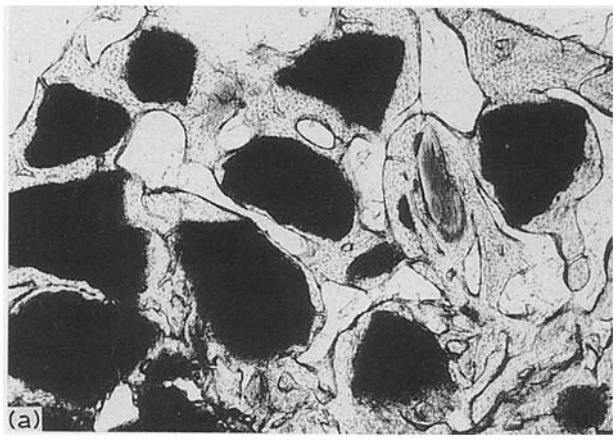
Four sockets lost their contents completely: two con-

taining Rh with saline within 1 week and 2 months (dogs 3 and 6, Table I); one containing Rh and one containing HA, both in gelatin, within 1 week. (dogs 1 and 2, Table I)

3.2. Histological evaluation

At a rough estimate, 27% of the sockets appeared to have lost 25% or more of the particles, leaving an open space in the socket adjacent to the still-concentrated retained particles that were present in sufficient quantity for investigation (Fig. 2). No difference in particle loss was estimated between sockets containing either Rh or HA.

In most sockets after 3 months the space between the Rh and HA particles applied in gelatin was filled with a well vascularized connective tissue but predominantly with woven bone. At many places HA particles, and to an estimated larger degree Rh particles, showed intimate contact with this newly formed bone. More Rh particles than HA particles were completely and directly surrounded by bone (Fig. 1a and 1b). No inflammatory reactions were met. The Rh particles had a less sharply edged, more rounded appearance than the HA particles. In general no resorptive cells were found. Occasionally an osteoclast-like cell was found at HA or Rh particles surrounded by connective tissue and positioned at or above the level of the alveolar crest.



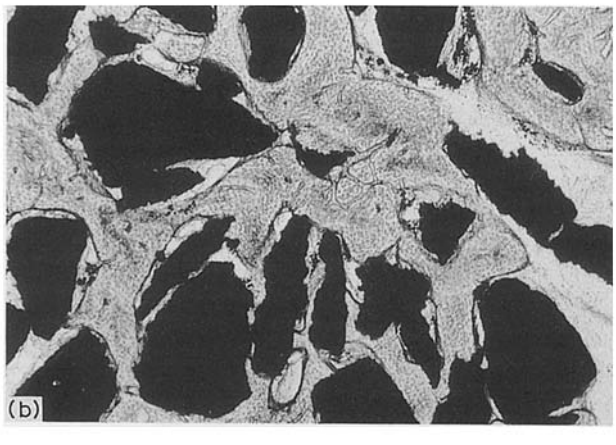


Figure 1 (a) Rh particles after 3 months. Many particles are completely surrounded by bone and are showing a more rounded appearance (× 10); (b) HA particles do have bone contact but less completely (× 10).

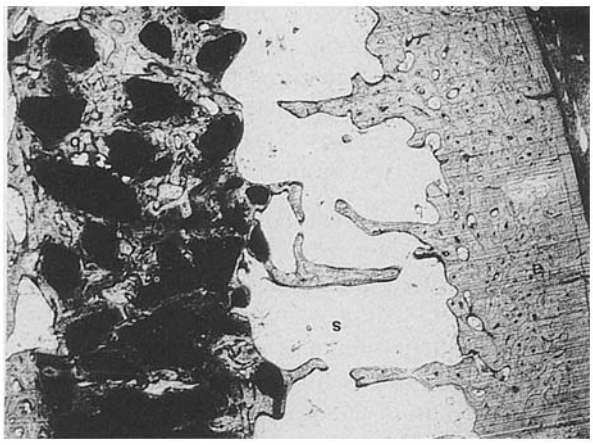


Figure 2 Where Rh particles are present new bone is formed around the particles. It is noticed that little trabecular bone is formed at those places where no particles are present in the socket.

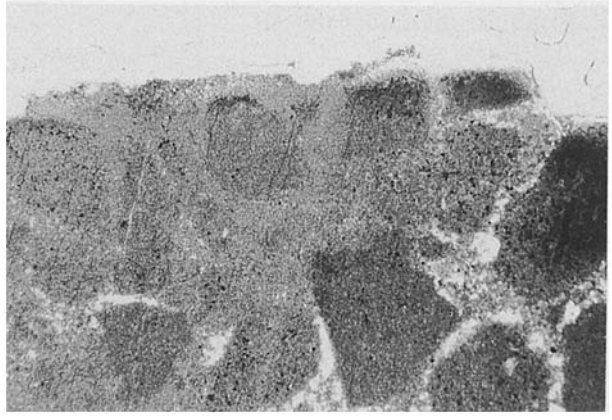


Figure 3 Clotting of Rh particles in saline. The separate particles are discernable but no space is left for bone growth between the particles (× 10).

In those cases where at places in the socket no particles happened to be present, new bone was hardly formed. When, however, only a few Rh or HA particles were present, bone was growing in between and was found in contact with the particles (Fig. 2). Marrow spaces were filled with fatty tissue and osteoblasts were found against newly formed bone.

Contrary to HA particles, in this period Rh particles in saline (one socket) appeared to clot to a dense mass (Fig. 3) and although the original contours of the particles were still discernable, no space was left for tissue ingrowth. Only at the border of this agglomerate was bony contact found. The HA particles in saline (two sockets) showed more spacing, though less when mixed with gelatin.

After 6 months an identical picture was observed, but now the newly formed bone had matured more. Bony contact with the particles had increased and more particles, especially Rh particles, were completely incorporated in bone. The one socket left in this period containing Rh in saline showed no dense clotting as was observed in the 3 month period. Space between the particles allowed ingrowth of bone.

3.3. Histomorphometry of bone deposition

The results of checking the reproducibility of the technique of tracing and measuring and of comparing bone deposition on the particles are given in Table II.

The mean percentages of bone deposition on the particles in each type of implant were:

Rh in saline in 1 premolar socket: 53.9%, s.e.m. = 5.6;
Rh in gelatin in molar sockets: 75.3%, s.e.m. = 3.6;
HA in saline in premolar sockets: 29.0%, s.e.m. = 4.2;
HA in gelatin in molar sockets: 38.5%, s.e.m. = 3.3.

3.4. Histomorphometry of particle resorption

The results of checking the reproducibility of tracing and measuring the surface area of the particles and of comparing the *in vitro* and *in vivo* applied implant materials are given in Table III.

3.5. Electron probe X-ray microanalysis

EPXMA of two unimplanted Rh particles resulted in a mean Ca/P ratio of 1.01 (Table IV) and absence of Mg (Table V). The Na content approximated closely that of Ca and P. Three and 6 months upon implantation the Ca/P ratio has risen to about 1.30. Table VI, as an example of Rh in gelatin, reveals that after 3 months Rh had taken up some Mg, which was also observed in the newly formed bone deposited on the particles. The Na content, compared with the unimplanted particles and in relation to the Ca and P content, had dropped drastically. As uptake of Mg and drop of Na content was observed with all other 3 and 6-month Rh implants, Table VI can be regarded as representative.

Paired t tests showed no significant differences in Ca/P ratio between centre and border of the particles, between bone deposited on the particles and bone further away, between Rh in gelatin and Rh with saline, and between 3 and 6-month particles.

3.6. X-ray diffraction

Rh particles in eight sections from the 3-month period showed a more or less advanced transformation into the apatite structure. XRD patterns of particles in seven sections from the 6-month period showed that peak positions were similar to those of hydroxylapatite (Fig. 4).

3.7. Infrared spectroscopy

To make a tablet for IR spectro-analysis Rh material was scraped with a small dental excavator from retrieved 6-month and PMMA embedded particles. Care was taken not to include surrounding bone. The IR spectrogram (Fig. 5) showed this Rh to contain carbonate that substituted the PO_4^{2-} ions, because the carbonate was only of the B-type.

TABLE II Statistics of bone deposition on Rh or HA particles in gelatin or saline: W = Wilcoxon's ranked sign test, MW = Mann and Whitney U-test, gel = gelatin, sal = saline

Line	Subject of test	Kind of test	Number of particles	p-value .... > ....
1	Reproducibility of tracing and measuring	W	40	0.31
2	Rh in gel versus Rh in sal	MW	61/17	0.016 gel > sal
3	HA in gel versus HA in sal	MW	71/29	0.099 gel > sal
4	Combination of 2 and 3	MW	132/46	0.0035 gel > sal
5	Rh in sal versus HA in sal	MW	17/29	0.001 Rh > HA
6	Rh in gel versus HA in gel	MW	61/71	< 0.001 Rh > HA

TABLE III Statistics of resorption of Rh and HA particles in gelatin or saline: MW = Mann and Whitney U-test, W = Wilcoxon's ranked sign test, gel = gelatin, sal = saline

Line	Subject of test	Kind of test	Number of particles	p-value
1	Reproducibility of tracing and measuring	W	40	0.26
2	Rh in sal versus Rh in gel, both *in vitro*	MW	12/36	0.91
3	HA in sal versus HA in gel, both *in vitro*	MW	16/5	0.94
4	Rh pool *in vitro* versus Rh in sal *in vivo*	MW	48/17	0.368
5	Rh pool *in vitro* versus Rh in gel *in vivo*	MW	48/61	0.395
6	HA pool *in vitro* versus HA in sal *in vivo*	MW	21/29	0.221
7	HA pool *in vivo* versus HA in gel *in vivo*	MW	21/72	0.401

TABLE IV Mean Ca/P ratios as measured by EPXMA of unimplanted Rh particles and implanted Rh particles at the centre and at the border against bone ongrowth; Ca/P ratio of ongrown bone; Ca/P ratio of newly formed bone further away from the particles. x = mean Ca/P , s.d. = standard deviation, n = number of particles. The figure between (. .) is the total number of X-ray spectra recorded in each series

Ca/P ratio	Not implanted 2 particles			3 months 3 implants from 1 dog			6 months 3 implants from 2 dogs		
	$\bar{x}$	s.d.	n	$\bar{x}$	s.d.	n	$\bar{x}$	s.d.	n
Centre particles	1.01 (10)	0.09	2	1.29 (5)	0.04	5	1.30 (17)	0.04	6
Border particles				1.31 (5)	0.05	5	1.31 (16)	0.03	6
Bone ongrowth				1.32 (4)	0.08		1.32 (7)	0.04	
Bone ingrowth				1.31 (3)	0.04		1.26 (6)	0.11	
Alveolar bone				1.33 (3)	0.06		1.39 (3)	0.04	

3.8. Lymph nodes

Light microscopy gave reason to believe that clusters of calcium phosphate grains were widely present (Fig. 6). Tiny black to brown/yellowish grains were commonly found in the tissue and cells as well. The nature of these grains was studied by several other techniques. Alizarine staining on calcium gave negative results. XRD and an average of 24 spectra by EPXMA in preselected areas in each of three sections where the grains were seen, did not demonstrate calcium phosphates. Instead more S and P was found, more characteristic of organic tissue, and sometimes traces of Si, Fe, Al and K. A control dog, however, used for other than implantation purposes, appeared to contain similar grains in excised submandibular lymph nodes.

4. Discussion

In this study resorption of Rh was not found whereas in a previous study [1] resorption of most Rh im-

TABLE V Composition (at %) of one unimplanted Rh particle measured by quantitative analysis of X-ray spectra recorded at different spots

Spot	Na	Mg	Ca	P	Ca/P
1	13.39	0.00	10.56	11.00	0.96
2	20.29	0.00	14.06	15.60	0.90
3	14.13	0.00	11.11	12.00	0.93
4	5.48	0.00	9.13	7.57	1.21
5	15.02	0.00	12.21	12.27	1.00

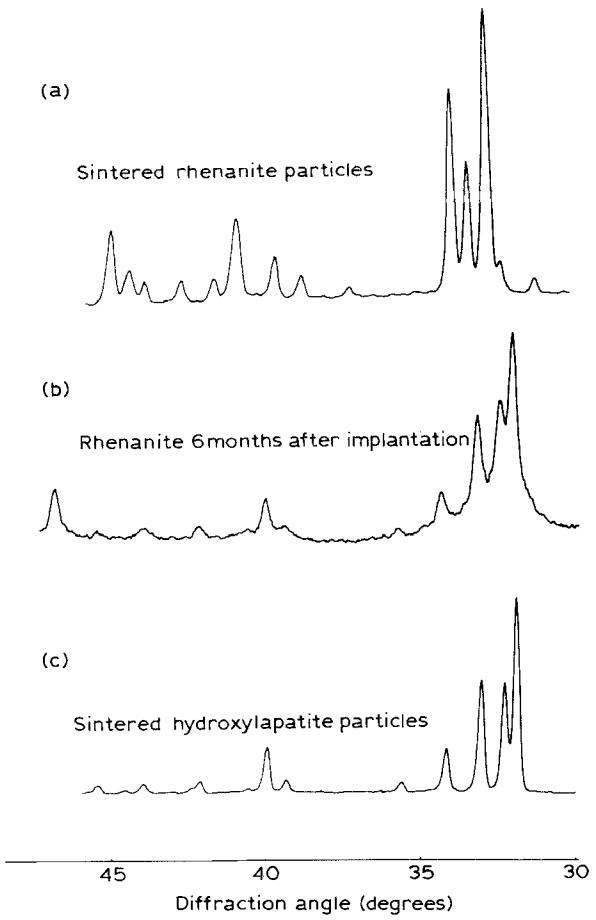


Figure 4 XRD patterns of Rh before implantation (a), Rh 6 months after implantation (b) and sintered HA (c). Peak position of the middle pattern shows more similarity with that of sintered HA than with Rh before implantation.

TABLE VI Quantitative composition (at %) at some spots in a section of Rh particles in gelatin after 3 months implantation

Spot	Position	Na	Mg	Ca	P	Ca/P
1	Centre particle 1	2.41	0.00	22.19	17.37	1.28
2	Border particle 2	1.36	0.37	19.86	15.33	1.30
3	Bone between 2 particles	0.71	0.24	14.13	11.25	1.26
4	Centre particle 2	3.14	0.23	25.72	20.27	1.27
5	Border particle 2	3.00	0.47	31.62	24.37	1.30
6	Bone ongrowth	0.65	0.48	22.75	16.46	1.38
7	Bone alveolar wall	1.34	0.47	27.07	19.34	1.40

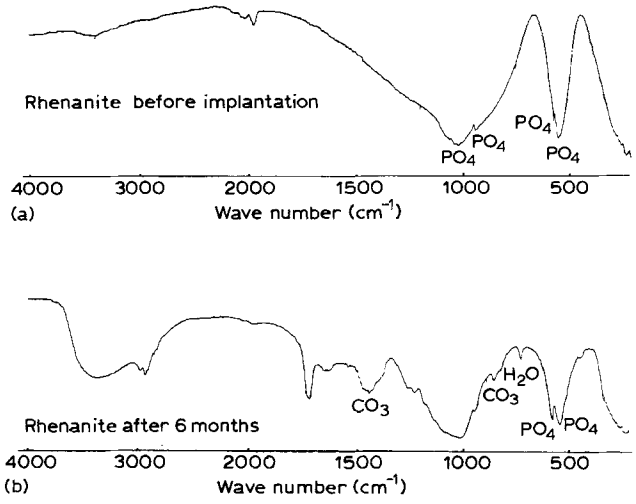


Figure 5 IR pattern of Rh before implantation (a) and Rh 6 months after implantation (b). The original Rh material now contains carbonate.

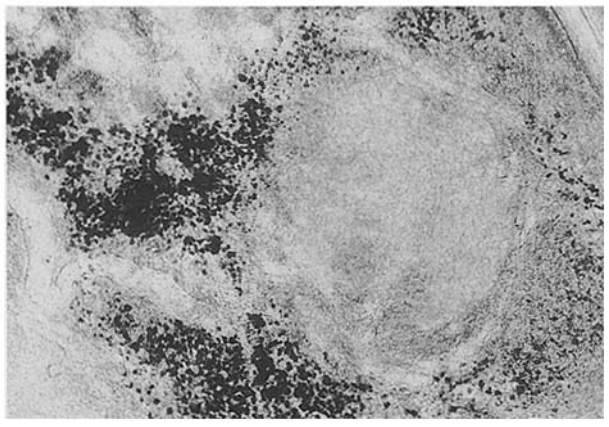


Figure 6 Lymph node tissue by light microscopy was thought to contain clusters (black) of calcium phosphates. The techniques applied to prove that the observed clusters (black) were really calcium phosphates, did not detect calcium phosphate (× 25).

plants was measured and histologically confirmed. The difference in Rh resorption between the two studies may be caused by factors mentioned in LeGeros *et al.* [5], such as the use of different kinds of animals of different ages, different implant sites and differences in porosity and shape of the Rh implant materials. The Rh particles upon implantation showed a more rounded shape than the HA particles. This obviously arose from wear during sieving (Rh is less wear-resistant than HA) and not from resorption. Histologic sections from *in vitro* filled artificial sockets showed a similar shape. Furthermore no resorptive cells were found in the *in vivo* sections and none of the applied techniques could demonstrate calcium phosphates in lymph node tissue. Histomorphometry gave no reason to believe that Rh or HA particles had undergone resorption during their 6-month *in vivo* stay.

In relation to porosity and shape it is hypothesized that the observed transformation of Rh into an apatite as reported in the previous study [2] and confirmed by this study, will come about more rapidly with 0.5–0.7-mm sized, 20% microporous Rh particles than with 5 mm wide, 10% microporous, more solid, Rh cylinders. By this rapid transformation, bioresorbability of the original Rh particles may be strongly reduced. Moreover, as Rh turns into an apatite containing carbonate, Na and Mg, it is more in correspondence with the chemical composition of bone mineral [5–7] than HA is often said to be. The transformed Rh particles may be recognized by the host as a more familiar material, which may account for the significantly higher rate of bone deposition when compared to HA. As a difference in Ca/P ratio between the 3 and 6-month periods was not found, transformation of Rh must have come about within 3 months and according to the previous study [2] even within 6 weeks. A follow-up study using shorter experimental periods may give evidence to this hypothetical explanation and may reveal an even shorter period of transformation.

As no significant difference in Ca/P ratio between centre and border of the sectioned Rh particles was found and both spots showed uptake of Mg (Table VI), it can be concluded that transformation of the Rh particles is through and through. XRD, showing apatite structures, may corroborate this conclusion. This suggests that the original Rh material is fully replaced by a biological apatite. The dynamics of this transformation process are not yet clear, but *in situ* replacement is conceivable.

The difference between the use of gelatin and saline is that Rh particles moistened with saline, in contrast to HA particles, tended to clot to a dense mass. Only in one socket from the 6-month period was enough space kept between the Rh particles to allow ingrowth of bone. In the literature, sufficient space is considered important to make ingrowth of bone possible at an early stage [8] and to allow the development of a normal spongeous bone structure with marrow spaces [9, 10].

Rh in the form used in this study distinguished itself from HA in the form used in this study. The difference in amount of bone deposition between Rh and HA particles, as was observed during the histological evaluation, was histomorphometrically measured. Although microscopically no difference in amount of bone deposition was estimated to exist either between the Rh particles in dogs 4 and 6 or between the HA particles in dogs 5 and 6, possible animal differences were excluded by taking the histomorphometrical measurements in only one dog, the dog that contained all types of implant materials (number 6, Table I). In that dog significantly more bone was found to be deposited on Rh particles, and more Rh particles than HA particles were completely incorporated in the newly formed bone. This means that in this study Rh by itself is found to be more stimulative to new bone formation than HA. As Rh particles in gelatin were also measured to have significantly more direct bone deposition than Rh particles in saline, increased spacing between the particles seems to have a great influence on the amount of ingrowth and deposition of bone. Furthermore, collagen or gelatin of different

origin may have had a positive effect on new bone formation, as has often been reported in the literature [10, 11, 12]. However, before concluding that Rh is more osteoconductive than HA, it should still be recalled that, in this study, particles are compared that differ slightly in shape, which may also have influenced bone deposition.

Complete closure of the extraction wounds to prevent particle loss is not effective enough. Additional measures should be taken to improve particle retention.

The purpose of filling tooth sockets is to preserve alveolar bone or alveolar ridges volume. When, during *in vivo* applications, Rh is commonly found to transform into a biological apatite more familiar to the host tissue, thereby positively stimulating new bone deposition, Rh can be regarded as a promising material for future application as a tooth socket or other defect filling material. It therefore deserves more research.

Acknowledgements

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