Osseointegration and osseoconductivity of hydroxyapatite of different microporosities

A. L. ROSA*, M. M. BELOTI, P. T. OLIVEIRA
Faculdade de Odontologia de Ribeirao Preto da Universidade de Sao Paulo, Av. do Cafe, S/N, 14040-904 Ribeirao Preto, SP, Brazil

R. VAN NOORT
School of Clinical Dentistry, University of Sheffield, Claremont Crescent, S10 2TA, Sheffield, England, UK
E-mail: adalrosa@forp.usp.br

The aim of this study was to determine the relationship between changes in microporosity and the osseointegration and the osseoconductivity of hydroxyapatite (HAp). HAp cylinders were manufactured by a combination of uniaxial powder pressing and different sintering conditions, with different percentages of microporosity: HAp-I with $3.96 \pm 0.75\%$ microporosity, HAp-II with $15.66 \pm 1.60\%$ microporosity, and HAp-III with $29.72 \pm 0.69\%$ microporosity. These HAp cylinders were surgically implanted in rabbit femurs. After 8 and 12 weeks, the femurs were removed, fixed, sectioned, ground, and stained by Stevenel's blue/Van Gieson for light microscopy and histomorphometry. Some ground sections were routinely processed for SEM. The osseointegration and the osseoconductivity were determined by means of image analysis and the data were submitted to ANOVA. In all cases the cortical bone was repaired and the HAp facing the medullary canal was lined with endosteum, which in some areas exhibited thin bone tissue formation. SEM observations showed no differences in the morphology of tissue-HAp interfaces for the three different porosities of HAp. There were no statistical differences between the groups related to either osseointegration or osseoconductivity. These results suggest that neither osseointegration nor osseoconductivity of HAp are influenced by changes in HAp microporosity.

1. Introduction

Hydroxyapatite (HAp) has been widely used in many clinical conditions as a bone substitute. Synthetic HAp for bone substitution can be produced with a range of porosities depending on the method of processing. The porosity can be classified according to pore diameter as macroporosity ($> 10\,\mu m$) and microporosity ($< 10\,\mu m$) and it has been suggested that these structural modifications influence the biocompatibility of HAp [1, 2]. Regarding the macropores, a number of studies have been conducted to evaluate the relationship between pore dimensions and tissue ingrowth to the HAp and there is a positive correlation between these parameters. The larger the macropores, the more tissue the ingrowth if the pore diameter is greater than $150\,\mu m$ [1, 3–5]. Recently, it has been suggested that the ideal pore diameter for bone ingrowth is between 300 and $400\,\mu m$ [6]. In contrast, little is known about the effect of microporosity, yet microporosity is often present in HAp samples as it results from incomplete densification of the HAp particles during processing [7]. According to Eggli *et al*. [1], micropores in HAp help to establish connections between the macropores, which allows interstitial fluid circulation through them. This event would facilitate blood vessel and tissue ingrowth into the HAp. Also, interconnected micropores are important because their presence allows the HAp to be machinable, that is, readily shaped with dental rotary instruments [8]. Dziedzic *et al*. [9] showed that HAp samples with different microporosities, implanted in rats, result in the formation of different bone–HAp interfaces. Preliminary results from *in vitro* studies using a primary culture of rat bone marrow cells and a rat osteosarcoma cell line have demonstrated that an increase in micropore percentage negatively influences HAp biocompatibility [2, 10].

Considering that the influence of microporosity on the *in vivo* biocompatibility of pure and highly crystaline HAp is not completely determined, the aim of this study was to determine the relationship between changes in microporosity and the osseointegration and osseoconductivity of HAp.

2. Material and methods

2.1. HAp samples

HAp cylinders (4.3 mm in diameter and 3 mm long) were manufactured by a combination of uniaxial powder

*Author to whom all correspondence should be addressed.

TABLE I Uniaxial load and sintering conditions of HAp samples

Sample	Load (ton)	Sintering temperature (°C)	Sintering time (h)
HAp-I	6	1250	3
HAp-II	2	1225	3
HAp-III	2	1150	3

pressing and different sintering conditions, as shown in Table I. This procedure allows the fabrication of HAp with both identical chemical composition and crystallinity but with different porosities [11, 12]. The cylinders were made from a high purity and crystalline HAp powder (Plasma Biotal, Tideswell, UK). Microporosity was calculated from the actual density compared with the theoretical density of HAp, and HAp surface was evaluated by scanning electron microscopy (SEM – JSM-5410, Jeol, MA, USA). Before implantation procedures, all specimens were cleaned in an ultrasonic bath and autoclaved.

2.2. Implantation procedures

Seven male rabbits (2–3 kg) received two HAp cylinders in each femur. Animals were anaesthetized with acepromazin (1 mg/kg), followed with a combination of ketamine and xylazine (50 and 3 mg/kg, respectively). After a local anesthesia with 2% mepivacaine plus adrenaline 1 : 100 000, the femoral shaft was aseptically exposed and the periosteum was gently scraped from the cortical bone. Two monocortical holes were drilled under copious irrigation with sterile saline using burs at low speed. The HAp cylinder was gently tapped into the hole until it was totally located beneath the external surface of the cortical bone. The wound was closed with sutures. Immediately after the implantation procedure, the animals were given diclofenac and were permitted weight-bearing during the whole post-implantation period. At 8 and 12 weeks following implantation, the animals were euthanized with a lethal dose of Nembutal and the femurs were harvested and processed for morphological and histomorphometric analyses.

2.3. Histological processing

The bone segments containing the HAp cylinders were ground and sectioned for light microscopy as previously described [13]. Briefly, immediately after harvesting the femurs, the bone segments containing the HAp cylinders were immersed in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.3) for 90 minutes. Following fixation, bone segments were dehydrated in graded concentrations of alcohol and embedded in LR White resin (London Resin Co., England). Following polymerization, resin blocks were radiographed to determine precisely the implantation sites of the HAp cylinders and then sectioned with a diamond knife (Microslice 2, Ultra Tec Manufacturing Inc., USA) to produce three samples per implant. Each sample was polished, and mounted on glass slides and the resulting 40 μm-thick mounted sections were further ground and polished to a thickness of 20 μm. Two samples were stained with Stevenel's blue and Van Gieson stain for light microscopy. The third sample was further polished and gold coated for SEM observation.

2.4. Histomorphometric analysis

Osseointegration was expressed as percentage of the HAp cylinder surface in close contact with the bone matrix of the cortical bone. Osseoconductivity was expressed as percentage of the HAp cylinder surface in close contact with the bone matrix in the medullary canal. Both these parameters were determined by means of an image analysis system using NIH Image software. The data were subjected to analysis of variance (ANOVA).

3. Results

3.1. HAp samples

Combination of uniaxial powder pressing and different sintering conditions produced HAp samples with different percentage of microporosity and they were classified into three groups: HAp-I with $3.96 \pm 0.75\%$ microporosity $(n = 10)$, HAp-II with $15.66 \pm 1.60\%$ microporosity $(n = 10)$, and HAp-III with $29.72 \pm 0.69\%$ microporosity $(n = 10)$. It was possible to observe from SEM micrographs that HAp samples presented pores with diameter less than 5 μm, i.e. micropores (Fig. 1).

3.2. Histological analysis

After 8 weeks of implantation, cortical bone regeneration was observed around HAp samples at the implantation sites (Fig. 2(a)). Bone tissue was characterized by mature bone composed of Haversian systems with wide Haversian canals. A layer of endosteum was observed in close contact to the HAp implants in the medullary canal. The endosteum was composed of elongated cells intermingled with bundles of collagen fibers located parallel to the HAp surface. In some areas there was evidence of mineralized bone matrix in close contact with the HAp surface, presenting osteocytes and osteoblasts (Fig. 2(b)). After 12 weeks of implantation, cortical bone appeared denser as indicated by a reduction in the diameter of Haversian canals. In the medullary canal, the amount of mineralized bone matrix contacting the HAp surface was greater than that observed after 8 weeks of implantation (Fig. 2(c)). Under polarized light microscopy, the parallel orientation to the HAp surface of the collagen fibers in the new bone matrix was clearly visible (Fig. 2(d)).

SEM observations showed close interfacial contact between the HAp and the new bone, with the mineralized matrix presenting a mirror image to the HAp surface (Fig. 2(e)). It was not possible to identify any differences in the HAp–bone interface morphology related to the amount of porosity in the samples. Small rounded structures were observed in both bone and HAp surfaces (Fig. 2(f)).

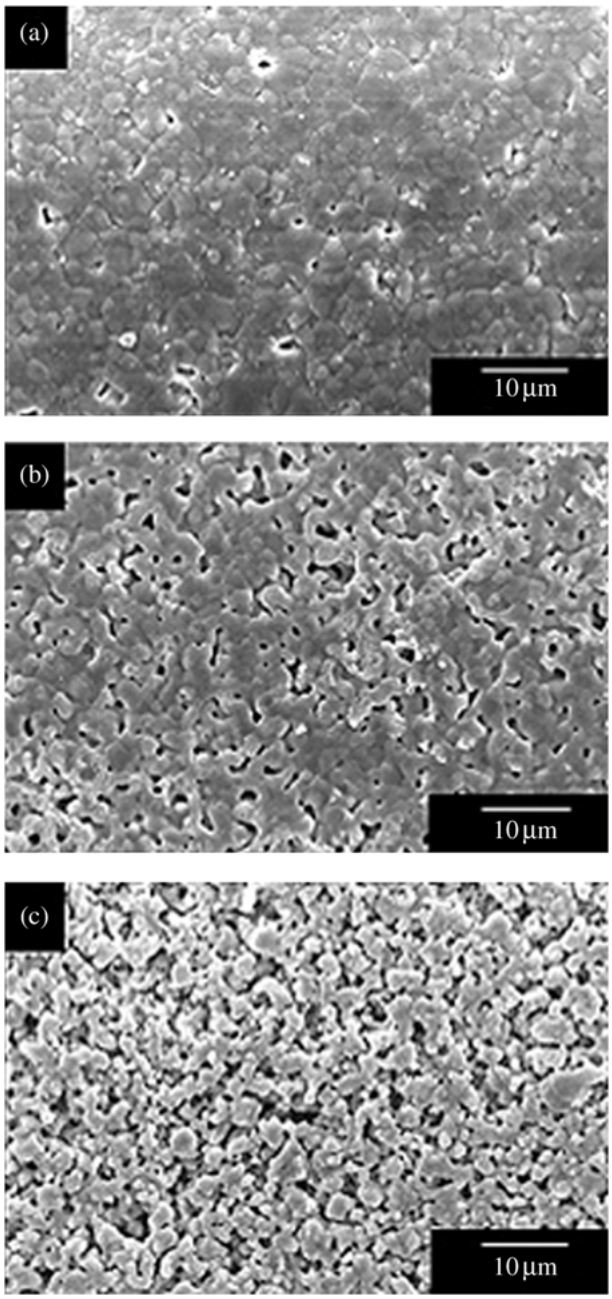

Figure 1 Scanning electron micrograph of HAp surfaces. (a) HAp-I; (b) HAp-II; (c) HAp-III.

3.3. Histomorphometric analysis

The percentage osseointegration of the samples is shown in Fig. 3. The mean percentage value of osseointegration was: 67.19 ± 6.12 $(n=5)$ after 8 weeks and 65.27 ± 9.03 $(n=4)$ after 12 weeks for HAp-I; 72.14 ± 8.04 $(n=5)$ after 8 weeks and 72.82 ± 18.98 $(n=5)$ after 12 weeks for HAp-II; and 62.79 ± 13.57 $(n=5)$ after 8 weeks and 77.63 ± 9.29 $(n=4)$ after 12 weeks for HAp-III. There were no statistically significant differences when either degree of porosity (ANOVA, $F=0.572$; D.F. $=2$; $p=0.572$), or implantation periods (ANOVA, $F=0.924$; D.F. $=1$; $p=0.346$) were compared.

The percentage osseoconductivity of the samples is shown in Fig. 4. The mean percentage value of osseoconductivity was: 36.50 ± 13.40 $(n=5)$ after 8 weeks and 56.24 ± 12.13 $(n=4)$ after 12 weeks for HAp-I; 31.10 ± 21.82 $(n=5)$ after 8 weeks and 31.41 ± 11.94 $(n=5)$ after 12 weeks for HAp-II; and 18.95 ± 7.30 $(n=5)$ after 8 weeks and 60.29 ± 25.41 $(n=4)$ after 12 weeks for HAp-III. There were no statistically significant differences when the degree of porosity was compared (ANOVA, $F=1.48$; D.F. $=2$; $p=0.248$); however, the percentage osseoconductivity was higher at the 12-week implantation period compared with 8 weeks (ANOVA, $F=7.77$; D.F. $=1$; $p=0.01$).

4. Discussion

The present results showed that different combinations of powder pressing technique and sintering produced HAp samples with different microporosity and, consequently, different surface appearance. Despite these differences, all HAp samples were biocompatible, as demonstrated by the quality of the HAp–tissue interface and by the capacity for osseointegration and osseoconductivity. In contrast to these findings, *in vitro* studies using rat bone cell culture systems showed that 30% microporosity HAp samples exhibited comparatively less biocompatibility [2, 10]. However, in these *in vitro* studies, the initial events of cell proliferation and protein production were evaluated while in this present *in vivo* study mature bone production was evaluated. Thus, it is possible that only the initial events are influenced by different percentages of microporosity in HAp.

After 8 weeks of implantation, all HAp samples implanted in rabbit femur cortical bone and medullary canal were completely lined either by endosteum or by mineralized bone matrix. In some areas in the medullary canal, mineralized bone matrix was observed in close contact to the HAp surface and this was apparently unconnected to the cortical bone. It is possible to speculate that in these areas there could be a greater stimulus for differentiation of endosteum cells into osteoblasts, which produced mineralized matrix earlier than other areas of endosteum. In favor of this hypothesis there is a study by Davies and Baldan [14] showing that HAp implanted in the medullary canal exhibited surface modifications in focal areas, characterized by micro-indentations, that would facilitate osteoblastic cell adhesion and spreading. Furthermore, it is also possible that endosteum fragments generated during the implantation procedure would adhere in some areas of HAp samples. These endosteum fragments would be in a more advanced stage of healing, therefore, providing more differentiated osteoblasts and would stimulate earlier new bone formation. In the remaining areas, the endosteum might have to become organized before lining the HAp surface.

The HAp–tissue interface exhibited areas in which mineralized bone tissue was in very close contact to the HAp surface. This suggests that bone was laid down in direct contact to the HAp surface and would appear to be corroborated by the SEM observations, showing the new bone to be a mirror image of the related HAp surface. In some areas it was possible to observe the formation of small rounded structures both at the bone surface and the HAp, which are morphologically similar to the globular extracellular matrix described by Davies [15]. There were no morphological differences at the HAp–bone interface with respect to the percentage of micropores on

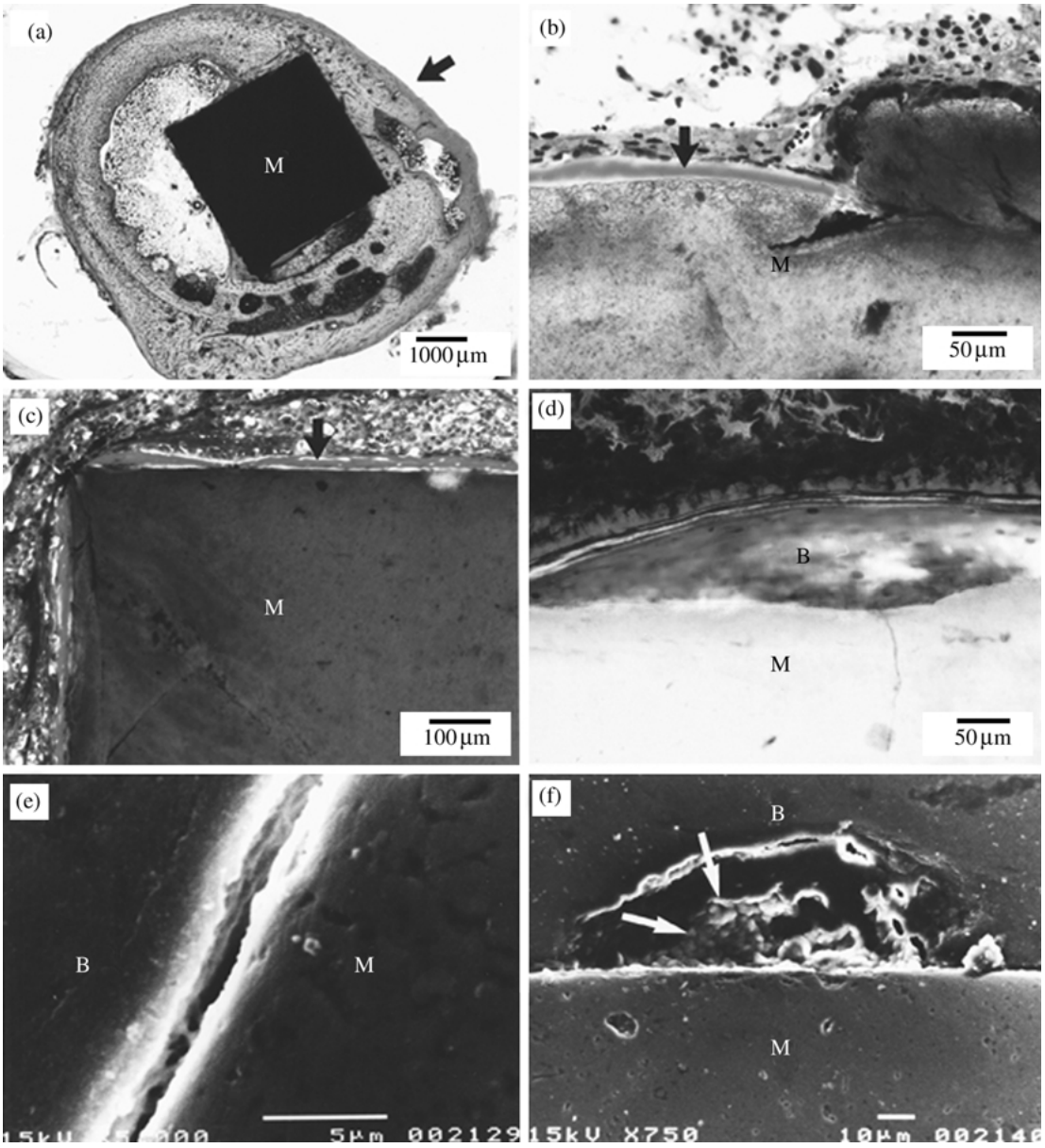

Figure 2 Ground sections of HAp implanted into rabbit femurs (Stevenel's blue/van Gieson stain). M–HAp, B–bone. (a) Cross section of HAp-I cylinder after 8 weeks of implantation. Arrow indicates the direction of cylinder implantation. (b) Hap-I–tissue interface showing the endosteum in close contact with the HAp implant in the medullary canal. Arrow indicates mineralized bone matrix. (c) HAp-III–tissue interface in the medullary canal after 12 weeks of implantation. Arrow indicates a continuing mineralized bone matrix in close contact with all the implant surface. (d) The parallel orientation to the HAp-III surface of the collagen fibers in the new bone matrix after 8 weeks of implantation (polarized light). (e) Close interfacial contact between the HAp and the new bone, with the mineralized matrix presenting a mirror image to the HAp-III surface (SEM). (f) Small rounded structures observed in both bone surface (arrows) and HAp-I surface (SEM).

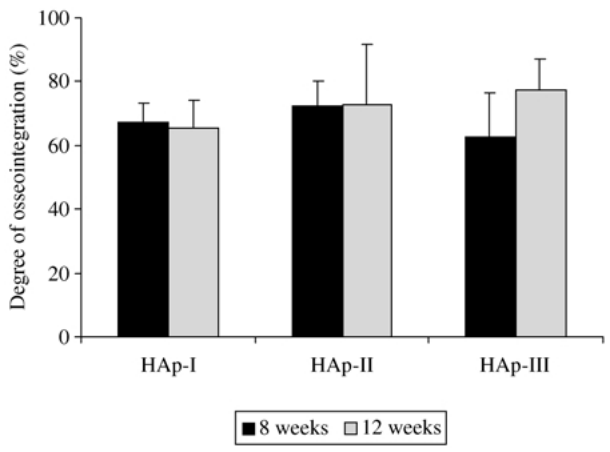

Figure 3 Degree of osseointegration of HAp samples. Data are shown as mean and bars are one standard deviation.

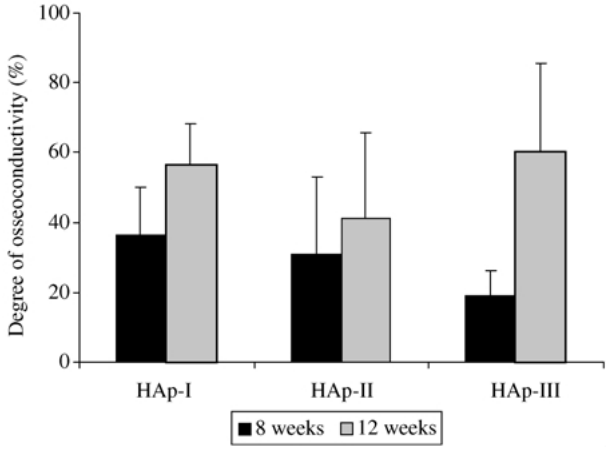

Figure 4 Degree of osseoconductivity of HAp samples. Data are shown as mean and bars are one standard deviation.

the HAp surface. In agreement with this result, Hing *et al*. [16] showed that the histological response to HAp implants with different densities was similar. Moreover, no interdigitation between bone tissue and the more porous HAp surfaces was observed, which is in disagreement with the results presented by Dziedzic *et al*. [9] after implantation of porous HAp in rat bone. Considering that in both studies pure and crystalline HAp samples with similar porosity were evaluated, these morphological differences could be due to the use of different experimental designs, such as animal species, periods of implantation and microscopic methods [17].

5. Conclusions

The biocompatibility of HAp has been well documented both *in vitro* and *in vivo*. In different experimental systems, it has been shown that a mineralized bone matrix can be produced in close contact with the HAp surface [16, 18–21]. However, little is known about the influence of microporosity on the HAp biocompatibility in relation to HAp capacity for osseointegration and osseoconductivity. Our results showed that neither osseointegration nor osseoconductivity were influenced by the amount of micropores present in HAp. Furthermore, after 8 weeks the osseointegration process was already completed as it did not increase from 8 to 12 weeks. On the other hand, the increase in the percentage osseoconductivity from 8 to 12 weeks and its low values would suggest that this process was not completed after 12 weeks of implantation. Thus, further studies should be conducted for evaluating in shorter periods the possible influence of microporosity on the osseointegration and osseoconductivity of HAp.

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