The effect of osteopenia on the osteointegration of different biomaterials: histomorphometric study in rats

M. FINI*, G. GIAVARESI, N. NICOLI ALDINI, P. TORRICELLI, G. MORRONE, G.-A. GUZZARDELLA, R. GIARDINO
Experimental Surgery Department and Chair of Surgical Pathophysiology, University of Bologna, Research Institute Codivilla-Putti, Rizzoli Orthopedic Institute (I.O.R.), Via Di Barbiano 1/10, 40136 Bologna, Italy

A. KRAJEWSKI, A. RAVAGLIOLI
Institute for Technological Research on Ceramics of CNR, Faenza-Italy

M. MATTIOLI BELMONTE, A. DE BENEDITTIS, G. BIAGINI
CIBAD Center for Innovative Biomaterials of Ancona, Italy
E-mail: giardino@alma.unibo.it

The osteointegration of Hydroxyapatite (HA), Titanium (Ti-6Al-4V: Ti), Zirconia (ZrO_2), Alumina (Al_2O_3) and 2 biological glasses (AP40 and RKKP) was comparatively investigated in normal and osteopenic rats by means of histomorphometry. Thirty-six Sprague Dawley female rats were left intact (Group C) while 36 were ovariectomized (Group OVX). Group C and OVX were further divided into 6 subgroups. After 16 weeks all animals were submitted to the femoral implant of nails made of the above-mentioned materials. Eight weeks after implantation the animals were euthanized, the femurs were harvested for histomorphometric analysis. The data showed that: (1) all the tested materials were biocompatible *in vitro*; (2) no significant differences existed in Affinity Index (AI) of Group C; and (3) results from paired comparison applied to the AI showed significant differences among the Groups C and OVX. The AI did not significantly change among intact groups, while it significantly decreased when some materials were implanted in OVX subgroups (AP40, ZrO_2 and Ti-6Al-4V: $p < 0.0005$, $p < 0.05$ and $p < 0.01$). It is confirmed that bone mineral density is a strong predictor of the osteointegration of an orthopedic implant and that the use of pathological animal models is necessary to completely characterize biomaterials.

1. Introduction

As a result of development and research during the last years, a variety of endosseous orthopedic and dental implant systems have become available with different designs, surface textures and construction materials [1]. It is well-known that both the outcome of an implant and the consequent osteointegration depend on a combination of determining factors. Among these, the patient's health status and the quality of host bone play an important role [1–6]. Whereas before, the improvement of physico-chemical characteristics of biomaterials was studied in healthy bone, nowadays also pathological models are developed, simulating the clinical environment in which biomaterials are positioned [7–15]. Moreover, many factors which may accelerate osteointegration rate are investigated too [16–18].

With aging, many structural and biological changes occur in human tissues. For example, bone mineralization, structure and biology are strictly influenced by age and disease [19–20]. In turn, the bone quality reflects this overall health status of the patient. It is well known e.g. that changes in calciotrophic hormone profiles are associated with loss in skeletal mass. Furthermore, it can be said that the association of age and diseases with poor nutritional habits, pathologies, medical therapies, decreased physical activity and so on, concurs in causing alterations of the skeleton. Histological, ultrastructural and microradiographic studies confirm an increase in intracortical porosity which is a consequence of an increase in the number and mean diameter of Haversian system [21]. This bone rarefaction could be a cause of the poor bone ingrowth at the bone materials interfaces even if many other factors may be at play. From a biological point of view, a limited source of osteoblasts, endothelial and inflammatory response cells is also accompanied by an impairment in chemical mediators of bone remodeling

*Author to whom all correspondence should be addressed.

[22]. Published histomorphometric data show that if only the pre-existing osteoblasts were to heal a fracture (or bone defect) they would require many years to make enough bone to do so [23]. Successful and prompt bone healing mandates increasing their number by many thousands and only the mediator mechanisms are able to do that [23]. The skeletal content of Insulin-like Growth Factor I (IGF-I) and Transforming Growth Factor-beta (TGF-β) in human bone is a function of age. A linear decline has been found in the skeletal content of IGF-I and TGF-β with increasing donor age [24]. Moreover, other authors found that the increase in DNA synthesis with different factors as parathyroid hormone, growth hormone, calcitonin, TGF-β, IGF-I, PDGF was significantly and negatively correlated with donor age in cultures obtained from the iliac crest bone of 50 to 70 year-old women [25].

So, the osteointegration of implants in osteoporotic and aged bones could be negatively influenced by a decreased capacity of osteoblasts to proliferate in response to systemic or locally released osteotrophic factors. For example, recent works confirmed the improvement of the bone-biomaterial interface after TGF-β local administration [26–28]. Because of this, the osteopenic animal model is not only used for research on osteoporosis pathophysiology, diagnosis, prevention and treatment, but has also been employed in surgical orthopedics, dental and maxillofacial fields. Researchers increasingly use metabolically altered models to improve the characterization of biomaterials and their related osteointegration processes after the implant in pathological bone [7–15].

The authors of this paper are now engaged in evaluating which are the overall effects, in terms of rate of success on bone repair and implants osteointegration, of both endocrine status and type of material (classified either bioinert or bioactive) [29]. The present study will illustrate the results of the influence of osteopenic bone on 3 important bioceramic materials (Hydroxyapatite: HA, Zirconia: ZrO_2 and Alumina: Al_2O_3), Ti-4V-6Al (Ti) and 2 biological glasses (AP40 and RKKP) osteointegration. In particular, this study does not aim to select the ''best'' material, but it focuses the attention on problems that may occur in cases where there is poor bone quality.

2. Materials and methods

2.1. Materials

Seventy-two cylindrical nails, 2 mm in diameter and 3 mm in length, were implanted. These nails were made with HA, ZrO_2 (Y-PST) and AL_2O_3 ceramics, with Ti-4V-6Al and with 2 biological glasses with similar composition (AP40 and RKKP) (Table I). HA powder was synthesized following the mechano-chemical method [30, 31]. A series of rods was produced with these powders by slip casting in a mould. The shaped samples obtained were sintered in a laboratory kiln at 1250 °C for 1 h, thus obtaining the required HA ceramic pieces to be implanted.

The other ceramic nails were manufactured by cutting, with a diamond wheel, longer rods obtained by extrusion. AL_2O_3 nails were fired at 1480 °C and ZrO_2 nails at 1530 °C. Both firings were carried out in a laboratory kiln.

The glasses were prepared by melting the starting products, in platinum crucibles, at 1450 °C for 60 min. Glass nails were manufactured by casting the just synthesized melts of the mentioned compositions from the platinum crucible into a graphite die of cylindrical shape. The diameter of the die was a bit larger, to take into account thermal shrinkage of the glass, in order to obtain the final diameter of 2 mm. The height was brought to the desired value by cutting and polishing.

The metallic rod was a Ti alloy (Ti6Al4V) with a concentration of $C < 0.08\%$ and $Fe < 0.25\%$[w].

The materials were sterilized by autoclaving at 120 °C for 20 min before *in vitro* and *in vivo* test.

2.2. *In vitro* test

The *in vitro* test was performed on an extract of the biomaterials according to ISO 10993-5. The extraction (0.9% NaCl) was performed using the following time and temperature conditions in a 5% CO_2-humidified atmosphere: 72 h at 37 °C; 72 h at 50 °C and 24 h at 70 °C. A 0.45% phenol solution was used as a positive control for extracts. After having incubated L929 fibroblast (NCTC CCL1) cultures with extracts in a 5% CO_2 humidified atmosphere at 37 °C for 24 h, cytotoxicity was determined by two quantitative tests : lactate dehydrogenase (LDH) activity and cell viability (MTT test).

LDH activity (lU^{-1}) was measured on supernatants using a kinetic assay (Boehringer Mannheim Automated Analysis for BM/Hitachi 717). The MTT test (tetrazolium salt test) was carried out by incubating cells with Dulbecco's modified medium and 5 mg ml^{-1} of MTT at 37 °C in 5% CO_2 for 4 h and solubilizing intracellular formazan crystals with dimethylsulphoxide. Finally, the absorbance of each sample, expressed as optical density $\times 10^{-2}$, was determined at a wavelength spectrophotometer.

2.3. Animal model

Seventy-two healthy 40-week-old retired breeder Sprague Dawley rats, weighing between 390 and 520 g, were used. They were randomly divided into 2 groups of 36 animals each: control (C) and ovariectomized (OVX). The OVX animals were anesthetized by means of a subcutaneous injection of 87 mg/kg ketamine (Ketavet, Farmaceutici Gellini, Aprilia Lt-Italy) and 13 mg/kg

TABLE I Chemical composition of the biological glasses mentioned in the text

Materials	SiO_2	P_2O_5	CaO	Na_2O	K_2O	MgO	CaF_2	Ta_2O_5	La_2O_3
AP 40	44.00	11.20	32.16	4.60	0.20	2.84	5.00	—	—
RKKP	43.84	10.27	31.93	4.55	0.19	2.79	4.94	0.99	0.50

xylazine (Rompun, Bayer Italy spa, Milano-Italy), and then underwent a bilateral ovariectomy through a lumbar access.

Sixteen weeks after, the left condyle of all 72 rats was exposed via a lateral skin incision under the same anesthesia and a cylindrical nail made of the above mentioned materials was implanted. The wounds were sutured in 2 layers and disinfected. We had 12 subgroups of 6 animals each: HA, ZrO_2, Al_2O_3, Ti, AP40 and RKKP nails were implanted in normal bone (Subgroups C: HA-C ; ZrO_2-C; Al_2O_3-C; Ti-C; AP40-C; RKKP-C) and in the osteopenic bone (Subgroups OVX: HA-OVX; ZrO_2-OVX; Al_2O_3-OVX; Ti-OVX; AP40-OVX; RKKP-OVX). Three animals were stabled per cage, fed with a standard pellet diet, and given water *ad libitum* under standard environmental conditions (T: 24 °C and RH: 55%). Eight weeks after the implant, the animals were pharmacologically euthanized under general anesthesia. Immediately after euthanization, the femurs were harvested for the histomorphometric study.

2.4. Histomorphometric study

The femoral condyles containing the implant were fixed for 24 h in 4% buffered paraformaldehyde, dehydrated in graded series of alcohol and embedded in methylmethacrylate resin. After polymerization, blocks were sectioned along a plane perpendicular to the bone surface by using a Leitz 1600 microtome and yielding undecalcified sections of 70–80 μm in thickness. The sections were stained with Fast Green and then observed with a Zeiss Axioskop transmission microscope. Histomorphometry was performed with a Kontron KS image Analysis system. Affinity Index (AI: the ratio of the length of the region in which bone is directly opposed to the implant without the presence of fibrous membrane divided by the total length of the bone-implant interface multiplied by 100) and the trabecular bone volume (BV/TV) in a defined area were calculated.

2.5. Statistical analysis

Data are reported as mean and standard deviations of the mean at significant level of $p < 0.05$. After evaluating the homogeneity of variance and the normality of data, ANOVA and Scheffè's multiple comparison tests were done in order to determine differences among the groups.

3. Results

The results of *in vitro* tests showed that all the tested materials were biocompatible with no significant differences observed when compared to negative control (Table II). Three rats died after the ovariectomy and were substituted. Neither intra- and post-operative nor general and local septic complications were observed after the femoral implants.

The BV/TV decreased significantly in OVX subgroups in comparison to the C subgroups (13.0 ± 4 vs 30.3 ± 5.1; $p < 0.01$). No significant differences were observed within the C or OVX Groups. In Table III the morphometric results on AI of the different biomaterials are shown. Results from paired comparison applied to AI are shown in Table IV. Figs 1, 2 and 3 show the histological appearance of Al_2O_3, RKKP and AP40 implants in normal bone respectively. The good histological osteointegration observed in these cases was superposable to the ones of all the tested materials. On the contrary, as shown in Figs 4 and 5, some differences exist between biomaterials when implanted in osteopenic bone. Some areas of a fibrous wall appear at the bone-implant interface and these are predominant in AP40-OVX subgroup.

4. Discussion

Based on the information available today, it is evident that osteoporosis is a public health issue that affects not only post-menopausal women or aged people, but also many chronic patients whose diseases cause secondary osteoporosis or as a result of their therapies [32]. Osteoporotic patients develop bone fractures that often require implantation of biomaterials (e.g. screws and prostheses) [33–35].

Because osteoporosis could cause poor bone ingrowth at the bone-biomaterial interface, some researchers decided to investigate the osteointegration of biomaterials in the osteopenic bone by means of animal models [7–15]. Also in a previous study, we observed different osteointegration rates for HA and Ti when implanted in osteopenic rats [15]. For the sake of completeness in the present paper we added another 4 materials that, together with HA and Ti, are very representative because of their clinical use and related research.

Even if at the present time, an experimental model that precisely mimics the pathophysiology of postmenopausal osteoporosis is unavailable, the estrogen deficient model of osteopenia in rats is considered to be a good model of decreased bone formation [36]. The right femur BV/TV evaluation (in OVX rats decrease of 57%; $p < 0.01$), together with biomechanical data (OVX rats 3 point bending test: decrease of 29.2% in maximum load; $p < 0.01$) and densitometric test (OVX rats: BMD decrease of 10%; $p < 0.05$) confirmed its reliability.

The use of a small animal model limited us to implant small cylindrical prototypes and consequently, the biomechanical tests on osteointegration could not be performed. Our group is investigating the sheep as

TABLE II *In vitro* test: LDH and MTT values (mean ± SD; $n = 5$ triplicates)

	HA	ZrO_2	Al_2O_3	Ti	AP40	RKKP	L929 (-)	phenol (+)
LDH (IU)	46.0	46.7	53.2	47.2	51.0	50.7	50.7	94.5
st. dev. (±)	5.0	2.2	2.8	2.5	0.8	1.2	1.9	5.0
MTT ($OD \times 10^{-2}$)	223.6	223.6	218.5	221.1	217.3	218.5	223.3	103.5
st. dev. (±)	2.8	2.8	3.4	3.7	5.7	3.4	1.6	13.1

TABLE III Affinity index values in normal (Control) and osteopenic (OVX) group (mean ± SD; $n = 6$)

Groups	HA	ZrO_2	Al_2O_3	Ti	AP40	RKKP
Control	77.0	58.2	65.0	61.2	53.6	65.0
st. dev. (±)	7.3	5.9	7.4	9.7	9.8	5.3
OVX	57.7	36.0	60.8	48.2	3.8	63.8
st. dev. (±)	11.5	6.7	9.6	6.7	2.6	7.9
% decrease	25.0	38.0	6.4	21.2	92.9	1.8

TABLE IV Results of the statistical analysis of affinity index % of the various materials tested

Groups	HA-C	HA-O	Zr-C	Zr-O	Al-C	Al-O	Ti-C	Ti-O	AP40-C	AP40-O	RKKP-C	RKKP-O
HA-C		n.s.	n.s.	$p<0.01$	n.s.	n.s.	n.s.	n.s.	n.s.	$p<0.0005$	n.s.	n.s.
HA-O	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	$p<0.0005$	n.s.	n.s.
Zr-C	n.s.	n.s.		$p<0.05$	n.s.	n.s.	n.s.	n.s.	n.s.	$p<0.0005$	n.s.	n.s.
Zr-O	$p<0.01$	n.s.	$p<0.05$		$p<0.05$	$p<0.05$	$p<0.05$	$p<0.0005$	n.s.	$p<0.0005$	$p<0.01$	$p<0.05$
Al-C	n.s.	n.s.	n.s.	$p<0.05$		n.s.	n.s.	n.s.	n.s.	$p<0.0005$	n.s.	n.s.
Al-O	n.s.	n.s.	n.s.	$p<0.05$	n.s.		n.s.	n.s.	n.s.	$p<0.0005$	n.s.	n.s.
Ti-C	n.s.	n.s.	n.s.	$p<0.05$	n.s.	n.s.		$p<0.01$	n.s.	$p<0.0005$	n.s.	n.s.
Ti-O	n.s.	n.s.	n.s.	$p<0.0005$	n.s.	n.s.	$p<0.01$		$p<0.05$	$p<0.0005$	n.s.	n.s.
AP40-C	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	$p<0.05$		$p<0.0005$	n.s.	n.s.
AP40-O	$p<0.0005$	$p<0.0005$	$p<0.0005$	$p<0.0005$	$p<0.0005$	$p<0.0005$	$p<0.0005$	$p<0.0005$	$p<0.0005$		$p<0.0005$	$p<0.0005$
RKKP-C	n.s.	n.s.	n.s.	$p<0.01$	n.s.	n.s.	n.s.	n.s.	n.s.	$p<0.0005$		n.s.
RKKP-O	n.s.	n.s.	n.s.	$p<0.05$	n.s.	n.s.	n.s.	n.s.	n.s.	$p<0.0005$	n.s.	

Al: Al_2O_3; C: Control; O: OVX and Zr: ZrO_2.

osteoporosis model in order to ameliorate the osteointegration study in osteopenic bone (e.g. insertion and extraction torque, pull-out or push out test). The experimental time of eight weeks was chosen by taking into consideration the published results of similar studies [10] in which this experimental time had the most important significant differences which were evident until 24 weeks time. After 24 weeks it was expected that material-bone bonding would have been completed and further intensive remodeling of the bone would not occur [37].

Pathological tissues are biological sites where the success of device implant is very difficult to achieve. The results of this work could suggest some effects coming from the interaction of bone with a material. These effects might be potentially detrimental or negative (e.g. for toxicity, interference in the growth rate of specific cells, etc.). However, they could be even adverse and difficult to be biochemically managed particularly in the absence of some specific substances which manage specific biochemical cycles. In any case, while a healthy tissue is able to bear these effects and

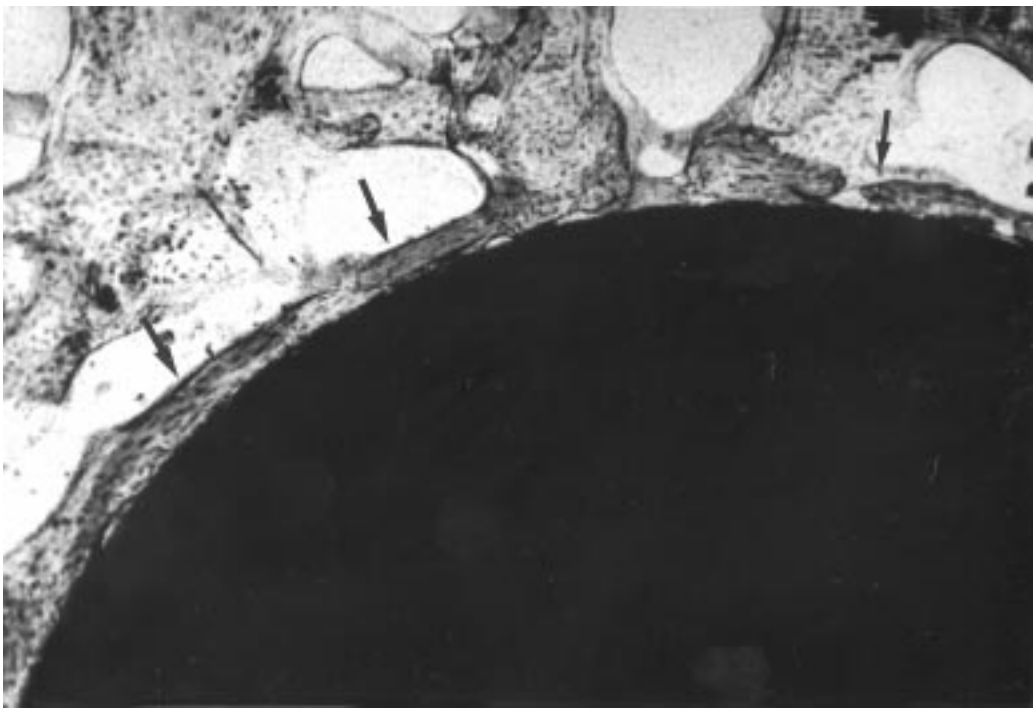

Figure 1 Al_2O_3 implanted in normal bone (Al-C). Trabecular bone (↑) directly apposed to the material surface without an evident interposition of fibrous tissue (undecalcified section, Fast Green, 10×).

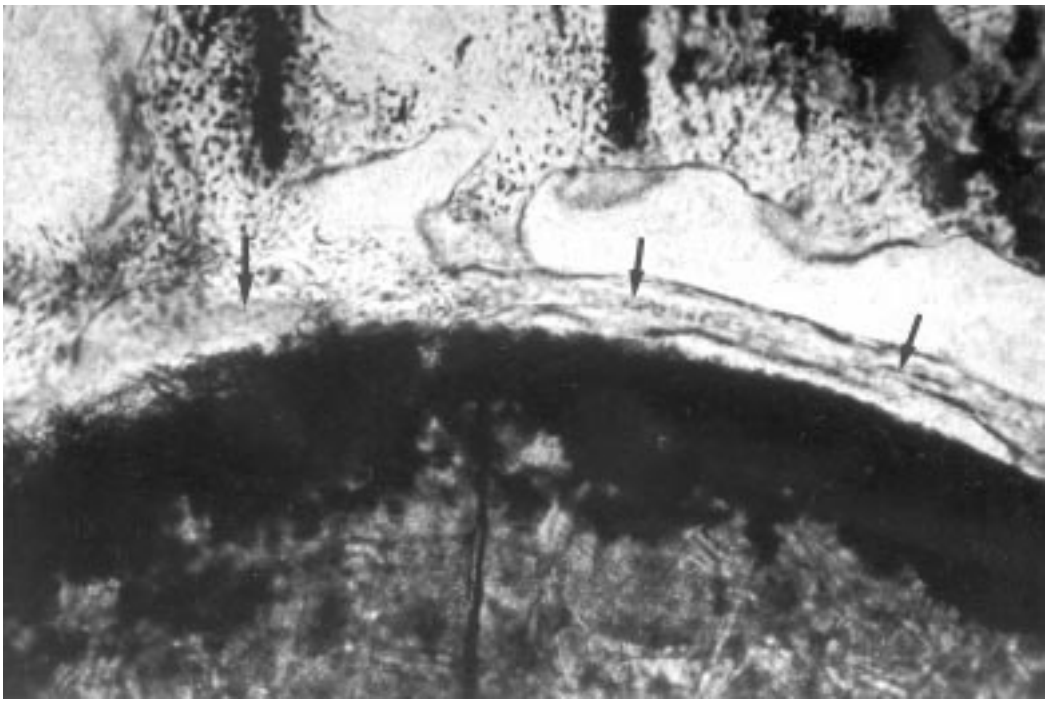

Figure 2 AP 40 implanted in normal bone (AP 40-C). The bone (↑) is directly apposed to the material surface without an evident interposition of fibrous tissue (undecalcified section, Fast Green, 10×).

provide for their overcoming with its own capabilities, a tissue already altered and eventually suffering from a lack of biochemical possibilities does not have the capability to overcome these effects that consequently become very marked and sometimes extreme. In fact, the results of this work display the detrimental effect of osteopenia due to estrogen deficiency on osteointegration of some biomaterials although they exhibit good performances in normal bone. No significant differences are visible at a first sight on Table III among the AI of metallic Ti and the ceramics (HA, ZrO_2, Al_2O_3) or biological glasses (AP40 and RKKP) implanted in normal bone (control). However, a more accurate recognition indicates that HA, Al_2O_3 ceramics and RKKP exhibit the highest AI values. Osteointegration properties of Ti, ZrO_2 and AP40 decreases significantly in osteopenic bone. Even HA ceramic, considered the material with highest affinity to bone, shows a significant decrease of its AI value in pathologic bone (25% decrease), although it is not far from the AI of the

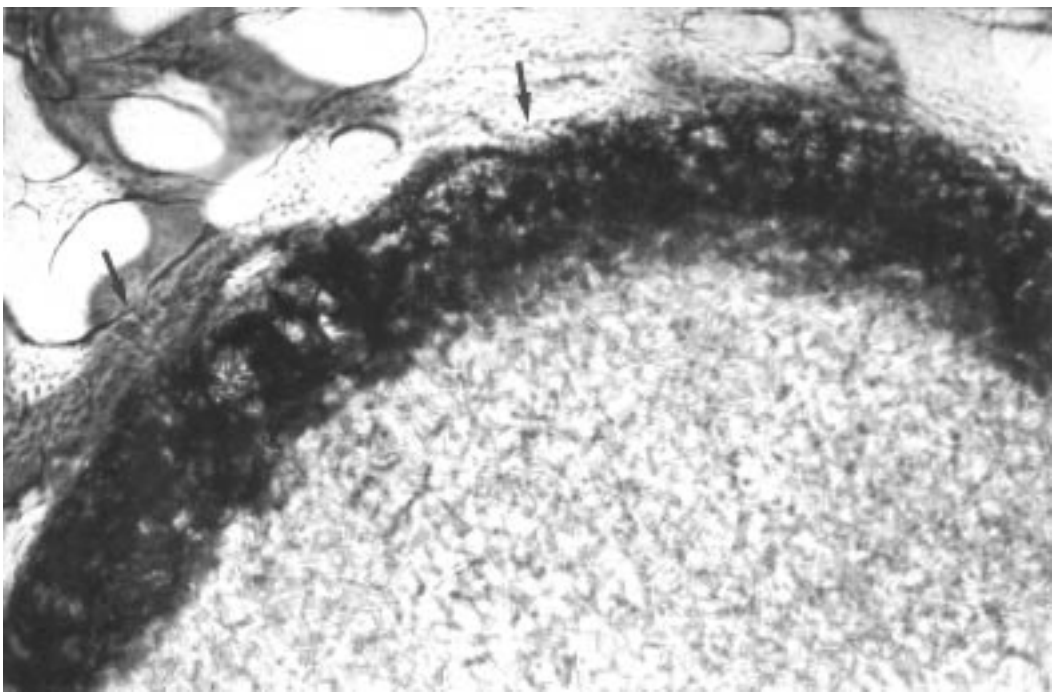

Figure 3 RKKP implanted in normal bone (RKKP-C). Good apposition of bone (↑) to the material surface without an evident interposition of fibrous tissue (undecalcified section, Fast Green, 10×).

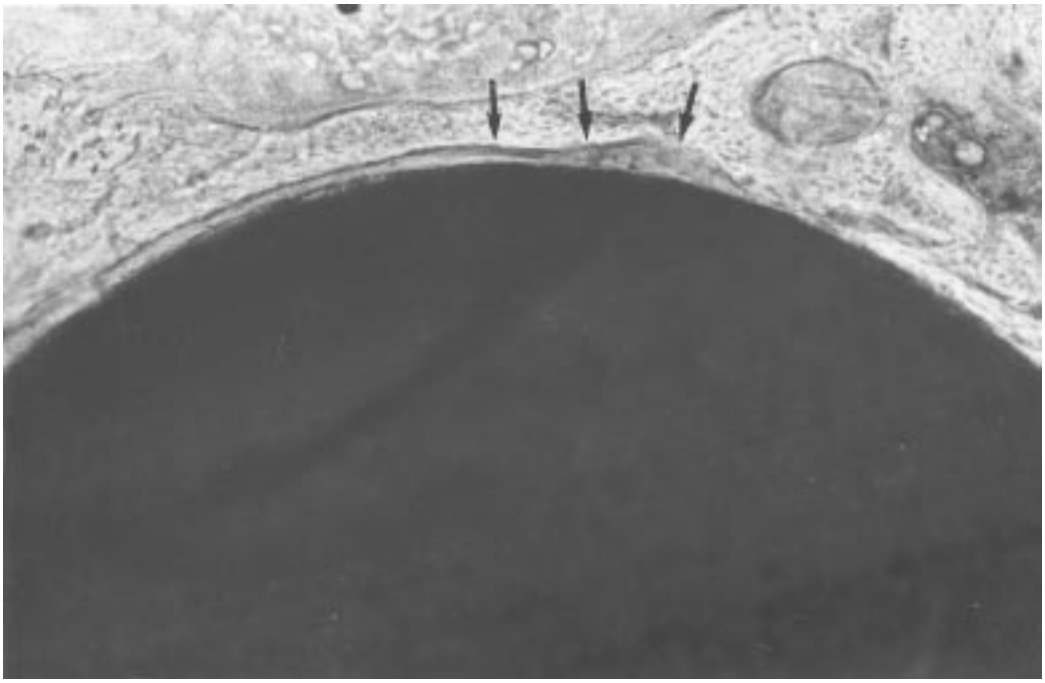

Figure 4 ZrO_2 implanted in osteopenic bone (Zr-OVX). At the bone-biomaterial interface, some areas are occupied by a fibrous wall (↑) (undecalcified section, Fast Green, 10×).

materials which remain the only with the best performances in the presence of osteopenia (RKKP and Al_2O_3). Moreover, AI of HA in osteopenic bone is quite superposable to that of Ti, ZrO_2 and AP40 in normal bone.

The dramatic decrease in osteointegration rate of AP40 in osteopenic bone, led us to suppose that there could be an influence of the local microenvironment of the bone bed also on the chemico-physical characteristics of the biomaterial itself. A preliminary X-ray micro-analysis study on the core of the materials and the bone surrounding them, showed bioglass modifications and an ionic release that was different from the one registered in case of implants in normal bone. Materials underwent modifications directly depending on the biological environment. The different rates of ionic exchange observed in the biological glass samples, confirm the existence, both in healthy and osteopenic bones, of different biochemical mechanisms that seem to influence biological glasses dissolution, precipitation and ion exchange reactions and, consequently, the osteointegration processes [38].

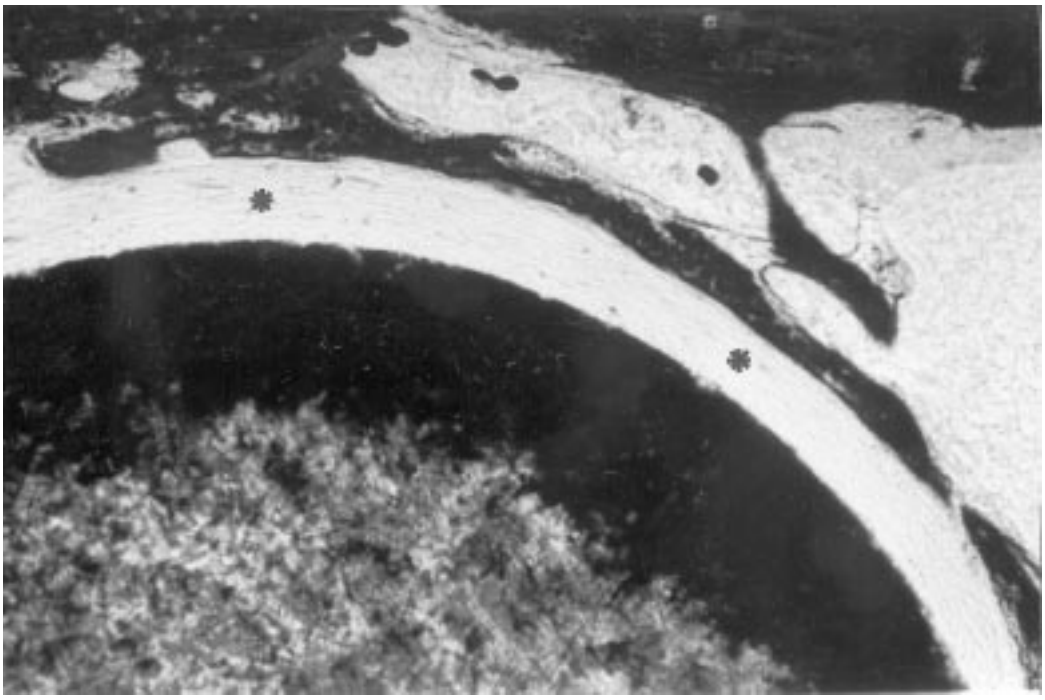

Figure 5 AP 40 implanted in osteopenic bone (AP 40-0VX). The external surface of the glass is completely occupied by a fibrous wall (*) (undecalcified section, Fast Green, 10×).

5. Conclusions

The results of this study are not to identify which material gives the best results; however, they should be taken into account by researchers and clinicians for the execution of the pre-clinical tests on materials and the selection of the ideal device for patients having biological drawbacks, respectively.

Researchers may improve and finalize pre-clinical studies with the adoption of suitable models.

Clinicians may take into account that not all of the biomaterials allow the same indications and potentialities. On the basis of the bone quality (an indicator is the mineral density), a selection of specific materials improving osteointegration processes may be made.

Acknowledgments

The authors appreciate the technical assistance of Patrizio Di Denia, Claudio Dal Fiume and Giovanna Caligiuri. This study was supported by grants from Istituti Ortopedici Rizzoli (research project no. 97044) and CNR (research projects no. 98.00004.34 and no. 96.00991.CT11)

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Received 27 August 1998
and accepted 10 August 1999