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Efecto de la hormona de crecimiento, el plasma rico en plaquetas y la brushita sobre la regeneración ósea

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Memoria para optar al grado de doctor presentada por

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Bajo la dirección del doctor Jose Mª Martínez González

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RESUMEN

Desde los tiempos míticos, el hombre ha soñado con la posibilidad de que sus tejidos dañados por la enfermedad, las heridas o la senescencia pudiesen ser regenerados y sustituidos por unos nuevos plenos de vida

La medicina regenerativa o Ingeniería Tisular es una de las ramas de la investigación biomédica que más se ha desarrollado en los últimos años y que al mismo tiempo presenta unas perspectivas de futuro prometedoras.

Desde un punto de vista un tanto academicista, que permite simplificar el abordaje del tema, consideramos que esta estrategia terapéutica se basa en tres pilares:

1. Lo que podríamos denominar en sentido amplio como Tisugénesis. Serían los aspectos ligados a las células. Una serie de células poco especializadas que desde los estudios de Friedenstein en el principio de los años 70 se ha dado en denominar Mesenchimal Stem cell o células madre mesenquimales. Estas células inicialmente identificadas en la médula ósea donde coexisten con las células progenitoras hematopoyéticas (también conocidas como Hematopoietic Stem cell) y que posteriormente han sido identificadas en muchos otros tejidos, tienen la capacidad de diferenciarse en células más específicas de un tejido. En realidad se trata de poblaciones celulares más o menos heterogéneas y que en su conjunto y mediante estímulos específicos –ya sea mecánicos, eléctricos o bioquímicos- son capaces de responder modificando su fenotipo y comportándose como células específicas y características de un determinado tejido. Estas células deben cumplir una serie de criterios conforme a lo que indica la International Society for Cellular Therapy (ISCT). Son células mononucleadas de aspecto fibroblastoide capaces de adherirse al plástico de los frascos de cultivo, con un perfil de marcadores de superficie que incluye ($CD73^+$ $CD90^+$ $CD105^+$ $CD34^-$ $CD45^-$ $CD11B^-$ $CD14^-$ $CD19^-$ $CD79a^-$ $HLA\text{-}DR^-$) y capaces de diferenciarse al menos a tres líneas celulares: óseas, condrocitos y adipocitos.
2. Tisuconducción. Este es un aspecto ligado al desarrollo de biomateriales. Estructuras que por sus características mecánicas, eléctricas de superficie, estructura porosa o composición, permiten a las células tener un soporte estructural que favorezca la dirección del crecimiento celular que lo asemeje

a la estructura del tejido que queremos mimetizar. Estos andamiajes han sido desarrollado bien como derivados orgánicos o directamente sintetizados en el laboratorio. Y en ambos casos pueden ser estructuras poliméricas o minerales. Entre las estructuras poliméricas, vemos como se han desarrollado las derivadas de proteínas como el colágeno, de azúcares como el chitosano o las caprolactonas o bien derivados de los ácidos láctico y glicólico. En cuanto a los derivados inorgánicos, y especialmente en cuanto respecta a los diseños encaminados a la obtención de soporte óseo, la utilización de derivados de los fosfatos cálcicos dopados con distintos iones es la más utilizada. Así la síntesis de diversos derivados de las hidroxiapatitas con contenidos variables en pirofosfatos, Sr, Mg, Si, e incluso la adición de polisacáridos como el condroitin sulfato permiten modular la respuesta mecánica y biológica de estos compuestos. La tendencia más actual es la de construir matrices compuestas en las que la nanotecnología y las nanofibras tienen una importancia parece que capital.

3. Tisuinducción. Este es el aspecto más ligado con el conocimiento de la fisiología de los organismos vivos. El conocimiento de los estímulos que van a poner en marcha los mecanismos de diferenciación celular, de modificación de su expresión genética y por tanto de apariencia morfofuncional. El conocimiento del mediador, de su receptor específico o general y la secuencia de actuación permite modular el proceso regenerativo. Factores como las proteínas morfogenéticas, hormonas y polipéptidos se han encontrado eficaces en estimular el cambio celular.

En la memoria que hemos redactado presentamos algunos de los trabajos realizados en estas direcciones, y que deberían naturalmente converger para construir un complejo en el que se combinaran las estructuras de soporte diseñadas como matrices híbridas fosfocálcicas y poliméricas en las que la reabsorción estuviese modulada por los tiempos de sustitución de matriz neoformada por las células multipotenciales consideradas anteriormente. También dedicamos un esfuerzo importante en el estudio de los factores que determinan o ayudan en la diferenciación celular.

En concreto presentamos cinco artículos que hemos ordenado cronológicamente.

El primero de ellos se refiere a la capacidad que presenta la hormona de crecimiento administrada en forma tópica en el lecho quirúrgico preimplantario para estimular el crecimiento de hueso. En un modelo experimental animal se realizó un análisis histomorfométrico del hueso obtenido en periimplantes colocados en tibias, frente al hueso obtenido en situación similar pero administrando tópicamente 4IU de hormona de crecimiento recombinante humana. Los resultados indican que se produce un aumento significativo del volumen óseo y del contacto del hueso con los implantes colocados.

En la segunda publicación abordamos el aspecto del soporte. Nuevamente en un modelo animal que modificamos para el estudio, analizamos la formación de hueso aplicando un cemento derivado del fosfato cálcico sintetizado por el grupo de trabajo que se reabsorbe en un tiempo más acorde con la velocidad de neoformación ósea. Los resultados muestran como la cantidad de hueso neoformado es significativamente superior con el nuevo material.

En la tercera publicación considerada, nos centramos en el plasma rico en plaquetas como medio de estimular la regeneración ósea. Realizamos un estudio comparativo entre dos sistemas para obtener el concentrado plaquetario evaluando la concentración obtenida y la ultraestructura (mediante microscopía electrónica de transmisión) del concentrado plaquetario activado.

Los dos siguientes trabajos analizan en un modelo animal el efecto de la aplicación del plasma rico en plaquetas en un defecto óseo crítico experimental. Los resultados indican que la aplicación del plasma rico en plaquetas aumenta la precocidad de la reparación tisular, que la aparición de osificaciones más precoz, pero que a medio o largo plazo no existe más regeneración ósea sino un mayor componente fibroso.

Las conclusiones finales a las que llegamos con estos trabajos hacen pensar que:

El diseño de nuevos biomateriales permite favorecer la regeneración ósea en cuanto a volumen. Que la aplicación de osteoinductores como la GH va a conseguir un efecto positivo en el volumen y calidad del hueso neoformado. Y que el plasma rico en plaquetas añadido a los lechos quirúrgicos en ausencia de un sustrato osteoconductor mejora inicialmente la regeneración, pero sin efecto significativo a medio o largo plazo.

ABSTRACT

From mythical times, man has dreamed of the possibility that their tissues damaged by disease, wounds or tissue senescence could be regenerated and replaced by new ones full of life.

Regenerative medicine or tissue engineering is one of the biomedical research branches which it has developed more in recent years and that at the same time present promising future prospects.

From a viewpoint somewhat academic, to simplify the approach to the issue, we believe that this therapeutic strategy is based on three pillars:

1. What we might be called broadly as Tisugénesis. Would the aspects linked to the cells. A series of little specialized cells that Friedenstein from studies in the early 70s has been called Mesenchimal Stem cell or mesenchymal stem cells. These initially identified in the bone marrow where they coexist with hematopoietic progenitor cells (also known as Hematopoietic Stem cell) and have subsequently been identified in many other tissues, cells have the ability to differentiate into more tissue-specific cells. Actually it is more or less heterogeneous cell populations and that as a whole and by specific stimuli-whether mechanical, electrical or biochemists are able to respond by changing their phenotype and behaving like cells and specific characteristics of a particular tissue. These cells must meet certain criteria under which indicates the International Society for Cellular Therapy (ISCT). They are looking fibroblastoid mononuclear cells able to adhere to plastic culture flasks, with a profile of surface markers including (CD73 + CD90 + CD105 + CD34- CD45- CD14- CD19- CD11B- CD79a- HLA-DR) and capable of differentiate at least three cell lines: bone, chondrocytes and adipocytes.

2. Tisuconducción. This is linked to the development of biomaterials appearance. By their structures, electrical surface porous structure or composition, mechanical characteristics allow cells to have a structural support that promotes cell growth direction close as the tissue structure we mimic. These scaffolds have been developed well as organic derivatives or directly synthesized in the laboratory. And in both cases may be mineral or polymeric structures. Among the polymeric structures, as we have developed derived from proteins such as collagen, sugars such as chitosan or caprolactones or derived from lactic and glycolic acids. As for the inorganic derivatives,

and in particular as regards to the designs aimed at obtaining bone support, the use of derivatives of calcium phosphates doped with various ions is most often used. Thus the synthesis of various derivatives of hydroxyapatites with variable contents in pyrophosphates, Sr, Mg, Si, and even the addition of polysaccharides such as chondroitin sulphate are used to modulate the mechanical and biological response of these compounds. The current trend is to build composite matrices in which nanotechnology and nanofibers have an importance seems that capital.

3. Tisuinducción. This is linked with the knowledge of the physiology of living organisms aspect. Knowledge of stimuli that will implement the mechanisms of cell differentiation, modifying their genetic expression and therefore morfofuncional appearance. Knowledge of the mediator, its specific or general receiver and modular actuation sequence allows the regenerative process. Factors such as morphogenetic proteins, hormones and polypeptides have been found effective in stimulating cell turnover.

In memory that we have written are some of the work done in these directions, and should naturally converge to build a complex in which the support structures designed as hybrid matrices phosphocalcic and polymer in which resorption were modulated by will combine the replacement times for the newly formed matrix multipotent cells considered above. We also spent a considerable effort in the study of the factors that determine or help in cell differentiation.

Specifically are five items that we ordered chronologically.

The first one relates to the ability that presents growth hormone administered topically in the surgical bed preimplantario to stimulate bone growth. In an animal experimental model analysis histomorphometric was performed of bone obtained in periimplantes placed in tibia´s, compared to bone obtained in a similar situation but topically administering 4IU of recombinant human growth hormone. The results indicate that it produces a significant increase in bone volume and bone contact with the implants placed.

In the second publication we boarded the appearance of the support. Again in an animal model for the study modify, analyze bone formation by applying a derivative of calcium phosphate cement synthesized by the working group that is reabsorbed in a line with the

speed of bone formation time. The results show that the amount of new bone is significantly higher with the new material.

In the third publication considered, we focus on the PRP as a means to stimulate bone regeneration. A comparative study between two systems for evaluating the platelet concentrate concentration obtained and the ultrastructure (transmission electron microscopy) activated platelet concentrate.

The next two papers analyze in an animal model the effect of the application of PRP in a critical experimental bone defect. The results indicate that application of platelet-rich plasma increases precocious tissue repair, the earliest appearance of ossification, but that medium to long term there is no more bone regeneration but more fibrous component.

Final conclusions arrived with this work suggest that:

The design of new biomaterials can promote bone regeneration in volume. The application of osteoinductive as the GH is going to get a positive effect on the volume and quality of new bone. And the PRP added to the surgical bed in absence of an osteoconductive substrate improves regeneration initially, but without significant effect at medium or long term.

INTRODUCCIÓN

Resolvió Zeus vengarse de Prometeo por su engaño, y negó a los mortales el último don que necesitaban para alcanzar la plena civilización: el fuego. Más, también aquí supo componérselas el astuto hijo de Japeto. Cogiendo el largo tallo del jugoso hinojo gigante, se acercó con él al carro del Sol que pasaba y prendió fuego a la planta. Provisto de aquella antorcha bajó a la Tierra y pronto la primera hoguera flameó hacia el Cielo….

…Después, Zeus dirigió su venganza contra Prometeo. Entregó al culpable a Hefesto y sus criados, Cratos y Bia (la coerción y la violencia), quienes hubieron de arrastrarle a las soledades de Escitia, y allí, sobre un espantoso precipicio, encadenarle con cadenas indestructibles al muro de roca del Cáucaso. Hefesto cumplió con desgano el mandato de su padre, pues amaba en el hijo de los Titanes al consanguíneo descendiente de su abuelo Urano, a un vastago de los dioses de tan alta alcurnia como Zeus. Con palabras llenas de piedad y bajo los improperios de sus brutales servidores, mandó a estos a que efectuaran el cruel trabajo….

…Y así hubo de permanecer Prometeo suspendido de la desolada peña, de pie, insomne, sin nunca poder doblar la cansada rodilla. «Exhalarás muchas inútiles quejas y suspiros —le díjo Hefesto—, pues la voluntad de Zeus es inexorable, y todos aquellos que llevan poco tiempo disfrutando de un poder usurpado son duros de corazón». En realidad, el tormento del cautivo debía durar eternamente, o por lo menos treinta mil años. Aunque suspirando y quejándose a voces, aunque llamando, como testigos de su dolor, a los vientos y a los ríos, a las fuentes y a las olas del mar, a la madre Tierra y a los astros del Zodíaco que todo lo ven, su. ánimo no se doblegó. «Debe soportar la decisión del Destino —dijo— todo aquel que sabe comprender la fuerza invencible ce la necesidad». Tampoco se dejó mover por las amenazas de Zeus a descifrar la oscura profecía de que un nuevo lazo matrimonia depararía al soberano de los dioses la perdición y la caída. Zeus cumplió su palabra: envió al prisionero un águila que, huésped diario, se nutría de su hígado, el cual, consumido, se regeneraba constantemente.[1]

La idea de la pervivencia y de la inmortalidad, ha ocupado siempre un lugar preponderante en la mente humana. La necesidad de ir más allá de la muerte se plasma en numerosos mitos y leyendas.

En este conocido pasaje de la mitología griega, Prometeo es castigado por Zeus y en su tortura aparece una referencia a lo que hoy entendemos como regeneración tisular.

La reconstrucción de los defectos óseos maxilofaciales constituye hoy en día uno de los mayores retos en el campo de la odontología, destacando su importancia en el tratamiento con implantes y en el área de la periodoncia. Aunque el tratamiento de la pérdida dentaria con implantes aloplásticos constituye en la actualidad una alternativa terapéutica con un excelente pronóstico, la necesidad de realizar modificaciones de los tejidos de soporte puede llegar a alcanzar un porcentaje elevado de los casos. Así Martínez2 considera que entre el 25% y el 72.3% de los casos presentan una reabsorción ósea subsidiaria de ser tratada mediante técnicas quirúrgicas avanzadas. En este sentido se han preconizado técnicas como la regeneración ósea guiada[3], la utilización de injertos[4,5,6], o la aplicación de biomateriales[7,8] para conseguir un mejor soporte óseo.

Bone Engineering de J.E.Davis es un texto esencial en el conocimiento sobre la regeneración del hueso y por extensión de los tejidos en general. En el prólogo de Vacanti[9] refiere (hace ya 15 años) el explosivo interés que tanto profesionales como público han desarrollado acerca de la promesa de nuevas estrategias terapéuticas basadas en la ingeniería de tejidos.

Podemos definir la ingeniería tisular como la parte de las ciencias biomédicas que desarrolla la regeneración de los tejidos mediante terapias basadas en el control del reclutamiento, la multiplicación y la diferenciación celular, su estímulo mediante factores de inducción y contención asociados al diseño de estructuras tridimensionales que permitan el soporte de la estructura celular remedando la estructura del tejido. Es decir la combinación de diseño de materiales bioactivos, la aplicación de sustancias tisuinductoras y la selección de estirpes celulares regeneradoras.

Los 100 trillones de células que tiene el organismo humano, se encuentran repartidos en unos 260 diferentes fenotipos que se asocian en el tiempo y el espacio para formar los tejidos y los órganos. Han seguido un proceso de diferenciación que consiste en adquirir un conjunto de características morfofuncionales y bioquímicas distintas al resto de los conjuntos celulares. Generalmente cuanto mayor es el grado de expresión de

características fenotípicas, menor es la capacidad de multiplicación de estas células. Sin embargo hay algunas células que manteniendo su capacidad de dividirse son capaces de expresar un morfotipo determinado bajo condiciones específicas de crecimiento.

Friedenstein, en los años 70, identificó en la médula ósea unas poblaciones celulares no hematopoyéticas capaces de renovarse y de dar lugar a células óseas. En 1991 Caplan utiliza el término de "células madre mesenquimales" al determinar la capacidad de diferenciarse también a otras líneas esqueléticas[3]. Fueron definidas como una población de células mononucleares derivadas de la médula ósea que cultivadas ex vivo se adherían al plástico, tenían una morfología fibroblástica, formaban colonias y eran capaces de diferenciarse en varias líneas celulares, en especial en hueso, cartílago y adipocitos. Posteriormente han sido identificadas también en muchos otros tejidos como músculo, tejido adiposo, pulpa dental, periodonto, etc.

En la actualidad la ISCT (International Society for Cellular Therapy) ha incluido como requisito, un patrón de expresión de marcadores antigénicos que responde a ($CD73^+$, $CD90^+$, $CD105^+$, y $CD34^-$, $CD45^-$, $CD11b^-$, $CD14^-$, $CD19^-$, $CD79a^-$, $HLA\text{-}DR^-$)[11]

Otra característica que las hace muy interesantes para su aplicación en investigación es su estabilidad genética. Por lo que a pesar de tener una gran capacidad de multiplicación son muy resistentes a la transformación. Sin embargo a medida que van envejeciendo, disminuye su capacidad diferenciadora.

El siguiente aspecto que debemos considerar, al menos desde un punto de vista un tanto académico y que simplifica la realidad biológica separando procesos que suceden de forma conjunta pero que para su mejor comprensión analizamos separadamente, es la influencia de distintos factores solubles sobre el comportamiento de estas células.

Según Scheller [12] El uso de señales solubles para la modulación de la formación ósea se ha convertido en un importante área de investigación en los últimos años. La primera caracterización de una BMP (bone morphogenetic protein) se realizó en 1965. Aunque su aplicación para mejorar el soporte óseo en implantología no se produce hasta 28 años más tarde en 1993. Diversos autores [13-14] han utilizado con éxito la BMP-2 recombinante humana para inducir la formación de hueso en modelos experimentales animales o clínicos.

Sin embargo hay otros muchos factores que pueden regular la osteogénesis como la hormona de crecimiento (GH)Prieto[15] . Se trata de un polipéptido de 191 aminoácidos secretado por las células acidófilas de la adenohipófisis y actúa a través de un intermediario, la IGF-I que se sintetiza en el hígado pero también en otros lugares como tejido condral y periostio bajo el propio estímulo de la GH. Actúa estimulando la proliferación y diferenciación osteoblásticas directa e indirectamente, según Kassem[16,17], a través de la citada IGF-I. En estudios experimentales en ratas viejas Bak[18] y Andreassen[19] encontraron que la GH estimula la formación de cortical ósea incrementando la mineralización desde el periostio y aumentando las propiedades mecánicas

PUBLICACIÓN 1

Effects of Local Administration of Growth Hormone in Peri-implant Bone: An Experimental Study with Implants in Rabbit Tibiae

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Purpose: *The objective of this study was to evaluate the qualitative and quantitative differences that could appear in newly formed peri-implant bone around Screw-Vent implants placed in rabbit tibiae when treated with local administration of growth hormone (GH).* ***Materials and Methods:*** *Eight New Zealand rabbits were randomly divided into 2 groups: the experimental group, which received 4 IU of GH in the form of lyophilized powder added to the ostectomy site before implant placement, and the control group, which did not receive GH before implant placement. Animals were sacrificed 2 weeks later, and histologic sections were obtained for histomorphometry and observation under light microscopy.* ***Results:*** *The sections in the GH-treated group presented enhanced growth of new trabeculae from the periosteal tissue, and the bone-to-implant contact in the experimental group was significantly greater ($P < .05$).* ***Discussion:*** *Local administration of GH stimulated a more dramatic effect than that seen previously with systemic GH administration, prompting growth from both the periosteum and endosteum.* ***Conclusions:*** *Local administration of GH at the time of implant placement could enhance peri-implant bone reaction.* INT J ORAL MAXILLOFAC IMPLANTS 2003;18:807–811

Key words: *animal studies, bone remodeling, dental implants, growth hormone*

Growth hormone (GH) is a peptide with 191 amino acids, secreted by the anterior pituitary gland, that stimulates the growth process, acting as a metabolic and mitogenic regulator. Its effects are mediated primarily by insulin-like growth factor I (IGF-I), a peptide of 70 amino acids that is synthesized in almost all tissues, but fundamentally in the liver and in chondral tissue[1] under GH stimulation.[2]

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GH is one of the substances that regulate bone growth and bone remodeling in vivo,[3] but it has only recently been accepted that GH may also act as a locally produced growth factor that can be secreted by various types of cells[4] and may exert both endocrine as well as paracrine and autocrine effects.

GH is able to stimulate bone growth by direct stimulation of the epiphyseal chondrocytes[5] and osteoblasts.[6] GH also increases synthesis of IGF-I and IGF-II,[7] which stimulates the proliferation and differentiation of osteoblasts.[8] In addition, GH is able to stimulate bone protein synthesis and mineralization[9] and increase bone turnover.[10]

Systemic GH has been used for stimulating experimental bone fracture repair in both young and old rats, showing an increase of up to 400% in biomechanical properties when compared with an untreated control group.[11–14] Recent studies have shown that GH can also have a local effect. Guicheux and coworkers[15] observed that local administration of GH, released from a calcium phosphate–type biomaterial carrier, was able to improve the substitution process of biomaterials by bone through an acceleration of the bone remodeling process.

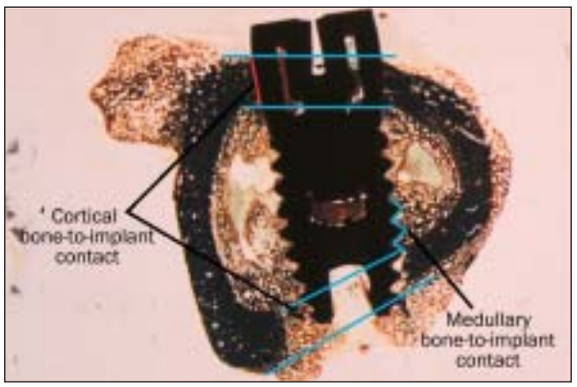

Fig 1 BIC contact at the cortical and medullary levels.

However, GH has rarely been applied locally during surgery to modify the osseointegration process.[16] The authors' working hypothesis has been that local administration of GH as a single dose at the time of implant surgery could accelerate the osseointegration process, inducing histologic differences compared to untreated control samples.

The objectives of the present study were the following:

1. To assess whether histologic differences appear in the peri-implant bone with the local administration of GH during surgery.
2. To evaluate quantitatively the peri-implant bone response with a morphometric analysis.

MATERIALS AND METHODS

A total of eight 3-month-old New Zealand rabbits, weighing 2.5 kg each, were used as experimental animals. Rabbits were randomly divided into 2 groups. Rabbits in both the control and experimental groups had one 3.3×8-mm Screw-Vent implant (Paragon Implant Company, Encino, CA) placed in the internal side of each tibia. In addition, the experimental animals received 4 IU of recombinant human GH (Saizen; Serono Laboratories, Madrid, Spain) in the form of lyophilized powder placed in the ostectomy site before placement of the implant.

After intramuscular anesthesia with ketamine (Imalgene 1000, 0.75 mg/kg; Merial, Lyon, France) and xylazine (Rompun, 0.25 mg/kg; Bayer, Leverkusen, Germany), an incision was made on the internal side of the tibia, at the union of the diaphysis/proximal metaphysis. After detachment of the cutaneous-periosteal tissues, the bone bed was prepared for implant treatment following instructions of the implant manufacturer, using internal/external cooling Paragon drills. The implants were placed and achieved primary stability; then, the periosteal flap was sutured with Dexon sutures (Davis & Geck, Wayne, NJ) and the skin with silk sutures. Oxytetracycline was administered orally to prevent postsurgical infection in both groups.

The animals were sacrificed 2 weeks after surgery. Both tibiae were dissected from their soft tissues, fixed in 10% buffered pH 7 formaldehyde, and embedded in 2-hydroxyethylmethacrylate resin, according to the Donath and Breuner method,[17] so as to cut undecalcified bone and titanium simultaneously with the Exakt microtome (Exakt Apparatebau, Norderstedt, Germany). The histologic analysis was conducted under a light microscope (Leica, Wetzlar, Germany) with sections stained with Masson stain and picrosirius.[18] These procedures were performed in the Department of Morphological Sciences and Surgery at the Medical School in the University of Alcalá de Henares, Madrid, Spain.

A morphometric study to quantify the newly formed bone around the implants was performed later with a MIP-4 imaging analyzer (a computerized system that performs area and volume measurements; Digital Image System, Barcelona, Spain). The parameter calculated was bone-to-implant contact (BIC), which is defined as the length of bone surface border in direct contact with the implant perimeter (×100%).[19] The BIC was measured at the cortical zone in contact with the implant (cortical level) and at the medullary zone in contact with the implant (medullary level) (Fig 1). These measurements were made with a 10× objective in all fields of each specimen by counting the number of intersections over the implant surface. Finally, the results were expressed as a percentage of the implant surface covered by bone at the cortical and medullary levels. For each sample, various sections were obtained and one of them was randomly used for the statistical evaluation.

The BIC mean values ± standard error of the mean (SEM) of each group were calculated. The groups were tested by the Student *t* test. The results and the statistical analysis were elaborated with the SPSS 11.0 computer system (SPSS, Chicago, IL).

RESULTS

At 2 weeks after implant placement and GH treatment, the experimental group sections demonstrated a greater periosteal and endosteal response than the control group sections. More newly formed trabeculae could be seen in the sections with GH. These trabeculae were thicker and more irregular than the control group sections (Figs 2 and 3).

Fig 2 Section obtained from a specimen without GH, showing poor periosteal and endosteal response (Masson; original magnification ×10).

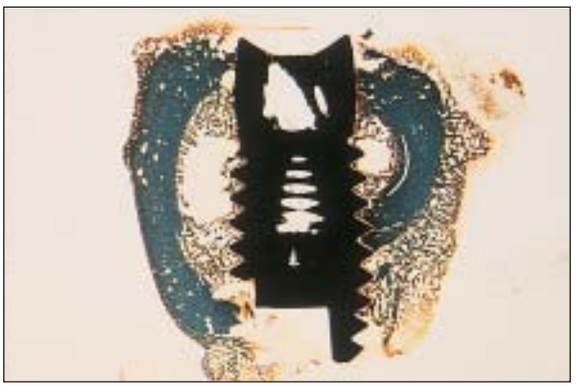

Fig 3a Section from a specimen with GH showing a greater periosteal and endosteal response. In this case the new trabeculae were mostly mineralized *(green)* and were perpendicular to the old cortex (Masson; original magnification ×10).

Fig 3b Section from another specimen with GH, with more endosteal and periosteal reaction than the section shown in Fig 3a. In this specimen, the new trabeculae were more disorganized and more irregular, and less mineralization had occurred than in the previous specimen (Fig 3a) (Masson; original magnification ×10).

Fig 3c Section from another specimen with GH, with an exaggerated reaction from the endosteum and fundamentally from the periosteum. The new periosteal trabeculae can be seen; some of them are perpendicular and mineralized. In another area, the new periosteal trabeculae were irregular, without zones of mineralization (Masson; original magnification ×10).

Birefringent neoformed collagen fibers were seen with the picrosirius stain, when polarized. More birefringent collagen fibers were seen in the sections with GH than in the sections without GH (Figs 4a and 4b).

The quantitative morphometric analysis obtained with the MIP-4 analyzer showed more BIC in the GH group, with statistical significance (Table 1; Figs 5 and 6). Mean BIC ± SEM at the cortical level was 66.67% ± 4.9% in the experimental group and 28.78% ± 2.6% in the control group, which was statistically significant ($P < .05$). Mean BIC ± SEM at the medullary level was 51.49% ± 6.9% in the GH group and 18.34% ± 2.5% in the control group, which was also statistically significant ($P < .05$).

DISCUSSION

In recent years, several substances have been used to improve peri-implant bone response: bone morphogenetic proteins,[20,21] growth factors,[22] and more recently hormones, such as GH.[16,23] Systemic administration of GH has been used to increase bone mass[14] and to improve the fracture repair processes.[11,12] Other authors have studied the stimulating effects of local GH on bone formation in rat mandibles[24] or the enhancing effects of local GH on formation of new bone and bone resorption using a phosphate/calcite matrix GH-releasing system.[15] The authors' previous data have also shown an increase in the peri-implant bone response with the local administration of GH in an osteoporotic rabbit model.[16]

The present histologic results showed an increase in newly formed bone trabeculae in the GH-treated group, derived from the periosteum and eventually from the endosteum. These findings are partially in disagreement with the results of most authors conducting research in this area, who propose that GH can stimulate the periosteal reaction without affecting the endosteum. Andreassen and coworkers,[14,25]

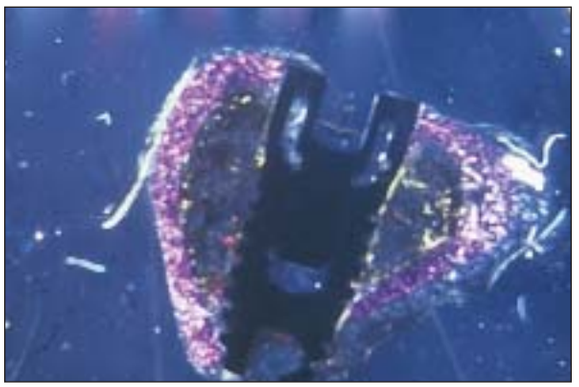

Fig 4a Section from a specimen without GH seen under polarized light, showing lesser birefringent neoformed collagen fibers (picrosirius; original magnification ×10).

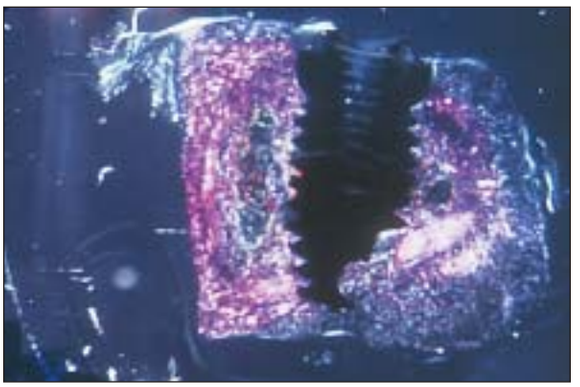

Fig 4b Section from a specimen with GH seen under polarized light, showing more birefringent neoformed collagen fibers (picrosirius; original magnification ×10).

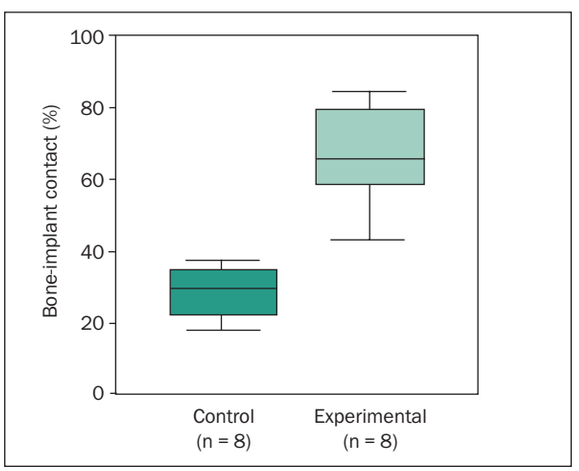

Fig 5 Cortical bone-to-implant contact. In the experimental group, mean BIC (± SEM) was 66.67% ± 4.9%, and in the control group, mean BIC was 28.78% ± 2.6%. These differences were statistically significant ($P < .05$).

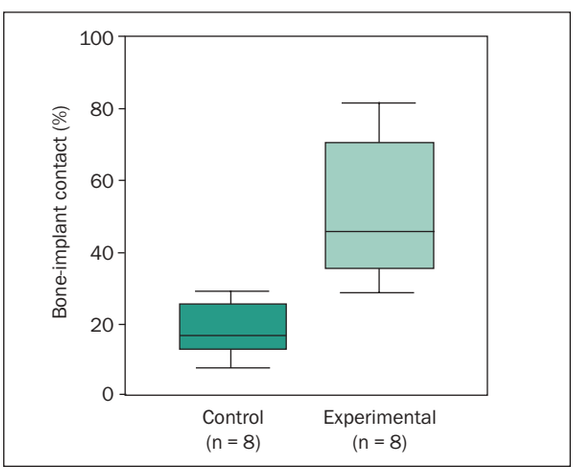

Fig 6 Medullary bone-to-implant contact. In the experimental group, mean BIC (± SEM) was 51.49% ± 6.9%, and in the control group, mean BIC was 18.34% ± 2.5%. These differences were statistically significant ($P < .05$).

Mosekilde and associates,[26] and Martínez and colleagues[27] showed that systemic GH was able to increase the cortical mass exclusively from the periosteum. In the present results, the endosteal reaction could have been induced by the high local levels of GH. The newly formed trabeculae were more irregular in the GH group. However, Jorgensen and coworkers[28] observed newly formed bone after the systemic administration of GH in growing rats and concluded that it had the characteristics of normal bone, with concentric lamellae forming osteons. The present results suggest that locally administered GH in a single dose of 4 IU (1.2 mg) could exert an "impulse effect" in the first hours of the process of osseointegration,[16] recruiting more preosteoblasts and thus leading to an acceleration of the process.

The morphometric data revealed that there was significantly greater BIC in the GH-treated group than in the control group. These results obtained with the local application of GH are similar to those of Lynch and associates,[22] who observed that local administration of platelet-derived growth factor and IGF-I were capable of stimulating the regeneration of bone around titanium dental implants in the early phases of healing. These data are in agreement with Cochran and colleagues,[20] who found more BIC in implants that were treated with local recombinant human bone morphogenetic protein-2.

CONCLUSION

The local administration of GH was able to enhance the peri-implant bone response around Screw-Vent implants placed in young rabbit tibiae at a statistically significant level.

Table 1 Bone-Implant Contact in Examined Species

Implant sample	Cortical BIC (%)	Medullary BIC (%)
Control group (no GH)		
1	18.11	16.03
2	33.94	29.22
3	35.96	16.21
4	26.82	18.06
5	27.71	22.68
6	38.03	10.72
7	31.70	25.88
8	18.00	7.98
Experimental group (with GH)		
1	52.48	47.91
2	43.03	35.32
3	64.31	43.11
4	65.09	35.31
5	66.02	28.62
6	80.75	68.61
7	77.31	71.37
8	84.44	81.67

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Bone augmentation in rabbit calvariae: comparative study between Bio-Oss® and a novel β-TCP/DCPD granulate

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Abstract

Aim: In the present in vivo study, we compare the bone regeneration capacity of a novel brushite cement synthesized in our laboratory (DTG) with Bio-Oss® using rabbits as an animal model.

Methods: The study was performed in a group of 14 adult New Zealand rabbits using the bone conduction model. Two titanium cylinders were fixed into perforated slits made on the parietal cortical bone of each rabbit. One cylinder was left empty (negative control) and the other was filled with either Bio-Oss® or brushite set-cement granules (test cylinder). Four weeks after the intervention, the animals were sacrificed and biopsies were taken. The following parameters were analysed: bone tissue augmentation, bone mineral density and biomaterial resorption. The comparison of data between the different groups was performed using the Mann–Whitney test with a significance level of $p < 0.05$.

Results: The mean bone mineral density and augmented mineral tissue inside the test cylinders were similar but higher than those of negative controls. Material resorption and bone tissue augmentation were significantly higher in the defects treated with the brushite-based set cement ($p < 0.05$).

Conclusions: Brushite cement granules were more resorbable and generated more bone tissue than Bio-Oss® inside the titanium cylinders placed in the rabbit calvaria.

Key words: Bio-Oss®; Brushite; β-tricalcium phosphate; glycolic acid; titanium bone cylinder; vertical bone augmentation

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Bone regeneration techniques constitute a valid surgical procedure for increasing bone quantity and quality in areas where insufficient bone volume prevents the stabilization of osteointegrated implants. Biomaterials for stimulating osseous regeneration should combine osteogenic, osteoinductive and osteoconductive properties. Besides, they should be resorbed and gradually replaced by newly formed bone (Giannoudis et al. 2005). The use of several bone substitutes has been described for bone regeneration but so far, only autografts (autologous bone grafts) re-unite all the mentioned properties, being the most suitable material. However, the limited availability of autografts in intra-oral areas and the post-operative morbidity associated with the use of extra-oral grafts forces the physicians to use other biomaterials in bone regeneration (Block & Kent 1997).

An alternative to autografts are allogenic biomaterials (grafts from another individual of the same species), such as human demineralized freeze-dried bone, provided by tissue banks. Unfortunately, there is some controversy regarding the osteoinductive capability of these materials (Schwartz et al. 1998); besides, they have the risk of immunological rejection and transmission of infections such as HIV and hepatitis that requires special manufacture measurements (Giannoudis et al. 2005). Currently, only bone morphogenetic proteins (BMP) seem to have osteoinductive properties but their use for dental practice is still in the experimental phase (Nevins 1996).

The osteoconductive properties offered by natural bone substitutes from animal origin, such as collagens and bovine hydroxyapatite Bio-Oss® (Geistlich Biomaterials, Wolhusen, Switzerland) overcome some of the autografts' limitations (Von Arx et al. 2001). For instance, Bio-Oss® chemical composition is very

similar to that of human bone hydroxyapatite (HA) as it contains a calcium/phosphate proportion of 1.67 identical to bone HA (Suzuki et al. 2000). Besides, its mineral matrix contains crystals of *ca.* 100 μm diameter, presenting morphological and structural properties very similar to those of the human bone (Rosen et al. 2002). Furthermore, Bio-Oss® rough topography favours osteoblastic anchorage, proliferation and synthesis of bone matrix on its surface (Acil et al. 2000), and is currently one of the most frequently used biomaterials in bone regenerative procedures. However, HA-based biomaterials are very slowly resorbed in vivo.

Third-generation biomaterials are designed with the aim to aid the body in self-healing. One desirable characteristic in bone materials is their ability to be remodelled, i.e. the biomaterial is resorbed by osteoclasts and subsequently replaced by newly formed bone through osteoblastic activity (Schilling et al. 2004). Biomaterials with a slow resorption rate, such as HA, interfere with bone growth, while biomaterials with a fast resorption rate, such as calcium sulphates, compromise the stability of the surgical site during the healing process (Stavropoulos et al. 2004, Lieberman et al. 2005). New biomaterials are designed to have resorption speeds that match bone growth rate. It is to be noted that brushite crystals have an in vivo resorption rate similar to human bone growth speed, *ca.* 20 μm/day, and this fact can be very important in stabilizing the newly formed bone. (Bohner et al. 2005).

Synthetic materials, such as β-tricalcium phosphate (β-TCP) and dicalcium phosphate dehydrate (brushite or DCPD) raise great interest as they are cheap, do not present immunologic or infectious problems (Giannoudis et al. 2005) and have a higher resorption rate in vivo than HA materials, allowing bone formation simultaneously with material resorption (Chow et al. 2003, Trisi et al. 2003).

In 1987, Mirtchi and Lemaitre introduced the first cement made from DCDP and β-TCP obtained from the reaction of a base (β-TCP) and an acid (monocalcium phosphate). The set cement composition presented a mixture of two minerals: β-TCP and DCDP (Mirtchi & Lemaitre 1989). The significance of this cement lies in its capacity to decompose in physiological environments and be resorbed by the body. Investigations performed on this cement showed that DCDP is resorbed up to three times faster than HA or β-TCP (Chow et al. 2003). This property seems to prove its feasibility in accelerating the substitution of the biomaterial by newly formed bone. Surprisingly, this biomaterial has barely been studied for dental implantology purposes, although it has proved to be useful in orthopaedic applications for stabilizing fractures or filling defects (Bohner et al. 2005).

Most investigations that evaluate the bone-forming capacity of biomaterials are performed in critical size defect (CSD) models (see Fig. 1a). However, in daily clinical practice, bone regeneration is often needed to grow vertically from the surface of the native bone, i.e. a vertical bone augmentation. The evaluation of bone-regeneration is better performed with the bone conduction model (Fig. 1b), introducing barriers in the bone defect that prevent lateral bone formation and allow bone growth only in the vertical direction (Lundgren et al. 1999, 2000).

The purpose of this study was to evaluate the bone-regenerative capacity of a novel brushite/β-TCP pre-set cement in granular form (DTG) and to compare its behaviour with the commercial bovine bone Bio-Oss®. The two biomaterials were implanted in rabbits using a calvaria, a titanium cylinder bone conduction model, and new bone tissue formation was analysed 4 weeks after the intervention.

Material and Methods

Before beginning the ''in vivo'' animal study, the protocol was approved by the ethical committee for animal experiments of the Complutense University of Madrid (UCM). Experiments were conducted in accordance with the guidelines laid down by the European Communities Council Directive of 24 November 1986 (86/609/EEC), and adequate measurements were taken to minimize pain and discomfort in the animals.

The preparation of the new biomaterial is explained elsewhere (Tamimi et al. 2005) but herein a brief description of the synthesis is given (Tamimi et al. 2005). The new biomaterial (DTG) was made from a brushite cement composed of monocalcium phosphate (0.8 g) and β-TCP (1.4 g) that sets in a glycolic acid aqueous solution (1 M) using a powder to liquid ratio of 1.7 g/l. The cement was left to set ex vivo into a hard material that was milled with a mortar and sieved to obtain granules with diameter ranging between 0.2 and 1.0 mm. The final composition of the granules was 87% DCPD and 17% β-TCP (Tamimi et al. 2005). Bovine bone regeneration biomaterial Bio-Oss® was acquired from Geistlich Biomaterials (Baden, Germany). The in vivo study was performed in a rabbit experimental model using a titanium bone conduction cylinder. The titanium cylinders (Apositos Sanitarios Aragoneses, Huesca, Spain) had an inner rough surface with the following dimensions: 4 mm height, 0.5 mm thickness and 9 mm inner diameter.

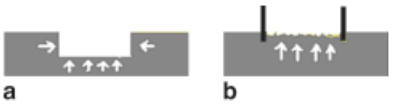

Fig. 1. Schematic representation of two possible methods for evaluating bone regeneration in vivo: (a) critical-size defect model in which bone regeneration occurs with undefined direction; and (b) bone-conduction chamber model for evaluating true vertical bone augmentation. Bone tissue is represented as the grey background, the directions of growth by the white arrows and the obstacles used for limiting bone growth are shown by the black stripes.

Fourteen healthy 6-month-old female New Zealand rabbits weighing between 3.9 and 4.4 kg were used as experimental animals in order to compare in vivo the bone augmentation capacity of Bio-Oss® with DTG (Tamimi et al. 2005). The animals were accommodated in the official stable for animal assays of the UCM at 22–24°C with 55–70% humidity, light cycles of 12 h, and air renewal 15 times/h. The rabbits were fed with a Panlab® (Panlab S.L., Barcelona, Spain) diet while drinking was permitted *ad libitum*. The rabbits were divided into two groups of seven each, the first group was to be treated with Bio-Oss® (group 1) and the second group with DTG (group 2).

Surgical procedure

The rabbits were anaesthetized with an intra-muscular dose of 0.75 mg/kg ketamine (Imalgene 1000®, Rhone, Merieux, France) and 0.25 mg/kg xilacine (Rompun®, Bayer, Leverkusen, Germany). Animals were placed in *sternal recumbency*, the head was shaved and the cutaneous surface was disinfected with a povidone iod solution before the operation. The calvaria bone was exposed through a skin incision

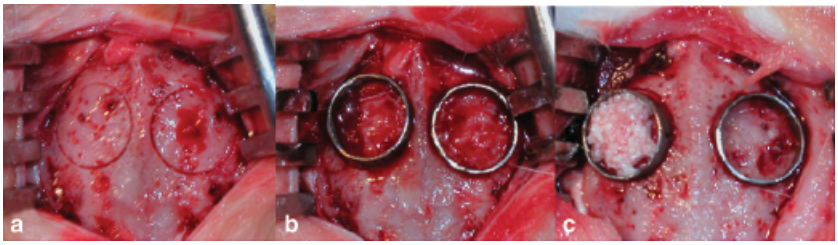

Fig. 2. (a): Photograph of rabbit calvaria with two slits for fixing the titanium cylinders. (b): photograph showing the fixation of the titanium cylinders on the slits. (c): photograph showing the right chamber grafted with Bio-Oss® while the left one was kept un-grafted for negative control.

approximately 4 cm in length over the *linea media*. A pair of tweezers was used to lift the skin before the periostium was also incised in the same place. A periosteal elevator was used for separating the periosteum from the bone surface. Two circular slits (0.5 mm thick × 9 mm inner diameter × 0.5 mm deep) were made in the parietal bone using a trephine on a slow-speed electric handpiece by applying 0.9% physiologic saline irrigation. The slits were made on each side of the median sagittal suture without crossing it. A titanium cylinder barrier was created by mechanically fixing the titanium cylinder on each slit applying slight pressure on it. The bone surface surrounded by the ring was slightly roughened with a rounded burr to promote bleeding (see Fig. 2). The tested biomaterials are stabilized inside the cylinders, and on each rabbit the right cylinder was grafted with 0.25 g of experimental biomaterial (Group 1: Bio-Oss® and group 2: DTG), while the contra-lateral chamber was filled with autologous blood clot as negative control (group 1: control 1 and group 2: control 2). Closure of periosteum and subcutaneous tissues was performed with resorbable Dexon® 3/0 sutures (North Haven, CT, USA), while the skin was relocated with (3/0) silk continuous sutures (Apositos Sanitarios Aragoneses, Huesca, Spain). Post-operative antibiotics were administered, Terramicina® (Pfizer, Madrid, Spain), in water for 7 days. The animals were sacrificed 4 weeks after the intervention with an overdose of sodium pentobarbital IV (Dolethal®; Vetoquinol, Lure, France).

Post-mortem, a surgical burr attached to a slow-speed electrical handpiece was used to harvest the bone blocks containing the titanium conduction cylinders from the animal's calvariae. Samples were then preserved fixed in formaldehyde 10% buffer solution at pH 7.0 before further analysis.

Densitometry

Post-mortem bone mineral density analyses were performed on the calvaria blocks using the XR-26 Norland® Densitometer (Norland Corp.; Fort Atkinson, WI, USA). In each calvaria block, a bone area of $0.5 \times 0.5\,cm^2$ was analysed inside both the experimental (Bio-Oss and DTG) and control (control 1 and control 2) chambers. The exploring resolution was $1.0 \times 1.0\,mm^2$, measuring resolution at $0.5 \times 0.5\,mm^2$ and exploration speed at 40 mm/s. The bone mineral content (BMC) values for the experimental and control samples were obtained from the densitometry analysis and the bone mineral density (BMD) was calculated using the formula:

$$BMD = \frac{BMC}{Area}$$

where the area is always $0.25\,cm^2$, BMC values are expressed in grams and BMD values in g/cm^2.

Histology and histomorphometry

Bone samples were dehydrated in ascending series of alcohol (60–100%), embedded in 2-hidroxy-ethyl-methacrylate, and then photopolymerized 6 h with UV light, 2 h with white light and 6 h with blue light into ready-to-cut sample blocks. A saw microtome Exakt® (Exact GmBH, Norderstedt, Germany) was used to cut coronal sections from the cylinders as described elsewhere (Donath & Breuner 1982, Slotte et al. 2003) 200 μm thick. The sections were then ground to a total thickness of 50–80 μm by means of a grinder Exakt® (Norderstedt) in order to achieve better histological visualization without risking the loss of the samples. Afterwards, surface staining was performed with haematoxylin & eosin (HE) and toluidine blue (TB; Donath & Breuner 1982). The histological evaluation of bone neoformation was carried out by means of optical microscopy. To perform the histomorphometric analysis, light micrographs (at magnification × 6) of the biopsy slices were captured with a digital camera and analysed with the histomorphometry software MIP-4 (Digital Image System, Barcelona, Spain). Six randomly selected slices were analysed for each biopsy. In each section, the area inside the cylinder was included for histomorphometric evaluation while the original cortical bone and the area outside the cylinder were excluded. The already existing bone was lamellar while the regenerated bone was woven and grew inside the cylinder so both types of bone could be easily differentiated in the histological observations.

The following measurements were taken from each cylinder: total sample volume, newly formed bone and remaining graft volume. These data permit to calculate the following parameters: average augmented bone volume formed in the cylinder (BV), volume of the remaining graft material (RG) and augmented mineralized tissue (AMT).

$$BV(\%) = \frac{\text{Newly formed bone volume}}{\text{total sample volume}} \times 100$$

$$RG(\%) = \frac{\text{Remaining graft volume}}{\text{total sample volume}} \times 100$$

$$AMT = BV + RG$$

Statistical analysis

A statistical software package (SPSS 7.0, Chicago, IL, USA) was used to analyse the histomorphometric and densitometry measurements by the Mann–Whitney test. Significance for the analysis was set at $p < 0.05$.

Results

There were no surgical complications during the preparation of the bone conduction cylinder and its filling with the experimental biomaterials. After the intervention, the animals recovered without post-operative signs of infection and no animals were lost during the study.

Densitometry

As shown in Table 1, the chambers grafted with Bio-Oss® and with DTG

had similar BMD values, which are significantly higher than those of the ungrafted negative controls (controls 1 and 2).

Histology

The observation of the histological sections taken from the different groups of bone conduction cylinders showed no inflammatory reaction. In the ungrafted chambers (controls 1 and 2) scarce bone formation activity was observed and only a few short and isolated trabeculae could be distinguished on the external surface of the cortical (Fig. 3). Neither osteoblasts nor osteoclasts were found in both control chambers.

In the chambers grafted with Bio-Oss® (Figs 4 and 5), implanted granules were observed distributed over the whole specimen area. Bio-Oss® was dyed red by HE and greyish with TB. Bio-Oss® particles presented lacunae free from osteocytes due to their animal origin; nevertheless, their artificial shape differentiate them from the bone trabecula growing around them. Bio-Oss® granules were mostly surrounded by a thin layer of fibrous tissue at the medium and upper tiers, and by bone trabeculae at the lower tier (corresponding to the area in contact with the calvaria). Bone regeneration was observed from the external surface of the cortical to approximately 1/3 of the height of the cylinder. However, signs of biomaterial resorption such as phagocytic cells forming Howship's lacunae on its surface, etching, pits or resorptive trail formation could not be identified on Bio-Oss® granules. From this observation, we infer that 4-weeks after implantation in rabbits' calvariae, Bio-Oss® showed osteoconductive properties but resorption did not take place.

In the cylinders grafted with DTG (Figs 6–9), the remaining granules were observed over the whole specimen area. DTG granules were intensely dark brown dyed by HE, while in TB the granules had a light grey colour. DTG particles presented a rounded morphology invaded by neoformed bone trabeculae growing from the external surface of the parietal bone, especially at the lower and medium tiers of the cylinder, and no inflammatory foreign body reaction was observed. Moreover, the surface of the biomaterial showed signs of resorption such as multinuclear/mononuclear phagocytic cells forming Howship's lacunae, surface pitting, resorption trail formation, and the remaining granules being perforated by the new bone (see Fig. 6). This material

Table 1. BMD values of each group

Group	BMD (g/cm^2)
Bio-Oss®	0.32 ± 0.05*
Control 1	0.15 ± 0.02
DTG	0.29 ± 0.07*
Control 2	0.14 ± 0.01

All data presented are mean values (± standard deviation).
*Significantly higher than all control groups ($p < 0.05$).
BMD; Bone mineral density values.

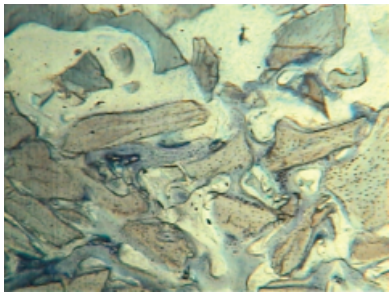

Fig. 4. Light microscope photograph of a toluidine blue-stained section from a bone conduction chamber grafted with Bio-Oss®. The photograph shows newly formed bone growing on the surfaces of Bio-Oss® granules (original magnification × 10).

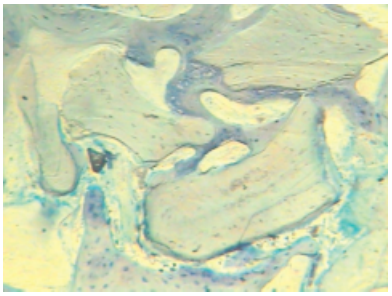

Fig. 5. Light microscope photograph of a toluidine blue-stained section from a bone conduction chamber grafted with Bio-Oss®. The photograph shows bone forming over the Bio-Oss® surface but no resorption pitting is observed on the granules, and their edges remain sharp (original magnification × 20).

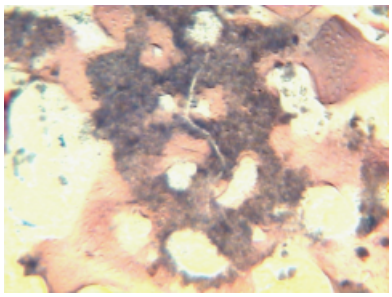

Fig. 6. Light microscope photograph of an haematoxylin & eosin-stained section from rabbit calvaria grafted with DTG. The micrograph shows a DTG granule (black) surrounded and perforated by the newly formed bone (red) (original magnification × 20).

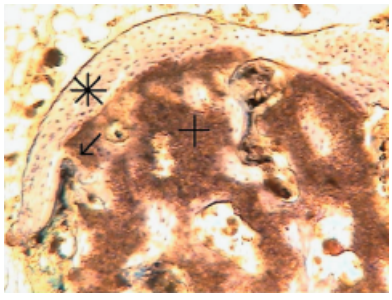

Fig. 7. Light microscope photograph of an haematoxylin & eosin-stained section from rabbit calvaria grafted with DTG. The biomaterial (+) is surrounded by bone tissue (*). Pitting resorption trail formation (arrow), Hawshipapos;s lacunae and rounding of the surface can be observed while the newly formed woven bone grows into the resorbing structure of the graft (original magnification × 20).

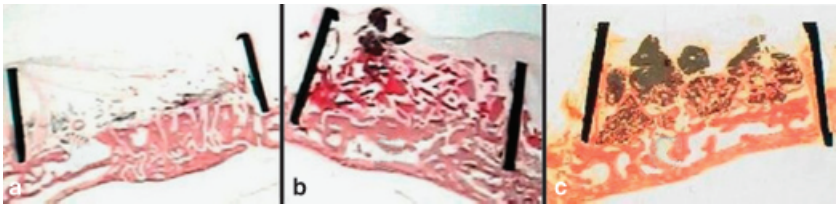

Fig. 3. Light microscope photograph of an haematoxylin & eosin-stained section from rabbit calvaria with titanium cylinders fixed on it (black strips). (a): unfilled cylinder (negative control), (b): cylinders grafted with Bio-Oss® granules. (c): cylinder grafted with DTG granules. The photograph shows newly formed bone growing into the Bio-Oss® and DTG granules, while no bone grows into the unfilled cylinder (original magnification × 2).

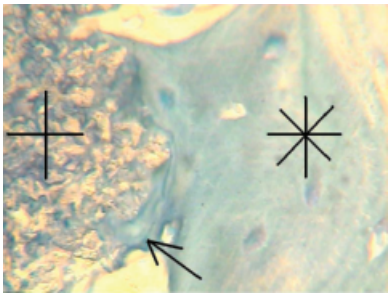

Fig. 8. Light microscope photograph of a toluidine blue-stained section from a bone conduction chamber grafted with the novel biomaterial (DTG). The photograph shows newly formed bone (*) in direct contact with the remaining monoclinic dicalcium phosphate dehydrate crystals (+). The interface between the biomaterial and the bone tissue is rough and a Howshipapos;s lacuna can be observed (arrow; original magnification × 40).

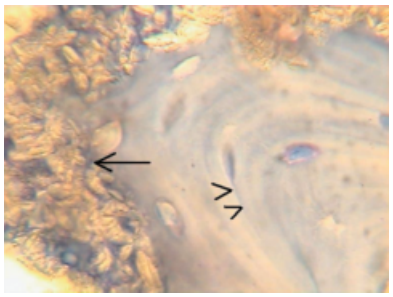

Fig. 9. Light microscope photograph of a toluidine blue-stained section from a bone conduction chamber grafted with the novel biomaterial (DTG). The photograph shows newly formed bone growing in a lamellar orientation (arrow heads) in direct contact with the remaining monoclinic dicalcium phosphate dedydrate crystals (arrow; original magnification × 40).

can be considered osteoconductive and bioresorblable.

Histomorphometry

The data obtained from histomorphometry analysis are shown in Table 2. The BV values are significantly higher for DTG-grafted cylinders, followed by Bio-Oss® cylinders and the negative controls. The RG values revealed that the chambers filled with Bio-Oss® presented less graft resorption than those filled with DTG. AMT values were similar for both Bio-Oss® and DTG cylinders, while negative controls had lower AMT.

Table 2. Results of the histomorphometry measurements

Group	AB (%)	RG (%)	AMT (%)
Bio-Oss®	11.7 ± 2.4*	37.2 ± 5.5	48.9 ± 10.0§
Control 1	5.7 ± 1.0	–	5.7 ± 1.0
DTG	16.7 ± 4.9†	22.4 ± 8.5‡	39.5 ± 4.9
Control 2	5.1 ± 1.1	–	5.1 ± 1.1

All data presented are mean values (± standard deviation).
*Significantly higher than all control groups ($p<0.05$).
†Significantly higher than all the other groups ($p<0.05$).
‡Significantly higher than Bio-Oss® group ($p<0.05$).
§Significantly higher than DTG group ($p<0.05$).
AB, augmented bone tissue; RG, Remaining unresorbed biomaterial; AMT, augmented mineral tissue

Discussion

Several authors consider that a 4-week period of implantation is enough time to observe angiogenesis and bone formation in several animal models, including rabbits, where experimental biomaterials are grafted into bone defects (Schmid et al. 1997, Boo et al. 2002, Herron et al. 2003). In our experiment, the histological changes that occurred in the grafted areas during the 4-weeks of implantation were pronounced and allow to evaluate the differences in bone regeneration capacity of both assayed biomaterials.

BMD values of the Bio-Oss® and DTG grafted cylinders were similar but significantly higher than the BMD values of the negative controls. Even though the densitometry analysis could be biased by the remaining unresorbed granules, the histomorphometric study revealed that in the control groups, no regenerated bone is formed. By contrast, both the Bio-Oss® and DTG groups offered significantly higher neoformed bone percentages.

In this study, there was no need to use histological apposition markers for identifying the newly formed bone because with the titanium cylinder model, the edges of the bone defect were clearly limited by the walls of the titanium cylinder (Slotte et al. 2003). On the other hand, the critical size defect model presents problems in identifying the original edges of the defects and apposition markers, such as tetracycline, are needed for recognizing the newly formed bone (Pautke et al. 2005).

The BV values obtained in samples treated with Bio-Oss® were comparable with that found in the literature (Slotte & Lundgren 1999) where Bio-Oss® was grafted into silicone cylinders on rats' calvariae (18.1%) or in titanium cylinders on rabbitsapos; calvariae (19.9%; Slotte et al. 2003). Nevertheless, the BV value obtained with the DTG granules was significantly higher than that achieved with Bio-Oss®. As the use of Bio-Oss® is widely spread in oral surgery, the result obtained with the DTG granules seems promising and worth continuous investigation (Tamimi et al. 2005).

Bio-Oss® is considered a non-resorbable material because it needs several years (3–6 years) of implantation before showing some slow in vivo resorption through osteoclast activity (Taylor et al. 2002). The presence of unresorbed granules within the newly formed bone is undesirable because it interferes with new bone growth and compromises the properties of the resulting tissue, affecting its osteointegration capacity for dental implants (Duda &Pajak 2004, Stavropoulos et al. 2004, De Boever & De Boever 2005, Zaffe et al. 2005). Other authors claim that biomaterials with slow in vivo resorption can interfere bone growth instead of enhancing it; however, we could not observe this effect in our study because the ungrafted negative control samples always had much less bone augmentation than the samples grafted with the experimental biomaterial (Stavropoulos et al. 2004).

In our study, Bio-Oss® showed no signs of graft resorption, and the biomaterial still occupied the whole area of the cylinder 4 weeks after the intervention. On the other hand, DTG-implanted granules were heavily penetrated by the newly formed bone through pitting and resorption trails confirming its bioresorption properties. This observation was supported by the RG values, which were significantly lower for the DTG grafted granules.

We attribute the high bioresorption of DTG to the presence of DCPD and β-TCP in its composition. β-TCP is moderately resorbable in vivo and needs only 12 weeks to be totally resorbed from bone defects created in animal models such as dogs, and 6–8 months when implanted in humans (Wiltfang et al. 2003, Suba et al. 2004). Furthermore, DCPD can be resorbed in vivo even faster than β-TCP because it is more soluble in water (Chow et al 2003, Herron et al. 2003, Tas & Bhaduri 2004). The combination of these two materials in the form of granules allows the diffusion of the ioninc species as well as the nutrients that would enhance the resorption of the material and the formation of new bone tissue. Bone growth was observed in the interior of our novel biomaterial inside the spaces where brushite resorption had already taken place; DTG appeared to be drilled by new bone formation while in the Bio-Oss® grafts, bone formation took place only around the granules.

This survey showed that both Bio-Oss® and DTG present good osteoconductive properties, achieving acceptable bone augmentation. However, the use of DTG offers faster in vivo resorption and increased bone neoformation when compared with Bio-Oss®.

Acknowledgements

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Clinical Relevance

Scientific rationale: Bio-Oss® granulate is an osteoconductive material that has been extensively used in bone regeneration despite its low resorption rate in vivo. In this study, we compare the bone augmentation capacity of Bio-Oss® with a brushite-based osteoconductive and bioresorbable material.

Principal findings: The novel biomaterial described in this study produces more vertical bone augmentation than Bio-Oss® and shows bioresortpion already at 4 weeks after implantation while Bio-Oss® remains practically unresorbed.

Practical implications: The novel brushite cement granules can be used for bone regeneration as an alternative to Bio-Oss®.

PUBLICACIÓN 3

A Comparative Study of 2 Methods for Obtaining Platelet-Rich Plasma

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Purpose: Double and single centrifugation are the most commonly used techniques for obtaining platelet-rich plasma (PRP) in dentistry. In this study, we used and compared 2 methods for obtaining PRP: double centrifugation (ACE system; Surgical Supply and Surgical Science Systems, Brockton, MA) and single centrifugation (Nahita system; Nahita, Navarra, Spain).

Materials and Methods: Blood samples were obtained from 30 random patients. Each blood sample was treated using the ACE system and Nahita system methods, after which the obtained material was analyzed by flow cytometry for platelet counts and by transmission electron microscopy (TEM) for ultrastructural analysis of the PRP gel.

Results: Platelet count analysis of the PRP obtained from both methods revealed that the ACE and Nahita systems accomplished platelet concentrations of (336%) and (227%), respectively. The platelet counting results obtained from the ACE system samples were more dispersed than their Nahita system counterpart. The ultrastructural (ie, TEM) study showed considerable alterations of the platelet aggregates in the ACE's PRP, especially when the samples were not mixed in the final stage of the procedure, whereas the Nahita aggregates always had a normal physiological appearance.

Conclusions: The ACE double-centrifugation method is able to achieve higher platelet concentrations than the single-centrifugation Nahita system, although the results obtained by ACE were more dispersed. Nevertheless, the ACE system provoked alterations in the PRP ultrastructure, and it was more sensitive to small errors during preparation.

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Platelet-rich plasma (PRP) techniques are widely used in many surgical procedures due to PRP's healing properties. Its high fibrin and growth factor concentration makes PRP very useful in plastic surgery and in stopping bleeding in patients with clotting disorders.[1-3] Fibrin glue was first described as a surgical adhesive by Matras[4,5] in the early 1980s, who recommended its use as a hemostatic and adhesive surgical agent that could promote tissue healing. In subsequent years, the discovery of transforming growth factor (TGF)-β1 in the platelets' alpha granules encouraged the possibility of using this blood derivative in bone and tissue regeneration.[6,7]

Recently, bone regeneration has become a very important subject in dental surgery. PRP, alone or in combination with other materials, including membranes and artificial or autologous bone grafts, are widely used to treat patients with bone volume deficiencies.[8-10] Moreover, PRP has been used in periodontal surgery, guided tissue regeneration, and dental implantology to produce bone and soft tissue regeneration in the periodontal and peri-implant regions.[11,12]

PRP is obtained by treating blood samples (usually taken from the same patient) with 1 of 3 methods: single centrifugation, double centrifugation, or apheresis. In all of these methods, PRP must be calcium-activated before being applied over the surgical site.[13-17]

Although the use of PRP as a bone regenerating material has been widely studied,[18-22] few studies have analyzed PRP at an ultrastrutural or cytological

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level to determine its actual effectiveness in providing growth factors for bone regeneration.[23,24] Transmission electron microscopy (TEM) allows evaluation of the cytological morphology with a very high resolution that cannot be achieved with other techniques. No studies analyzing the ultrastructural morphology of the PRP clots used in dentistry exist in the literature. Consequently, we initiated the present study to compare the platelet count and ultrastructure of PRP obtained by 2 methods commonly used by dentists: the single-centrifugation method (Nahita system; Nahita, Navarra, Spain) and the double-centrifugation method (ACE system; Surgical Supply and Surgical Science Systems, Brockton, MA).[20,21,25] The PRP samples obtained by each method were analyzed by TEM and flow cytometry platelet counting.

Materials and Methods

Blood samples were obtained from 30 patients, chosen randomly from the Oral and Maxillofacial Surgery Department at the "Doce de Octubre" University Hospital, Madrid. The patient group comprised 19 males and 11 females ranging in age from 17 to 84 years (mean, 48.33 ± 20.81 years). Informed consent was obtained from each patient, and the hospital's Ethics Committee approved the study protocol.

Blood samples were extracted from the right basilic veins into test tubes containing dextrose citrate acid as an anticoagulant. To obtain platelet concentrates, blood samples were treated with 2 different PRP methods using a specific centrifuge machine, the ACE double-spin system (ACE 170-0002) or the Nahita single-spin system (2610). The time taken to obtain PRP by each system was registered, and the samples were analyzed by the platelet-counting device and TEM for ultrastructural analysis.

TECHNICAL DESCRIPTION

ACE Double-Centrifugation System for PRP Processing

An 18-gauge needle (Introcam Safety 186; Braun Medical, Bethleham, PA) connected to a 3-way key (BD Conecta Plus 3; Becton Dickinson, Helsingborg, Sweden) was used to extract the blood into 3 10-mL Vacutainer tubes (Becton Dickinson). Each tube contained 1.5 mL of an anticoagulant solution (trisodium citrate, citrate dextrose acid), allowing a net blood volume of 8.5 mL per tube to be obtained. The tubes were gently sloped several times to mix the blood with the anticoagulant solution.

The 3 test tubes were introduced into an ACE centrifuge machine and subjected to a force of 160 *g* (1,300 rpm) for 10 minutes. In this way, the blood was separated into a red inferior phase, containing most of the blood cells (first-centrifugation PRP), and a superior plasma supernatant phase (first-centrifugation platelet-poor plasma [PPP]). The superior PPP layer was aspirated away using a 5-mL syringe with a 63-mm needle and placed into a new test tube without anticoagulant. The blood derivatives obtained from the first test tube—first-centrifugation PPP and first-centrifugation PRP—were labeled samples B and C, respectively, and saved for the analysis.

The remaining contents of the other 2 test tubes (first-centrifugation PRP) were treated with a second centrifugation, using a 400-*g* force (2,000 rpm) for 10 minutes, which separated the plasma into a superior yellowish PPP layer and an inferior reddish PRP layer. The PPP was extracted with a 5-mL syringe attached to a 63-mm needle and labeled sample D (second-centrifugation PPP). The 1.5 mL of PRP remaining in the original test tube was gently homogenized by aspirating and evacuating the contents of the test tube 3 times, using a 5-mL syringe with a 76-mm needle. This final homogenized sample was labeled sample E (second-centrifugation PRP). Many practitioners who use the double-centrifugation ACE method do not mix the plasma in the final stage; for this reason, a sample of unmixed second-centrifugation PRP was also taken for TEM examination.

Nahita Single-Centrifugation System for PRP Processing

Blood was extracted into 3 5-mL citrated tubes (Venojet; Terumo MR, Tokyo, Japan) containing 0.5 mL of trisodium citrate, citrate, and acid citrate dextrose (ACDA) as anticoagulants. The test tubes were gently slopped until mixing of the blood with the anticoagulant was complete. One of the 3 test tubes was used for whole blood analysis and initial platelet counting (sample A). The remaining 2 test tubes were centrifuged with a 280-*g* force (1,500 rpm) for 7 minutes.

After centrifugation, the plasma in the test tube was divided into 3 equal layers, which were placed in 3 different sterile test tubes using a 500-μL pipette (Nahita system) in an attempt to minimize turbulence during aspiration. The most superficial layer was placed in the first test tube and labeled sample F (PPP). The middle layer, platelet medium plasma (PMP), was placed in the second tube and labeled sample G. The lowest layer, which had the highest platelet content (PRP), was placed in the third tube and designated sample H.

Sample Labeling

Each sample was labeled with a letter as follows:

A: Whole blood
B: ACE centrifugation system, first-centrifugation PPP

C: ACE centrifugation system, first-centrifugation PRP
D: ACE centrifugation system, second-centrifugation PPP
E: ACE centrifugation system, second-centrifugation PRP
F: Nahita centrifugation system, PPP
G: Nahita centrifugation system, PMP
H: Nahita centrifugation system, PRP.

The following parameters were analyzed: platelet count of each sample, time needed to complete each method, and ultrastructure of the obtained PRP clots.

PLATELET COUNTING AND STATISTICAL ANALYSIS

Platelet counting analysis was performed on all of the samples using a flow cytometry device (ADVIA 120 Hematology System; Bayer, Leverkusen, Germany) in the Hematology Department of "Doce de Octubre" University Hospital. Samples E and H were the most relevant because they are the final products obtained by the ACE and Nahita systems, respectively. Sample C is also interesting, because it should be similar to sample H. For this reason, 2 other parameters were calculated for these 3 samples: platelet concentration and platelet increase over whole blood baseline. These values were calculated using the following equations:

$$\text{Platelet increase over blood baseline} = \frac{\text{Platelet count of (PRP)} - \text{Platelet count of whole blood}}{\text{Platelet count of whole blood}}$$

and

$$\text{Platelet concentration} = \frac{\text{Platelet count of PRP}}{\text{Platelet count of whole blood}}$$

The platelet increase over whole blood baseline value is better for describing the increase in platelet counts. Nevertheless, the platelet concentration value is the more commonly used value in the literature; therefore, it was calculated for comparing our results with the data published by other authors. The data thus obtained were analyzed using a parametrical Student *t* test with SPSS software (SPSS Inc, Chicago, IL).

Ultrastructure Analysis (TEM)

Preparing, cutting, and visualizing the samples were done using the following devices and reagents: ultramicrotome (Ultra Cut-E; Reitchert-Jung, Wetzlar, Germany); resin trimmer (Ultratrim; Reitchert-Jung); Knife Maker (Reitchert-Jung); transmission electron microscope (JEOL 1010; JEOL, Tokyo, Japan), NaCl (Sigma-Aldrich, Schnelldorf, Germany); KCl, KH_2PO_4 (Merck, Whitehouse Station, NJ); Na_2HPO_4 (Probus, Barcelona, Spain); $CaCl_2$ solution (Roche, Basel, Switzerland); Glutaraldehyde (Tosimis; Rockville, MD); Spurr's Taab embedding resin (Taab Laboratories, Berkshire, UK), and Eppendorf tubes (Daslab; Nirco SA, Barcelona, Spain).

Plasma samples were activated with a 0.0025 M $CaCl_2$ solution and were kept at body temperature for 40 minutes, accomplishing complete clot jellification. Clot fragments were fixed with glutaraldehyde solution 25%, protected from the light with an aluminum foil, and stored in a refrigerator for 6 hours at 4°C. Samples were then washed with a phosphate-buffered saline (PBS)(pH 7.4; 4°C) every 30 minutes for a total of 5 times and cut with a surgical blade n°15 into small fragments that were placed in Eppendorf tubes. The PBS was carefully removed and replaced by a 1% osmium tetraoxide solution that was withdrawn 1.5 hours later, after which the samples were washed with tri-distilled water every 15 minutes a total of 4 times. Because osmium tetraoxide is very toxic, all of the contaminated materials were disposed of in safe containers. At this point, samples were dehydrated by placing them in increasing concentrations of acetone solutions: 30%, 50%, 70%, 80%, 90%, and 95%. All samples were kept 30 minutes in each concentration before being moved to the next one. Then they were placed in pure acetone for 60 minutes to achieve complete dehydration. During this process, each acetone solution was renewed every 15 minutes.

After dehydration, samples were imbibed with different Spurr's resin-to-acetone solution ratios. First, a resin-to-acetone solution of 1:3 was applied for 1 hour, followed by a 1:1 resin-to-acetone solution for another hour and then a 3:1 resin-to-acetone solution for 2 hours. Finally, the samples were introduced in a 100% resin for 10 hours, after which the 100% resin was renewed, and the resin-imbibed samples were placed in an incubator at 60°C for 3 days to complete the resin polymerization.

The samples containing resin-polymerized blocks were removed from the Eppendorf tubes and trimmed into adequate parallel shapes with the Ultratrim device. A microtome blade was prepared using the Knife Maker device, and the samples were cut into preliminary 75-μ-thick slices. These slices were dyed with methylene blue for assaying with light microscopy. When preliminary slices were satisfactory, 50-nm-thick cuts for TEM were made with the Ultramicrotome. These cuts for TEM were fixed with uranilium citrate 2%, and contrast was prepared with lead citrate. Finally, the samples were placed on the Stubbs for TEM analysis.

Table 1. PLATELET COUNTING OF THE SAMPLES

Sample	A	B	C	D	E	F	G	H
Platelets/μL (mean)	187.230	173.960	426.000	23.930	630.200	196.300	222.860	425.600
Standard deviation	57.650	72.010	134.930	11.580	269.960	73.120	81.690	152.110

Platelet counting of the samples: A, whole blood; B, ACE centrifugation system, first centrifugation PPP; C, ACE centrifugation system, first centrifugation PRP; D, ACE centrifugation system, second centrifugation PPP; E, ACE centrifugation system, second centrifugation PRP; F, Nahita centrifugation system, PPP; G, Nahita centrifugation system, PMP; H, Nahita centrifugation system, PRP.

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Results

PLATELET COUNTING

The average hematocrit and initial whole blood platelet count (sample A) of the patients included in the study were 36.68 ± 6.71% and 187,233 ± 57,658 platelets/mm^3, respectively. Platelet counts for samples C (426,000 ± 134,932 platelets/mm^3), E (630,200 ± 269,969 platelets/mm^3) and H (425,600 ± 152.111 platelets/mm^3), were higher than that for sample A (Tables 1 and 2, Fig 1).

TEM micrographs showed regular aggregations of nearly empty oval-shaped platelets with intact cell membranes, slightly touching each other at the center of the aggregate. Inside the platelets, mitochondria, an open canalicular system, and very few dense and alpha granules were seen. All organelles were oriented toward the center of the aggregate. In a similar manner, platelet secretions were also concentrated in the middle of the aggregate. Some myelin bodies were noted, caused by cell injury and the glutaraldehyde-fixing process. There were some erythrocytes and filaments of polymerizing fibrin extending outward from the center of the aggregate (Fig 2).

Sample C: ACE First-Centrifugation PRP

Compared with sample B, the sample C micrograph showed an obvious increase in the concentration of platelets in each aggregate as well as in the amount of fibrin (Fig 3).

Sample E: ACE Second-Centrifugation PRP

As shown in Figure 4, in the unmixed samples, numerous packed red blood cells (PRBCs) surrounded the platelet aggregates. Despite being deformed and compacted by centrifugation, these cells maintained a continuous space between each other, due to the electrical repulsion of the adjacent positively charged membranes. Platelet aggregates had a compacted round shape that made it difficult to spot the platelets, although their membranes, mitochondria, and open canalicular system remained intact. Platelets showed a high degree of activation caused by the loss of membrane potential, marked by such features as high-grade depletion of alpha and dense granules, formation of vacuoles, and prolongation of pseudopodia between the red cells. Most fibrin agglomerates were totally trapped within platelet aggregates, although some agglutinated fibrin was observed outside the aggregate in the spaces between the PRBCs. Fibrin agglomerates did not touch the cell membranes. The sample showed higher amounts of fibrin and platelets compared with the other PRP

Table 2. COMPARISON BETWEEN ACE AND NAHITA SYSTEMS IN THE FOLLOWING PARAMETERS: PLATELET RICH PLASMA CONCENTRATION, PLATELET INCREASE OVER WHOLE BLOOD BASELINE, BLOOD VOLUME NEEDED AND TIME NEEDED

	Double Centrifugation (ACE system)		Single Centrifugation (Nahita system)
Parameters	C	E	H
Platelet concentration	(227%)	352%	232%
Platelet increase over whole blood baseline	(127%)	236%	128%
Blood volume	25.5 mL		13.5 mL
Time needed	25 min		12 min

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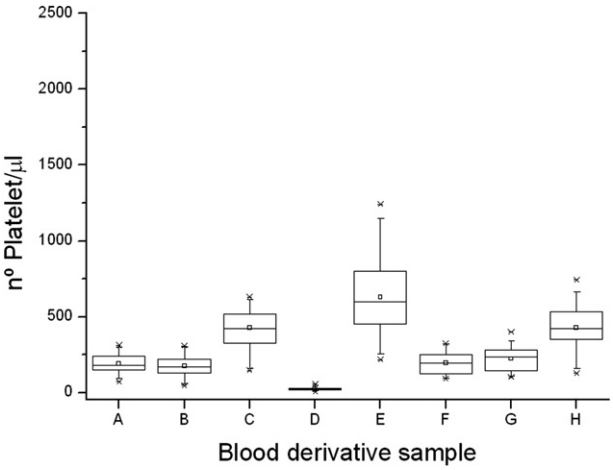

FIGURE 1. Relationship between blood derivative sample and platelet concentration.

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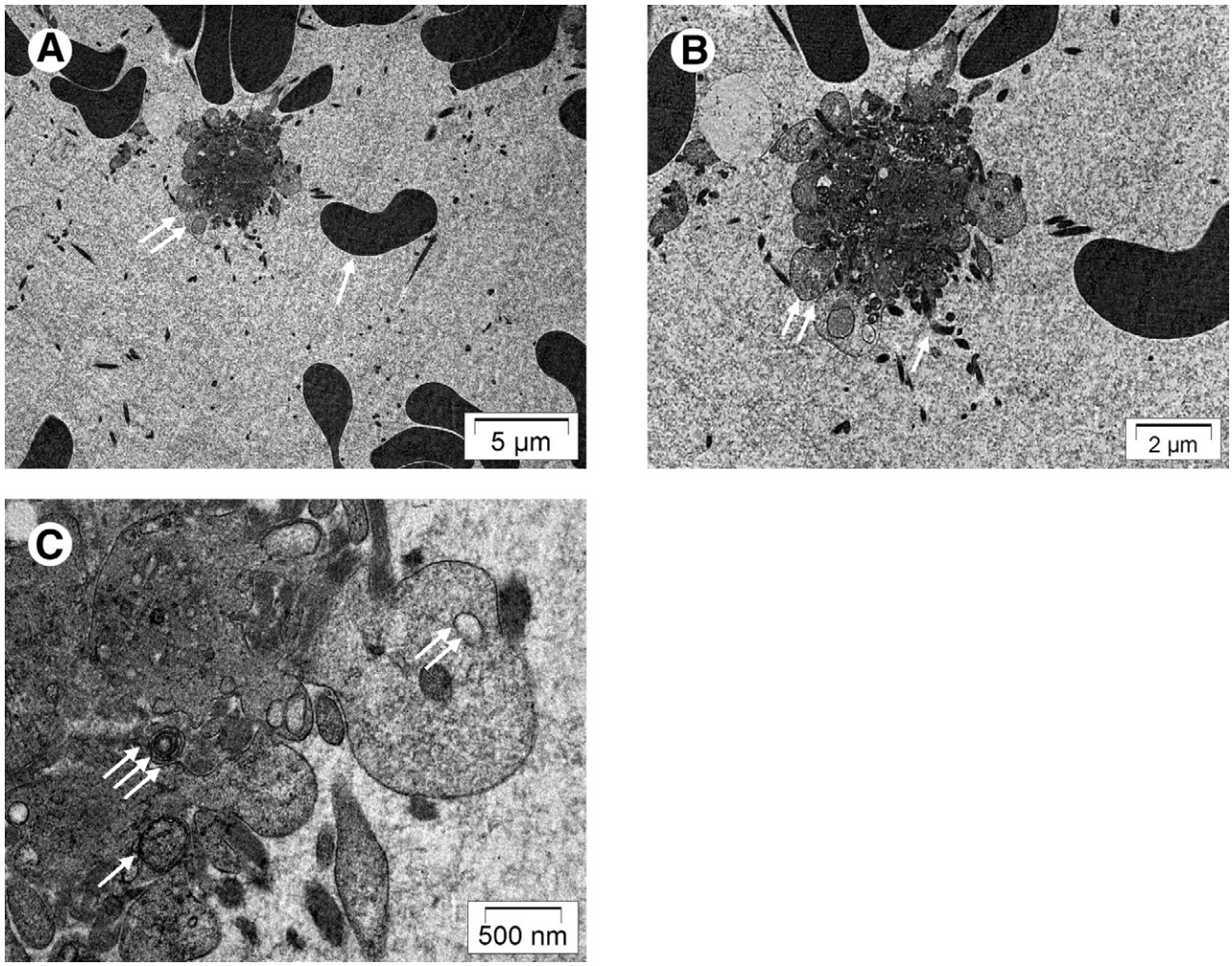

FIGURE 2. *A*, Sample B, ×5000. The image shows some erythrocytes (*1 arrow*), regular platelet aggregations (*2 arrows*). *B*, Sample B, ×10,000. Platelets are touching each other at the aggregate center portion, leaving their outer surfaces free (*1 arrow*). Platelet secretions are concentrated in the middle of the aggregate. Polymerizing fibrin filaments prolongate out of the aggregate center (*2 arrows*). *C*, Sample B, ×40,000, ACE first-centrifugation PRP. The micrograph shows nearly empty oval-shaped platelets with intact cell membranes. Mitochondria (*1 arrow*), remnants of the open canalicular system (*2 arrows*), and some myelin bodies are noted (*3 arrows*).

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samples. Some lymphocytes were seen in the clot, and these had increased cytoplasm due to activation of the immune response. This phenomenon is compatible with an increased synthesis of cytokines by white blood cells, which could prematurely activate platelet degranulation, causing a premature loss of growth factor proteins in the PPP layer. On the other hand, when the sample was mixed with the pipette, normal heavy populated platelet aggregates were free of PRBCs (Fig 5).

Sample F: Nahita Single-Centrifugation PRP

This sample exhibited few platelet aggregates, with some fibrin threads that polymerized inside the aggregate projecting outward. Platelets were rounded with an intact continuous membrane that contained some organelles, granules, irregular vacuoles, and electrodense bodies (probably glycogen). All of these cellular bodies were concentrated in the platelet aggregate pole (Fig 6).

Sample H: Nahita Single-Centrifugation PRP Layer

This sample also contained some fibrin threads and erythrocytes along with the platelet aggregates, which had less fibrin and fewer platelets compared with the double-centrifugation PRP samples. Inside the platelets, dense canalicular system, mitochondria, cell pseudopodia, and electrodense bodies were seen. But platelets were nearly absent in most of the granules, with only very few alpha granules remaining undelivered (Fig 7).

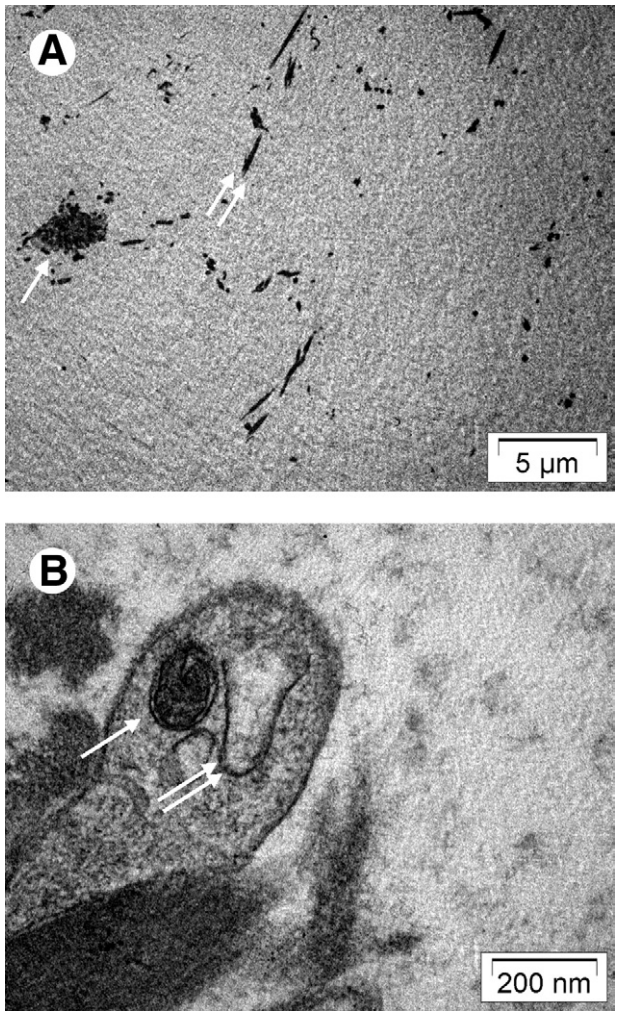

FIGURE 3. *A*, Sample C, ×4000. The micrograph shows a platelet aggregate (*1 arrow*) and fibrin filaments (*2 arrows*). *B*, Sample C, ×120,000. The micrograph shows cell membrane integrity, with an evident mitochondria (*1 arrow*) and open canalicular system (*2 arrows*) within platelets.

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Discussion

PLATELET COUNTS

Due to the increasing use of implants and the consequent need for sufficient osseous tissue, more efficient bone graft techniques are being investigated. One of the more recent advances in this area is the use of autologous platelet gel to promote healing and regeneration of soft and hard tissues. This gel, a combination of PRP and calcium ions, was first used as a sealing, homeostasis, and wound healing promotion material.[4]

Marx et al[19] demonstrated that the use of PRP techniques in surgery produce an increase in platelet concentration, and their application on bone defects increases bone formation and density within 6 months, enhancing osseous quality and quantity. These authors also proved the presence of 3 growth factors (platelet-derived growth factor [PDGF], TGF-β1, and TGF-β2) with their respective membrane markers in the PRP.

According to Marx,[22] the working definition of PRP is a concentration of 1,000,000 platelets/μL in a 5-mL volume of plasma (PRP concentration, 338%). Lower concentrations are reportedly unreliable in enhancing wound healing, although higher concentrations have not been shown to further enhance wound healing. Marx et al[19] stated that a double-centrifugation technique is necessary to truly concentrate platelets from autologous blood. The first spin separates the red blood cells from the plasma, which contains the platelets, white blood cells, and clotting factors. The second spin finally separates the platelets and white blood cells, along with a few red blood cells, from the plasma. This "soft spin" produces a PRP free from the obstruction due to a large number of red blood cells. Marx et al[19] stated that obtaining PRP with a single-spin technique would produce not a true PRP, but rather PRP–PPP mixture with a disappointingly low platelet count.

On the other hand, Anitua[21,26] supported using a single-spin technique (although he did not report the platelet concentrations obtained by this procedure). Other authors have reported obtaining platelet concentrations of 356% using the single-spin technique.[27]

In this study, the PRP concentrations of platelets obtained by both techniques after the first centrifugation were quite similar, 232.4% for the Nahita system and 227.8% for the ACE system. The PRP concentrations obtained from the ACE second-centrifugation protocol revealed an increased platelet concentration of 352%. These results are similar to the minimum 338% proposed by Marx,[22] but well below the 713% mentioned by Gonshor in his study of the ACE system.[25]

Recent studies have confirmed that double-centrifugation techniques can yield concentration values equal or higher than those cited by Marx. Weirbrich et al[23,24] obtained increases of 370% using the Curasan system (Curasan AG, Kleinostheim, Germany) and 761% with the PCCS system (3i Implant Innovations, Inc, Palm Beach, FL) in a comparative study between the 2 protocols. In other studies, Appel et al[28] managed to obtain a concentration of (501%) with the PCCS system and (1170%) with the Curasan system.

The platelet concentrations obtained with ACE technique were superior to those obtained by the Nahita system. This finding was expected because the ACE system uses a double-centrifugation technique. Nevertheless, the ACE results were more dispersed than the Nahita results; thus, occasionally the concen-

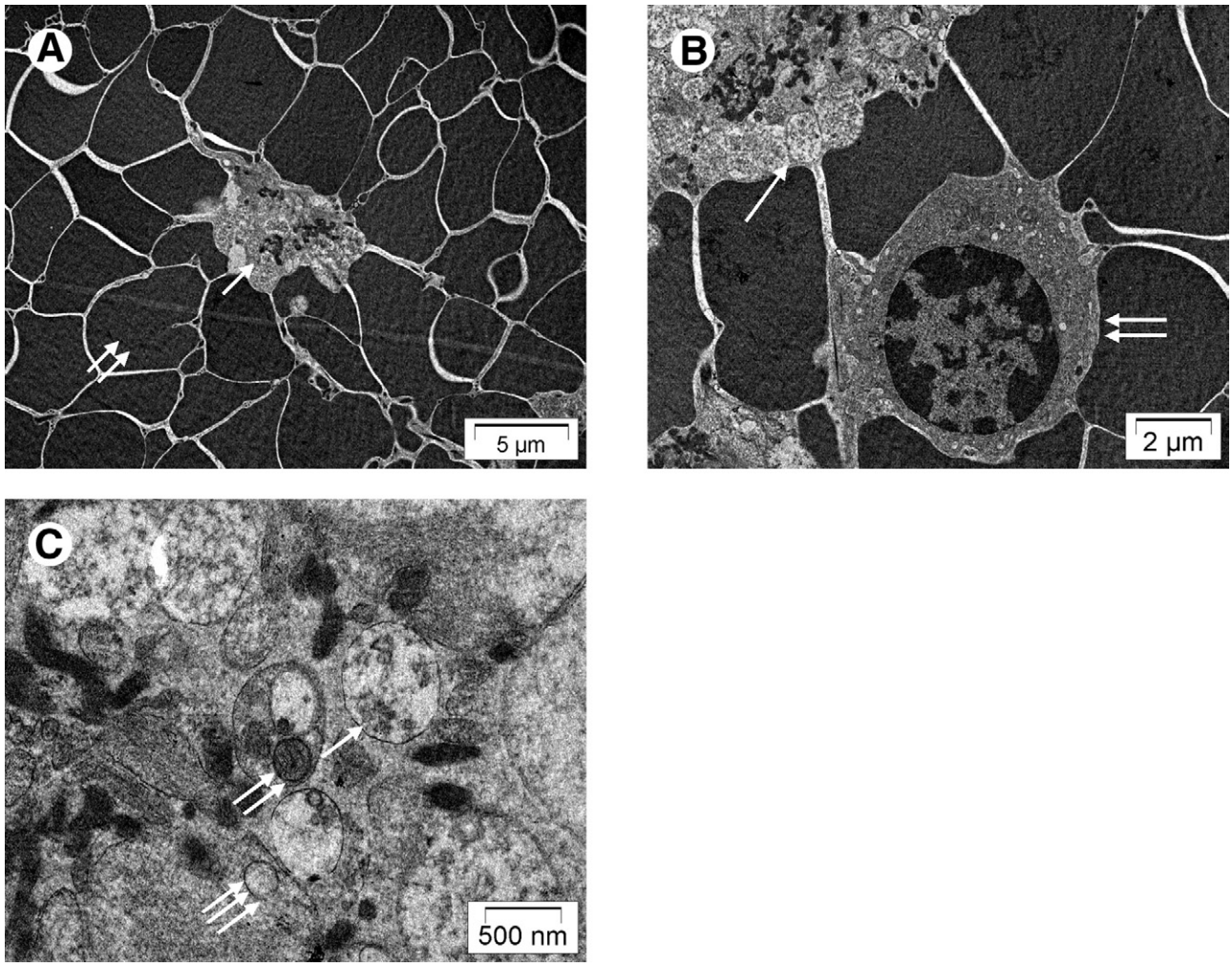

FIGURE 4. *A*, Sample E (nonmixed sample), ×5000. The micrograph shows numerous PRBCs (*2 arrows*) surrounding the platelet aggregate (*1 arrow*). Red blood cells maintain a continuous space between each other. Platelet aggregates have a compacted appearance. Most fibrin agglomerates are agglutinated within the platelet aggregates. Fibrin agglomerates are not touching the PRBC membranes. *B*, Sample E (nonmixed sample), ×10,000. This figure shows round-shaped deformed platelets (*1 arrow*) and lymphocytes with increased cytoplasm (*2 arrows*). *C*, Sample E (nonmixed sample), ×40,000. Although the platelet membranes remain intact, they are compacted and difficult to spot. Vacuoles (*1 arrow*), mitochondria (*2 arrows*), and an open canalicular system (*3 arrows*) are seen. Fibrin (*black patches*) is seen within platelet aggregates.

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trations obtained by the Nahita system were higher than those obtained with the ACE system for the same patient. This lack of precision could be due to the delicacy of the ACE technique. Furthermore, the ACE system consumed more time and required larger volumes of blood.

Transmission Electron Microscopy

Using TEM for examining blood derivative clots revealed several phenomena regarding the PRP ultrastructure that would not have been detected using the more common techniques of immunohistochemistry or light microscopy. Furthermore, TEM can be even more cost-effective than inmunohistologic analysis, such as enzyme-linked immunosorbent assay or flow cytometry techniques, because it does not require expensive immunologic markers.

Comparing the micrographs of the samples obtained with the 2 PRP techniques revealed that in both techniques, platelets had liberated nearly all of the alpha and dense granules' contents. We concluded that a period of 30 minutes of Ca^{++} incubation was easily sufficient to make the platelets empty all of their contents. Despite the centrifugation procedures, most platelet organelles could be differentiated in all of the samples, and several signs of platelet function and vitality, such as intact membranes and reaction to Ca^{++} by pseudopodia prolongation and degranulation, could be seen. Therefore, it could be stated that centrifugation did not harm platelets in both the single- and double-centrifugation techniques.

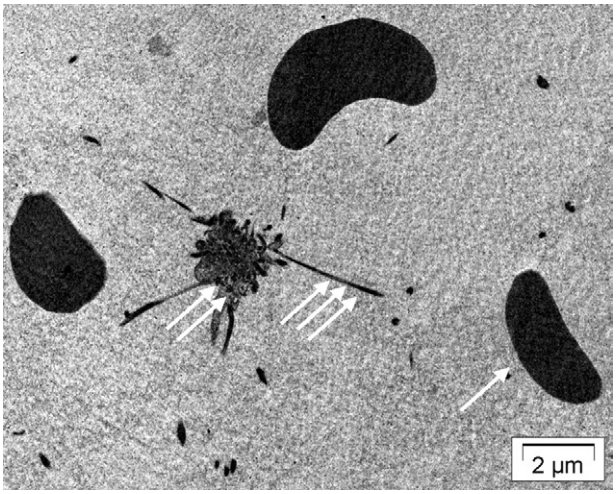

FIGURE 5. Sample E (mixed sample), ×8000. The figure shows some normal erythrocytes (*1 arrow*) and a heavy populated platelet aggregate (*2 arrows*) with fibrin threads spreading from it (*3 arrows*).

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Other authors have confirmed this fact, explaining that it occurs due to the low gravity force used in both techniques.[29]

In the double-centrifugation ACE technique, higher amounts of erythrocytes were observed. When mixing of the PRP clot was not performed, deformed PRBCs were predominant. Furthermore, there was no direct contact between the PRBCs. This phenomenon was explained in other works by the membrane-positive charge repulsion produced through the sodium potassium pump.[30] Therefore, the PRBCs maintained their membrane potential and vitality despite the centrifugation procedure. On the other hand, platelets could adhere to each other and to the erythrocytes due to membrane depolarization.[31]

Polymerized fibrin projected outward from the platelet aggregate in all samples except the nonmixed sample E, in which fibrin was trapped within the platelet aggregates. This variation can be explained by the repulsion of the positively charged fibrinogen proteins from the also positively charged erythrocyte membranes. This phenomenon could be accentuated by the RBCs' anaerobic metabolism. Due to their lack of mitochondria, RBCs produce high amounts of lactic acid under in vitro conditions, resulting in decreasing media pH. Because the isoelectric points of the different fibrinogen proteins range between 4 and 7, acidification of the media will result in positively charged fibrinogen chains.[32] A fibrin-positive charge will repel it from the RBC membrane, causing agglutination and entrapment of fibrine inside the clot. In a similar manner, growth factors with basic isoelectric points, such as PDGF (pH 10-11) and TGF-β1 (pH 9.5), would also have a positive charge in this acidic environment that could repel them from the RBC membrane, compromising their availability for tissue regeneration.[33,34] Furthermore, activated lymphocytes were detected in sample E (nonmixed). Special attention should be paid to the fact on activation, leukocytes release cytokines that activate the platelets prematurely. Platelets can also be activated by excessive pipetting, centrifugation of the samples, the use of metallic or glass instruments, the use of a tourniquet over the vein to be punctured for blood extraction, or when not eliminating the first 2 mm of the extracted blood.[35,36] This premature platelet activation leads to an early release of growth factors. So when the blood sample is centrifuged, the growth factors will move to the most superior part. This way, the obtained PRP will be poor in growth factors. Dugrillon et al[37] reported that the number of platelets

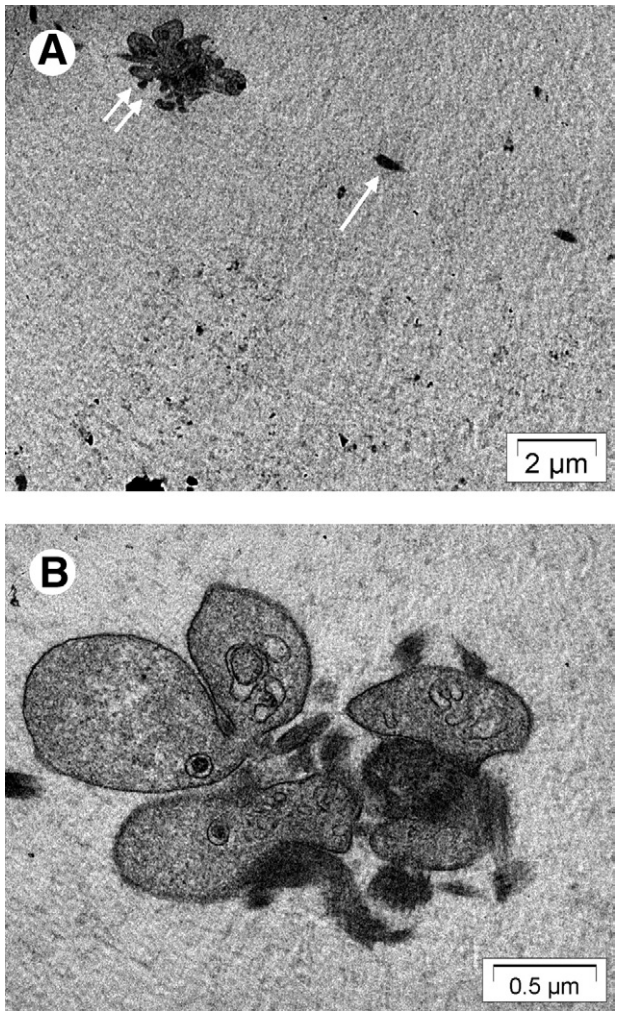

FIGURE 6. *A*, Sample F, ×10,000. The micrograph shows a platelet aggregate (*2 arrows*) and very few fibrin threads (*1 arrow*). *B*, Sample F, ×50,000. Rounded platelets with an intact continuous membrane holding organelles are seen.

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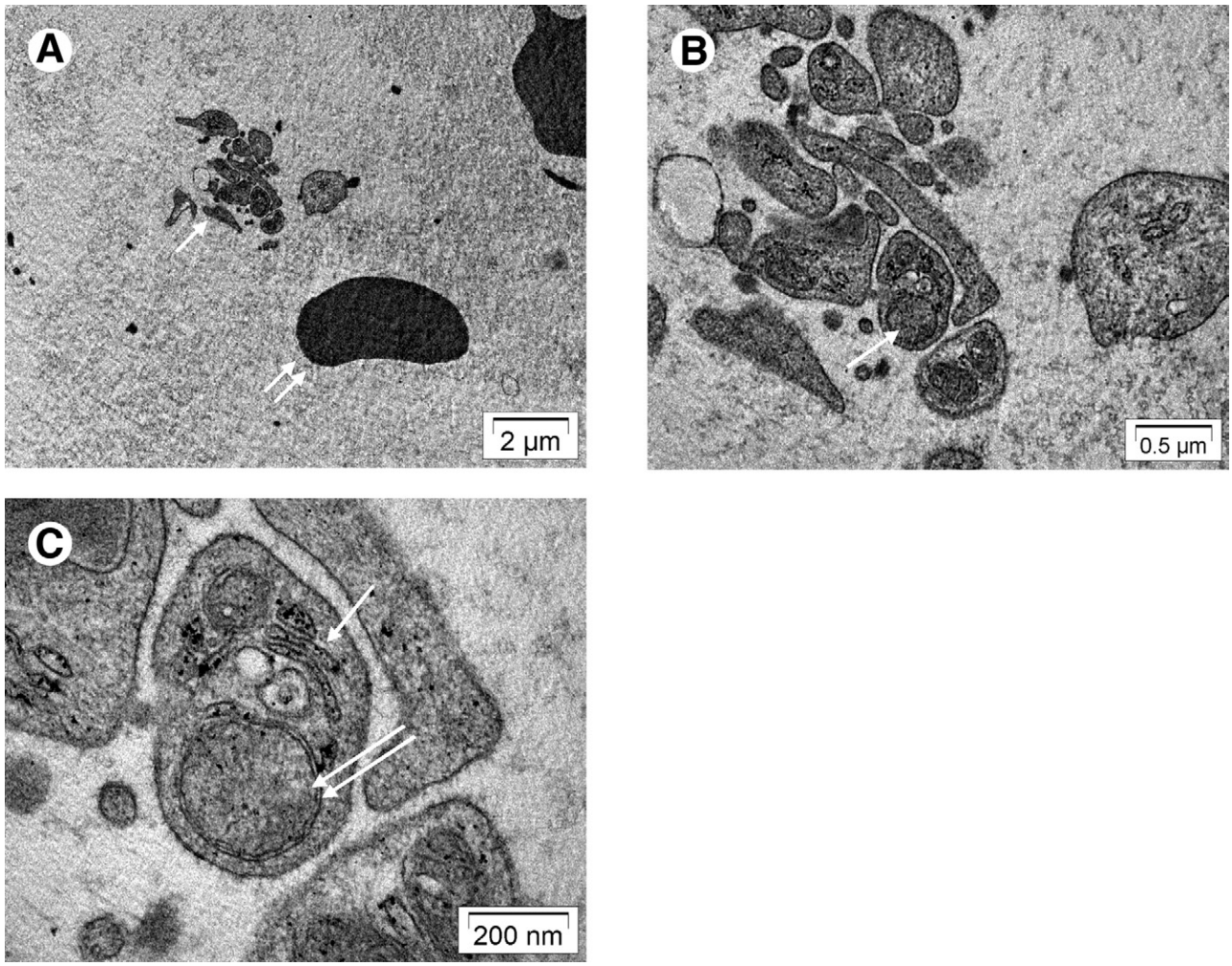

FIGURE 7. *A*, Sample H, ×10,000. A platelet aggregate (*1 arrow*) surrounded by few erythrocytes (2 arrows). *B*, Sample H, ×40,000. The figure shows a platelet aggregate. The platelet membranes remain intact, with organelles, such as alpha granules, easily seen (*1 arrow*). *C*, Sample H, ×120,000. The figure shows a platelet with its dense tubular system (*1 arrow*), and an alpha granule (2 arrows).

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is not always proportional to the growth factors' quantity, so more attention should be paid to the quality of PRP than to the number of platelets concentrated. Their study proved that the TGF-β1 and platelet concentration are proportionally related to the centrifugation forces when these forces are less than 800 *g*. Above 800 *g*, the TGF-β1 becomes inversely related to the centrifugation force. In our study, the centrifugation forces were 280 *g* for the Nahita system and 160 *g* and 400 *g* for the first and second spins, respectively, of the ACE system. These *g* forces are far below the 800 *g* reported by Dugrillon et al[37]; thus, both Nahita and ACE *g* forces are within the safe limit, although a slight increase could be performed in the *g* forces to get higher concentrations of platelets, which needs to be confirmed with platelet viability tests in future studies.

To summarize our findings, both the Nahita (single-centrifugation) and ACE (double-centrifugation) systems are easy to use, can concentrate platelets without damaging them despite the centrifugation process that the platelets suffer, and are valid for bone and tissue regeneration. Nevertheless, the ACE system achieves higher platelet concentrations and larger platelet aggregates than the Nahita system, although the Nahita system is simpler and less time-consuming than ACE system. On the other hand, PRBCs and fibrin agglutination were found in ACE double-centrifugation PRP samples in which final mixing was not performed. Future studies should use immunohistochemistry techniques to confirm that this fibrin agglutination phenomenon can also affect platelet growth factors. Nonetheless, mixing of the final clot obtained by the ACE double-centrifugation technique is an important step that should not be skipped. TEM high-resolution analysis for blood elements has proven to be a very accurate and useful tool for PRP ultrastructural investigations.

Acknowledgments

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PUBLICACIÓN 4

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PUBLICACIÓN 5

Effect of Solely Applied Platelet-Rich Plasma on Osseous Regeneration Compared to Bio-Oss®: A Morphometric and Densitometric Study on Rabbit Calvaria

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ABSTRACT

Background: The use of platelet-rich plasma (PRP) in bone augmentation procedures is well documented; however, the exact benefit of this material is not yet established.

Purpose: This study aimed to evaluate the benefits of using PRP, when only used, and compare it to Bio-Oss® (Geistlich Biomaterials, Wolhusen, Switzerland) in vertical bone augmentation capacity.

Materials and Methods: The study was performed in calvaria of eight adult female New Zealand rabbits using titanium bone conduction cylinder. Two titanium cylinders were fixed into perforated slits made on the parietal bone of each rabbit. On each rabbit, one chamber was grafted with Bio-Oss, and the contralateral was filled with PRP. Animals were sacrificed 4 weeks after intervention and biopsies were taken. Densitometric, histological, and histomorphometric analyses were performed to evaluate bone mineral density, vertical bone augmentation, and remaining graft volume, respectively. Statistical analyses were performed with Mann–Whitney test, using a significance level of $p < .05$.

Results: Densitometric and histomorphometric data analysis revealed that mean bone mineral densities and bone augmentation were significantly lower in the cylinders treated with PRP ($p < .0001$) 4 weeks after implantation.

Conclusion: This study showed no beneficial effect of using PRP on osseous regeneration. In addition, it was emphasized that Bio-Oss presents good osteoconductive properties by achieving suitable bone volume values.

KEY WORDS: Bio-Oss, bone regeneration, PRP, rabbits

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Ideal bone graft material should combine osteoconductive, osteoinductive, and osteogenic properties; however, only autologous bone grafts gather all these three properties.[1] Several osteoconductive biomaterials such as allografts, xenografts, and alloplastic grafts have been evaluated for bone regeneration purposes.[2–9] Bio-Oss® (Geistlich Biomaterials, Wolhusen, Switzerland) xenografts are hydroxyapatite granules from bovine origin that have been extensively used in bone augmentation procedures.[3–6] The lack of osteoinductive properties in this biomaterial encourages researchers to find ways for improving its in vivo behavior. Recently, the use of growth factors for stimulating bone regeneration raised great interest.[10–16] Multiple studies had pointed out the advantages of using platelet-rich plasma (PRP)

for stimulating bone regeneration.[17,18] PRP is a platelet concentrate easily obtained by centrifugation of autologous blood and enriched with growth factors. These growth factors were described to be released from alpha granules when platelet activation is induced by Ca ions from the extracellular media. Platelet growth factors play an important role in angiogenesis and tissue regeneration by controlling cell migration, differentiation, and proliferation.[19–21] PRP had been used in combination with other biomaterials such as bovine hydroxyapatite or autologous bone grafts.[22–25]

However, there might be possible benefits from using PRP, especially when it is not combined with other biomaterials. Therefore, the present study aimed to evaluate the benefits of using PRP alone, and compare it to Bio-Oss, as control, in vertical bone augmentation capacity.

MATERIALS AND METHODS

In this animal study, the protocol was approved by the ethical committee for animal experiments of Complutense University of Madrid (UCM). Experiments were conducted in accordance with the guidelines laid down by the European Communities Council Directive of November 24, 1986 (86/609/EEC), and adequate measurements were taken to minimize pain and discomfort to animals.

Eight healthy 6-month-old female New Zealand rabbits of about 3.5 kg were used in this study. The animals were accommodated in the official stable for animal assays of the UCM at 22 to 24°C with 55 to 70% humidity, light cycles of 12 hours, and air renewal 15 times per hour.

Preparation of PRP and Platelet Counting

All rabbits were anesthetized with an intramuscular dose of 0.75 mg/kg ketamine (Imalgene 1000®, Rhone Merieux, Toulouse, France) and 0.25 mg/kg xilacine (Rompun®, Bayer, Leverkusen, Germany). Immediately before surgery, 10 cc of whole blood was withdrawn via ear venous aspiration into 4.5 cc test tubes. Directly, it was mixed with a 3.8% sodium citrate solution at a ratio of 1 cc sodium citrate solution to 5 cc whole blood, achieving anticoagulation through calcium binding. The blood was then centrifuged with a Nahita® 280G centrifuge (AUXILAB S.L., Navarra, Spain) into three basic components: red blood cells (RBCs), PRP, and platelet-poor plasma (PPP). Because of differential densities, the RBC layer usually presents at the lowest level, the PRP layer in the middle, and the PPP layer at the top. A pipette (Gilson, Villiers-le-Bel, France) was used to separate each layer from the less dense to the denser. Therefore, PPP was separated first (about 2.25 cc) and PRP second (about 0.9 cc), leaving the residual RBCs (about 2.25 cc).

Platelet counting of the obtained PRP was measured with a flow cytometry device (ADVIA 120, Hematology system, Bayer, Leverkusen, Germany). Before its surgical application, PRP (0.2 mL) was activated with a 30% $CaCl_2$ solution. The preparation of PRP was performed simultaneously during the surgical procedures.

Surgical Procedure

In the present study, titanium bone conduction cylinder method was applied. The titanium cylinders (Laboratorios Aragoneses s.a., Madrid, Spain) had an inner rough surface with dimensions of 4 mm height, 0.5 mm thickness, and 9 mm diameter. Upon collecting the PRP, animals were placed in sternal recumbency, the head was shaved, and the cutaneous surface was disinfected with povidone iod solution prior to the operation. Surgical steps followed in this protocol were similar to those applied in a previous study.[26] The calvaria bone surface was exposed through a skin incision of approximately 4 cm in length over the linea media. The defects were made on each side of the median sagittal suture without crossing it. A titanium bone conduction chamber was created by fixing a 4-mm-high titanium ring on each slit, and slightly removing the cortical bone surface surrounded by the ring with a rounded burr in order to promote bone regeneration. On each rabbit, one chamber was grafted with Bio-Oss and the contralateral was filled with PRP. Closure and relocation of periosteum, subcutaneous tissues, and skin were achieved according to a previous protocol.[26] Terramicina® (Pfizer, Madrid, Spain) in water was given for 7 days as a postoperative antibiotic. The animals were sacrificed 4 weeks after the intervention with an overdose of sodium pentobarbital IV (Dolethal®, Vétoquinol, Lure, France).

In postmortem phase, tested bone sites were harvested and samples were then preserved fixed in formaldehyde 10% buffer solution at pH 7.0.

Densitometry

Densitometry for the analysis of bone mineral density (BMD) was achieved on the calvaria blocks using the

XR-26 Norland® densitometer (Norland Corp., Fort Atkinson, WI, USA). A bone area of 0.5×0.5 cm^2 was analyzed inside both the PRP and the Bio-Oss control cylinders with an exploring resolution of 1.0×1.0 mm^2, a measuring resolution of 0.5×0.5 mm^2, and an exploration speed of 40 mm/s. BMD was then calculated.[26]

Histology and Histomorphometry

Sample dehydration, embedding, sectioning, and staining were performed according to a previous protocol.[26–28] The histological evaluation of bone neoformation was carried out by means of optical microscopy. Light micrographs (at magnification ×6) of the biopsy slices were captured with a digital camera and analyzed with the histomorphometry software MIP-4 (Digital Image System, Barcelona, Spain). Six randomly selected slices were analyzed for each biopsy sample. The area inside the cylinder was included for histomorphometric evaluation, while the original cortical bone and the area outside the cylinder were excluded. The already-existing bone was lamellar, while the regenerated bone was woven and grew inside the cylinder so both types of bone could be easily differentiated in the histological observations.

Total sample volume, newly formed bone, and remaining graft (RG) volume were measured in each cylinder. Collected measurements would enable calculating the average percentage of augmented bone volume (BV) formed in chamber and percentage volume of the RG material. The total of BV and RG is the augmented mineralized tissue (AMT).[26]

Statistical Analysis

Statistical analyses were achieved using statistical software package (SPSS 7.0, Chicago, IL, USA) for analyzing densitometry and histomorphometric measurements by Mann–Whitney test ($p < .05$).

RESULTS

No operative or postoperative complications were noticed, and no animal loss occurred before scarification.

Platelet Counting

Platelet counts confirmed that the PRP preparation technique used in this study produced a source of highly concentred platelets. The average peripheral blood platelet count was 144,000/mm^3 with a range from 70,000 to 260,000. The average PPP count was 45,000/mm^3 with a range of 10,000 to 50,000. The average PRP platelet count was 1,050,000/mm^3 with a range of 625,000 to 1,465,000.

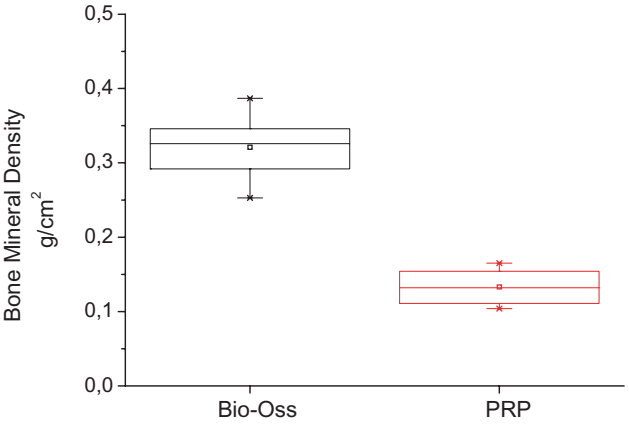

Figure 1 Box-plot chart of bone mineral density values of inside the cylinders.

Densitometry

BMD mean values of PRP and Bio-Oss chambers were 0.317 ± 0.051 and 0.128 ± 0.024 g/cm^2, respectively. High statistical significant difference ($p < .0001$) was revealed as Bio-Oss had significantly high mineral density (Figure 1).

Histology

No inflammatory reactions were observed in the histological slices in bone conduction cylinders for both tested materials. In both groups, bone growth was more pronounced on the titanium walls and occurred from the original bone surface, but not from the periosteum. This was observed more in Bio-Oss cylinders (Figure 2).

In cylinders grafted with PRP, bone formation was scarce, and few isolated newly formed trabeculae were seen on the external cortical surface. The trabeculae

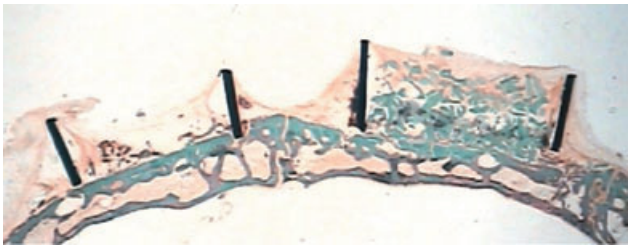

Figure 2 Higher degree of new bone formation and lesser numbers of interconnecting bone trabeculae noticed in samples of platelet-rich plasma (left cylinder) compared to those grafted with the Bio-Oss (right cylinder) (original magnification × 2).

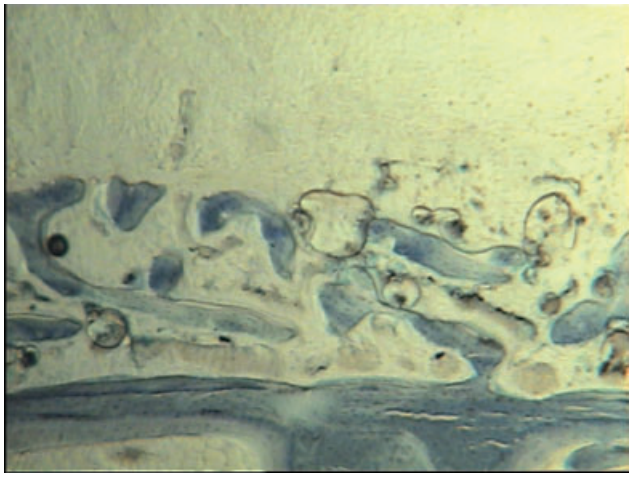

Figure 3 Few isolated newly formed trabeculae were seen on the external cortical surface (original magnification × 10).

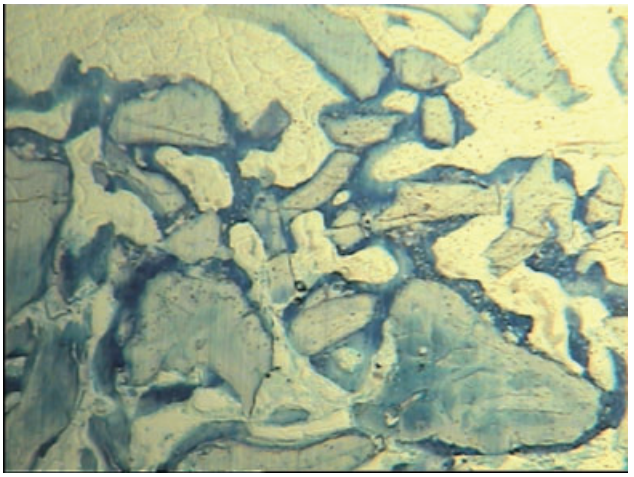

Figure 4 Micrograph of bone conduction chamber grafted with Bio-Oss showing the second half of the bone conduction chamber away from the original bone surface. Bio-Oss granules (*) are surrounded mainly by fibrous tissue (+) and scarce newly formed bone (arrowhead) (original magnification × 10).

were often connected to the original bone surface. No osteoblast activity was observed (Figure 3).

Regarding chambers grafted with Bio-Oss, a large number of the implanted granules was observed distributed over the whole specimen area. Acidophilic Bio-Oss was dyed gray-blue with TB. Bio-Oss particles present lacunae free from osteocytes because of their animal origin; nevertheless, their artificial shape differentiates them from any bone trabeculae that might be growing around. Bio-Oss granules were mostly surrounded by a thin layer of fibrous tissue, especially at the medium and upper tiers, while in the lower tier (corresponding to the area in contact with calvaria) bone trabeculae were observed surrounding the smooth surface of the Bio-Oss particles (Figure 4). Bone regeneration was observed from the external surface of the cortical bone to approximately one-third of the height of the cylinder confirming the osteoconductive properties of Bio-Oss (Figure 5). However, typical signs of biomaterial resorption such as osteoclasts forming Howship's lacunae on its surface, etching, pits, or resorptive trail formation could not be identified on Bio-Oss granules.

Histomorphometry

The data obtained from histomorphometry analyses are shown in Table 1. Obtained BV, RG, and AMT values were significantly lower for PRP chambers ($p < .0001$).

DISCUSSION

A clear differentiation was revealed by the result of the present study between the effect on osseous regeneration of solely applied PRP and Bio-Oss. The investigated densitometric and histomorphometric parameters showed statistically significant high influence of the control (Bio-Oss) when compared with PRP (see Figure 1 and Table 1).

In recent studies, PRP had been found to influence bone matrix protein expression during early stages of bone regeneration, and a significant increase in bone formation occurs 2 weeks after its implantation.[29,30] A possible role in local regulation of fracture healing and bone regeneration was suggested and that might be probable because of the synergic effect of growth

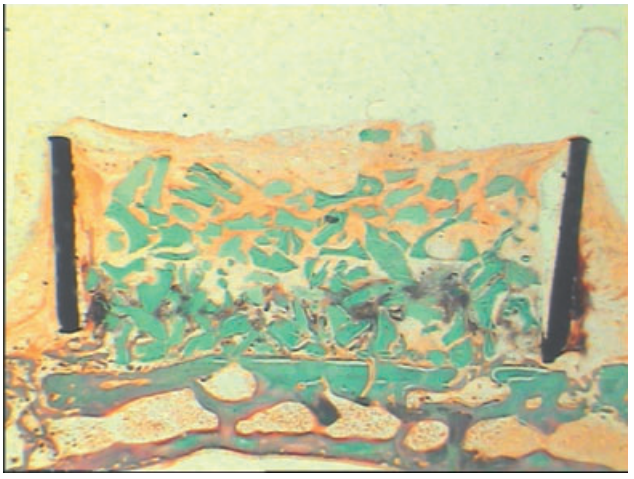

Figure 5 Bone regeneration was observed from the external surface of cortical bone to approximately one-third of the height of the cylinder (original magnification × 3).

TABLE 1 Results in Percentage of Histomorphometric Analysis for Both Tested Materials

Tested Material	BV % (±SD)	RG % (±SD)	AMT % (±SD)
PRP	6.24 (±1.87)	0.00	6.24 (±1.87)
Bio-Oss	13.18 (±4.09)	35.31 (±6.48)	46.81 (±10.55)

AMT = augmented mineralized tissue; BV = bone volume; PRP = platelet-rich plasma; RG = remaining graft.

factors present in the alpha granules of PRP.[31–33] Similarly, several authors claimed PRP capacity to improve bone regeneration quality and quantity.[17] It has been suggested that PRP improves bone regeneration in its early phases, between 3 and 6 weeks after implantation.[34] In addition, it has recently been reported that PRP alone could help in increasing bone augmentation in sinus lifting procedures.[35] However, in this experiment, the aforementioned could not be confirmed when PRP was solely applied. The present study came in agreement with other recent studies that reported no benefits from using PRP on bone regeneration in bicortical defects on rabbit calvaria.[30,36] It was reported that PRP has no effect in early bone healing, and its combination with collagen shows no benefits on bone regeneration over using collagen alone.[30] Moreover, in another study, PRP was combined with collagen sponges and used to fill critical size defects on rabbit calvaria, but no significant improvement on the bone regeneration was observed.[36] Yamada and colleagues[37] stated that PRP is only useful when it is used combined with mesenchymal cells; otherwise, it has no better effect than controls.

Nevertheless, the histomorphometric results of the present study reveal little bone formation in PRP groups. These results resemble those reported elsewhere in the literature, where only a 6.24% augmented BV was obtained after 4 weeks of locally applying peripheral blood in a similar-guided bone regeneration model.[38] The BV values obtained in samples treated with Bio-Oss were comparable to that found in the literature where Bio-Oss was grafted into silicone cylinders on rat's calvaria (18.1%),[39] or in titanium cylinders on rabbits' calvaria (11.7[26] and 19.9%[27]) (see Table 1).

Most of the animal studies that evaluate bone regeneration obtained by using PRP were done in critical-sized defect models.[40,41] In the evaluation of osteoconductive materials, the defect created should be large enough to challenge the adjacent bone with a space that can hardly fill spontaneously. However, the effects of growth factors on bone conductive materials are usually seen at an early stage during bone ingrowth. It is then difficult to find the right time to measure these effects, if new ingrown bone rapidly filled the defect.[42] The "bone conduction chamber" (or cylinder) appeared to be a useful tool for quantifying bone regeneration under the most variable conditions in both rats, goats, and rabbits.[27,41,42] This bone chamber is unlikely to be completely filled with bone. Therefore, the effects of growth factors, processing of bone and biomaterials within these chambers, can be evaluated as differing final amounts of bone are formed. Moreover, using bone conduction chambers, vertical bone augmentation obtained by tested biomaterials can be evaluated.

Several authors consider that a 4-week period of implantation is enough time to observe angiogenesis and bone formation in several animal models, including rabbits, where hydroxyapatite polymers or brushite were grafted into bone.[26,38,43,44] In the present experiment, histological changes that occurred in grafted areas during the 4 weeks of implantation were pronounced and permit to evaluate the differences in bone regeneration capacity of both assayed biomaterials.

Regarding signs of biomaterial resorption such as osteoclasts forming Howship's lacunae on its surface, etching, pits, or resorptive trail formation could not be identified on Bio-Oss granules. From the present observations, it can be inferred that after 4 weeks in rabbits' calvaria, Bio-Oss resorption did not take place.

CONCLUSIONS

Within the limits of the present study, it can be concluded that no beneficial effect of using PRP on osseous regeneration as it was solely applied. In addition, it was emphasized that Bio-Oss presents good osteoconductive properties by achieving suitable BV values.

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DISCUSIÓN

CONCLUSIONES

1. El cemento granulado de brushita es más reabsorbible y genera más hueso que la hidroxiapatita de origen bovino.

2. El diseño de materiales basados en cementos fosfocálcicos dopados, mejora sus propiedades biológicas.

3. La administración tópica de GH produce un aumento de la neoformación ósea y mejora el índice de contacto hueso-implante.

4. Para la obtención de plasma rico en plaquetas parece más eficaz la técnica de centrifugado simple.

5. La aplicación de PRP en lechos quirúrgicos óseos en modelos experimentales animales acelera ligeramente la reparación tisular, pero no mejora la formación de hueso a medio plazo.

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