Article

Effectiveness of Silicon Platelet-Rich Fibrin and Autologous Bone on Bone Regeneration in Rabbit Calvarian Defects: A Radiological and Histological Study

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Abstract: Repairing bone defects in oral surgery often requires the use of bone regeneration techniques. Silicon is an element that has been employed as regeneration material in several studies. In our study, silicon was combined with autologous bone and platelet-rich fibrin (PRF) membranes to analyse the behaviour of this element in bone regeneration. Four circumferential defects were created in the cranial vault of five New Zealand rabbits. The following elements were applied to the regeneration of the defects: (P): PRF; (S): silicon and (B): autologous bone, with the following distribution of study groups: Group 1 (PSB); Group 2 (PS); Group 3 (SB) and Group 4 (CONTROL): unregenerate group. The animals were sacrificed after 3 weeks. Computed microtomography studies (μ-CT) were carried out, as well as histomorphometric ones. The ANOVA statistical test was used with a Bonferroni post-hoc test to compare the results ($p \leq 0.05$). Radiologically, groups PSB and SB were better as far as quantity and percentage of healthy bone observed, but not significantly compared to the control group. The PS group was significantly worse. The histological test revealed that the PSB group was the one to present the largest area, percentage and perimeter of mineralised bone. On evaluating the forming bone (osteoid), no difference was observed across the groups with the exception of the bone perimeter, where the SB group was significantly better. The bone height variable showed no significant differences. In conclusion we can state that the combination of PRF, autologous bone and silicon provides good results at 3 weeks whilst the PS group shows the worst results. This highlights the importance of autologous bone forming part of the graft material in order for the bone to mineralise.

Keywords: bone regeneration; silicon; platelet-rich fibrin; PRF; autologous bone

1. Introduction

Bone defects in oral surgery are common and sometimes difficult to repair, requiring bone regeneration techniques to ensure the success of restorative treatment. Scientific research continues to make advances in the understanding of the biological and physiological processes involved in bone healing, regeneration and remodelling [1]. In vivo tissue engineering covers regeneration and reconstruction of tissues and organs in the body itself. The basic premise is that controlled manipulation of the extracellular microenvironment

may lead to controlling the ability of the cells to organise themselves, grow, differentiate, and form a functioning extracellular matrix and finally new functional tissue [2].

Regenerative techniques have become routine procedures and with proven clinical results, but their predictability and wait time are still some of the drivers for continuing to investigate the development of new and improved materials for bone regeneration. Autologous bone and platelet-rich plasma are two widely used materials in the field of implantology for regenerating soft and hard tissue. However, a material that combines all the advantages of each of them separately has not yet been developed.

The use of autologous bone is widely described in the literature since it is considered the gold standard for bone regeneration. Notwithstanding, despite the fact that it has excellent biological properties of osteoinduction, osteoconduction and osteogenesis, autograft presents considerable morbidity and an elevated and unpredictable resorption rate [3] but has shown that its behaviour improves on combination with other bone substitutes, obtaining a more abundant and consistent volume over time and quicker bone healing [4,5].

Together with autologous bone, platelet-rich blood derivatives are also frequently used in tissue regeneration. Amongst these derivatives is platelet-rich fibrin [PRF]. It is a regenerative biomaterial widely used in various medical applications. Furthermore, it has gained greater popularity compared to platelet-rich plasma [PRP] because it a single-step technique to which no chemical substances need to be added. Another advantage is greater gradual release of growth factors over time [6]. A single fibrin membrane contains a large amount of growth factors and cytokines involved in bone regeneration and soft tissue maturation [7]. It is an easily manipulated biomaterial which in oral surgery is applied in periodontal therapy, alveolar preservation, surgical bone augmentation procedures and, in combination with bone grafts, in maxillary sinus elevation surgery [8–11]. Its latest application, still not clinically tested, is as a decontaminant for rough titanium surfaces due to its antimicrobial properties [12,13].

Finally, there are several publications which state that bioinorganic ions such as zinc, manganese, magnesium or silicon are essential in bone metabolism [14,15]. Silicon is the second most abundant element in the Earth's crust and in the human body it is found mainly in areas of bone mineralisation and growth [16]. It plays a vital role in bone and connective tissue biology, and although its mechanism of action is not understood exactly, thanks to the research by Carlisle and Schwarz it is known that it acts by producing greater mineralisation on bone matrix through the synthesis and stabilisation of the crosslinking of collagen fibres [17–20]. Silicon is an initiating factor in bone mineralisation since it is present in elevated concentrations in the osteoid matrix of immature bone and, as the bone matures, silicon concentration is diminished by the concentration of calcium [17,21]. It has been used over the years as bone regenerative material in the form of dental implant coating as well as being incorporated in ceramic biomaterials [hydroxyapatite, tricalcium phosphate or glass ceramics] providing good results regarding bone regeneration in in vitro and in vivo studies [22–24]. This good behaviour is due to its strong bond with bone because of the formation of a biologically active layer, similar to apatite, on its surface [25–27].

Silicon has been employed together with other biomaterials, such as glass ceramics [28] or apatite [29,30] or with tricalcium phosphate (TCP) [28] to enhance the biological properties of the materials with which it bonds. However, as far as we know, no studies have been found on the use of silicon in combination with autologous bone or with platelet-rich fibrin membranes for use in bone regeneration.

In our study, silicon was combined with autologous bone and PRF membranes in order to analyse the role of this element in bone regeneration when combined with other widely used biomaterials. The main aim was to assess the bone regeneration obtained in the different groups analysed (these being (P): PRF; (S): silicon and (B): autologous bone, with a study group distribution as follows: Group 1 (PSB); Group 2 (PS); Group 3 (SB) and Group 4 (CONTROL): unregenerate group) over a period of 3 weeks in an animal testing model.

2. Materials and Methods

2.1. Test Animal Specimens

The experimental study was carried out on the parietal bone of 5 laboratory New Zealand rabbits aged 6 months and weighing between 3.5–4 kg. The animals were fed daily (ad libitum) with a laboratory animal diet using Harlan-Teklad (2030). The animals were subjected to surgery under general anaesthesia at the Jesús Usón Minimally Invasive Surgical Centre, Cáceres, Extremadura, Spain. The experiment was conducted in accordance with the guidelines of the Spanish National Health Institute (NIH) and European Directive 86/609/EEC on the care and use of experimental animals. The study also complied with European Directive 2010/63/EU on the protection of animals used for experimental purposes and all local laws and regulations. The researchers obtained approval of the Ethical Committee of the Institution (CCMI-Ref 028/16). Identification of the animals comprising the groups to be evaluated was undertaken using a chip. During the experimental period the specimens were kept in individual cages.

2.2. Surgical Procedure

Before initiating the surgical procedure, immobilisation of the rabbits was performed, and their vital signs were recorded. The anaesthetics employed were intravenous midazolam (0.25 mg/kg) and propofol (5 mg/kg) and inhaled 2.8% sevoflurane gas. Two analgesics were used: tramadol (3 mg/kg) and ketorolac (1.5 mg/kg). After sedation, a retro-orbital blood sample was drawn from each rabbit using a butterfly needle. Said samples were placed in test tubes without anticoagulant and were centrifuged for 12 min at a speed of 2700 rpm at room temperature to obtain PRF membranes. Once the membranes had been obtained, these were cut into small portions of approximately 2 mm in diameter to be divided into three parts which were used subsequently for the three experimental groups. A total of 0.01 mg per cc silicon versenate was used. (Natural Energy Laboratory of Venezuela, Caracas, Venezuela).

Four non-self-healing bone defects were achieved (diameter: 9 mm; depth: 3 mm approximately, until reaching the dura mater) on the parietal bone, on each side of the median line of the cranium, using a trephine (Helmut-Zepf Medical GmbH, Seitingen, Germany) mounted on a surgical micromotor at 2000 rpm under irrigation with saline solution. Piezoelectric instruments were used to remove the internal table and medullary bone of each defect. Depth was controlled with a periodontal probe. Once the defects had been undertaken, the bone obtained was ground and the material obtained was divided into two equal parts. The configuration of the groups was the following: Group 1 (PSB): mixture of platelet-rich fibrin membrane (P) + silicon (S) + autologous bone (B); Group 2 (PS): platelet-rich fibrin membrane (P) + silicon (S); Group 3 (SB): silicon (S) + autologous bone (B) and lastly Group 4 (CONTROL) in which no regenerative material was placed. The distribution of the groups in parietal bone can be seen in Figure 1.

After stitching, anti-inflammatory and analgesic agents were administered (carprofen 1 mL/12.5 kg and buprenorphine 0.05 mg/kg). Finally, the animals were sacrificed using an intravenous overdose of potassium chloride after 3 weeks. The surgical procedure can be seen in Figure 2.

The samples obtained of the cranial vault of each specimen were cut on the anatomical sagittal plane, and after separation from the cerebral mass and washed in physiological saline solution, the pieces were cut and marked individually. Each of the samples was immersed in a 10% formalin solution for tomographic and histomorphometric analysis [31].
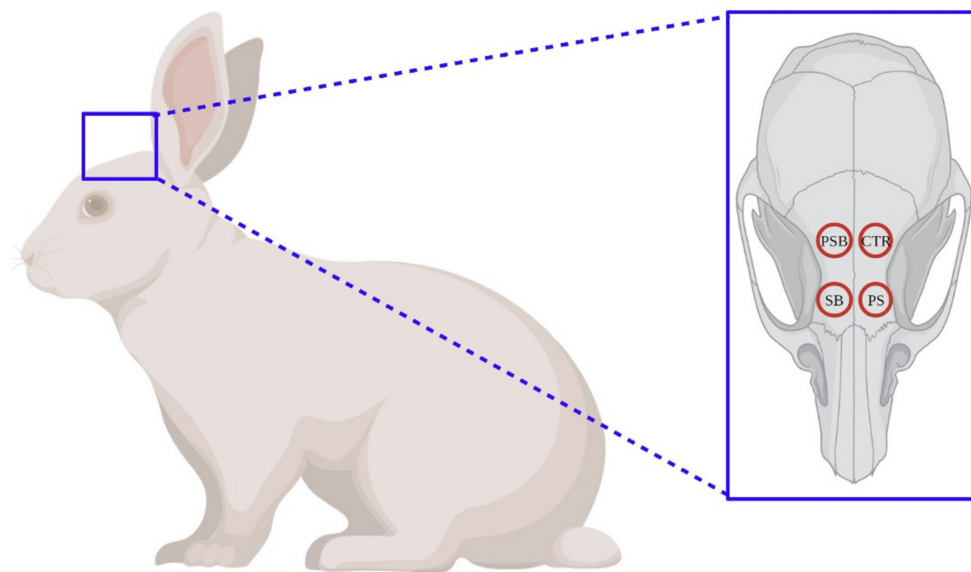
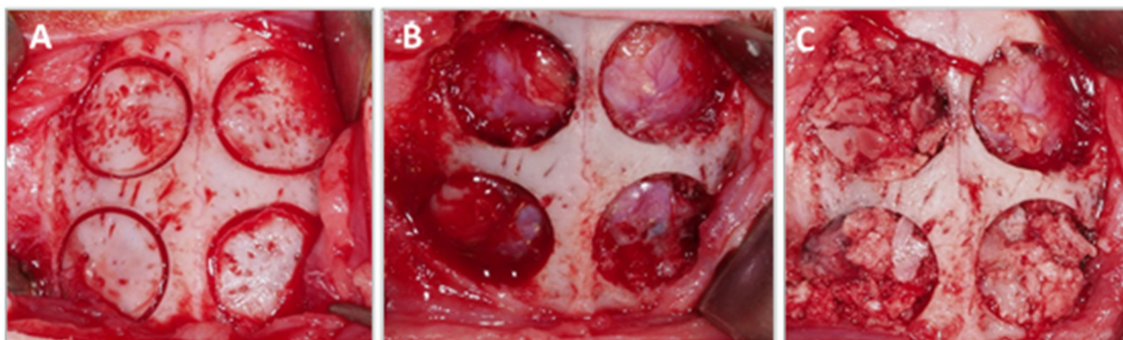**Figure 1.** Distribution of study groups in parietal bone defects.

Figure 2. Summary of the surgical procedure followed in the study undertaken. (**A**) Bone defects made with trephine. (**B**) Removal of the tablet bone exposing the dura mater. (**C**) Filling of the defects (upper left PSB; upper right Control group; bottom left SB group and bottom right PS group).

2.3. Microcomputed Tomography (Micro-CT)

The samples were analysed using Computed Tomography (CT) with a CT Bruker Albira scanner (Bruker Co., Billerica, MA, USA). Acquisitions were made using the following parameters: 1000 image, in 360° radiographic projection, at 45 kV and 30 min acquisition time. Tomographic reconstruction was carried out using Albira Suite software (Bruker Co., Billerica, MA, USA) and standard reconstruction parameters [32] to generate 2D and 3D volumes with 8.3 voxels resolution/mm. The average bone density measured in Hounsfield Units (HU) was evaluated using PMOD software (Bruker Co., Billerica, MA, USA), positioning spherical volumes of interest [VOI] of 2 mm in a rosette formation within each lesion. High resolution reconstructions of a 10 mm^3 volume were performed in each lesion using Albira Suite software, giving rise to volumes with a resolution of 20 voxels/mm. For each of the volumes the following variables were evaluated: (1) The sum total of the Hounsfield values of all the voxels (TOTAL SUM), (2) the average of all the Hounsfield values of the voxels (Averaged) in Hounsfield units and (3) the percentage of healthy bone (%).

These same variables were evaluated for air—as a measurement to establish the background noise of the images—and for surrounding bone, in which the percentage of expected healthy bone is 100%.

2.4. Histological Processing of the Sample

Samples were obtained from the cranium of each sample, cutting along the anatomical sagittal plane. The desiccated specimens were immediately submerged in a solution of 4% formaldehyde and 1% calcium embedded in acrylic resin and they were processed for cutting following the Donath and Breuner method for obtaining histological cuts of 5 µm in thickness [33]. The samples were stained with Von Kossa (VK) 5% silver nitrate (Sigma-Aldrich Chemical Co., Poole, UK) to view the mineralised bone after 3 weeks and they were observed using an Olympus BXB61 optical microscope (Olympus, Tokyo, Japan) with 1.5 and 20× lenses. The images were taken using a DSP DS-Fi1 digital signal processor (Nikon, Tokyo, Japan) in conjunction with NIS-Elements 4.0 BR software (Nikon, Tokyo, Japan). An image was taken of each bone defect. One week before sacrificing the specimens (at 2 weeks) a fluorescent marker was administered to the rabbits to observe calcein deposition on the recently deposited bone matrix. The fluorescent images were taken using a DSP DS-Fi1 camera [Nikon, Tokyo, Japan] in conjunction with NIS-Elements 4.0 BR software [Nikon, Tokyo, Japan].

For both kinds of stainings (VK and immunofluorescence) four variables were analysed: bone height (only on VK) (mm) (BH), bone area (μm^2) (BA), percentage of bone area (%) (BA) and bone perimeter (µm) (BP).

2.5. Statistical Analysis

For the statistical analysis of the results obtained the ANOVA t-test was applied with a subsequent Bonferroni test to compare the results obtained in both study groups, using STATVIEW F-4.5 software. The results were expressed as median $\pm$ standard deviation for all the variables analysed. The significance level was set at $p \leq 0.05$.

3. Results

Results for Radiological Variables

Using Albira Suite software, high definition 2D and 3D reconstructions were obtained. Images of the peripheral pristine bone (PPB) were taken and used as a reference for radiological analysis (Figure 3). Bone quantification variables were measured, in which after 3 weeks there were no statistically significant differences across the PSB, SB and CONTROL groups in the Total SUM, Averaged and % Healthy Bone variables. The PS group was the one which obtained the least significant values for these three variables (Tables 1 and 2).

Three weeks later histological evaluations were made of the four groups (Table 3 and Figure 4). The bone height variable showed no statistically significant differences across the groups after 3 weeks of healing.

Two histological stainings, Von Kossa and fluorescence, were carried out (Figure 5). The first was used to observe mineralised bone. The bone area [BA], area percentage [%BA] and bone perimeter [BP] were observed. The Von Kossa staining revealed that the PSB group presented statistically higher BA and BP than the other groups. The %BA was higher in the CONTROL group although not significantly compared to all the groups, the sample size being very small.

The fluorescence staining was employed to measure the aforementioned variables in newly deposited bone. No statistically significant differences were observed across the groups for the BA and %BA variables after 3 weeks. In the SB group the bone perimeter was significantly higher, the PSB group being the one which showed a significantly lower value.
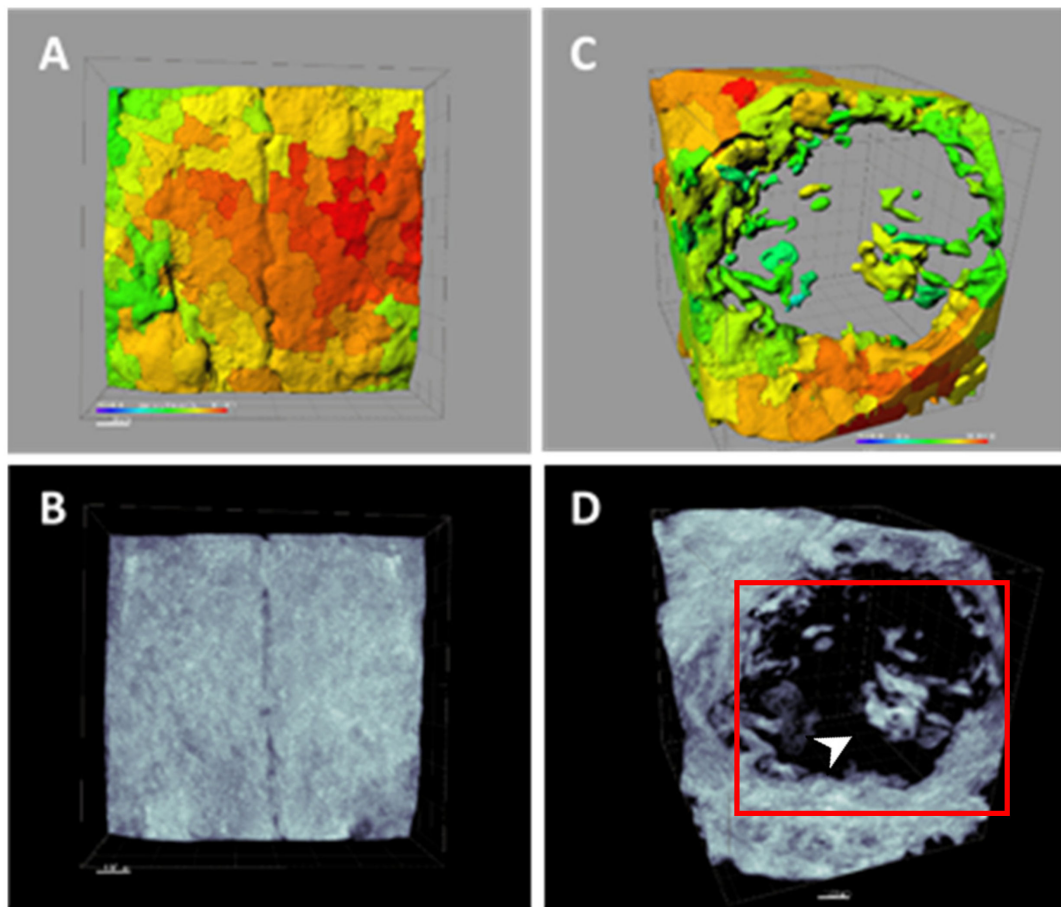
Figure 3. 3D reconstructions of the PPB (**A**,**B**) and SB group after 3 weeks (**C**,**D**). Region of interest (ROI) on image D with new bone formation inside the defect (arrow). The images present a calibration of 1 μm. The colour range oscillates between 250–1000 HU.

Table 1. Radiological results (total HU of bone density of the area studied per group, median of said measurement per group and % of regenerated bone per group) after 3 weeks. The statistically significant differences are shown in the same row by pairs of similar symbols (*****, ******; $p < 0.05$).

	PSB	PS	SB	CONTROL
Total [SUM]	624,660.31 ± 2,250,130.64 **	−1,639,444.22 ± 2,777,161.44 * **	133,306.39 ± 3,121,502.01 *	−75,755.14 ± 2,975,902.80
Averaged [HU]	105.17 ± 378.54 **	−276.21 ± 467.589 * **	22.33 ± 524.53 *	−12.58 ± 501.389
% Healthy Bone	68.44 ± 25.11 *	44.65 ± 29.38 * **	61.15 ± 31.60	61.98 ± 33.69 **

Table 2. Radiological results after 3 weeks of bone and air. Both groups have been analysed for reference use but were not compared with the rest of the study groups.

	BONE	AIR
Total [SUM]	3,760,926.76 ± 858,429.84	−5,942,171.43 ± 15,026.42
Averaged [HU]	632.91 ± 144.35	−1000 ± 0.00
% Healthy Bone	100 ± 6.34	0.00 ± 0.00

Table 3. Histological results after 3 weeks (BH, bone height; BA, Bone area; BP, Bone perimeter. The statistically significant differences are shown in the same row by pairs of similar symbols (*, **, ***, $, $$; $p < 0.05$).

|		**PSB**	**PS**	**SB**	**CONTROL**
Von Kossa	BH [mm]	0.55 ± 0.68	0.45 ± 0.39	0.32 ± 0.53	0.36 ± 0.70
	BA [μm^2]	$6,282,673.45 \pm$ $8,253,714.11$ * ** ***	$5,062,533.54 \pm$ $3,969,879.62$ **	$1,692,143.97 \pm$ $1,984,710.21$ *	$3,221,432.66 \pm$ $3,969,747.89$ ***
	%BA	16.04 ± 8.45 * ** ***	16.08 ± 5.53 **	13.05 ± 11.63 *	16.55 ± 8.245 ***
	BP [μm]	$510,190.71 \pm$ $424,927.16$ * ** ***	$184,974.83 \pm$ $164,277.64$ **	$153,907.889 \pm$ $194,164.45$ *	$208,103.83 \pm$ $191,808.85$ ***
Fluorescence	BA [μm]	$50,955.48 \pm 17,668.89$	$31,502.58 \pm 971.37$	$85,634.73 \pm$ $105,370.49$	$43,814.77 \pm 5869.72$
	%BA	8.62 ± 3.39	8.58 ± 2.33	9.04 ± 8.36	8.1 ± 1.44
	BP [μm]	0.09 ± 0.04 * $ $$	0.17 ± 0.06 ** $	0.55 ± 0.54 * ** ***	0.21 ± 0.03 *** $$
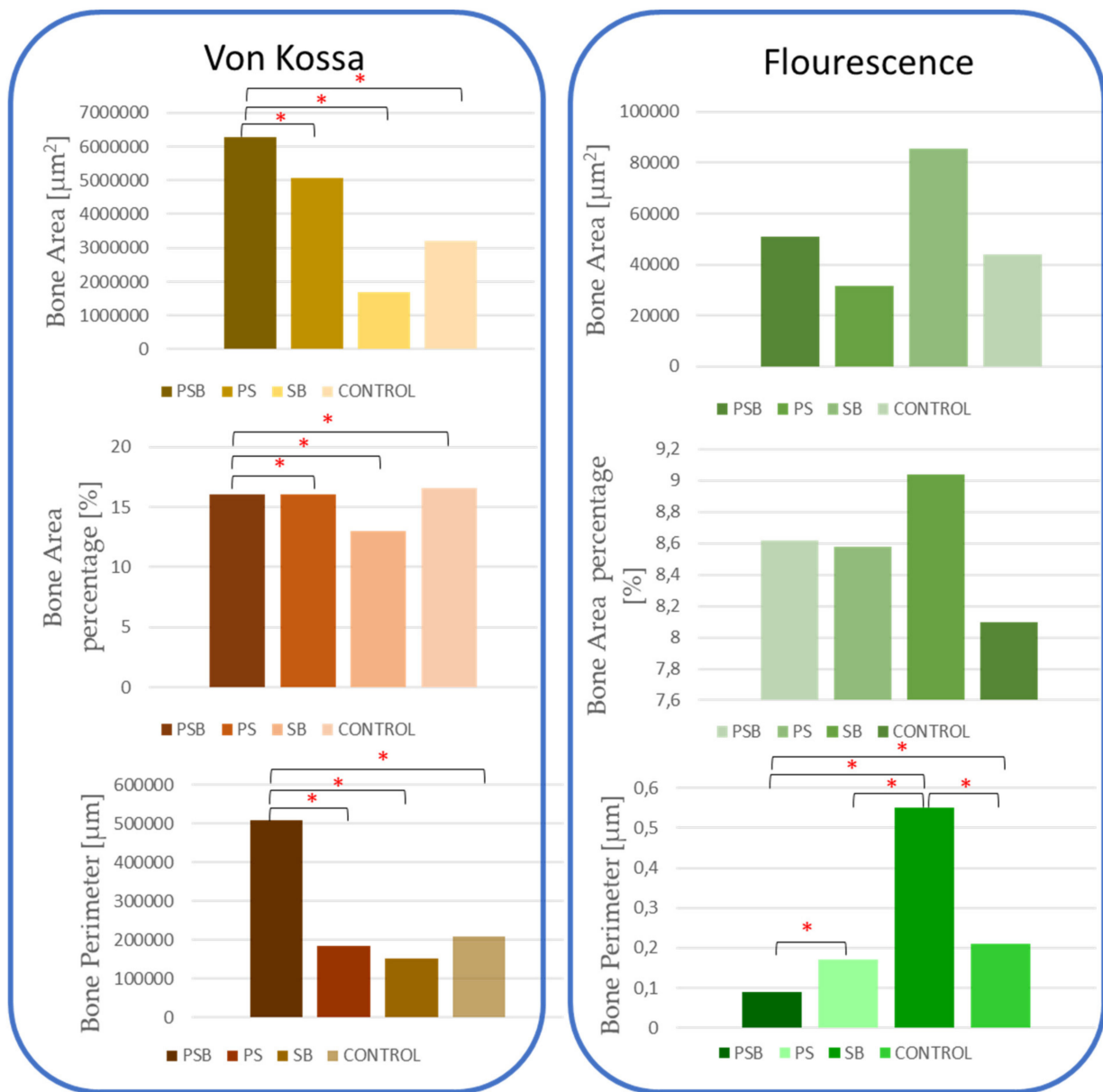
Figure 4. Graph chart of the three variables measured with Von Kossa and fluorescence stains after 3 weeks. The statistically significant differences between groups are shown with (*) symbol ($p < 0.05$).
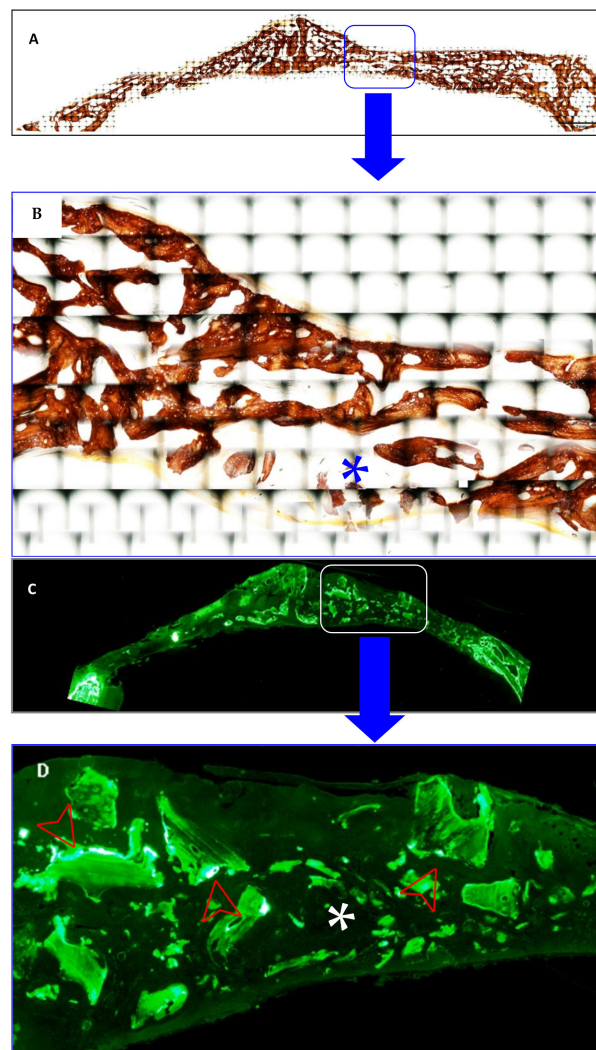
Figure 5. Images of histological sections made using Von Kossa (**A**,**B**) and fluorescence staining (**C**,**D**) after 3 weeks. Blue and white asterisks indicate new bone formation in the defect. Red pointers indicate areas of calcein deposition around the bone defect. The images present a calibration of 2 mm.

4. Discussion

In this radiological and histological study, we observed the bone formed after the placing of different bone regenerative materials to which silicon was added in order to evaluate its role as a stimulator of bone formation.

In the radiological study (Table 2) it was observed that groups PSB and SB showed higher values in terms of the amount and percentage of healthy bone observed. However, these differences were not statistically significant with regard to the CONTROL group. On the other hand, the PS group did present significantly lower values compared to the rest of the groups.

The histological study (Table 3) revealed that the PSB group was the one that presented the greatest area, percentage and perimeter of mineralised bone compared to the other groups. Conversely, on evaluating calcein deposition on the bone matrix, no difference was observed across groups with regard to bone area and area percentage. The SB group presented a significantly larger bone perimeter. The bone height variable showed no differences across the groups.

Silicon is a bioinorganic ion which has been employed as a regenerative material in several studies, usually with other inorganic biomaterials such as calcium phosphate or hydroxyapatite [34]. The advantages they offer are a low cost and a longer useful life [35]. The bioactivity of silicon will depend on the accumulation of silicon ions when it is exposed

to body fluids, such as blood as in the case of this study. This phenomenon was to give rise to the formation of a layer similar to biologically active apatite [36].

As far as we know, there is no evidence in the literature of the use of silicon versenate as a regenerative material, thus the comparison with other studies must be undertaken with caution.

The method usually employed is carried out by substituting hydroxyapatite or calcium phosphate with silicon by precipitation methods, enabling regenerative biomaterials with better properties to be obtained [37,38]. Several in vitro studies have shown that silicon is a biocompatible and bioinert element [36]. Furthermore, it promotes angiogenesis, osteoblastic differentiation and increases bone mineral density, by accelerating its formation [16,17,22,23,27,39,40]. In a recent study defects were regenerated on the cranial vaults of rats and, after 4 weeks, it was observed that significantly larger bone formation occurred when compared with a similar mixture to which silicon had not been added [30].

Likewise, mixed in vitro and in vivo studies have been undertaken in which it has been possible to compare the behaviour of silicon in both situations. Mao et al. carried out a study in which silicon was added to a mixture of calcium phosphate and glass ceramic to regenerate cranial vault defects in rabbits. Cellular viability was observed similar to the one which presented in spongy bovine bone, but with a greater rate of cellular proliferation. All this was attributed to the inclusion of silicon ions, which was to also boost other cellular functions such as cellular adhesion [41]. In the histological study undertaken, increased new bone formation was observed in the experimental group.

In accordance with the reviewed literature, a greater amount of mineralised bone would be expected to be found in the groups containing silicon. In our study, the PS and SB groups showed no difference compared to the control group, the combination of silicon, platelet-rich fibrin and autologous bone [the PSB group] being the one which enabled a significantly higher level of mineralised bone than the rest.

Autologous bone is still deemed the gold standard material for regeneration since it is the only one with osteogenic, osteoinductive and osteoconductive properties and this gives it an advantage in comparison with the rest of available materials. Notwithstanding, its limited availability, morbidity and its higher resorption rate have prompted the search for new biomaterials [35].

In our study, autologous bone combined with silicon [SB] showed no significant difference compared to the PS and control groups with regard to calcified bone. However, it was, after the PSB group, the one which presented a greater amount of bone and the one which presented greater bone perimeter of osteoid bone. There are no publications in which any of the study groups have employed autologous bone with silicon; and our preliminary results suggest that we would need to see how the autologous bone behaves over longer periods.

Fibrin-rich plasma has been used before in bone regeneration as the only filler material [42–44], or in combination with autologous bone [45], xenografts [46] or alloplastic grafts [47] providing better results regarding bone regeneration and faster healing compared to negative control groups. However, there is limited evidence on the combination of silicon and platelet-rich concentrates. Only two studies have been found in the literature which combine both materials. Their results differ and with ours as well. In the first study a combination of PRP/silicon was subsequently gelled with calcium chloride. It was applied on a cell culture of osteoblasts, and greater proliferation, greater cell viability and greater cell deposition of calcium was observed in the experimental group than in the group without silicon [48]. Conversely, in the second study a cell-generating factor (CGF) was combined with sodium orthosilicate on human osteoblasts. No statistically significant differences were observed in respect of cellular proliferation or growth compared to the groups in which only orthosilicate or only platelet concentrate was administered. Conversely, a greater production of type I collagen was observed [49]. In both studies it was concluded that the osteoconductive properties of the silicon would be boosted by the growth factors in the CGF. Both results differed in our study, in which the PS group was the

one that obtained significantly lower radiological values, although histologically it showed no difference compared to the control group. The comparison with both publications must be made with caution because they were in vitro studies with a different material. Notwithstanding, our results would be in accordance with two trials undertaken on rabbit bone in which it was observed that the PRF did not significantly improve bone regeneration when compared with the negative control group [47,50].

In our study, greater formation of mineralised bone occurred in the PSB group and this could be due to two factors: the first of these is that the silicon and the PRF would boost its osteoconductive capacity on combining with each other and at the same time it would accelerate bone mineralisation. This could explain the better performance of the PSB group in our study. The second factor is the addition of autologous bone to the biomaterial which, as mentioned earlier, is deemed the reference material in bone regeneration.

As limitations of the study, it is worth noting the low number of specimens in the sample, as well as the time period evaluated. The PSB and SB study groups offered good regeneration results, however, radiologically they did not present a significant difference compared to the control group. A way of improving the study would be to evaluate longer time periods, which would perhaps offer more revealing results on the behaviour of these regenerative materials. Furthermore, the use of a positive control group (filling the defect with autologous bone without silicon or a mixture of just autologous and heterologous bone) could have been contemplated. Another limitation of the study is the difficulty in comparing due to the type of material employed (silicon versenate). Silicon is an element that is employed in very different forms in the literature such as silicon dioxide [51], silicon acetate [52] and sodium silicate [27], and there is still no validated protocol. Granulated silicon was used in our study. Under physiological conditions, bio-available silicon is found in the form of orthosilicate acid, whilst in the studies in which hydroxyapatite substituted with silicon has been employed, the silicon used is in the form of SiO_4, although, on the other hand, it has recently been shown that silicon in its physiological format could not be concentrated at the inorganic mineral stage of bone without metabolising it beforehand to orthosilicate [53].

In the radiological study, no statistically significant difference was observed across the study groups in comparison with the control group and this could be due to the lack of membrane use or fixing agents to coat the defect so as to stabilise coagulation and prevent delay in complete ossification of the area, although the period studied (3 weeks) would be insufficient to observe it.

5. Conclusions

Silicon is a promising element as a material to be included with other bone regenerative materials due to its low cost and good properties observed in vitro and in vivo, although the definition of its properties and optimal conditions of use are still being investigated [54,55]. The combination of platelet-rich fibrin, autologous bone and silicon offers goods results after 3 weeks. The PSB and SB groups being the ones offering good results, the PSB group presents greater bone mineralisation speed compared to SB and this could be due to the inclusion of platelet-rich plasma and silicon. The results obtained in this study reveal the importance of autologous bone forming part of the graft material in order for the bone to mineralise.

Within the limits of this research, the histological analysis of regenerated tissues could provide useful information on the nature and amount of bone formed with the use of silicon, platelet-rich fibrin and autologous bone. Further studies are needed to gain knowledge of the true regenerative potential of bioinorganic ions, such as silicon.

Institutional Review Board Statement: The experiment was developed in accordance with the guidelines of the European Directive 2010/63/EU about the protection of animals used for scientific purposes and with all local laws and regulations. Animals were adequately housed; food and water were provided daily ad libitum with rabbit-maintenance Harlan-Teckland Lab Animal Diets (2030). The researchers obtained the approval of the Ethics Committee of the Institution (CCMIJU-Ref 028/16).

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

PRF   Platelet-Rich Fibrin
PRP   Platelet-Rich Plasma
TCP   TriCalcium Phosphate
VK   Von Kossa
BH   Bone Height
BA   Bone Area
%BA   Bone Area percentage
BP   Bone Perimeter

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