Society For Biomaterials

Autologous bioscaffolds based on different concentrations of platelet rich plasma and synovial fluid as a vehicle for mesenchymal stem cells

Ane Garate,[1]* Pello Sánchez,[1,2,3]* Diego Delgado,[1] Ane Miren Bilbao,[4] Emma Muiños-López,[5] Froilán Granero-Moltó,[5] Gorka Orive,[2,3] Felipe Prosper,[5,6] José Luis Pedraz,[2,3] Mikel Sánchez[1,4]

[1]Advanced Biological Therapy Unit- UTBA, Hospital Vithas San Jose, Vitoria-Gasteiz, Spain
[2]NanoBioCel group, Laboratory of Pharmaceutics, University of the Basque Country, School of Pharmacy, Vitoria-Gasteiz, Spain
[3]Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria-Gasteiz, Spain
[4]Arthroscopic Surgery Unit-UCA, Hospital Vithas San Jose, Vitoria-Gasteiz, Spain
[5]Cell Therapy and Experimental Orthopedics, University of Navarra Clinic, Pamplona, Spain
[6]Hematology and Cell Therapy department, University of Navarra Clinic, Pamplona, Spain

Received 12 July 2017; revised 8 September 2017; accepted 21 September 2017
Published online 00 Month 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.36247

Abstract: In the field of tissue engineering, diverse types of bioscaffolds are being developed currently for osteochondral defect applications. In this work, a novel scaffold based on platelet rich plasma (PRP) and hyaluronic acid with mesenchymal stem cells (MSCs) has been evaluated to observe its effect on immobilized cells. The bioscaffolds were prepared by mixing different volumes of synovial fluid (SF) with PRP from patients obtaining three formulations at PRP-SF ratios of 3:1, 1:1 and 1:3 (v/v). The live/dead staining revealed that although the cell number of each type of bioscaffold was different, these this constructs provide cells with a suitable environment for their viability and proliferation. Moreover, immobilized MSCs showed their ability to secrete fibrinolytic enzymes, which vary depending on the fibrin amount of the scaffold. Immunohistochemical analysis revealed the positive staining for collagen type II in all cases, proving the biologic action of SF derived MSCs together with the suitable characteristics of the bioscaffold for chondrogenic differentiation. Considering all these aspects, this study demonstrates that these cells-based constructs represent an attractive method for cell immobilization, achieving completely autologous and biocompatible scaffolds. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2017.

Key Words: bioscaffold, platelet rich plasma, fibrin, synovial fluid, MSCs

How to cite this article: Garate A, Sánchez P, Delgado D, Bilbao AM, Muiños-López E, Granero-Moltó F, Orive G, Prosper F, Pedraz JL, Sánchez M. 2017. Autologous bioscaffolds based on different concentrations of platelet rich plasma and synovial fluid as a vehicle for mesenchymal stem cells. J Biomed Mater Res Part A 2017:00A:000–000.

INTRODUCTION

The treatment of diseases affecting the articular cartilage has become a key challenge for orthopedic research because of the limited self-repair capacity of this tissue. Focal lesions in articular cartilage are one of the main pathologies in traumatology, in which tissue fragments are removed leaving exposed underlying bone. These lesions can be chondral or osteochondral defects depending whether they affect only the cartilage or bone tissue, and evolve into more severe injuries or even osteoarthritis if the treatments are not suitable.[1]

Current therapies encompass from "pure" surgical techniques such as chondroplasty and microfractures to therapies involving the use of scaffolds and cells to encourage tissue regeneration. The formers try to clean the injured area and tackle the underlying bone to cause bleeding in order to promote the regeneration of the cartilage. However, the created tissue is mainly fibrocartilage that presents different biomechanical properties from the original cartilage. Thus, although the results are positive in a short term, the new repaired tissue suffers deterioration over time.[2] To overcome these drawbacks, other techniques such as cell-based therapies have been employed promoting a better regeneration of damaged tissue. Autologous chondrocyte implantation (ACI) is so far the treatment that has most evolved, although some studies showed failure in a considerable percentage of patients.[3] Currently, the matrix-assisted chondrocyte transplantation (MACT) is being studied with promising results, but further long-term clinical trials are needed.[4]

The development of therapies based on mesenchymal stem cells (MSCs) has opened a new range of alternatives for the treatment of these pathologies. MSCs present characteristics promoting regeneration that could lead to the formation of new tissue like proliferation, differentiation and modulation of the environment, as it has been observed in promising

Additional Supporting Information may be found in the online version of this article.
*These authors contributed equally to this work
Corresponding to: M. Sanchez; e-mail: mikel.sanchez@ucatrauma.com

preclinical studies. Nevertheless there are still some issues that require be standardized like which cell sources are more appropriate.[5] MSCs can be obtained from different sources of the organism, but bone marrow and adipose tissue are the main niches to collect these cells for therapeutic purposes.[6] However, in recent years the idea of using progenitor cells related to target tissue has emerged as an attractive fount for cartilage regeneration. In fact, several studies suggest that synovial membrane and synovial fluid (SF) are promising sources of MSCs especially when some condition exists like meniscal tears or osteoarthritis.[7–9] In addition, MSCs from these reservoirs seem to exhibit an increased chondrogenesis so their use in cartilage repair can be a promising alternative.[10]

Another key factor in the MSCs cellular therapy for cartilage repair is the method to implement them in the defect. Thus, the use of scaffolds is essential to ensure at least the survival of implanted cells. These scaffolds also must meet the appropriate characteristics to promote the optimal therapeutic effect of immobilized cells, such as ensuring its adhesion or promoting chondrogenic differentiation.[11] Finally, the presence of cellular signals that stimulate the implanted cells to perform their biological action would complete the requirements for an optimal tissue engineering comprising scaffold, cells, and biological signals.[12]

For that purpose, in this study a bioscaffold for the treatment of cartilage defects has been developed, based on the combination of two main components. On one hand, platelet rich plasma (PRP), which provides fibrin and signals constituted by growth factors, and on the other hand SF, which supplies hyaluronic acid (HA) and MSCs (SF-MSCs). The HA of the SF together with the fibrin formed after PRP activation allows the formation of a complete autologous scaffold, while maintain a release of growth factors supplied by PRP over time, being an appropriate biological stimulus for SF-MSCs. Both, fibrin and HA have the ability to release MSCs and promote their chondrogenesis, thus the combination of these components could be beneficial for this therapeutic purpose.[13,14]

In summary, this work is intended to study the effect of the immobilization of SF-MSCs, in a complete autologous and biocompatible scaffold based on HA and fibrin for the treatment of cartilage defects.

MATERIALS AND METHODS

The institutional review board approved this study, and informed consents were obtained from every patient to whom biological samples were extracted.

PRP preparation

PRP was prepared from the peripheral blood of healthy donors. Eighty milliliters of venous blood was extracted from each patient in order to prepare the PRP and withdrawn into 8 mL tubes containing 3.8% (w/v) sodium citrate. Blood was centrifuged at 1200 *g* for 8 min at room temperature. After centrifugation, the plasma fraction located above the sedimented red blood cells, but not including the buffy coat, was collected in a tube. This plasma fraction preparation contained a higher concentration of platelets (1–2 times the concentration of platelets compared with peripheral blood, depending on the platelet count and size as well as the hematocrit) and an absence of erythrocytes and leukocytes. According to PAW classification system proposed by DeLong et al. this PRP classified as P2-x-Bβ.[15]

Collection of SF

SF was collected from the joint space of healthy donors. A 21 G needle was placed into the joint space and SF arthrocentesis was carried out. At least 3 mL of SF from the same donor was collected in a tube for the elaboration of bioscaffolds.

Elaboration of bioscaffolds based on PRP and SF

To prepare bioscaffolds based on PRP and SF, both components were mixed using different volumes and obtaining three different formulations for each patient: 3:1 (1.5 mL PRP and 0.5 mL SF), 1:1 (1 mL PRP and 1 mL SF), and 1:3 (0.5 mL PRP and 1.5 mL SF). The cell-polymer constructs contained fibrin, platelets, and growth factors from PRP component, and on the other hand, HA, MSCs, and leucocytes (Monocytes and Lymphocytes) from SF (Figure 1). After mixing of both components, calcium chloride (10% w/v/1.05 mM) was added in order to activate the platelets of PRP and trigger subsequent formation of fibrin scaffold with HA present in SF, maintaining the mixtures at 37°C for 20 min. After that time, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was added to each bioscaffold and maintained at 37°C in 5% CO_2/95% air atmosphere during the study. Reagents were purchased from Life technologies, USA.

Viability and metabolic activity of immobilized cells

To assess the viability, cells immobilized into different scaffolds were dyed with the LIVE/DEAD kit (Life technologies, USA) following manufacturer's indications. After 30 min, fluorescence micrographs were taken by inverted confocal microscopy (Olympus Fluoview 500 Confocal Microscopy, Olympus, Japan). A fibrin scaffold without cells was used as a control to correct the autofluorescence.

The cellular activity of the entrapped cells in the three types of formulations was evaluated *in vitro* at days 1, 7, 14, 21, and 28 by a sensitive colorimetric assay (CCK8) using WST-8 (2-(2-methocy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2-4-disulphenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-Methosy PMS. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium. Briefly, 500 μL of CCK-8 (Sigma-aldrich, St Louise, MO) solution was added to a 5 mL media with the bioscaffold (10% v/v) and incubated at 37°C for 4 h in humidified conditions. The resulting orange formazan produced is directly proportional to the metabolic activity, and this product that is soluble in tissue culture medium was read 5 min later on a microplate reader (Infinite M200, Tecan, Switzerland) at 450 nm with 650 nm as the reference wavelength ($n = 5$), being the metabolic activity determined by the measurement of this absorbance. From the

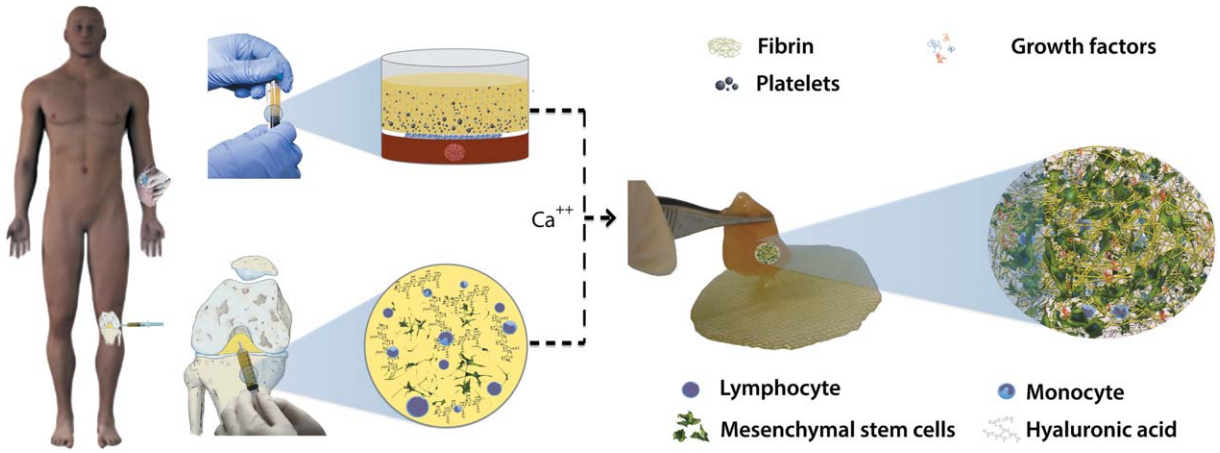

FIGURE 1. Schematic illustration of autologous bioscaffold elaboration, mixing the synovial fluid (SF) and Platelet-Rich Plasma (PRP) from the patient. After the combination of the PRP obtained from the patient's blood and the SF extracted from the knee, the activation and formation of the fibrin matrix is carried out by adding calcium chloride. The resulting bioscaffold entraps the molecular and cellular components of both compounds.

curves obtained with the absorbance data over time, pharmacokinetic variables were determined using WinNonlin in order to find differences between formulations overtime. Peak metabolic activity (Cmax), and time to peak metabolic activity (Tmax) were determined directly from the absorbance versus time curve derived from the observed data.

Measurement of U-plasminogen activator secretion of immobilized cells

To quantify U-plasminogen activator (uPA) secretion from immobilized cells, all types of scaffolds were maintained in the culture medium during 14 days, changing the media after 7 days of culture. The media which maintained the bioscaffolds between 7 and 14 days was collected and the uPA Quantikine ELISA was realized according to manufacturer's instructions (R&D Systems, Minneapolis, MN). Standards and samples were run in duplicate. uPA of three different patients was measured in all formulations ($n = 3$). For this assay, a fibrin scaffold without SF-MSCs was used as a control group.

Histological and immunohistochemistry differentiation analysis

All types of bioscaffolds were maintained in culture medium for 21 days. Next, cell-polymer constructs were fixed in 4% phosphate-buffered formalin, embedded in paraffin, and cut into 4 μm sections. In the three types of scaffolds, sections were stained with Hematoxylin and Eosin H&E (Merck, Darmstadt, Germany) and with GIEMSA staining to reveal the histological structure.

Chondrogenic differentiation was evaluated by immunohistochemistry (IHC) for type II collagen. For that purpose, sections were hydrated in grade ethanol and subjected to antigen unmasking by sequential 15 min treatments of hyaluronidase (4 mg/mL in PBS) and pepsin (4 mg/ml in 0.01 N HCl solution) at 37°C. Endogenous peroxidase activity was blocked by H_2O_2 treatment (3% H_2O_2 in PBS). Samples were incubated overnight at 4°C with a mouse monoclonal antihuman type II collagen (0.5 μg/mL; Clone II-4CII, MP Biomedicals). Staining was visualized with DAB using EnVision chromogenic kit (DAKO) according to the manufacturer's instructions ($n = 3$).

Statistical analysis

Results are expressed as mean ± SD. Statistical analysis was performed with PASW Statistics 18.0 (SPSS®, Chicago, IL). Normal distribution of samples was assessed by the Shapiro-Wilk test and homogeneity of variance by the Levene test. The different treatments were compared with ANOVA test. In case the data did not fit the normal distribution, or the variances were not homogeneous, the nonparametric Kruskal-Wallis one-way analysis of variance was applied. Differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

Elaboration of three types of formulations and macroscopic characteristics

The composition of formulations was similar, since the three types of scaffolds were prepared based on two components. On one hand, PRP, with fibrin, platelets, and growth factors and on the other hand, SF, with HA, MSCs, and leucocytes (monocytes and lymphocytes) (Figure 1). However, the mixture of the components with different proportions results in three distinct scaffolds, with different volume, consistency and appearance. Formulation 3:1 (1.5 mL PRP and 0.5 mL SF) was the group with the highest consistency, since it had more fibrin than others scaffolds. On the other hand, the scaffold 1:3 (0.5 mL PRP and 1.5 mL SF) was the smallest one (Figure 2).

In order to assess the evolution of bioscaffolds during 28 days, pictures were collected at different time points. The macroscopic analysis revealed differences in the volume and consistency among the three types of bioscaffolds, showing that the scaffold with formulation 3:1 maintained the greatest consistency until the end of the study (Figure 2). In contrast,

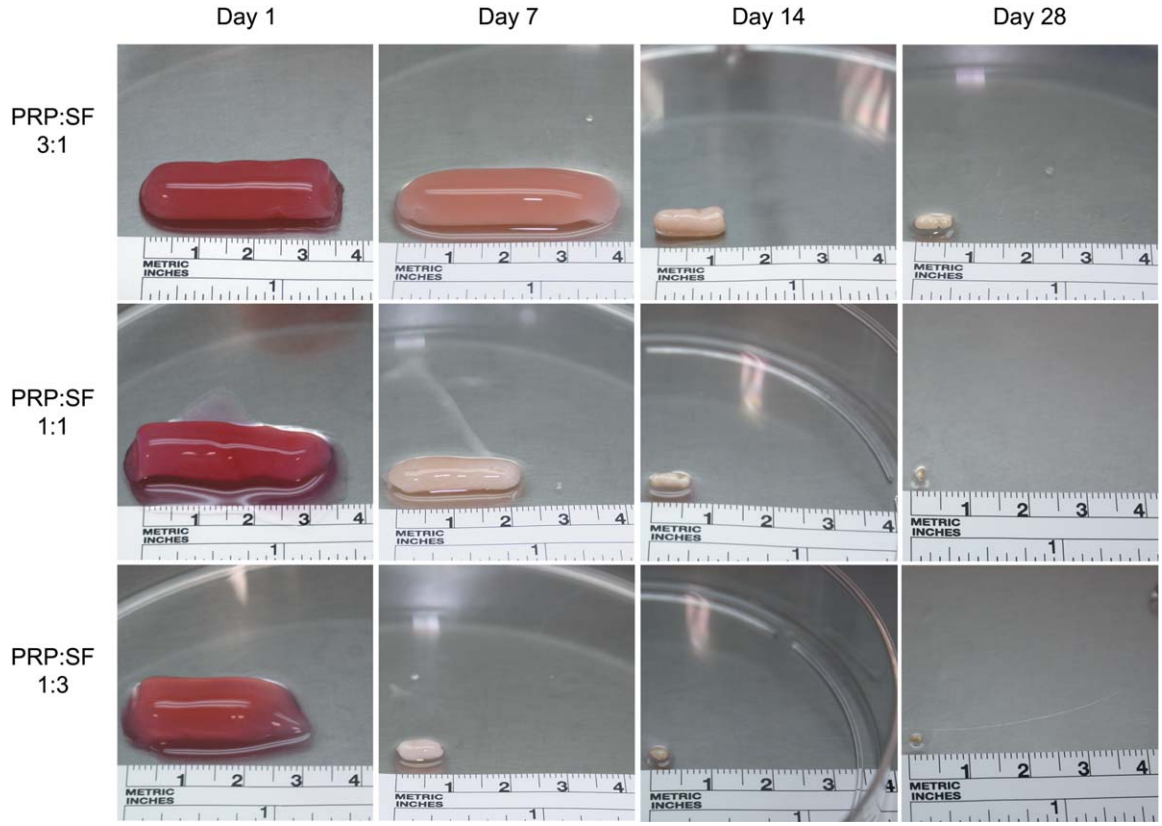

FIGURE 2. Macroscopic images of bioscaffolds. The bioscaffolds were formulated at different ratios of PRP:SF (3:1, 1:1, and 1:3) and their degradation were evaluated at different time points up to 28 days.

there was an abrupt size reduction in the case of the formulation 1:3, achieving the smallest bioscaffold at day 28. Table I collects reduction rates of different types of scaffolds. As it can be observed, depending on the volume of PRP or SF, and the maintenance time in the culture media, the reduction of the scaffold is different, achieving the most similar rate in all types of formulations between 7 and 14 days of study (Table I).

Viability of immobilized cells

The fluorescence micrographs demonstrated that immobilized cells maintained their viability and increase the cell number in all formulations. Although at day 1 a small cell number could be observed in all types of formulations, at day 7 the number of living cells increased in all cases, obtaining the highest viability or cell number with the formulation 1:3. At day 14, all bioscaffolds showed the highest number of living cells, demonstrating the proliferative capacity of immobilized cells during all the study (Figure 3).

Metabolic activity of immobilized cells

For a better understanding of the behavior of immobilized cells, the metabolic activity was measured in all types of bioscaffolds by measuring the emitted absorbance. As it can be observed in the Figure 4, the formulation containing the same volume of PRP and SF (1:1) maintained a similar metabolic activity at different time points during the study, while the other bioscaffolds achieved more variable activity values. According to pharmacokinetic values obtained from metabolic activity curves, all formulations reached similar peak metabolic activity (Cmax) [Figure 4(B)]. However, the formulation containing more SF (1:3) together with the formulation 1:1 reached its peak metabolic activity at day 7 (Tmax), whereas the other formulation with more PRP (3:1) achieved the maximum activity at day 21 ($p < 0.05$) [Figure 4(C)].

Measurement of uPA secretion of immobilized cells

To quantify the amount of secreted fibrinolytic enzymes between 7 and 14 days of the study, uPA quantikine ELISA

TABLE I. Reduction rates of elaborated bioscaffolds during the study

	Day 1 to Day 7	Day 7 to Day 14	Day 14 to Day 28
3:1 (PRP:SF)	1 (No reduction)	3.3 (4 cm/1.2 cm)	2 (1.2 cm/0.6 cm)
1:1 (PRP:SF)	1.75 (3.5 cm/2.1cm)	3.5 (2.1 cm/0.6 cm)	3 (0.6 cm/0.2 cm)
1:3 (PRP:SF)	3.75 (3 cm/0.8 cm)	2.7 (0.8 cm/0.3 cm)	1.5 (0.3 cm/0.2 cm)

PRP: platelet rich plasma; SF: synovial fluid.

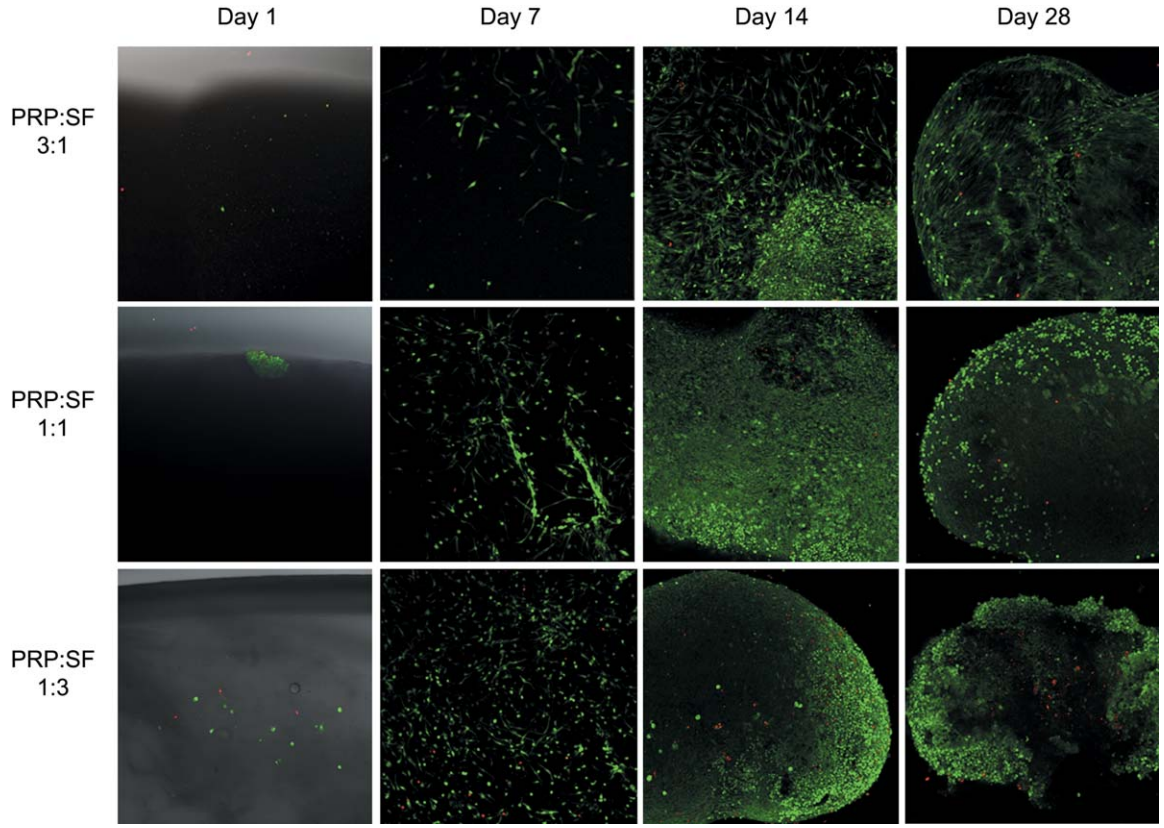

FIGURE 3. Microscopic images of cells immobilized within bioscaffolds. The bioscaffolds were formulated at different ratios of PRP:SF (3:1, 1:1, and 1:3) and viability of embedded cells into the three different bioscaffolds was evaluated at different time points up to 28 days using live/dead dye (green: living cells, red: dead cells).

was carried out. The obtained results proved that depending on the formulation, the secretion of this fibrinolytic enzyme may vary. In this case, the cells immobilized in the bioscaffold with the most fibrin amount (3:1) achieved the highest uPA secretion, while the cells immobilized in the formulation 1:3, obtained the lowest values for human uPA ($p < 0.05$) (Figure 5).

Cell morphology analysis by histology

After 21 days in culture, Hematoxilin and Eosin staining showed the morphology of immobilized cells. To provide more accurate data of this characteristic, the supporting information also showed the morphology of cells at day 7 and 14, based on the Giemsa staining. As shown the pictures, there are not significant differences between the morphology of cells entrapped into different scaffolds. On the other hand, immunohistochemical analysis revealed the positive staining for collagen type II, being the 3:1 the group with the lowest signal (Figure 6).

DISCUSSION

In the last years, the damage of articular cartilage has become a common problem in diverse population sectors, becoming a challenge for orthopedic research due to the limited self-repair capacity of this tissue.[1] This fact has sharpened and amplified the methods to provide damaged articular cartilage with a suitable environment, developing increasingly sophisticated bioscaffolds to improve the regeneration potential of the tissue. Besides their composition, the cellular component of these scaffolds is of crucial importance. Currently, therapies employing MSCs are gaining special attention because of their several positive properties, including their antiinflammatory effects and their differentiation potential. Moreover, in the last years, the sources to obtain MSCs are constantly increasing, and each reservoir presents its own benefits and disadvantages.[2,5,6] Concerning joint pathologies, MSCs from synovium and SF (SF-MSCs) are being increasingly studied. This type of MSCs has a high proliferative and chondrogenic potential, according with other groups that demonstrated superior cartilage formation of SF-MSCs compared to other sources.[16,17] In addition, they can be obtained from the patient using minimally invasive techniques such as arthrocentesis, and the HA present in the SF provide a suitable environment for cell immobilizatio.[18]

Bearing this in mind, this work describes a novel bioscaffold for its application in chondral defects based on two main components. First, the SF that contains HA and SF-MSCs as we proved and described in a previous work.[19] Second, PRP, which provides consistency due to the fibrin and an optimal microenvironment with cellular signals thanks to the growth factors. PRP has been employed for articular cartilage treatments for several reasons, including homeostasis of joint tissues, antiinflammatory, anabolic or immunomodulatory effects, pain reduction, and the

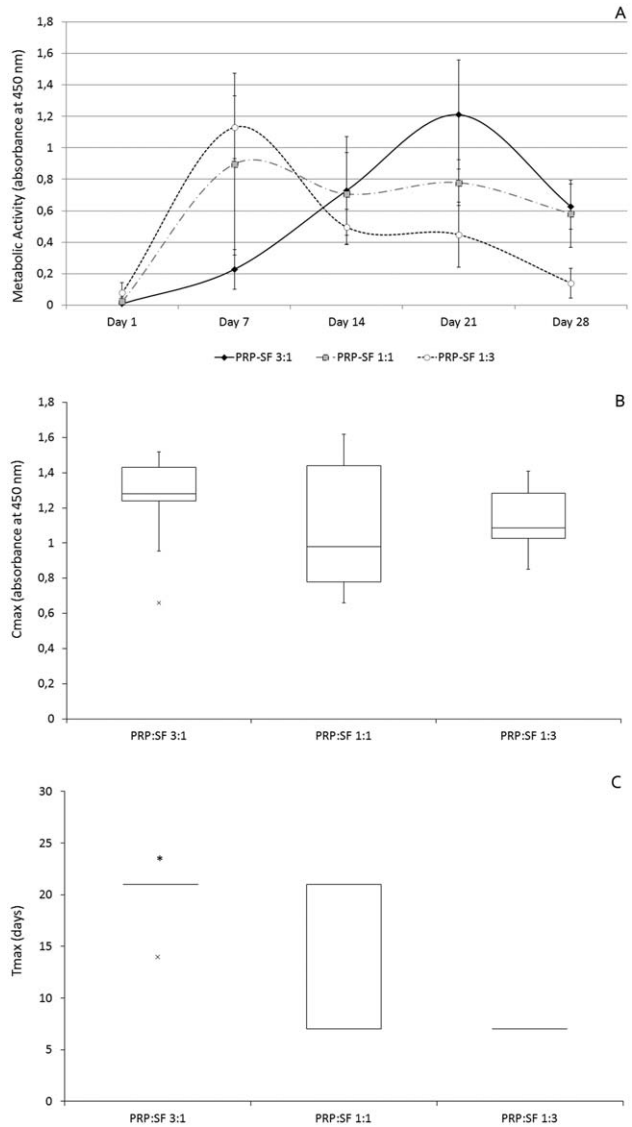

FIGURE 4. Metabolic activity (A), Cmax (B), and Tmax (C) of the bioscaffolds were formulated at different ratios of PRP:SF (3:1, 1:1, and 1:3) and metabolic activity of embedded cells into the three different bioscaffolds was evaluated at different time points up to 28 days by a sensitive colorimetric assay (CCK8) ($n = 5$). *$p < 0.05$ with respect to other formulations. x = outlier. Cmax = Peak metabolic activity; Tmax = time to peak metabolic activity.

improvement of physical function.[20,21] The present work is intended to combine the benefits of PRP with its biological and functional characteristics with the SF-MSCs and HA from the SF from the same patient, achieving a complete autologous bioscaffold.

The different proportions selected to prepare scaffolds based on PRP and SF (PRP:SF), allowed the formation of the constructs, obtaining a suitable niche for cell immobilization. However, as expected, depending on the volume of PRP or SF used in each formulation, the reduction rate (Table I), the size and the consistency of each construct type varied (Figure 2). Thus, the formulation 3:1 which contained the highest amount of PRP, and consequently, the highest fibrin proportion, had the most consistent appearance and its size was higher during all the study. Contrariwise, the formulation 1:3 was the bioscaffold with the smallest size in those 28 days. It has to be noted that this formulation contained the lowest amount of fibrin due to the lower volume of PRP, but also the highest SF-MSC number. This fact must be considered, since according to Neuss S. et al. MSCs, as other cells like endothelial cells, fibroblasts or mesothelial cells, also exhibit a fibrinolytic capacity due to the expression of several fibrinolytic enzymes, facilitating the reduction of fibrin mesh.[22] Furthermore, a study carried out by Zahn et al. showed that the shrinkage of PRP constructs was dependent of the number of MSCs.[23] Although the shrinkage of this fibrin matrix could represent a drawback, it is necessary to address the use of this novel formulation with a different approach. Despite currently in the clinical field it is intended to obtain a complete and stable filling of chondral defects with biodegradable scaffolds, the approach in this study is different. The matrix described in this work provides support to autologous MSC, protecting them against an aggressive and inflammatory environment. This formulation, attached in the bottom of the defect thanks to the adherent properties of fibrin, could release both growth factors attached to fibrin and biomolecules produced by the MSCs. They would generate not only a biological microenvironment suitable for regeneration and formation of new tissue but also a chemotactic effect.[22] In order to achieve this "*in situ*" production of proteins, the early fibrin degradation and matrix shrinkage is mandatory, which would be replaced by new tissue and the healing of the defect. Moreover, the integration of fibrin matrix in the defect and its subsequent shrinkage due to MSC activity could be a key factor in the formation of new tissue according to Takebe et al. who suggested this condensation is a natural step in the formation of tissue and organs via MSCs.[24]

The differences in the cell number of different formulations were corroborated with live/dead staining, showing more green fluorescence after 7 days of study within the formulation containing the highest amount of SF (1:3) comparing with the other types of scaffolds (Figure 3). In

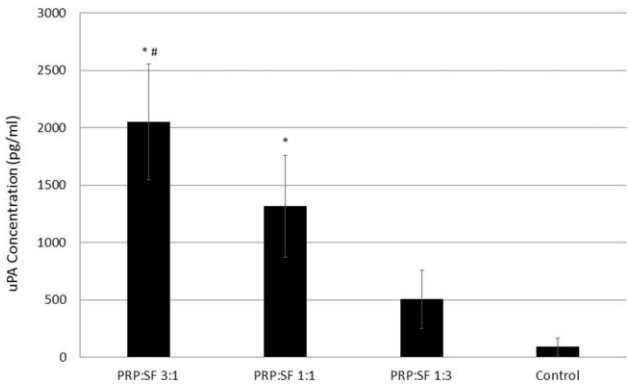

FIGURE 5. U-plasminogen activator (uPA) secretion. The bioscaffolds were formulated at different ratios of PRP:SF (3:1, 1:1, 1:3) using fibrin as a control group. uPA secretion of the three different bioscaffolds was evaluated at day 14 by ELISA assay ($n = 3$). *$p < 0.05$ with respect to control, $^{\#}p < 0.05$ with respect to formulation of ratio 1:3.

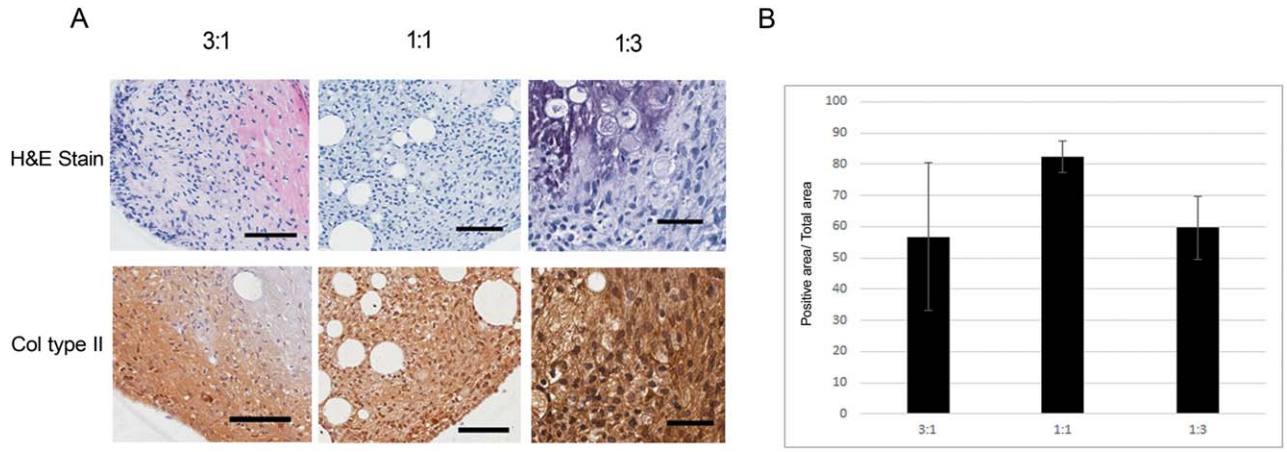

FIGURE 6. Histological and immunohistological analysis of 21 day cultured bioscaffolds. Cells in bioscaffolds 3:1 and 1:1 showed a chondrogenic morphology (upper row). The highest presence of type II Collagen quantified was corresponded with the bioscaffold ratio 1:1.

contrast, the formulation 3:1, which contained the lowest proportion of SF, revealed a low cell number at day 7, although until the end of the study all formulations achieved high viability of immobilized cells. Indeed, although the use of SF as MSC source may provide some advantages respect other reservoirs, the number of MSCs obtained in this niche is very low.[25] However, as it can be observed in the Figure 3, the bioscaffolds prepared in this work provide a suitable platform for SF-MSCs immobilization, allowing their proliferation over time and obtaining a high cell number in all type of constructs during the study.[26]

These results come along with the metabolic activity of immobilized MSCs, where the formulation 3:1 achieved the highest absorbance values later than other types of bioscaffolds because of the lower initial cell number as compared to other ratios (Figure 4). At the end of the study, the formulations 3:1 and 1:1 maintained a high absorbance value, while the formulation 1:3 revealed a clear drop since the day 7 until the day 28. This conduct could be explained due to the elevated cell number achieved in that formulation at the end of the study. The cell number of this type of scaffold along with the high proliferation activity of immobilized MSCs could induce a lack of space for immobilized cells in the small constructs[26] (Figure 2).

As mentioned above, MSCs facilitates the reduction of fibrin mesh.[22] With the aim of evaluating the fibrin clot invasion by the immobilized SF-MSCs, we also measured the uPA, since among others, it is believed to be a key component of the fibrinolysis system. uPA is synthesized by several cell types, including MSCs, facilitating the degradation of the fibrin mesh. The fact that SF-MSCs express key components of the fibrinolytic cascade, in this case uPA, suggests that hMSCs have the capacity to indirectly regulate the activity of the main executor enzyme of fibrinolysis (plasmin) in their immediate adjacent.[22,27] Levels of uPA were determined close to day 14 of culture, since according to metabolic activity it was the optimal time to achieve the similar cell activity in all types of scaffolds (Figure 4), and as observed in the Table I, this day the reduction rate also was similar in all constructs. According to the results represented in the Table I, the lowest uPA secretion was collected in the group 1:3, the formulation prepared with the lowest fibrin amount, while other formulations showed a higher uPA secretion (Figure 5). Although the formulation 1:3 contained the highest number of cells at day 0 and it could mean a higher uPA secretion, the characteristics of the scaffolds between day 7 and day 14 justify these uPA levels. Indeed, in this period the formulation 1:3 showed a size limitation, with very little fibrin, which could influence in cell growth as can be observed in the decrease of metabolic activity from day 7 to day 14, meaning low levels of uPA. In contrast, during this period, the group 3:1 exhibited a larger size with much more amount of fibrin, which allows the cells to continue their growth, being observed in the ascending metabolic activity, which translates into higher levels of uPA. Our findings come along with other studies that reported the secretion of fibrinolytic enzymes such as uPA from degradation of fibrin clots with embedded MSCs. In addition, these enzymes could promote the migration and mobilization of near MSCs, which would enhance the production and release of more biomolecules involved in tissue repair processes, promoting the tissue regeneration.[22,27]

The typical fibroblast-like phenotype of SF-MSCs entrapped into the PRP-SF bioscaffolds as well as their distribution in the edges of the scaffold can be observed in the Figure 6 and supporting information. After some days in the culture medium, MSCs proliferate and moved to the extreme of the constructs, being their presence more evident than in the center. Figure 6 also showed a positive staining for collagen type II in the immunohistochemistry analysis, proving the chondrogenic differentiation potential of SF-MSCs. In addition to the intrinsic chondrogenic differentiation capacity of these cells, it should be noted that the biological activity of PRP employed for the elaboration of the bioscaffolds could induce the differentiation of SF-MSCs into chondrogenic phenotype. Indeed, the fibrin network formed with

the activation of liquid retains some of the plasmatic and platelet growth factors achieving an optimal molecular environment. The sustained release of these growth factors entrapped in the fibrin clot includes the presence of TGF-β, whose action on SOX9 pathway entails the production of collagen type II.[28] This biologic action together with the suitable characteristics of the bioscaffold and the HA from the SF may lead to a chondrogenic differentiation of immobilized SF-MSCs. Other groups also described the potential of combining PRP and HA to induce chondrogenesis, among other benefits,[27,29] and according with Amman et al.[30] our results also corroborated that the formulation which contains the highest HA amount (1:3), expressed more collagen type II than other formulations.

The main limitation of this study is that the initial number of cells is unknown, although previous studies showed that the concentration of MSCs in SF was 1–10 MSCs/μL.[19]

CONCLUSION

The results collected in this study indicate that the MSCs coming from the SF maintained a high viability, proliferation, and metabolic activity in the autologous bioscaffolds. Moreover, the secretion of uPA from immobilized cells and the degradation of formed fibrin clot put in evidence the fibrinolytic capacity of SF-MSCs together with the chondrogenic differentiation potential provided by the bioscaffold. Although further studies will be needed, autologous scaffold made from PRP and SF could be an alternative to the treatment of chondral lesions.

ACKNOWLEDGMENTS

This work was supported by the Provincial Council of Alava through the AlavaInnova Program and Basque Government through the GAITEK Program. The authors wish to thank A. Iriondo, M. Montoya, M. L. González, M.J. Arnaiz, and C. Pérez de Arrilucea for their efficient sample collection, and M.A. Solinís and A. Mateo for her technical support.

REFERENCES

1. Benders KE, van Weeren PR, Badylak SF, Saris DB, Dhert WJ, Malda J. Extracellular matrix scaffolds for cartilage and bone regeneration. Trends Biotechnol 2013;31:169–176.
2. Huang BJ, Hu JC, Athanasiou KA. Cell-based tissue engineering strategies used in the clinical repair of articular cartilage. Biomaterials 2016;98:1–22.
3. Minas T, Von Keudell A, Bryant T, Gomoll AH. The John Insall Award: A minimum 10-year outcome study of autologous chondrocyte implantation. Clin Orthop Relat Res 2014;472:41–51.
4. Kon E, Filardo G, Gobbi A, Berruto M, Andriolo L, Ferrua P, Crespiatico I, Marcacci M. Long-term results after hyaluronan-based MACT for the treatment of cartilage lesions of the patellofemoral joint. Am J Sports Med 2016;44:602–608.
5. Murphy MB, Moncivais K, Caplan AI. Mesenchymal stem cells: Environmentally responsive therapeutics for regenerative medicine. Exp Mol Med 2013;45:e54.
6. Grässel S, Lorenz J. Tissue-engineering strategies to repair chondral and osteochondral tissue in osteoarthritis: Use of mesenchymal stem cells. Curr Rheumatol Rep 2014;16:452.
7. Garcia J, Wright K, Roberts S, Kuiper JH, Mangham C, Richardson J, Mennan C. Characterisation of synovial fluid and infrapatellar fat pad derived mesenchymal stromal cells: The influence of tissue source and inflammatory stimulus. Sci Rep 2016;6:24295.
8. Ando W, Kutcher JJ, Krawetz R, Sen A, Nakamura N, Frank CB, Hart DA. Clonal analysis of synovial fluid stem cells to characterize and identify stable mesenchymal stromal cell/mesenchymal progenitor cell phenotypes in a porcine model: A cell source with enhanced commitment to the chondrogenic lineage. Cytotherapy 2014;16:776–788.
9. Nakagawa Y, Muneta T, Kondo S, Mizuno M, Takakuda K, Ichinose S, Tabuchi T, Koga H, Tsuji K, Sekiya I. Synovial mesenchymal stem cells promote healing after meniscal repair in microminipigs. Osteoarthritis Cartilage 2015;23:1007–1017.
10. Mak J, Jablonski CL, Leonard CA, Dunn JF, Raharjo E, Matyas JR, Biernaskie J, Krawetz RJ. Intra-articular injection of synovial mesenchymal stem cells improves cartilage repair in a mouse injury model. Sci Rep 2016;6:23076.
11. Richter W. Mesenchymal stem cells and cartilage in situ regeneration. J Intern Med 2009; 266:390–405.
12. Fortier LA, Barker JU, Strauss EJ, McCarrel TM, Cole BJ. The role of growth factors in cartilage repair. Clin Orthop Relat Res 2011; 469:2706–2715.
13. Zhang Y, Heher P, Hilborn J, Redl H, Ossipov DA. Hyaluronic acid-fibrin interpenetrating double network hydrogel prepared in situ by orthogonal disulfide cross-linking reaction for biomedical applications. Acta Biomater 2016;38:23–32.
14. Snyder TN, Madhavan K, Intrator M, Dregalla RC, Park D. A fibrin/hyaluronic acid hydrogel for the delivery of mesenchymal stem cells and potential for articular cartilage repair. J Biol Eng 2014;8:10.
15. DeLong JM, Russel RP, Mazzocca AD. Platelet-rich plasma: The PAW classification system. Arthroscopy 2012;28:998–1009.
16. Jones BA, Pei M. Synovium-derived stem cells: A tissue-specific stem cell for cartilage engineering and regeneration. Tissue Eng Part B Rev 2012;18:301–311.
17. Chiang CW, Chen WC, Liu HW, Chen CH. Application of synovial fluid mesenchymal stem cells: Platelet-rich plasma hydrogel for focal cartilage defect. J Exp Clin Med 2014;6:118–124.
18. Lee JC, Min HJ, Park HJ, Lee S, Seong SC, Lee MC. Synovial membrane-derived mesenchymal stem cells supported by platelet-rich plasma can repair osteochondral defects in a rabbit model. Arthroscopy 2013;29:1034–1046.
19. Muiños-López E, Delgado D, Sánchez P, Paiva B, Anitua E, Fiz N, Aizpurua B, Guadilla J, Padilla S, Granero-Moltó F, Prósper F, Sánchez M. Modulation of synovial fluid-derived mesenchymal stem cells by intra-articular and intraosseous platelet rich plasma administration. Stem Cells Int 2016;2016:1247950.
20. Anitua E, Pelacho B, Prado R, Aguirre JJ, Sanchez M, Padilla S, Aranguren XL, Abizanda G, Collantes M, Hernandez M, Perez-Ruiz A, Penuelas I, Orive G, Prosper F. Infiltration of plasma rich in growth factors enhances in vivo angiogenesis and improves reperfusion and tissue remodeling after severe hind limb ischemia. J Control Release 2015;28:202–231.
21. Sanchez M, Anitua E, Delgado D, Prado R, Sanchez P, Fiz N, Guadilla J, Azofra J, Pompei O, Orive G, Ortega M, Yoshioka T, Padilla S. Ultrasound-guided plasma rich in growth factors injections and scaffolds hasten motor nerve functional recovery in an ovine model of nerve crush injury. J Tissue Eng Regen Med 2017;11:1619–1629.
22. Neuss S, Schneider RK, Tietze L, Knüchel R, Jahnen-Dechent W. W. Secretion of fibrinolytic enzymes facilitates human mesenchymal stem cell invasion into fibrin clots. Cells Tissues Organs 2010;191:36.
23. Zahn J, Loibl M, Sprecher C, C,Nerlich M, Alini M, Verrier S, Herrmann M. M. Platelet-rich plasma as an autologous and proangiogenic cell delivery system. Mediators Inflamm 2017;2017:1075975.
24. Takebe T, Enomura M, Yoshizawa E, Kimura M, Koike H, Ueno Y, Matsuzaki T, Yamazaki T, Toyohara T, Osafune K, Nakauchi H, Yoshikawa H, Taniguchi H. Cell Stem Cell 2015;16:556–565.
25. Jones EA, Crawford A, English A, Henshaw K, Mundy J, Corscadden D, Chapman T, Emery P, Hatton P, McGonagle D. Synovial fluid mesenchymal stem cells in health and early osteoarthritis: Detection and functional evaluation at the single-cell level. Arthritis Rheum 2008;58:1731.
26. Sadeghi-Ataabadi M, Mostafavi-poyr Z, Vojdani Z, Sani M, Latifi M, Talaei-Khozani T. Fabrication and characterization of platelet-rich plasma scaffolds for tissue engineering applications. Mater Sci Eng C Mater Biol Appl 2017;71:372–380.

27. Vallabhaneni KC, Tkachuk S, Kiyan Y, Shushakova N, Haller H, Dumler I, Eden G. Urokinase receptor mediates mobilization, migration, and differentiation of mesenchymal stem cells. Cardiovasc Res 2011;90:113–121.
28. Vadalá G, Russo F, Musumeci M, Este MD, Cattani C, Catanzaro G, Tirindelli MC, Lazzari L, Alini M, Giordano R, Denaro V. Clinically relevant hydrogel-based on hyaluronic acid and platelet rich plasma as a carrier for mesenchymal stem cells: Rheological and biological characterization. J Orthop Res 2016; doi:10.1002/jor.23509.
29. Chen WH, Lo WC, Hsu WC, Wei HJ, Liu HY, Lee CH, Tina Chen SY, Shieh YH, Williams DF, Deng WP. Synergistic anabolic actions of hyaluronic acid and platelet-rich plasma on cartilage regeneration in osteoarthritis therapy. Biomaterials 2014;35:9599–9607.
30. Amman E, Wolff P, Breel E, van Grienven M, Balmayor ER. Hyaluronic acid facilitates chondrogenesis and matrix deposition of human adipose derived mesenchymal stem cells and human chondrocytes co-cultures. Acta Biomater 2017;52:130–144.