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Bone regeneration in osteoporosis by delivery BMP-2 and PRGF from tetronic–alginate composite thermogel

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ABSTRACT

As the life expectancy of the world population increases, osteoporotic (OP) fracture risk increase. Therefore in the present study a novel injectable thermo-responsive hydrogel loaded with microspheres of 17β-estradiol, microspheres of bone morphogenetic protein-2 (BMP-2) and plasma rich in growth factors (PRGF) was applied locally to regenerate a calvaria critical bone defect in OP female rats. Three systems were characterized: Tetronic® 1307 (T-1307) reinforced with alginate (T-A), T-A with PRGF and T-A-PRGF with microspheres. The addition of the microspheres increased the viscosity but the temperature for the maximum viscosity did not change (22–24 °C). The drugs were released during 6 weeks in one fast phase (three days) followed by a long slow phase. In vivo evaluation was made in non-OP and OP rats treated with T-A, T-A with microspheres of 17β-estradiol (T-A-βE), T-A-βE prepared with PRGF (T-A-PRGF-βE), T-A-βE with microspheres of BMP-2 (T-A-βE-BMP-2) and the combination of the three (T-A-PRGF-βE-BMP). After 12 weeks, histological and histomorphometric analyzes showed a synergic effect due to the addition of BMP-2 to the T-A-βE formulation. The PRGF did not increased the bone repair. The new bone filling the OP defect was less mineralized than in the non-OP groups.

1. Introduction

Alginate is a natural polymer widely studied for pharmaceutical preparations as a drug delivery system (Jain and Bar-Shalom, 2014) and for cell encapsulation (Martín del Valle et al., 2009; Orive et al., 2009; Wang et al., 2009). In addition, alginate sponges and hydrogels have been proposed as scaffolds for in vitro cell proliferation and differentiation and in vivo tissue regeneration and at the same time, as delivery systems for different active substances (De la Riva et al., 2009). Poloxamines, marketed as Tetronics, are X-shaped copolymers with four poly(ethylene oxide)- poly(propylene oxide) (PEO-PPO) arms that form gel structures depending of the concentration, pH and temperature. Poloxamine-based hydrogels have been studied for bone regeneration purposes because of their in vitro intrinsic osteogenic activity (Rey-Rico et al., 2011). Despite that the observed in vitro osteogenic capacity of Tetronic 908 (T-908) and Tetronic 1307 (T-1307) was not evident in vivo, their physico-chemical properties made them very interesting biomaterials for bone scaffolds and as carriers for active microparticles (Del Rosario et al., 2015b; Rodríguez-Évora et al., 2014). The sol-to-gel transition of T dispersions occurs as previously determined approximately at 26 °C (Rodríguez-Évora et al., 2014) and the viscosity increased when the temperature was raised to 37 °C. However, the blends of these poloxamines and alginates have not been studied yet. By contrast, preparation and optimization of blends of alginates with other in situ gelling polymers such as Pluronic® have been reported (Barba et al., 2014; Grassi et al., 2006). Pluronic-alginate blends were proposed as drug delivery carriers of some drugs by different routes of administration including pilocarpine for ophthalmic application (Lin et al., 2004) nucleic acid based drugs for injectable arterial endoluminal delivery (Abrami et al., 2014) and as a controlled release transdermal system for seleginine (Chen et al., 2011).

On the other hand, many traumas and diseases (tumors, osteomyelitis, osteoarthritis) may lead to large bone defects requiring surgery. As the size of these defects exceed the auto-repair capacity of the bone, new approaches that stimulate osteogenesis are needed. The filling of the defect with autologous bone is the gold standard, but in the case of the regeneration of bone defects in the osteoporotic (OP) population it is necessary to add to the limitations of this technique, those

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of the osteoporotic bone. In general, most of the studies in ovariectomized (OVX) animals supported that the healing time in fractures of osteoporotic bones is delayed, the risk of non-union and failure of implant fixation are higher than in healthy bones (Kubo et al., 1999; Namkung-Matthai et al., 2001; Oliver et al., 2013). All stages of the repair process are impaired in osteoporotic bone: during the early phase, progenitor cell recruitment, differentiation, and proliferation; angiogenesis and vasculogenesis and the expression of estrogen receptor; during the mid-phase, the capacity of extracellular matrix production and callus formation; and at later phase the callus remodeling capacity (Cheung et al., 2016). All these facts show that it is necessary to activate the process to repair the OP bone successfully. Many growth factors (GFs) are involved in the bone healing cascade including bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), platelet derived growth factor (PDGF-AB), and insulin-like growth factor (IGF) among others.

A natural source of human-based proteins and growth factors is blood and more specifically human plasma and platelets. In fact, it is well reported that human platelets contain several growth factors and cytokines stores in their alpha-granules. Just by using a standardized technique known as plasma rich in growth factors (PRGF), it is possible to prepare from each patient a small volume of plasma enriched in a wide range of biologically active mediators (Anitua et al., 2015). The latter has been successfully applied in the repair and regeneration of different types of injuries in many medical fields including dentistry, traumatology, ophthalmology and dermatology, among others (Anitua et al., 2008, Anitua et al., 2012, Anitua et al., 2013). On the other hand, BMP-2 has the capacity to stimulate fracture healing and to promote bone formation. In fact, many studies are dedicated to the formulation and evaluation of BMP-2 to repair bone defects in healthy animals (Del Rosario et al., 2015a,b; Rodríguez-Évora et al., 2014, 2013). Despite the BMP-2 potential application, this GF has not been widely studied in OP. The few studies reported have been developed in different animals and BMP-2 has been incorporated in different carriers, which makes it difficult to obtain solid conclusions (Segredo-Morales et al., 2017). In general, the doses are varied and the release kinetics is not determined or is not an objective to be optimized. Overall, the limited reports on animal experiments suggest that the local administration of BMP-2 stimulated fracture healing and improved the repair of certain bone defects in OP animals (Kyllönen et al., 2015; Li et al., 2010; Park et al., 2013; Wu et al., 2011; Zarrinkalam et al., 2013). Assuming this, the present study aims to prepare and characterize a novel injectable thermo-responsive hydrogel as local drug delivery system and apply it in the context of regeneration of an osteoporotic bone defect. The hydrogel system is composed of a poloxamine, (T-1307) reinforced with alginate crosslinked with calcium chloride. PRGF and BMP-2 in microspheres of poly(D,L-lactide-co-glycolide) 50:50 (PLGA) combined with poly(D,L-lactide) (PLA-S) microspheres of 17β-estradiol were selected as bone active substances for local delivery in the defect. The impaired bone repair process in OP might improve by a sustained supply of 17β-estradiol that lack in post-menopausal OP, combined with an osteogenic inductor such as PRGF containing a GFs cocktail or with the BMP-2 alone. The synergism of the PRGF and BMP-2 combination was also investigated. These combinations with a suitable delivery strategy might be a good approach suited for the clinical management of osteoporotic fractures. Local drug delivery would significantly reduce the risk of adverse effects and increase the efficiency of osteoporotic fractures healing.

2. Materials and methods

The materials were processed under aseptic conditions. All lab instruments and liquid components, except BMP-2 (GenScript, Piscataway, USA) with ED50 of 0.85 μg/mL, measured by its ability to induce alkaline phosphatase production by C2C12 cells and 17β-estradiol (βE, Sigma-Aldrich), were autoclaved (121 °C, 30 min, Auster Selecta, Spain). Poloxamine, T-1307, was provided by BASF Corporation, (Ludwigshafen, Germany). Sodium alginate, Pronova® UP MVG, was supplied by Novamatrix (Sandvika, Norway) and calcium chloride (Merck, Germany) was used as alginate crosslinker. PLA-S (Resomer® R 203 S) and PLGA (Resomer® RG 504) were purchase from Evonic Industries (Darmstadt, Germany).

2.1. Evaluated systems and preparation conditions

Five types of Tetronic-alginate hydrogel systems were prepared under aseptic conditions: a) plain Tetronic-alginate gel (T-A), b) Tetronic-alginate gel with PLA-S microspheres containing 17β-estradiol (T-A-βE), c) Tetronic-alginate-PRGF gel with PLA-S microspheres containing 17β-estradiol (T-A-PRGF-βE) d) Tetronic-alginate gel with a combination of PLA-S microspheres containing 17β-estradiol and PLGA microspheres containing BMP-2 (T-A-βE-BMP) and e) Tetronic-alginate-PRGF gel with a combination of PLA-S microspheres containing 17β-estradiol and PLGA microspheres containing BMP-2 (T-A-PRGF-βE-BMP).

2.1.1. PRGF preparation and characterization

PRGF preparation was carried out as described (Anitua et al., 2015), blood samples were voluntarily taken from the authors of this study and divided into tubes containing 3.8% (w/v) sodium citrate. Then, the pool of blood was centrifuged at 580*g* for 8 min and the plasma column was divided into fraction 1(F1) and fraction 2 (F2). F1, platelet poor fraction, was discarded and F2, defined as the 2 cm^3 platelet rich plasma just above the leukocyte buffy coat, was used in the study. The release of growth factors and morphogens was provoked by the addition of a PRGF activator (calcium chloride) to the platelet concentrate. In order to characterize the PRGF, platelet enrichment and growth factors content of the PRGF were determined. Platelet count in peripheral blood and in F2 fraction was measured with a hematology analyzer (Micros 60, Horiba ABX, Montpelier, France). Additionally, the supernatant released after platelet activation was collected by centrifugation at 1000*g* for 10 min. The following growth factor levels were measured by commercially available Enzyme-linked immunosorbent assay kits (ELISA) (R&D Systems, Minneapolis, MN): TGFβ1, PDGF-AB, IGF and VEGF (Okada et al., 2016).

2.1.2. Microspheres preparation and characterization

The microspheres of BMP-2 were prepared by a double emulsion (w/o/w) method. Briefly, 200 μL of a BMP-2 (900 μg/mL) aqueous solution in 0.2% poly-vinyl alcohol (PVA) was stirred in vortex for 30 s (Genie®2, position 10) with 1 mL of a 150 mg/mL solution of PLGA in methylene chloride (DCM). Afterwards, 10 mL of PVA 0.2% (w/v) was added and vortexed for 15 s (Genie®2, position 10) to be poured into 100 mL of a 0.1% PVA aqueous solution and left under magnetic stirring for 1 h. For later characterization some batches of microspheres were prepared using ^{125}I-BMP-2. BMP-2 was labeled with ^{125}INa (Perkin-Elmer) according to the iodogen method (Fraker and Speck, 1978), as described (Del Rosario et al., 2015b).

The microspheres of 17β-estradiol were prepared with PLA-S by the solvent evaporation method. Briefly, 0.5 mL of 17β-estradiol (4 mg) and PLA-S (150 mg) solution in methanol (MeOH) MeOH:DCM 20:80, was emulsified with 4 mL of 1% PVA aqueous solution using vortex stirring (Genie®2, position 10) for 1 min and poured into 100 mL of 0.15% PVA aqueous solution and left under magnetic stirring for 1 h (Segredo-Morales et al., 2018a).

Both types of microspheres were collected by filtration (Supor®-450 filters, Pall Corporation, 47 mm) and lyophilized. Microspheres were characterized in terms of morphology by Scanning Electron Microscopy (SEM, Jeol JSM-6300) and size by laser diffractometry (Mastersizer 2000, Malvern Instruments). Encapsulation efficiency was determined in three aliquots of each microspheres batch. Encapsulation efficiency

of BMP-2 microspheres was determined in ^{125}I-BMP-2 batches with a gamma counter (Cobra® II, Packard) (Rodríguez-Évora et al., 2014), while the content of 17β-estradiol in the microspheres was determined spectrophotometrically at λ = 280 nm previously dissolved in a mix of MeOH:DCM (20:80).

2.1.3. Preparation and characterization of the hydrogel systems

Solutions of T-1307 at 30% (w/w), alginate UP at 6% (w/w) and $CaCl_2$ at 26.5% (w/w) were made in sterilized water (Mili-Q). T-1307 and alginate solutions were prepared by magnetic stirring overnight at 4 °C and RT respectively and were kept at 4 °C until use. To prepare the T-A hydrogel, the solutions were mixed in an ice bath to maintain a liquid state and diluted to lead a final concentration of 21% of T-1307, 1.5% of alginate and 1% of $CaCl_2$. Briefly, T-1307 solution was mixed with the alginate solution stirring in vortex (position 10) for 3 min. The mix was maintained cold in the ice bath for 5 min. Then the $CaCl_2$ solution was added and stirred in vortex (position 10) for 3 min. Lastly the final particulate system was extemporaneously prepared by mixing the hydrogel with the microspheres (18 mg/100 μL).

The systems containing PRGF were prepared as above and, after the addition of the $CaCl_2$, the gel was lyophilized and reconstituted with the PRGF at 0.77 g/g of gel.

To check the gel formation the system was kept in the oven at 37 °C during 15 min, after which the tilting test was applied. Furthermore, the dose uniformity was also tested using ^{125}I-BMP-2 microspheres. Briefly, a 1 mL syringe was loaded with the T-A-PRGF-βE-^{125}I-BMP system in cold, after 10 min at RT the radioactivity of 5 doses of 100 μL was measured in the gamma counter.

Surface morphology and porous structure of the hydrogel were analyzed by scanning electron microscopy (SEM, Jeol JSM-6300) of the freeze-dried formulations. Chemical elements on the surface were analyzed by SEM-EDX (Energy Dispersive X-ray spectroscopy).

The porosity of the hydrogel was calculated as Eq. (1).

$$Porosity(\%) = \left(1 - \frac{p_{app}}{p_{true}}\right) \cdot 100 \quad (1)$$

where ρ_{true} (measured with a helium pycnometer, AccuPyc 1330, Micromeritics) and ρ_{app} (calculated by dividing mass by external geometrical volume) are the real and apparent densities of the lyophilized hydrogel.

The viscoelastic characteristics of the T-A, T-A-PRGF and T-A-PRGF-blank microspheres were tested in triplicate using a Bohlin CVOD 100 rheometer equipped with a Peltier temperature control system and using a cone-plate geometry (diameter 40 mm, angle 4°) leaving a gap of 1 mm. The dependence of the viscosity with temperature was recorded at 10 s^{-1} in the range of 0–80 °C.

To check the hydrogel stability 100 μL of the T-A was placed in a cylindrical mold of approximately 8 mm (internal diameter) × 1.5 mm (high) that simulate the calvarial bone defect. The hydrogel in the mold was incubated in a 50 mL flask with 25 mL of water (mili-Q) at 37 °C during 4 weeks. The samples were withdrawal at 1, 2, 3 and 4 weeks and lyophilized. Then the surface morphology and porous structure of the samples were visualized and photographed with a stereo microscope (Leica M205 C, Leica LAS, v3 software) and with the SEM.

The in vitro 17β-estradiol release was assayed in triplicate (37 °C). The 17β-estradiol is a poorly soluble drug and to maintain the sink conditions the release assays were carried using a release medium of water:MeOH (50:50), as previously reported (Birnbaum et al., 2000, Wischke and Schwendeman, 2008). To study the influence of the PRGF on the 17β-estradiol release kinetics, the release profiles were assayed by dispersing the microspheres in the hydrogel with and without PRGF (T-A-PRGF-βE and T-A-βE). The amount of 17β-estradiol released was determined by the spectrophotometric method.

2.2. In vivo experiments

The protocol was approved by the Ethical Committee of the University of La Laguna. The animal experiments were carried out in conformity with the EC directive on Care and Use of Animals in Experimental Procedures (2010/63/UE). All the surgical procedures were carried out under aseptic conditions.

2.3. Animal model

50 Female Sprague-Dawley rats weighing 225–250 g divided in two groups of 25 each, were used in the study. The experimental osteoporosis was induced in one group (OP) by bilateral ovariectomy (OVX) under isoflurane anesthesia, via a dorsal approach. In addition, two weeks post-ovariectomy, 0.3 mg/kg body weight of dexamethasone-21-isonicotinate (Deyanil retard, Fatro Ibérica, Spain) were administered subcutaneously once in two weeks (Govindarajan et al., 2013) for up to the time of sacrifice. The remaining 25 rats were used as sham healthy controls. As the OVX rats, the sham group, underwent to the same surgical process except the ovariectomy to avoid differences due to the surgery.

Five different systems of thermogel (100 μL) with a mixture of PLA-S/PLGA microspheres and containing 200 μg of 17β-estradiol, 4 μg of BMP-2, 730 μL of PRGF or a combination of these active substances, as described in the evaluated systems section, were assayed. 4 months post-OVX, critically sized circular (8 mm) cranial defect was surgically created with a trephine burr in the rats under isoflurane as previously described (Rodríguez-Évora et al., 2013). The five systems were injected into the defects of 10 groups of rats, 5 groups of sham and others 5 groups of OP, the wound was closed and sutured. Then, the animals were allowed free movement and food and water uptake. Analgesia consisted in buprenorphine administered subcutaneously (0.05 mg/kg) before surgery and paracetamol (70 mg/100 mL) in the water, during 3 days post-surgery. The animals were sacrificed 12 weeks post-surgery and defect enclosing segments were resected from the calvariae to analyze the bone refilled.

2.3.1. In vivo BMP-2 release assay

The BMP-2 release experiments were carried out in two groups of 5 rats each. One group was implanted with 100 μL of Tetronic-alginate gel with a combination of PLA-S microspheres containing 17β-estradiol and PLGA microspheres containing ^{125}I-BMP-2 (T-A-βE-BMP) and the other group was implanted with the same hydrogel system but now lyophilized and reconstituted with the PRGF (T-A-PRGF-βE-BMP). The remaining ^{125}I-BMP-2 at the bone defect was measured periodically using an external probe-type gamma counter (Captus®, Nuclear Iberica) as previously described and validated (Delgado et al., 2006).

2.3.2. Histology and histomorphometrical evaluation

To determine the capacity of the bone active substances to regenerate the critical size defect practiced in the calvaria of the rats, samples of the 10 groups of 5 rats each were examined.

Samples were fixed (4% paraformaldehyde solution), decalcified in Histofix® Decalcifier (Panreac, Barcelona, Spain) and prepared for histological analysis as previously described (Hernández et al., 2012). New bone formation was identified by hematoxylin-erythrosin staining. Bone mineralization was assessed with VOF trichrome stain, in which red and brown staining indicates advanced mineralization, whereas less mineralized, newly formed bone stains blue (Martinez-Sanz et al., 2011). Sections were analyzed by light microscopy (LEICA DM 4000B). Computer based image analysis software (Leica Q-win V3 Pro-image Analysis System, Barcelona, Spain) was used to evaluate all sections. A region of interest (ROI) within the defect (50 mm^2) for quantitative evaluation of new bone formation was defined. New bone formation was expressed as a percentage of repair with respect to the original defect area within the ROI. The osteoporosis-like condition was

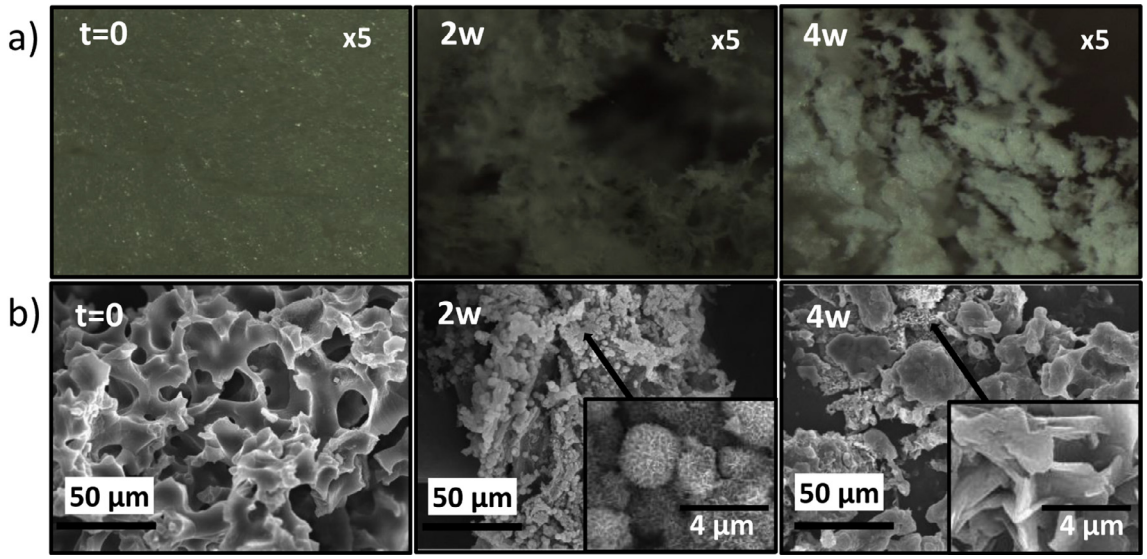

Fig. 1. System evolution. Images of the Tetronic-alginate and its evolution throughout 4 weeks of incubation in water at 37 °C. a) Images from the stereomicroscope at 5x magnification; b) SEM images showing the internal structure of the hydrogel. The inserts display details of the structure at higher magnification.

confirmed histologically by determining the bone thickness and studying the bone microarchitecture in cross sections of calvaria. From the total bone repair, the areas of mature bone (MB) and immature bone (IB) were determined and the MB/IB ratio for each experimental group as well as between sham and osteoporotic-like animals, was calculated.

For immunohistochemical analysis, sections were deparaffined and rehydrated in Tris-buffered saline (TBS) (pH 7.4, 0.01 M Trizma base, 0.04 M Tris hydrochloride, 0.15 M NaCl), which was used for all further incubations and rinse steps. Sections were incubated in citrate buffer (pH 6) at 90 °C for antigen retrieval, followed by incubation in 0.3% hydrogen peroxide in TBS buffer for 20 min. After a rinse step, sections were blocked with 2% FBS in TBS–0.2% Triton X-100 (blocking buffer). The indirect immunohistochemical procedure was carried out by incubating the sections with collagen type I and osteocalcin antiserum (1/100) (Millipore, Barcelona, Spain) in blocking buffer overnight at 4 °C. Sections were rinsed three times, then incubated with biotin-SP-conjugated donkey anti-rabbit F(ab0) fragment (1/200) (Millipore, Barcelona, Spain) in blocking buffer for 1 h followed, after another rinse step, by incubation in peroxidase-conjugated streptavidin (1/300) (Millipore, Barcelona, Spain) for 1 h. Peroxidase activity was revealed in Tris–HCl buffer (0.05 M, pH 7.6) containing 0.04% of 4-chloro-1-naphtol (Sigma, Poole, UK) and 0.01% hydrogen peroxide. Reaction specificity was confirmed by replacing the specific antiserum with normal serum or by pre-adsorption of the specific antiserum with the corresponding antigen.

Collagen type I and osteocalcin staining was evaluated using computer-based image analysis software (ImageJ, NIH, Bethesda, MD). Collagen type I and osteocalcin staining was measured by applying a fixed threshold to select for positive staining within the ROI. Positive pixel areas were divided by the total surface size (mm^2) of the ROI. Values were normalized to those measured from blank scaffolds and are reported as relative staining intensities.

Statistical analysis was performed with SPSS.21 software. We compared the distinct treatments by means of a one-way analysis of variance (ANOVA) with a Tukey multiple comparison post-test. Significance was set at $p < 0.05$. Results are expressed as means ± SD.

3. Results

3.1. System characteristics

The mean volume diameter of the microspheres of PLGA containing BMP-2 was 108.9 µm, (10% < 54.8 µm and 90% < 139.3 µm) and 83.2 µm, (10% < 32.6 µm and 90% < 117.8 µm) for the PLA-S microspheres containing 17β-estradiol. The encapsulation efficiency was 70% and 81% for BMP-2 and 17β-estradiol, respectively. The porosity of the hydrogel was 78% ± 1.35. Micrographs of the hydrogel systems, obtained with the stereo microscopy and with the SEM, showed that the T-A hydrogel has a porous surface. The hydrogel was formed by a regular porous structure of a network of polymer with pores interconnected that evolves to a less uniform and more porous structure after its incubation in water (Fig. 1). The T was lost by dilution in the medium and the skeleton of the alginate increased its porosity by erosion (Fig. 1). The EDX analysis indicated calcium located on the surface of the hydrogel, after 4 weeks incubation the atomic% of calcium was reduced 7-fold.

Temperature dependence of the viscoelastic behavior of T-A dispersions alone and reconstituted with PRGF (T-A-PRGF) together with the final injectable system (T-A-PRGF with blank microspheres) is shown in Fig. 2. The maximum viscosity of the three hydrogels was detected in the range of 22–24 °C. The T-A and T-A-PRGF profiles were similar, the value of maximum viscosity was in the range of 30–40 Pa·s. By contrast the addition of the microspheres prompted an important increase in the viscosity of the hydrogel system up to 50–60 Pa·s. Although the temperature of the maximum viscosity was approximately the same, the presence of PRGF, but above all, the addition of microspheres, shifted the curves to lower temperatures. Mean values (± sd) of the parameters that defined the PRGF characteristics are reflected in Table 1.

3.2. 17β-estradiol and BMP-2 release kinetics

The in vitro 17β-estradiol release profiles reflected that the PRGF did not affect the release kinetics of the 17β-estradiol from the microspheres (Fig. 3). Only the first day the percent of 17β-estradiol was slightly reduced by the PRGF, 12% against 18%. After the 6 weeks that the assay lasted, 80% of the drug was released. On the other hand, BMP-2 release from the gel, with and without PRGF, was assessed at the defect site (Fig. 3). No difference between both of the ^{125}I-BMP-2

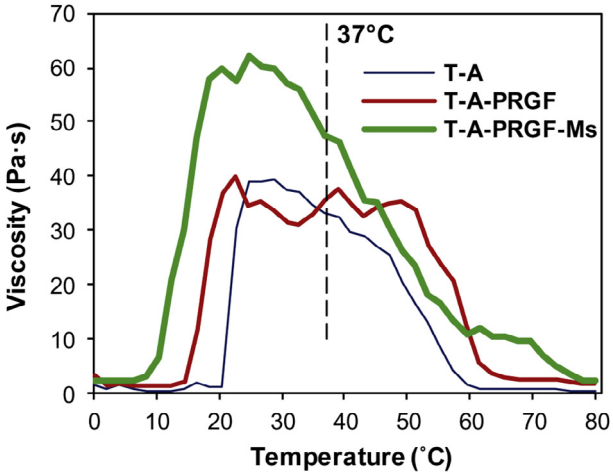

Fig. 2. System evolution. Evolution of the viscosity with the temperature of the Tetronic-alginate hydrogel (T-A) freshly prepared and after freeze-drying and reconstitution with PRGF (0.77 g/g of gel) (T-A-PRGF) and containing a mixture of PLA-S/PLGA microspheres (180 mg/mL of gel) (T-A-PRGF-Ms).

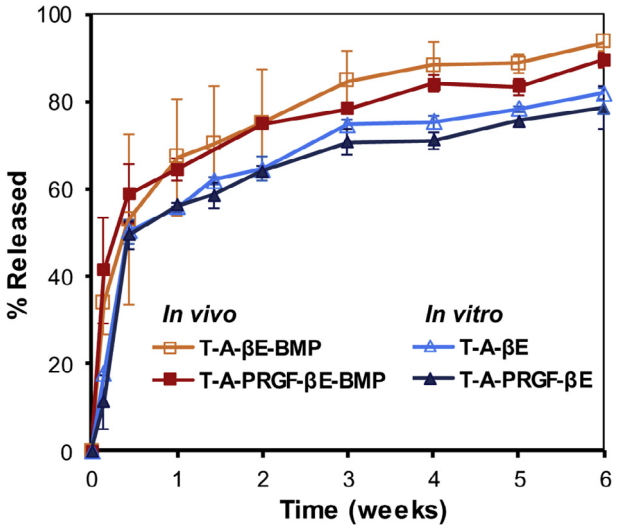

Fig. 3. Release assay. Release profile of ^{125}I-BMP-2 from PLGA microspheres included in T-A-βE-BMP and T-A-PRGF-βE-BMP systems, after implantation in the rat calvaria defect (n = 5) and release profile of 17β-estradiol from PLA-S microspheres incorporated in the T-A-βE and T-A-PRGF-βE systems (n = 3) in a mixture of Water:MeOH (50:50) at 37 °C.

profiles was detected. Again, the effect of the PRGF only showed during the first day, increasing the BMP-2 released from 34% to 41%. Ultimately, 90% of the encapsulated BMP-2 was delivered during the 6 weeks.

3.3. Histological, histomorphometrical and immunohistochemical analysis

The histological analysis of the calvaria samples in the non-OP and OP animals four months after ovariectomy and the treatment with dexamethasone showed clear differences in the thickness of the bone, the calvaria bone being between 30% and 40% thicker in the non OP animals (Fig. 4).

The analysis of the samples twelve weeks after the implantation of the systems with the different treatments, revealed no repair in blank groups non-OP and OP, with a poor bone formation at the margins of the defect (Fig. 5A). Connective tissue occupied a great part of the defect area in some animals of both blank groups (Fig. 5A). However, higher proportion of adipose tissue mixed with connective tissue was observed in OP animals (Fig. 5A).

In the groups treated with 17β-estradiol (T-A-βE) and with the combination with PRGF (T-A-βE-PRGF), the repair rate was between 24% and 32%, being slightly higher in OP animals than in non-OP. The new bone was irregularly distributed, little at the edges and somewhat more at the center of the defect. In OP groups, a high proportion of connective tissue was observed as well as some adipose tissue. (Fig. 5A and B).

The highest repair rates, between 50% and 58%, were detected in the groups treated with BMP-2, both in combination with 17β-estradiol and in combination with 17β-estradiol and PRGF. Both groups showed significant differences with respect to the blank groups in OP and non-OP animals (Fig. 5A and B). The groups treated with both BMP-2 combinations showed better repair responses than the groups without BMP-2. However, only the non-OP groups treated with the combinations of BMP-2, T-A-βE-BMP and T-A-βE-PRGF-BMP showed significant differences with the non-BMP-2 groups (T-A-βE and T-A-βE-PRGF) (Fig. 5A and B).

In summary, no significant differences were observed between non-OP and OP animals for any of the treatments tested. Furthermore, PRGF did not improve the response induced by 17β-estradiol alone or by the combination of 17β-estradiol and BMP-2 (Fig. 5A and B)

By contrast, the estimated ratio (MB/IB) between mature bone (more mineralized bone) and immature bone (less mineralized bone) was significantly higher in non-OP against OP groups, independently of the treatment. The ratio MB/IB in the OP groups treated with the BMP-2 combinations was slightly better than the other groups (Fig. 6).

The detection of collagen type I immunoreactivity, a marker of early osteogenesis, in the newly formed bone, showed significant differences in relative staining intensities only between blank groups and BMP-2 groups in both non-OP and OP animals (Fig. 7).

Also, significant differences in osteocalcin relative staining intensity between blank and treated groups in both non-OP and OP animals, were detected (Fig. 8). In addition, in non-OP, BMP-2 induced a significant increase of the osteocalcin relative staining intensity (Fig. 8). High variability was observed in the OP groups treated with BMP-2.

For both markers studied, no differences were observed in any of the treatments between non-OP and OP rats (Fig. 8).

4. Discussion

T-1307 is a gel-forming poloxamine at above room temperature, near to body temperature, which was previously studied as an injectable system for bone regeneration (Rodríguez-Évora et al., 2014). The T-1307, once exposed to physiological temperatures, formed a soft gel, the addition of the alginate, once crosslinked, reinforced the structure and formed a hard gel at lower temperature. The alginate increased the viscosity of the T-1307 and reduced its transition sol-to-

Table 1
Platelet number (Plt) in blood and in the platelet rich plasma fraction from six donors. Concentration of each growth factor in the PRGF. Doses of the growth factors (pg) injected in 100 μL of the hydrogel system.

Plt/μL × 10^3 (blood)	Plt/μL × 10^3 (plasma)	Plt enrichment	PDGF-AB (pg/mL)	IGF (pg/mL)	TGF (pg/mL)	VEGF (pg/mL)
191 ± 56	384 ± 81	2.1 ± 0.5	24456 ± 7175	122784 ± 36027	25 ± 7.5	293 ± 86
Dose of GFs injected in the defect (pg/100 μL gel)			1883 ± 94.2	9454 ± 472.7	1.99 ± 0.10	22.5 ± 1.1

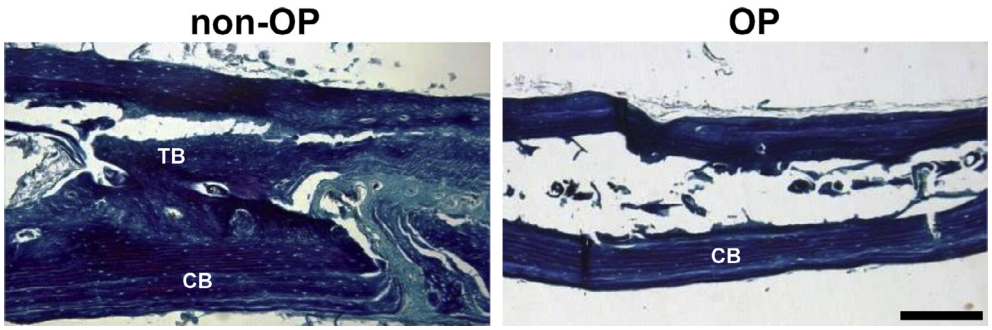

Fig. 4. Osteoporosis model. Representative images in transversal section of calvarial bone in non-OP and OP rats showing the differences in bone microarchitecture. Observe the difference in bone thickness, as well as the absence of trabecular bone in OP rats (VOF staining). CB: compact bone, TB: trabecular bone. Scale bar = 180 μm.

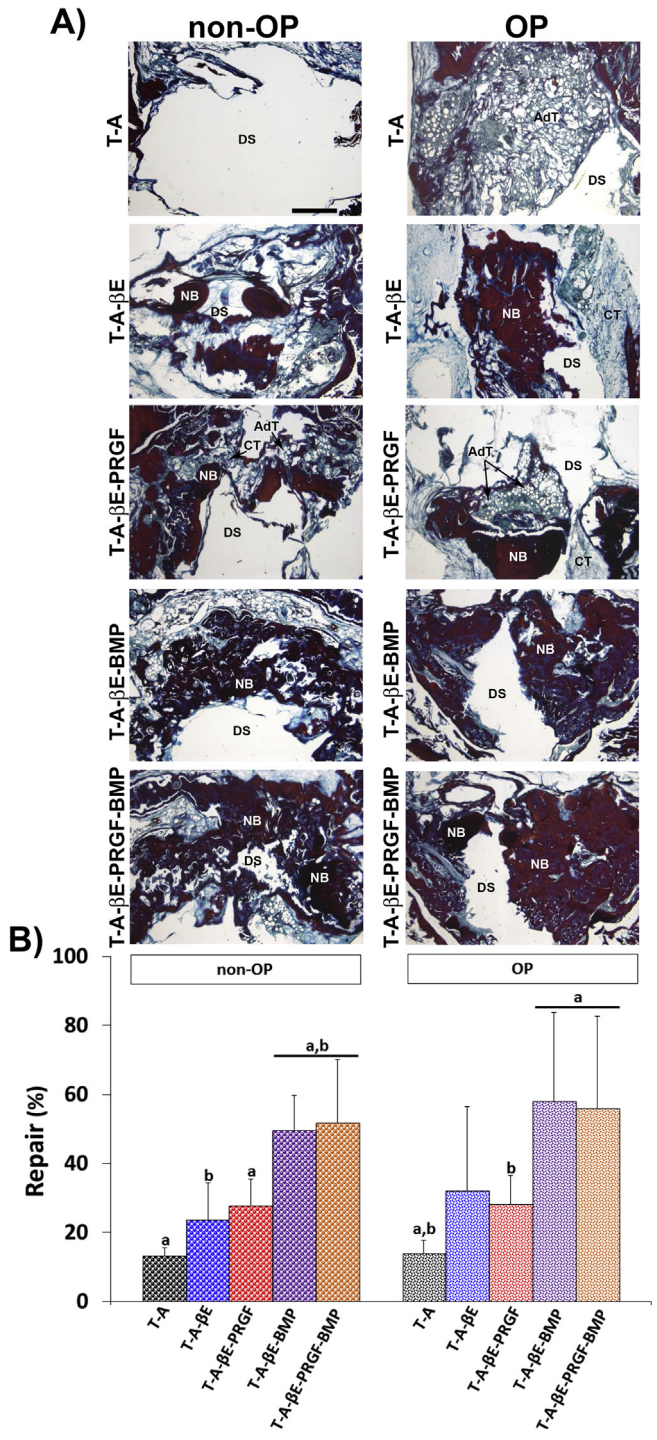

Fig. 5. Bone repair. Representative images in horizontal section of calvarial critical size defects in non-OP and OP rats showing the repair response at the defect level in the different experimental groups 12 weeks postimplantation A). Histomorphometrical analysis comparing of the degrees of repair (%) among the different experimental groups in non-OP and OP rats 12 weeks post-implantation B). Bars represent means ± SD (n = 3), $p < 0.05$. The identical letter on different bars indicates significant differences. AdT: Adipose tissue, CT: connective tissue, NB: newly formed bone, DS: defect site. Scale bar = 1.5 mm.

gel temperature. The systems used in this study were prepared and stored in cold to keep flowing because of the transition sol-to-gel temperatures were lower than RT. However, the gel was easy to handle and did not pose any problem when injecting. In fact, the dose content

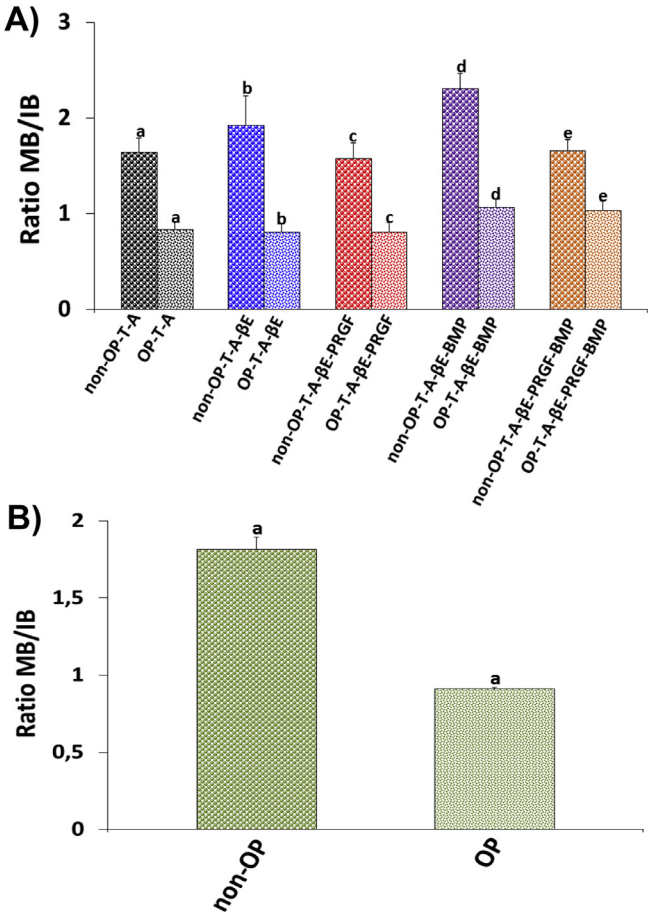

Fig. 6. Ratios MB/IB. Histomorphometric analysis showing the ratio between mature bone and inmature bone (MB/IB) among the different experimental groups A) and between non-OP and OP rats B), estimated using VOF staining. (n = 3), $p < 0.001$.

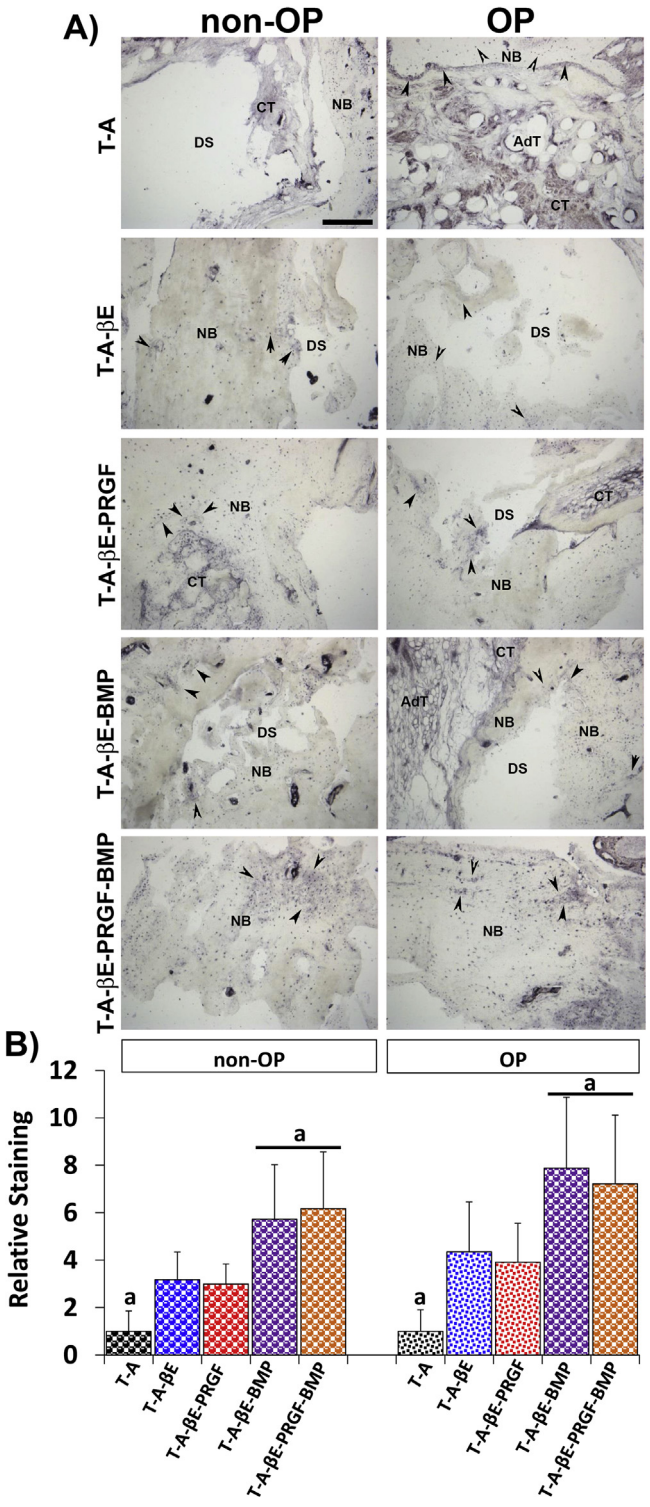

Fig. 7. Collagen type I. Representative images in horizontal section of calvarial critical size defects in non-OP and OP rats showing collagen type I immunoreactivity (ir) at the defect level in the different experimental groups 12 weeks postimplantation A). Histomorphometric analysis showing the relative staining values for collagen type I-ir B). Arrowheads indicate immunolabeling in osteoblasts, osteocytes, and areas of the extracellular matrix of new bone. Bars represent means ± SD (n = 3), $p < 0.05$. The identical letter on different bars indicates significant differences. AdT: Adipose tissue, CT: connective tissue, NB: newly formed bone, DS: defect site. Scale bar = 300 μm.

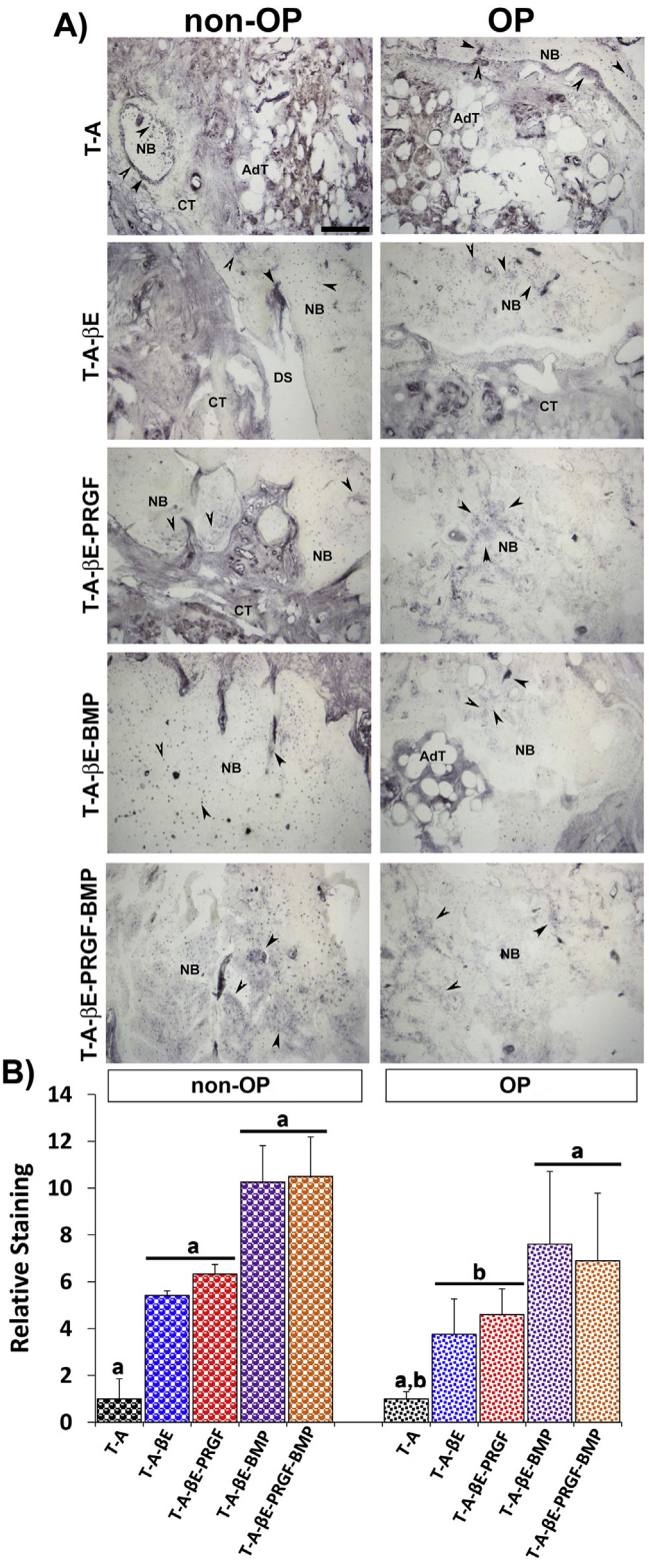

Fig. 8. Osteocalcin. Representative images in horizontal section of calvarial critical size defects in non-OP and OP rats showing osteocalcin immunoreactivity (ir) at the defect level in the different experimental groups 12 weeks postimplantation A). Histomorphometric analysis showing the relative staining values for osteocalcin-ir B). Arrowheads indicate immunolabeling in osteoblasts, osteocytes, and areas of the extracellular matrix of new bone. Bars represent means ± SD (n = 3), $p < 0.05$. The identical letter on different bars indicates significant differences. AdT: Adipose tissue, CT: connective tissue, NB: newly formed bone, DS: defect site. Scale bar = 300 μm.

was approximately 90% of the calculated dose after 10 min at RT, which supports its syringeability. In addition, the formation of the gel at temperatures below 37 °C was an advantage for implantation and reduced the time of surgery. Moreover, as was expected during incubation, the Tetronic was cleared up while the hard alginate structure remained and eroded, slowly increasing the porosity. In fact, taking into account that the process of regeneration of the bone defect in OP seems to be slower than in non-OP, the addition of the alginate was aimed to prolong the permanence of the scaffold in the defect, to act as an anchor and guide to improve the new tissue formation. The active ingredients as PRGF and the microspheres of the two drugs modified the rheological properties. Although an increase of the maximum viscosity by the addition of the PRGF to the system preparation (T-A-PRGF) was expected, the maximum viscosity was similar to the T-A prepared with water. The PRGF provoked a reduction of the transition sol-to-gel temperature. By contrast, the addition of the microspheres led to an increase of the viscosity and a further decrease of the transition temperature due to the hydrophobic interaction of the PLA-S and PLGA with the PPO blocks of the T-1307 as was previously observed in a Tetronic-pluronic-α-cyclodextrine hydrogel (Segredo-Morales et al., 2018a). Once the injectable system was characterized, the next objective was to validate that the local administration of 17β-estradiol and its combinations with PRGF or with BMP or the combination of the three agents can accelerate the regeneration process in a calvaria critical size defect in an OP rat model. Local delivery is appealing to avoid adverse effects of the systemic administration and to reduce the required dose of drugs. This study showed that the effect of 17β-estradiol alone or 17β-estradiol combined with PRGF slightly improved the defect repair process in both non-OP and OP animals, inducing minimal bone formation at the margins of the defect, with respect to the blank groups. The addition of BMP-2, a potent osteoinductor, to both combinations (T-A-βE-BMP and T-A-βE-PRGF-BMP), greatly improved the repair effect, the percentage of repair more than doubled with respect to the combinations without BMP-2. In this case, the formation of new bone was observed not only in the margins of the defect, but intramembranous ossification zones were observed inside it.

The histological and histomorphometrical analysis showed that new bone in non-OP rats was more mineralized than in OP groups, independently of the treatment. In fact, 17β-estradiol alone and its combination with PRGF and BMP-2, improved the repair percentage in a variable degree in both, non-OP and OP groups, with respect to the blank groups, but did not improve the mineralization as demonstrated the MB/IM ratio. The main actions of 17β-estradiol in bone through the estrogen receptor β, involves gene expression and synthesis of new proteins related to osteosynthesis and mineralization (Alhodhodi et al., 2017; Liao et al., 2016; Qiu et al., 2014; Wang et al., 2016). The effects of 17β-estradiol not only increase the bone matrix production by enhancing the transcription and translation of bone sialoprotein and Runx2, but also promoted the calcium deposition (Li et al., 2014; Liao et al., 2016). For all this, the addition of 17β-estradiol aimed to restore the local hormonal state in OP rats to balance bone synthesis/resorption and consequently improve the processes of regeneration and mineralization.

However, as in a previous study (Segredo-Morales et al., 2018b), the combination of 17β-estradiol with BMP-2 significantly improved the percentage of bone repair in OP rats, but did not improved the mineralization of the new bone. The explanation for the low mineralization observed in the newly formed bone in OP rats may be due to an ineffective action of 17β-estradiol as a consequence of a low concentration in the defect site. In this sense, the importance of the concentration levels of 17β-estradiol on the differentiation of other cell types, as chondrocytes, has been shown (Shi et al., 2017). Predicting the concentration of 17β-estradiol in the defect is difficult because 17β-estradiol is very poorly soluble in water and the release medium for in vitro tests is composed of 50% MeOH. In addition, as the PRGF did not affect the release kinetics of both 17β-estradiol and BMP-2, it was expected that 17β-estradiol release rate in vivo was slower than in vitro, in contrast to the release of BMP-2 (Segredo-Morales et al., 2018b). Therefore, in order to improve the previously observed 17β-estradiol release profile and increase the speed in the second phase in the present study, the polymer concentration, in the preparation of the microspheres, was slightly reduced. In contrast, the polymer concentration was doubled in the BMP-2 microspheres preparation to prolong the release of the protein (Segredo-Morales et al., 2018b). In this way it was intended to supply BMP-2 during or at least in the first stage of the regenerative process, which is delayed in the OP. Although the PRGF exerts its action in the first stages of the regeneration process, when incorporated into the hydrogel it was expected to increase the residence time in the defect and favour its action. However, in this study, PRGF did not produce any improvement in the regeneration of the defect and did not even potentiate the effect of 17β-estradiol or BMP-2, probably, because the dose of the GFs from the PRGF in the volume of the injected system in the defect was much lower than the BMP-2 in the microspheres. In any case, the conditions present in the environment of the defect are probably not the most appropriate. The cells at the edge of the defect would be the only ones available to interact with the agents included in the system and probably much of the amount released at short times was wasted because it cannot be used by them. Even, the 17β-estradiol might act through another subtype of receptor such as αER or GPR130 leading to a different cellular response. At last, as was reported, the efficacy of plasma fractions as PRGF depend on several factors, and the outcome of therapy is variable and unpredictable in orthopedic patients. The PRGF has been used successfully in different injuries (Anitua et al., 2012; Rodríguez-Agirretxe et al., 2017; Vaquerizo et al., 2017). However, even accepting its contribution in the repair of some tissue defects, its role in complicated bone injuries is not proven (Oryan et al., 2015). The present study, carried out in an OP rat model by combination of ovacteriomy and treatment with steroids, demonstrated its lack of efficacy in compromised bone lesions.

5. Conclusions

The addition of alginate to Tetronic reinforced the structure of the thermoresponsive gel increasing the scaffold stability. The gel-forming temperature of the T-A system below the body temperature reduced the surgery time. Moreover, this formulation maintained the gelling properties when reconstituted with water or PRGF after being freeze-dried. The bone repair induced by the different treatment in OP and non-OP groups were not significantly different. The addition of BMP-2 increased the response to 17β-estradiol but no change was observed with the PRGF. However, the mineralization of the new bone formed in OP groups was notably inferior than in non-OP groups.

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Declarations of interest:

None.

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