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Thermosensitive eyedrops containing platelet lysate for the treatment of corneal ulcers

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ABSTRACT

Corneal lesions cause significant pain and visual impairment and, in many cases, are unresponsive to conventional treatments. Platelet lysate (PL) is an haemoderivative rich in growth factors (GFs) that are released by platelets after freeze-thawing destruction of platelet rich plasma (PRP). The aim of the present work was to develop thermosensitive and mucoadhesive eyedrops to maintain and prolong the contact of platelet lysate (PL) with cornea ulcers.

A sterile vehicle based on chondroitin sulphate sodium (CS) and hydroxypropylmethyl cellulose (HPMC) was developed. An extemporaneous loading of the vehicle with PL was performed and the obtained formulation was able to quickly thermogelify at about 32 °C and was characterized by good mucoadhesive properties. ELISA evidenced that the growth factor PDGF AB was compatible with the vehicle and stable in the formulation up to 15 days of storage at 2–8 °C.

In vitro wound healing and proliferation test (performed using rabbit corneal epithelial cells (RCE)) showed that the formulation enhanced cell growth and put in evidence a synergistic effect of CS and PL in stimulating cell proliferation.

The overall results indicate that PL loaded in thermosensitive and mucoadhesive eyedrops can be profitably employed to treat corneal lesions.

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1. Introduction

Cornea is an epithelium lacking in blood vessels. It is composed of three cell layers: epithelial cells, stromal cells and endothelial cells. Additionally, it contains Descemet's membrane, a thick basement membrane between the stroma and endothelium. In humans there is also Bowman's layer, a thickened acellular collagenous zone between the epithelium and stroma (Yu et al., 2010). The corneal epithelium forms a protective barrier and serves as the main refractive element of the visual system. The healing of corneal wounds, that can be caused by injuries or surgical interventions or disease such as keratitis and keratopathy, is normally fast to re-establish epithelial barrier function (Lu et al., 2001).

Successful wound healing involves a number of processes including cell migration, cell proliferation, re-stratification, as well as matrix deposition and tissue remodelling. Particularly critical are cell migration and proliferation, which are driven by growth factors released into the injury sites. 12–24 h after cornea damage, the depleted stroma is repopulated by means of keratocytes, fibroblasts and possibly myofibroblasts. In the meantime, within the first 24 h of injury, apoptotic and necrotic debris is phagocytized due to stromal infiltration by macrophages/monocytes, T cells and polymorphonuclear cells. One to 2 weeks following injury, myofibroblasts, which derive from keratocytes responding to TGF-β (transforming growth factor-beta), appear and provide to remodelling collagen and extracellular matrix through production of collagen, glycosaminoglycans, collagenases, gelatinases and MMPs (matrix metallo proteinases). Myofibroblasts slowly disappear over the ensuing weeks, although the process may continue for months to years (Dupps and Wilson, 2006).

Persistent corneal epithelial defects (CEDs) are associated with decreased production of tears or reduced corneal sensitivity. CEDs cause significant pain and visual impairment, are often unresponsive to conventional treatments and are therefore still difficult for ophthalmologists to treat (Yamada et al., 2008).

Although many therapies have been proposed for the treatment of corneal lesions, the management of these conditions remains problematic and the healing with the standard protocols is often unattainable (Geremicca et al., 2010).

Platelets are specialized secretory cells that release from intracellular alpha granules, in response to activation, a large number

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of biologically active substances and in particular growth factors (GFs). These play an important role in healing process by starting and by amplifying the lesion resolution (Geremicca et al., 2010).

The most intensively investigated GFs derived from platelets are PDGF (platelet derived growth factor), TGF-α and β (transforming growth factors alpha and beta), FGF (fibroblast growth factor), EGF (epidermal growth factor), VEGF (vascular endothelial growth factor). Some of these GFs are available in purified form, but since tissue repair cannot be effectively mediated by a single agent, GF pool obtained by platelets is able to furnish multiple signals essential to complete the regeneration process (Anitua et al., 2006). Moreover it is also recognized that the efficacy of the GFs critically depends on the way they are made available to the injured tissue.

Recently platelet lysate (PL) which is an haemoderivative based on bioactive molecules (especially GFs) released by platelets after freeze-thawing destruction usually starting from a platelet rich plasma (PRP), has been proposed in clinical practice for the treatment of oral mucositis (Del Fante et al., 2011).

The development of suitable therapeutic vehicles is of paramount importance to deliver growth factors according to the repairing requirements. This represents a challenge especially when the application site is the cornea where the residence time and the contact between formulation and injured tissue could be too short to exert the therapeutic effect due to the blinking and the lachrymation (removal mechanisms).

In a previous work of ours (Sandri et al., 2011) platelet lysate (rich in growth factors and other bioactive molecules involved in the healing process) was loaded in a mucoadhesive vehicle based on polyacrylic acid or chitosan (well known mucoadhesive polymers) as viscous eyedrops. The formulation based on chitosan and platelet lysate was able to enhance cell growth whilst polyacrylic acid was less efficient.

Chondroitin sulphate (CS) is a sulphated glycosaminoglycan (GAG) that is covalently bond to a variety of protein cores to form proteoglycans. A function of GAG is the sequestering of secreted growth factors at cell membranes useful for tissue morphogenesis (Lin, 2004). Negatively charged GAG including heparin, heparan sulphate and CS are capable of electrostatic interaction with positively charged GFs and in particular with basic fibroblast growth factor (bFGF), insulin like growth factor (IGF), VEGF, PDGF and TGF-β, resulting in stabilization and reduced degradation to stabilize and to prevent degradation of growth factors in solutions (Lim et al., 2011).

Given these premises the aim of the present work was to develop thermosensitive and mucoadhesive eyedrops containing platelet lysate for the treatment of corneal lesions. The formulation was based on CS associated with hydroxypropylmethylcellulose (HPMC), which exhibits thermosensitive gelation with sol–gel transition depending on temperature (Sarkar, 1995). The in situ gelling properties should increase precorneal retention time. The proposed vehicle was intended to be extemporaneously loaded with platelet lysate (PL). The formulations were subjected to rheological characterization to put in evidence the thermogelling properties. Mucoadhesion was studied by means of tensile test. Compatibility and stability of PL in the formulation was determined by means of ELISA of PDGF AB, assumed as representative of platelet GFs. The bioactive properties of PL loaded formulation were assayed by means of in vitro proliferation and in vitro wound healing on rabbit corneal epithelium cell cultures (RCE).

2. Experimental part

2.1. Materials

The following polymers were employed:

- Chondroitin-6-sulphate sodium (CS) (Bovine 100 EP, Bioiberica, S, kind gift from Prochifar s.r.l., Milan, I).
- Hydroxypropylmethyl cellulose (HPMC) (Metolose 60 SH-4000 Shin-Etsu, J).

Platelet lysate (PL) was obtained by Apheresis Service of Immunohaematology and Transfusion Service Center for transplant immunology (Fondazione IRCCS Policlinico S. Matteo, Pavia, I) employing a sterile connection technique. Aliquots of hyperconcentrate platelets (high platelet concentration in small plasma volume and minimal leukocyte contamination) were obtained from apheresis performed on regular blood donors. Platelet pool was frozen at −80 °C for 5 h and subsequently defrozen in a sterile water bath at 37 °C. An automated platelet count and tests for aerobial, anaerobial and fungi contamination after saline dilution were performed.

2.2. Methods

2.2.1. Preparation of formulations

CS and HPMC have been solubilized in saline solution (NaCl 0.9%, w/v) in ice cold bath. CS and HPMC solutions were mixed at 1:1 weight ratio to obtain final formulations containing HPMC at final concentration of 2% (w/w) and CS at concentrations of 2 and 4% (w/w).

PL loaded formulation was prepared by mixing 3 parts of a concentrated vehicle (2.7%, w/w HPMC and 5.3%, w/w CS) with 1 part of PL to obtain the same final polymer concentrations as previously set.

2.2.2. Rheological characterization

The rheological analysis was carried out by means of a rotational rheometer (Rheostress RS600, Haake, Karlsruke, G), equipped with a cone plate combination (C35/1: diameter = 35 mm; angle = 1°) as measuring system.

The formulations were subjected to oscillation measurements, performed at constant shear stress value that was chosen in the linear viscoelastic region, previously experimentally determined for each sample using stress sweep test in which increasing shear stress at low (0.1 Hz) frequency at 32 °C was applied. The viscoelastic response of the sample obtained in the oscillation test was expressed by the storage (G') modulus. The measurements were performed at a constant value of frequency (0.1 Hz) and at temperature values ranging between 8 and 42 °C (heating rate: 1 °C/min), to evaluate the gelation temperature of the samples (equilibrium time: 180 s). A steep increase in G' indicates sol–gel transition.

The formulations were characterized also for gelation time at gelation temperature previously determined: G' was recorded at 0.1 Hz frequency as function of time: the time required to obtain G' plateau value indicates gelation time.

PL loaded formulation was also characterized for viscosity at $10\,s^{-1}$ and G' at 1 Hz frequency at different temperatures: 25, 32, 35 °C to confirm the influence of thermogelation behaviour at room temperature and after application site temperature.

2.2.3. Mucoadhesion measurements

Mucoadhesion measurements were performed using TA.XT plus (Texture analyser, ENCO, Spinea, I) equipped by 1 kg load cell, cylinder probe of 1 cm and the measuring system A/MUC (mucoadhesion test ring) (Szűcs et al., 2008). The measuring system A/MUC consists of a ring in which the biological support can be fixed. In this case the support is a filter paper disc wetted with 100 μl of 8% (w/w) mucin dispersion (mucin type: type II crude, Sigma–Aldrich, Milano, I) in simulated lachrymal fluid (pH 7.4). Artificial lachrymal fluid was prepared by dissolving $NaHCO_3$ 2.2 g/l, NaCl 6.26 g/l, KCl 1.79 g/l, $CaCl_2{\cdot}2H_2O$ 0.0735 g/l, and $MgCl_2{\cdot}6H_2O$ 0.0964 g/l in distilled water

(Bonferoni et al., 2007). 20 mg of each formulation were applied on the cylinder probe. Sample and biologic substrate were put in contact with a preload of 6000 mN for 3 min. Cylinder probe was moved upward at a prefixed speed of 2.5 mm/min up to the complete separation of the mucoadhesive interface (mucin-sample).

The force of detachment as a function of displacement was recorded and the parameter work of adhesion (mN mm) (AUC) was calculated as area under the "force vs displacement" curve (Caramella et al., 1994).

2.2.4. Assay of growth factors

The concentration of platelet derived growth factor PDGF AB in the PL and in the formulations was assayed by means of ELISA (Human PDGF AB Quantikine PharmPak, R&D Systems, Minneapolis, MN, USA; assay range: 31.2–2000 pg/ml). The concentration of PDGF AB in the formulation was related to the concentration of PDGF AB in the platelet lysate employed for their preparation at time zero and a parameter "% PDGF AB" was calculated as

$$\frac{\text{concentration of PDGF AB in the formulation}}{\text{concentration PDGF AB in PL}} \times 100$$

The formulation and PL were stored at 2–8 °C for 15 days and PDGF AB concentration in both samples was determined at fixed times (0, 1, 4, 7, 11 and 15 days).

2.2.5. Proliferation test

RCE cell line (rabbit corneal epithelial cells) was obtained from the European Cell Culture Collection (N° 95081046, ECACC, Salisbury, UK). Cells with passage numbers 8–12 were used.

RCE were grown in a medium having the following composition: Dulbecco's Modified Eagle Medium (DMEM) mixed 1:1 with Ham's nutrient mixture F12, supplemented with L-glutamine (1%, v/v, 2 mM), a mixture of penicillin (100 IU/ml), streptomycin (0.1 mg/ml) and amphothericin B (0.25 μg/ml), foetal bovine serum (15%, v/v), epidermal growth factor (10 ng/ml) and insulin (5 μg/ml) (Sigma, Milan, I). The cells were incubated at 37 ± 0.5 °C in a humidified atmosphere, containing 5% CO_2.

20 μl of RCE suspension were seeded in each well of 96-well plates (area of 0.34 cm^2) at density of 7500 cells/well. 200 μl of each sample were simultaneously put into the well. The cells were co-seeded in the following media: complete growth medium (standard growth conditions), minimal medium Mm (not supplemented with foetal calf serum) for the control; Mm containing PL diluted 1/20 (5% (w/w) final concentration); CS at 4% (w/w) diluted 1/20 in Mm; CS at 4% (w/w) containing PL diluted 1/20 in Mm; HPMC at 2% (w/w) diluted 1/20 in Mm; HPMC at 2% (w/w) containing PL diluted 1/20 in Mm; formulation without PL diluted 1/20 in Mm; formulation containing PL diluted 1/20 in Mm.

Well plate was kept at 37 °C in an atmosphere of 95% air and 5% CO_2 and 95% of relative humidity for 24 h. After 24 h, the cells were subconfluent and attached to the well bottom and Neutral Red (NR) test (Tox Kit 4, Sigma–Aldrich, Milano, I) was performed. This determines the accumulation of NR supravital dye in the lysosomes of viable, uninjured cells. Cell membrane or lysosomes damages cause a poor or no capability to pick up NR.

Each well was washed with saline phosphate buffer (PBS) to remove surnatants. 200 μl of NR solution (0.33 mg/ml in DMEM) were put in each well for 2 h of contact time. Cell substrates were then washed with PBS and the fixing medium (1% $CaCl_2$ and 0.5% formaldehyde aqueous solution), to wash NR not entrapped in the cells and to fix the substrate. Such a fixing solution was then removed and a solubilizing solution (1% of acetic acid in ethanol) was added to each cell substrate to cause cell disruption and to release NR captured by viable cells. The NR solution absorbance was determined by means of a plate reader (Perkin Elmer, Milan, I) at wavelength of 490 nm with 650 nm reference wavelength. The absorbance read for each sample was compared with that of cells in complete growth medium as positive control (growth in standard conditions), that was considered as 100% viability (Sandri et al., 2011).

2.2.6. In vitro wound healing test

In vitro wound healing test is based on the employment of Petri μ-Dish (Ibidi, Giardini, Milan, I) in which an insert is enclosed. The insert is made of 2 chambers with a growth area of 0.22 cm^2 divided by a septum corresponding to a cell free gap of 500 ± 50 μm.

RCE were seeded in each chamber at 10^5 cells/cm^2 and grown at confluence in standard conditions as previously described. After 24 h cells reach confluence and the insert is removed displaying 2 areas of cell substrates divided by the prefixed gap. Cell substrates were put in contact with 200 μl of formulation diluted 1/20, with PL at 1/20 concentration and with the complete medium. At prefixed times (0, 24, 48, 72, 98 h) microphotographs were taken to evaluate the invasion and cell growth in the gap.

2.2.7. Statistical analysis

Statistical differences were determined using Mann–Whitney *W* test (Stat Graphics 5.0, Statistical Graphics Corporation, Rockville, MD, USA). Differences between groups were considered to be significant at $p < 0.05$.

3. Results and discussion

3.1. Rheological characterization

Fig. 1 reports *G*′ (elastic modulus) profiles vs temperature of unloaded formulations based on CS and HPMC. The following samples were evaluated: mixtures of CS at concentrations of 2% (w/w) and 4% (w/w) with HPMC at fixed concentration of 2% (w/w) and HPMC at 2% (w/w) alone. As reported in literature (Talasaz et al., 2008) HPMC gelation is induced by temperature variations and is directly related to polymer concentration. This is caused by hydrophobic interaction between polymeric chains containing methoxy substitution. At low temperatures, the macromolecules are hydrated, and polymeric chains scarcely interacts whilst simple entanglements occur. At higher temperature, when a partial dehydration of the polymer occurs, polymer–polymer chain association takes place, and HPMC solution becomes gel with a network structure, occurrence indicated by sharply increase in viscosity (Talasaz et al., 2008). It is reported that the presence of salts decreases the gelation temperature by means of salting-out mechanisms: in particular 1% (w/w) HPMC gelation passes from 61 °C in water to 56.3 °C in NaCl 0.2 M (Liu et al., 2008).

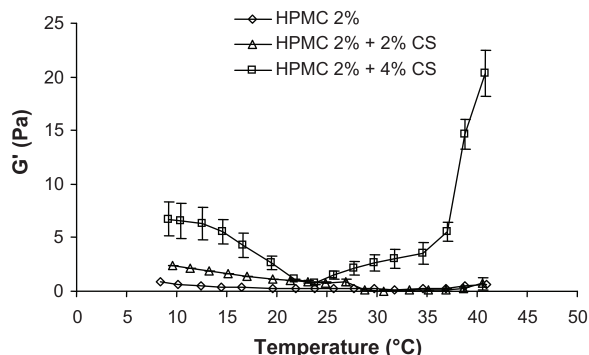

Fig. 1. *G*′ (elastic modulus) profiles vs temperature of unloaded formulations based on CS and HPMC. In particular the following samples were evaluated: mixtures of CS at concentrations of 2% (w/w) and 4% (w/w) and HPMC at fixed concentration of 2% (w/w) and HPMC at 2% (w/w) alone (mean values ± sd; *n* = 6).

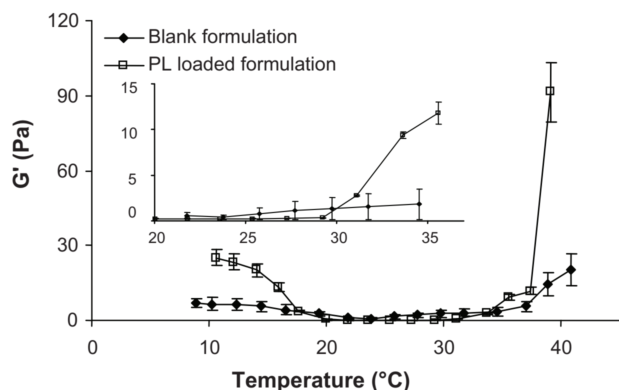

Fig. 2. *G'* (elastic modulus) profiles vs temperature of unloaded and PL loaded formulations based on CS 4% and HPMC 2% (mean values ± sd; *n* = 6).

The increase of CS concentration from 2 to 4% (w/w) decreased the gelation temperature of HPMC dissolved in NaCl 0.9% (w/v). In particular the unloaded formulation based on HPMC 2% (w/w) and CS 4% (w/w) was characterized by a slight *G'* increase at about 25 °C and a further sharp *G'* increase at temperature of about 35 °C. Moreover it is reported that SO_4^{2-} groups of CS (ions of Hofmeister series or lyotropic series), are able to dramatically influence HPMC chain hydration and their interaction with medium ions: this mechanism, not yet entirely clear, is however normally used to precipitate proteins by means of salting out and it seems to be related to specific interactions between ions and macromolecules in competition with the interactions between ions and the water molecules.

Fig. 2 reports *G'* (elastic modulus) profiles vs temperature of unloaded and PL loaded formulations based on CS 4% and HPMC 2%. PL loaded formulation was characterized by a sharp increase of elastic parameter at 32–35 °C. PL loading decreased the sol–gel transition temperature of HPMC to the physiological ophthalmic range. This could be due to the assistance of PL component to the HPMC chain dehydration.

The increase of *G'* modulus was quite moderate with respect to typical thermogelifying systems. However this seems suitable for ophthalmic application, as a moderate hardening of the gel is conceivable to prolong formulation precorneal residence without causing patient's discomfort, blurred vision and eventual nasolachrymal occlusion.

Fig. 3 reports *G'* profiles vs time of unloaded and PL loaded formulations based on CS 4% and HPMC 2%. The unloaded and the PL loaded formulations presented constant *G'* profiles as a function of time (Kruskall–Wallis test, Post hoc Box and Whisker plot) to

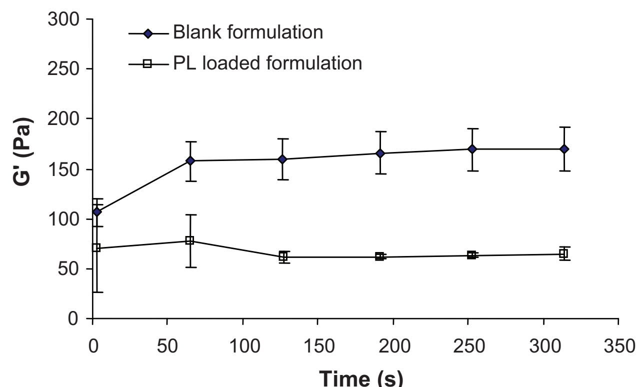

Fig. 3. *G'* profiles vs time of unloaded and PL loaded formulations based on CS 4% and HPMC 2% (mean values ± sd; n = 6).

Table 1
Values of viscosity and *G'* evaluated for PL loaded formulation at 25, 32, 35 °C.

Temperature °C	Viscosity (mPa s) (at 10 s^{-1}) (mean values ± sd, *n* = 6)	*G'* (Pa) (at 1 Hz) (mean values ± sd, *n* = 6)
25	146 ± 8	7 ± 4
32	192 ± 22	763 ± 148
35	345 ± 31	750 ± 95

indicate that sol–gel transition instantly occurred: this is a crucial aspect as fast gelation is an advantage for formulation that must resist to the removal mechanisms of ophthalmic region (blinking and lachrymation) that are active in the first few seconds after application.

In Table 1 the viscosity and *G'* values of PL loaded formulation are reported. The viscosity and *G'* of PL loaded formulation was significantly higher at 32–35 °C with respect to 25 °C to indicate that the application/administration can determine an increase of formulation consistency caused by gelation.

3.2. Mucoadhesion properties

In Fig. 4 the results of mucoadhesion measurements are reported for unloaded and PL loaded formulations. Both the formulations were characterized by good mucoadhesive properties as it is shown by values of the parameter work of adhesion significantly higher in presence of mucin substrate with respect to those of blank measurements, moreover, since the results obtained for PL loaded formulation were superimposable to those obtained for the unloaded one, the presence of platelet lysate did not impair the mucoadhesive properties of the polymers.

3.3. Assay of growth factors

Fig. 5 illustrates the results of ELISA assay of PDGF AB, assumed as representative of PL growth factors in PL after loading in the formulation. This quantification was performed during 15 days of storage at 2–8 °C to simulate in use conditions.

The profile of % PDGF AB was close to 100% for the PL loaded formulation up to 15 days. PL stored in the same conditions presented a significant decrease in % of PDGF AB after 24 h and a concentration that remained afterward close to 80%. Formulation seems to be characterized by stabilization properties towards PDGF AB. This can be conceivably argued also for other growth factors especially for the basic ones, as it is described in the literature that CS, being a GAG, is able to bound growth factors via electrostatic interactions (Deepa et al., 2002; Lim et al., 2011; Macri et al., 2007). This can explain the observed increase PL growth factor stability and compatibility with polymeric vehicle.

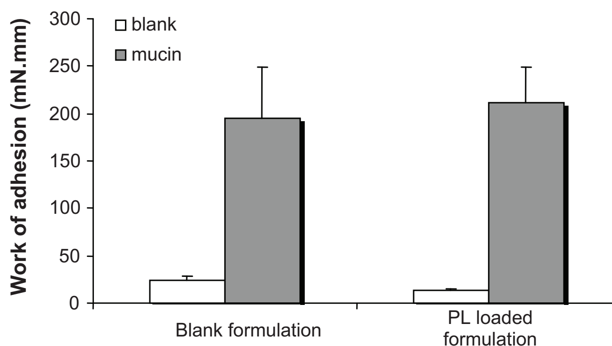

Fig. 4. Values of the mucoadhesion parameter work of adhesion evaluated for unloaded and PL loaded formulations (mean values ± sd; n = 9).

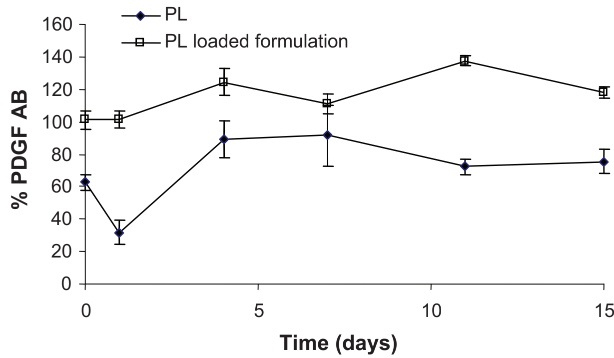

Fig. 5. % values of PDGF AB of formulation and PL as such, calculated with respect to fresh PL evaluated for 15 days of storage at 2–8 °C (in use conditions) (mean values ± sd; n = 6).

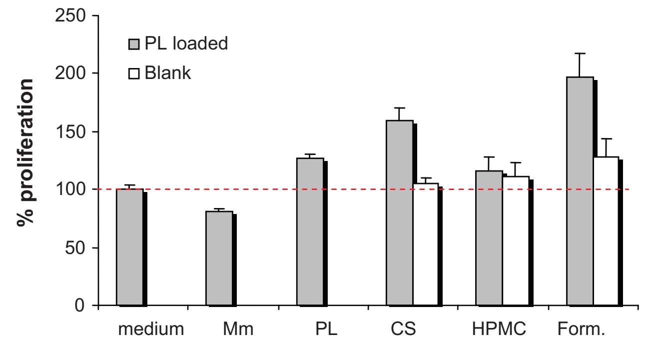

Fig. 6. % proliferation values evaluated for formulation and each component of the formulation (HPMC and CS, at the same concentrations) in presence and without PL, in comparison with PL, with a negative control medium without serum (Mm) and complete medium (medium) (mean values ± sd; n = 6). Statistical evaluation (Mann–Whitney W test): Medium vs Mm $p < 0.001$; Mm vs PL $p < 0.001$; medium vs PL $p < 0.001$; PL vs CS PL $p = 0.08$; PL vs HPMC PL $p = 0.460$; PL vs Form PL $p = 0.004$; Mm vs CS $p < 0.001$; Mm vs HPMC $p = 0.021$; Mm vs form $p = 0.002$; CS with PL vs (w/o) PL $p < 0.001$; HPMC with PL vs (w/o) PL $p = 0.787$; form with PL vs (w/o) PL $p < 0.023$.

3.4. Proliferation properties

In Fig. 6 the % proliferation values evaluated for formulation and each component of the formulation (HPMC and CS, at the same concentrations) in presence and without PL are reported for RCE cells. The properties were compared with that of PL as such and with a negative control medium without serum (Mm) and complete medium (medium).

Although lacking of GFs, the polymeric samples (CS, HPMC and unloaded formulation) presented proliferation performances significantly higher than those of Mm. This is in line with the data from literature in the case of CS (Zou et al., 2009) whilst represents quite a new finding in the case of HPMC. However the addition of PL to HPMC sample did not increase proliferation properties, whilst CS was able to enhance PL driven cell growth, confirming the stabilizing effect of such polymer on growth factors. This effect, conceivably due to CS, was still visible in the PL loaded formulation even if CS was associated with HPMC.

3.5. Wound healing properties

In Fig. 7 the microphotographs of RCE substrates with gap, put in contact with control (medium not supplemented Mm), PL, unloaded formulation (b form), and PL loaded formulation are reported for different times: time 0 (just after the contact with the samples), 24, 48 h. PL was diluted to have the same final concentration as in the PL loaded formulation.

At time zero (t = 0) for all substrates the cell gap can be seen in the pictures. After 24 h of contact (t = 24) with PL loaded formulation, cells showed a subconfluent growing filling the gap, whilst, in the substrates in contact with both the control and the PL, a reduced but still detectable gap was visible. The unloaded formulation behaviour was comparable to that of control: in both cases the

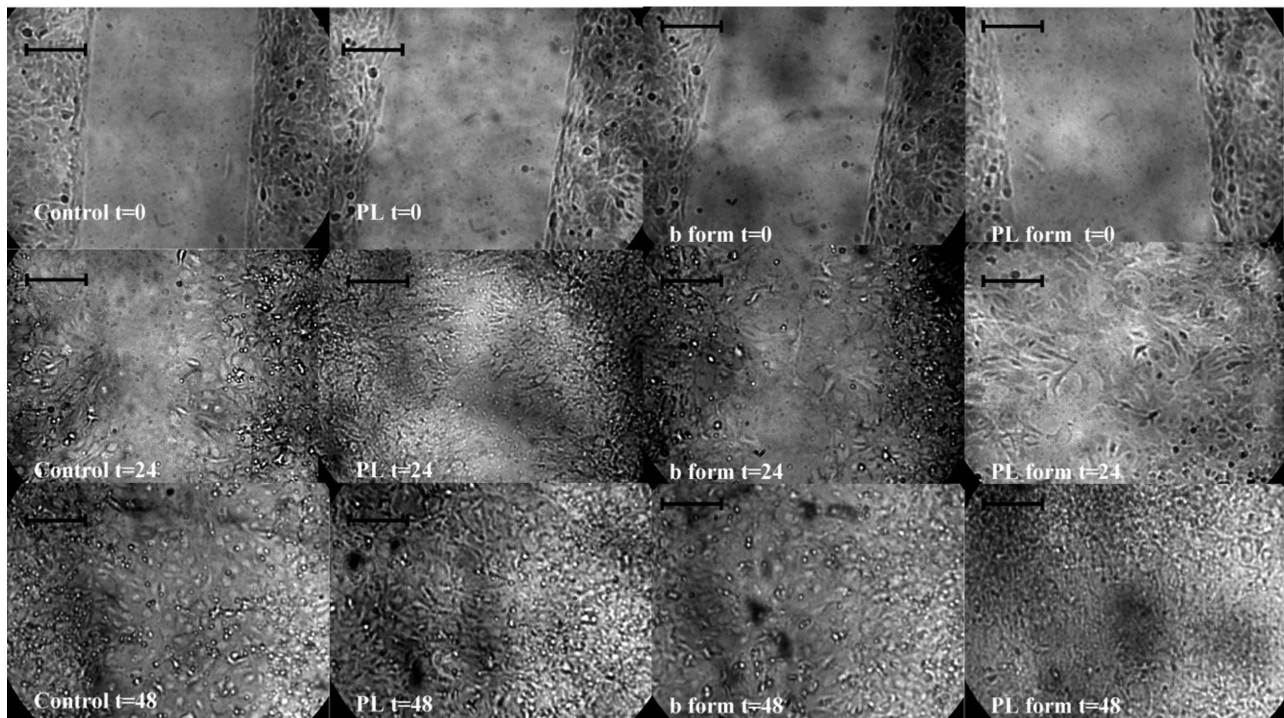

Fig. 7. Microphotographs of RCE substrates with gap, put in contact with Mm (medium not supplemented), PL, unloaded formulation (b form), and PL loaded formulation for different time: time 0, 24, 48 h. Bars = 200 microns.

cell growth was probably promoted by the cell medium in which the unloaded formulation was diluted. PL loaded formulation was as able to promote cell growth more efficiently than PL alone. This indicates that the formulation was able to enhance cell growth in presence of PL, conceivably due to stabilizing effect of CS on growth factors present in PL.

4. Conclusions

The thermosensitive eyedrops based on HPMC 2% and chondroitin sulphate 4% loaded with platelet lysate showed a sol–gel transition at 32–35 °C. The association of CS to HPMC in saline solution determined a lowering of gelation temperature of HPMC by means of salting-out mechanisms caused by NaCl of saline solution and by SO_4^{2-} of CS. Salting-out decreased the sol–gel transition temperature due to partial dehydration of HPMC chains. The increase in viscosity due to gelation was relatively low: this should have the advantage to avoid the discomfort of a rigid system, such as an insert, in the precorneal region. Moreover, at the same time, it should allow a higher resistance of the formulation towards eye removal mechanisms (lachrymation and blinking). This was also supported by the good mucoadhesion behaviour, not impaired by platelet lysate components.

In particular gelation and mucoadhesion properties should favour the permanence of PL in contact with corneal lesions in the precorneal area.

Eyedrops based on CS and HPMC demonstrated good compatibility with PDGF AB up to 15 days (in use stability) probably due to interactions of CS and growth factors, as described in literature (Deepa et al., 2002; Lim et al., 2011; Macri et al., 2007) which should protect growth factors from degradation.

The eyedrops formulation was able to promote in vitro RCE cell proliferation and migration: this should promote in vivo tissue reparation. Further investigations should be conducted to evaluate in vivo properties.

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