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Preparation, fabrication and biocompatibility of novel injectable temperature-sensitive chitosan/glycerophosphate/collagen hydrogels

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Abstract This paper introduces a novel type of injectable temperature-sensitive chitosan/glycerophosphate/collagen (C/GP/Co) hydrogel that possesses great biocompatibility for the culture of adipose tissue-derived stem cells. The C/GP/Co hydrogel is prepared by mixing 2.2% (v/v) chitosan with 50% (w/w) β-glycerophosphate at different proportions and afterwards adding 2 mg/ml of collagen. The gelation time of the prepared solution at 37°C was found to be of around 12 min. The inner structure of the hydrogel presented a porous spongy structure, as observed by scanning electron microscopy. Moreover, the osmolality of the medium in contact with the hydrogel was in the range of 310–330 mmol kg^{-1}. These analyses have shown that the C/GP/Co hydrogels are structurally feasible for cell culture, while their biocompatibility was further examined. Human adipose tissue-derived stem cells (ADSCs) were seeded into the developed C/GP and C/GP/Co hydrogels (The ratios of C/GP and C/GP/Co were 5:1 and 5:1:6, respectively), and the cellular growth was periodically observed under an inverted microscope. The proliferation of ADSCs was detected using cck-8 kits, while cell apoptosis was determined by a Live/Dead Viability/Cytotoxicity kit. After 7 days of culture, cells within the C/GP/Co hydrogels displayed a typical adherent cell morphology and good proliferation with very high cellular viability. It was thus demonstrated that the novel C/GP/Co hydrogel herein described possess excellent cellular compatibility, representing a new alternative as a scaffold for tissue engineering, with the added advantage of being a gel at the body's temperature that turns liquid at room temperature.

1 Introduction

The fundamental principle underlying the concept of tissue engineering lays in the ability to inoculate functional cells into a three-dimensional (3D) scaffold/extracellular matrix substitute to render it a specific performance, and then use it to replace or repair injured tissues in human recipients that require cell therapies [1–3]. These 3D scaffolds are mainly classified into two categories: porous (or "hard") and injectable (or "soft") scaffolds [4]. The latter type has been the focus of an increasing attention due to the advantages of in situ gelation, such as its ease of operation, molding pattern, lower trauma and allow avoiding surgeries [5–7].

3D scaffolds made from or with biological chitosan have been widely used in basic biomedical fields and clinic therapies due to their well-known biocompatibility, non-immunogenicity, and biodegradability [8]. Recent studies have shown that a mixture of chitosan and β-glycerophosphate (GP) can form injectable scaffolds [9, 10] due to their temperature-sensible properties that change from

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liquid at room temperature to a gel after injection into the body. The high concentration of ions in the GP molecule, however, leads to a relatively low cellular biocompatibility that limits the application of such hydrogel. Cho et al. [11] grafted isopropyl acrylamide onto hydrosoluble chitosan to obtain hydrosoluble chitosan-g-polyisopropyl acrylamide copolymer. It becomes an opaque and loose hydrogel at 32°C, although the in vivo biodegradability and biocompatibility of isopropyl acrylamide still requires further research. Another type of hydrogel is poly system (ethylene oxide-propylene oxide- ethylene oxide), which has been shown to not only biologically degrade, but demonstrated certain toxicities in rat in vivo experiments [12].

Being one of the most ideal matrices to support cellular growth known up to now and the first natural biological material applied in cell culture [13], collagen has the advantages of having low immunogenicity, special biocompatibility and biological degradability. Collagen has a unique molecular identifying signal system that can improve cellular adhesion, proliferation and differentiation, hence providing a suitable scaffold bed for cellular expansion and differentiation [14]. Moreover, this protein can decompose into several types of amino acids in vivo, being nontoxic, nonirritant and having been proven not to trigger immunoreactions. Collagen also possesses the added advantage of gelating at the body's temperature, although with the disadvantages of having a fast degradation rate and a relatively low mechanical strength.

In order to develop a novel temperature-sensitive injectable hydrogel scaffold, we have optimized a blend mixture of chitosan, β-glycerophosphate and collagen, which was further studied for its biocompatibility using human adipose-derived stem cells (ADSCs) and carried out the fabrication of hydrogel-ADSCs constructs in vitro.

2 Materials and methods

2.1 Materials and equipment

The materials used to perform the experiments herein reported are as follows: Rat tail collagen (Chuang'er Biotech. China); chitosan (Deacetylation 93%, Haidebei halobios engineering Ltd., Jinan); β-glycerophosphate and collagen type I, phosphate buffered saline (PBS) and fetal bovine serum (FBS) (Sigma, USA); h-Dulbecco's Modified Eagle Medium (h-DMEM, GIBCO, USA); fetal cattle serum (Minhai Ltd., Lanzhou, China); kit-LIVE/DEAD Viability/Cytotoxicity (Invitrogen, USA). The instruments used were as follows: inverted fluorescence microscope (IX71, Olympus); digital color camera system (Sony-3 CCD); Image analysis system (Image-pro-plus); microplate reader (Bio-Rad, USA); Acid base indicator (Benchtop); pH meter (Beckman, USA); osmometer (Wescor, USA) and SRKLYOVAC GT2-9 freezer dryer (LABCONCO, USA).

2.2 Cell culture

The adipose tissue was provided by Dr. Sha from the Cosmetic Plastic Surgery Clinic in Dalian in China with informed consent from the patients. In brief, approximately 400–600 mg of human subcutaneous adipose tissue was washed several times in D-hank's buffer. The adipose tissue was minced finely using a surgical scissor and incubated in digestion buffer at 37°C with constant agitation for about 20 min. Once digested, the liquid separated into three layers: the upper layer contained yellow oily lipocytes, the intermediate layer adipose tissue and the bottom layer the buffer and mononuclear cells. The buffer from the bottom layer was then carefully collected and the digestion was completed in a centrifuge tube containing h-DMEM with 10% fetal bovine serum. The mixture was centrifuged at 1500 rpm for 10 min and then trypsin-collagenase was added to the remnant adipose tissue again. The described steps were repeated for 2–3 times, until the adipose tissue was completely digested. Cell pellets were afterwards suspended in medium (DMEM+10%FBS) and plated in T-25 T-flasks. To avoid the influence of red blood cell lyse to the growth of ADSCs, the commonly used KRB (Krebs-Ringer-Bicarbonate) or NH_4Cl weren't applied. Cells were cultured at 37°C and 5.0% CO_2 in a humidified incubator, with 100% of the media replaced every 3 days. Purified ADSCs at passage 4 were prepared for later use.

2.3 Quantification of surface markers by flow cytometry

Cells were collected by collagenase and lysozyme treatment of the T-flasks, and stained using the antibodies anti-CD34-FITC, anti-CD44-FITC, anti-CD45-PE, anti-CD105-FITC and anti-HLA-DR-PE for 20 min in the dark alongside the corresponding isotype and positive controls. After staining, the cells were washed twice in PBS and analyzed using a standard Becton-Dickinson FACS Aria instrument (BD, USA). The data were acquired and analyzed using the FACS DiVa software (BD, USA). All antibodies and isotype controls were purchased from BD Biosciences (USA).

2.4 Preparation of chitosan/glycerophosphate (C/GP) hydrogel

A solution of 2.2% (v/v) chitosan was prepared by dissolving the required amount of chitosan powder in 0.1 mol l^{-1} acetic acid as the methods described in references [15–17], followed by a filter-sterilization and storage

in an ice bath. Similarly, 50% (w/w) β-glycerophosphate solution was prepared by dissolving β-glycerophosphate powder into tri-distilled water to make up for the required volume and concentration and then the solution was filter-sterilized. Afterwards, β-glycerophosphate solution was added into the previously prepared chitosan solution according to different volume ratios inside the ice bath, and finally the two solutions were fully mixed in order to form the C/GP hydrogel.

2.5 Determination of the pH and gelation time of the C/GP hydrogels

The measured pH and gelation time of the C/GP hydrogels were different depending on the volume ratios between chitosan and β-glycerophosphate. A pH meter was used to detect the pH values of different volume-ratio solutions while the time to gelate was controlled using a stopwatch. Briefly, 1 ml of each C/GP solution was dropped into a small tube, placed in an incubator at 37°C, and then was examined every 2 min by turning over the tube until the liquid didn't flow, time at which it was recorded the gelation time.

2.6 Preparation of the chitosan/β-glycerophosphate/collagen (C/GP/Co) hydrogels

A collagen solution with a concentration of 2 mg/ml was prepared by adding 400 μl collagen into 480 μl ice-cold tri-distilled water, then mixed with 24 μl of 0.1 mol/l NaOH solution and 100 μl of 10× PBS, and finally stored in an ice bath until further use. The C/GP/Co hydrogel was prepared by mixing C/GP hydrogel solution with the collagen solution at the following volume ratios of C/GP:Collagen—7:3, 5:5 and 3:7.

2.7 Osmolality of the culture media in contact with the hydrogels

A volume of 30 μl of each hydrogel solution (C/GC and C/GP/Co) were added into separate wells of a 96-well plate. They were then allowed to gelate for 1 h at 37°C, after which 100 μl of culture media was added into each plate and left overnight at 37°C. The osmolality of the supernatant media in contact with each hydrogel was assessed through an osmometer, and were compared with that of standard culture medium.

2.8 Biocompatibility of hydrogels and fabrication of hydrogel-ADSCs constructs

A volume of 10 μl of either C/GP/Co or C/GP hydrogel solutions were added into separate wells of 96-well culture plates (each hydrogel filling 21 wells). Then the plates were put into an incubator at 37°C to form the hydrogel. ADSCs collected after passage 9 were suspended at a density of 8×10^4 cells/ml, and 100 μl of this cell suspension was added into each well (a total of 0.8×10^4 cells/well) containing the hydrogels and into other 21 blank wells without hydrogels [18]. Following to this, the samples were cultured for 7 days in an incubator at 37°C with a 100% humidified chamber and 5% CO_2, with full media replacement every 3 days. The biocompatibility of the hydrogels and growth status of the hydrogel-ADSCs constructs were observed under inverted fluorescence microscope at the end of the culture period. The absorbance values of 3 wells from each group were determined using cck-8 kit through a microplate reader throughout the whole culture period.

To assess cellular viability, 50 μl of either the C/GP/Co or the C/GP hydrogel solutions were added into separate wells of a 24-well plate, and allowed to gel at 37°C. ADSCs collected after passage 9 were then suspended at a density of 8×10^4 cells/ml and 650 μl of this solution were added per well (a total of 5.2×10^4 cells/well). The hydrogel-ADSCs constructs were then stained using a Live/Dead Viability/Cytotoxicity Kit at the end of 3 and 7 days of culture, respectively, and observed under a fluorescence inverted microscope.

2.9 Observation by scanning electron microscopy (SEM)

Prepared solutions of the C/GP and C/GP/Co hydrogels and the ADSCs-C/GP/Co constructs were placed into separate wells of a 96-well plate, at a volume of 120 μl per well. Afterwards, each well was allowed to gelate at 37°C for 15 min, pre-frozen for 2 h in a −80°C fridge, and then freeze-dried for 48 h. After this, the samples were transferred into centrifuge tubes and fixed with 2.5% glutaraldehyde (pH 7.2–7.4) for 3 h at room temperature, and washed two times with PBS. The supernatant was then removed and 1 ml of 30% ethanol was added to each sample tube. After 5 min, 0.4 ml of the supernatant was replaced with a 50% solution of ethanol and then a gradient-elution protocol was used until reaching a final concentration of 100% ethanol (50/75/85/95/100% ethanol in water). Afterwards, tertiary butyl alcohol (TBA) gradient-elution was performed the same way as previously described for ethanol until reaching a final concentration of 100% TBA, and finally the samples were vacuum dried, ion sputtered and observed under a scanning electron microscope (SEM).

2.10 Statistical analysis

Each experiment was repeated three times and the statistical analysis of the results obtained from three tests was

performed using OriginPro7.5 software and is shown as the mean ± standard deviation (SD).

3 Results and discussion

3.1 Cellular morphology and membrane surface protein expression by flow cytometry analysis

The initially adherent cells grew into spindle- or stellate-shaped cells, which then developed into visible colonies after 7 days of the initial plating as shown in Fig. 1a. The cells began to proliferate rapidly and were passaged by trypsinization every 3–4 days when they were 100% confluent. After the second passage, hADSCs appeared to adopt a more uniform fibroblast-like shape with directionality and regularity (Fig. 1b–c), similar to bone marrow stromal cells. We examined the expression of CD34, CD44, CD45, CD105 and HLA-DR on the membrane surface of the ADSCs, and the results are shown in Fig. 1h–l. The antibodies CD44 and CD105 were positively expressed, while CD34, CD45, and HLA-DR were absent.

3.2 Variation of the pH and gelation time with the ratio of C: GP in the hydrogel

The pH and gelation time of the C/GP solution prepared with chitosan and β-glycerophosphate according to different volume ratios are shown in Table 1. It was possible to observe that by increasing the ratio of β-glycerophosphate in the hydrogel the pH of the resulting solution was higher while the gelation time decreased. This result is consistent with that obtained by Chenite et al. [19, 20]. In addition, it was also found that when a weak base solution such as disodium hydrogen phosphate was added, a white precipitate appeared when the pH was greater than 6.3, and such solution would gelate at room temperature. This demonstrated that the glycerol-group in the glycerophosphate plays an important role towards the gelation process. The underlying mechanism is that the poly-alcohol group of β-glycerophosphate cuts off the chitosan chain, accelerating the formation of a hydrophilic shell around the chitosan molecule, and thus improving the chitosan chain protective hydration, which prevents the associative effects at low temperatures and neutral pH. However, with an increase of the temperature, the hydrophilic interaction and hydrogen bonding start playing an important role that triggers a physical cross-link throughout the whole solution, originating its gelation.

The results have shown that for a volume ratio of chitosan: β-glycerophosphate of 5:1 the pH was 7.16 with a gelation time of 10 min at 37°C, which satisfies the requirements for an injectable scaffold. It was observed that the presence of collagen has a negligible effect to the gelation time and pH, hence the C/GP solution used for the following experiments were done with this proportion. This finding was not consistent with investigation from Wang et al. [17], they thought that high chitosan/ collagen ratios decreased both pH and gelation time.

3.3 Preparation of the C/GP/Co hydrogel

The C/GP hydrogel and the collagen solution were mixed at three different volume ratios of C/GP: Collagen—7:3, 5:5, and 3:7. All solutions were observed to be liquid at room temperature, and gelate at 37°C, as shown in Fig. 2a–b (for demonstration purposes only it is shown a volume ratio of C/GP: Collagen of 5:5). The gelation times were 12, 12 and 14 min, respectively. It was estimated that the higher the collagen volume ratio in the solution, the faster the hydrogel would depredate. Taking this into consideration and the gelation times described, the hydrogel prepared for the following experiments all adopted a volume ratio of chitosan: glycerophosphate: collagen is 5:1:6.

3.4 Morphology of the hydrogels

The prepared hydrogels of C/GP and C/GP/Co were observed by scanning electron microscopy (SEM) as shown in Fig. 2c–d. The inner structure of the two hydrogel systems both showed a spongy porosity. The pore canal in the C/GP/Co hydrogel was intensive, uniform and coherent, with diameters in the range of 100–200 μm. On the other hand, the C/GP hydrogel was loose, irregular and with a larger average diameter. This difference in their inner structure could be attributed to the presence of collagen in the hydrogel that promoted a stabilization of its structure following to the polymerization of chitosan. As the temperature of the hydrogel solution rises during heating, the chitosan molecules start to become hydrophobic, aggregating into larger bundles of molecules, which then separate out from the liquid solution leading to a decrease of the chitosan molecular dispersion, hence forming larger interspaces. With the presence of collagen, which doesn't take part in the polymerization of chitosan and fills the interspaces left by the gradual cross-linking of chitosan molecules, these spaces are more protected and hence result in smaller and more cohesive pore canals.

In addition, this study also discovered that the C/GP hydrogels had an increased strength after gelation when their structure was included with collagen, and that was intimately related to the different inner structures observed in either C/GP or C/GP/Co hydrogels.

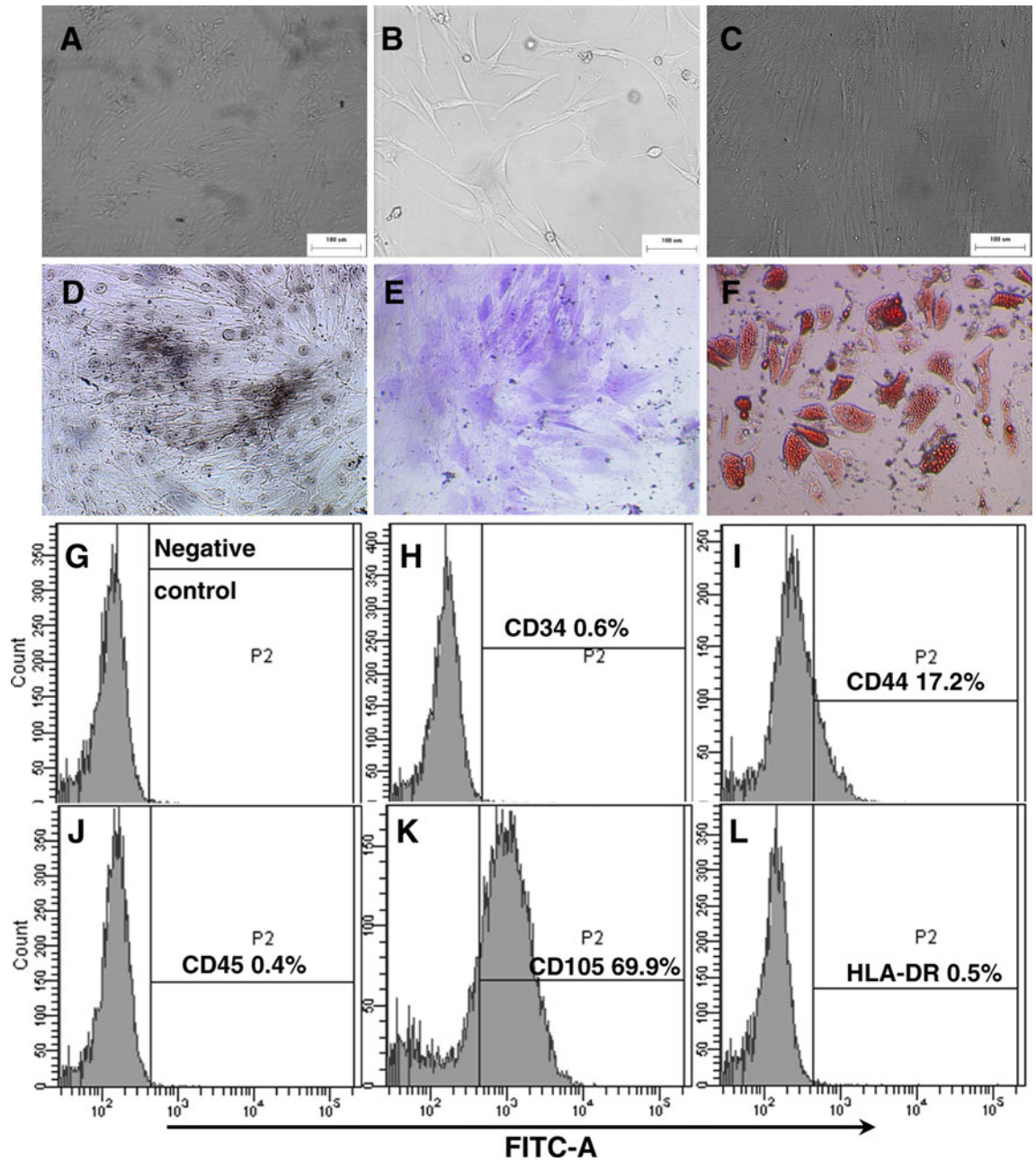

Fig. 1 Morphology, multipotential differentiation assays and flow cytometry analysis of hADSCs from subcutaneous fat tissue cultured in vitro. After being plated in tissue culture flasks for 7 days, the initially adherent cells grew into spindle-shaped cells (**a**); Passage 7 cells at day 3 (**b**) and day 7 (**c**). Multi-differentiation potential of hADSCs towards the mesodermal and endodermal lineages was assessed through staining with ALP, which stains for the early stage osteogenic-differentiated ADSCs (**d**); ADSCs under chondrogenesis induction media developed into chondrocytes, with differentiated cells stained using Toluidine Blue (**e**); hADSCs differentiated towards the adipogenic lineage and formed lipid vesicles, which were stained using Oil red-O (**f**). Immunophenotypic characterization of ADSCs (**g–l**), with cells positively expressing the antigens CD44 (**i**) and CD105 (**k**), while negatively expressing the antigens CD34 (**h**), CD45 (**j**) and HLA-DR (**l**). *Scale bars* = 100 μm

3.5 Osmolality of the hydrogel cultures

This experiment also monitored the osmolality of standard culture medium and of supernatant medium collected from cultures with C/GP and C/GP/Co hydrogels, whose results are shown in Fig. 3a. Taking into account that β-glycerophosphate is a soluble salt, it was expected that a large amount of ions would dissociate and dissolve into the surrounding solution. The osmolality of the media in contact with the C/GP hydrogel was the highest of the three groups, with a value above the 370 mmol kg^{-1}, which is higher than the optimal range for cellular growth

Table 1 The pH value and gelation time of chitosan and GP mixed with different volume ratios

C/GP volume ratios	pH	Gelation time/min
0:1	9.13	–
4:1	7.23	7
5:1	7.16	10
7:1	7.12	12
8:1	7.05	12
16:1	6.81	60
1:0	5.3	–

(240–370 mmol kg^{-1}). Collagen doesn't dissociate in aqueous solutions, and its stabilization of the structure of the C/GP/Co hydrogel led to a relatively lower osmolality, mainly due to the ionic strength within the surrounding solution of C/GP/Co hydrogels would decrease, while that of the standard culture medium was the lowest. Although the presence of the C/GP/Co hydrogels increased the osmolality of the medium (when compared to the control group), it was still within a suitable range to support cellular growth.

3.6 Biocompatibility of hydrogel and fabrication of hydrogel-ADSCs constructs

3.6.1 Morphology of cells cultured within the hydrogels

The morphology of the ADSCs was observed under an inverted microscope on the 7th day of culture as shown in Fig. 3b–d. It was seen that in all the three groups the cells have grown into a long spindle shape. As previously mentioned, β-glycerophosphate, used to produce both hydrogel types, has a moderate toxicity for the cells, and hence the ADSCs in the control group presented the best morphology and the largest expansion. However, it was also possible to observe that the cells cultured within the C/GP/Co hydrogels were in better shape and higher number than those in contact with the C/GP hydrogels.

3.6.2 Proliferation of ADSCs in the hydrogels

The proliferation of cultured ADSCs was assessed by cck-8 and the results are shown in Fig. 4a. The absorbance value obtained for the control group was the highest, while the group cultured with the C/GP/Co hydrogel has shown an increase with time, and was higher than the absorbance observed in the C/GP group, which in turn decreased slightly with time. Correlating these results with the cellular proliferation, it can be concluded that the control group has shown the best performance, while the cells in the C/GP/Co group could propagate continuously with better results than those for the C/GP group, and finally that the number of ADSCs in the latter group decreased with time.

3.6.3 Assessment of cellular viability

The cellular viability of the cultured ADSCs under different conditions was assessed through a Live/Dead Viability/Cytotoxicity kit and is shown in Fig. 5. The green

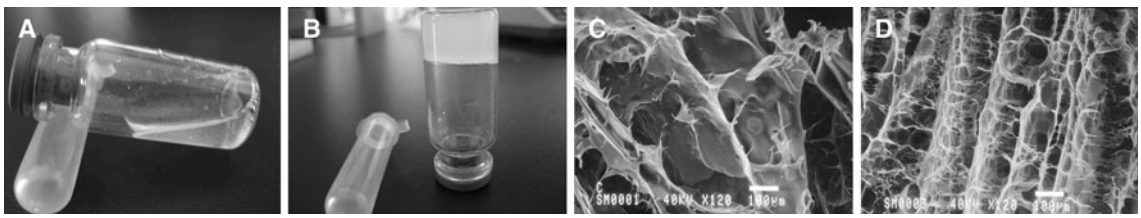

Fig. 2 Photographs of thermo-sensitive C/GP/Co hydrogels and SEM photographs of C/GP and C/GP/Co gels. This is liquid at room temperature (**a**) and gel after 12 min at 37°C (**b**), (**c**) C/GP and (**d**) C/GP/Co hydrogels

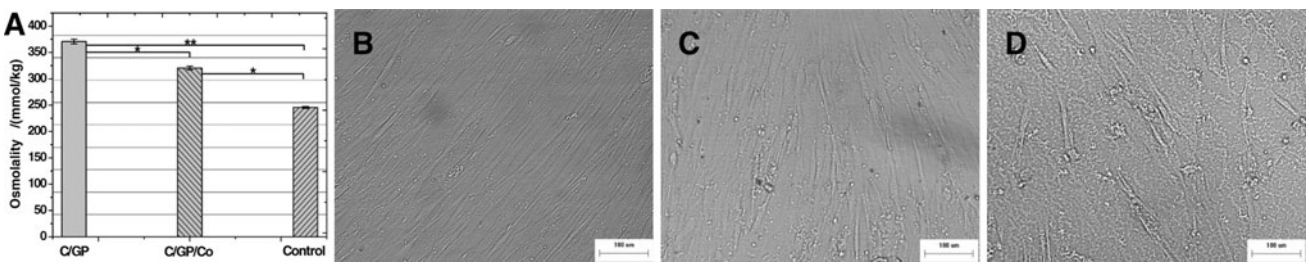

Fig. 3 Osmolality (**a**) of the media in different hydrogels and control group and morphology of ADSCs cultured in different hydrogels after 7 days of culture. Osmolality (**a**) of the media, (**b**) Control group and (**c**) C/GP and (D) C/GP/Co hydrogels, respectively. * $P < 0.05$, ** $P < 0.01$

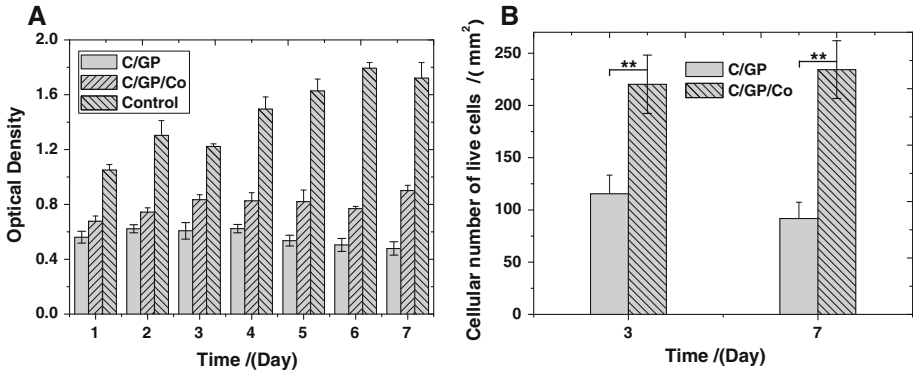

Fig. 4 Viability of ADSCs cultured in different hydrogels measured using cck-8 and total cell number of live cells in the different hydrogels. Viability (**a**) of ADSCs and total cell number (**b**) of live cells. ** $P < 0.01$

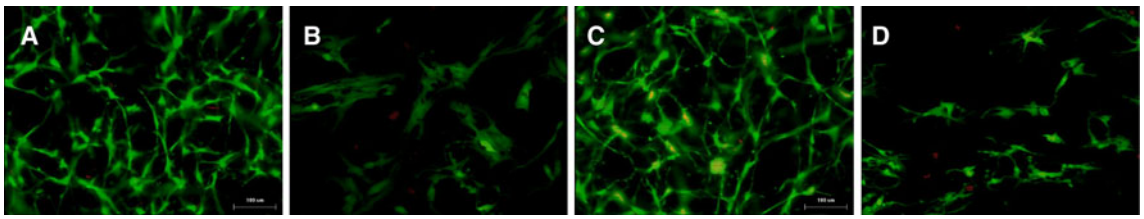

Fig. 5 Photographs of ADSCs cultured in different hydrogels after live/dead staining. C/GP/Co (**a**) and C/GP (**b**) hydrogels at day 3; C/GP/Co (**c**) and C/GP (**d**) hydrogels at day 7. *Scale bars* = 100 μm

spindle-shaped cells are live cells, whilst the red and round cells are dead. On the third day of culture, the ADSCs from the C/GP/Co group appeared with a fusiform shape, with only a minority of the cells apoptotic; on the other hand, those collected from the C/GP group presented only a small amount of cells with an extended state, while some of them were dead (it's worth to note that part of the dead cells might have been washed away by the buffer solution during the staining procedure due to their characteristic lack of adhesion). After 7 days of culture, the number of dead cells observed on the C/GP/Co group had increased slightly, but the living cells had also proliferated into higher numbers, presenting an extended shape. However, in the C/GP group, the few live cells presented an abnormal state with a declined tendency to grow, and a visibly higher cell death than that observed after 3 days of culture.

Live cells cultured in both hydrogels were scored and analyzed by 8 random views under the microscope, whose results are shown in Fig. 4b. It can be seen that the number of living cells collected from the C/GP/Co hydrogels didn't vary much from the 3rd day up to the 7th day of culture, although it was higher than that observed in the C/GP group, which in turn presented a slightly decrease in the number of live cells. The statistical results were consistent with the cck-8 measurements.

The results have also indicated that the high-concentration ions released by the C/GP hydrogel were poisonous to the cells up to some extent, and that the cellular affinity to chitosan is weaker than to collagen. This could help explaining the reason for having found a better behavior in terms of cellular growth and viability by the C/GP/Co hydrogels than those prepared only with C/GP. As one of the favored materials to design scaffolds for tissue engineering applications, collagen offers a good adhesive surface that promotes cellular attachment, proliferation and expansion [21], even despite its low strength and fast degradation rate that usually affect cellular growth. Hence, the affinity of the C/GP hydrogel system towards supporting the growth of ADSCs could be enhanced through the addition of collagen, by providing a better supporting matrix, while the degradation of the latter molecule also benefitted from this interaction: being chitosan a positively charged molecule and collagen carrying an opposite charge, the electrostatic interaction therefrom resultant could increase the stability of collagen in culture [22].

After 14 days of culture with adipose-inducing culture media, stained cells with Oil-Red-O were observed under an inverted microscope (Fig. 6a–b). It is possible to observe a large amount of lipid droplets emerging within the stem cells, with the majority of them presenting typical characteristics of adipocytes, while the number of droplets increased with time. It was thus demonstrated that the ADSCs cultured on the surface of the C/GP/Co hydrogels still maintained their differentiation potential after the culture period. Finally, the SEM photographs of the hydrogel-ADSCs constructs (Fig. 6c–d) show a great number of ADSCs spreading and intercrossing with each other and good cellular viability and morphology.

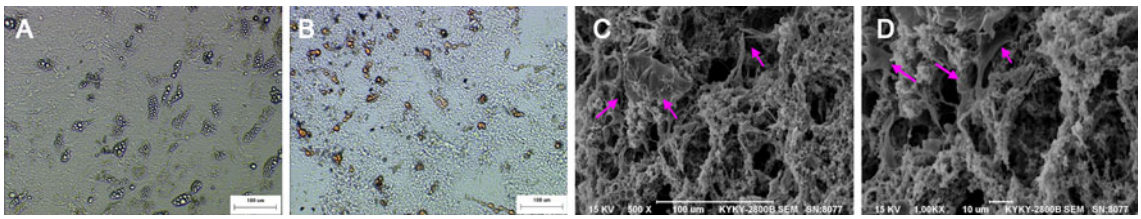

Fig. 6 Adipogenic potential of ADSCs after being cultured using the C/GP/Co hydrogel for 14 days and SEM images of encapsulated ADSCs within it (**c** and **d**). Phase contrast observation (**a**) and Oil Red-O staining (**b**); and SEM pictures with magnifications of 500× (**c**) and 1000× (**d**). The *pink* arrows represented the ADSCs within the hydrogels

4 Conclusions

A novel hydrogel composed of Chitosan, β-glycerophosphate and collagen (C/GP/Co) was herein proposed. This has the advantages of being liquid at room temperature and gel at the body's temperature and when compared to hydrogels composed of only Chitosan and β-glycerophosphate (C/GP) to contain a lower cytotoxic, better cellular compatibility and more uniform pore structure to support cellular growth. In sum, C/GP/Co hydrogels are amenable for use as injectable scaffolds in tissue engineering applications.

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