***Characterization of human adipose tissue-derived stem cells in vitro culture
and in vivo differentiation in a temperature-sensitive chitosan/ β -
glycerophosphate/collagen hybrid hydrogel***

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Abstract: In this study, the interaction of human adipose tissue-derived stem cells (ADSCs) with chitosan/ β -glycerophosphate/collagen (C/GP/Co) hybrid hydrogel was test, followed by investigating the capability of engineered adipose tissue formation using this ADSCs seeded hydrogel. The ADSCs were harvested and mixed with a C/GP/Co hydrogel followed by a gelation at 37°C and an *in vitro* culture. The results showed that the ADSCs within C/GP/Co hydrogels achieved a 30% of expansion over 7 days in culture medium and encapsulated cell in C/GP/Co hydrogel demonstrated a characteristic morphology with high viability over 5 days. C/GP/Co hydrogel were subcutaneous injected into SD-rats to assess the biocompatibility. The induced ADSCs-C/GP/Co hydrogel and non-induced ADSCs-C/GP/Co hydrogel were subcutaneously injected into nude mice for detecting potential of adipogenic differentiation. It has shown that C/GP/Co hydrogel were well tolerated in SD rats where they had persisted over 4 weeks post implantation. Histology analysis indicated that induced ADSCs-C/GP/Co hydrogel has a greater number of adipocytes and vascularized adipose tissues compared with non-induced ADSCs-C/GP/Co hydrogel.

Key words: Adipose-derived stem cells; Chitosan; Hydrogel; 3-dimensional fabrication; Adipogenic differentiation

Introduction

The adipose tissue-derived stem cells (ADSCs) obtained from adipose tissue has a strong proliferative capacity and multi-lineage differentiation potential as bone mesenchymal stem cells (BMSCs) [1, 2]. Compared with BMSCs, the ADSCs are more available due to their large quantities with minimal possibility of morbidity and discomfort clinically [3, 4]. In view of those practical advantages, ADSCs have recently received a widespread attention in tissue engineering.

In the tissue engineering research, stem cells must be combined with a scaffold material to form cell-material complex, cultured in vitro or in vivo under suitable conditions to allow stem cells grow, differentiate in the 3D space and eventually form desired functional tissues [5]. The ideal scaffold material should have good biodegradability, biocompatibility and 3D space structure as compared to natural extracellular matrix (ECM) [6, 7]. The hydrogel is one of the favorable 3D materials which can mimic the native ECM. Chitosan and β -sodium glycerophosphate (GP) were mixed by Chenite [8] to produce chitosan/sodium glycerophosphate (C/GP) hydrogel---a kind of thermal sensitive hydrogel which does not gel until temperature rises to 37°C or so. This characteristic of the synthetic hydrogel are probably due to the coordination of interaction forces including hydrogen bonding, electrostatic and hydrophobic interactions. Compared to normal process of implantation, thermal sensitive hydrogel shows huge advantages as it can be introduced into patients by injection and form gel which minimize pain and side-effects [9]. Due to its great property, thermal sensitive hydrogel is an ideal biological scaffold material for the tissue engineering. Chitosan materials have showed good biocompatibility [10, 11], low immunogenicity and accepted biodegradable rate in previous

studies [12]. The raw rubber materials are added to the system by Song *et al* [13] to reduce the toxicity of C/GP/Co hydrogel plastic which has better biocompatibility. There are many reports demonstrating that ADSCs can be seeded into scaffolds followed by cultivation and cell differentiation [14-17]. However, few reports were on ADSCs growing in 3D space and differentiated in vivo.

In the present study, the ADSCs which harvested from human are composited with C/GP/Co hydrogels to investigate the cells growth, proliferation and spreading activity inside the material and to assess the possibility of engineered adipose tissue formation. ADSCs-hydrogel was then subcutaneously injected into nude mice for studying the potential of the adipogenic differentiation and the possibility of adipose tissue formation in vivo for further clinical application.

Materials and methods

Isolation and culture of human ADSCs

The adipose tissue was provided by Dr. Sha from the Cosmetic Plastic Surgery Clinic, Dalian, China with consent. In brief, approximately 2-3g of human subcutaneous fat was washed for several times in D-hank's buffer, mincing and then incubated in digestion of collagenase and trypsin buffer(1:1w/w) at 37°C for 20 min. After incubation, the suspension was separated into three layers: upper layer containing yellow oily lipocytes, intermediate layer adipose tissue and the bottom layer of 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-Dulbecco's Modified Eagle Medium (h-DMEM, GIBCO, USA) with 10% fetal bovine serum (Sigma, USA). The mixture was centrifuged at 1500 rpm for 10 min prior to addition of trypsin-collagenase to the remnant adipose tissue for few times until adipose tissue was completely digested. Cell pellets were re-suspended in medium (DMEM+10%FBS) and plated in tissue culture-flasks. To avoid the contamination of red blood cell, NH_4Cl or KRB (Krebs-Ringer-Bicarbonate) were not used. Cells were cultured at 37°C and 5.0% CO_2 humidified incubator with media replaced every 3 days. Purified ADSCs at passage 4 were prepared for later use [18].

Quantification of surface markers by flow cytometry

ADSCs at passage 5 were collected, 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 with corresponding isotype and positive controls (BD, USA). 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).

Multiple differentiation potential of ADSCs

The cellular growth and morphology was observed under an inverted microscope in bright field and a confocal laser scanning microscopy (CLSM), respectively.

Osteogenic differentiation: ADSCs at passage 5 were seeded at cell density of 4×10^4 cells/mL. In the 24-well culture plate, 3 wells containing ADSCs and alkaline phosphatase (ALP) staining, another 3 wells for von-Kossa staining, and the remaining 6 wells were used as a negative control group. The culture medium was replaced with osteogenic differentiation media when the cells began to adhere to the wells followed by media replacement every 3 days. At 2 and 4 week's time point, cells were stained by ALP and von Kossa, respectively.

Chondrogenic and adipogenic differentiations: ADSCs collected at passage 5 were also seeded at density of 4×10^4 cells/mL for studying chondrogenic and adipogenic differentiations. ADSCs were cultured at standard condition, while media were changed to chondrogenic and adipogenic differentiation media when cell adhesion occurred. Morphology of induced cells were characterized, and attached and differentiated cell were also stained by toluidine blue and oil red (Sigma, USA) after 2 weeks of inductions, respectively.

Preparation of C/GP and C/GP/Co hydrogel

Chitosan (Deacetylation 93% v/v, Haidebei, Jinan) solution (2.2% v/v acetic acid solution) and β -glycerophosphate solution (50%w/w aqueous solution) were mixed in ratio of 5:1 v/v to prepare chitosan/glycerophosphate (C/GP) solution. Rat tail type I collagen (700 μ L, Chuang'er Biotech, China) solution was prepared prior to mix with 30 μ L of 0.1 mol/L NaOH solution and 100mL of 10 $\times$ D-Hanks used as collagen solution. C/GP/Co hydrogel solution as the

experimental group was then prepared by mixing equal volume of Rat tail collagen type I solution (1mg/mL) with C/GP (C: GP=5: 1, V/V) solution in ice bath [13, 19]. A hydrogel solution without collagen was also prepared as control group. The feedstock used for hydrogel preparation had already been sterilized with a filter [19, 20].

Osmolality of the culture media in contact with the hydrogels

C/GP/Co and C/GP hydrogel solution (30 μ L) were added into 96-well culture plate, respectively, gelation for 1 h at 37°C, following by adding 100 μ L of culture media into each plate and remained at 37°C overnight. The osmolality of the supernatant media in contact with each hydrogel was assessed through an osmometer (Wescor, USA), in comparison with standard culture medium.

Morphology of the hydrogels

Prepared solutions of the C/GP and C/GP/Co hydrogels were placed into separate wells of a 96-well plate, at a volume of 120 μ L per well. Each well was then allowed to gelatinize at 37°C for 15 min, pre-frozen for 2 h in a -80°C fridge prior to freeze-dry for 48 h. Dry samples were fixed with 2.5% glutaraldehyde (pH 7.2-7.4) for 3 h at room temperature, washed twice in PBS followed by dehydration using a series of ethanol dilutions(30-100%). Samples were vacuum dried, ion sputtered and scanned using scanning electron microscope (SEM) (S2520, Hitachi) [21, 22].

The prepared C/GP hydrogel scaffold and C/GP/Co hybrid hydrogel scaffolds were placed on a 96-well culture plate with a dose of 120 μ L each well respectively. After 15 min of gelation at 37°C, the sample was put into -80°C refrigerator for 2 hours of refrigeration, then it was dried for 48 h in a freeze drier, the middle part of the scaffold was chosen to be observed under a

SEM with an acceleration voltage at 40kV.

Rheological measurement of hydrogel

The gelation temperature of C/GP and C/GP/Co thermosensitive hydrogel was measured through the temperature scanning of rheology using AR2000ex rheometer (TA Company, USA).

Determination of C/GP/Co hydrogel scaffold degradation in vitro

Degradation of C/GP/Co hybrid hydrogel scaffold was assessed via an established method. Briefly, 1mL mixed solution at different ratio of C/GP and collagen was added into a tube. After 1 h of gelation at 37°C, lysozyme and collagenase mixture in PBS (pH7.4)(0.01M) was added and then incubated at 37°C with stirring at 50 rpm. Dry sample weight was measured at different time points. The degeneration rate was calculated by the following formulation.

$$\text{Degeneration rate \% (t)} = [\text{Wd (0)} - \text{Wd (t)}] / \text{Wd (0)}$$

Here, Wd (0) is initial weight of sample, Wd (t) is the weight of sample at time t.

Production of ADSCs-hydrogel constructs

ADSCs (passage 5) cell at density of 4×10^4 cells/mL suspension was prepared prior to mix with prepared collagen solution resulting in ADSCs-C/GP solution at 7×10^6 cells/mL. According to different requires of experiment, the solutions were added into separate well of 96-well culture plates (40μL per well) and 24-well plates (250μL per well), respectively, and allowed to form gel at 37°C for 20 min. Thereafter, 150μL and 1mL culture media was added respectively into those two kinds of culture plates and cultured for 7days at 37°C with a 100% humidified chamber and 5% CO₂, with medium replaced every day. In control group, 30μL 10×D-Hanks and 600μL cell suspension were added into another 1.4 mL C/GP and then cultured at same condition. For the thermal-resistance characteristic of hydrogels and type I collagen from rat tail,

the treating process should be strictly conducted in ice-bath [23].

Cell proliferation

Cell proliferation was assessed. At day 1, 3, 5, 7, cell culture media in both experimental and control group were replaced by 100 μ L fresh media and incubated with 10 μ L CCK-8 at 37°C for 5h. The absorbance values were measured using a microplate reader (Bio-Rad, USA) at wavelength of 450. The growth curve was calculated by plotting absorbance as a function of time. The growth curve: ordinate-cell numbers (thousand) in every well averagely, abscissa-days (d) [24].

Cell apoptosis

ADSCs apoptosis in hydrogel constructs were assessed using a Live/Dead Viability/Cytotoxicity Kit (Invitrogen, USA). At 2 and 5 days post culture samples were incubated with reagents at 37°C for 15 min, and then observed under a fluorescence inverted microscope (IX71, Olympus) [25].

Morphology of ADSCs-hydrogel constructs

ADSCs-hydrogel constructs at day 5 were dehydrated using 4% ethanol and then freeze-dried for 24h. Specimens were then mounted on aluminum sample stubs and sputter coated with gold prior to examination in the SEM at a voltage of 40kV.

In vivo biocompatibility of hydrogel model

The *in vivo* biocompatibility of ADSCs-hydrogel constructs were studied using a rat model. 6 pathogen-free, male SD rats, aged 8 weeks weighing 250 ± 3 g and BALB/CA nude mice at aged 4 weeks, weighing 120 ± 5 g were acquired and studied under a protocol approved by Ethics Committee of Dalian University of Technology with Experimental Animal License

(2001-545). SD rats were used for investigating the biocompatibility of blank hydrogels, while nude mice were recruited for studying in vivo adipogenic differentiation in hydrogel.

Each SD rats was conducted surgical peritoneal endometrial with random 4 spots near four limbs. C/GP/Co hydrogel (0.3mL) was subcutaneously injected into SD rats using C/GP hydrogel [26]. In comparison, 6 SD rats were injected the same volume of C/GP hydrogel as negative control. At 4 weeks post injection, 3 rats in experimental group and the control group were sacrificed for histology analysis.

Hydrogel constructs with surrounding tissue were fixed in 4% paraformaldehyde for 48h. All samples were then dehydrated and embedded in paraffin. The 5 μ m sections were deparaffinated in xylene and stained with Haemotoxylin and Eosin (H&E) and Masson's trichrome staining [27].

In vivo adipogenic differentiation of ADSC-hydrogel constructs

3 BALB/CA-nu nude mice of 4 weeks age were anesthetized individually by intra-peritoneal injection of phenobarbital sodium followed by injection of 0.2mL ADSCs-hydrogel subcutaneously at neck. In control group, mice were only received hydrogel injection. 4 weeks later, ADSCs-hydrogel and blank hydrogel were removed, respectively. The biopsies were then fixed and stained with H&E and Masson's Trichrome for observation.

Statistical analysis

Results of degeneration time, cell proliferation were reported as mean $\pm$ SD. Statistical analysis (n=3) were performed using OriginPro8.0 software for a significance level of $p < 0.05$.

Results and Discussions

Cellular morphology and surface markers by flow cytometry analysis

In cell culture, colonies of ADSCs were visible in shape of spindle or stellate (Fig1A1). Thereafter, cells began to proliferate rapidly, fibroblast-like shape of ADSCs (Fig1A2). Toluidine blue and Oil red O staining confirmed the differentiation tendency of ADSCs towards chondrocytes and adipocytes (Fig1A3, 1A4). Single ADSC at passage 7 was observed under inverted microscope (IX71, Olympus) (Fig.1B1). The cellular morphology can then be observed after mitochondria and cellular nucleus staining (Fig.1B2-1B4). Expression of CD34, CD44, CD45, CD105 and HLA-DR on the membrane surface of the ADSCs was further examined and results showed that antibodies CD44 and CD105 were positively expressed, while CD34, CD45, and HLA-DR were negative in agreement with that ADSCs are not belong to hematopoietic stem cell lines.

Morphology of the hydrogels

SEM examination of C/GP and C/GP/Co hydrogels revealed porous spongy surface morphology. C/GP/Co hydrogel exhibited an intensive, uniform and coherent porous internal connected canal with pore size in the range of 100–200 μ m. Comparing to C/GP/Co hydrogel, C/GP hydrogel revealed a fairly loose and irregular pores with a larger pore diameter. The difference of internal surface morphology of between two difference hydrogel could be attributed to the presence of collagen which enhanced the stabilization of hydrogel structure following by 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 reduction of the chitosan molecular dispersion, hence forming larger interspaces. Collagen was not involved in polymerization of chitosan but filled in the interspaces and encapsulated via gradual cross-linking of chitosan molecules, resulting in more stable structure with small cohesive pore canals [28].

When C/GP solution mixed with the collagen solution at the ratio of 7:3, 5:5, 3:7 v/v, all final solutions were able to gelatinized at 37°C (Fig. 2E, F). The volume ratio of C/GP solution and collagen solution in the mixed solution shown in the picture was 5:5. However, the gelation time was varied from 10min, 12min and 14min for the ratio of 7:3, 5:5 and 3:7 between C/GP and collagen, respectively. The addition of collagen in the solution has no effects on chitosan gelation, thus the gelation can still be processed with the presence of collagen, the structure and characteristic of collagen fibers are determined by both electrostatic and hydrophobic forces of adjacent collagen molecules. Hydrophobic interaction and electrostatic attraction of side chains of the amino acid of collagen fibers were both forced by the increased temperature and pH, which is the reason the collagen fibers were hardened.

Rheological characterization of hydrogel scaffold

Within the temperature range of 20 ~ 55°C, the storage modulus G' and loss modulus G'' of different ratios of C/GP (4:1 and 5:1) and C/GP/Co thermosensitive solution (5:1:6) were tested using AR2000ex rheometer under the heating rate of 1°C/min (Fig. 3). The literature [29] indicates that the temperature in which G' curve increased dramatically and deviated from G'' curve sharply was identified as the gelation temperature. In this study, the gelation temperature observed from the G' and G'' curve was probably in the range of 40 ~ 42°C, but when we enlarged the curve in the range of 32 ~ 42°C, it was found that the values of G' and G'' had

risen starting from about 36 °C. On behalf of the storage modulus, G' reflected the deformation behavior of the fluid, therefore from the drawing of partial enlargement, it can be seen that the critical value of deformation was about 36 ~ 38 °C, namely the hydrogel began to form. Particularly, the biomaterial for cell culture was required a certain porosity, degree of swelling and degradation rate. As the temperature increased, the water evaporated and the gel state became denser, in some sense, excessive gelation can be detrimental to cell growth and degradation of the material. Furthermore, the previous experiments were repeated utilizing the remaining sample after rheological detection, once again the gel occurred at 37 °C, therefore it indicated that the rheological testing and experiment were in good agreement, which also indirectly explained the thermosensitive hydrogels prepared in vitro can formed gelation in situ.

The figure.4A showed the pictorial diagram of prepared scaffold before gel at 20 °C. From the left the first two was the mixture of chitosan and β -glycerophosphate (the ratio was 5:1 and 4:1), and the third was the mixture of chitosan, β -glycerophosphate and collagen (the ratio was 5:1:6). Figure.4B-D was the view of formulated sample placed horizontally. Figure.4B-C was the mixture of chitosan and β -glycerophosphate mixture. The larger proportion of β -glycerophosphate made the cross-linking with chitosan higher, therefore at room temperature the two samples has been observed showing a viscous gel state. And although the addition of collagen did not affect the crosslinking, the increase of moisture decreased the viscosity of scaffold (Figure.4D). Figure.3E-G displayed the gelation state of the remaining sample at 37 °C water bath after rheology detection. Compared with Figure.4B-D, apparently, the rheological behavior of the material has changed with the variation of temperature.

Hydrogels scaffold degeneration

The degradation time of C/GP/Co hydrogels scaffold was varied based on the different ratio of C/GP solution and collagen solution. The result (Fig.5A) revealed all three ratios of C/GP had initial burst degeneration in the first 40 hours with approximately 15 % to 45 % degeneration for ratios: 7:3, 5:5, and 3:7 followed by a plateau phase from 40h to 100 h.

The degeneration time of scaffold prepared in this research was faster compared to C/GP injectable hydrogels scaffold as reported in previous literature [30]. In the literature collagen was not added into the injectable C/GP hydrogel. Increased collagen content in the final hydrogel scaffolds accelerated hydrogels scaffold degenerated because the degradation rate of collagen itself was faster than chitosan. Hence, to achieve a balance between biocompatibility and degeneration, the volume ratio of C/GP solution and collagen solution of following C/GP/Co hydrogels scaffold at 5:5 was employed in the present study which resulting in Chitosan/Glycerol Phosphate/Collagen at ratio 5: 1: 6.

Osmolality of the hydrogel cultures

The osmolality of standard culture medium and of supernatant medium collected from cultures with C/GP and C/GP/Co hydrogels were monitored. As expected, the results (Fig.5B) showed that a large amount of ions was dissociated and dissolved into the surrounding solution, as b-glycerophosphate is a soluble salt. The osmolality of the media which in contact with the C/GP hydrogel was measured at 370 mmol kg⁻¹, the highest in all three groups, slightly higher than known optimal range for cellular growth (240-370 mmol kg⁻¹) but still acceptable. Collagen is not soluble in aqueous solutions, but it take an important role to stabilize the structure of the C/GP/Co hydrogel, resulting in 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.

Cell morphology cultured within the hydrogels

ADSCs-C/GP/Co hydrogel remaining as liquid with good flowability at low temperature, thus enable for injection. After 20 min gelatinizing at 37°C, ADSCs-C/GP/Co suspension transformed from liquid to gel which had good mechanical strength and maintained its integrity in culture media over 5 days. Microscope observations indicated that the cells well distributed through the entire hydrogel (Fig.6A). It can be seen in light image that ADSCs attached and stretched inside the C/ GP/Co hydrogel 2 days post seeding and cells remain its characteristic fusiform shape when cell grew in the hydrogel (Fig.6B). 4 weeks later, the C/GP/Co hydrogel still remained its integrity and no significant fragmentation and degradation was noticed (data not shown), suggesting its good biophysical property as 3D cell culture environment.

Proliferation of ADSCs in the hydrogels

The optical density (OD) of CCK-8 hatching solution is a linear function of the number of cells. A higher OD value indicates a greater number of cells. As shown in Fig.6C, OD value of cell number of ADSCs cultured in C/GP/Co hydrogel was significantly increased over the time with approximately 2.23 ± 0.21 at day 1, 2.55 ± 0.10 at day 3 and constantly increased to 2.88 ± 0.08 until day 7. In contrast, ADSC grew inside hydrogel without collagen (C/GP) showed cell number increased only until day 3, following by OD value dramatically drop to 1.72 ± 0.04 . These results demonstrated that C/GP/Co hydrogel is less toxic and it provides a more favorable environment.

Previous study [31] suggested that the cell toxicity of C/GP hydrogel was known by

sodium- β -glycerophosphate (GP). The higher the concentration of GP, the worse cell toxicity is. This is due to the water solubility of GP which is the main component of hydrogel. Furthermore, GP avoidably dissolve and diffuse into surrounding culture media or tissue fluid, causing osmosis pressure changes, which will in turn lead to cell activity decrease and even cell death. Type I collagen is a natural material with favorable biocompatibility, low cell toxicity and no immunity resistance [32, 33] and it has been widely used in cell culture system such as coating the culture plate, therefore collagen is added in C/GP hydrogel in the present study and our results confirmed that collagen is capable to reduce GP concentration of the hydrogel system and hence minimize cell toxicity.

Assessment of cellular viability

Calcein and Hoechst were chosen to stain live cells and dead cells respectively. 2 and 5 days post cell culture in vitro; Hoechst staining indicated that massive ADSCs encapsulated in C/GP/Co hydrogel (Fig.6D). Staining using calcein showed that most of encapsulated ADSCs were positively stained, emitted strong green fluorescence, which further confirmed that they were live cells (Fig.6E). Moreover, there were thimbleful dead cells being identified by PI emitting red fluorescence (Fig.7A₄). In the experimental group and control group, dead cells were accounted for only 2-3% of the total number of cells. These results indicated that ADSCs maintain good growth activity in C/GP/Co hydrogel and no sign of stressed or dead cells after being cultured 5 days.

Previous research reported that [34,35] ADSCs, an adherent growing cells, have a special fusiform morphology, suggesting that initial well attachment and expansion can contribute to ideal cell growth and proliferation. In the present study, we found that ADSCs remain its ability

to expand in the C/GP/Co hydrogel scaffold. Subsequently, ADSCs was noticed to stretch in to all directions after 2 days seeding in the C/GP/Co hydrogel (Figs.7A₁₋₅). We also observed that these encapsulated cells were in its characteristic fusiform morphology and extraordinary extend in only 5 days in cell culture (Figs.7B₁₋₅). Compared to ADSCs in C/GP/Co hydrogel, ADSCs in C/GP hydrogel (control group) was found hardly expand with spherical morphology on both day 2 and day 5, suggesting C/GP does not provide a suitable condition for ADSCs (Figs.7C₁₋₅, D₁₋₅).

In our study, SEM analysis showed that ADSCs proliferated and migrated on the surface of C/GP/Co hydrogel (indicated by arrow in Figs.8A&B) and favorably interacted with the surrounding materials. Although ADSCs that cultured on the surface of Petri dish showed the characteristic fusiform morphology, cell growth and expansion were limited by two dimensions (Fig.6D-F). Interestingly, ADSCs remained fusiform morphology when they cultured in 3D environment, which further revealed that the 3D culture environment in C/GP/Co hydrogel was suitable for the growth of ADSCs.

The histocompatibility of hydrogel

In our in vivo study, C/GP/Co solution was able to inject subcutaneously, set and formed a gel with diameter of approximately 1.5-2cm protruding at injection site. No lacking or disoperation of the injected hydrogel was observed. In addition, no severe inflammatory reaction such as tissue necrosis, edema, hyperemia, hemorrhaging were observed during the study. 15 minutes later, implants would form hard gel and set subcutaneously (Figs.9A&B).

The animal behavior was monitored for 4 weeks study, no signs of pain/distress, restlessness, depresses and lack of appetite were noticed for all rats. On site of injection, no

bleeding, severe inflammation reactions were observed, demonstrating that the C/GP/Co gel injection was well tolerated by rats. 4 weeks post injection, all gel implants were absorbed and bio-degraded with approximately half gel were left over and well encapsulated by surrounding tissue (Figs.9C&D), fibroblasts and neovascularization (Fig.9E). At the end of 4 weeks, all rats were culled and implants biopsy was collected for histology analysis.

The result in figure 8, H&E staining and Masson's staining, showed that there were granulocytes and macrophages on the boundary of C/GP/Co hydrogel after 2 weeks; In addition, there were a large number of achrocytes which trended to infiltrate the gel (Fig.10A), and massive fibroblasts (Fig.10B). All the above phenomena indicated that implants initiated low-grade chronic inflammatory reaction and foreign body reaction *in vivo* [36]. 4 weeks post-injection, heterophil granulocyte and macrophage were not seen in H&E staining but many achrocytes were present (Fig.10C) with lymphatic infiltration (Fig.10D).

In vivo subcutaneous injection study confirmed that C/GP/Co hydrogel has low immune resistance, favorable biological properties and biocompatibility for using as frame materials in tissue engineering [37]. In contrast, an interface were observed between tissue and the C/GP hydrogel 4 weeks post in the control group , while the hydrogel was encapsulated by huge amount of fibroblasts (Figs.10E&F), indicating a strong rejection to foreign substance [36].

Adipogenic differentiation in vivo experiments

The ultimate goal of tissue engineering is to repair damaged tissues or organs and recover their functions by artificial construction. Therefore, it is necessary to explore differentiation behavior of the stem cells after being embed into the scaffold materials and implanted into organism. In our study, ADSCs which have been induced to differentiate into adult adipose

cells were also embedded into C/GP/Co hydrogel. The C/GP/Co hydrogel was injected into athymic mice to investigate the ability of adult adipose cells differentiation [38].

As described above in vivo study, the ADSCs-C/GP/Co hydrogel constructs were injected into athymic mice, the implants formed protrusions in situ (Fig.11A), no free or dispersion phenomenon was observed, and could completely into gel about 15min. The activities of rats were fine during 4 week cultured period. There were no bleeding and implants were not observed to be exuded at the injection site. 4 weeks later, implants were partially degraded and the sizes of implants reduced approximately 5%(Fig.11B) and biopsy with implants was collected for histology analysis. We first noticed that ADSC seeded hydrogel showed different characters of interplant compared to blank hydrogel (Fig.11C). The blank hydrogel was tight, while ADSCs- C/GP/Co hydrogel was soft and very similar to adipose tissue.

Oil red staining showed intensive red staining inside the hydrogel using adipogenic-induced ADSCs (Fig.11D), indicating lipid were formed in insert position. H&E staining showed that there were considerable fibroblasts and mesenchyme distributing in frame and intensive vacuolus like lipid droplets (Fig.11F). This finding indicated that ADSCs in C/GP/Co hydrogel had differentiated into adult adipose cells with function to form lipid droplets [39]. In comparison, no lipid droplets as (Fig.11E) were seen in non-induced ADSCs-hydrogel. Taken together, the result manifested that ADSCs could not spontaneously differentiate into adult adipose cells in C/GP/Co hydrogel. As spontaneous and random differentiation of stem cells may induce carcinogenesis [40], the experiments therefore further confirmed that ADSCs should be directionally induced to avoid carcinogenesis before the compound of cell and hydrogel was inserted into organism.

Pervious study [41] showed that neovascularization spontaneously formed in frame was one of significant preconditions to form artificially construct tissue. The blood vessel can continuously provide necessary nutrients for cells in frame materials so that the cambium could implement functions. Over the past decades in tissue engineering, neovascularization has also become an important criterion to determine if the cell-scaffold is feasible and functional in vivo. For the adipose tissue, its internal blood flow and vascular density is 2 to 3 times than skeletal muscle [42].

In the present research, H&E staining photographs of interplant indicated that new vessels grow into the hydrogel (Fig.11F) after cell-scaffold being implanted in nude mice in 4 weeks, which showed that ADSCs were obviously trended to differentiate into adult adipose cells and grow well in C/GP/Co hydrogel and had the potential to form functional adipose tissue ultimately. However, the experimental session was limited, and interplant was dislodged for observation after 4 weeks. But it can presume that if the culture time is prolonged, the compound of cell and hydrogel will have the potential to sequentially grow and ultimately form functional adipose tissue.

Conclusion

The hydrogel scaffold was produced using chitosan, β -glycerophosphate and collagen. This novel hydrogel is in liquid phase at low temperature and is gelatinized at 37°C. The new hydrogel provides ADSCs a favorable 3D environment with highly maintain cell proliferation, cell morphous and cytoactive. In vivo study further confirmed that ADSCs seeded hydrogel is able to differentiate into adipose tissue, indicating great histocompatibility and favorable ability of adipogenesis. This attractive property of the C/GP/Co hydrogel points to its value as an excellent scaffold material for tissue engineering.

Conflict of interest

The authors have no conflicts of interest.

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Tab.1 pH value and gelation time of chitosan, GP and Co mixed with different volume ratios

C: GP volume ratios	pH	Gelation time/min	C/GP: Co ratios	pH	Gelation time/min
4: 1	7.23	7	7: 3	7.16	12
5: 1	7.16	10	5: 5	7.16	12
16: 1	6.81	60	3: 7	7.16	14

Fig.1 Morphology, multi-potential differentiation, cell viability and flow cytometry analysis of ADSCs from subcutaneous fat tissue cultured in vitro. After being plated in tissue culture flasks for 7 days, the cells at Passage 7 grew into spindle-shaped cells (A₁). Multi-differentiation potential of ADSCs towards mesodermal and endodermal lineages was assessed through ALP staining, which stains for the early stage osteogenic-differentiated ADSCs (A₂); differentiated cells stained with toluidine blue under chondrogenic induction (A₃); differentiated cells towards adipogenic lineage and formed lipid vesicles, which were stained using Oil red O (A₄). Single ADSC at passage 7 (B₁); mitochondria (B₂) of single ADSC stained by MitoTracker® Red CMXRos; cellular nucleus (B₃) of single ADSC stained by SYTO® green fluorescent nucleic acid stains; overlay (B₄) of B₂ and B₃; C₁–C₆, expression of cell surface CD markers of ADSCs at passage 7. A₁₋₄: ×100; B₁₋₄: ×1000.

Fig.2 SEM photos of C/GP and C/GP/Co hybrid hydrogels and photographs of thermo-sensitive C/GP/Co hydrogels. (A&B): SEM photos of C/GP hybrid hydrogel (×60, ×300); (C&D): SEM photos of C/GP/Co hybrid hydrogel (×60, ×300); Liquid C/GP/Co hybrid materials at room temperature (E) and gel after 12 min at 37 °C.

Fig.3 Curve of the storage modulus G' and loss modulus G'' of different ratios of C/GP (4:1 and 5:1) and C/GP/Co thermosensitive solution (5:1:6) within the temperature range of 20 ~ 55 °C, using AR2000ex rheometer under the heating rate of 1 °C/min; And the drawing of partial enlargement of G' and G'' curve in the range of 32 ~ 42 °C.

Fig.4 Pictorial diagram of prepared scaffold before and after gelling. A showed prepared scaffold before gel at 20 °C. From the left the first two was the mixture of C/GP (5:1 and 4:1), and the third was C/GP/Co (5: 1:6); B-D was the view of formulated sample placed horizontally

(B-C: C/GP, D: C/GP/Co); E-G displayed the gelation state of the remaining sample at 37°C water bath after rheology detection(E: C/GP/Co, F: C/GP 4:1, G: C/GP 5:1).

Fig.5 Degradation rate and osmolality assay of C/GP/Co hybrid hydrogels. (A): degradation rate of C/GP/Co gels with different volume ratios of C/GP and Co; (B): osmolality assay of culture media in C/GP and C/GP/Co hydrogels, respectively.

Fig.6 Morphology, expansion and viability of ADSCs in cell-C/GP/Co hybrid hydrogel constructs. Cell morphology of ADSCs-C/GP/Co hydrogel constructs (A): $\times 40$ magnification (B): $\times 100$; Expansion of ADSCs in C/GP/Co hydrogel (C): $*P < 0.05$ (D): Hoechst 33324 stain; (E): Calcein-AM stain; F: Overlay of D and E.

Fig.7 Morphology, distribution and viability of ADSCs cultured in C/GP/Co and C/GP hydrogels. ADSCs cultured in C/GP/Co hydrogels, in bright field (A_1), stained by Hoechst 33324 (A_2), stained by Calcein-AM (A_3) and PI (A_4), overlay by (A_3) and (A_4) after 2 days (A_5) and 5 days (B_{1-5}) of culture, respectively; ADSCs cultured in C/GP hydrogels, in bright field (C_1), stained by Hoechst 33324 (C_2), stained by Calcein-AM (C_3) and PI (C_4), overlay by (C_3) and (C_4) after 2 days (C_5) and 5 days (D_{1-5}) of culture, respectively; A_1 - D_5 : $\times 100$.

Fig.8 SEM assay for cell morphology and distribution of ADSCs within C/GP and C/GP/Co hydrogels after 7 days fabrication, respectively. (A) cell-C/GP hydrogel constructs $\times 500$; (B) cell- C/GP/Co hybrid hydrogel constructs $\times 1000$.

Fig.9 Photograph of the subcutaneous injection of C/GP and C/GP/Co hydrogels into SD rats. (A-B): C/GP/Co hydrogel, 0 day after injection; (C-E): C/GP/Co hydrogel, 28 days after injection; (F): size of different hydrogels, C/GP hydrogel; C/GP/Co hydrogel; C/GP: Co=2:1, C/GP: Co=1:1.

Fig.10 Immunostaining analysis of ADSCs-C/GP/Co and ADSCs-C/GP hydrogel implants and surrounding tissues. (A), (C) and (E): H&E staining; (B), (D) and (F): Masson staining. (A&B): ADSCs encapsulated with C/GP/Co hybrid hydrogel, after 2 weeks injection; (C&D): ADSCs encapsulated with C/GP/Co hybrid hydrogel, after 4 weeks injection; (E&F): ADSCs encapsulated with C/GP hydrogel, after 4 weeks of injection.

Fig.11 Photograph of the subcutaneous injection of induced ADSCs-C/GP/Co hydrogel into nude mice and biological assays of cell-hydrogel complex implants. (A). 0 day after injection; (B&C): 28 days after injection. Oil red O staining of cell-hydrogel implants after 4 weeks in vivo culture (D) Induced ADSCs (E) Non-induced ADSCs (F) H&E immunostaining of adipose-induced ADSCs-C/GP/Co hybrid hydrogel complex implants after 4 weeks in vivo culture.

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