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The effect of PLGA sphere diameter on rabbit mesenchymal stem cells in adipose tissue engineering

Yu Suk Choi · Si-Nae Park · Hwal Suh

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Abstract To investigate the effect of injectable PLGA sphere's diameter on adipose tissue engineering, rabbit mesenchymal stem cells were attached to various diameters of injectable PLGA spheres (<75; 75–100; 100–150; 150–200; and 200–250 μm). These five groups were cultured in adipogenic media for 2 weeks in vitro and injected into necks of nude mice. Prior to in vivo study, cell proliferation and adipogenic differentiation were determined by hexosaminidase assay and Oil red O staining after 2 weeks. Group C (100–150 μm) showed the highest adipogenic differentiation and the proliferation capacity of Group B (75–100 μm) was significantly higher than that of any other group. We harvested newly formed tissues from necks of nude mice after 1 and 4 weeks. Although PLGA spheres have not been degraded and there was no significant histological difference among various sizes of spheres after 1 week, well-organized fat pads (PLGA spheres were completely degraded) could be observed, and the histology of the 100–150 μm groups resembled that of native tissue after 4 weeks. Based on these experiments, we could conclude that the optimal size of PLGA spheres for adipogenesis was 100–150 μm.

Y. S. Choi · S.-N. Park · H. Suh (✉)
Department of Medical Engineering, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, South Korea
e-mail: hwal@yuhs.ac

S.-N. Park · H. Suh
BK21 Project Team of Nanobiomaterials for the Cell-based Implants, Yonsei University, Seongsanno 250, Seodaemun-gu, Seoul 120-752, South Korea

1 Introduction

Adipose tissue is widely distributed in human body, comprising 15–25% of the body weight in adults. Among its various distinct functions, its mechanical function as padding and filler, caused adipose tissue to be previously classified as connective tissue. As a depot of high-energy material, adipose tissue plays an important role in metabolism. Due to its ability to bind large amounts of fluid, adipose tissue influences the water balance. Finally, adipose tissue has good insulating properties; therefore it is connected with thermo-regulation [1].

In plastic and reconstructive surgery for regeneration from soft tissue defect [2], autologous transplantation of fat grafts has been clinically performed for depressed regions or scars in breast and facial areas [3, 4]. However, this treatment often has problems, such as the absorption and fibrosis of tissues grafted [5–7]. A promising substitute for the tissue engineering would be artificially induced formation of adipose tissue at a defect site.

In tissue engineering, a temporary scaffold is required to serve as an adhesive substrate for implanting cells and as a physical support to induce the formation of the new tissue. The temporary scaffold shall be biocompatible and biodegradable [8]. Additionally, in plastic and reconstructive surgery, minimal post-operation scars are ideal. Conventional sheet- or sponge-type scaffolds leave scars when transplanted via incision. Conventional injectable materials (gel-type) leave no scars, but do not provide appropriate rigidity. We designed injectable poly(D,L-lactic-co-glycolic acid) (PLGA) spheres, which provide appropriate rigidity as well, to meet these requirements in our previous studies [9–11]. Not only should the diameter of injectable PLGA spheres be smaller than that of the gauge of the syringe, but it must be large enough to adhere to MSCs [12, 13].

The injectable PLGA spheres used in this study automatically aggregated (data not shown), and functioned similarly to porous scaffolds in vivo. Porous, three-dimensional scaffolds have been used extensively as biomaterials in tissue engineering for in vitro and in vivo studies [9–11, 14]. These scaffolds of aggregated PLGA spheres serve as a physical support structure, just as conventional three-dimensional porous scaffolds do. Pore size of scaffold significantly influences the cell morphology and phenotypic expression [15, 16]. Diameter of injectable PLGA spheres is directly related to the pore size on scaffold of aggregated PLGA spheres. Finally, diameter of injectable PLGA spheres influences cell activity.

The aim of this study is to evaluate the effect of the diameter of injectable PLGA spheres on adipose tissue engineering.

2 Materials and methods

2.1 Fabrication of PLGA spheres

Poly (D,L-lactic-co-glycolic acid) [85:15] (Mw=90,000, Purac) was dissolved in dichloromethane to yield a 12:100 weight/volume(w/v) solution. This was added drop wise to stirred solution of 0.27% poly(vinyl alcohol) (PVA; Mw=30,000–70,000, SIGMA). The resulting PLGA/PVA emulsion was stirred for 72 h, resulting in complete evaporation of the solvent. Spheres were isolated by filtration, washed with deionized water, air-dried for 2 h, and then vacuum-dried for an additional 24 h [9, 17]. Using commercially available sieves, PLGA spheres were separated into the following size ranges: <75; 75–100; 100–150; 150–200; and 200–250 μm. Produced spheres were kept desiccated until further use.

2.2 Isolation and culture of rabbit mesenchymal stem cells

Rabbit mesenchymal stem cells (MSCs) were obtained from adult female white New Zealand rabbits (2.5–3.3 kg), aged between 8 months and 1 year (Samtaco Experimental Animals, Seoul, Korea), following a previously reported method [18]. In brief, heparinized bone marrow was fractionated over a Ficoll-Paque™ Plus solution (Amersham Biosciences, Uppsala, Sweden), and mononuclear cells were plated at a concentration of 2×10^6 nucleated cells/ml. Non-adherent cells were removed by changing the media. Cultured cells were maintained at 37 °C in a 5% CO_2 air atmosphere, with media changes every 3 or 4 days [19]. Then, we cultured these cells in adipogenic media supplemented with 1 μM dexamethazone (Sigma, St. Louis, MO, USA), 0.1 mM indomethacin (Sigma, St. Louis, MO, USA), 0.5 mM 3-isobutyl-1-methylzanthine (IBMX) (Sigma, St. Louis, MO, USA), and 10 μM insulin (Sigma, St. Louis, MO, USA) for 7 days (adipoMSCs).

2.3 Green fluorescence protein (GFP)-transfection of MSCs

MSCs were transfected by GFP for purposes of cell tracking. The primary MSCs were transfected with GFP as a cell marker, by GFP Fusion TOPO^R TA Expression Kits (Invitrogen, Carlsbad, CA, USA) and FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to manufacturer's instructions [9]. The efficiency of cell transfection with GFP was approximately 20%.

2.4 Cell seeding

PLGA spheres (<75; 75–100; 100–150; 150–200; and 200–250 μm) for in vitro studies were exposed to UV light on all surfaces for 24 h, swelled in 70% ethanol, and washed thoroughly. Spheres were spread on 24-well plates and seeded with adipoMSCs (MSCs were cultured in adipogenic media for 7 days) at a concentration of 5×10^5cells/well (Group A:adipoMSCs attached to PLGA spheres sized <75 μm; Group B: 75–100 μm; Group C: 100–150 μm; Group D: 150–200 μm; and Group E: 200–250 μm). Cells were allowed to adhere to the spheres for 12 h on a shaker. Sufficient amount of media was added to completely immerse the spheres, for 14 days of culture in adipogenic medium [9, 20].

2.5 Scanning electron microscope (SEM)

Morphology for five groups of adipoMSCs-PLGA spheres were characterized by scanning electron microscopy (SEM; Hitachi S-800, Tokyo, Japan). Samples were fixed with glutaraldehyde solution in PBS (2% w/v, 4 °C) for at least 24 h. Thereafter, samples were dehydrated with methanol, coated by an ultra-thin layer (300 Å) of gold, and their morphology was examined [9].

2.6 Adipogenic differentiation of MSCs

After cell seeding, five groups of adipoMSCs-PLGA spheres were allowed to adhere for 24 h in complete media at 37 °C in a CO_2 incubator. Then MSCs-PLGA spheres were cultured in adipogenic media. Adipogenic differentiation was

assessed in media supplemented with 1 μM dexamethazone (Sigma, St. Louis, MO, USA), 0.1 mM indomethacin (Sigma, St. Louis, MO, USA), 0.5 mM 3-isobutyl-1-methylzanthine (IBMX) (Sigma, St. Louis, MO, USA), and 10 μM insulin (Sigma, St. Louis, MO, USA) for 1, 3, 5, 7, 10, and 14 days [20]. Cell proliferation was assessed by hexosaminidase assay, and differentiation was confirmed by Oil red O staining.

2.7 Hexosaminidase assay

Conventional hexosaminidase assays were performed to evaluate proliferation [9, 21]. In brief, five groups of adipoMSCs-PLGA spheres were washed thoroughly, and soaked into 100 μl of hexoaminidase substrate (3.75 mM ρ-nitrophenyl-*N*-acetyl-β-D-glucosaminide, 0.25% Triton X-100, 0.05 M citrate buffer, pH 5.0) in each well of a 48-well plate. After 1 hour incubation in humidified atmosphere containing 5% CO_2 at 37 °C, 75 μl of stop-development buffer (5 mM EDTA, 50 mM glycine, pH 10.4) was added to each well. Quantitative data was obtained by an ELISA reader at a wavelength of 405 nm.

2.8 Oil red O staining

Five groups of adipoMSCs-PLGA spheres cultured in adipogenic media for 14 days were fixed in 10% formaldehyde solution (Sigma, St. Louis, MO, USA) in aqueous phosphate buffer for at least 1 h, washed with 60% isopropanol (Sigma, St. Louis, MO, USA), and stained with Oil Red O solution (in 60% isopropanol) for 10 min, followed by repeated washing with water. Solution was distained in 100% isopropanol for 15 min [9].

2.9 In vivo study

In vivo study involved the injection of the five groups of adipoMSCs-PLGAs into nude mice for 1, 4 weeks ($n = 6$ for each time point). Nude, athymic mice (BALB/c-nu, 8 weeks) were operated on under inhalation anesthesia with aseptic conditions. Spheres for in vivo studies were exposed to UV light on all sides for 24 h, swelled in 70% ethanol, and thoroughly washed. Spheres were spread on 24-well plates, and seeded with adipoMSCs at a concentration of 5×10^5 cells/well. Cells were allowed to adhere to spheres for 12 h on a shaker. Sufficient amount of media was added to completely immerse the spheres, which were then cultured for 12 h [9].

Five groups of adipoMSCs-PLGAs were injected into necks of nude mice. Newly formed tissues were retrieved at week 1 and week 4. To evaluate the histology, tissues were assessed via Oil red O staining with frozen sections. Frozen sections were constructed by slicing the samples into 50 μm sections using a cryostat, and then embedding on cover slips. Slides were examined under both fluorescence and light microscope (Olympus BX60, IX70 Olympus Optical Co., Tokyo, Japan). The population of GFP-positive cells was evaluated by fluorescence microscopy. Among GFP-positive cells, those, which had fully differentiated into adipose tissue were confirmed by Oil red O staining [9].

3 Results

3.1 In vitro study

Figure 1 shows the morpholoy for five groups of adipoMSCs-PLGA spheres after cell seeding. Diameters of PLGA spheres were <75; 75–100; 100–150; 150–200; and 200–250 μm. MSCs were evenly attached in all spheres, and some cells spread from one PLGA sphere to another one (Fig. 1a, b).

Five groups were cultured and observed in adipogenic media for 1, 3, 5, 7, 10 and 14 days in vitro. Adipogenic differentiation was determined by Oil red O staining (Fig. 2a). Group A (<75 μm) showed the poorest results in differentiation, compared with other groups. Results for Group B (75–100 μm) and Group E (200–250 μm) improved, but were not significant when compared with the remaining two groups, C and D. Though Group C (100–150 μm) and Group D (150–200 μm) differentiated at a similar rate until the seventh day, differentiation of Group D (150–200 μm) was arrested afterwards, while Group C (100–150 μm) continued to differentiate with constant speed.

Capacity of proliferation was determined by hexosaminidase assay (Fig. 2b). No change in proliferation capacity was observed in Group A (<75 μm) and Group E (200–250 μm) for 2 weeks. Proliferation capacity of Group B (75–100 μm) rapidly increased for the second week. Although similar tendency was showed in Group D (150–200 μm), it was still comparatively less than Group B. In Group C (100–150 μm), proliferation stopped at day 10 but suddenly started to increase very rapidly again on day 10.

3.2 In vivo study

Four weeks after PLGA spheres were injected into nude mice, well-organized fat pads could be observed in the neck portion (Fig. 3a, b). Figure 3c is gross image of the extracted fat pad. The fluorescence micrograph was

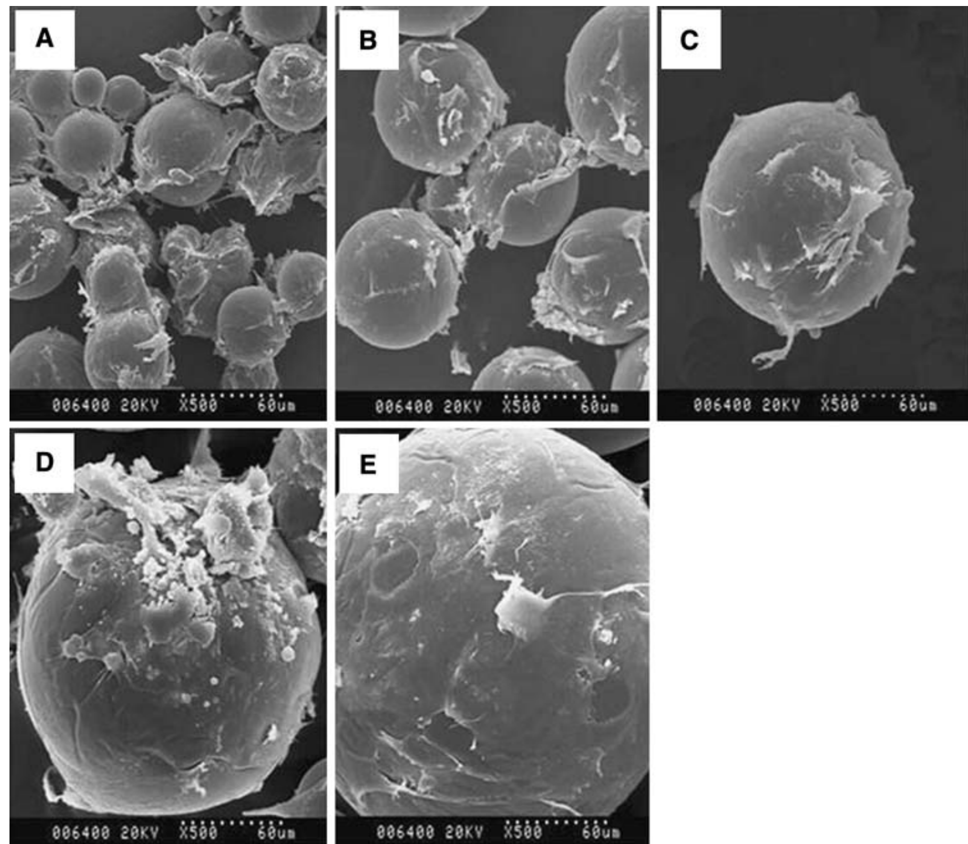

Fig. 1 SEM image of adipogenic induced MSCs attached to various sized PLGA spheres. <75 μm (**a**), 75–100 μm (**b**), 100–150 μm (**c**), 150–200 μm (**d**), 200–250 μm (**e**)

GFP-positive (Fig. 3d), indicating that the newly formed tissue was originated from injected MSCs.

Figs. 4 and 5 are Oil red O staining images of newly formed adipose tissue on the first and fourth week. On the first week, although adipose tissue formation could be observed, PLGA spheres were not yet degraded, (arrows in Fig. 4) and there was no significant difference among various sizes of the spheres. On the fourth week, PLGA spheres were completely degraded, and Group C (100–150 μm) of Fig. 5 had the most histological resemblance to native tissue (Fig. 5f). Morphologically, Group B (75–100 μm) was similar to adipose tissue, but the extent of staining was weak.

4 Discussion

The main disadvantage in previous adipose tissue regeneration studies was that the implanted cells were not well-organized as native adipose tissue and were eventually degenerated. This was due to matured adipocytes and the inability to induce angiogenesis. To address this problem, pre-adipocytes were transplanted instead of mature adipocytes or tissue fillers. The strategy was to use potent cells for proliferation and differentiation to form adipose tissue. It is true that pre-adipocytes derived from adipose tissue are widely used to study human adipogenesis; however, these cells are already committed to the adipogenic lineage. Pre-adipocytes have reduced proliferation ability, unpredictable variability based on anatomical sites, and limited availability. In addition, with regard to primary preadipocyte cultures, differentiation capacity is clearly donor-dependent and significantly decreases with age [22–26].

In this study, MSCs attached to injectable PLGA spheres with different diameters were examined in vitro and in vivo. MSCs are multipotent, and possess ability to proliferate into a mass of cells with only a single aspiration of bone marrow [27]. Capacity of MSCs to differentiate or proliferate is similar among most donors, rendering autologous transplantation possible in most patients [28]. By exploiting these well-known advantages, this study provides evidence that MSCs can principally be used in the development of an adipose tissue engineering technique, which will enable the creation of a three-dimensional soft tissue.

It has already been reported that pore size of the scaffold influences cell activities. On porous silicon nitride scaffolds, endothelial cells preferentially bind to scaffolds with pores smaller than 80 μm, while fibroblasts preferentially bind to larger pores (>90 μm). In PLLA scaffolds, vascular smooth muscle cells preferentially bind to one range of pore sizes (63–150 μm) while fibroblasts bind to a wider range (38–150 μm) [29, 30].

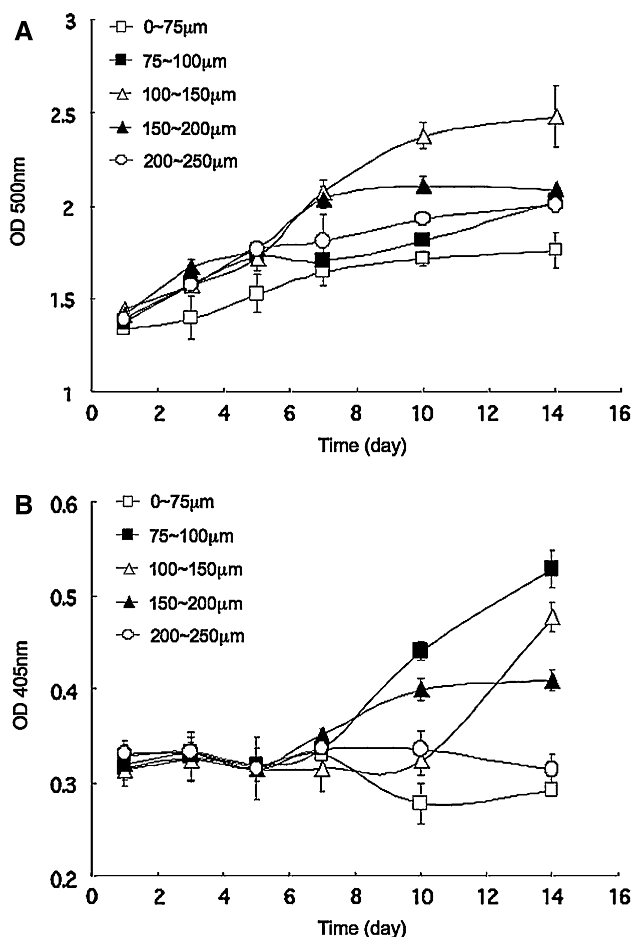

Fig. 2 Cell proliferation and adipogenic differentiation test of APLGA groups of various sizes in vitro for 2 weeks. Cell proliferation was determined by hexosaminidase assay. Differentiation of various sized APLGA groups in vitro for 2 weeks. Adipogenic differentiation was determined by Oil red O staining. ANOVA test of 5 groups and 6 time course using S-PLUS software. $p < 0.001$

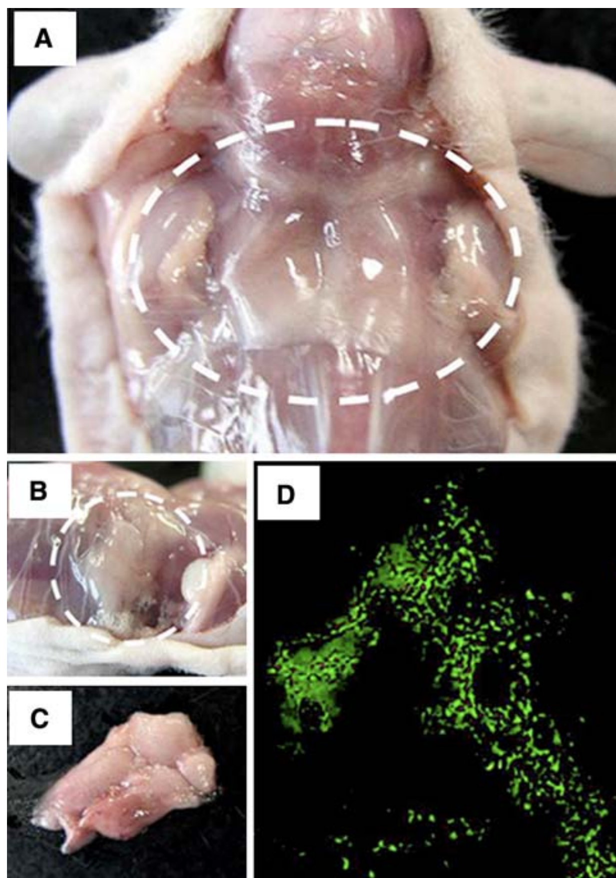

Fig. 3 PLGA derived adipose tissue was well organized in the nude mouse subcutis. A fat pad was formed in the neck portion of the nude mouse. Top view (**a**) and lateral view (**b**). Newly formed adipose tissue in a nude mouse injected with 100–150 μm PLGAs (**c**). A fluorescence micrograph of newly formed tissue in a nude mouse 4 weeks after injection (magnification × 100) (**d**)

To evaluate optimal size of PLGA spheres for adipose tissue engineering, MSCs-PLGA spheres were injected in necks of nude mice. The ideal material for adipose tissue regeneration should lead appropriate differentiation as well as proliferation. As seen in Fig. 2, Group C (100–150 μm) differentiated rapidly at a relatively constant rate for

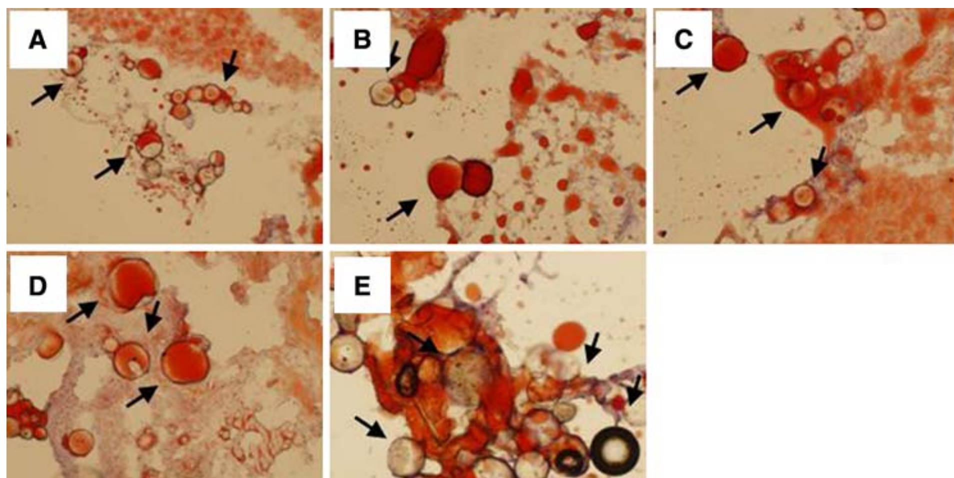

Fig. 4 Oil red O staining of newly formed adipose tissue after 1 week. <75 μm (**a**), 75–100 μm (**b**), 100–150 μm (**c**), 150–200 μm (**d**), 200–250 μm (**e**). PLGA spheres were not yet degraded (black arrow) (magnification × 200, Bar = 100 μm)

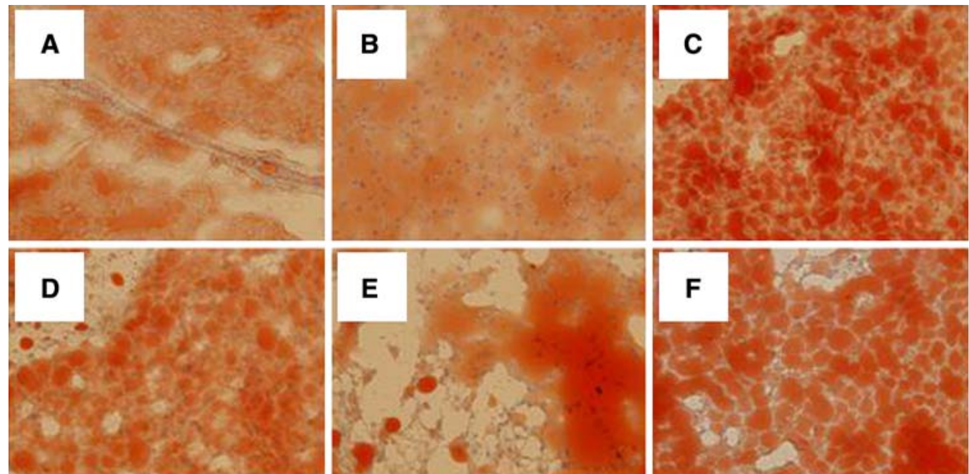

Fig. 5 Oil red O staining of newly formed adipose tissue after 4 weeks. <75 μm (**a**), 75–100 μm (**b**), 100–150 μm (**c**), 150–200 μm (**d**), 200–250 μm (**e**), native adipose tissue (**f**) (magnification × 200, Bar = 100 μm)

14-day period, but showed a unique pattern in proliferation. This pattern of proliferation can be explained by the fact that cells differentiate better in a more confluent environment. When comparing the differentiation and proliferation curves of Group C (100–150 μm), we can see that the differentiation rate drops on the tenth day, while the proliferation rate suddenly increases at a significant rate on the same day. This may be caused by the sudden proliferation of remaining undifferentiated cells [31]. From this experiment, diameter of 100–150 μm was considered as the optimal size for adipogenesis. Moreover, PLGA spheres were completely degraded on the fourth week. Group C (100–150 μm) had the greatest histological resemblance to native tissue (Fig. 5f) on the fourth week, which indicates that Group C (100–150 μm) showed the best results for in vitro study.

It has been reported that MSCs can differentiate into adipocytes only under confluent conditions [32]. MSCs attached to 100–150 μm PLGA spheres recognized the environment formed by Group C (100–150 μm) as the most confluent one among the 5 groups.

In conclusion, various sizes of PLGA spheres were tested. Adipogenesis-induced MSCs attached to PLGA spheres were cultured in vitro and in vivo. Proliferation and differentiation tests were performed in vitro, and Group C (100–150 μm) showed the best results among the 5 groups. MSCs survived after transplantation into nude mice and fully differentiated at the subcutaneous fat. Histological aspects of Group C (100–150 μm) were comparable to native adipose tissue. Despite these favourable outcomes, reasons are not clarified. Further studies on the interaction between MSCs and various sizes of PLGA spheres shall be performed for better understanding of the micro-environment needed for adipose tissue regeneration in vivo. In addition, studies on neovascularization originating from MSCs, and the long-term volume maintenance of newly formed adipose tissue shall be performed.

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