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ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

Locally Increased Level of Inorganic Phosphate Induced Nodules or Calcification After Bolus Fat Grafting

Shangshan Li[1] · Wenyue Liu[1] · Deni Kang[1] · Hao Cheng[1] · Zifei Li[1] · Jun Qi[1] · Dali Mu[1] · Chunjun Liu[1] · Minqiang Xin[1] · Su Fu[1] · Jie Luan[1]

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Abstract

Background Nodules or calcifications have been a common complication after breast augmentation with fat grafting, especially in cases with partial bolus fat grafting. There are some clinical preventive measures, but mechanisms related to this complication have not been elucidated yet. Inorganic phosphate (PI), being a product of fat metabolism, is a well-known stimulus of other kinds of pathological calcification such as vascular calcification. We aimed to determine whether PI had a similar effect on formation of nodules after fat grafting.

Methods Nodules or calcification after fat grafting models using nude mice were created by bolus fat injection. Levels of PI of necrotic liquid located in the central zone and mineralization deposition of graft were examined 1 week, 2 weeks, 1 month, 2 months and 7 months after bolus fat injection. External high phosphate solution was injected 3 times a week to the fat grafts for 2 months, and mineral deposition was examined. In addition, adipose-derived stem cells (ADSCs) were treated with high phosphate osteogenic differentiation medium in various concentrations and times. ADSCs were also treated with osteogenic differentiation in addition to tetramisole which could reduce the level of PI. Mineral depositions of the cells were examined. The central necrotic liquid was extracted from patients who found palpable nodules after breast augmentation with fat grafting. The level of PI of this necrotic liquid and normal lipoaspirates from patients who received normal liposuction for body contouring was compared.

Results The in vivo study indicated that the local PI concentration of the necrotic zone increased significantly 2 months after large volume bolus fat injection. Calcification was not formed after 2 months, but was formed after 7 months, indicating that the effect of PI on calcification was time-dependent. In addition, with the effect of external injection of high phosphate solution into the fat graft, calcification was formed after 2 months, indicating the effect of PI on calcification was dose-dependent. The in vitro study also indicated PI could induce calcification of ADSC in a time- and dose-dependent manner. The study in humans indicated that the level of PI in the necrotic zone of nodules after fat grafting was higher than that in normal lipoaspirates.

Conclusions This study indicated that the level of PI in the central necrotic zone was elevated after bolus fat injection, which could provide an environment to induce calcification of surrounding tissue.

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Keywords Inorganic phosphate · Calcification · Autologous fat grafting

Introduction

Autologous fat grafting has been widely used in breast augmentation or reconstruction. Calcification, which might occur in 25% of cases [1], is a complication that has never

✉ Jie Luan
luanjieplastic@126.com

[1] Department of Aesthetic and Reconstructive Breast Surgery, Plastic Surgery Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, 33 Badachu Road, Shijingshan District, Beijing, China

been completely solved. Although calcifications resulting from fat necrosis were quite easily distinguished from those associated with cancer by radiologists in most cases [2], sometimes the calcifications imitated malignity and required biopsy to clear the diagnosis [3]. Furthermore, sometimes large palpable nodes might occur after fat grafting, decreasing the satisfaction rates. Currently, partial bolus fat injection could be one of the reasons for the formation of calcification and various studies indicated that multilayer, multipoint, multitunnel with small amounts per cannula injection might be preventive for the formation of these nodules [4, 5]. However, there is a lack of evidence that illustrates the mechanisms of formation of this kind of calcification.

There were two main extracellular mineral ions, inorganic phosphate (PI) and calcium (Ca) that have a synergic effect on the formation of pathological calcification mainly consisting of hydroxyapatite [6–10]. A lot of studies indicated that inorganic phosphate (PI) had an effect on pathologic calcifications such as vascular calcification in a dose and time manner [11–13]. Phospholipid was hydrolyzed by phospholipase to phosphatidic acid which liberated PI under the effect of alkaline phosphatase (ALP) [14], which indicated Pi was one of the products of fat metabolism.

In this study, we aimed to determine whether the level of PI in the central necrotic zone was elevated in the process of large volume bolus fat injection, and whether this high PI environment had a promoting effect on calcification which could contribute to the formation of palpable nodules.

Materials and Methods

In Vivo

Oil Cysts After Fat Grafting Animal Model

Animal care and the study protocol were approved by the Animal Care and Ethics Committee of Peking Union Medical College. All applicable institutional and/or national guidelines for the care and use of animals were followed. Balb/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). Human lipoaspirates were obtained from healthy Chinese female volunteers who underwent liposuction for body shaping. All volunteers signed informed consents. Female mice (n = 20) with an age of 6–8 weeks were used for this study. Tumescent fluid containing 0.04% lidocaine and 1 mg/L epinephrine was injected, and liposuction was done by using negative pressure from one human volunteer (female, age 19, body mass index BMI 24.5 kg/m^2, thigh liposuction). After 15 min of decantation of lipoaspirates, the bottom layer with bloody tumescent fluid and top layer with oil were discarded. For imitating the clinical effect of mass injection that might cause nodules, bolus injection of 4 mL lipoaspirates was administered on the dorsal flank of nude mice to make the necrotizing zone as large as possible (Fig. 1). After 1 week, 2 weeks, 1 month, 2 months and 7 months, respectively, four mice were sacrificed each time and grafts including central necrotizing zones were harvested (n = 4). Central necrotic liquid was harvested through aspirating the central zone of fat grafts using a 18-gauge syringe needle, and then, the remaining fat grafts were harvested through an incision around the grafts. Levels of PI in the central necrotic liquid of 1 week, 2 weeks, 1 month and 2 months were examined, and mineralization deposition of grafts of 2 months and 7 months was examined by hematoxylin–eosin (HE) staining and Von kossa staining, (These methods were described later in the methods section.) If capsules were visually present at the time of fat harvest, their firmness was assessed by palpation.

Treatment of Fat Grafting with High Inorganic Phosphate Solution

Lipoaspirates were obtained with the same method from another volunteer (female, age 23, BMI 23.4 kg/m^2, upper arm liposuction). Bolus injections of 4 mL lipoaspirates were administered on the dorsal flanks of nude mice (n = 8). For the experimental group (n = 4), high inorganic phosphate (10 mM) solution was injected locally 3 times a week into grafted fat. For the control group (n = 4), saline was injected locally 3 times a week into grafted fat. After 2 months, nude mice were sacrificed. Levels of Pi in the

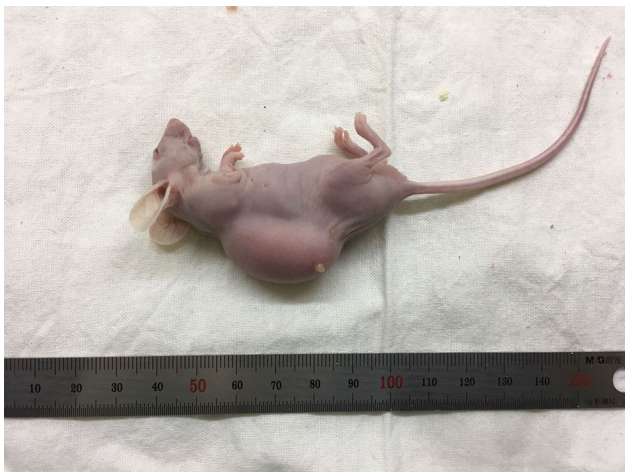

Fig. 1 Bolus injection of 4 mL lipoaspirates was administered on the dorsal flank of nude mice to make the necrotizing zone as large as possible

central necrotic liquid were examined and mineralization deposition of grafts was examined by hematoxylin–eosin (HE) staining and Von kossa staining.

In Vitro

Human Adipose-Derived Stem Cells (ADSCs) Culture

Human adipose tissue was collected after liposuction surgery from Plastic Surgery Hospital, Peking Union Medical College. Lipoaspirates were collected and digested with collagenase I (Sigma, New York, NY, USA). Then, cells were cultured with mesenchymal stem cell medium (MSCM) containing 1% penicillin–streptomycin, 5% fatal bovine serum (FBS) and 1% mesenchymal stem cell growth supplement (ScienCell Research Laboratories, Carlsbad, CA, USA). All cells were cultured at 37 °C in 5% CO_2 humidified environment. The culture medium was replaced in the first two days and every three days thereafter.

Treatment of ADSCs with High Phosphate Osteogenic Differentiation Medium

To investigate whether the high PI solution had a promoting effect on calcification of ADSCs, human mesenchymal stem osteogenic differentiation medium kits, containing β-glycerophosphate (10 mM), ascrobate, dexamethasone and glutamine [15] were purchased from Cyagen Biosciences. ADSCs were cultured in osteogenic medium for 7 days, 14 days and 28 days, respectively. ADSCs were cultured in different concentrations of β-glycerophosphate in osteogenic differentiation medium (1 mM, 3.3 mM and 10 mM) and were checked at two different time points (14 days and 28 days). Mineral deposition was examined by Alizarin Red S staining and quantification of calcium deposition was also examined.

Effect of Tetramisole (Levamisole) on Cells with High Phosphate Osteogenic Differentiation Medium

The effects of tetramisole (levamisole), a specific inhibitor of ALP [16], with various concentrations, were examined on induction of ADSCs calcification with osteogenic differentiation medium. After treating in vitro ADSC samples with osteogenic differentiation medium, various concentrations of levamisole were administered (10^{-5} M, 10^{-4} M and 10^{-3} M) for 14 days and samples were assessed for mineral deposition using Alizarin Red S staining.

Clinically: Examination of Removed Human Oil Cysts or Palpable Nodules

Clinically, central necrotic liquid was extracted from patients who needed to remove palpable nodules that were found after breast augmentation with fat grafting. The samples of lipoaspirates were collected from other patients who received liposuction for body contouring as a control group. All the patients signed informed consents. Levels of PI of the central necrotic liquid and that of lipoaspirates were examined. Histologic examination was done in one nodule removed from a patient who had palpable nodules after fat grafting.

Histologic Examination

All tissues removed from nude mice were fixed and embedded. These samples were sectioned at 5 μm and hematoxylin–eosin (HE) staining, Von kossa staining or Alizarin Red S staining were conducted for calcification visualization.

Quantitative Measurements of Inorganic Phosphate, Ionized Calcium and Calcification

The oil, central liquids in necrotizing zones or lipoaspirates were suspended in saline at a 1:10 ratio, and the supernatant was used for measurement of PI concentrations. PI concentration was measured using the phosphomolybdic acid method (PI kit, Nanjing Jiancheng, China). Briefly, a working solution containing molybdic acid was added to the sample for 30 min at 37 °C. Then, the whole solution was cooled to room temperature and absorbance was measured at 660 nm. The PI concentration of the sample was calculated by the relationship with the value of the standard PI solution.

Cells were decalcified with 0.6 N HCl for 24 h. The calcium content of supernatants was determined colorimetrically by the o-cresolphthalein complex one method (Calcium kit; Sigma) as previously described [17]. After decalcification, the cells were washed with PBS and solubilized with 0.1 N NaOH/0.1% SDS. The protein content was measured with a BCA protein assay kit (Solarbio, China). The calcium content was normalized to protein content and expressed as μmol calcium/mg protein [12].

Statistical Analysis

Data analysis was conducted using Student's -test and one-way ANOVA test by Prism 6 software (Graphpad, San Jose, CA, USA). Data are presented as mean ± SD (standard deviation), and p values less than 0.05 were considered statistically significant.

Results

In Vivo

The level of PI in oil cysts and calcium contents of grafts were significantly elevated 2 months after fat grafting. Significant calcifications were found in the fat grafts 7 months after bolus fat grafting.

After bolus injection of human lipoaspirate into nude mice and harvesting them at 1 w, 2 w, 1 m and 2 m, we found that the central necrotic liquid that was extracted 2 months after injection was thicker in consistency than that extracted 2 weeks or 1 month after injection. However, after 7 months, the central necrotic zones were semisolid, not liquid (Fig. 2).

For the central necrotic zones that were semisolid after 7 months, we just extracted the central liquid samples from 1 week, 2 weeks, 1 month and 2 months. We found that the levels of PI of the necrotic liquid did not change significantly in the first month after fat grafting (0.805 mM at 1 week, 0.906 mM at 2 weeks, 0.769 at 1 month, $p > 0.05$). However, the levels were significantly increased at 2 months after grafting (1.924 mM at 2 months, $p < 0.05$, Fig. 3). Meanwhile, firmer capsules were found in the fat grafts at 2 months and 7 months.

Fat grafts at 2 months and 7 months were taken out for HE staining and Von kossa staining. Few significant positive results for calcification were presented in the first 2 months. However, after 7 months, significant calcification was found (Fig. 4).

These results indicated that the levels of PI did not change significantly during the first month which might be related to the balance between generation and absorption.

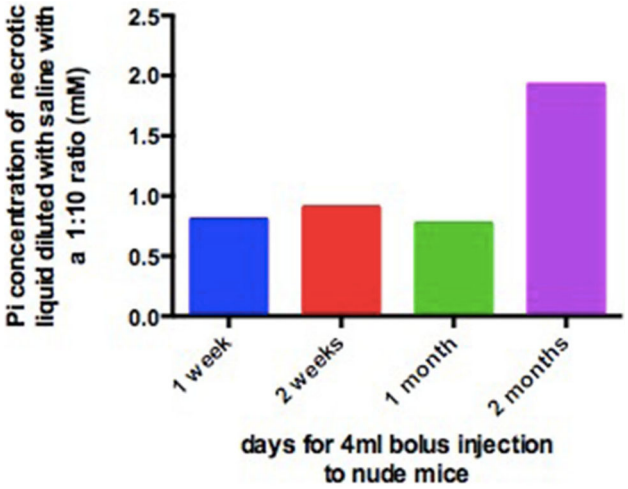

Fig. 3 PI concentrations of liquids in central zones diluted with saline with a 1:10 ratio at various times after 4 mL bolus injection of lipoaspirates

The level of PI was elevated significantly at 2 months which might be related to the formation of a firm capsule that isolated the central necrotizing zone from the surrounding area that inhibited the absorption of the necrotic liquid. However, the levels of calcification were still not large enough for HE or Von kossa staining at 2 months after fat grafting. After 7 months, the necrotic zone was semisolid and significant calcifications were found which was in accordance with the clinical experience that calcification occurred at least several months after fat grafting.

Calcification was found in the group of bolus fat injection in addition to local injection of high phosphate solution for 2 months, while there was no significant

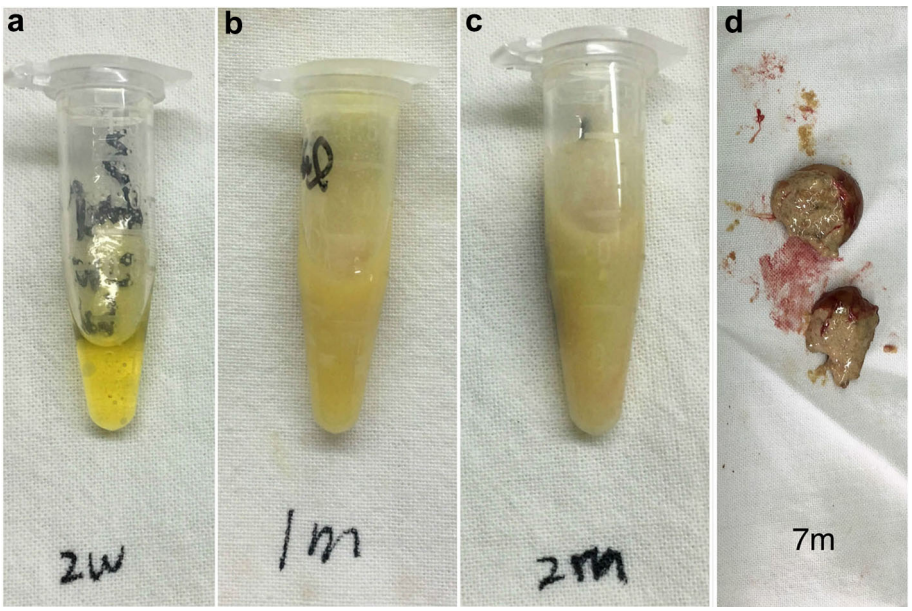

Fig. 2 Liquid characters of necrotizing zone. **a** 2 weeks after fat grafting. **b** 1 month after fat grafting. **c** 2 months after fat grafting. **d** 7 months after fat grafting

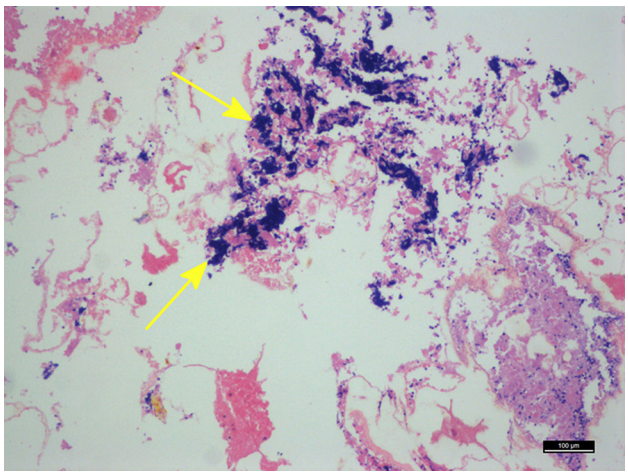

Fig. 4 HE staining of fat grafting at 7 months after bolus fat injection. Calcification was indicated as yellow arrows

calcification in the control group that was injected bolus fat adding saline instead.

The results of HE staining and Von kossa Staining indicated that significant calcification was found in the experimental group that was injected with high phosphate solution 3 times a week for 2 months (Fig. 5). However, in the control group, no significant calcification was found.

In Vitro Study

PI Induces Calcification of ADSCs in a Dose-Dependent and Time-Dependent Manner

To confirm the effect of high phosphorus on induction of calcification, ADSCs were treated with high phosphorus osteogenic differentiation medium for 7, 14 and 28 days. As shown in Fig. 6, the results of Alizarin Red S staining indicated that mineral deposition was increased in a time-dependent manner. The results of quantitative calcium deposition were 0, 4.795, 5.256, 9.454 μmol/mg protein at 0, 7, 14, 28 days (Fig. 7). ADSCs were also treated in osteogenic differentiation medium with 1, 3 and 10 mM β-glycerophosphate for 14 days and 28 days. The results of Alizarin Red S staining (Fig. 8) indicated that higher a concentration of β-glycerophosphate induced higher levels of calcification. The results of quantitative calcium deposition were 0.052, 0.088, 1.255, 2.523 μmol/mg protein at 14 days; 0.001, 0.023, 4.620, 7.690 μmol/mg protein at 28 days (Fig. 9). These results indicated that PI induced calcification of ADSCs in a dose-dependent and time-dependent manner.

Tetramisole (Levamisole) Inhibited High Phosphorus Induction of Calcification

To further confirm the effect of PI on calcification, ADSCs were treated with high phosphorus osteogenic differentiation medium in the presence of various concentrations (10^{-5} M, 10^{-4} M, 10^{-3} M) of tetramisole, a specific inhibitor of ALP [16], for 14 days. The results of Alizarin Red S staining indicated that 10^{-3} M tetramisole significantly reduce calcification of ADSCs (Fig. 10).

Clinical Study

The level of PI in necrotic zones of nodules that were removed from patients who received fat grafting before was significantly higher than that in normal lipoaspirates.

Palpable nodules were found from seven breasts of patients who received breast augmentation with fat grafting (age 23–36, BMI 17.2–25.2 kg/m^2, donor site included thigh or waist or abdomen). The time periods from fat grafting to palpable nodules were 6 months to 2 years. The character of extracted necrotic liquid (Fig. 11) was as thick as the fat grafting animal model at 2 months after injection.

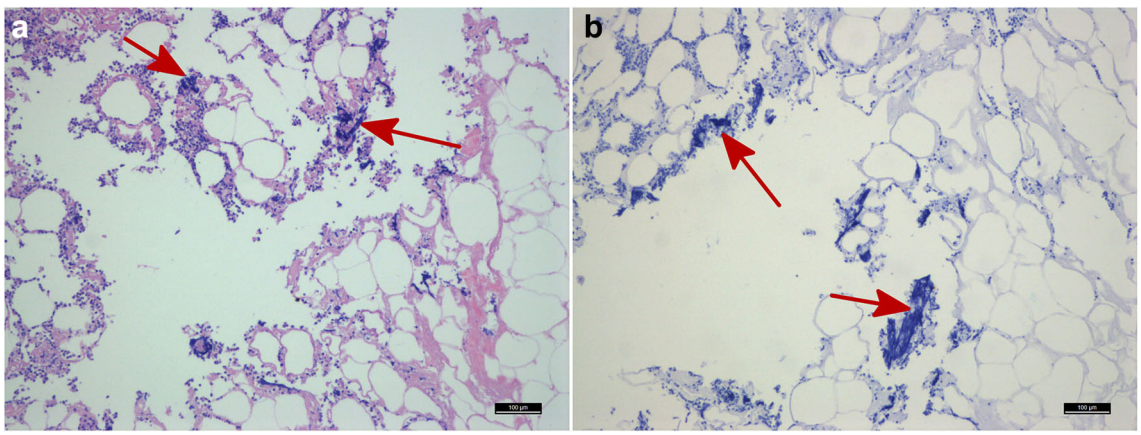

Fig. 5 Bolus fat injection in addition to high Pi solution for 2 months. **a** HE staining. **b** Von kossa staining. Calcifications were indicated as red arrows

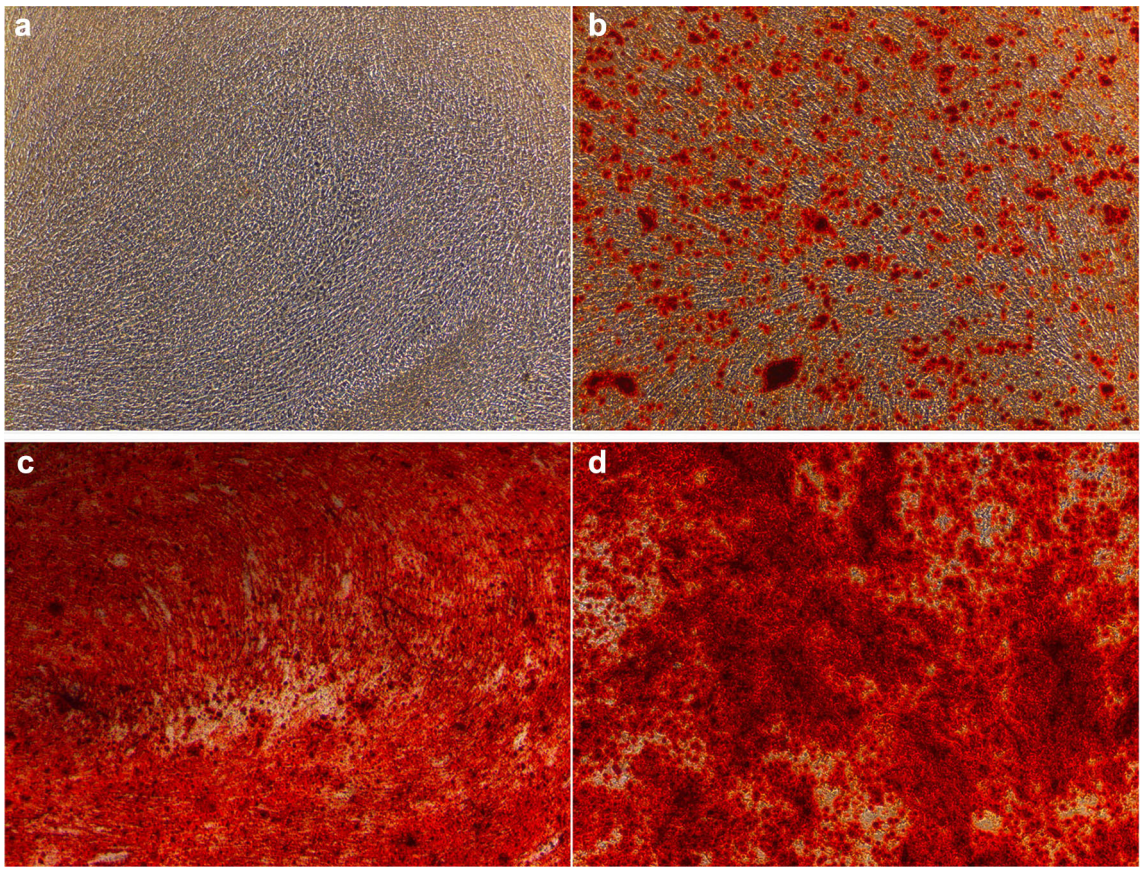

Fig. 6 Alizarin Red S staining of the ADSCs induced with various times of osteogenic differentiation medium. **a** ADSCs induced with 0 day of osteogenic differentiation medium. **b** ADSCs induced with 7 days of osteogenic differentiation medium. **c** ADSCs induced with 14 days of osteogenic differentiation medium. **d** ADSCs induced with 28 days of osteogenic differentiation medium. Red part was explained as part of calcification

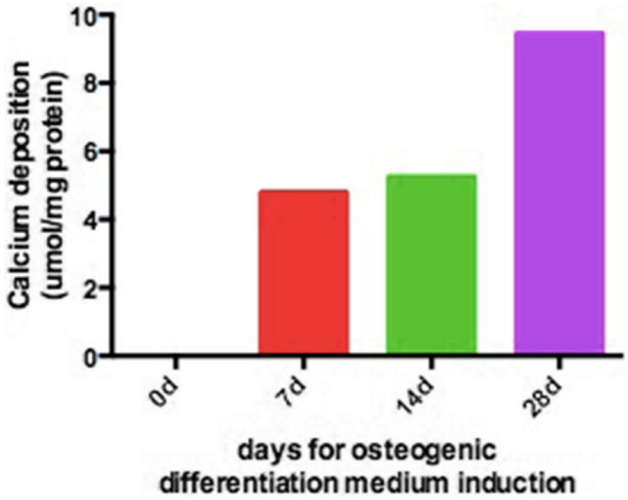

Fig. 7 Quantitative measurement of calcium of ADSCs induced with 0, 7, 14, 28 days of osteogenic differentiation medium

Liposuction was conducted for body contouring in another five patients (age 23–40, BMI 18.4–21.8 kg/m^2, donor site included thigh or waist or abdomen). The mean level of PI was significantly higher in liquids from nodules than in normal lipoaspirates (1.304 mM vs. 0.801 mM, $p < 0.05$).

In one of these cases, a woman (age 36, BMI 25.2 kg/m^2) received thigh liposuction and breast augmentation with fat grafting 1 year ago, and palpable nodules were found 6 months after fat grafting. A large range of nodules were removed but most of them were solid, and little visible central liquid was found inside them. The results of alizarin Red S staining indicated that calcifications were found in the cyst wall (Fig. 12). In these specimens, we found that some calcification occurred just in the partial capsule of the wall but some calcification, being substantive, occupied the whole capsule, inferring that this kind of process was accumulative which was in accordance with the experiments in vitro. We also found that most calcification occurred inside the wall (Fig. 12), indicating that the environment inside the wall might have an effect on formation of calcification and the recruited cells also contributed to formation of calcification.

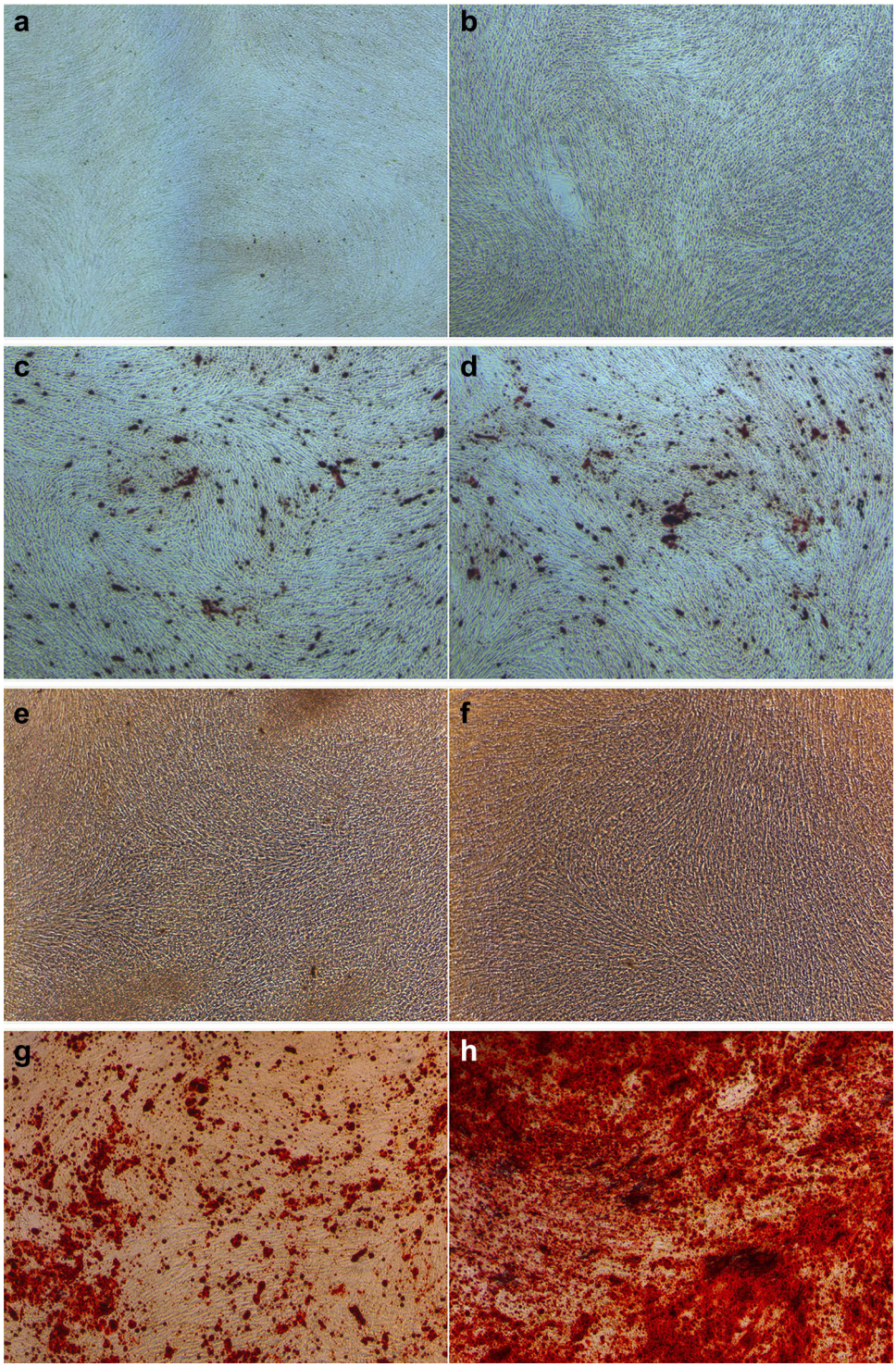

Fig. 8 Alizarin Red S staining of the ADSCs induced with osteogenic differentiation medium with 1, 3 and 10 mM β-glycerophosphate. **a** ADSCs induced with 0 mM β-glycerophosphate medium for 14 days. **b** ADSCs induced with 1 mM β-glycerophosphate medium for 14 days. **c** ADSCs induced with 3.3 mM β-glycerophosphate medium for 14 days. **d** ADSCs induced with 10 mM β-glycerophosphate medium for 14 days. **e** ADSCs induced with 0 mM β-glycerophosphate medium for 28 days. **f** ADSCs induced with 1 mM β-glycerophosphate medium for 28 days. **g** ADSCs induced with 3.3 mM β-glycerophosphate medium for 28 days. **h** ADSCs induced with 10 mM β-glycerophosphate medium for 28 days. Red part was explained as part of calcification

Discussion

Formation of nodules or calcifications has been a common complication after breast augmentation with fat grafting [1, 18, 19]. After fat grafting, the innermost zone was the fat necrotizing zone, which might be correlated with oil cysts or even calcification [20]. Liponecrosis could be related to excessive bolus injection into a single area, inadequate blood supply in the recipient tissues, or less commonly, mechanical trauma caused by the blunt cannula [3]. Calcification might be related to inflammatory reactions but the mechanisms have not been clearly elucidated [21]. At first, capsules were developed but could not be detected on mammography [3, 22] and became visible on

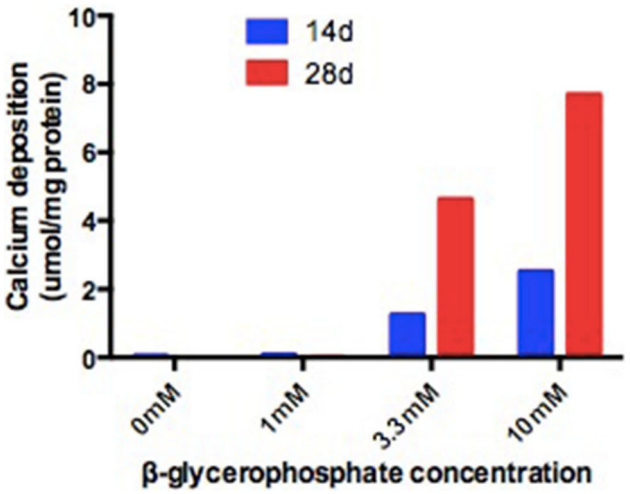

Fig. 9 Quantitative measurement of calcium of ADSCs induced with osteogenic differentiation medium containing 0, 1, 3.3, 10 mM β-glycerophosphate for 14 days and 28 days

mammography after a few months [1, 23]. To figure out the mechanisms of this kind of nodules or calcification, we created an animal model using bolus fat injection to nude mice. In our animal model, we found that significant calcification was found at 7 months and the capsules at 2 months and 7 months after fat injection were much thicker and firmer than capsules at 2 weeks and 1 month.

Previous studies indicated that PI stimulated pathologic calcification such as vascular calcification in a dose- and time-dependent manner [11–13]. Elevated phosphate resulted in loss of smooth muscle markers and elevated expression of osteochondrogenic markers [12, 13, 24, 25]. Another study indicated that in the process of fat metabolism, phospholipid was hydrolyzed to produce phosphatidic acid which liberated inorganic phosphate under the effect of ALP [14]. However, few previous studies directly correlated PI with calcification after fat grafting.

In this study, we firstly created an animal model to imitate the formation of nodules or calcification after fat

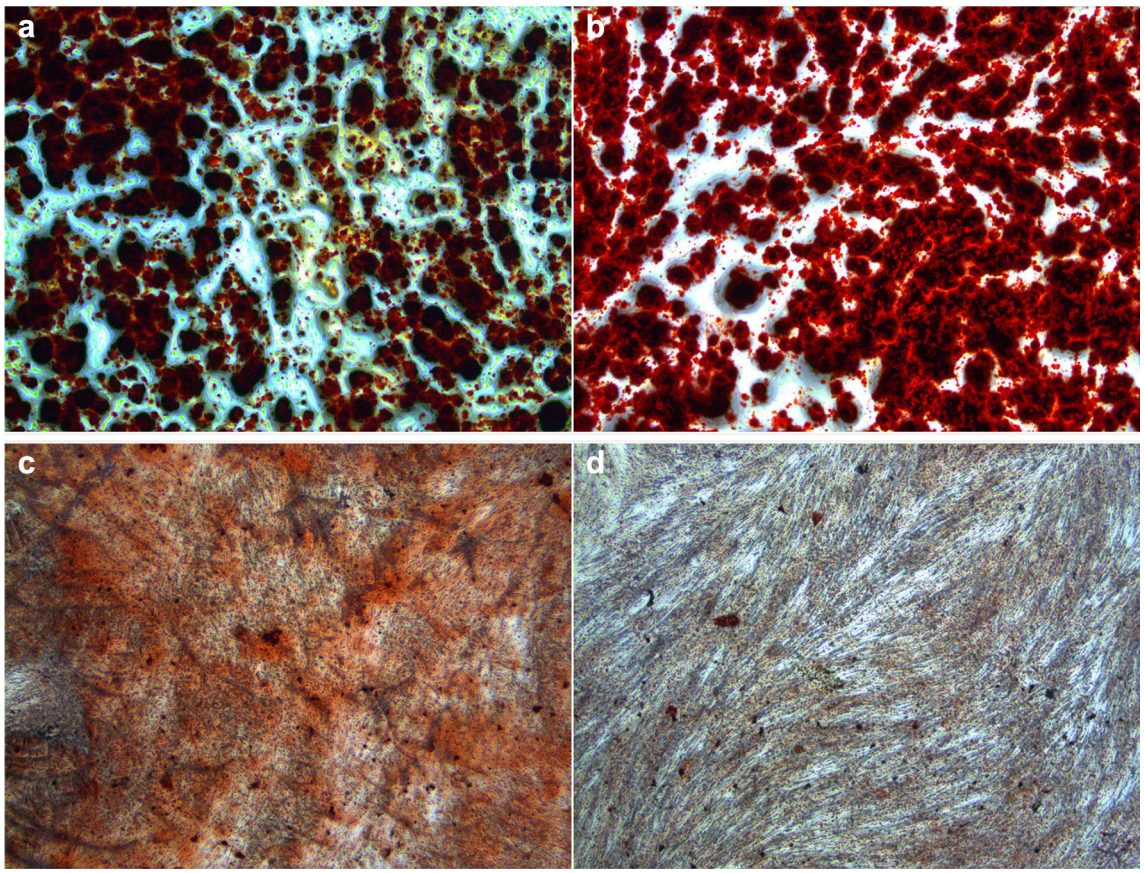

Fig. 10 Alizarin Red S staining of the ADSCs induced with osteogenic differentiation medium in addition to various concentrations of tetramisole (levamisole) for 14 days. **a** ADSCs induced with osteogenic differentiation medium without levamisole. **b** ADSCs induced with osteogenic differentiation medium in addition to levamisole(10^{-5} M). **c** ADSCs induced with osteogenic differentiation medium in addition to levamisole (10^{-4} M). **d** ADSCs induced with osteogenic differentiation medium in addition to levamisole (10^{-3} M). Red part was explained as part of calcification

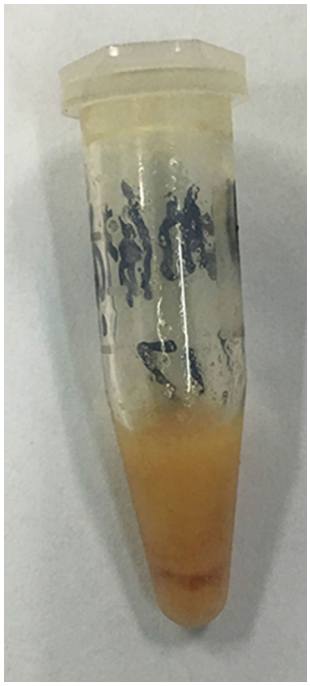

Fig. 11 Liquid characters of necrotic liquid removed from a patient

grafting. We attempted to inject the lipoaspirates as much as possible in confined spaces in nude mice so that large nodules could be formed. We found that if we injected more than 4 ml in one point, the grafts were dispersed to surrounding areas so we chose 4 ml as our volume of bolus injection. Our results indicated levels of PI did not change significantly in the first month after fat grafting. But at 2 months after fat grafting, the levels of PI in oil cysts were significantly elevated along with the formation of thick and firm capsules. According to these findings, we hypothesized that during the first month, there was a PI metabolic balance which might be related to an incomplete capsule, but after the formation of thick capsules that occurred at approximately 2 months, the oil cysts were isolated from the surrounding area, the absorption of PI was reduced which resulted in locally elevated concentration of PI.

Although no calcification was found after 2 months of 4 mL bolus fat injection to nude mice, significant calcification was found after 7 months, indicating that formation of calcification was accumulative and needed several months, which was in accordance with clinical experience. However, with the effect of external injection of high phosphate solution, calcification was found after 2 months, indicating that PI was actually a stimulator of calcification. These results indicated that the effect of PI on calcification was time- and dose-dependent.

Our studies in vitro indicated that high PI caused calcification of ADSCs in a dose-dependent and time-dependent manner, indicating that calcifications were not formed instantaneously but formed after a period of time that the effect of PI accumulated both in concentration and time. It also corresponded with our study in vivo and clinical observations that palpable nodules or calcifications were usually detected at several months after fat grafting. Previous studies stated that ALP hydrolyzed various monophosphate with a release of PI [26, 27]. Our research indicated that tetramisole, an inhibitor of ALP, could significantly reduce calcification of ADSCs induced by high PI osteogenic differentiation medium indicating that decreasing levels of PI could reduce the range of calcification. These experiments further verified that high levels of PI could be one of the stimuli that induce calcification of ADSCs. As ADSCs and vascular cells could both occur in the surrounding area of fat grafting [28], combined with the former study that PI could contribute to calcification of vascular cells [12], these cells including ADSCs and vascular cells could be potential cells that could be affected by the high phosphate environment and contribute to the calcification after fat grafting.

This study firstly created animal models that imitated nodules or even calcification after fat grafting, which could be useful for further research of this kind of unresolved complication after fat grafting. We also firstly determined that locally high levels of PI could be one of the reasons that contribute to the formation of calcification after fat grafting.

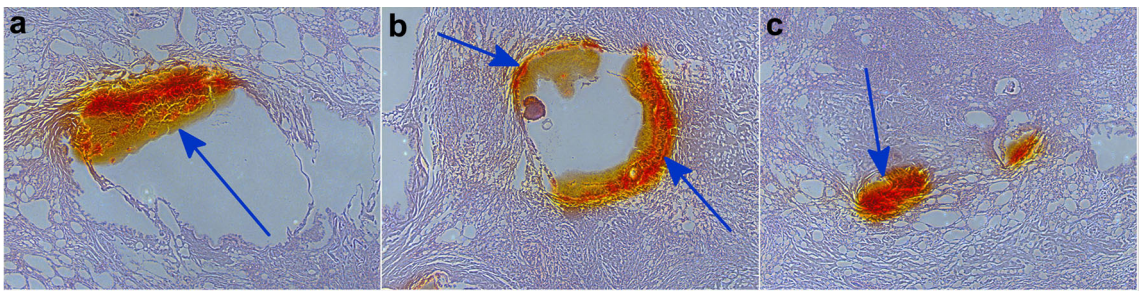

Fig. 12 Alizarin Red S staining of human palpable nodules after autologous breast augmentation with fat grafting. **a** Calcification occurred in a small part of capsule wall. **b** Calcification occurred in most capsule wall. **c** Substantial calcification occurred in the whole cyst

As some studies stated that phospholipid could liberate PI under the effect of phospholipase and ALP [14], the inhibitors of these enzymes could be potential inhibitors for formation of calcification. Tetramisole (levamisole) was indicated as a specific inhibitor of ALP [16]. U73122 could be an inhibitor of phospholipase C and phospholipase D in rat myocardial membranes [29]. Rolipram could inhibit the effect of phospholipase A_2 and phospholipase D in some conditions [30]. Further experiments in vivo should be done to verify if these potential inhibitors could decrease the level of PI in this animal model and consequently inhibit formation of calcification.

However, there were some limitations in this study. This study did not find out the reason for PI elevation in animal models, in other words, which molecules contribute to the formation of PI in vivo. Previous studies indicated that phospholipase and alkaline phosphatase (ALP) might contribute to the formation of PI from fat metabolism [14], or maybe due to release of PI in the process of fat destruction, further studies should be done to verify if these enzymes are potential contributors of PI formation after fat grafting which might be helpful for preventing nodules or calcifications after fat grafting. This study also could not figure out which kind of surrounding recruited cells could mainly contribute to formation of calcification under the effect of high PI environment in necrotic zones. Further experiments should be done to verify these issues.

Conclusions

This study indicated the mechanism that the level of PI in the central necrotic zone was elevated, which could stimulate the calcification of surrounding tissue. Further experiments could be done to figure out the mechanisms of PI elevation and which specific kind of surrounding tissue or cells could be affected by high levels of PI for formation of calcification. More studies could be done to decrease local PI concentration to prevent nodules or calcifications after fat grafting.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest to disclose.

Ethical Approval "All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards."

Informed Consent Informed consent was obtained from all patients.

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