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| Feature Article |

Tamoxifen-Loaded Polylactide Beads for Breast Tissue Engineering Following Lumpectomy

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Abstract : Breast cancer ranks second among cancer deaths in women. Lumpectomy as an important surgical treatment is often subsequently followed by plastic and reconstructive surgery to repair the soft tissue defects resulting from the tumor resection. Since lumpectomy cannot remove all cancer cells, neoadjuvant chemotherapy is required for most cases. The objective of this study was to engineer an injectable sized composite both for breast tissue reconstruction and for neoadjuvant chemotherapy. The injectable sized polylactide (PLA) beads incorporating tamoxifen (TAM) was manufactured using an emulsion-solvent evaporation technique. The TAM loading rate of the beads was about 0.33%. The encapsulation efficiency was about 6.9%. TAM was gradually released from the TAM-loaded PLA beads. Both human mesenchymal stem cells (hMSCs) and human breast cancer cells MCF-7 were cultured with various concentrations of TAM (0~20 μM) for 4 days. Cell viability results suggested that TAM was less toxic to hMSCs compared to MCF-7 cells. The 50% inhibitory concentration (IC_{50}) of TAM for hMSCs and MCF-7 cells were 12.5±1.6 μM and 5.3±0.8 μM, respectively. After hMSCs were cultured with TAM-loaded PLA beads directly in the well for 8 days, the cell viability of hMSCs was not negatively affected both on Day 4 and on Day 8. However, when MCF-7 were cultured either directly with TAM-loaded PLA beads in the well or indirectly with beads in a transwell insert, the cells viabilities were significantly inhibited ($P < 0.05$). TAM alone at various concentrations (1~ 4 μM) had not effect on hMSCs adipogenic differentiation. When hMSCs were co-cultured with TAM-loaded PLA beads in the transwell inserts, TAM-loaded PLA beads had no effect on hMSCs adipogenic differentiation. Our results suggest that the TAM-loaded PLA beads may be useful as cell microcarrier for injectable tissue engineered composites, while providing an anti-cancer therapeutic for neoadjuvant chemotherapy.

Key words: *tamoxifen, polylactide, microcarrier, tissue engineering*

1. Introduction

Breast cancer is the most frequently diagnosed cancer in women. An estimated 209,060 new cases of invasive breast cancer and an estimated 40,230 breast cancer deaths are expected to occur among women in the United States in 2010. Breast cancer ranks second among cancer deaths in women (after lung cancer).[1] Currently, surgical treatments for breast cancer include breast-reserving lumpectomy or radical mastectomy; the different types of surgery are based on the stage of the cancer. Either mastectomy or lumpectomy are often subsequently followed by plastic and breast reconstruction to repair the soft tissue defects resulting from the tumor resection.[2]

Common strategies for breast reconstruction are collagen injections, autologous tissue transfers as breast implants and fillers;[3-5] however, there is no single filler material that will satisfy all clinical needs. Fat tissue is found in excess amounts all over the human body and is easily obtained by excision or liposuction. Transplantation of this fat tissue to target locations, however, has not been consistently successful in patients.[6] When autologous fat tissue is transplanted from one location to the defect site, the common occurrence is significant resorption of the transplanted tissue over time, resulting in 40~60% of the graft volume loss.[7] This might be explained by the insufficient tissue vascularization that limits the supply of oxygen and

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nutrients to the tissue, and therefore, the long-term tissue survival.[8]

A series of smaller porous constructs might enhance vascularization potential; however, there still remains the problem of implanting multiple, easily dispersed and easily compressed materials. In order to overcome the limitations, a small, porous collagen beads as cell microcarrier, combing the cellular constructs with alginate gel to form an injectable material were used in our lab. The positive attributes from both materials were combined to produce a new family of tissue engineered implants, injectable composite for use as a reconstructive option following lumpectomy.[9-10] Further mesenchymal stem cells were used to generate adipose tissue in tissue-engineering strategies.[11-12]

Cancer cells in II or III stage breast cancer patients have invaded surrounding epithelial tissue of mammary glands and even metastasized distally, the lumpectomy or mastectomy cannot remove all cancer cells and neoadjuvant chemotherapy is required for most cases.[13-15] The objective of this study was to engineer an injectable sized composite both for breast tissue reconstruction and for neoadjuvant chemotherapy. The tissue engineering composite for breast reconstruction was used to incorporate anti-cancer therapeutics so that the anti-cancer therapeutic-loaded tissue engineered composite could both provide anti-cancer therapeutic to remnant cancer cells while serves as useful cell microcarriers.

2. Materials and Methods

2.1 Materials

Polylactide (PLA, weight average M_W 212,000 Da) was purchased from Dow Chemical, USA. Tamoxifen (TAM), PVA (polyvinyl alcohol, 87-89% hydrolyzed, typical Mw 13,000 ~ 23,000), and glutaraldehyde were purchased from Sigma-Aldrich (St.Louis, MO, USA). Dichloromethane (CH_2Cl_2, HPLC grade) was purchased from Mallinckrodt Chemic (Philipsburg, NJ, USA). Isopropanol was purchased from VWR Internationals (West Chester, PA, USA). Hexanediamine was purchased from ACROS Organics (NJ, USA). Collagen was purchased from INAMED Biomaterials (Fremont, CA, USA). Live/Dead assay kit was purchased from Molecular Probes (Invitrogen, Eugene, OR, USA).

Poietics™ human mesenchymal stem cells (hMSCs), hMSCs growth medium and adipogenic differentiation-bulletkit were purchased from Lonza (Lonza Walkersville, Inc., Walkersville, MD, USA). Human breast cancer cells MCF-7 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Eagle's Minimum Essential Medium (EMEM, ATCC) with 10% FBS, 10 μg/ml Insulin and 100 unit/ml Penicillin G/100 μg/ml streptomycin sulfate (Gibco/Invitrogen Corporation, Grand Island, NY, USA).

2.2 TAM-Loaded PLA Beads Preparation and Characteristic Analysis

TAM-loaded PLA beads were manufactured using an emulsion-solvent evaporation technique[16-17] with some modification. Briefly, 0.1 g TAM and 2 g PLA were dissolved in 20 ml CH_2Cl_2 to obtain transparent PLA/CH_2Cl_2 solution. The solution was then poured into 100 ml deionized water containing 0.5% (w/v) PVA under agitation with a rate of 400 rpm by a mechanical stirrer. The O/W ratio is 1:5. The agitation was continued for 24 hr at 25°C to evaporate the organic solvent. The produced beads were collected by a membrane filtration, extensively washed with deionized water, and dried under reduced pressure at 35°C for 3 days. The surface of beads was aminolyzed by immersing in 6% hexanediamine/isopropanol solution at 60°C in water bath for 10 min. They were then extensively washed with deionized water and dried at room temperature under reduced pressure. The aminolyzed beads were moistened in 1% glutaraldehyde solution at room temperature for 3 hr to transfer the NH_2 groups into CHO groups. After washing extensively, the beads were surfaced coated with collagen by immersing in 0.5% collagen/3% acetic acid solution at 4°C for 24 hr with occasional shaking. After filtering, the beads were washed with pure water directly. PLA beads without TAM were also manufactured as the same procedure and used as control in cell culture experiments.

Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) spectrometry was used to examine the bead surfaces before and after collagen coating. ATR spectra were collected using a Thermo-Nicolet Magna 550 FTIR spectrometer equipped with a Thermo Spectra-Tech Foundation Series Diamond ATR. Samples and background collections were performed using 16 scans at a resolution of 4 cm^{-1}. Data was analyzed and ATR and baseline corrections were applied using the Thermo Ominc E.S.P. version 6.1a. software.

Differential Scanning Calorimeter (DSC) 7 system (PerkinElmer Instruments, Shelton, CT, USA) was used to conduct thermal analysis of TAM-loaded PLA beads and PLA beads.

After being dried at room temperature under reduced pressure, the beads with a diameter of around 300 μm were separated by standard sieves. Finally the beads were sterilized with ethylene oxide and placed under house vacuum for a minimum of 24 hr before use in cell culture experiments (Fig 1).

TAM was dissolved in CH_2Cl_2 and made a series of

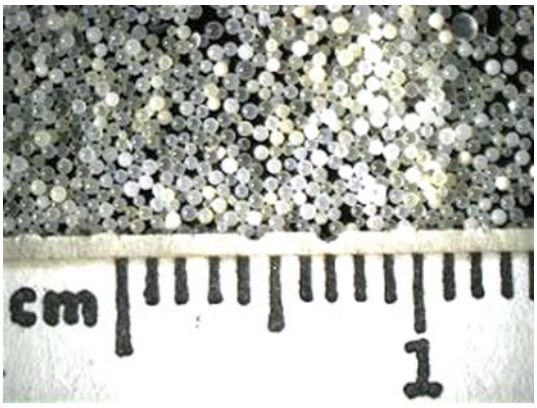

Figure 1. TAM-loaded PLA beads, ranged from 300-450 μm. TAM in the amount of 0.3 mg was loaded into 100 mg of beads. Encapsulation efficiency of TAM was 6.9%. The cumulative percent of TAM released from beads was about 30%.

concentration of TAM to prepare standard curve. The wavelength was fixed at 300 nm. The absorbance was measured with DU 640B Spectrophotometer (Beckman, USA). TAM-loaded PLA beads were dissolved in CH_2Cl_2 and measured the absorbance at 300 nm. The TAM loading rate was calculated by TAM content over given beads amount. The encapsulation efficiency was calculated by TAM content over given TAM content in the beads. Another standard curve was made by dissolving TAM in 0.02 M HCl. The TAM-loaded PLA beads were placed in 0.02 M HCl on the shaker for 60 days to study the release profile.

2.3 Effect of TAM Alone and TAM-Loaded PLA Beads on Cell Viability

Poietics™ hMSCs and MCF-7 were seeded at 2.1×10^4/cm^2 and 2.5×10^4/cm^2, respectively, in 24-well plates overnight, and then treated with various concentrations of TAM (0~20 μM) alone for 4 days. Cell viability was evaluated with an alamarBlue® assay.

TAM-loaded PLA beads (50 mg/well) were moistened in PBS for various times (30, 15, 5 and 0 days) before used for cell culture. For hMSCs, the beads were transferred into new well of 12-well plate. Then hMSCs were seeded at 2.1×10^4/cm^2 into the well and cultured with beads for 8 days. The cell viability was assayed on Days 4 and 8 using an alamarBlue® assay. The hMSCs grown on the bead surfaces were observed with Live/Dead assay kit.

For MCF-7 cells, the moistened beads were either transferred into new well of 12-well plate directly with cells or put in transwell inserts (ϕ12 mm) indirectly with cells. MCF-7 were seeded at 2.1×10^4/cm^2 and cultured for 8 days. The cell viability was assayed on Days 4 and 8 using an alamarBlue® assay. The MCF-7 cells grown on the bead surfaces were observed with Live/Dead assay kit.

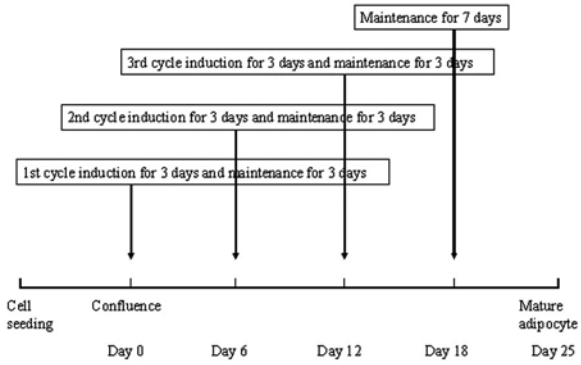

Figure 2. Experimental flowchart detailing the induction of hMSCs to adipocyte.

2.4 Effect of TAM Alone and TAM-Loaded PLA Beads on hMSCs Adipogenic Differentiation: Triglyceride Level

First, Poietics™ hMSCs were seeded at 2.1×10^4/cm^2 in 12-well plates and grown to confluence. The post-confluent cells were induced into adipocyte according to the protocol (Fig 2). The cells were treated with various concentration (0, 1, 2, 4 μM) of TAM for 3 days beginning on the first induction cycle. The cells were induced and maintained for 3 cycles for 18 days and then maintained for additional 7 days to be mature adipocyte. On the last day (Day 25), the cells were collected to measure triglyceride content.

Second, Poietics™ hMSCs were seeded at 2.1×10^4/cm^2 in 12-well plates and cultured to confluence. TAM-loaded PLA beads (50 mg/well) were placed in transwell insert (ϕ12 mm). PLA beads were used as control. The post-confluent cells were induced into adipocyte differentiation according to the protocol (Fig 2). The cells were induced and maintained for 3 cycles for 18 days and then maintained for 7 days to be mature adipocyte. On the last day (Day 25), the cells were collected to measure triglyceride (TG) content.

2.5 Effect of MCF-7 Cells with or without PLA Beads or TAM-Loaded PLA Beads on hMSCs Adipogenic Differentiation: Fatty Acid Composition

Poietics™ hMSCs were co-cultured with MCF-7 cells as following. First, hMSCs were seeded at 2.1×10^4/cm^2 in 12-well plate and cultured to confluence. MCF-7 cells were seeded at 2.5×10^4/cm^2 in the transwell insert (ϕ12 mm) on the day before confluence of hMSCs. After MCF-7 cells attached to the inserts overnight, TAM-loaded PLA beads or PLA beads were added

into the transwell inserts and placed into each well which hMSCs had been grown to confluence. The group with inserts containing no beads and no MCF-7 cells was set as control. All inserts were renewed at the end of each cycle of induction/ maintenance of hMSCs. On the last day (Day 25), the cells were collected to analyze fatty acids composition.

2.6 Cell Viability Assay

The cell viability of each cell line was measured quantitatively using an alamarBlue® assay (BioSource International, Inc., Camarillo, CA, USA).[18] Briefly, 100 μl of water-soluble alamarBlue® was added to each well and incubated for 2 h on an orbital shaker. 150 μl of each sample was pipetted into individual wells of a clear, flat bottom 96-well microplate. The fluorescence was read with excitation wavelength at 544 nm and emission wavelength at 590 nm using an ELISA reader. The fluorescence generated is directly proportional to the number of viable cells in a culture medium. All samples were assayed in triplicate. Results were reported as cell viability ratio (average OD/average negative control OD ± standard deviation (SD)).

2.7 Live/Dead Assay

Briefly, removed the Live/Dead assay reagents (Molecular Probes) from the freezer and allow warming to room temperature. Warm the PBS also. 2 ml of sterile PBS was put into 15 ml centrifuge tube for 2 wells. 4 μl of 2 mM EthD-1 stock was added and mixed thoroughly by vortexing. This gave a 4 μM EthD-1 solution. Then 5 μl of the 4 mM calcein AM stock was added to the 4 μM EthD-1 solution in the 15 ml tube and mixed thoroughly by vortexing. This gave a 10 μM calcein solution. The beads, which had the cells on the surface, were placed into a well of a 12-well plate and rinsed with room temperature PBS to remove possible serum esterase activity.

2.8 Triglyceride Measurement

The amount of triglyceride (TG) produced by the differentiating hMSCs was determined using a Human Adipocyte Differentiation Assay Kit (Zen-Bio Inc., Research Triangle Park, NC, USA). Briefly, the cells were rinsed with Dulbecco's PBS and aspirated. The cells were subjected to three freeze-thaw cycles, and then incubated in 1 ml of 1% Triton®-X-100 solution (Sigma-Aldrich) at room temperature for 30 min. After incubation, the wells were scraped with a cell scraper and the Triton solution and cell debris were placed in microtube to be centrifuged at 3000 rpm. for 10 min. Triplicate mixtures of 100 μl of cellular lysate and 100 μl of triglyceride reagent (Thermo Electron Corp., Melbourne, Australia) were prepared. Meanwhile, glycerol standard solution (Sigma-Aldrich) was used to make a standard curve ranging from 0 μM to 200 μM. The 96-well plate was incubated for 15 min at room temperature, and the absorbance of all samples and standards were detected at 490 nm (Dynex Technology, Chantilly, VA, USA).

2.9 Cell Morphology

Image of the D1 cells were captured using an Axiovert 135 inverted microscope (Zeiss, Germany), Image-Pro 5.0 software (Media Cybernetics, Silver Spring, MD, USA), and a ProRes™ $C10^{Plus}$ digital camera (Chori Imaging Corporation, Yokohama, Japan). All cellular images were photographed at total magnification of 320×.

2.10 Fatty Acids Composition Analysis

The fatty acid composition profile was determined using a gas chromatography as described by Folch *et al*[19]. Briefly, the cells were rinsed with PBS and scraped into a tube. Total lipid was extracted using chloroform/methanol (2:1 vol/vol) plus 0.58% sodium chloride. After evaporation to dryness, methylene chloride and sodium methoxide in methanol was added into the lipid residue to conduct transmethylation. The internal standard (ISTD C23:0, Sigma) was added and the samples were run on GC for long chain fatty acids measurement. The percentage of fatty acid was calculated according to the formula:

$$\text{mg Fatty acids} = (\text{Total area} - \text{Area}_{ISTD})/ (\text{Area}_{ISTD}/0.5\ \text{mg});$$

$$\text{Fatty acid percentage} = ((\text{Area}_{\text{fatty acid}}/\text{Area}_{ISTD}/0.5\ \text{mg})/\text{mg fatty acids}) \times 100$$

2.11 Western Blot Analysis

The hMSCs were collected and lysed in sample buffer (Bio-Rad, Hercules, CA, USA). The protein concentration was quantified using a BCA Total Protein Assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein extracts from each sample were loaded on SDS-PAGE gels for electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The various proteins were analyzed using a chemiluminescence (Roche Diagnostics, IN) analysis kit. The target proteins included fibronectin and β–actin (Santa Cruz Biotechnology, Santa Cruz, CA).

2.12 Statistical Analysis

All cell culture data were expressed as the mean ± standard deviation (SD) for n=3. Statistical significance was assessed using a factorial analysis of variance (ANOVA), and a two-tailed LSD test (SAS 9.0 for Window, SAS, Cary, NC, USA).

3. Results

3.1 ATR-FTIR and DSC Analysis and Beads Characteristics

ATR-FTIR analysis verified that collagen was successfully coated on the surface of the beads. Before collagen coating, both TAM-loaded PLA beads and control PLA beads had no absorbance at the range of 1652-1657 nm and 1548-1556 nm. However, after collagen coating, either beads indicated absorbance at the above-mentioned two wavelengths range.

The glass transition temperature of TAM-loaded PLA beads and control PLA beads assayed by DSC 7 system were 44.29 and 47.09°C, respectively. The melting points of TAM-loaded PLA beads and control PLA beads were 152.20 and 154.37°C, respectively. The results suggested the physical characteristics were same.

A standard curve was prepared to assay the content of TAM in beads: A= 0.0263C - 0.014, r = 0.999. TAM-loaded PLA beads (0.11g) were dissolved in 5 ml of (1:4) CH_2Cl_2/HCl. The TAM concentration was 72.22 μg/ml. The TAM content was 361.12 μg. The TAM loading rate was about 0.33%. The encapsulation efficiency was about 6.9%. The standard curve of TAM in 0.02M HCl was: A=0.0168 – 0.0025, r = 0.999. TAM was gradually released from the TAM-loaded PLA beads on the shaker for 60 days. The accumulative TAM release was 87.2 μg. The final release percentage was 27%.

3.2 Effect of TAM and TAM-Loaded PLA Beads on Cell Viability

Both hMSCs and MCF-7 were cultured with various concentrations of TAM (0~20 μM) for 4 days. Cell viability results suggested that TAM was less toxic to hMSCs compared to MCF-7 cells. The 50% inhibitory concentration (IC_{50}) of TAM for hMSCs and MCF-7 cells were 12.5±1.6 μM and 5.3±0.8 μM, respectively.

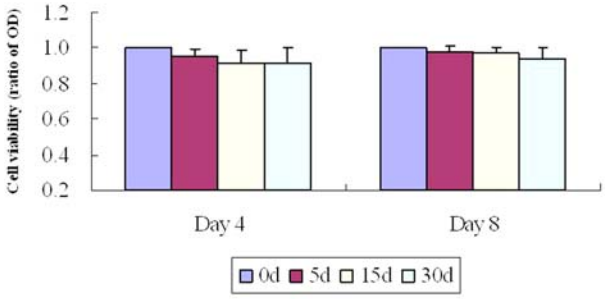

Figure 3. hMSCs cell viability was not affected by TAM-loaded PLA beads.

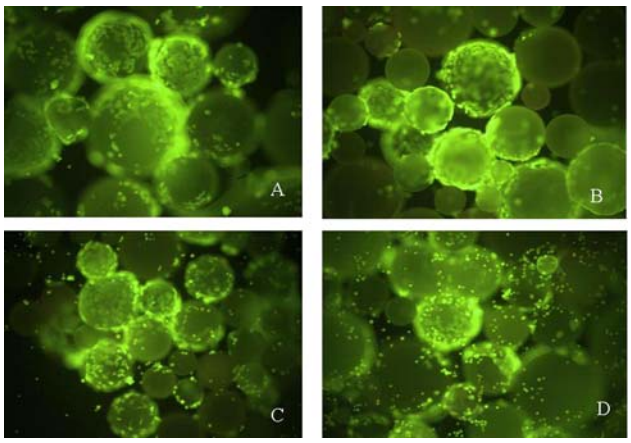

Figure 4. hMSCs grown on the TAM-loaded PLA beads surface. (A) hMSCs on TAM-loaded PLA beads moistened in PBS for 0 days. (B) hMSCs on TAM-loaded PLA beads moistened in PBS for 5 days. (C) hMSCs on TAM-loaded PLA beads moistened in PBS for 15 days. (D) hMSCs on TAM-loaded PLA beads moistened in PBS for 30 days.

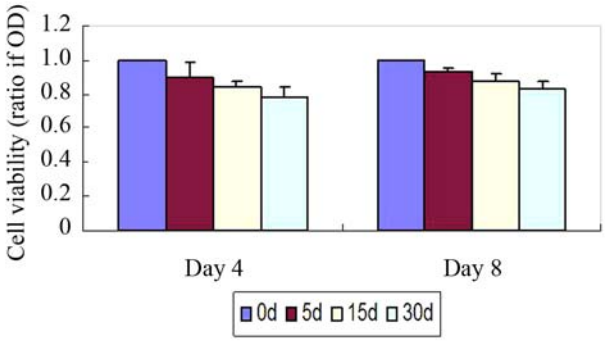

Figure 5. MCF-7 cell viability was inhibited when cultured directly with by TAM-loaded PLA beads in well plate.

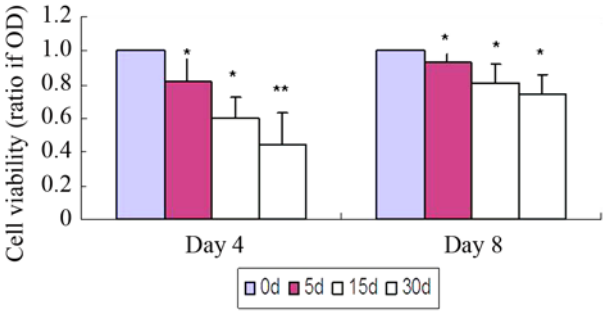

Figure 6. MCF-7 cell viability was inhibited when cultured indirectly with by TAM-loaded PLA beads in transwell inserts.

After hMSCs were cultured with beads directly in the well for 8 days, the cell viability of hMSCs was not negatively affected both on Day 4 and on Day 8 (Fig 3). HMSCs grew well on the surface of TAM-loaded PLA beads which were

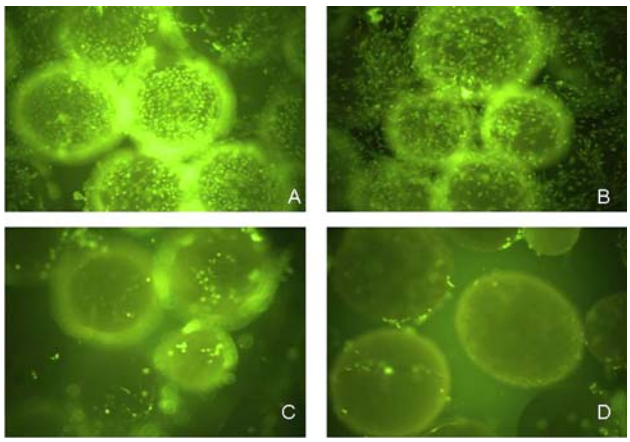

Figure 7. MCF-7 cells grew well on the surface of TAM-loaded PLA beads. (A) MCF-7 cells on TAM-loaded PLA beads moistened in PBS for 0 days. (B) MCF-7 cells on TAM-loaded PLA beads moistened in PBS for 5 days. (C) MCF-7 cells on TAM-loaded PLA beads moistened in PBS for 15 days. (D) MCF-7 cells on TAM-loaded PLA beads moistened in PBS for 30 days.

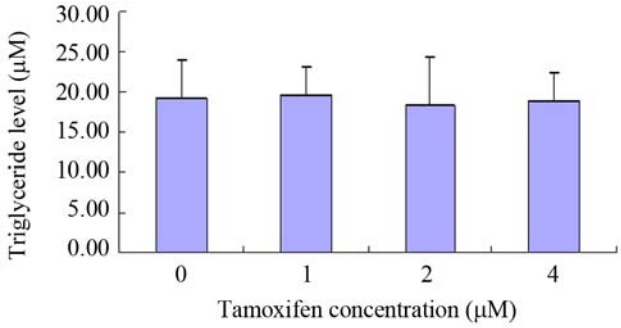

Figure 8. TAM at various concentrations (1~4 μM) had not effect on hMSCs adipocyte differentiation.

moistened in PBS for various days (Fig 4). However, when MCF-7 were cultured either directly with beads in the well or indirectly with beads in a transwell insert, the cell viabilities were significantly inhibited ($P < 0.05$) (Fig 5 and 6). MCF-7 cells grew well on the surface of TAM-loaded PLA beads which were moistened 0 days but bad on the surface of beads which were moistened for 30 days (Fig 7). The inhibiting effect of TAM-loaded beads on MCF-7 cell viability was dependent on the moistening time of beads.

3.3 Effect of TAM alone and TAM-Loaded PLA Beads on hMSCs Differentiation

On the last day of adipocyte induction, the cells were collected to measure triglyceride content. The results showed

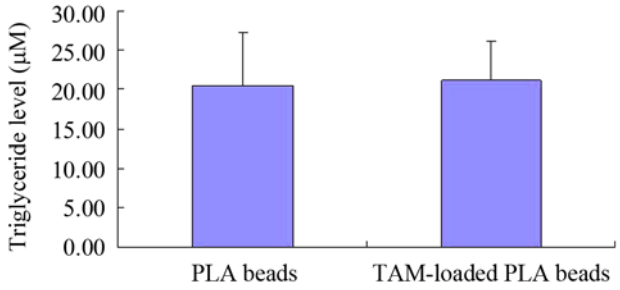

Figure 9. PLA beads and TAM-loaded PLA beads had not effect on hMSCs adipocyte differentiation.

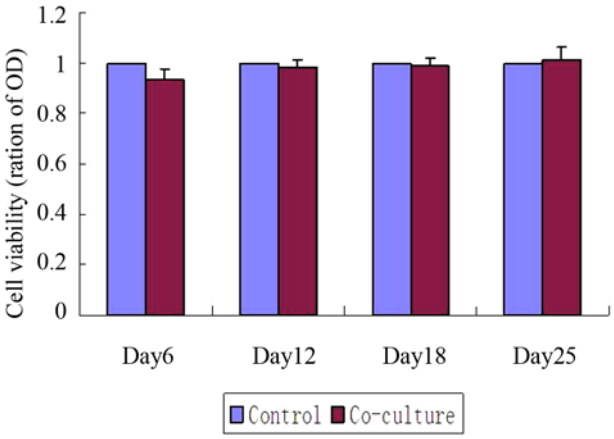

Figure 10. hMSCs viability was not affected by co-cultured MCF-7 cells.

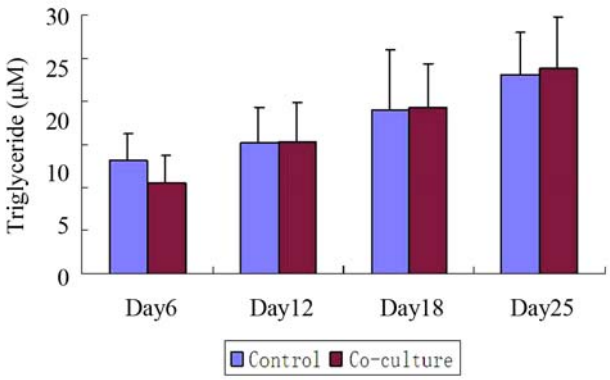

Figure 11. Adipocyte differentiation of hMSCs was not affected by co-cultured MCF-7 cells.

that TAM at various concentrations (1~4 μM) had not effect on hMSCs adipocyte differentiation (Fig 8). When hMSCs were co-cultured with PLA beads without TAM or TAM-loaded PLA beads in the transwell inserts, the triglyceride levels were

Table 1. Fatty acid composition (%) of the human stem cells co-cultured with PLA beads and TAM-loaded PLA beads with or without MCF7 cells in transwell inserts.

In inserts	-	-	-	MCF-7	MCF-7	MCF-7
	-	PLA beads	TAM-loaded PLA beads	-	PLA beads	TAM-loaded PLA beads
Lauric	0.65±0.07	0.63±0.08	0.56±0.03	0.57±0.25	0.78±0.18	0.72±0.17
Myristic	7.37±1.10	6.94±0.99	6.55±1.14	6.13±2.27	8.65±2.80	7.93±2.52
Palmitic	34.39±3.97	34.77±3.43	33.80±4.58	32.02±6.07	36.16±1.63	34.75±2.97
Stearic	1.84±1.44	2.71±2.68	2.27±2.25	3.32±2.09	3.13±3.21	3.45±3.90
Oleic	11.29±1.50	12.65±3.07	11.61±2.39	16.39±2.80	11.87±3.30	12.00±4.16
Linoleic	0.62±0.70	0.77±0.93	0.64±0.68	1.09±1.01	0.78±0.78	0.65±0.59
Linolenic	0.14±0.10	0.16±0.13	0.15±0.11	0.30±0.12	0.21±0.16	0.21±0.16
Total	56.30±1.01	58.64±3.01	55.57±1.11	59.81±2.46	61.59±3.72	59.73±4.33

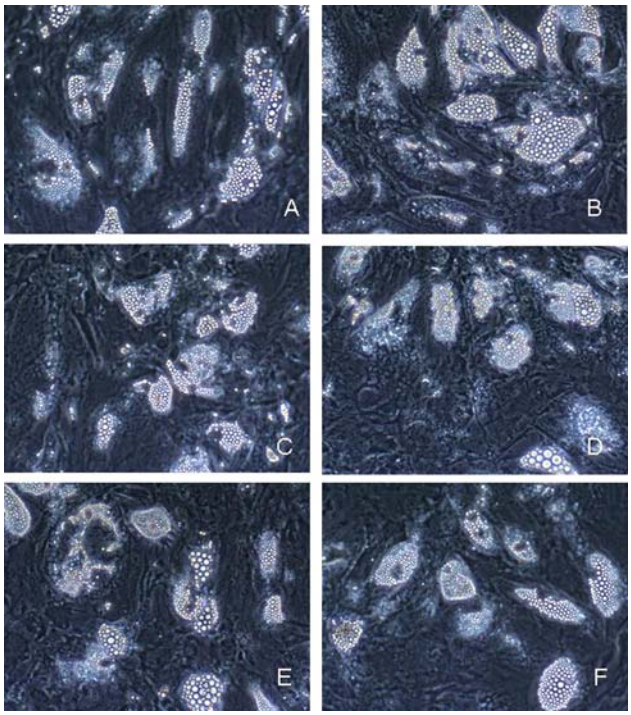

Figure 12. Morphologic observations showed that on no significant differences were seen among the 5 treated groups and one control group. (A) control, (B) PLA beads in insert, (C) TAM-loaded PLA beads in insert, (D) MCF-7 cells in insert, (E) MCF-7 cells plus PLA beads in insert, and (F) MCF-7 cells plus TAM-loaded PLA beads in insert.

not significant different (Fig 9).

3.4 hMSCs Adipogenic Differentiation Profile When Co-Cultured with MCF-7 Cells with Beads

On day 6, 12, 18 and 25, hMSCs viability was measured to evaluate the effect of co-cultured MCF-7 cells. The alamarBlue results showed that hMSCs viability was not affected by co-cultured MCF-7 cells (Fig 10). On day 6, 12, 18 and 25, hMSCs was collected to measure the triglyceride content. TG levels

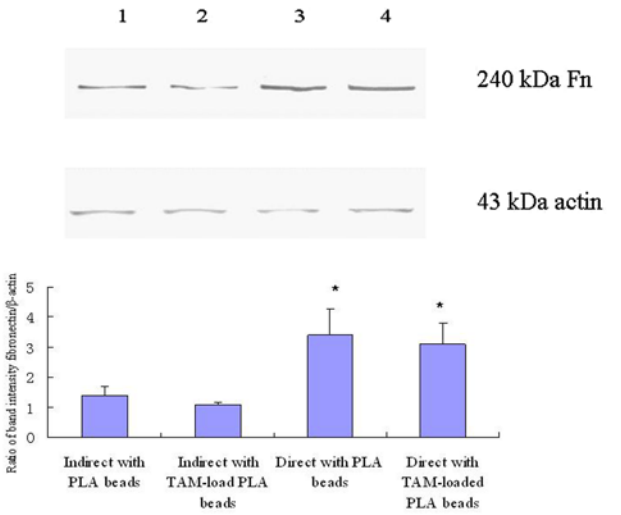

Figure 13. Relative ratio of bands intensity fibronectin/β-catin.

assay showed that the adipocyte differentiation of hMSCs was not affected by co-cultured MCF-7 cells (Fig 11).

On day 25, fatty acid composition analysis showed that there was no significant difference when hMSCs were co-cultured with or without MCF-7 cells in transwell inserts combined with PLA beads and TAM-loaded PLA beads (Table 1). Morphologic observations showed that on no significant differences were seen among the 5 treated groups and one control group (Fig 12).

3.5 Western Blot Results

Western blotting results showed that fibronectin expressions of MCF-7 cells treated directly with PLA beads was enhanced compared to that of cells treated indirectly with PLA beads (Fig 13) .

4. Discussion

Great progress has been made in fabricating various three-

dimension scaffolds.[20] Beads have been paid attention in tissue engineering because of their unique properties for scaling up 3-D cell culture, loading capacity for drugs or growth factors, and most importantly, for tissue regeneration as injectable composites. Cell culture on injectable sized beads may produce a larger amount of cells during a relatively short period. It is much convenient if the cells-attached beads are used directly as injectable cell microcarriers. When used *in vivo*, biodegradable beads are especially appreciated since traditional cell microcarriers, such as collagen-coated Sephadex beads (Cytodex-3) and Cultispher series, are less or even non resorbable. Retinoic acid-loaded beads of poly (lactide-co-glycolide) (PLGA) have shown a function to modulate the differentiation of pluripotent stem cells. [21] In this present work, we engineered a TAM-loaded PLA beads for breast tissue engineering following lumpectomy.

In the MCF-7 cell culture, the cytotoxic effect of tamoxifen was observed at level of more than 5 ìM after treatment for 4 days. TAM acts primarily through estrogen receptors by modulation of gene expression. The pharmacological concentration (above 5 μM) of TAM has been found to induce apoptosis of breast cancer cells.[22] When the MCF-7 cells were cultured directly with TAM-loaded beads in the same well or indirectly in the transwell inserts for 8 days, the cell viability inhibition were concentration-dependent. The inhibiting effect of TAM-loaded beads on Day 4 was much more compared with that on Day 8, indicating that the mechanism of tamoxifen action is at the early stages.[23] However, our study showed that TAM at the same concentration (5 μM) has no effect on human mesenchymal stem cells meanwhile high concentration of tamoxifen (at least 10 μM) had inhibiting effect on hMSCs viability. The inhibition is due to the proapoptotic effect of TAM in estrogen receptor-negative breast cancer cells and other cell types that lack estrogen receptors such as those in malignant gliomas, pancreatic carcinomas, ovarian cancers and melanomas.[24-26] And TAM-loaded PLA beads had almost no effect on differentiation of hMSCs. The results suggested that the growth and differentiation of human stem cells is independent of estrogen receptor status. This laid the experimental foundation for engineering TAM-loaded PLA beads for breast tissue engineering following lumpectomy.

In this study human mesenchymal stem cells were induced into adipocytes. The fatty acid composition analysis of mature adipocytes showed that lauric (0.56~0.78%), myristic (6.13~8.65%), palmitic (32.02~36.16%), stearic (1.84~3.45%), oleic (11.29~16.39%), linoleic (0.62~1.09%) and linolenic (0.14~0.30%) acids accounted for about 60% of the triglyceride fatty acids. There were no significant differences in the average fatty acid composition among various treatment groups. Calder and co-workers compared the fatty acid composition of triglycerides from 15 distinct adipose depots taken from each of 7 adult male human subjects.[27] They found that there were large differences in the average fatty acid composition between individual subjects. However, there was no site-specific differences in the proportions of myristic (3.8~4.7%), palmitic (23~29%), linoleic (6.7~9.8%) acids. While most human adipose depots differ little in fatty acid composition, some sites in particular the calf had site-specific properties. These indicated that there might be a little difference of fatty acid composition in between differentiated adipocyte and adipose depots. Rautalahti and co-worker studied the fatty acid composition of human breast adipose tissue in 10 cases of breast cancer.[28] The relative fatty acid composition of adipose tissue samples taken from human breasts was lauric (0.8%), myristic (5.2%), palmitic (24.5%), stearic (6.7%), oleic (39.4%), linoleic (7.4%) and linoleic (0.6%) acid. Zhu and co-workers further analyzed fatty acid composition of triglycerides in breast adipose tissue both in female breast cancer patients and patients with benign breast disease.[29] The fatty acid composition in the entire group of cancer patients did not differ significantly from that in the benign group. No significant difference was observed in the proportion of fatty acids such as myristic (5.71~6.41%), palmitic (24.86~26.78%), stearic 5.96~6.35%), oleic (42.23~43.47%), linoleic (8.46~10.31%) and linolenic (0.08~0.12%) between the two groups. Nesaretnam and co-workers investigated whether there was a difference in tocopherol and tocotrienol concentrations in malignant and benign adipose tissue using fatty acid levels in breast adipose tissue as a biomarker of qualitative dietary intake of fatty acids.[30] No differences were evident in the fatty acid composition in breast adipose tissue between the two groups of patients (benign and malignant). However, there was a significant difference in the total tocotrienol levels between malignant (13.7±6.0 μg/g) and benign (20±6.0 μg/g) adipose tissue samples. The above-mentioned studies revealed that the possible difference of fatty acid composition in breast cancer tissue as compared with normal tissue was influenced by dietary intake and that adipose tissue was a dynamic reservoir of fat soluble nutrients. The fatty acid composition of mature adipocytes in our study was similar to those reported in above-mentioned studies,[27-30] which indicated that human mesenchymal stem cells could be induced to adipocytes for breast tissue reconstruction.

There was a little difference between the cell viability of MCF-7 cells after treated indirectly with TAM-loaded PLA beads and directly with TAM-loaded PLA beads. We

postulated it might be related with the direct contact of beads surface on cells. Our western blotting results indicated that when MCF-7 cells were cultured directly with TAM-loaded PLA beads and PLA beads, the surface of beads would enhance fibronectin expression compared to that MCF-7 cells cultured indirectly with beads. Fibronectin is one of the main components of the extracellular matrix[31] and could enhance cell proliferation and cell viability[32-33]. However, the mechanism underlying the beads surface enhancement on fibronectin expression is not clear and worthwhile to study further.

5. Conclusion

The results suggest that the TAM-loaded PLA beads may be useful as cell microcarriers in injectable tissue engineered composites, while providing an anti-cancer therapeutic.

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