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Effect of Biodegradable Shape-Memory Polymers on Proliferation of 3T3 Cells

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This article evaluates the in vitro biocompatibility for biodegradable shape-memory polymers (BSMP) invented by the authors. 3T3 cells (3T3-Swiss albino GNM 9) of primary and passaged cultures were inoculated into two kinds of carriers: the BSMP carrier and the control group carrier. Viability, proliferation, and DNA synthesis (the major biocompatibility parameters), were measured and evaluated for both the BSMP and naked carrier via cell growth curve analysis, MTT colorimetry and addition of ^{3}H-TdR to culture media. The results showed that there was no difference between the BSMP carrier and the control dish in terms of viability, proliferation, and metabolism of the 3T3 cells. Overall, the BSMP carrier provides good biocompatibility and low toxicity to cells in vitro, and could indicate future potential for this medium as a biological material for implants in vivo.

Keywords 3T3-Swiss albino GNM 9, biocompatibility, biodegradable shape-memory polymers, biomaterial

1. Introduction

The exploration into biodegradable and absorbable implants was unveiled as early as the late 1960s. The pins, bars, and screws made of polyglycolic acid (PGA) and polylactic acid have already been commercialized, along with other widely used biodegradable materials. These have been established as biocompatible without severe, acute, or subacute histological reactions or toxic effects over three decades of experience (Ref 1).

In the interest of applying absorbable polymers in minimally invasive procedures, American and German experts jointly developed the biodegradable and absorbable shape-memory copolymer in 2002 (Ref 2). Following this, the authors of this article invented the absorbable shape-memory material made of polycaprolacton in 2006 (Ref 3), opening up a door for further application of the absorbable materials into medicine. This study evaluated biocompatibility of the biodegradable shape-memory polymers (BSMP) invented by the authors by using cytological methodology in order to study viability, proliferation, and metabolism of cells seeded on BSMP in vitro.

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2. Experimental Procedures

2.1 Cell Culture and Passage

3T3 mouse embryonic fibroblasts (Shanghai Cell Bank of the Chinese Academy of Science) were used to perform the cell culture passage. Cells were cultured in DMEM culture medium supplemented with trypsase, type-II collagenase (Gibco Co., Ltd.), FBS (HyClone Co., Ltd.), and penicillin-streptomycin for cell culture (20,000 μ/mL) (Shandong Lukang Pharmaceutical Co., Ltd.). Cells were seeded on 6-well (Corning Co., Ltd.) tissue culture plates. When the cells were 80% confluent, 0.1% trypsase was added to allow digestion at 37 °C for 3-5 min. An inverted microscope was used to observe digestion closely. The moment that the cells began to shrink and become round and the intercellular gap began to enlarge, DMEM culture solution containing a little amount of 10% FBS was added to inactivate the trypsase. Subsequently, the cells were suspended by pipetting and were diluted to a concentration of 1×10^5/mL after the digestion. Following this, cells were inoculated into a 35 mm 6-well plate for culture in an incubator at 37 °C, 10% CO_2, with saturated humidity. The cells of the fifth and sixth passage that grew in a log growth phase were applied to the study, entering into the BSMP group or the control group. (BSMP group: every bottom of the 6-well tissue culture plates were covered with one piece of BSMP (Ref 3) with a thickness of 1 mm and a diameter of 35 mm, sterilized by epoxy ethane. Control group: the bottoms of the 6-well tissue culture plates were naked, covered with no materials.)

2.2 Immunohistochemistry Assay

Instant immunocytochemistry via the SP method was used. The 3T3 cell suspension was inoculated to a piece of cover glass (24 × 40 mm) and subject to the experimental procedures.

Cells were cultured for 1 week, cover glass was then removed, and cells were fixed using cool 95% acetone for 20 min, followed by washing three times with PBS. After drying, slides were dewaxed, condensed, rinsed, and blocked. Primary monoclonal antibody for Vimentin (Chemicon, US) was then added at a 1:100 dilution and then slides were transferred to a wet box overnight at 4 °C. Cells were rinsed with PBS, followed by Biotin secondary antibody staining (Chemicon, US). Results were developed with DAB, re-stained with hematoxylin, and then mounted for observation. The expression of Vimentin was observed under 400 objective amplification microscope.

2.3 Growth Curve

The cell suspension was inoculated to six wells preset with BSMP with a dose of 1 mL/well. Following this, the suspension was changed every 24 h. Cell morphology and growth were observed at different time points under an inverted microscope. Viability was tested by 0.5% trypan blue staining, and cells were counted using a blood cell count plate for 7 consecutive days. Cell growth curves were plotted according to the data collected.

2.4 MTT Colorimetry

When the cells were 80% confluent, they were mixed with the digestion solution for 3-5 min at 37 °C. Cells were inoculated to a 96-well plate (1×10^4/well in 200 μL, $n = 5$) and cultured in an incubator for 24 h after the inoculation. At this point, culture medium was discarded by aspiration and 20 μL of MTT (Amresco Co., Ltd.) was added into each well. Cells were then cultured again in medium for 4 h. The supernatant was then slowly aspirated and 200 μL of DMSO was added into each well, followed by vibration for 10 min on a micro-oscillator in order to completely solve the MTT. OD was determined at 490 nm (A_{490}) for each well using an ELISA analyzer. Statistical analysis was performed using the t test.

2.5 ^{3}H-TdR Addition

When the cells covered about 80% of the plate, they were subject to serum starvation for 72 h. The supernatant was discarded and freshly prepared 37 MBq/L culture solution supplemented with ^{3}H-TdR (The Atomic Energy Institute of the Chinese Academy of Science) was added into both the BSMP group and control group. Cells were cultured for 24 h, rinsed with PBS twice, digested and collected. DNA was precipitated using precooled 10% trichloroacetic acid, and extracted using 0.5 mL of 1 M NaOH. The solution was then neutralized using 0.5 mL of 1 M HCl, and DNA was filtered using a piece of 0.2 μm Glass Fiber Filter Paper. DNA was dried and transferred to a scintillation bottle with scintillation liquid. After 10 h, radioactive impulse number was determined every minute using a liquid scintillation counter.

3. Results

3.1 Morphological Observation

Observation under the inverted microscope showed that the cells from the BSMP carrier and the naked dish did not differ from each other in terms of cellular morphology at all time segments. Both groups grew well along the dish walls on the second day. The cells were arranged sparsely and the cytosomes appeared in long-shuttle shapes or irregular triangles with transparent cytoplasms and 2-3 long or short protrusions (Fig. 1). On the fourth day, the cells covered about 70-80% of the plate. On the seventh day, the cells coalesced to 100% of the bottom, ranging densely in a radial, weaving, whirling, or overlapping pattern (Fig. 2). The cells had good refraction: no fragments or particles, nor disfigured, atrophied or necrotic cells. The cell nuclei were small and elliptical and were in the center of the cell without abnormal mitotic figures. Under transmission electron microscopy, the cells from the BSMP carrier on the seventh day showed affluent rough endoplasmic reticulum in the cytoplasm with a big nucleus, obvious nucleolus, and short protrusions. These cells also showed many microvilli and ruffles on the cell surface (Fig. 3).

3.2 Growth Curves of the BSMP Cells and the Control Group Cells

1 h immediately after the inoculation, cells of passage 5 and 6 of both groups presented adherent growth, reaching a live cell rate of 96.5% (control group cells) and 97.2% (BSMP cells) 24 h later. The most frequent growth pattern was radial from a single cell, and the cells mostly were elliptical and even, and formed relatively big clones. 6-8 days later, the cells covered the entire bottom of the well in a single layer. Few cells floated in the culture medium. On the fifth day, the live cell rates were 90.5%

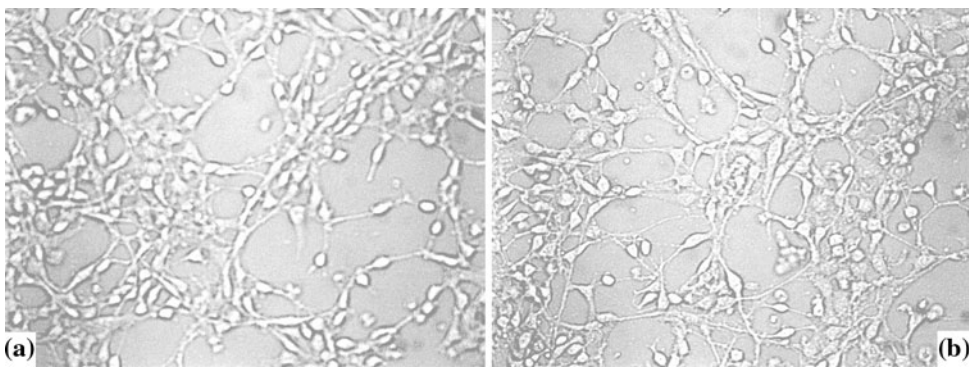

Fig. 1 Morphological observation of the 3T3 cells on day 2 (20×). (a) BSMP cells and (b) control group cells

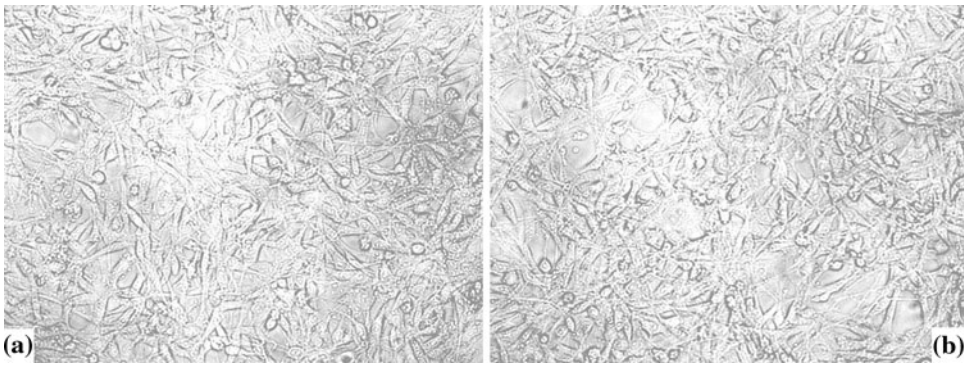

Fig. 2 Morphological observation of the 3T3 cells on day 7 (20×). (a) BSMP cells and (b) control group cells

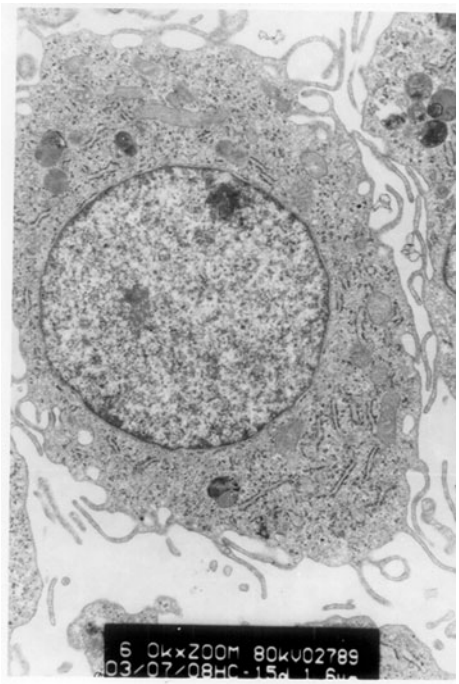

Fig. 3 Transmission electron microscopy (6×10^3) of the cells from the BSMP wells on the seventh day showed affluent rough endoplasmic reticulum in the cytoplasm with a big nucleus, and obvious nucleolus. Short protrusions, many microvilli and ruffles can be seen on the cell surface

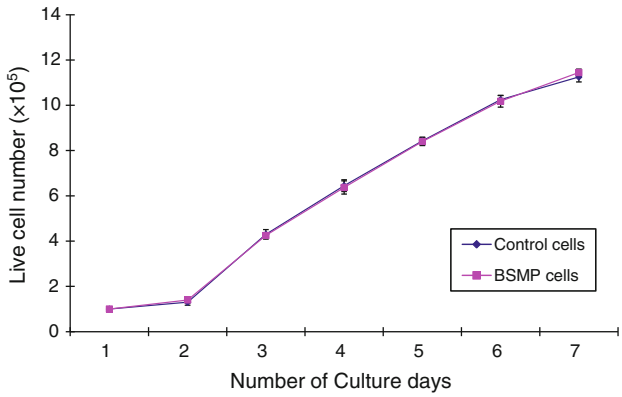

Fig. 4 Cell growth curves calculated by cell counting

(control group cells) and 91.2% (BSMP cells). At day 7, live cell rates were 86.3% (control group cells) and 87.1% (BSMP cells). The cell growth curves (Fig. 4) derived from Table 1 indicate that BSMP has no effect on growth of the 3T3 cells.

3.3 BSMP's Effect on Cell Proliferation

There was no significant difference between A_{490} values of the BSMP cells and control group cells during days 1-7 (Table 2). There was also no significant difference between the two groups ($p > 0.05$) in terms of cell growth, further indicating BSMP has no effect on cell growth.

Addition of the ^{3}H-TdR into the BSMP cells and the control group cells kept increasing on days 1, 3, 5, and 7 (Table 3). The seeping amount did not differ between the two groups ($p > 0.05$), indicating that the two groups are equal in terms of DNA synthesis level. Furthermore, BSMP has no effect on cell proliferation.

4. Discussion

The use of biological materials for medical purposes should ideally have good biocompatibility for consideration for clinical use, apart from their physical and chemical traits. Currently, there are two approaches to evaluate biocompatibility: in vivo experiments, i.e., embedding the materials into the body, performing histological examinations phase by phase, and observing pathological transition of the materials and surrounding tissues; and in vitro experiments, i.e., observing the effect of the materials or material extraction on cell growth, metabolism, and proliferation. Johnson et al. (Ref 4) showed that the in vitro experiment was more sensitive than in vivo embedding in evaluating toxicity and cellular influence for biological materials.

In contrast to previous evaluations of material biocompatibility that laid emphasis on morphological and amount changes, current appraisals of biocompatibility valued by more and more scholars stress the materials' effect on cell growth, adhesion, proliferation, and metabolism. This technique allows for the analysis of live and functional cells by using cell proliferation as a biocompatibility evaluation parameter.

Table 1 Cell count ($\times 10^5$) of the control group cells and the BSMP cells at different time points

Culture day	1	2	3	4	5	6	7
Control cells							
Well 1	1	1.2	4.5	6.9	8.6	10.2	11.1
Well 2	1	1.2	4.1	6.4	8.5	10.2	11.2
Well 3	1	1.4	4.1	6.5	8.3	10	11
Well 4	1	1.2	4.6	6.2	8.6	10.2	11.6
Well 5	1	1.3	4.3	6.2	8.4	10.4	11.4
Well 6	1	1.5	4.2	6.5	8.2	10.5	11.2
Mean ± SD	1.00 ± 0.00	1.30 ± 0.13	4.30 ± 0.21	6.45 ± 0.26	8.43 ± 0.16	10.25 ± 0.18	11.25 ± 0.22
BSMP cells							
Well 1	1	1.3	4.2	6.8	8.4	9.8	11.6
Well 2	1	1.5	4.3	6.2	8.5	10.5	11.4
Well 3	1	1.4	4.1	6.4	8.6	10.1	11.2
Well 4	1	1.3	4.2	6.2	8.1	10.3	11.4
Well 5	1	1.3	4.4	6.6	8.3	10.4	11.6
Well 6	1	1.6	4.3	6	8.5	10	11.5
Mean ± SD	1.00 ± 0.00	1.40 ± 0.13	4.25 ± 0.10	6.37 ± 0.29	8.4 ± 0.18	10.18 ± 0.26	11.45 ± 0.15

Table 2 A_{490} ($\bar{x} \pm s$, $n = 7$) values of the BSMP cells and control group cells at different time points

Day	1	2	3	4	5	6	7
Control cells	0.83 ± 0.05	0.87 ± 0.06	1.06 ± 0.04	1.36 ± 0.11	1.69 ± 0.08	1.89 ± 0.09	2.28 ± 0.07
BSMP cells	0.85 ± 0.07	0.91 ± 0.03	1.08 ± 0.08	1.32 ± 0.08	1.62 ± 0.06	1.91 ± 0.07	2.34 ± 0.04

Table 3 Addition of the ^{3}H-TdR into the BSMP cells and the control group cells at different times ($\bar{x} \pm s$, $n = 7$)

Day	1	3	5	7
Control cells	132.5 ± 23.6	265.5 ± 17.1	367.1 ± 31.4	512.8 ± 36.4
BSMP cells	124.9 ± 16.8	275.9 ± 13.6	382.9 ± 25.8	306.4 ± 22.5
$p > 0.05$				

For example, the direct touching method has the highest cytotoxicity sensitivity, since it is able to detect even weak cytotoxicity, thus, playing a leading role in in vitro biocompatibility evaluation (Ref 5). This method directly places the cells onto the biological materials for cell culture, observing the morphological and amount changes caused by release of toxins as well as the adhesion of the cells onto the materials. Usually, a good biocompatible material is reflected by easy adhesion and quick proliferation, and the result of the direct touching method can be used to estimate aftermath of the materials after being embedded. Some in vitro laboratory experiments are able to measure cellular viability and proliferation including radioisotope (^{3}H-thymine, ^{3}H-leucine) uptake, fluorescence stain (CFSE), and flow cytometry.

This study evaluated BSMP's biocompatibility in vitro by cultivating 3T3 cells on a BSMP-preset dish and observing BSMP's effect on growth and proliferation of the 3T3 cells, allowing toxicity potential of the BSMP toward the cells to be evaluated. This study employed the Thiazolyl Blue Tetrazolium Bromide (MTT) stain method of photoabsorption and ^{3}H-TdR addition to observe BSMP's effect on 3T3 proliferation. The MTT colorimetric assay, formerly applied in immunology research, was raised by Mosroann in 1983 to be used as a biocompatibility evaluation technique. Since then, it has been applied to biocompatibility evaluations by some scholars. The live cells can uptake MTT added into the culture medium with a certain dose, transforming MTT to blue Formazan using mitochondrial succinic acid dehydrogenase. The intensity of the blue stain is proportional to the live cell amount. ^{3}H-TdR addition is proportional to cellular DNA synthesis speed and proliferation level. Thymine is a raw material for DNA synthesis and can be mixed into live cells for DNA synthesis, labeling the cells in phase S and reflecting speed of DNA synthesis with the addition extent. In this study, the 3T3 cells grew and proliferated on the BSMP carrier at the same rate as that on the control group under different circumstances following adhesion, indicating that BSMP does not have any suppressive effect on 3T3 cell growth and proliferation in vitro and does not affect their biological behaviors.

5. Conclusion

3T3 cells have active biological behaviors on the BSMP carrier, indicating the biodegradable shape-memory polymers have good biocompatibility in vitro.

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