Effects of Collagen Nano-Spheres on Cell Cultures

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Abstract: A conventional means of repairing a damaged organ or tissue is to introduce cultured cells or tissues to the corresponding defect site. Three factors affecting the success of such repair include cell culturing, scaffolding and the signaling factors between the cells and scaffold. Enhanced binding of cell adhesion molecules, such as integrin, to specific molecules of the extracellular matrix (such as RGD sequences on the collagen) can generally induce cytoplasmic signal transduction cascades and activate gene expressions, subsequently achieving rapid cell proliferation and differentiation. This study investigates how type I and type II collagen nano-spheres in the culture medium influence hepatocytes and chondrocytes cultures. Experimental results indicate that hepatocytes demonstrated better viability and function after adding a higher concentration of type I collagen nano-spheres (5×10^{-2} mg/ml) to the culture medium than with lower concentration collagen particles (5×10^{-4} mg/ml) and the control group. The hepatocytes cultured with type I collagen nano-spheres also had a slightly higher level of albumin secretion in static dish cultures than that of the control group. Additionally, hepatocytes cultured in a stir bioreactor by adding type I collagen nano-spheres for 5 days formed a cell spheroid that is approximately 5 mm in diameter. In chondrocytes cultures, the chondrocytes displayed a higher secretion of glucosaminoglycan (GAG) than that of the control group (without adding collagen nano-spheres). Chondrocytes could form a large amount of spheroids rapidly with adding type II collagen nano-spheres to the medium in a 1-day rotary bioreactor culture. Histological staining and SEM observations revealed that these chondrocytes still retained their original phenotype and secreted collagen around the cells. In summary, hepatocytes and chondrocytes cultured in a rotary bioreactor while adding a moderate concentration of collagen nano-spheres could rapidly form cell pellets, maintaining their *in vivo*-like morphologies and specific functions.

Keywords: Nano-spheres, collagen, hepatocytes, chondrocytes, bioreactor.

INTRODUCTION

Binding of cell membrane receptors (e.g., integrin) to the specific molecules on extracellular matrix (such as RGD or DGEA sequences on the collagen) can induce cytoplasmic signal transduction cascades, thereby activating the gene expressions [1]. Extracellular matrix (ECM) thus profoundly impacts the migration, proliferation and differentiation of cells [2].

As a protein, collagen forms most of ECM, explaining its extensive use as a biomaterial in carrier systems to deliver drugs, proteins and genes or as scaffold construction in tissue engineering. As is expected, biomaterials design that can regulate cell behavior such as proliferation and differentiation is of priority concern in fabricating tissue engineering scaffolds. The structure of the scaffold material for facilitating cell attachment significantly contributes to their morphology, proliferation, and functioning, as well as the subsequent tissue organization [3]. The collagen matrix that influences the cellular behaviors has been extensively studied, with those results demonstrating that cells cultured in these collagen matrices grow and exhibit differentiation behaviors close to those of an *in vivo* environment [4]. However, the collagen matrices prepared are normally the standard fibrillar forms or sponge forms. Our recent study developed a high-voltage electrostatic field system, in which controlling the electrostatic field strength, environmental temperature and collagen concentration can produce collagen particles with sizes in the nanometer range [5]. To extend the medical applications of these collagen nano-spheres, this study evaluates how adding type I and II collagen nano-spheres, rather than the growth factors, onto the culture medium affects hepatocytes and chondrocytes grown in a rotation bioreactor. For comparison, exactly how these added collagen nano-spheres affect the viability and biochemical function analysis of hepatocytes and chondrocytes is also evaluated. Cell morphologies are also observed and examined by an optical microscope and scanning electron microscopy (SEM).

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MATERIALS AND METHODS

1. Preparation and Characterization of Collagen Nano-Spheres

Our previous study developed a high-voltage electrostatic field system [5]. Briefly, two parallel plate electrodes (made of copper sheets, 18 cm × 6 cm) with a gap distance of 2 cm were used. A high voltage was supplied by a DC power supply system (as provided by Bertan, series 230, U.S.A.) and controlled by a functional generator (Hewlett Packard, 33120A, U.S.A.) (Fig. **1**). 1 ml of iced collagen solution (0.2 mg/ml) was poured into a plastic Petri-dish and then placed at the center point of two parallel electrodes. The preparation parameters were set by type I and type II collagens. The reaction time was set to 3 hr. Thereafter, a portion of the treated collagen solution (20 μl) was immediately placed on formvar-coated copper grid and then negatively stained with 1 % phosphotungstic acid (Fluka, U.S.A). Finally, this dry grid was observed under transmission electron microscope (TEM) for ensuring the formation of collagen nano-spheres (Philips, Model CM 200, the Netherlands).

3. Hepatocytes Isolation and Culture

Hepatocytes were isolated from Sprague-Dawley rats (250-350 g) by using a modified collagenase perfusion protocol [6]. Briefly, the portal vein was cannulated with a 25-guage needle and the liver perfused with warm, calcium free HBSS (Hanks' Balanced Salts) with 0.01% EDTA at a rate of 10 ml/min for 10 min. Once visibly cleared of blood, the liver was excised and minced into small pieces and placed in a dish containing a HBSS medium supplement with 0.5 mg/ml collagenase (collagenase for heaptocyte isolateion, Sigma). This dish was incubated at 37 °C for 3 h and shaken once

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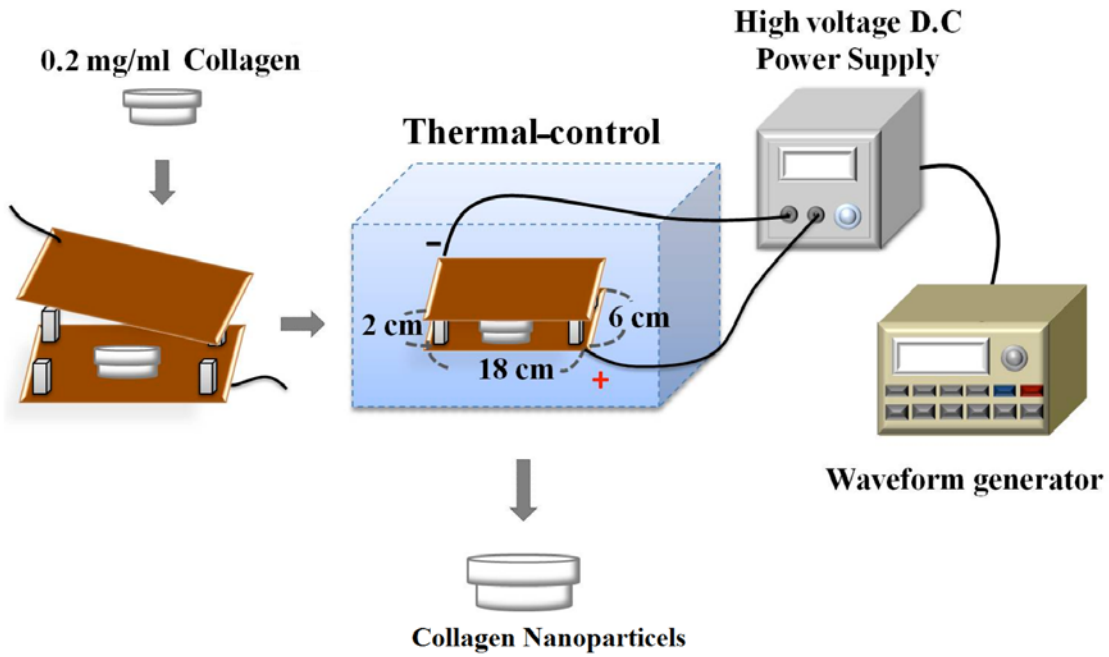

Fig. (1). Schematic presentation of the experimental electrostatic field system and collagen nano-spheres preparation procedures.

every 30 min. The liver cells suspension was then filtered and centrifuged at 500 rpm for 5 min. After the supernates were discarded, the precipitates (isolated hepatocytes) were further purified by centrifugal elutriation with PBS solution three times before culturing.

3. Isolation and Culturing of Chondrocytes

Chondrocytes were isolated from the knee cartilage of New Zealand rabbits (2-3 weeks of age) by using a modified collagenase perfusion protocol. The cartilage was sliced and minced into approximately 2 mm^3 pieces. After rinsing twice with cold phosphate buffered saline (PBS), the sliced cartilage was placed in a dish containing F12 medium supplement with 0.2% protease K. This dish was incubated at 37 °C for 2 h and shaken once every 30 min. After 2 h of digestion, the sliced cartilage was placed into a dish containing F12 medium supplement with 0.2% collagenase type II (Sigma). This dish was then incubated at 37 °C for 3 h and shaken once every 0.5 h. Next, the chondrocytes suspension was filtered and centrifuged at 500 rpm for 5 min. After the supernates were discarded, the precipitates (isolated chondrocytes) were further purified by centrifugal elutriation with PBS solution three times. Finally, the collected chondrocytes were re-suspended (in 75-cm^2 flasks) in F12 (GIBCO) supplemented with 10 % fetal bovine serum and antibiotic (200U/ml penicillin/streptomycin) and cultured at 37°C in a humidified incubator containing 5% CO_2.

4. Hepatocytes Cultured with Type I Collagen Nano-Spheres in Bioreactor

The isolated hepatocytes (10^4 cells/ml, a final cell density) mixed with type I collagen nano-spheres (5×10^{-4} mg/ml) in a centrifuge tube were incubated at 37°C in 5% CO_2 incubator for 3 h [7]. 100 ml of the cell-collagen mixture was then transferred and poured into a stir bioreactor (Corning, U.S.A). This bioreactor was placed inside a 37°C incubator. Finally, the cultured medium was evaluated daily by albumin secretion analysis and MTT assay.

5. Chondrocytes Cultured with Type Ii Collagen Nano-Spheres in aBioreactor

The isolated chondrocytes (10^4 cells/ml, a final cell density) mixed with type II collagen nano-spheres (5×10^{-4} mg/ml) in a centrifuge tube were incubated at 37°C in 5% CO_2 incubator for 3 h. 100 ml of the cell-collagen mixture was then transferred and poured into a stir bioreactor. This bioreactor was placed inside a 37°C incubator. Finally, the cultured medium was evaluated daily by glycosaminoglycans secretion analysis and MTT assay.

6. Cell Morphology and Histological Observation

For histological analysis, all specimens were fixed with 10% neutral buffered formaldehyde (pH 7.2) and decalcified with 15% neural EDTA. The samples were embedded in paraffin and sectioned at a thickness of 5μm. The sections were stained with hematoxylin and eosin and Masson's, and observed under light microscopy. The samples were also observed in a scanning electron microscope.

7. Albumin Secretion Analysis

Aliquots of media were removed from the monolayer cultures and stored at -20 °C before determining the albumin concentration in a medium. Finally, the samples were analyzed using a QuantiChrom™ BCP Albumin Assay kit (DIAP-250, U.S.A), for the colorimetric quantitative determination of albumin at 610 nm on an ELISA spectrophotometer.

8. Content Analysis of Glycosaminoglycans

Aliquots of media were removed from the bioreactor cultures and stored at -20 °C before determining the glycosaminoglycan concentration in the medium. The samples were analyzed using a Blyscan™ glycosaminoglycans Assay (B1000, U.K), to quantitatively determine the colorimetric feature of glycosaminoglycans at 656 nm on an ELISA spectrophotometer.

RESULTS AND DISCUSSION

1. Observations of Collagen Nanospheres

Figs. (**2**(**a**) and (**b**)) show the prepared type I and type II collagen nano-spheres, indicating that the obtained type I collagen nano-spheres closely aggregated together in large numbers (cluster of nano-spheres), resulting in a larger and irregular outer shape. Additionally, increasing the treated temperature to 37°C led to spontane

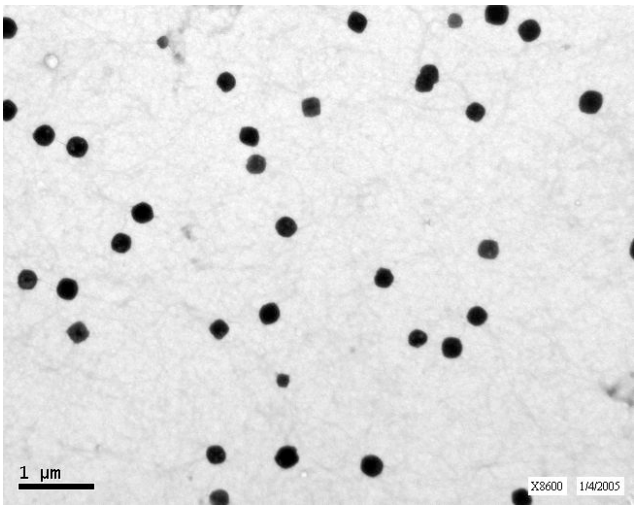

Fig. (2). (**a**) Transmission electron micrograph of type II collagen nano-spheres.

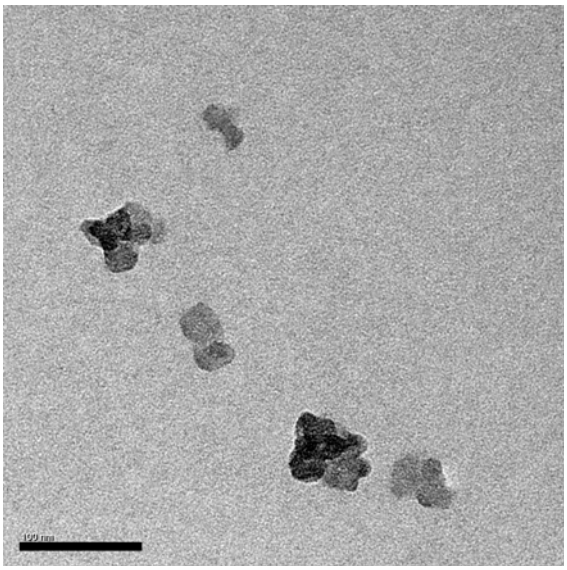

Fig. (2). (**b**) Transmission electron micrograph of type I collagen nano-spheres.

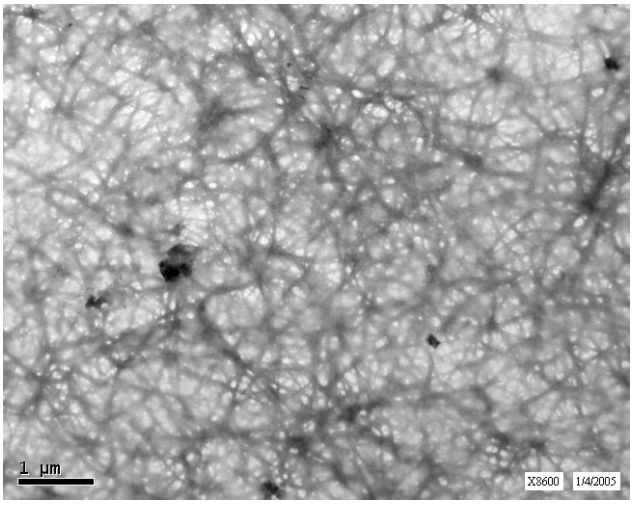

Fig. (2). (**c**) Transmission electron micrograph of type I collagen fiber.

ous fibrillogenesis of collagen (Fig. **2** (**c**)). Conversely, type II collagen nano-spheres with good sphericity was more easily prepared than type I collagen under the same high voltage electrostatic system.

2. Hepatocytes Co-Cultured with Type I Collagen Nano-Spheres

Exactly how type I collagen molecules affect primary rat hypatocytes viability and function was assayed by mitochondrial activity (MTT assay) test and albumin secretion analysis. Also, the hepatocytes mitochondrial function decreased by approximately 50 % within 72 h of isolation and increased progressively to a level similar to the initial activity within 10 days. Moreover, the hepatocytes co-cultured with type I collagen nano-spheres had a slightly higher level of albumin secretion than that of the control group without nano-spheres (Fig. **3**). In stirring bioreactor cultures, the stirring environment inhibited the attachment of hepatocytes to the glass substratum and, conversely, facilitated cell-cell interactions or cell-nanospheres bindings, aggregation and subsequently formed a cell pellet, also referred to as a spheroid. The size of hypatocytes spheroids was increased more rapidly after adding type I collagen nanosheres into the medium than in the control group. The hepatocytes spheroids increased to approximately 5 mm in diameter after a 5-day culture. Closely examining the SEM and H&E stained sections revealed that the hepatocytes retained their original phenotype and some collagen matrix formed besides the hepatocytes, indicating that the functions of hepatocytes remained unchanged after adding collage nano-spheres (Fig. **4**).

3. Chondrocytes Co-Cultured with Type II Collagen Nano-Spheres

Similarly, many chondrocytes spheroids formed after adding type II collagen nano-spheres in a 2-day culture in a rotary bioreactor (Fig. **5a**). SEM observations indicated that the spheroids contained well-differentiated chondrocytes (Fig. **5b**). The chondrocytes cultured with type II collagen nanospheres secreted more glycosaminoglycan than that with the control group (Fig. **6**). This finding suggests that the added collagen nano-spheres not only maintained the proliferation and differentiation of chondrocytes, but also improved the ability of chondrocytes to secrete glycosaminoglycan.

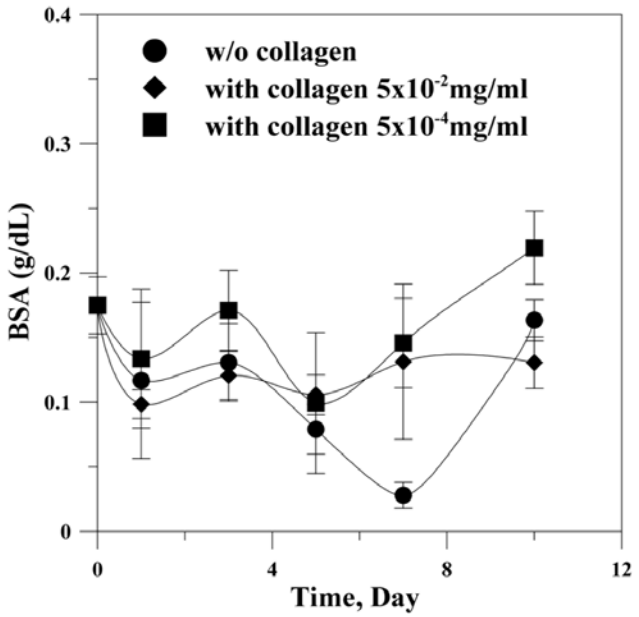

Fig. (3). Effects of type I collagen nano-spheres on the albumin secretion of hepatocytes. Data were presented as mean ±S.D. for all groups (n=3).

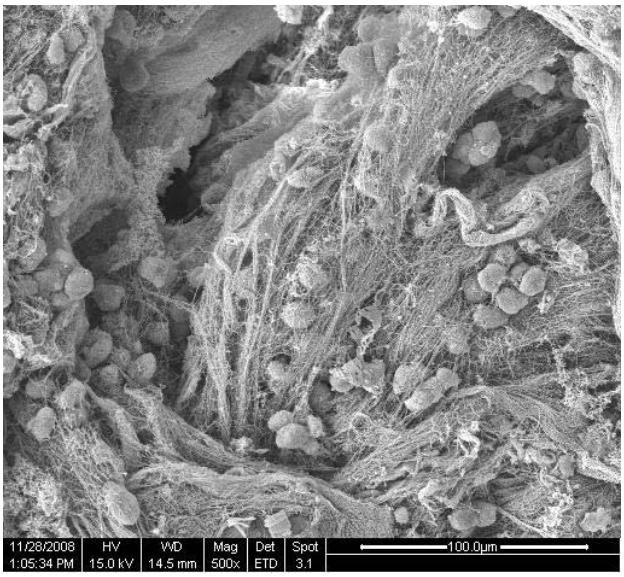

Fig. (4). (**a**)SEM observation of hepatocytes spheroid.

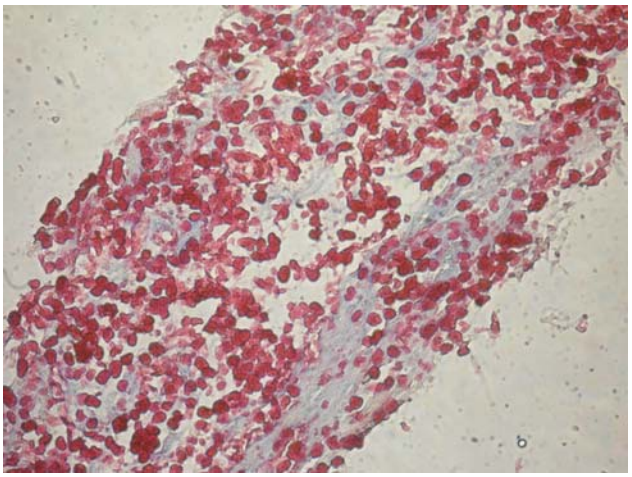

Fig. (4). (**b**)H&E staining of sectioned hepatocytes spheroid.

However, detailed interactions of mechanisms or events of the collagen nano-spheres and cells are still under evaluation in our laboratory.

CONCLUSIONS

Hepatocytes and chondrocytes cultured in a rotary bioreactor, while adding a moderate concentration of collagen nano-spheres, can rapidly form cell spheroids in a short period culture. These spheroids can maintain their *in-vivo*-like morphologies and specific functions, including albumin secretion and glycosaminoglycan secretion. This ability can comply with tissue engineering requirements, thus providing rapidly a large amount of cultured cells or tissues onto defect sites to repair damaged organs or tissues

This study developed a high-voltage electrostatic field system to prepare nano-sized collagen molecules, which were applied in cell culturing as an ingredient of the culturing medium. Experimental results demonstrated that the added collagen nano-spheres contributed to the cell proliferation and functions. As mentioned earlier, binding of cell membrane receptors (e.g., integrin) to the specific molecules on extracellular matrix (e.g., RGD or DGEA

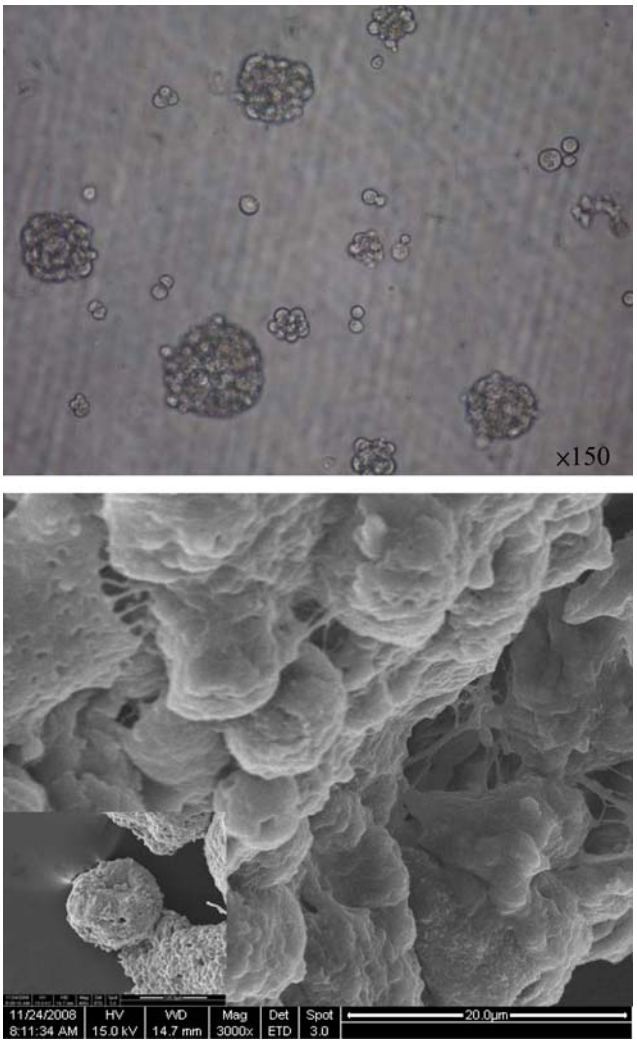

Fig. (5). (**a**) The chondrocytes pellets in 3-day culture with 10^{-4} mg/ml collagen nano-spheres in rotation bioreactor. (**a**) OM; (**b**) SEM observations.

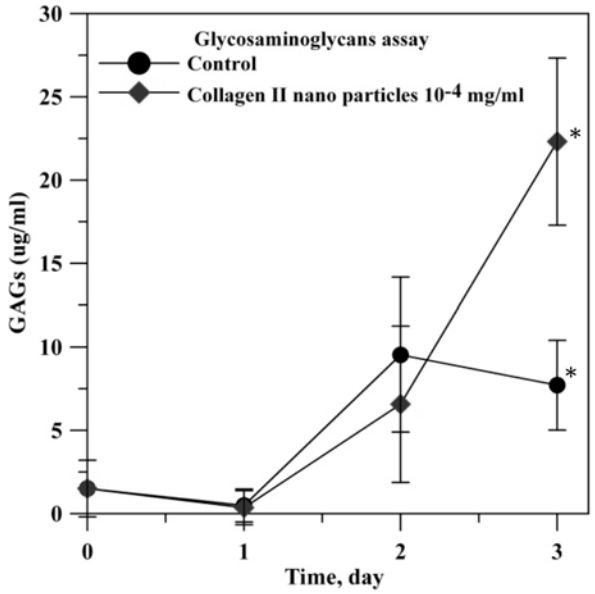

Fig. (6). GAGs content test for chondrocytes cultured with 10^{-4} mg/ml collagen nano-particles in bioreactor. $p<0.05$.

sequences on the collagen) could induce cytoplasmic signal transduction cascades, thereby activating the gene expressions. Therefore, the collagen nano-spheres could possibly collide (interact) with cells rapidly and easily in a stirring environment, thus initiating the proliferation and differentiation of cells. Efforts are underway to find evidence of cytoplasmic signal transduction cascades between the cells receptors and collagen by immunofluorescence focal adhesion kinase (FAK) staining assays, thus paving a new direction for cell cultures and tissue engineering.

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