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Original Article

The Homing of Spermatogonial Cells in the Cavities of a Novel Nanoscaffold

Saeide Nezami Saridar[1] M.Sc., Seyedhossein Hekmatimoghaddam[2*] M.D., Mona Yadegar[3] M.Sc.

[1]Department of Biology, Islamic Azad University, Ashkezar Branch, Ashkezar, Yazd, Iran.
[2]Department of Laboratory Sciences, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
[3]Department of Biology, Islamic Azad University, East Tehran Branch, Tehran, Iran.

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Background and Aims: In this lab trial, the effect of scaffold based on human serum albumin (HSA) and hydroxyapatite nanoparticles (HA NPs) on mouse spermatogonial cell line (SCL) was investigated.

Materials and Methods: To synthesize HA NPs, calcium nitrate and diammonium phosphate at pH 13 were gently added and heated at 100 ºC for 24 hours. Then serial concentrations of HA NPs was separately added to 500 mg/mL of HSA, and immediately placed in the 100 ºC water bath. Then, all scaffolds were cut, and incubated with SCL for 6h, 12h, and 24h at 37 ºC. Finally, the cell count was read, and homing of the cells was examined by optical microscopy.

Results: It was found that the quantity of cells did not change by increase in concentration of HA NPs. On the other hand, increased incubation time led to decrease in cell count. Light microscopic observation of scaffold cavities after incubation showed the homing of spermatogonial cells.

Conclusions: This promising scaffold must be more investigated in vitro and in vivo, and may be suitable for making artificial testis.

[*] **Corresponding Author:** Department of Laboratory Sciences, School of Paramedicine Shahid Sadoughi University of Medical Sciences, Yazd, Iran. **Email address**: shhekmati2002@yahoo.com **Tel**: 0098-3536285406; **Mobile Phone**: 0098-9133518314; **Fax**: 0098-3537246541

Introduction

Haploid gametes are obtained from spermatogonial cells through meiosis [1, 2]. This phenomenon can take place in vitro, at laboratory conditions [3,4].Sperm preservation and testis tissue cryopreservation are common protocols for some male infertility diseases, e.g. pre-pubertal cancer [5], but this approach has some challenges [6,7]. In vitro spermatogenesis is a new regenerative medicine strategy, especially by the 3-dimensional scaffolds. It has been found that artificial scaffolds can mimic the natural extracellular matrix (ECM) [8], and provide biological niche for cells [9]. The scaffolds provide important physical and chemical signals necessary for cell spreading and homing. The pores of scaffolds may help in remodeling of tissue structures [10]. Nowadays, various scaffolds have been used for tissue engineering [11-17]. Rafeeqi et al. used carbon nanotubes (CNTs) as scaffold for spermatogonial cell, and found that the cells maintained their shape and function at least 21 days on the scaffolds. Also, they declared that the scaffold had high biocompatibility, and can be applied for in vivo studies [18]. Eslahi et al. investigated the impact of poly-L-lactic acid (PLLA) scaffold on the mouse spermatogonial stem cell; significant increase in the cell clusters was seen. Importantly, they found differentiation of spermatogonial stem cells to sperm cells [19].

Based on previous studies and also our team expertise in manufacture of bioscaffolds, we aimed at evaluation of the effect of a novel scaffold based on human serum albumin (HSA) and hydroxyapatite nanoparticles (HA NPs) on mouse spermatogonial cell line (SCL).

Materials and Methods

Materials

Calcium nitrate, diammonium phosphate, HSA, trypsin containing 5% ethylenediamine tetraacetic acid (EDTA), Roswell Park Memorial Institute (RPMI) 1640 medium, and Fetal calf serum (FCS) were provided from Sigma-Aldrich Chemical Co, (St Louis, MO, USA).

Synthesis of HSA/HA NPs scaffold

12 g of diammonium phosphate ($(NH_3)_2HPO_4$) was dissolved in 375 mL of distilled water (DW), and 36.15 g of calcium nitrate ($Ca(NO_3)_2$) was dissolved in 525 mL of DW. Then, 50 mL of calcium nitrate solution and 50 mL of diammonium phosphate solution, previously adjusted to pH 13, were gently added and heated 24 hours at 100 ºC. After incubation, the precipitate was rinsed with DW, dried at 37 ºC, and hardly ball-milled for one hour. Then, HA NPs were characterized by scanning electron microscopy (SEM) (S-2400, Hitachi, Japan), and dynamic light scattering (DLS) (Molvern, Italy).Serial concentrations of HA NPs (100, 50, 25, 12.5 mg/mL) were separately added to 4 mL of 500 mg/mL HSA. The mixture was hardly blended for one minute, and straightaway held in the 100 ºC water bath for 10 minutes. After

coagulation of the mixture, HSA/HA NPs scaffold was frozen at -20 ºC, and then held in the 37 ºC water. So, four HSA/HA NPs scaffolds were prepared, in which the concentration of HA NPs was variable, i.e. the final concentrations of HA NPs in the scaffold were 1.2, 2.5, 5, and 10 mg/mL. To investigate the size of the scaffold cavities, all the scaffolds were cut (near 100 µm×2 mm), and observed by optical microscope. Finally, each scaffold was cut (2 mm×2 mm), and placed into sterile wells of a 96-well microculture plate.

The preparation of SCL

At first, one mL of RPMI 1640 enriched with 10% FCS and penicillin/streptomycin antibiotics (RPMI 1640-10% FCS-antibiotic) was added to mouse SCL obtained from the Pasteur Institute of Iran. Then, they were incubated for 4 days at 37 ºC. After incubation, the cells were washed and transferred to a culture flask containing 10 mL of RPMI 1640-10% FCS-antibiotic, and incubated for 14 days at 37 ºC. To release adhered cells, one mL of 100 mg/mL trypsin containing 5% EDTA was added and incubated for 30 minutes. Then, the cells were washed three times by RPMI 1640-10% FCS, and the concentration of cells was adjusted to 10000 cells/mL.

The main experiments

As mentioned, each scaffold was cut (2 mm×2 mm), and placed into sterile well of a 96-well microculture plate. Then, 100 µL of the cell suspension was added to each well, and incubated for 6 h, 12 h, and 24 h at 37 ºC. After incubation, the scaffold pieces were removed, and the quantity of cells was read by cell counter and hemacytometer slide. All data were normalized to control, according to Formula 1.

Formula 1: The cell count=A/B

A is the cell count in test tube, and B is the cell count in control tube. In this study, the cells not exposed to any scaffolds were considered as negative control.

Statistical analysis

All tests were carried out three times, and the results were shown as mean±standard deviation. Then, Student's *t-test* was done by SPSS software, V.16.0 for Windows (SPSS Inc., USA), and $P<0.05$ was considered as the level of significant difference.

Results

Characterization

The SEM image of HA NPs is shown in Figure 1. Also, the DLS graph is observed in Figure 2. It was demonstrated that HA NPs were approximately rod-like and agglomerated, and their size distribution was near 50-250 nm.

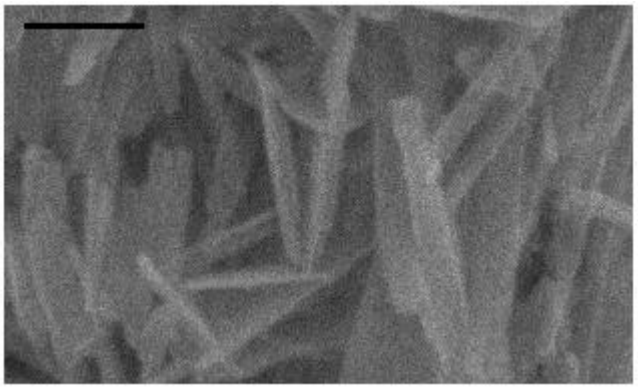

Fig.1. The SEM image of HA NPs. The scale bar is 100 nm

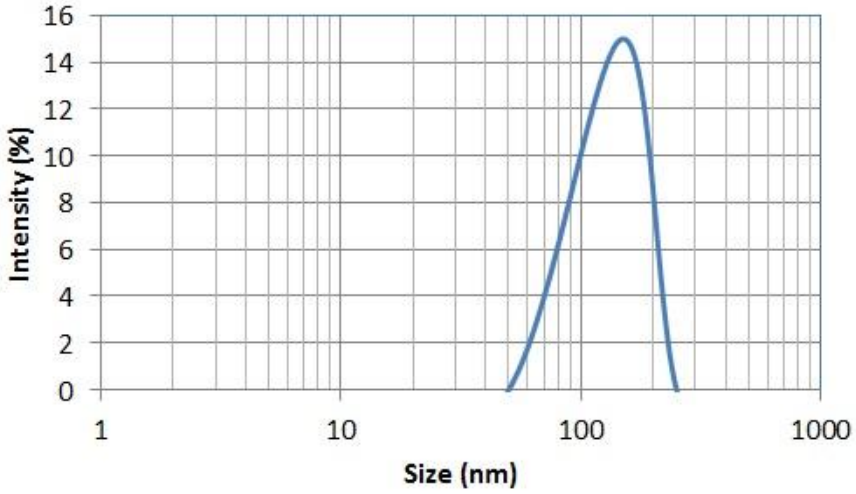

Fig.2. The size distribution of HA NPs, obtained by DLS.

The attachment assay

Figure 3 shows the count of cells when exposed to the scaffolds. This parameter was not affected by concentration of HA NPs. But, by increase in incubation time, a decrease in cell count was obtained.There were significant differences between the cell counts at 24 h vs. 12 h, and between the cell counts at 24 h vs. 6 h. Each cavity measured 40-500 micrometers. Figure 4 demonstrates the homing of spermatogonial cells in a scaffold cavity, obtained by light microscope. As seen, many spermatogonial cells are adhered to surface of the scaffold.

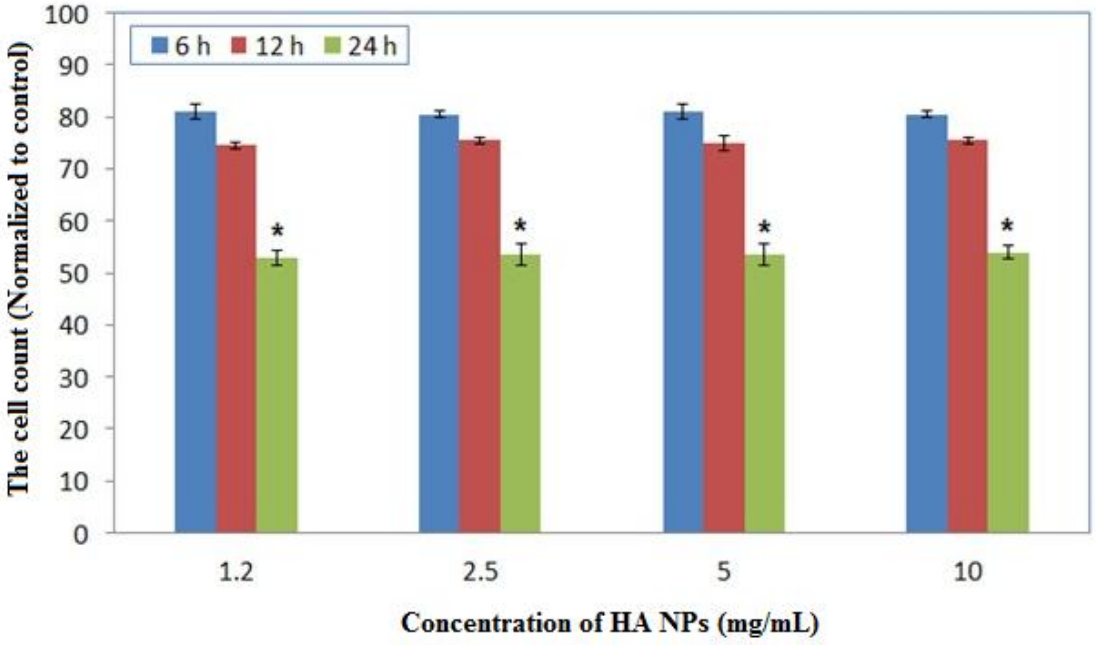

Fig.3. The cell count after incubation with the scaffold, obtained by cell counter. *P<0.05 compared with 6 h and 12 h, n=3. All data were normalized to control.

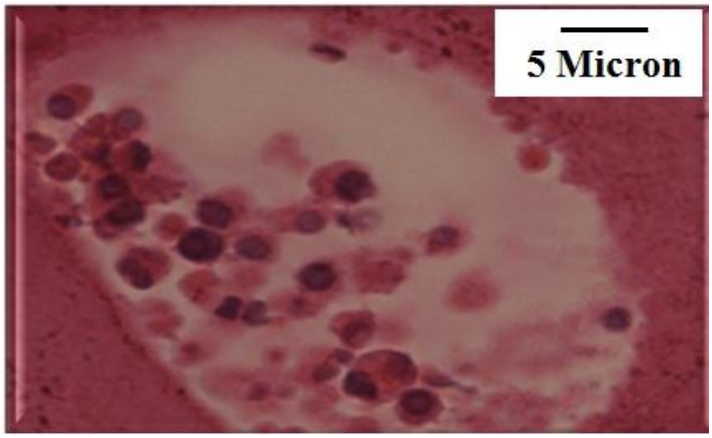

Fig.4. The homing of spermatogonia in scaffold cavity, obtained by light microscope.

Discussion

Here, a novel scaffold was synthesized. This was based on HSA and HA NPs. Then, the attachment of spermatogonial cells on the scaffold was investigated. In this study, HSA was used because the molecule is highly biocompatible, and many cells can uptake it [20]. HA NPs were used because they are highly biocompatible and biodegradable, similar to HSA., HA NPs have added advantages, e.g. high surface size, pore size, and wettability, in comparison with HA

microparticles. There are reports about high adsorption, bioactivity, and mechanical properties of HA NPs [21]. It was speculated that the scaffold (HSA/HA NPs) could provide adhesive surface for cells, in addition to seeding, proliferation, and differentiation on it. In this study HSA/HA NPs scaffolds with different concentrations of HA NPs were synthesized, and then their impact on the SCL was assessed. It showed that the cell count was not affected by concentration of HA NPs, but increase in incubation time led to decrease of cell count. Also, the homing of spermatogonial cells in scaffold cavity was observed. Spermatogonial cells could adhere to surface of the scaffold.

It must be mentioned that the initial suspension of HA NPs higher than 100 mg/mL could not be prepared, because of rapid precipitation of HA NPs. As mentioned in the material and methods section, the ratio of HA/HSA was 0.25 (v/v). Based on our experiment, this ratio could not be increased, because higher HA volumes led to decrease in strength. Although the final concentrations of HA NPs in the scaffolds were 10, 5, 2.5, and 1.2 mg/mL, but the authors propose higher and lower concentrations to be tested in future studies. It must be mentioned that bad nutritional conditions may lead to toxic effects of the scaffolds at longer incubations, which is a common problem in the cell culture [18, 19]. In the future studies, the cells within the scaffold should be incubated and evaluated for more than 24 h. On the other hand, in the future works, other biocompatibility tests such as hemolysis, irritation, sensitization, and implantation must be considered, in order to obtain a general scope of the process.

Here, HSA protein was used because it is natural, biocompatible and adsorbent. Moreover, previous studies have demonstrated that it could be taken up by receptor-mediated endocytosis, and is finally degraded within the lysosome [22]. Furthermore, it can be covalently labeled with several molecules such as drugs or fluorescent dyes [20]. It has been found that albondin, a gp60 protein of endothelium is an agent of binding of albumin to cells [23]. Kuchar et al. applied the albumin-binding domain as a part of cellular matrix. They showed that the domain was expressed by gram-positive bacteria, and also can be applied for protein purification [24]. Based on previous studies, albumin is the good material to be used in the scaffolds. A novel HSA scaffold was designed by Ferrero-Gutierrez et al. for axon regeneration. They showed that the scaffold could be seeded by adipose tissue-derived stem cells and olfactory ensheathing cells [25]. Gallego et al. found that albumin scaffold was a favorable and non-toxic material to grow and differentiate osteoblasts [26, 27]. Weszl et al. showed that although the coating of scaffolds with different proteins of extracellular matrix could improve cell proliferation, but HSA is the best substance for this purpose [28]. Nseir et al. found that electrospun albumin scaffold could be used as an artificial biomaterial, and showed that the scaffold was capable of adsorbing different serum proteins, such as laminin. They proposed that the albumin scaffold could be modified by different

molecules for different applications [29]. Albumin-derived peptide scaffold which was from the sequence of HSA was developed by Luisi et al. They demonstrated that the scaffold had high compatibility and could bind to various molecules [30]. Tripathi et al. investigated the physical, mechanical, and biological properties of a novel porous hydroxyapatite scaffold. The study showed that human osteoblasts could adhere, proliferate and migrate into the scaffold [21]. Wu et al. worked on the biological properties of hydroxyapatite scaffolds, and showed its good biocompatibility. They declared that its surface metrology did not have any change after 21 days [31]. Dey et al. designed a new collagen/hydroxyapatite scaffold, and demonstrated in vitro seeding and attachment of cells onto the scaffold. Also, they found that the cells could proliferate, migrate and differentiate into the scaffold [32]. Leukers et al. applied hydroxyapatite scaffold made by 3-D printing. It was found that the cells proliferated into the scaffold, and differentiated in close contact to the structure [33]. Kwon et al. built porous hydroxyapatite scaffold through polymer replication method and solid freeform fabrication. They confirmed their biocompatibility, safety, and efficacy by cytotoxicity assays, hemolysis, irritation, and implantation [34].

This scaffold must be more investigated in vitro and in vivo, and its efficacy should be studied at actual conditions. In the future studies, the proliferation and differentiation of spermatogonia must be evaluated at different conditions. We propose that the scaffold may be good for synthesis of artificial testis.

Conclusion

It can be concluded that 24 h is needed for homing and establishment of cells in HSA/HA NPs scaffold. Also, increased concentration of HA NPs cannot change cell quantity. On the other hand, increase in incubation time leads to decrease in cell count. This scaffold must be more investigated in vitro and in vivo, and may prove suitable for making artificial testis.

Conflict of Interest

No conflict of interest is addressed.

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