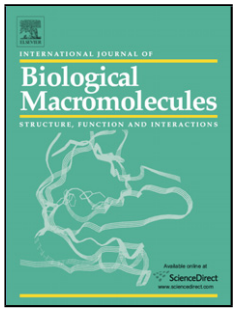

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Evaluation of alginate hydrogel cytotoxicity on three-dimensional culture of type A spermatogonial stem cells

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Abstract

The culture of spermatogonial cells for future transplantation, based on the specific biology of these cells is important and necessary. Recently, the use of scaffolds especially alginate for culturing stem cells has been the focus of many researchers. The aim of this study was to evaluate the cytotoxicity of alginate hydrogels to cultures of type A spermatogonial stem cells. Spermatogonial stem cells of 6 day-old immature mice were isolated by surgery; thereafter, the cells were purified by MACS using antibodies against thy-1 and C-kit and cultured on a layer of laminin. After purification, spermatogonial stem cells were encapsulated in alginate hydrogels. After one month of encapsulation and culture in DMEM culture medium containing 10 ng/ml GDNF, cells were removed from hydrogel and were examined for viability, cell morphology and structure, cytotoxicity and expression of apoptosis genes Fas, P53, Bax, Bcl2, Caspase3 by staining with trypan blue, scanning electron microscopy, LDH test, and Real time PCR, respectively. The encapsulation did not change the morphology and viability of spermatogonial stem cells. Investigations showed that spermatogonial stem cells preserve by the high viability (74.08%) and cytotoxicity of alginate hydrogel was estimated to be 5%. Expression of Fas gene increased in main group compared with the control group, and expression of Bax and P53 was reduced in main group compared with the control group. Expression of Bcl2 and Caspase3 genes did not show any significant difference between the main group and the control group. Considering the lack of cytotoxicity and antioxidant properties of alginate hydrogel scaffold and high viability of cells, this three-dimensional scaffold is applicable for culturing and encapsulation of spermatogonial stem cells.

Keywords: Spermatogonial stem cells, encapsulated, alginate hydrogel, apoptosis

Introduction

Tissue engineering is a multidisciplinary field that exploits principles of both engineering and life sciences to provide biological alternatives which can restore, maintain or improve functions of a tissue or an entire organ. In a general view, tissue engineering is a triangle with three vertices of cells, scaffolds and biological signals, with cells being the most important vertice [1].

Stem cells are a group of cells capable of dividing into quite similar cells, with the capabilities to produce and differentiate into more specialized cells [2]. In several cases, to achieve therapeutic purposes, stem cells are required much more than the amount that could be isolated from a patient and this highlights the need for *in vitro* culture systems for the expansion of primary cell population. Although the quality and purity of expanded stem cells are also important in addition to the number [3]. The need for culture and expansion of stem cells in various diseases such as male infertility was very important for researchers. Male germ cells are a collection of differentiated cells which altogether comprise spermatogonial. In primates and humans, these cells are of two lines namely; spermatogonial cell type A and type B. Type A is sub classified into A_{dark} and A_{pale} groups. A_{dark} group comprise a population of about 1% of spermatogonial cells, and are, in fact, spermatogonial stem cells (SSCS) with low mitotic divisions [4]. Given that the number of SSCS cells in the testes is usually very low, devising a method for proliferation and survival of germ cells during the culture and proliferation and enrichment of SSC cells *in vitro* could be an important strategy which will help in detailed study of SSCS and subsequently provides a higher chance of success in the SSC transplantation *in vivo* [5]. Since one of the reasons for male infertility is due to loss of sexual germ cells as a result of anti-cancer treatments, such as chemotherapy and radiotherapy. Due to the increasing survival rate of cancer patients after therapy, especially children, and attaining the age of fertility, the importance of

treating infertility after cancer treatment in these patients comes into light. Due to lack of active spermatogenesis in children, maintaining the testes or germ cells in different ways has been considered by researchers. One of these methods is the use of freezing-thawing technique for freezing the cells before chemotherapy, and finally melting after the patient has recovered and transplanting the cells into the testes. However, using cryoprotectants in this technique, due to their cytotoxicity and free radical formation during melting, can damage the cells. For this reason, researchers are searching for other methods such as preserving and proliferation of these cells in culture during patient's treatment period, which can be eventually transplanted into the patient after treatment. A lot of concerns exist about the quality of these cells for transplantation after treatment. Recently, in the field of tissue repair and cell culture, there has been great attention towards the combination of scaffolds and cells to simulate physical and biological properties of normal tissues in the body [6]. Alginate is a natural biopolymer mainly extracted from brown algae and to a lesser extent from bacteria. In fact, in the extracellular matrix of the algae, alginate is in combination with calcium, magnesium and sodium cations. It is available in the form of dry powder, and is convertible to alginate gel *in vitro*. This hydrogel makes a three-dimensional scaffold which on one hand, provides more surface area available for cell proliferation and on the other hand, facilitates nutrient distribution in medium and thus facilitates cell growth. Monomeric compounds, structural sequences and the rate of alginate gel formation influence the rate of nutrient diffusion, porosity, swelling rate, strength and biocompatibility of the gel [7, 8]. Encapsulation of cells in hydrogels causes uniform distribution of cells in a gel matrix, and the permeability of the hydrogel leads to the proper release of oxygen, nutrients and biochemical stimuli in the surrounding environment. Moreover, the controllable rigidity of hydrogel is a kind of physical stimulus by itself [9]. The aim of this study is to evaluate the

interaction between alginate hydrogel and spermatogonial stem cells type A during three-dimensional culture.

Materials and Methods

Mice surgery and isolation of murine spermatogonial stem cells

In this study, 20 NMRI mice purchased from Medicinal Plants Research Center of Khorram-Abad were used for the experiment. In 6 day-old neonatal mice, after surgery, testes were gently removed and placed in a Petri dish containing DMEM culture medium (Gibco) with 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco). The tunica albuginea and epididymis were completely removed under stereo microscope. Then, Milazzo enzymatic digestion was used to isolate the cells and prepare cell suspension [10]. The excess tissues were removed from the testicles; then they were placed into 2 μg/ml collagenase IV (Sigma) and 5 μg/ml DNAse I (Sigma) and incubated for 15 min at 37 ° C and 5% CO_2, they were centrifuged for 5 min at 800 rpm. For proper isolation of cells, 1ml trypsin EDTA (Sigma) was added to cell pellets obtained from the previous step and pipetting was carried out to split apart and disperse the cells; then the cells were incubated for 5 min. Trypsin was inactivated by DMEM containing 10% FBS. The resulting suspension was passed through a 70 μm nylon mesh (FALCON, USA) and the number of cells and cell viability was assessed by Hemocytometer (fig.1)

Figure 1.

Purification of spermatogonial stem cells by Magnetic Activity Cell Sorting (MACS):

The cells were placed in a Petri dish (60 mm) coated with a layer of 20 μg/ml laminin and then placed in an incubator for one night. Thereafter, the supernatants were removed and the laminin containing Petri dish was washed with PBS buffer. To prevent the binding of gross cells, it was

incubated with a solution of 0.5 mg/ml BSA for one hour at 37 ° C, and then rinsed with PBS buffer. MACS method was used for the purification of SSCs cells. In this method, a specific marker of SSCs, namely Thy-1 + (CD90.1 MicroBeads, 130-094-523 murine antibody) as a positive control, C-Kit (CD117 MicroBeads, 130-091-224 murine antibody) as the negative control and MS, LS, XS MACS columns were used. Each 10^7 cells were centrifuged for 10 min at 300 rpm. Obtained cell pellets were re-suspended by adding 90 µl buffer solution. The buffer solution contained: PBS, 0.5% BSA, pH 7.2 and 2 mM EDTA. The MACS BSA stock solution (# 130-91-376) was diluted by the ratio of 1:20 with auto MACS Rinsing solution (# 130-091-222). A volume of 10 µl CD90.1 MicroBeads was added to this buffer solution. The resulting suspension was refrigerated for15 min, then cells were washed by adding 1-2 mM buffer and centrifuged for 10 min at 300 rpm. Each 10^8 cells were re-suspended in 500 µl; then the cell suspension was passed through MACS column for cells to be separated in the magnetic field.

Encapsulation of spermatogonial stem cells in alginate

After preparing a solution of sodium alginate by dissolving 1.25 g of powdered alginate (Sigma Aldrich, Germany) in 150 mmol NaCl at pH 7.4, it was added to the cell pellet, then the cell-alginate solution was slowly added to 135 mmol/L calcium chloride dropwise, such that cell-alginate MicroBeads were created. Ten minutes later, calcium chloride was removed by washing the MicroBeads with 0.9% NaCl (Merck-Germany).

Culture of spermatogonial stem cells

MicroBeads in Falcon tube were slowly transferred into a flask. Spermatogonial stem cells were cultured for 30 days in DMEM containing 10% FCS, and GDNF growth factor (10 ng/ml), and

were kept in an incubator (37 ° C, 90% humidity and 5% CO_2). During this period, culture medium was changed every three days.

Depolymerization of cell-alginate solution

For this purpose, a solution of 119 mmol /L sodium citrate was utilized. MicroBead containing solution was placed for about thirty minutes in the incubator and was centrifuged at 1800 rpm for 8 min; then 1 ml DMEM medium was added to cell pellet.

Evaluation of cell viability using trypan blue: Spermatogonial stem cells were counted at two stages, before encapsulation in alginate hydrogels, and after depolymerization. To evaluate cell viability, Trypan blue method (Sigma-America) was utilized.

Cytotoxicity assay by measuring LDH

Lactate dehydrogenase enzyme is usually released from damaged cells. With the measurement of this enzyme, valuable information could be realized on the effects of drugs on cells [11]. LDH level was measured three times according to the manufacturer's instruction (Roche company).

Real-time PCR

First of all, total RNA was extracted by Jana Bioscience pp-210s Kit (Qiagene America) according to the manufacturer's instruction. The concentration of RNA was measured with Nano drop (Biochrom WPA Biowave ll) at a wavelength of 280-260 nm. CDNA synthesis from extracted RNA was performed by AccuPower CycleScript RT PreMix kits (dN6), (BIONEER, Korea), according to the manufacturer's instructions. In this study, specific primers for apoptosis genes, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the reference gene were obtained from Gene Bank and are shown in Table 1. GAPDH is a reference gene that was added

as an internal control to perform a normal PCR reaction. Real time PCR (RT-PCR) was carried out utilizing synthesized cDNA, primers, Master Mix 2X (Jena Bioscience kit, Germany) and under thermal conditions of 95 ° C for 2 min followed by 45 cycles at 60 ° C for 45 s.

Table 1.

Scanning Electron Microscopy (SEM)

Santana freeze dried method was used to process alginate hydrogel microbids and encapsulated spermatogonial stem cells after one month 3D culture [12].

Results

Morphologic investigation of alginate capsules and spermatogonial stem cells

Capsules were spherical with uniform margins and the cells were homogeneously distributed throughout the capsule. Images showed that the capsules have retained their structural integrity and spherical shape even after 30 days. The encapsulated cells remained enclosed in the alginate matrix until day 30. Spermatogonial stem cells were circular in alginate capsules while not binding to the surface. Therefore, in this study, the process of encapsulating the cells did not alter the morphology of SSCs.

Figure 2.

Evaluation of encapsulated stem cell survival

Recently, three-dimensional alginate scaffold because of easy preparation and its ability to encapsulate cells has attracted much attention. Alginate hydrogel has several useful features such as biocompatibility and being non-immunogenic that is likely to be related to its hydrophilic properties [13]. In order to determine the effects of encapsulation in alginate hydrogels, cell

survival and viability was assessed by trypan blue staining. The mean survival rate of freshly isolated spermatogonial stem cells was calculated as 96.9% of statistically significance level ($P < 0.001$). The mean survival rate of spermatogonial stem cells encapsulated in alginate hydrogels after one month at statistically significant level ($P < 0.001$) was obtained as 74.08%, which shows the high viability of the cells.

Assessment of cytotoxicity of spermatogonial stem cells encapsulated in alginate hydrogels

Another important way to verify the toxicity with evaluation of membrane integrity is analysis of serum lactate dehydrogenase (LDH). If the cell membrane is damaged, it can be founded by examining the changes in absorption of NADH in the presence of LDH at wavelength of 492 nm and reducing the solution absorption in this wavelength. Alginate hydrogels are abundant, cheap and non-toxic[14]. Alginate is a biocompatible hydrogel for the encapsulation of cells to protect them from the host's immune system. Small molecules such as glucose, oxygen and waste products are able to pass freely through the gel matrix [15]. The rate of cytotoxicity of alginate hydrogels after one month was calculated as 5% by measuring the amount of LDH released by cells encapsulated in alginate hydrogels according to the manufacturer's instruction (Roche, Germany) using the corresponding formula in it (by ELISA reader at wavelength of 492 nm).

The relative gene expression report of apoptosis genes

To evaluate the expression of apoptosis genes in the two groups of freshly isolated spermatogonial stem cells and cells encapsulated in alginate hydrogel, Real time PCR was performed three times. Generally, there are several ways to do quantified PCR using RT-Real Time PCR. In this research, gene expression of apoptosis (Bax, Fas, Casspase3, Bcl2, P53) were investigated using DNA-binding agents such as SYBR green. The dye is connected alternatively

in the small gap of DNA. With the increase of double-stranded DNA, the attached SYBR Green is increased and as a result, more fluorescent light is emitted that is measured by the device. Table 2 indicates the comparison of apoptosis gene expression in both control and experiment groups using Real time PCR method.

Table 2.

Bax: The level of Bax gene expression showed a significant difference between the main group and the control group, in the presence of the reference gene (P value = 0.000), the expression level in the main group was lower than the control group (0.114). **Fas:** The expression level of this gene showed a significant difference between the main group and the control group, in the presence of the reference gene (P value = 0.000), the expression level in the main group was higher than the control group (2.464). **Bcl2:** The expression of this gene showed no significant difference in the main group and control group in the presence of the reference gene (P value = 0.341). **P53:** The expression level of this gene showed a significant difference between the main group and the control group, in the presence of the reference gene (P value = 0.000), the main group showed a lower expression level than the control group (0.341). **Caspase3**: The expression of this gene showed no significant difference between the main group and the control group in the presence of a reference gene (P value = 0.169).

Figure 3.

In this graph, as the gene expression level approaches 1, it indicates that there is no significant difference in expression level of the corresponding gene between the test group and the control group.

Electrophoresis

Since, the biological macromolecules such as DNA and proteins can be charged; therefore, they can be separated by placing in an electric field are based on the physical properties such as spatial shape t, molecular weight and electrical charge. For this purpose, a technique called electrophoresis is used. PCR products for each gene were loaded on electrophoresis gel and the image of electrophoresis gel represents the bands relevant to the specific amplification of the desired fragments (fig.4).

Figure 4.

SEM analysis

SEM capture images showed that cell morphology and density, and scaffold structural have preserved after 30 days. Figure 6 showed that SSCs spreading onto the scaffold surface and proliferate in alginate hydrogel during 3D culture (Fig.5).

Figure 5.

Discussion

In this study, the cytotoxic effect of alginate hydrogel on type A spermatogonial stem cells of 6-day-old mice was studied. The results of this study indicated the proper biocompatibility of alginate hydrogels for the cells and that the encapsulated cells were not damaged. By measuring the expression of apoptosis genes (Caspase3, BAX, P53, Bcl2, FAS) in the group of alginate hydrogel encapsulated cells in comparison with the control group, it seemed that alginate hydrogel, with its antioxidant properties, does not induce cell death and cellular damage. SEM micrographs showed that cell morphology and spreading and proliferation preserved. Given that half of the medical problems of infertile couples is related to male factors, and to improve the level of life expectancy after treatment for cancer patients, especially patients below the age of

puberty, and since using cryopreserved sperm cells is not applicable, taking advantage of one's own spermatogonial stem cell transplantation, in order to restore fertility is very promising. In recent years, much attention has been focused on converging scaffolds and cells in the field of tissue repair, in order to simulate the biological and physical properties of natural tissues of the body. Alginate hydrogel is one of the biological scaffolds that can be used in the field of tissue engineering. Its hydrated three-dimensional network allows cell adhesion, distribution, migration and interaction with other cells. This advantage makes the hydrogel a good option for cultivation and differentiation of cells in three-dimensional environment [16, 17]. Spermatogonial stem cells were isolated and purified for the first time in 1977 by Bilway et al from 6-day old mice by mechanical separation from tubule and enzymatic digestion (using collagenase enzymes, trypsin and hyaluronidase). They obtained a cell suspension containing 90% spermatogonial stem cells [18]. Suitable characteristics of alginate such as biodegradability, bioactivity, appropriate porosity, nutrients release and oxygen release, increases ECM production by cultured cells on the scaffold [19]. Stevens et al, by culturing N.P. cells (The central part of the intervertebral disc) on alginate scaffold reported that the alginate scaffold supported further proliferation of N.P. cells and increased secretion of ECM by these cells [16]. Some studies have also shown that cells isolated from human and rabbit intervertebral discs, secreted more collagen type 2, aggrecan and glycosaminiglycans after culturing on alginate scaffold [20]. With regard to the viability and survival of germ cells in this study, after thirty days from their encapsulation, an average of 74.08% significance level ($p < 0.001$) was observed. This result is similar to the study of Wang et al. [21]; they showed that cells in alginate encapsulation process have no effect on the survival of embryonic stem cells. After 10 days of encapsulation of cell, viability still remains at a high level and was about 72%. In addition, by measuring lactate dehydrogenase enzyme which is secreted

from the membrane of damaged cell; three times, cytotoxicity was calculated as 5% using the formulas included in the kit. The low level of cytotoxicity represents a low cell membrane damage of spermatogonial cells by environmental factors[22].reported that in spermatozoids with excellent quality which could be frozen and thawed, the enzymatic activity of GOT, ALT and LDH is low [23]. In a study carried out in 2013 on the use of alginate capsules as a three-dimensional scaffold for the differentiation of Wharton's jelly mesenchymal stem cell to definitive endoderm, it was demonstrated that alginate is a suitable non-fatal composition for encapsulating Wharton's Jelly mesenchymal stem cells [19]. Almqvist et al [23] showed that chondrocytes encapsulated in alginate incur less damage during freezing. Massie et al [22] showed that alginate reduces the toxic effects of freezing material during freezing of hepatocytes.

Apoptosis is a form of programmed cell death that has its own biochemical and morphological characteristics. Induction and occurrence of apoptotic events is promoted by several signaling pathways. Two major pathways are intrinsic or mitochondrial pathway (by Bax, Bcl2, P53, Caspase3, etc. genes) and the extrinsic pathway or pathway of death receptor on cell membrane (by Fas, Fas-L, etc. genes) [24, 25]. From the results of the present study, a comparison of cells encapsulated in alginate hydrogels after one month with the freshly isolated cells, shows that encapsulated cells decreased the expression of Bax and P53 and the expression of Bcl2 and Caspase3 had no significant difference, but the expression of Fas was increased. Investigations have shown that the alginate prevents cell death by preventing oxidation through its anti-apoptotic properties. The results of studies by Kostski et al (2009), Lu et al (2008), Chidamanduih et al (2007) indicate the antioxidant effect of alginate coating [26]. A research by Toosi et al (2011) showed that alginate prevents neuronal cell death by blocking the formation of free radicals [27]. Encapsulation of cells in alginate microcapsules for a long time makes them

non-permeable and reduces insulin release and cell death [28]. Encapsulation of stem cells allows cells to sense the external environment and release small proteins such as growth factors and does not allow large proteins such as antibodies to enter the cell. With regard to the gene expression levels of apoptosis genes in cells encapsulated in alginate, it seems that alginate mostly inhibits the mitochondrial apoptosis pathway and provides a higher biocompatibility through the proper distribution of oxygen and other nutrients.

Conclusion

It seems that alginate capsules, by providing inward flow of adequate amounts of nutrients and oxygen and outward flow of cellular metabolites, does not interfere in cell viability. This is particularly true about the cells in the center and periphery of the capsules. In addition, it can be concluded that the small size of the capsules plays a role in two-way transmission of compounds.

The study showed that alginate is a perfect and non-toxic compound to encapsulate spermatogonial stem cells, and this compound does not affect the viability and morphology of stem cells.

It seems that all of these are due to the chemical properties of alginate hydrogels. Non-adhesive nature of alginate supports cell-cell interaction that is important for maintaining cell survival and improving cell function characteristics. Since the three-dimensional hydrogel networks are very hydrated, they provide a structure similar to the extracellular matrix. Besides, gelation and cross-linking processes do not damage the cells. High hydrophilicity of alginate facilitates the distribution of nutrient to the structure which enhances cell survival and ion and other nutrients exchange by cells to the culture medium [29]. Alginate hydrogels are highly porous structures which facilitate macromolecules distribution and its preparation as a scaffold does not need toxic

activators or alteration of temperature. It seems alginate provides an environment that promotes cellular activity and metabolic pH [15]. The encapsulation of stem cells allows cells to sense the external environment and to retain small proteins such as growth factors while large proteins such as antibodies cannot pass through the capsule. The best defense mechanism against free radicals and apoptosis is through antioxidant defense [30].

Due to the antioxidant properties of alginate, it has no cytotoxic effect on these cells, and does not lead the cells to apoptosis.

It seems that alginate by inhibiting mitochondrial apoptosis pathway and preventing the activation of the cytochrome c and its release from mitochondria prevents apoptotic genes expression and cellular destruction [31].

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Table 1. Forward and reverse primers of apoptosis genes and reference genes for RT-PCR

Gene	Primer sequences (5´-3´)	Size (bp)	Reference Gene bank
P_{53}	**F:5´ GTTTCCTCTTGGGCTTAGGG 3´ R:5´ CTTCTGTACGGCGGTCTCTC 3´**	**255**	**NM 011 640**
Caspase3	**F:5´ CAGCACCTGGTTACTATTCCT 3´ R:5´ GTTAACGCGAGTGAGAATGTG 3´**	**125**	**NM 004 346**
BAX	**F:5´ CGAGCTGATCAGAACCATCA 3´ R:5´ GAAAAATGCCTTTCCCCTTC 3´**	**277**	**NM 007 527**
FAS	**F:5´ GAGAATTGCTGAAGACATGACAATCC 3´ R:5´ GTAGTTTTCACTCCAGACATTGTCC 3´**	**314**	**NM 004 104**
BCL2	**F:5´ TAAGCTGTCACAGAGGGGCT 3´ R:5´ TGAAGAGTTCCTCCACCACC 3´**	**344**	**NM 007 741**
GAPDH	**F:5´ CAATGTGTCCGTCGTGGATCT3´ F:5´ GTCCTCAGTGTAGCCCAAGATG3´**	**208**	**NM 008 084**

Gene expression level was analyzed by Rotor gene Q and Rest 2009 software.

Table 2. Apoptosis genes: a comparison between the control group (freshly isolated) and cells encapsulated in alginate hydrogels (test group)

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
GAPDH	**REF**	**0.7725**	**1.000**				
BAX	**TRG**	**0.7575**	**0.114**	**0.111 - 0.118**	**0.111 - 0.118**	**0.000**	**DOWN**
FAS	**TRG**	**0.795**	**2.464**	**2.072 - 2.952**	**1.928 - 3.156**	**0.000**	**UP**
BCL2	**TRG**	**0.825**	**0.931**	**0.847 - 1.023**	**0.831 - 1.043**	**0.341**	
P53	**TRG**	**0.785**	**0.341**	**0.287 - 0.407**	**0.280 - 0.417**	**0.000**	**DOWN**
CASPASE3	**TRG**	**0.8225**	**0.511**	**0.469 - 0.557**	**0.459 - 0.569**	**0.169**	

REF: reference, TRG: target.

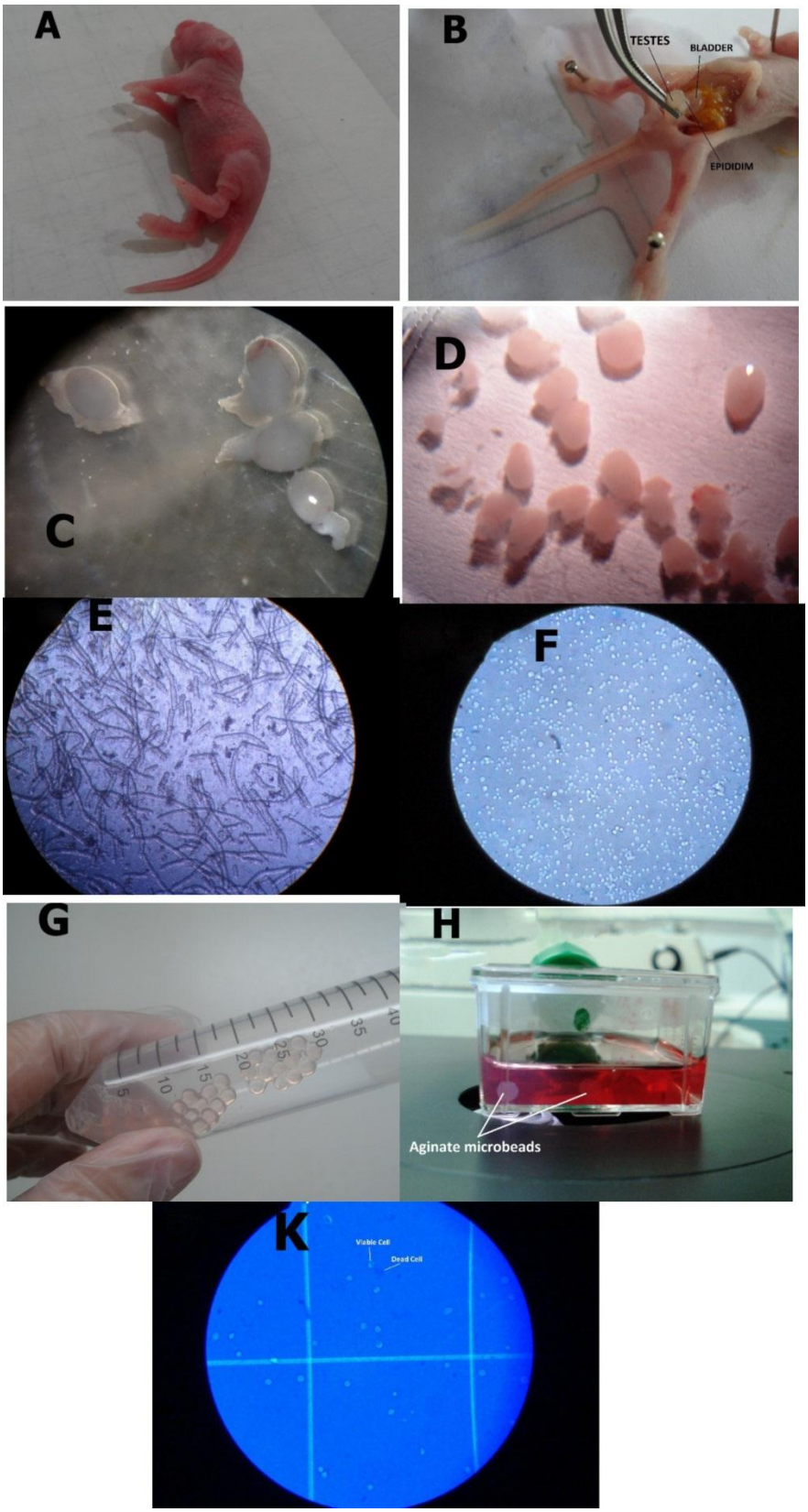

Figure 1. A; 6 day-old neonatal mice, B; Testis harvested from mice, C; testis with tunica albuginea and epididymis floated in PBS buffer, D; The tunica albuginea and epididymis were completely removed under stereo microscope, E; Seminiferous tubuls after testes digested with 2 μg/ml collagenase IV (Sigma) and 5 μg/ml DNAse I (Sigma) and incubated for 15 min at 37 ° C and 5% CO_2, F; SSCs after enzymatic digestion using trypsin EDTA at 100x magnification, G; SSCs encapsulated alginate microbieds, 3D culture of encapsulated, K; viable SSCs.

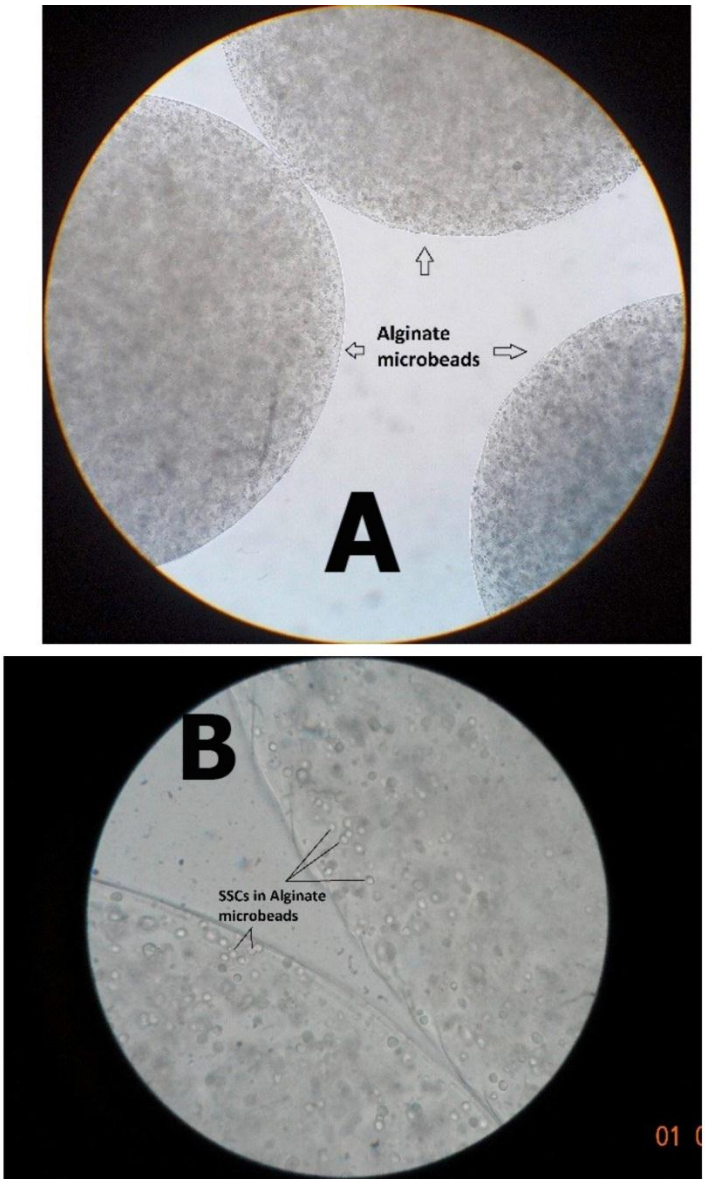

Figure 2. A; Microscopic image of alginate capsules containing spermatogonial stem cells (40x magnification): spherical capsules with a uniform margin. B; Microscopic image of cells encapsulated in alginate at 100x magnification: distribution of spermatogonial stem cells in alginate capsules' three-dimensional environment, cells are circular while not binding to the surface and all have the same morphology

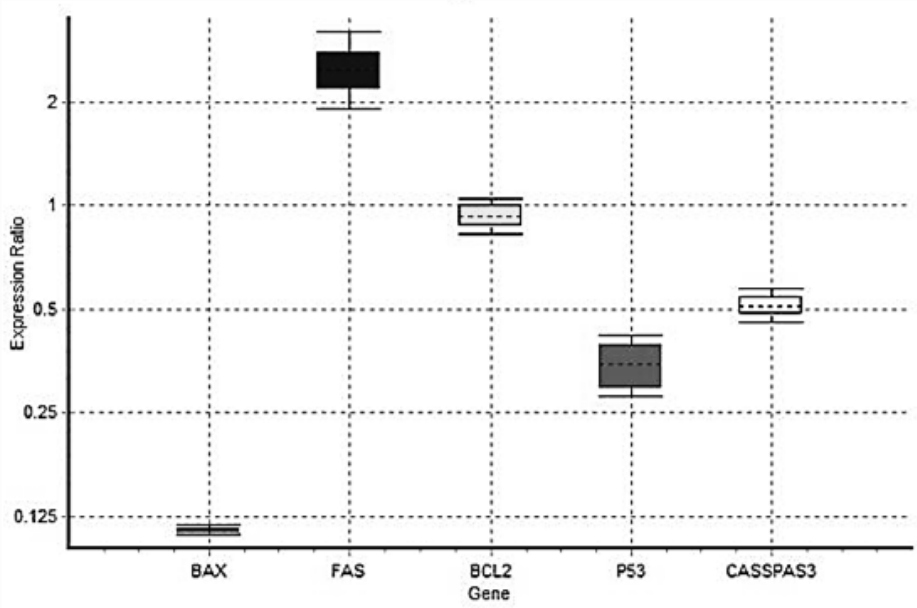

Figure 3. The diagram shows the average ratio of apoptotic gene expression, dotted lines represent the average gene expression and continuous lines above and below the diagram represents the maximum and minimum gene expression observed.

Figure 4. Electrophoresis of RT-PCR products of apoptotic genes Bax, Fas, Bcl2, Caspase3 and P53 on 1.5% agarose gel.

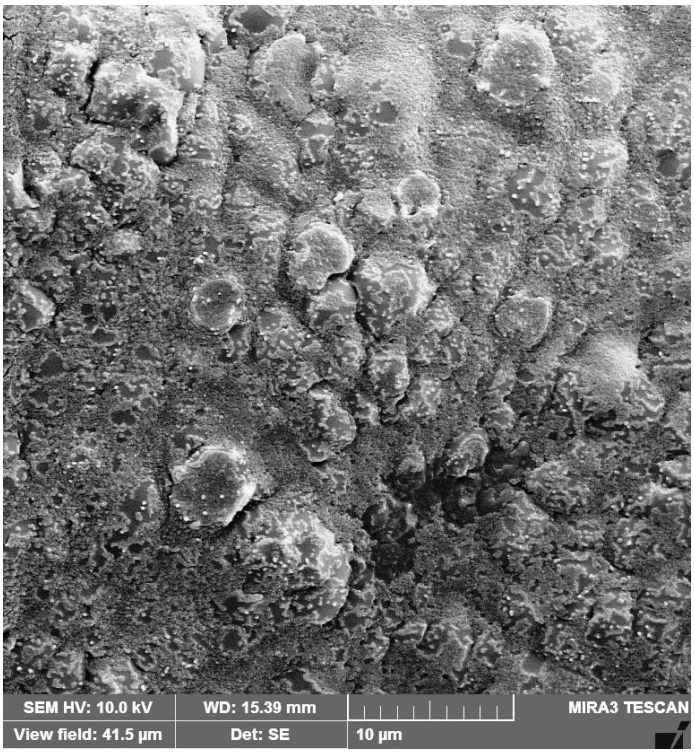

Figure 5. Micrographs obtained by SEM after one month 3D culture. Spherical shaped SSCs can be seen attached to the surface of hydrogel scaffold one month after culture.