

ORIGINAL ARTICLE

Characterization of embryonic stem-like cells derived from mouse spermatogonial stem cells following low-intensity ultrasound treatment



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Received 26 December 2019; accepted 26 May 2020

Available online 23 December 2020

KEYWORDS

Spermatogonial stem cells;
ES-like cells;
Low-intensity ultrasound;
Mouse

Abstract

Objective: Spermatogonial stem cells (SSCs) are able to form embryonic stem-like cells (ES-like cells) and embryonic bodies (EBs). Low-intensity ultrasound stimulation (LIUS) has positive effects on the growth and differentiation of the different cells. In this study, we tried to investigate the effects of LIUS on SSC differentiation to ES-like cells.

Materials and methods: SSCs were isolated from neonatal mice and their identification was confirmed by tracking of PLZF, Oct-4, and C-Kit proteins. The SSCs and Sertoli cells were co-cultured in DMEM/F12 supplemented with 15% FBS and LIF. SSCs stimulated by LIUS with 200 mW/cm² intensity. Characterization of obtained ES-like cells was confirmed with Sox2, Oct-4, and SSEA-1 immunofluorescence staining. Also, real-time PCR was performed to analyse the expression of c-Myc and Nanog genes in ES-Like Cells and Stra8, Piwil2 and Plzf genes in SSCs after 21 days of the in vitro culture.

Results: Our results showed c-Kit, PLZF and Oct-4 proteins were expressed positively in SSCs and Sox2, Oct-4, SSEA-1 in the ES-like cells by immunocytochemistry. The results of flow cytometry showed a significant increase in expression of c-Myc and Nanog in ES-like cells compared to SSCs ($p < .05$), whereas the Stra8, Piwil2, and Plzf became down-regulated during 21 days of culture. ES-like markers cell SSEA-1, Sox2 and Oct-4 were increased in the LIUS group compared to the control group ($p < .05$).

Conclusion: The results indicated that ES-like cells with pluripotency characteristics were derived from SSCs.

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PALABRAS CLAVE

Células madre
espermatogoniales;
Células similares a
ES;
Ultrasonido de baja
intensidad;
Ratón

Caracterización de las células madre embrionarias derivadas de células madre espermatogoniales de ratón, después del tratamiento con ultrasonido de baja intensidad

Resumen

Objetivo: Las células madre espermatogoniales (SSC) pueden formar células embrionarias de tipo madre (células similares a ES) y estructura de cuerpos embrionarios (EB). La estimulación por ultrasonido de baja intensidad (LIUS) tiene efectos positivos sobre el crecimiento y la diferenciación de las diversas células. En este estudio, tratamos de investigar los efectos de LIUS en la colonización y la viabilidad de células similares a ES.

Materiales y métodos: Las SSC se aislaron de ratones neonatales y su identificación se confirmó mediante el seguimiento de las proteínas PLZF, Oct-4 y c-kit. Las SSC y las células de Sertoli se cultivaron conjuntamente en DMEM/F12 suplementado con 15% de FBS y LIF. Las SSC estimuladas por LIUS continúan con una intensidad de 200 mW/cm². La caracterización de las células similares a ES obtenidas, se confirmó con tinción con inmunofluorescencia Sox2, Oct-4 y SSEA-1. Además, se realizó una PCR en tiempo real para analizar la expresión de los genes *c-Myc* y *Nanog* en células similares a ES y genes *Stra8*, *Piwi12* y *Plzf* en SSC, después de 21 días de cultivo *in vitro*.

Resultados: Nuestros resultados mostraron que las proteínas c-kit, PLZF y Oct-4 se expresaron positivamente en SSC y Sox2, Oct-4, SSEA-1, en las células similares a ES por inmunocitoquímica. Los resultados de la citometría de flujo mostraron un aumento significativo en la expresión de *c-Myc* y *Nanog* en células similares a ES, en comparación con las SSC ($p < 0,05$), mientras que *Stra8*, *Piwi12* y *Plzf* disminuyeron en 21 días. Las células marcadoras similares a ES, SSEA-1, Sox2 y Oct-4 se incrementaron en el grupo LIUS, en comparación con el grupo control ($p < 0,05$).

Conclusión: Los resultados indicaron que las células similares a ES, con características de pluripotencia, se derivaron de las SSC.

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Introduction

SSCs (spermatogonial stem cells) are the base of sperm production and male fertility. Similar to other stem cells, SSCs are rare in testis tissue, representing only 0.03% of all germ cells in rodent testis.¹ Neonatal and adult mice SSCs are able to develop into multipotent cells when cultured under specific conditions *in vitro*.^{2–5} In addition, the efficient and long-term culture of SSCs is now possible allowing sufficient cells for regenerative therapy to be generated.⁶ Recently, some groups of researchers have independently found successfully formation of ES-like cells (embryonic stem-like cells) by exposing human testicular cells to specific ES conditions *in vitro*.^{7–9} Another study shows that ES-like cells derived from testis are capable to differentiate into neuroepithelial like cells that may provide a cellular reservoir usable for neurodegenerative disorders.¹⁰ Pluripotency potential of these ES-like cells has been demonstrated by their capacity to contribute to germ-line chimeras and differentiation into tissues that belong to all three embryonic germ layers. ES-like cells express a blend of germ line and embryonic stem cell markers.^{2,3,11,12} Recent studies show that new methods such as mechanical waves, especially LIUS (low-intensity ultrasound stimulation) have an influence on the proliferation and colony formation of cells. Now, the positive effect of these waves enhances growth, proliferation, differentiation and etc. These waves

increase the proliferation of human umbilical cord-derived mesenchyme stem cells,¹³ hematopoietic stem cells¹⁴ and Adipose-derived stem cells.¹⁵ Also, these waves increase the proliferation of chondrocytes^{16,17} and fibroblast.¹⁸ Another study shows that The LIUS of mouse SSCs increased of Integrin- $\alpha 6$ and Integrin- $\beta 1$ gene expressions after Continuous and pulsed LIUS.¹⁹ In relation to previous results showing the pluripotency of ES like cells derived from testis, the aim of this study was to evaluate the differentiation capacity and alteration in gene expression patterns during the *in vitro* differentiation of mouse SSCs into ES like-cells following exposure to LIUS.

Materials and methods**Generation and culture of ES-like cells**

In this experimental study, ES-like cells were generated from 3 to 5 days neonatal mouse testis.²⁰ All of procedures were according to animal ethical clearance of Tarbiat Modares University. Both testes of neonatal mouse were removed and were decapsulated. After mechanical dissection, testes were done under two-step enzymatic digestion with DMEM/F12 (Pan-Biotech, UK) which contained; 0.5 mg/ml Collagenase-Dispase (Sigma, USA), 0.5 mg/ml Trypsin (Sigma, USA), and 0.05 mg/ml DNase (Sigma, USA) at 37 °C for 30–45 min. The obtained

dissociated cells that included SSCs and Sertoli cells were incubated in differential plate method for SCs (Spermatogonial Cells) purification. After SCs purification, these SCs were co-cultured with sertoli cells at 34°C and 5% CO₂, and in a humidified atmosphere, in DMEM/F12 (Pan-Biotech, UK) supplemented with 15% FBS (Gibco, UK), 1mM L-glutamine (Gibco, UK), 0.1mM Nonessential Amino Acids (Gibco, UK), 0.1mM β mercaptoethanol (Sigma-Aldrich), and 1000 units/ml LIF (Sigma-Aldrich).^{10,20,21}

Ultrasound stimulation design

Our ultrasound device (PHYSIOMED, Germany) was designed with the following parameters: frequency: 1 MHz, intensity: 200 mW/cm², Time: 200 s, duration: 5 days. These design parameters were chosen based on the previous biological and clinical studies.^{14,19} LIUS applied by a transducer to SSCs cultured in an enclosed sterile conventional 3.5 cm tissue culture plate in an incubator with 32°C temperature and 5% CO₂. It was transmitted through the bottom of the well via coupling gel between the transducer and the Plate.¹⁹ After LIUS stimulation, the cells cultured for 21 days. To investigate the proliferation rate, the mean number of whole cells per volume on day 7th was considered. The colonization of ES-like cells was assessed on 7th day with number and diameter of colonies by Invert-phase microscope (Zeiss, Germany) equipped with ocular grid.

Quantitative real-time polymerase chain reaction (RT-qPCR)

To confirm generation of ES-like cells, Real-Time PCR was performed to analyze the expression of a subset of pluripotency markers as well as germ cell-specific genes. *Nanog* and *C-Myc* were assessed as pluripotency genes in ES-like cells and *Stra8*, *PLZF*, and *Piwill2* were assessed in SSCs as stem cell markers on day 21. Total RNA was extracted from the cells using RNX Plus™ (Cinnagen, Iran) according to the manufacturer's recommendations. In order to remove genomic contamination, RNA was treated with DNase I using a kit from Fermentas (Fermentas, Lithuania). Concentrations of RNA were determined using UV spectrophotometer (DPI-1, Qiagen). The cDNAs were synthesized from 500 ng DNase treated RNA samples with a Revert Aid™ first-strand cDNA synthesis kit (Fermentas, Lithuania) using oligo dT primers. For PCRs, primers were adapted from others²¹⁻²⁵ and synthesized by Cinnagen Company (Iran). PCRs were carried out using Master Mix (Cinnagen, Iran) and SYBR Green I (Fluka, Switzerland) in a Rotor-Gene 3000 thermo cycler (Corbett, Australia). The PCR program started with an initial melting cycle, 4 min at 94°C, to activate the polymerase and followed by 40 cycles as follows: a melting step (20 s at 94°C), an annealing step (30 s at 57°C), and an extension step (30 s at 72°C). After completing the PCR run, the quality of the reactions was confirmed by melting curve analyses. Efficiency was determined for each gene using a standard curve (the logarithmic dilution series of testis cDNA). For

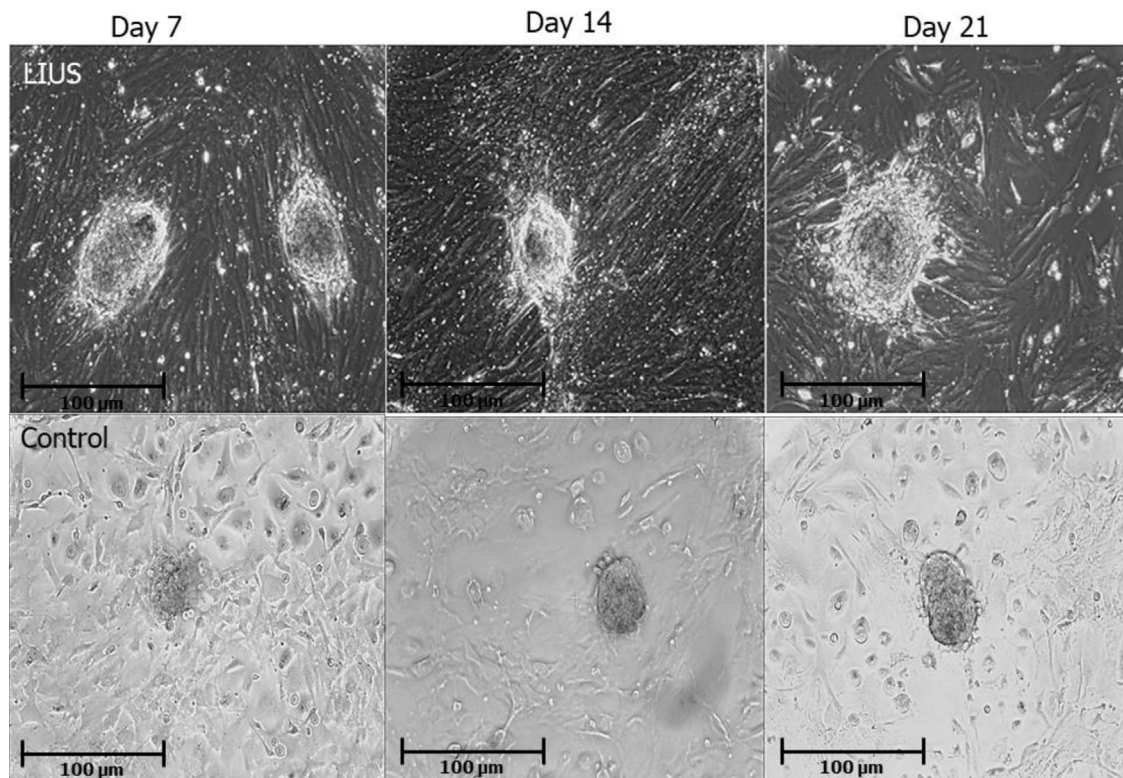


Figure 1 SSCs were culture on the sertoli cells feeder layer in two conditions: LIUS treatment (upper row) and control (without LIUS treatment) (lower row). The morphology of ES-Like cells colonies derived from SSCs On in LIUS group were more shaped and expanded with sharp edge, which is not similar to control group.

each sample, the reference gene (b2M) and the target gene were amplified in the same run. Ratio of gene expression was determined using the Comparative CT (cycle threshold) method.

Immunocytochemistry analysis

For immunocytochemistry, cells in each group were washed with phosphate-buffered saline (PBS) at PH 7.4 and fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature (RT). Fixed cells were permeabilized with 0.2% Triton X-100 (MP Biomedicals, USA) for 10 min at RT followed by 3 times washing with PBS. To block unspecific binding of the antibody, cells were incubated with 10% goat serum

(Vector Laboratories, USA) for 30 min at RT. Then, cells were incubated with primary antibodies overnight at 4°C. Primary antibodies including: Rabbit Polyclonal antibody Oct-4, SSEA-1, Sox2; the following day, cells were washed twice with PBS and incubated with the appropriate secondary antibody. Antigens were visualized using appropriate fluorochrome-conjugated secondary antibodies including: PE: Donkey to Rabbit IgG (200:1; Abcam) and FITC: Goat anti Rabbit IgG (200:1; Abcam). After two times washing with PBS for 5 min, cells were mounted with 4, 6-diamidino 2-phenylindole (DAPI)/PBS and Images were captured with an Olympus phase contrast microscope (BX51, Olympus, Tokyo, Japan).

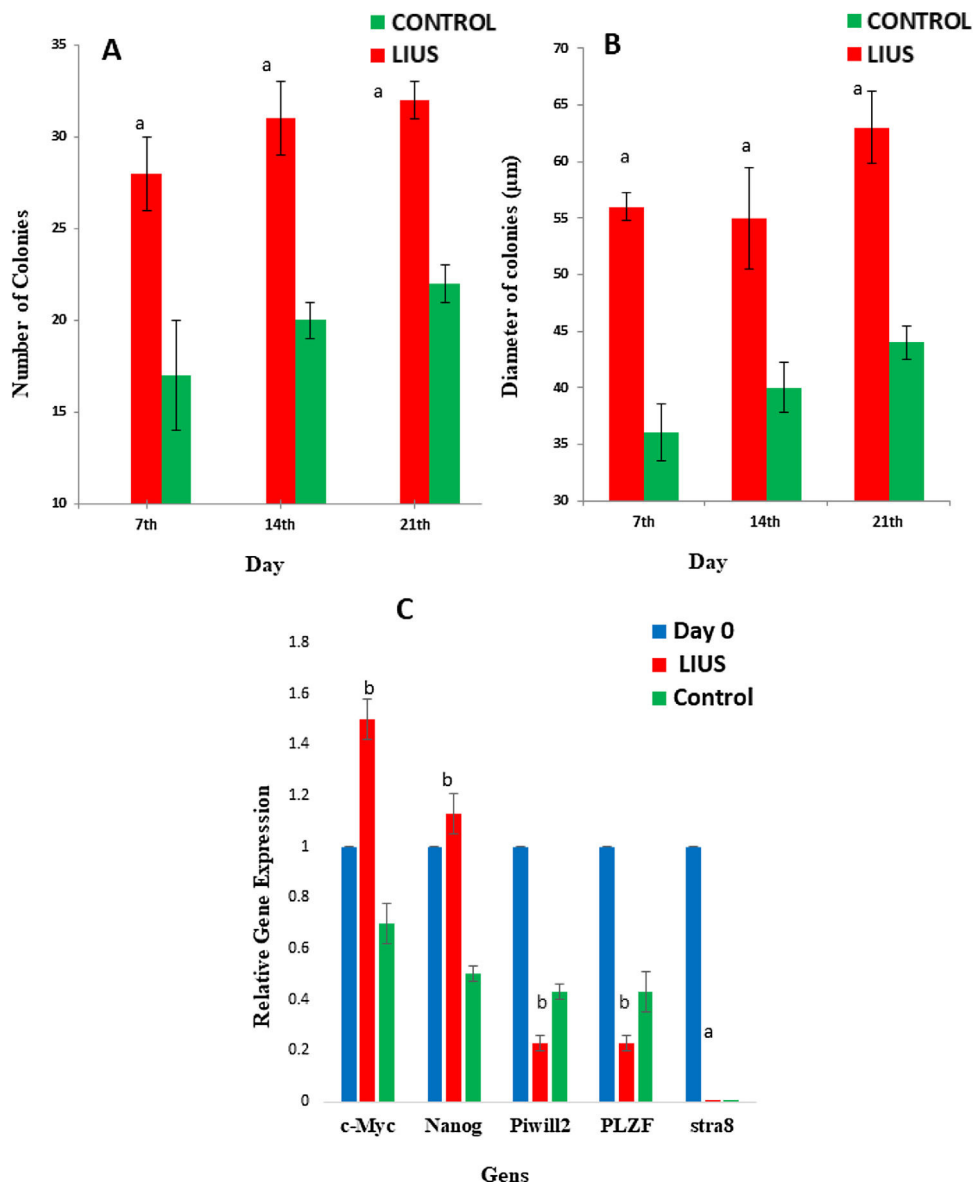


Figure 2 Evaluation of colonization in LIUS and control groups in different days includes the number of colonies (A) and the diameter of colonies (B) was showed. And (C) is the ratio of pluripotency genes expression: *Nanog*, *C-Myc*, and spermatogonial genes: *Stra-8*, *Plzf*, and *Piwill2* in ES-Like cells in LIUS and control groups on day 21st. Relative expression was shown as the ratio of target gene to 0th day. (a) Significant differences compared to control group ($P < 0.05$). (b) Significant differences compared to control group and day 0 ($P < 0.05$).

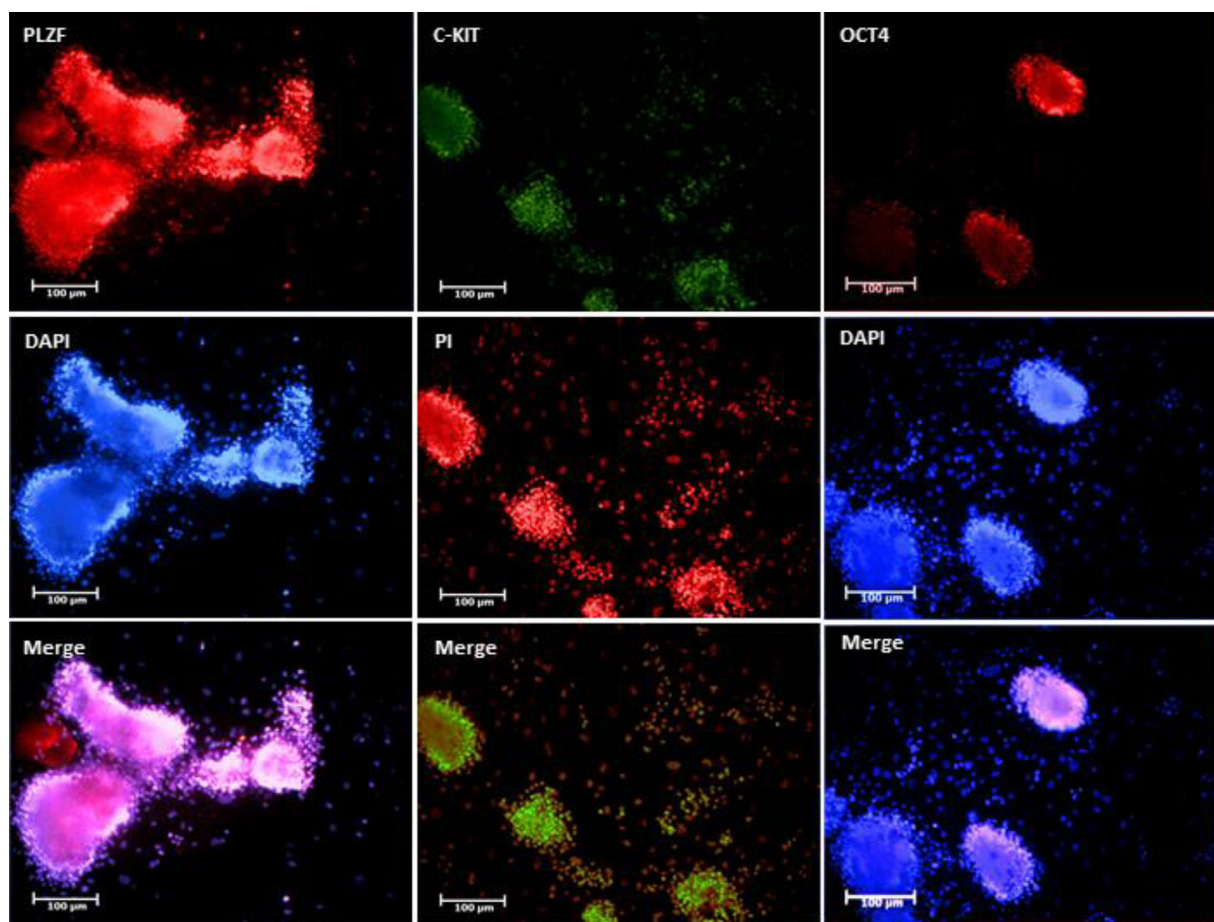


Figure 3 Identification of isolated SSCs was confirmed by Immunofluorescence staining of PLZF, C-KIT and OCT-4 proteins in Colonies derived from SSCs culture on day 7th. The most of SSCs derived colonies have positive reaction to PLZF, C.KIT and OCT-4 anti-bodies.

Flow cytometry

Cells were washed with PBS and treated with trypsin/EDTA (Sigma, USA) for 5 min to form single cells. The suspension collected by centrifugation at 2000 RPM for 5 min, then were fixed in 4% PFA for 10–15 min at 4°C for stabilizing proteins, followed by permeabilizing of cells in detergent (0.2% Triton X-100). Fixation/permeabilization procedures had to be on ice. Then, it was washed by adding 2 ml of PBS, and centrifuged at 2000 RPM for 5 min. The supernatant was discarded and the cells were suspended in goat serum for 45 min to block nonspecific antibody binding. Cells were labeled with primary antibodies overnight at 4°C in dark room. Antibodies including: Rabbit Poly Clonal Antibody Oct-4, SSEA-1, And Sox-2: (1:50; Abcam) and Rabbit Poly Clonal Antibody Oct-4, PLZF, C-Kit: (1:50; Abcam). Then they were washed three times by PBS. The following day, cells were incubated with the appropriate secondary antibody and antigens were visualized using appropriate fluorochrome-conjugated secondary antibodies included: PE, Donkey to Rabbit IgG (200:1; Abcam) and FITC, Goat anti Rabbit IgG (200:1; Abcam). This incubation had to be done in dark, followed by three times washing by PBS. Analysis was performed as soon as possible using PARTEC

CyFlow space flowcytometer (GERMANY) and FlowJo software (WinMDI 2.9, J. Trotter).

Data analysis

Each data point represents the average of three separate experiments with three repeats in each experiment. The one-way ANOVA and Tukey post hoc tests used to determine the statistical significance of observed differences in the mean values among our groups using the SPSS statistical software (SPSS 16.0 Production Mode Facility). P value less than 0.05 was considered significance.

Ethical consideration

Current study was conducted under the protocol approved by the animal experimentation committee of Medical Sciences Faculty in Tarbiat Modares University.

Results

After purification of SSCs and sertoli cells, we culture these cells as co-culture. Most of sertoli cells were attached to

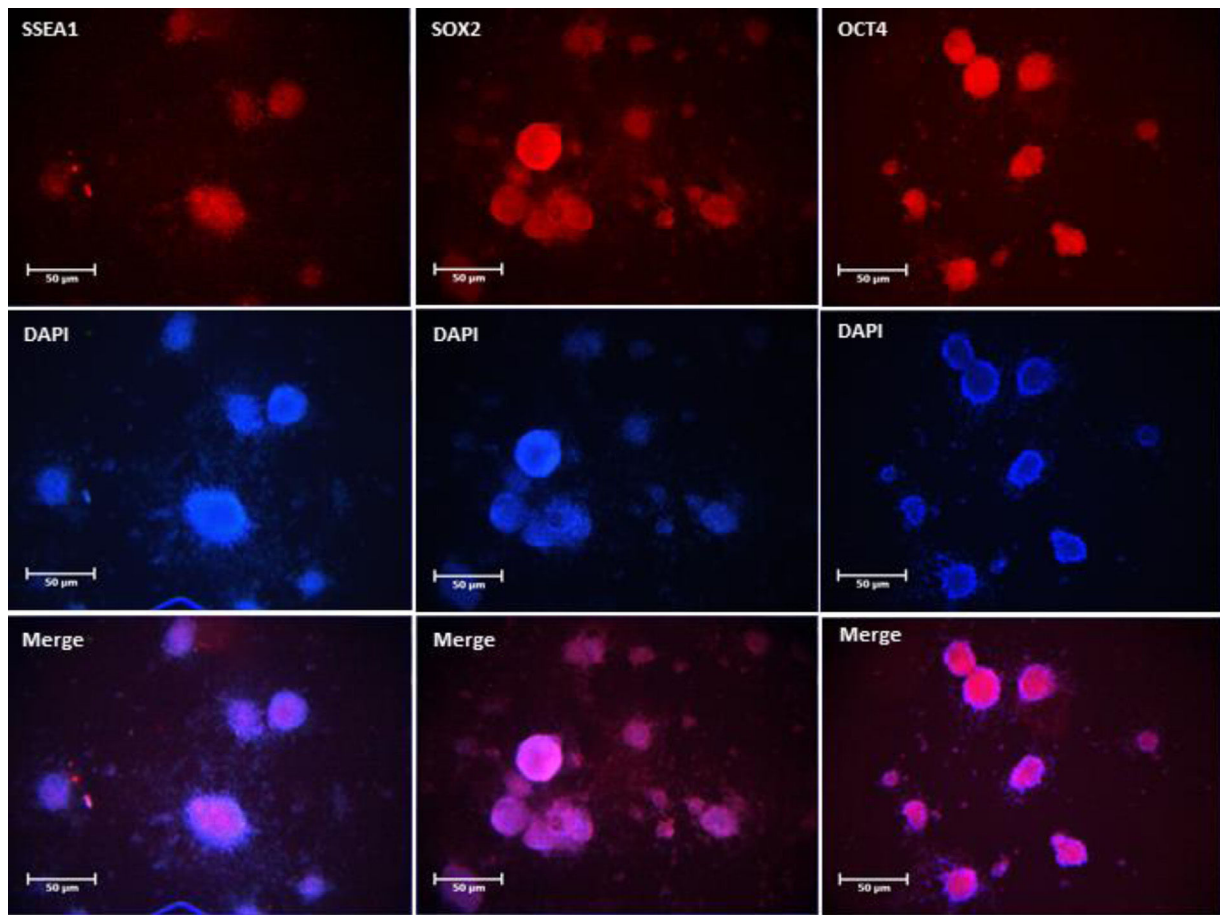


Figure 4 Immunofluorescence staining of ES-Like cell colonies derived from SSCs treated by LIUS for pluripotency markers SSEA1, SOX2 and OCT4 on day 21st.

the growing surface and SSCs were attached in the sertoli cells 48 hr of post plating. These cells proliferated and created a monolayer of cells. Our result showed after several days, SSCs formed colonies and these colonies were passaged every 7 days (Fig. 1). After ultrasound stimulation, ES-like colonies with sharp edge that resembled ES-cell appeared within 3 weeks (at passages 3rd). ES-like cells were confirmed by immunofluorescent & Immunocytochemistry staining and quantitative real-time q-PCR analysis.

On days 7th, 14th, 21st the findings showed that the colonization increased in LIUS groups compared to control (without LIUS treatment) groups ($P < 0.05$). The average number of colonies in 7th, 14th, 21st days in LIUS group was 28 ± 3 , 3 ± 1 , 32 ± 1 and in control group were 17 ± 2 , 202 , 221 , respectively. Regarding the diameter of colonies, there were significant differences between groups ($P < 0.05$) on days 7th, 14th, 21st (Fig. 2 A). Also, the average diameters of colonies in LIUS groups were $56 \pm 1/2$, $55 \pm 4/5$, $63 \pm 3/2$ and in control group were $36 \pm 2/5$, $40 \pm 2/2$, $44 \pm 1/5$ (Fig. 2 B).

Immunofluorescent staining

Isolated SSCs were positive to PLZF, Oct-4 and c-Kit protein detecting respectively (Fig. 3). The obtained ES-like cell colony expressed the SSEA-1, Sox2 and Oct 4 marker (Fig. 4). On day 21st the findings showed that the expres-

sion of a subset of pluripotency markers increased in LIUS group compared to control groups ($P < 0.05$) (Fig. 5). Result showed that in control group ($54/66 \pm 4/4$) % of ES-like cells were positive to Oct4 and ($72/92 \pm 5/4$)% in LIUS groups. Also, ($46/60 \pm 3/9$)% of ES-like cells were positive for sox2 in control group and ($52/92 \pm 4/4$)% were positive in LIUS group. Furthermore, ($53/95 \pm 4/3$)% of ES-like cells had positive reaction for SSEA-1 in control groups and ($58/71 \pm 5/4$) % in LIUS groups (Fig. 5). The whole results of flowcytometry were presented in Fig. 5.

Results of real-time RT-PCR

These results demonstrated that the expression of pluripotency genes *Nanog*, *C-Myc*, *Plzf* and *Piwill2* had significantly increased in LIUS group's compared to control groups on day 21st ($P < 0.05$). The expression of *Stra8*, gene had no significant differences between groups ($P > 0.05$) (Fig. 2D).

Discussion

New researches have shown the derivation of pluripotent stem cells from neonatal and adult mouse testis that can differentiate to all cell types.^{22–24} These studies proved that ES-like cells were generated without genetic manipulation but under specific culture conditions.^{10,20} Our

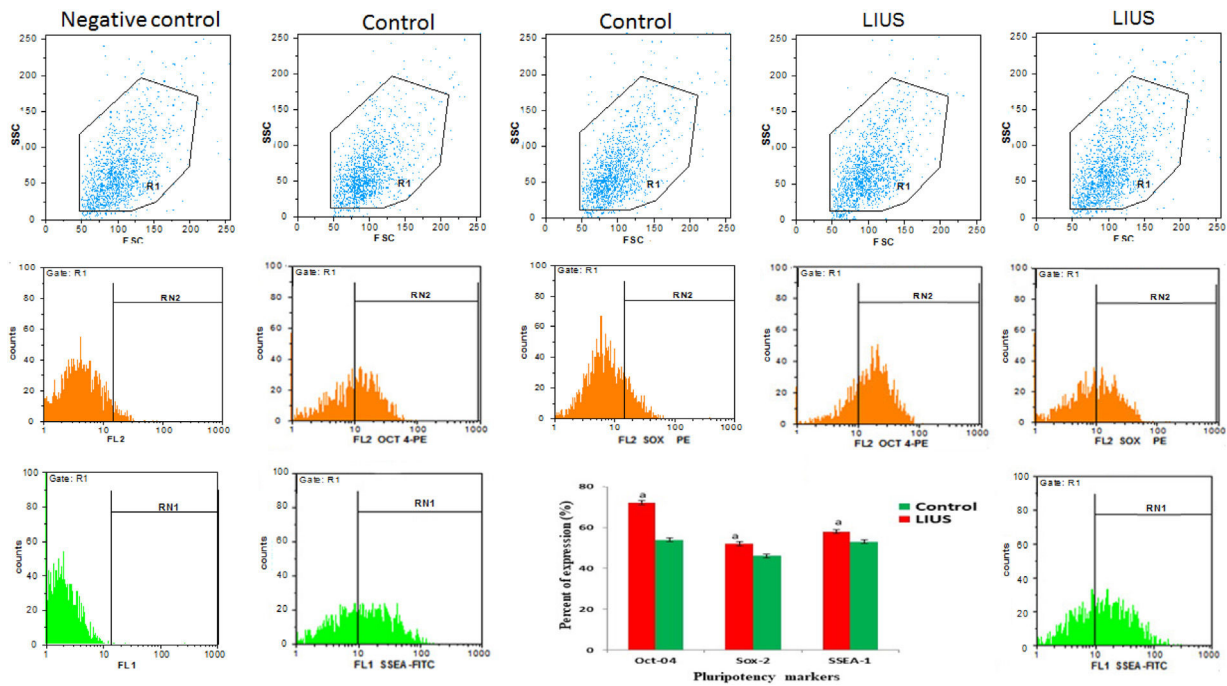


Figure 5 The whole results of Flow Cytometry of ES-Like cells on 21st day for Oct4, Sox2, SSEA-1 markers as pluripotency potential of these cells. The results were shown in both LIUS and control groups. The quantitative results of flow-Cytometry were shown in chart. (a) Significant differences compared to control group ($P < 0.05$).

research demonstrated that ES-like cells originated from SSCs, express high levels of the pluripotency genes *Nanog* and *C-Myc*. Furthermore, germ cell-specific genes such as *Stra8*, *Mvh*, and *Piwill2* are downregulated which confirms previous results.^{2,10,20} Our results showed that ES-like cells derived from SSCs share many molecular and cellular characteristics with embryonic stem cells; on cellular level, these cells have the same morphology with the sharp edged colonies and can form embryonic body structure after transfer to bacteriological Plate.²⁵ In this study, we investigated the effects of LIUS on germ cell specific and pluripotency genes as same as colonization, proliferation and survival rates of cells during 21 days of culture in vitro. Our results showed that SSCs can proliferate and form colonies on the feeder layer of sertoli cells in related to previous studies.^{2,10,20} Consistent with present data, an earlier study demonstrated that stimulating osteoblasts and chondrocytes with LIUS transiently increased the expression of specific integrins, namely $\alpha 5$ and $\beta 1$ integrin²⁶ and $\alpha 6$ and $\beta 1$ integrin.¹⁹ Our findings obtained from in vitro study strongly revealed that LIUS could improve the number of mouse ES-like cells and their colonies. While viability rates in LIUS treatment decreased in compared to control group ($P < 0.05$). Mohaqiq et al (2018) stimulated mouse SSCs by LIUS and LIUPS (Low Intensity Ultrasound Pulsed Stimulation) and reported that the proliferation and colonization rates were increased during 21 days of culture in vitro. Integrin family proteins provide a link between extra cellular matrix and intracellular cytoskeletal components as well as actin filaments. Integrin proteins were thought to have function by undergoing conformational changes that activate them and reveal their ligand binding site. Integrins can bind to cytoskeletal components and other signaling

molecules, while they activate several intracellular signaling pathways in response to mechanical stress such as sound waves, thus enabling the cells to react to changes in their physical environment.²⁷ Integrin proteins family act as sensitive mechanoreceptors on the surface of cells. Ultrasound waves produce mechanical stimulation which has been transferred to adherent cells via interactions with the ECM. Increase in integrin expression was observed in the cells after LIUPS treatment. It was shown to activate a number of downstream kinases including focal adhesion kinase (FAK), phosphatidylinositol 3-kinases (PI3K) and mitogens activate protein kinase,²⁸ indicating that LIUS with their effect on transmembrane proteins, such as integrins, might be able to stimulate more SSCs to divide and it can enter these cells to mitotic process through regulation of self-renewal or differentiation pathway. Our results of LIUS effects on ES-like cells showed that these waves had a useful short time effect on proliferation and colonization during 21 days culture.

Conclusion

We have demonstrated a novel continuous LIUS mediated effect on ES-like cells proliferation, colonization and survival rates during 21 days culture. We also concluded that LIUS unregulated ES-like cell genes and proteins expression.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics

committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Funding

This study is sponsored financially by the Tarbiat Modares University.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgment

The present study is part of the MSc thesis of Anatomical Sciences at Tarbiat Modares University, Medical sciences faculty and sponsored by Tarbiat Modares University.

References

1. Tegelenbosch RA, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutat Res.* 1993;290:193–200.
2. Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, et al. Generation of pluripotent stem cells from neonatal mouse testis. *Cell.* 2004;119:1001–12.
3. Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, et al. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature.* 2006;440:1199–203.
4. Seandel M, James D, Shmelkov SV, Falcatori I, Kim J, Chavala S, et al. Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. *Nature.* 2007;449:346–50.
5. Kanatsu-Shinohara M, Lee J, Inoue K, Ogonuki N, Miki H, Toyokuni S, et al. Pluripotency of a single spermatogonial stem cell in mice. *Biol Reprod.* 2008;78:681–7.
6. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, et al. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod.* 2003;69:612–6.
7. Conrad S, Renninger M, Hennenlotter J, Wiesner T, Just L, Bonin M, et al. Generation of pluripotent stem cells from adult human testis. *Nature.* 2008;456:344–9.
8. Golestaneh N, Kokkinaki M, Pant D, Jiang J, DeStefano D, Fernandez-Bueno C, et al. Pluripotent stem cells derived from adult human testes. *Stem Cells Dev.* 2009;18:1115–26.
9. Kossack N, Meneses J, Shefi S, Nguyen HN, Chavez S, Nicholas C, et al. Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. *Stem Cells (Dayton, Ohio).* 2009;27:138–49.
10. Nazm Bojnordi M, Movahedin M, Tiraihi T, Javan M. Alteration in genes expression patterns during in vitro differentiation of mouse spermatogonial cells into neuroepithelial-like cells. *Cytotechnology.* 2013;65:97–104.
11. Izadyar F, Pau F, Marh J, Slepko N, Wang T, Gonzalez R, et al. Generation of multipotent cell lines from a distinct population of male germ line stem cells. *Reproduction (Cambridge, England).* 2008;135:771–84.
12. Glaser T, Opitz T, Kischlat T, Konang R, Sasse P, Fleischmann BK, et al. Adult germ line stem cells as a source of functional neurons and glia. *Stem Cells (Dayton, Ohio).* 2008;26:2434–43.
13. Yoon JH, Roh EY, Shin S, Jung NH, Song EY, Lee DS, et al. Introducing pulsed low-intensity ultrasound to culturing human umbilical cord-derived mesenchymal stem cells. *Biotechnol Lett.* 2009;31:329–35.
14. Xu P, Gul-Uludag H, Ang WT, Yang X, Huang M, Marquez-Curtis L, et al. Low-intensity pulsed ultrasound-mediated stimulation of hematopoietic stem/progenitor cell viability, proliferation and differentiation in vitro. *Biotechnol Lett.* 2012;34:1965–73.
15. Jiang T, Xu T, Gu F, Chen A, Xiao Z, Zhang D. Osteogenic effect of low intensity pulsed ultrasound on rat adipose-derived stem cells in vitro. *J Huazhong Univ Sci Technol Med Sci.* 2012;32:75–81.
16. Zhang ZJ, Huckle J, Francomano CA, Spencer RG. The effects of pulsed low-intensity ultrasound on chondrocyte viability, proliferation, gene expression and matrix production. *Ultrasound Med Biol.* 2003;29:1645–51.
17. Korstjens CM, van der Rijt RH, Albers GH, Semeins CM, Klein-Nulend J. Low-intensity pulsed ultrasound affects human articular chondrocytes in vitro. *Med Biol Eng Comput.* 2008;46:1263–70.
18. Zhou S, Schmelz A, Seufferlein T, Li Y, Zhao J, Bachem MG. Molecular mechanisms of low intensity pulsed ultrasound in human skin fibroblasts. *J Biol Chem.* 2004;279:54463–9.
19. Mohaqiq M, Movahedin M, Mokhtari M, Mazaheri Z. Upregulation of integrin- α 6 and integrin- β 1 gene expressions in mouse spermatogonial stem cells after continues and pulsed low intensity ultrasound stimulation. *Cell J (yakhteh).* 2018;19:76.
20. Asadi MH, Javanmardi S, Movahedin M. Derivation of ES-like cell from neonatal mouse testis cells in autologous sertoli cells co-culture system. *Iran J Reprod Med.* 2014;12:37–46.
21. Mohaqiq M, Movahedin M, Mazaheri Z, Amirjannati N. In vitro transplantation of spermatogonial stem cells isolated from human frozen-thawed testis tissue can induce spermatogenesis under 3-dimensional tissue culture conditions. *Biol Res J.* 2019;52:2–9.
22. Nayernia K. Stem cells derived from testis show promise for treating a wide variety of medical conditions. *Cell Res.* 2007;17:895–7.
23. Mohaqiq M, Movahedin M, Mazaheri Z, Amirjannati N. Successful human spermatogonial stem cells homing in recipient mouse testis after in vitro transplantation and organ culture. *Cell J.* 2019;20:513–20.
24. Mohaqiq M, Movahedin M, Mazaheri Z, Janati NA. Following in vitro spermatogenesis with long-term preserved spermatogonial stem cells. *Pathobiol Res.* 2016;19:1–15.
25. de Rooij DG, Mizrak SC. Deriving multipotent stem cells from mouse spermatogonial stem cells: a new tool for developmental and clinical research. *Development (Camb, Engl).* 2008;135:2207–13.
26. Riester D, Hildmann C, Grunewald S, Beckers T, Schwienhorst A. Factors affecting the substrate specificity of histone deacetylases. *Biochem Biophys Res Commun.* 2007;357:439–45.
27. Henikoff S. Histone modifications: combinatorial complexity or cumulative simplicity? *Proc Natl Acad Sci USA.* 2005;102:5308–9.
28. Hartzog GA, Tamkun JW. A new role for histone tail modifications in transcriptional elongation. *Genes Dev.* 2007;21:3209–13.