Investigation on the Effect of Low Intensity Ultrasound Stimulation on Mouse Spermatogonial Stem Cell Proliferation and Colonization

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ABSTRACT

Introduction: Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis. New procedure such as sound wave especially low intensity ultrasound (LIUS) can be effective on increasing the number of cells. In this study, the effect of LIUS stimulation on mouse SSCs proliferation and colonization was investigated.

Methods: Isolated SSCs from neonatal mice were cultured in DMEM culture medium with 10% Fetal Bovine Serum (FBS). In the first phase of study, temperature controlled by LIUS of plate containing culture medium and in the next phase, SSCs stimulated by LIUS with 3 different Intensity doses (100, 200 and 300 mW/cm2) for 5 days and SSCs proliferation and colonization assessed at day 7.

Results: The LIUS treatment of mouse SSCs increased the proliferation and colonization of SSCs in experimental groups compared to the control group. The average of proliferation in 100, 200, 300 mW/cm2 and control group were 1.96 ± 0.03, 2.26 ± 0.03, 1.73 ± 0.03 and 1.66 ± 0.03, respectively. The average number of colonies in 100, 200,300 mW/cm2 and control group were 45 ± 1.2, 53 ± 2.4, 28 ± 1.2 and 20 ± 0.8, respectively. The average diameters of colonies in 100, 200, 300 mW/cm2 and control group were 235.3 ± 6.8 µm, 204.6 ± 12.3 µm, 203.6 ± 5.6 and 214.3 ± 9.1 µm, respectively. The results showed significant increase in proliferation rate and number of colonies in experimental groups compared to control group ($P<0.05$), whereas there were no significant difference between groups regarding to diameter of colonies.

Conclusion: The results suggested that LIUS treatment can be an efficient and cost-effective method to improve the proliferation and colonization of SSCs during culture.

Key Words:
Proliferation,
Colonization,
Mouse,
Stem Cell,
Ultrasound.

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1. Introduction

Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and male fertility. Similar to other tissue specific stem cells, SSCs are rare, representing only 0.03 percent of all germ cells in rodent testis [1]. This is because SSCs are heavily outnumbered by the differentiating spermatogonia, spermatocytes, spermatids and sperm that they produce. Like all other stem cells, SSCs defined by their ability to balance self-renewing divisions and differentiating divisions. This balance maintains the stem cell pool and meets the proliferative demand of the testis to produce millions of sperm each day [2]. Knowing the spermatogenesis in vitro is very important and can be useful in treatment of infertile men. One way of knowing this process is isolation, culture, reservation and proliferation of spermatogonial cells in vitro. Several studies show that in 40 percent of infertility cases, there are some problems such as azoospermic, oligospermic or decreasing of sperm motility. Oligospermic in some cases is due to the decrease of sperm cells germ or SSCs [3]. Decrease of SSCs viewed in some cases of infertility. Enrichment, proliferation and differentiation of SSCs in vitro, is important and colonization of spermatogonial cells prepare retardants pool of germ cells that it is very important for treatment of infertility, germ cell gene change, cell transfect and differentiation of SSCs in vitro [4]. Recent studies show that new procedures such as sound waves, especially low intensity ultrasound (LIUS) can influence the proliferation and increase the number of cells. Now, positive effect of these waves enhances the growth, proliferation, differentiation and etc. These waves increase the proliferation of human umbilical cord-derived mesenchymal stem cells [5], hematopoietic stem cells [6] and Adipose-derived Stem Cells [7]. Also these waves increase the proliferation of chondrocytes [8 and 9] and fibroblast [10]. However, the effect of LIUS stimulation on SSCs, which have very important function in male fertility, has not yet been explored. In this study, on the effect of LIUS on SSCs proliferation and colonization was investigated.

2. Materials & Methods

Isolation and culture of SSCs: The method of SSCs isolation was according the previous studies [11] modified by Javanmardi et al [12]. Briefly, in each isolating, 8- 12 mouse's testes during 10 min were collected and after once washing in Dulbecco's Minimum Essential Medium (DMEM), placed in new medium. The present study conducted under the protocol approved by the animal experimentation committee of medical sciences faculty of Tarbiat Modares University. After removal of the capsule, testes were cut to smaller pieces and placed in culture medium containing Collagenase IV (0.5 mg/ml, Sigma, USA) and incubated in 37°C for 20 min, then centrifuged for 5 min with 1500 rpm Speed then medium of above on plaque exchanged with PBS and centrifuged 2 times and each time 3 min with 1000 rpm speed. This phase led to removal of interstitial tissue from testis pieces. Then, Trypsin (0.5 mg/ml, Sigma, USA) added to this solution for 2 minutes and centrifuged for 5 min with 1500 rpm speed. Eventually, obtained cells were pooled. Obtained mixture, commonly included two kinds of cells: sertoli and spermatogonial cells.

Immunocytochemistry of PLZF: Using immunocytochemistry, PLZF protein (marker for SSCs) was detected in the spermatogonial cell-derived colonies. The protocol of immunocytochemistry was performed according to the previous study [13]. Briefly, before rinsing with PBS, the cells grown on the glass slides were fixed for 20 minute in 4% paraformaldehyde at room temperature. After permeabilization by 0.2% Triton X-100 (MP Biomedicals, Irvine, California, United State) for 1 hour to facilitate antibody penetration, the slides were washed with PBS supplemented with 0.2% bovine serum albumin. Nonspecific antigens blocked with 10% normal goat serum (Vector Laboratories, INC. Burlingame, California, the United States). Afterwards, the slides incubated overnight at 37º C with a mouse monoclonal anti-PLZF antibody (Santa Cruz Biotechnology, diluted 1:100). After washing the slides with PBS, the second antibody (goat Texas red-conjugated anti mouse IgM, diluted 1:100; Sigma USA) applied for 2 hours at room temperature in the dark.

Ultrasound stimulation design: Our ultrasound device (PHYSIOMED, Germany) was designed with the following parameters: ultrasound frequency = 1 MHz, intensity= 100, 200 and 300 mW/cm2, Time: based on the results of the first step of the study (Temperature Control), Duration: 5 days. These design parameters were chosen based on the previous biological and clinical studies [14 and 15]. Ultrasound stimulation applied by a transducer to spermatogonial cells 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.

Treatment of SSCs: the Cells were maintained in Dulbecco's Minimum Essential Medium (Gibco, UK) supplemented with 10% (v/v) fetal bovine serum (Gib-

co, UK). The cells exposed to LIUS with 100, 200 and 300 mW/cm2 intensities as experimental groups. The control group cultured in 10% FBS in DMEM medium. After different stimulation intensities, SSCs cultured for 7 days. To investigate the proliferation, the mean number of whole cells per volume on day 7th was considered. Obtained colonies from spermatogonial cells were assayed on 7th day with Invert- phase microscope (Zeiss, Germany) equipped with ocular grid.

Temperature control: Already, in the first step of this study, the temperature of plate containing culture medium was controlled by a microtermometer during LIUS stimulation with 100, 200 and 300 mW/cm2 intensities.

Data analysis: The data of proliferation rates and colonization of ultrasound stimulated cells in different intensities are presented as mean ± standard deviation. 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.

3. Results

SSCs isolation and culture: After two steps of enzymatic digestion, the obtained cells (spermatogonial cells and sertoli cells) cultured in DMEM medium supplemented with 10% FBS. These cells were aggregated and started division and formed colonies (Fig 1). In addition, PLZF protein was detected in these colonies (Fig 2).

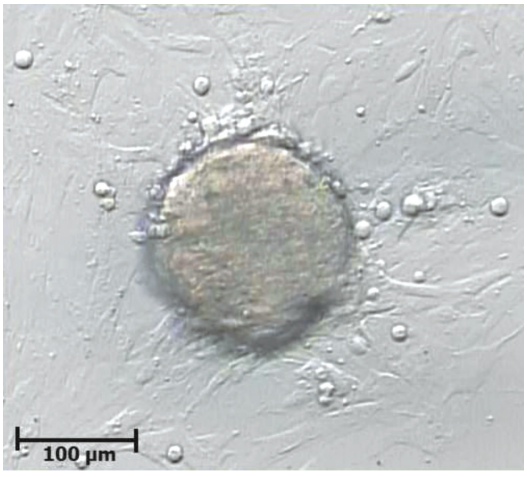

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Figure 1. Spermatogonial cells formed a typical colony

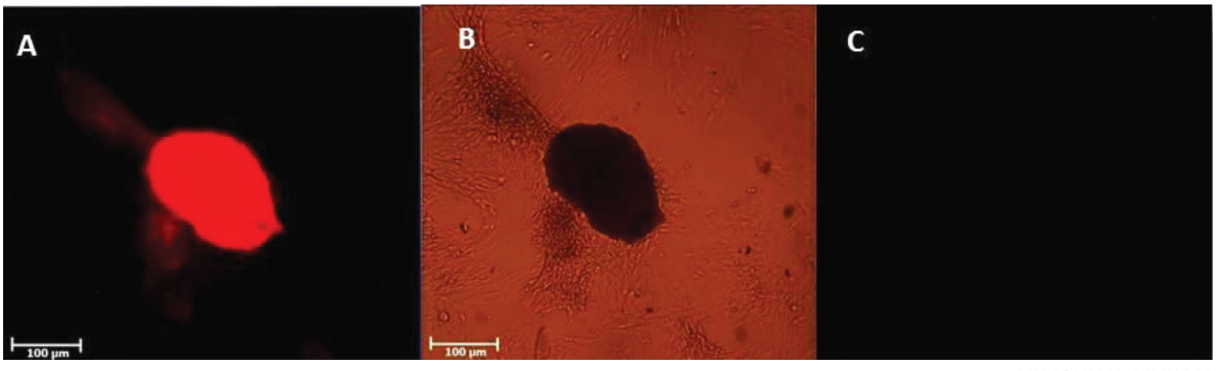

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Figure 2. Immunoflurecent staining of SSCs, detection PLZF Positive Cells.
A: red florescent cells are PLZF positive observed under Immune fluorescence microscope on obtained colonies.
B: Colonies observed under inverted phase contrast microscope.
C: The negative control group, cells observed under Immune fluorescence microscope.

Temperature control: In this step, the data showed that with increase of intensity, the time for hyperthermia decreased (Fig 3). The average time of 1° C hyperthermia in 100, 200 and 300 mW/cm2 was 360, 200 and 90 second, respectively. In the next step of this study, the time and intensity index used in cells stimulation .

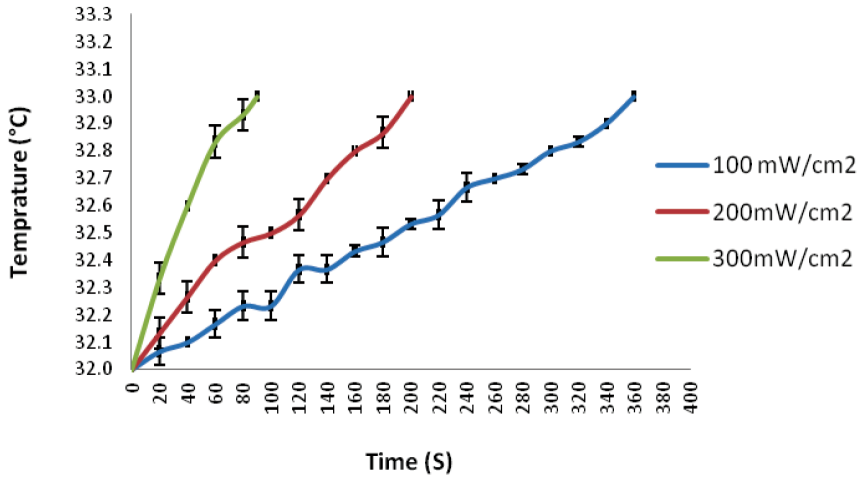

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Figure 3. The Enhancement curve of culture media temperature after exposure to ultrasound stimulation with different Intensities. Data are means ± SD, n=3.

Treatment of Cells: In this study, LIUS was applied to SSCs cultured in DMEM supplemented with 10% (v/v) FBS at intensities of 100, 200 and 300 mW/cm2 for 360, 200 and 90 second, respectively for 5 days. On day 7th, it was found that the proliferation rate increased in 100 and 200 mW/cm2 intensities compared to 300 mW/cm2 intensity and control groups ($P<0.05$) (Fig 4 and 5). that the findings showed that the colonization increased in experimental groups compared to control groups ($P<0.05$). However, stimulation with 200 mW/cm2 intensity LIUS resulted in a better proliferation and colonization of SSCs compared to 100 mW/cm2 intensity ($P<0.05$). The proliferation rate in 100, 200 and 300 mW/cm2 and control groups were 1.96 ± 0.03, 2.26 ± 0.03, 1.73 ± 0.03 and 1.66 ± 0.03, respectively. The average number of colonies in 100, 200 and 300 mW/cm2 and control groups were 45 ± 1.2, 53 ± 2.4, 28 ± 1.2 and 20 ± 0.8, respectively. Regarding the diameter of colonies, there were no significant differences between groups (Fig 6). The average diameters of colonies in 100, 200 and 300 mW/cm2 and control groups were 235.3 ± 6.8 µm, 204.6 ± 12.3 µm, 203.6 ± 5.6 and 214.3 ± 9.1 µm, respectively.

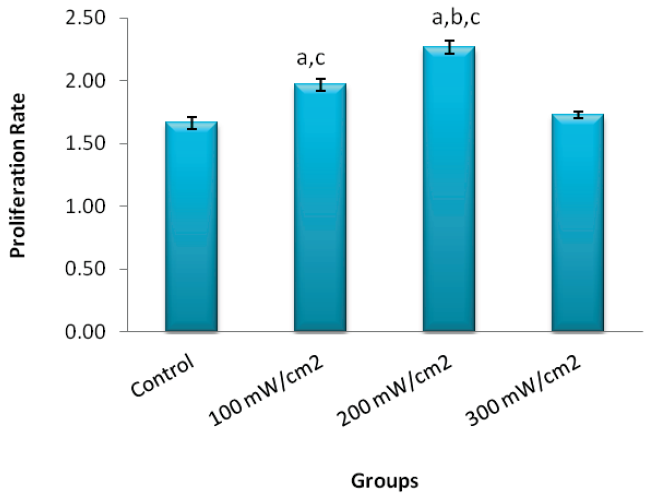

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Figure 4. The proliferation rate in the experimental and control groups in day 7th. Data are means ± SD, n=3
a: significant differences compared to the control group ($P<0.05$).
b: significant differences compared to 100 mW/cm2 group ($P<0.05$).
c: significant differences compared to 300 mW/cm2 group ($P<0.05$).

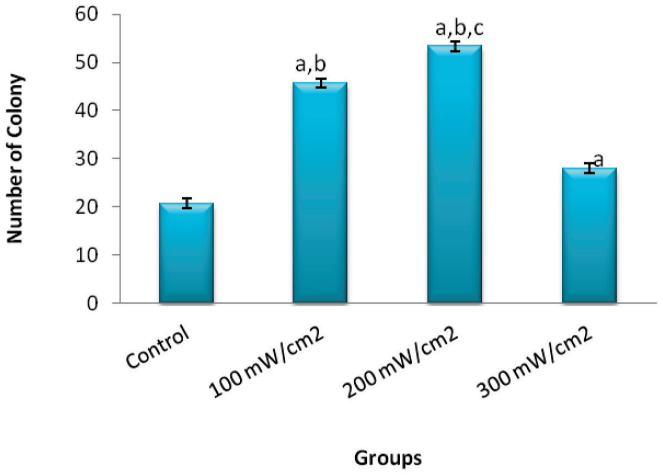

Figure 5. The Number of colonies in the experimental and control groups in day 7th. Data are means ± SD, n=3
a: significant differences compared to the control group (P<0.05).
b: significant differences compared to 100 mW/cm2 group (P<0.05).
c: significant differences compared to 300 mW/cm2 group (P<0.05).

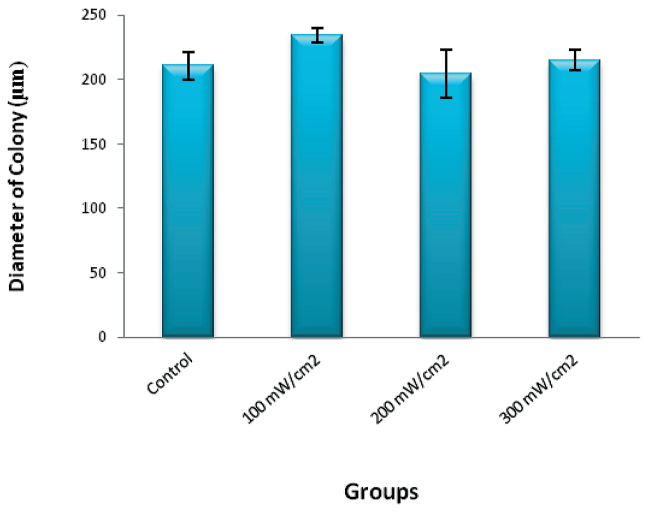

Figure 6. The diameter of colonies in experimental and control groups in day 7. Data are means ± SD, n=3

4. Discussion

Enrichment, proliferation and differentiation of SSCs in vitro, is very important. Colonization of spermatogonial cells prepare retardants pool of germ cells that can be used in treatment of infertility, germ cell gene change, cell transfect and differentiation of SSCs in vitro [3]. The purpose of this study was to investigate the effect of LIUS on SSCs proliferation and colonization. Recent studies show that new procedures such as sound waves, especially low intensity ultrasound can be effective in proliferation and increasing the number of cells. However, the effect of LIUS stimulation on SSCs, which have very important function in male fertility, has not yet been explored. In this study, LIUS stimulation was applied to SSCs cultured in DMEM supplemented with 10% FBS at intensities of 100, 200 and 300 mW/cm2 for 360, 200 and 90 second, respectively for 5 days. On 7th day, it was found that the proliferation rate and number of colonies increased in the experimental groups. However, the stimulation with 100, 200 and 300 mW/cm2 intensity LIUS resulted in a better colonization of SSCs compared to the unstimulated SSCs. Additionally, the SSCs stimulated with 200 mW/cm2 showed the highest proliferation rate

and colonization compared to the another experimental group. This suggests that 200 mW/cm2 is the optimal ultrasound intensity for the proliferation and colonization of SSCs that is consistent with our present data. An earlier study demonstrated that LIUS increased the proliferation and colonization of hematopoietic stem/progenitor cells (HSPCs) [5]. They were stimulated HSPCs with low intensity of ultrasound for 4 days and reported that the enhanced proliferation rate and burst forming unit-erythroid colony formation. Some studies showed the mechanisms of action of LIUS on cells growth, proliferation and colonization [16 and 17]. Tang et al observed that stimulating osteoblasts and chondrocytes with low intensity of ultrasound transiently increased the expression of specific Integrins, namely α5 and β1 [16]. Integrins provide a link between the extracellular matrix (ECM) and the intracellular cytoskeletal components and actin filaments. Integrins are thought to function by undergoing conformational changes which activate them and reveal their ligand binding site. Subsequently, it enables the integrins to bind to cytoskeletal components and other signaling molecules to activate several intracellular signaling pathways [17]. These pathways are normally activated in response to mechanical stress enabling the cells to react to changes in their physical environment. Therefore Integrins act as sensitive mechanoreceptors on the surface of cells. US waves generate pressure transferred to adherent cells via interactions with the ECM. So it may be suggested that LIUS with their effect on transmembrain proteins such as integrins can stimulate more SSCs to divide and can enter these cells to mitotic process in order to self-renewal or differentiation pathway.

5. Conclusion

In conclusion, this report establishes that acoustic energy induces proliferation and colonization of spermatogonial stem cells in vitro. Hence, LIUS stimulation could be a good strategy for improving culture and an outcome of stem cell enrichment.

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