European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 08, Issue 1, 2021

Studying the effect of co-culture system of Mesenchymal Stem Cells on development of follicles in mice ovary in vitro

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Abstract

Objective:

Using cell culture and tissue culture techniques, many researchers are working to elucidate the hormonal factors necessary for the structural differentiation and functional activities of ovarian tissue. Therefore, in this study, we have studied ovarian tissue culture on mesenchymal stem cells (MSCs) derived from bone marrow. Hence, in this study, we examined ovarian tissue culture on mesenchymal stem cells isolated from cultured adipose tissue.

Materials and Methods:

In this study, mesenchymal stem cells became isolated from adipose tissue of the mouse. After four days and monolayer formation of Mesenchymal stem cells, obtained ovarian was placed on the cultured Mesenchymal stem cells' monolayer for 7 days.

Results:

The results of this study show that numbers of mature follicles for treat group compared to the control group were 61/2±1/2 and 38/1 ± 9/9, respectively which had a significant increase compared to control group (P<0/05). This shows the efficiency of tissue culture in co-culture with Mesenchymal stem cells. Also, the amount of internal solo layers of treat group (31/2 ± 5/2) had no significant difference compared to the control group (24/1 ± 7/9).

Conclusion:

According to the results yielded regarding the number and quality of obtained follicles, it seems that this method is efficient and of high importance in producing mature follicles, and subsequently high-quality oocytes and embryo.

Keywords: Tissue culture, Cell culture, Ovarian tissue, Mesenchymal stem cells, Mice.

Introduction

MSCs are the spindle shaped plastic-adherent cells derived from bone marrow, adipose, and other tissue sources that have the capability to self-renew and undergo multipotent differentiation in vitro (1).

MSCs attach to HSCs by adhesion molecules such as N-cadherin and β integrins. Cytokines released by MSCs such as KIT-L, SDF-1, and Ang1 support the growth and differentiation of HSCs by binding to Kit, CXCR4 and Tie2 receptors. While HSCs are attached to MSCs, the expression of Notch ligands (Jagged and Delta-like) in MSCs is enhanced through the Went signaling pathway. Expression of Notch receptors in HSCs is enhanced by sonic hedgehog (Shh) in HSCs and MSCs in vitro and decreases the repertoire of HSCs in vivo (1-2).

Stem cells derived from adipose tissue express high concentrations of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), Beta transforming growth factor (TGF-β), and semi-insulin growth factor (GF) (2).

Takahara et al. (2013) examined the therapeutic effects of mesenchymal stem cells in the ovaries of mice treated with cyclophosphamide and found the harmful effects on ovarian tissue treated with mesenchymal stem cells were less than those in the control group. Besides, the number of antral follicles in the mesenchymal stem cells treated group was insignificantly higher than the control group (3).

Many researchers are trying to clarify necessary hormonal factors for structural distinguishability and functional activities of ovarian tissue. There are techniques used in many studies to culture ovarian organ or isolated follicles in vitro environment. In order to collaborate sequence of follicles' creation and hormonal conditions, to activate inactive follicles, and to grow and sprinkle follicles, total ovarian tissues of the fetus or newborn rodents have been incubated in organ culture systems.

Materials and Methods

In this experimental study, 40 *NMRI* mice with approximate age of 4 to 6 weeks were used, they were killed by cervical vertebrae method and then disinfected with 70% alcohol. Adipose tissue was isolated from back of the groin and abdominal area of mice and washed by phosphate-buffered saline sterile solution (PBS). Then, under sterile conditions adipose tissue was cut with scalpel into millimeter pieces under the hood laminar Class II; connective tissue and blood vessels were separated from.

In order to break down adipose tissue, 1.5 mg collagenase enzyme (Sigma type) was applied per 1 gr adipose tissue. After the addition of a suitable amount of enzyme, samples were incubated at 37 ° C for 60 minutes. After ensuring complete breakdown of tissue, the same volume as the enzyme solution used in cultured area, DMEM (Gibco) containing 10% FBS (sigma) and 1% penicillin / streptomycin (Gibco) was added to cell suspension in order to neutralize enzyme activity. Afterward, the suspension was centrifuged in 15ml Falcon tubes for 10 minutes at 1400rpm; and the supernatant liquid was evacuated along with adipocytes cells.

5 ml culture media of DMEM +% 10FBS was added to the resulting cell deposition; and lubricating buffer solution with ammonium chloride (Merck), potassium bicarbonate (Merck) and (Merck)EDTA was used in order to breakdown red blood cells in cell sediment. After 10 minutes, the same volume as lysis buffer, 5 ml DMEM +% 10 FBS medium was added and then centrifuged for 10 minutes at 1400rpm. After smoothing with filters having 200 micrometer holes, it was recentrifuged in the same condition. Finally, the cells were cultured in 25 square centimeters flask in DMEM +% 10FBS +% 1penicillin / streptomycin incubator media at 37 ° C, 5% CO2, and relative humidity.

After a few hours, mononuclear cells began to stick to the bottom of flask. Additional cells were evacuated by replacing the medium after 24 hours. After 4-5 days, cells were proliferated and occupied the surface of the flask (Confluent). After the proliferation of cells in primary culture and occupancy of at least 60% of the flask floor, after 4-5 days, cells were transferred to several wells. The cells were poured in Eppendorf after trypsin and centrifuge and then washed with PBS. Thereupon, they were centrifuged for 10 minutes at 2000rpm.

Conjugated CD90 primary antibodies were added to PE (50 ml Fico Eritrean) and CD31 conjugate was added to FITC (fluorescence ISO thiocyanate) and refrigerated for 30 minutes; ABS PE-IgG2a and FITC- IgG2b anti bodies were used to negative control. Next, cells were washed with PBS and centrifuged for 10 minutes at 2000rpm. Afterward, 1c formalin fixing buffers were added to cells which the obtained one was analyzed with Flow cytometry device (Beckton Dickinson, Germany).

40 ovary pairs of 40 female mice with 5 weeks' age were separated. After removing fat and washing them for 2 times in Hanks balanced salt solution (HBSS), one of the ovaries was transferred to organ culture container and the other one was fixed in Bouin solution to investigate control samples. In the phase between the Penny 1%, 3 mg / ml BSA, %10 FBS, DMEM environment (liquid culture containing Sylsn- streptomycin) and air, it was incubated in 5% CO2 incubator containing 95% air at 37 ± · / 1 ° C for 6 days.

The medium was changed every day. Microscopic evaluation of theca cells, granulosa, and counting healthy and damaged Ovarian follicles were carried out under Hematoxylin - eosin staining. Data were analyzed by SPSS software; and a paired T was used to compare data means. P <0/05 was considered significant.

Results

In order to prove the mesenchymal nature of these cells, in addition to their adhesion to the culture dish, another way to identify them is to use surface marker which was actually studied by flow cytometry method after the first passage and the separation of mesenchymal stem cells from other cells. In this study, mesenchymal stem cells isolated from fat were evaluated through CD90 markers. Results of CD90 markers on mesenchymal stem cell expressed the adipose tissue of this marker at 82% (Figure 1)

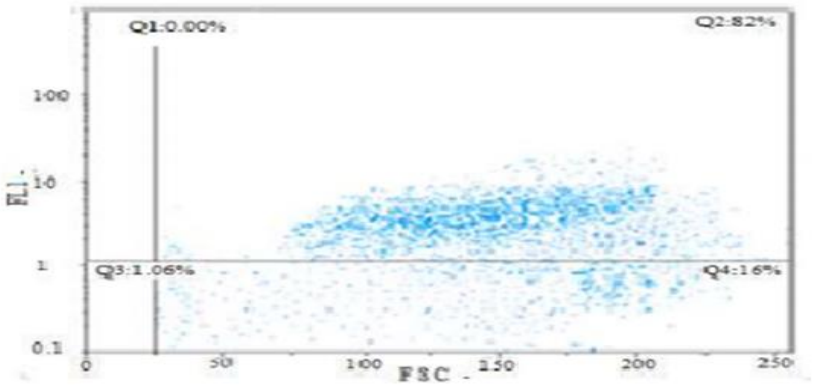


Figure 1. Mesenchymal stem cells of adipose tissue that expressed surface marker as 82%.

The average number of primary follicles between control group and co-culture group with mesenchymal stem cells had no significant difference. The average number of primary follicles is shown in Table 2 and Figure 2. Based on the results, the mean number of primary follicles in the in vivo control group at the beginning and end of the period was not significantly different from other studied groups (p <0.05) (**Figure** 2).

According to the results of the cultured ovaries in control group compared to co-culture group, there were no significant differences in the average number of secondary follicles (p <0.05) (**Figure** 4). Thenceforth, there is a significant difference in the mean number of mature follicles in control culture group compared to treated group with mesenchymal stem cells (p <0.05)

Increase in the number of mature follicles suggest effectiveness of medium compared to secondary follicular development (**Figure** 3) After microscopic examination of the tissues of the cultured control

and treatment groups, the mean number of preantral follicles did not show a significant difference between the control group (**Picture** 1).

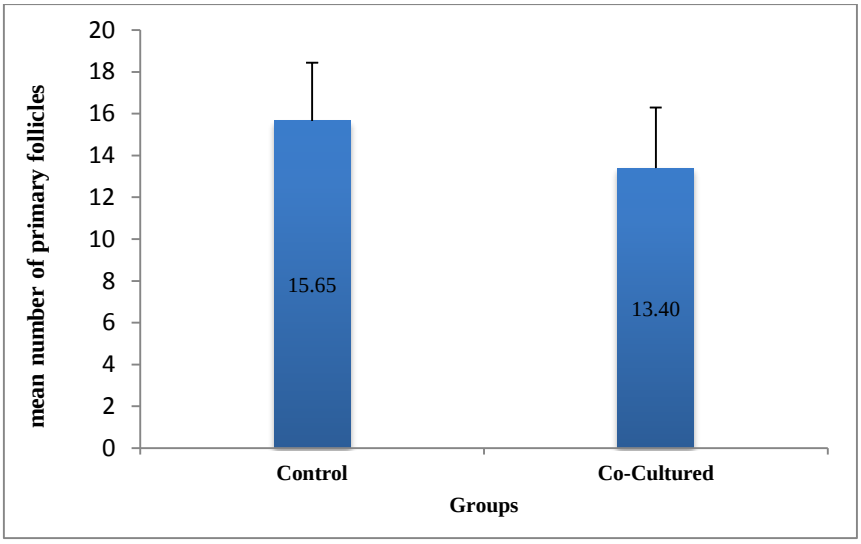


Figure 2- comparing mean number of primary follicles in control and treat group.

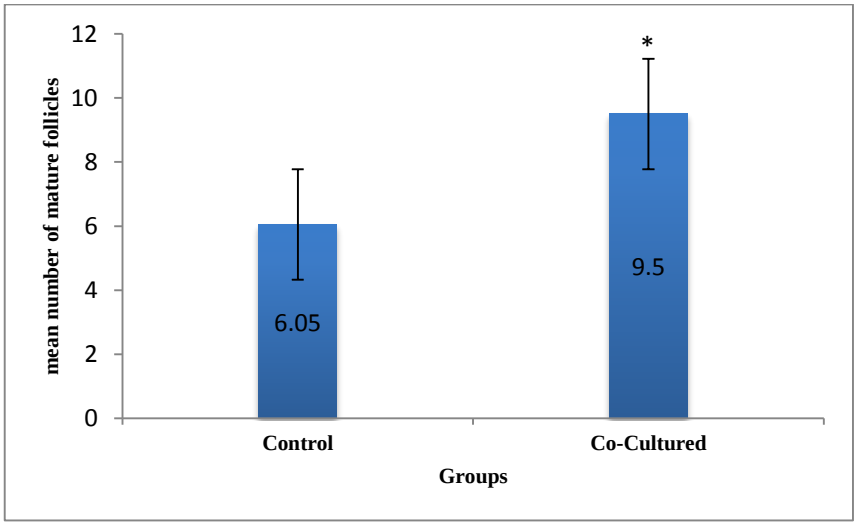


Figure 3- Comparing mean number of mature follicles in control and treat group.

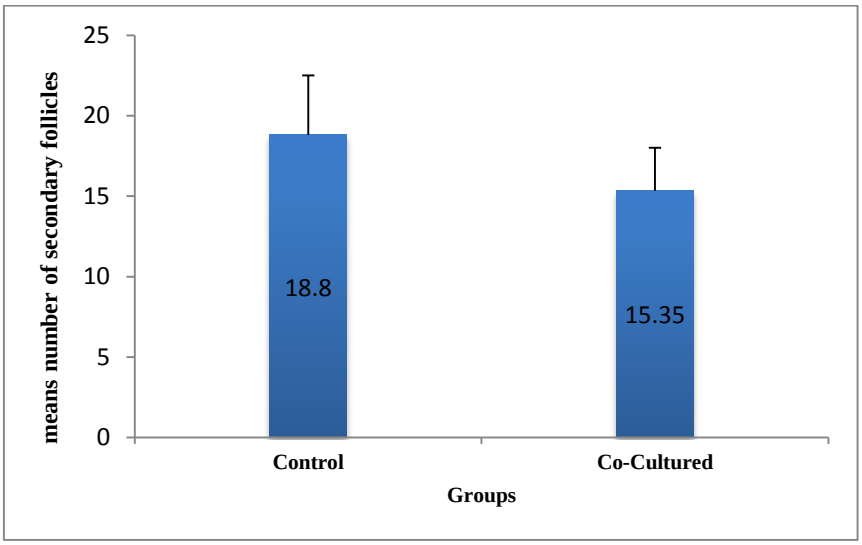
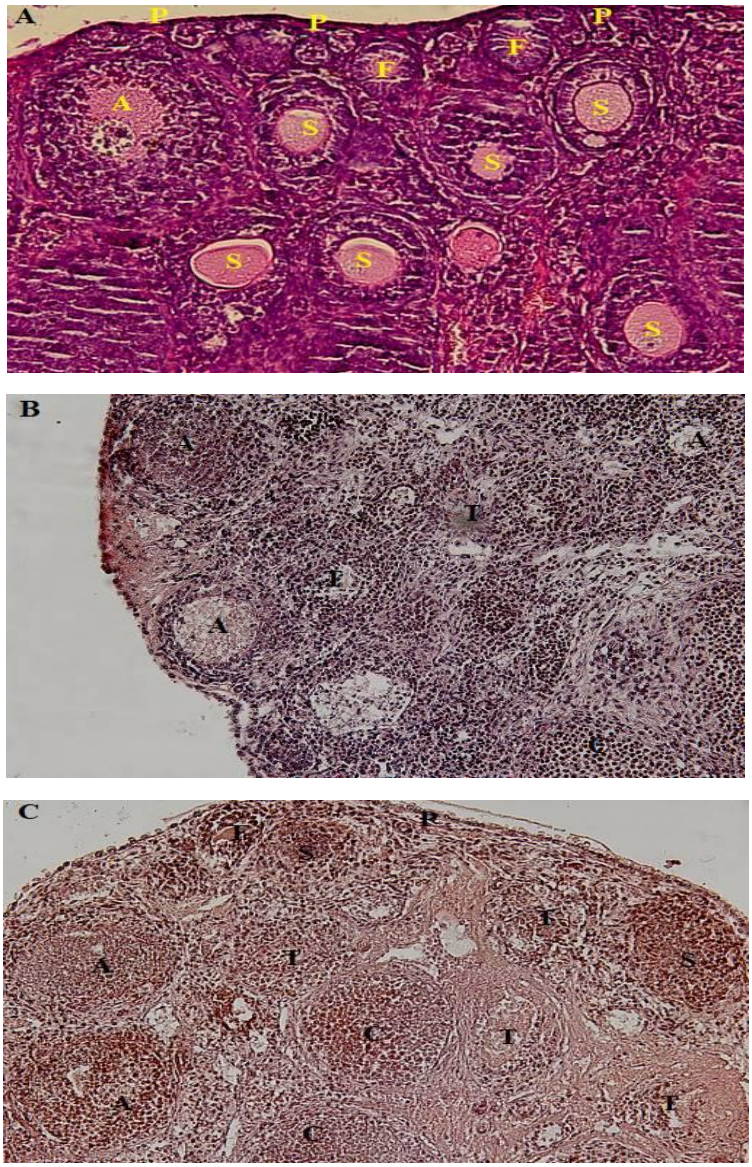


Figure 4- Comparing mean number of secondary follicles in control and treat group.



Picture 1. Ovarian image of different groups. **A:** Control Group. H&E staining. 800X
B: treatment with Co-culture Group, Masson's trichrome staining. 400X. **C**: treatment Group. PAS staining.400X.
P: Primordial follicle. F: Primary follicle. S: Secondary follicles. A: preantral follicles. T: Atretic follicle. C: corpus luteum.

Discussion

In the present study, we investigated co-culture effect of ovarian tissue with mesenchymal stem cells in culture medium and in vitro environment. There have been motivating attempts invested to improve culture systems and better tissue culture and various body organs. Furthermore, application of variant techniques for successful ovarian tissue culture was investigated back for more than 60 years (4, 5). Studies were carried out on ovarian tissue of mouse, hamster, and cows and human that have been successful in 50 days' incubation of ovaries with no sign of necrosis (6). In most of the culture systems, placing ovarian tissue on a physical protector in culture wells, glass or hour glass is possible with minimum immersion; and access to sufficient quantities of culture medium under the protector is also

feasible. Incubation conditions are 37 ° C and 5% CO2 though various studies reported optimization of culture conditions (7).

Culture systems of entire ovary or a piece ovary have numerous applications such as determination of steroid synthesis in cultured tissue. These types of experiments in different species lead to better understanding of growth time and ovary enzyme functionality and the response of Theca and granulosa cells to Gonadotropins from fetus growth till maturity and adulthood (8).

Monitoring the development of follicles or changes in steroid production and also identifying the express time of FSH and LH receptors in the ovaries during growth in the tissue culture system were investigated. Adult mesenchymal stem cells have the potential to grow and differentiate into chondrocytes. Previous studies clearly show that mesenchymal stem cells cause inhibition of immune response cells. Accordingly, they are ideal candidates for co-culture (9,10,11). Based on the results obtained in this study, it has been identified that mesenchymal stem cells co-culture has significantly increased Heca cell size and number of follicles ($P < 0.05$). Studies in vivo and in vitro determined the stem cells effects for induction of Antral follicle growth and ovulation in cultured mouse ovary pieces.

Eppig et al. (1996) studied the effect of injecting Mesenchymal stem cells into cancer ovarian tissue, in vivo condition, maximum number of mature oocytes and the progression of meiosis induce ovulation (12). Results of our study also confirms the mentioned potential. The human ovary contains insulin receptors which show the role of peptides in ovarian regulation. In vitro studies show that insulin can directly stimulates production of androgens by ovarian stroma (13,14,15). Moreover, a good ovarian culture system, which can repeat ovary condition, has a pivotal and main role to study Oogenesis and follicle genesis and provides an unlimited supply of oocyte medical use. More than the past 20 years, mice oogenesis has been studied by experimental culture technology. Primary ovaries in Bx follicles of newborn mouse ovary are able to enter into the second meiotic division and have full maturity Mice in vitro condition. Recently, Mice oogenesis has been studied in several laboratories by culturing in vitro condition.

In the present study, ovarian tissues were cultured only for up to six days, which was a good source for prolonged tissue culture in co-culture with mesenchymal stem cells. In addition, considering the study used stem cells differentiated by Fibroblasts have not had a beneficial effect on cell apoptosis, as studies have shown that the amount of culture period by using mesenchymal stem cells is much longer and has been evaluated even with apoptosis induction treatments. Finally, the best culture period for investigating tissue culture, including tissues that require many substances and hormones for survival, has been reported as six to eight days. Therefore, in this study, the duration of ovarian tissue culture was prolonged for seven days.

In 2008, Ling et al. examined the growth of follicles as well as the maturation of mouse eggs in co-culture with mesenchymal stem cells. They reported that the optimized culture medium used for follicle and oocyte maturation with mesenchymal stem cells significantly increased follicle length and significantly increased the resulting oocytes' maturation and subsequent fertilization (16). In our study, the growth of primary and secondary follicles, as well as the development of mature follicles, were observed in the co-culture group with mesenchymal stem cells, which is consistent with the studied research, which is probably due to the released signals of mesenchymal stem cells and its effect on adjacent cells

Conclusion

According to the results regarding the number and quality of obtained follicles, it seems that this method is efficient and important in producing mature follicles and subsequent oocytes. Due to growth of infertility as well as reproductive disorders, more widespread researches are needed in this area.

Acknowledgment

The authors appreciate the vice president for research and technology of Ferdowsi University of Mashhad.

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