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Investigation of Stem Cell Applications on *In Vitro* Fertilization in Rats

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ABSTRACT: We aimed to search the effects of bone marrow-derived mesenchymal stem cell-conditioned media on in vitro fertilization by investigation of lifetime of germ cells cleavage, degeneration rates and embryo quality. For this purpose, firstly MSCs were isolated from femurs and tibias of the rat, and cells were cultured until the fourth passage. Sperm and oocytes were collected from male and female rats. Oocytes were added in Human Tubal Fluid Media (HTFM), Single Step Media (SSM), Alpha-MEM Media (AMM) and Bone Marrow-Derived Mesenchymal Stem Cell-Conditioned Media (CM). Thousand sperm were added into the media which including oocytes. Embryos were allowed to produce by IVF. The development of the embryos was followed until the 11th day, and the arrest, degeneration rates and alive embryos were established. The embryos reached 2, 4, 8, 16 cells stages and morula stage in the CM. While AMM had a negative effect on fertilization and embryo development, the most favourable effect was shown to be caused by CM in comparison with the other medias. These results have shown that the beneficial effects of CM in IVF would be a significant increase in the rate of fertility and development of embryos.

Keywords: Bone Marrow Mesenchymal Stem Cell, In Vitro Fertilization, Oocyte, Sperm, Superovulation.

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INTRODUCTION

Infertility has become an important problem for both men and women in recent years, and approximately 8-12% of married couples of reproductive stage have infertility problems. In the literature, the definition of infertility is as follows: It is the absence of pregnancy for 12 months or more during an unprotected sexual intercourse (Gunes et al., 2020). Considering the infertility rates of couples who apply to IVF centers, the rate of infertility caused by women is 33-41%, while the rate caused by men is approximately 25-39%. The infertility problem caused by both women and men is 9-39%. These rates of infertility may differ according to the geography and socio-economic status of the countries (Agarwal et al., 2020; Wasilewski et al. 2020).

When the reasons of infertility are investigated, it has been determined that the diseases that cause the dysfunction in the reproductive organs, such as hormone dysregulation, uterine or cervical diseases or cancers, endometriosis, polycystic ovary syndrome, premature ovarian insufficiency, may result in infertility. In men, hormone and sperm motility disorders, hypogonadism, infection or varicocele are the causes of infertility. The rate of unexplained cases in infertility is 30% (Agarwal et al., 2020; Del Giudice et al., 2020; Wasilewski et al. 2020).

Fertilization is a complex event in which many molecular signaling pathways come from oocyte and spermatozoa are involved (Yao et al., 2019). On the other hand, it is important to provide a suitable media or microenvironment in the in vitro fertilization (IVF) laboratory together with appropriate physical conditions. Since the conventional IVF procedures are insufficient in some cases of infertility, there is tendency to alternative methods (Osman et al., 2018). One of these is mesenchymal stem cell (MSC) applications (Zhu et al., 2020; Karimaghai et al., 2018; Virant-Klun et al. 2019; Manuel, 2020). Stem cells hold promise in many areas due to its plasticity and ability to differentiate into other cells, and also they secrete many growth factors (Yang et al., 2019). Despite this potential, clinical studies on reproductive organs in humans are limited because of ethical reasons. In a clinical research of stem cells, adipose tissue-derived mesenchymal stem cells (ADSCs) were used to trigger oogenesis in the ovary. While only 2 oocytes were harvested as a result of ovarian stimulation treatment with hormones from a woman diagnosed with low idiopathic ovarian response, it has been reported that 14

oocytes were obtained after the application of ADSCs with hormone therapy (Estuardo et al. 2020). Mozafar et al., stated that bone marrow derived mesenchymal stem cell (BMSC) application showed an effective result by stimulating spermatogenesis in mice created azoospermia model (Mozafar et al., 2018). A similar study was carried out with hamsters and in the azoospermia model created with busulfan, it was found that ADSCs repaired the tissue damage in the testicle and induced the spermatogenesis (Karimaghai et al., 2018).

As can be seen, the stem cell itself was used in these studies. Therewithal, it has been reported that the conditioned medium (CM) of mesenchymal stem cells could support the maturation of oocytes and sperm motility. Because, the MSCs secreted the cytokines and growth factors into the CM, and it is thought that the CM will have an improvement effect on the reproductive cells and organs (Ullah et al., 2015; de Olivera Bezerra et al., 2019; Yang et al., 2019). Bader et al., reported that the CM of ADSCs raised the sperm motility in vitro condition, whereas Akbari et al., appointed that CM of human umbilical cord mesenchymal stem cells promoted the oocyte maturation and the formation of blastocyst (Akbari et al., 2017; Bader et al., 2018).

In our study, we intended to reveal whether some growth factors secreted by stem cell into the medium have an effect on IVF and embryo development. And, we aimed to search the effects of BMSC-conditioned media on IVF and compare with other conventional mediums by investigation of lifetime, germ cells cleavage, embryo quality, and degeneration rates.

MATERIALS AND METHODS

Informed consent was obtained from all individual participants included in the study. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (Manisa Celal Bayar University (date: 27.11.2013, ethic approval number: 77.637.435-71).

Experimental Animals

In this study, we realized our animal experiment by receiving animal ethics certification approval from Manisa Celal Bayar University (date: 27.11.2013, ethic number: 77.637.435-71). Rats were provided by Experimental Animal Centre from Manisa Celal Bayar University. We used 20 female Wistar albino rats and 10 male Wistar albino rats which has $250 \pm$

50 gr weight and without inbred animals. Rats have waited in 25°C room temperature, 12 hours dark and 12 hour light condition. Rats were fed by *ad-libitum*. The subjects were kept in a separate room in the other isolated laboratory to avoid stress due to factors such as noise and heat.

BMSC Isolation and Characterization

BMSCs which are collected from rat tibias and femurs, and cells were cultured in alpha-MEM medium (F0915, Biochrom, Berlin, Germany) containing 15% fetal calf serum (S0113, Biochrom, Berlin, Germany), 50 µg/ml gentamycin (A2712, Biochrom, Berlin, Germany), 100 UI/ml. penicillin and 100 UI/ml. streptomycin (A2213, Biochrom, Berlin, Germany), amphotericin (A2612, Biochrom, Berlin, Germany) and 200 mM L-glutamine (K0282, Biochrom, Berlin, Germany) at 37°C and 5% CO₂. Then, non-adherent cells were removed from the flask, and adherent cells were maintained until passage 4 (P4) by changing the media every two days (Aydemir et al., 2018).

BMSCs were dyed immunocytochemically for characterization using Stro-1, CD45, CD90 and CD105 markers at P4 (Demirayak et al., 2016). Cells were passaged into the 35mm² petri dishes, and were seeded to be 70-80% confluent. After fixation with 4% paraformaldehyde (1.04004, Merck, Darmstadt, Germany), cell were permeabilized with 0.1% Triton-x100 (T8787, Sigma, St. Louis, USA), they were washed in phosphate buffer saline (PBS). The blocking serum was applied to the cells for 1 h, and cells were incubated in primary antibodies, Stro-1 (MAB4315, Millipore), CD45 (ab10558, Abcam), CD90 (ab92574, Abcam) and CD105 (ab11414, Abcam) for 18 h. For the negative control, some of cells were treated with PBS instead of primary antibodies. Then cells were treated with biotin-streptavidin hydrogen peroxidase secondary antibody (85-9043, Invitrogen®-Histostain Plus Bulk Kit, CA, USA) for 30 min. Diaminobenzidine (DAB, 00-2020, Zymed, CA, USA) was used to make the immunoreactivity visible, and the counterstaining was performed with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany). After washing in distilled water, cells were mounted using aqueous media AML060, Scytek, Logan, Utah, USA) (Özdal-Kurt et al., 2016). The immunoreactivities were evaluated under a light microscope (BX43, Olympus, Japan) according to H-score method. The intensity of immunoreactivity was determined as; weak (+), moderate (++) and strong (+++) respectively, and the stained cells were counted in five fields

for each intensity. The H-score values were obtained using the formula: P_i (intensity of staining +1). The percentage of stained cells was indicated as P_i (varying from 0% to 100%) (Aydemir et al., 2018).

Following the characterization, BMSCs at P4 were cultered and after two days, the media taken from the flask and it was used to experiments. This media called as Conditioned Media (CM).

Ovary and Oocyte Collection

Superovulation

For the superovulation (SO) method, the female rats at estrous cycle were identified by analyzing vaginal smear. The vaginal smear was performed between 15.00-16.00 p.m. the smear samples were dyed with Giemsa, and the samples were examined under a light microscope (Aydemir et al., 2018). 10 Female rats at estrous cycle were applied to the superovulation process, Follicular Stimulating Hormone (FSH, Pregon, Organon) ve Human Chorionic Gonadotropin (HCG, Pregnyl, Organon). Firstly, the 25 IU FSH was injected to the them, after 48 hours, superovulation was completed by doing 20 IU HCG injection. After 48 hours from HCG injection (intraperitoneally (ip)), the ovaries were dissected from female rats under anesthesia (75 mg/kg ketamine and 10 mg/kg xylazine by ip). One of two ovaries was used for oocyte collection, other ovary was used for the histological examination (Honda et al., 2019).

Histochemistry

After superovulation, one of two ovaries was evaluated for the histological structure and formation of follicles. The ovaries were fixed in the 10% formalin for 48 h and the routin paraffin embedding protocol was applied. 5 µm thick sections were cut paraffin blocks. The samples of ovary were dyed with routin heamatoxylin and eosin (H&E), and they were examined in terms of the presence of oocyte cumulus complex (OCC) and number of follicles under a light microscope.

Oocyte collection

One of two ovaries was used to obtain oocytes. The ovaries with tuba uterina were removed by using sterile forceps and scissors and then were put in RPMI (Biochrom 0844B) medium. We put tuba uterine and ovary in Petri dishes (No. 1016 falcon Petri dishes). To collect oocytes, the ovaries and tuba uterina from the RPMI medium were shredded by ppd injectors

in laminar airflow. Shredded ovary and tuba uterine samples were taken outside and then oocytes were collected with Pasteur pipette (Isolab 225 mm, cat. no: 084.01.002) under a stereomicroscope (Olympus SZX12, Shanghai, China). Oocyte cumulus complex (OCC) came out of the tuba uterina. Oocytes were collected with cumulus complexes (aid with Pasteur pipette) (Hino et al., 2020). These groups were divided into four groups which are Alpha-MEM Media (AMM), Human Tubal Fluid Media (HTFM), Single Step Media (SSM) and Conditioned Media (CM) obtained from BMSCs.

Sperm Collection and IVF Method

Male rats were anesthetized (ketamine and xylazine) and sacrificed with the method of cervical dislocation in laboratory conditions. After sacrifice, we removed male rat testes and put in RPMI media which included 10% fetal bovine serum (FBS). The testis samples were put in Petri dishes in laminar airflow. Excess fat around of testes was removed. After we removed excess fat tissue, testes were put in fresh media. The end of epididymis from testis was cut with a sterile surgical knife and then testis was milked (Kumar et al., 2016). Later, sperm in the medium was collected and centrifuged at 1000 rpm for 3 minutes. After centrifugation, sperm from supernatant portion was spread on a slide (we only take 5 µl to count) and sperm count was made. After we saw sperm on the slide, sperm which is in 15 ml tube was collected with 1000 µl pipette and then 1000 sperm (almost 30 µl) were put in drops which include oocytes. Then we investigated embryo development at 0, 3, 6 and 9. day.

Statistical analysis

The findings were analyzed statistically with one-way analysis of variance (ANOVA) by Tukey-Kramer multiple comparisons test on GraphPad (GraphPad Software, San Diego, CA, USA). using one-way analysis of variance (ANOVA). The results were given as mean ± standard deviation. The statistical significance was considered as $P \leq 0.05$ (Aydemir et al., 2018).

RESULTS

BMSCs characterization

Under cell culture laboratory conditions, BMSCs which were collected from rat tibia and femur were cultured until its confluent in AMM containing other supplements. The **characterization** of BMSCs were made immunocytochemically at the P4. After immu-

nocytochemistry assay, we stated that the positivities of Stro-1 and CD105 (** $p < 0.001$), and also negativities of CD45 and CD90 (** $p < 0.001$) (Figure 1).

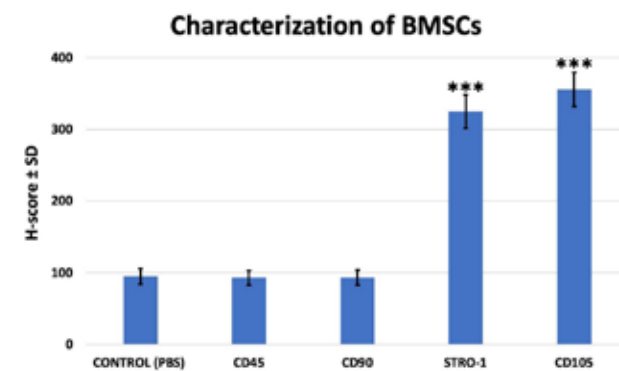


Figure 1. The H-score results of BMSCs characterization using CD45, CD90, Stro-1 and CD105

Histological evaluation

The ovary samples from 10 female rats treated with SO, were examined under a light microscope, and the follicles were counted from each rat ovary samples (five area) (Figure 2). It was seen that SO significantly increased the number of follicles ($16 \pm 3,1$) compared to non-treated with SO ($5 \pm 1,4$) method (Figure 3). However, the OCCs were found in all follicles.

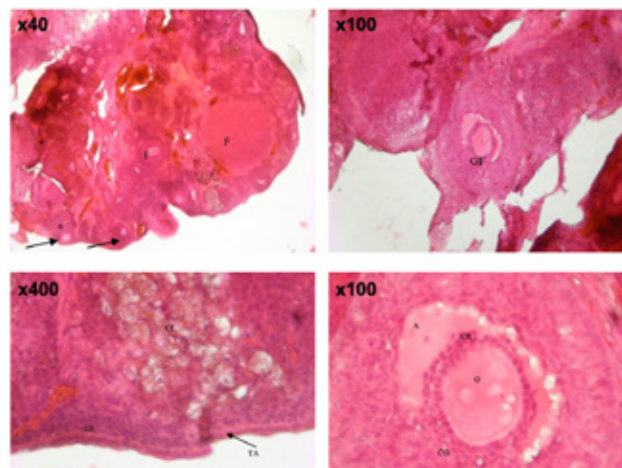


Figure 2. The images of ovaries of female rats that have been given FSH and HCG. We have shown that ovaries which have been induced with FSH and HCG application was increased the follicles and bloody supply amount. H&E staining. F: Follicle, primordial follicles are indicated by the arrow, GF: Graaf Follicle, CL: Corpus Luteum, O: Oocyte, TA: Tunika Albuginea, GE: Germinal Epithelium, CR: Corona Radiata, CO: Cumulus Oophorus, A: Antrum

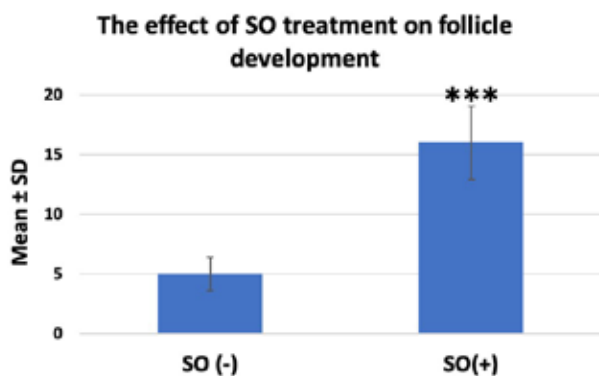


Figure 3. The evaluation of development of ovarian follicles after the treatment of SO.

Evaluation of IVF

After SO procedure, we collected oocyte and put in HTFM, SSM, AMM ve CM media which have been incubated one night before. Firstly, we determined the cell division stages in the oocytes as prophase I (PI), metaphase I (MI), and metaphase II (MII) (Figure 4).

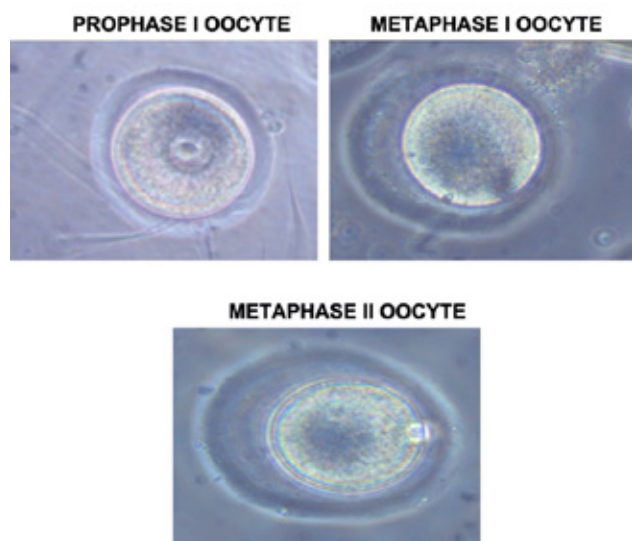


Figure 4. Rats Prophase I (PI), Metaphase I (MI) and Metaphase II (MII) oocytes examples. Magnification: x200.

After we added sperm, we took sperm and oocytes photos (Figure 5). We have indicated that PI oocytes in CM were matured into MII oocytes (Figure 6). Whereas oocytes in AMM weren't seen fertilization, oocytes in CM, SSM ve HTFM were seen fertilization. (Figure 6 and 7).

We observed that oocytes and embryos in HTFM were been healthy at the end of the 6th day. Degeneration was observed in 20% of oocytes in HTFM. On the contrary HTFM and SSM, oocytes and embryos in CM were seen healthy (Figure 8). We have shown that all oocytes in AMM degenerated on the 9th day.

Moreover, oocytes and embryos in SSM and HTFM degenerated. Only one oocyte in SSM was seen as healthy. We have indicated that almost half of embryos and oocytes in CM were degenerated (Figure 9). Oocyte maturation and healthy process were indicated at (Table 1).

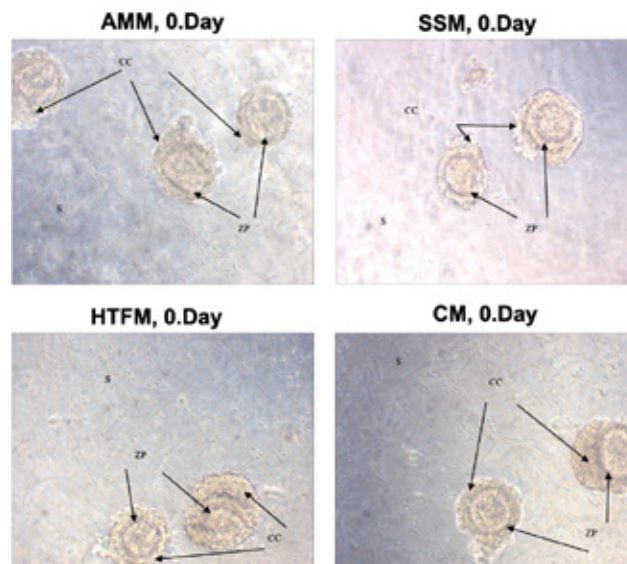


Figure 5. Images of oocytes which were collected from SO+ ovary in a different medium. Morphological differences between sperm and oocyte were not observed. CC: Cumulus Cells, ZP: Zona Pellusida, S: Sperm, Magnification: x100.

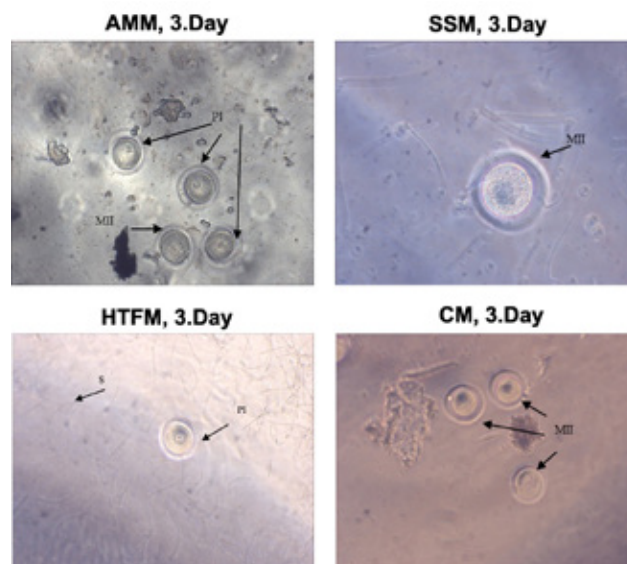


Figure 6. Images of non-fertilized oocytes on culture condition. PI: Prophase I, MII: Metaphase II, S: Sperm. Magnification: x100.

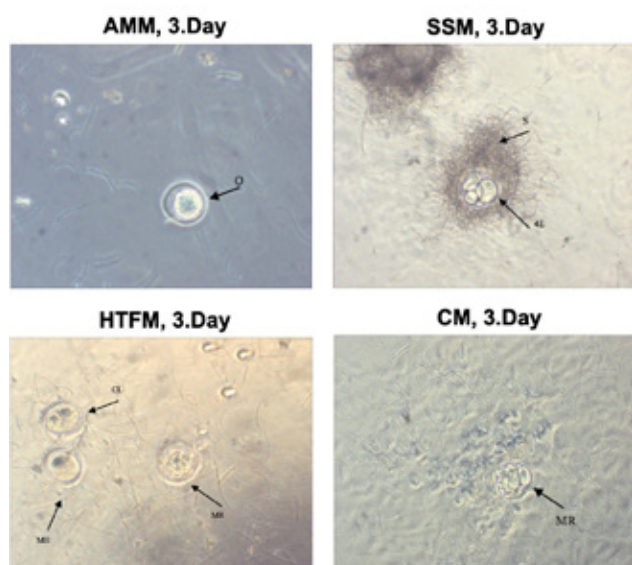


Figure 7. Images of oocyte and embryo at the end of the 3th day. Oocytes in AMM haven't been fertilized. One oocyte in CM has been fertilized. We showed that it has reached the morula stage. We also showed that one oocyte in HTFM was fertilized and reached the compaction stage. 4E: 4 Cell Embryo, S: Sperm, MII: Metaphase II Oocyte, O: Oocyte, MR: Morula, CL: Embryo from cleavage. Magnification: x100.

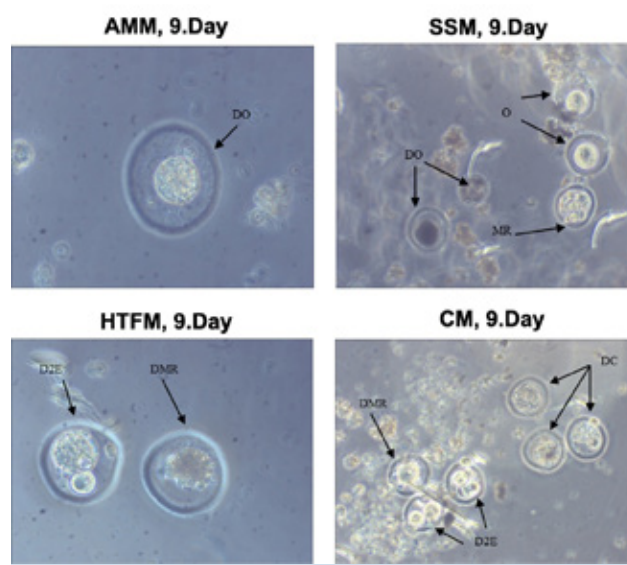


Figure 9. Images of oocytes and embryos on the 9th day. We showed that all embryos in HTFM and SSM degenerated on the 9th day. Only four oocytes in SSM were seen healthy. All oocytes in AMM were degenerated. Nine oocytes in BMSCM were seen healthy whereas three oocytes in HTFM were seen healthy. In addition to, three embryos in BMSCM were arrested at compaction stage. MR: Embryo from morula, D2E: Degenerated 2 cell embryo, DC: Degenerated embryo from compaction, MII: Metaphase II oocyte, DO: Degenerated Oocyte, O: Oocyte, DMR: Degenerated embryo from morula stage. Magnification: x100.

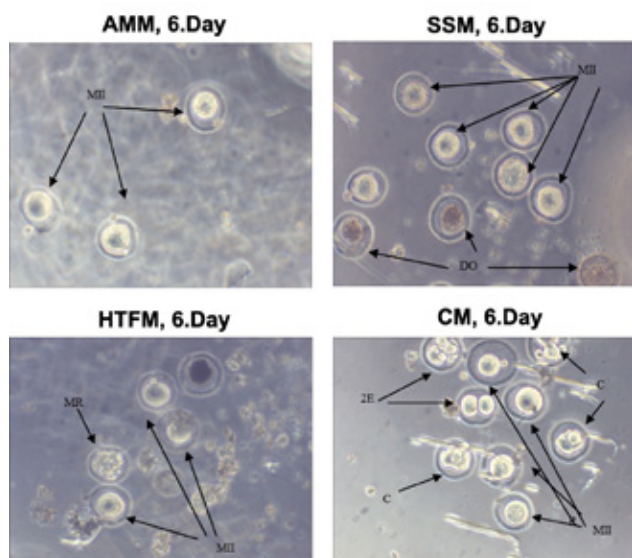


Figure 8. Images of oocytes and embryos on the 6th day. Oocytes in AMM were seen healthy. Whereas two oocytes in CM were degenerated, other oocytes in CM were healthy. We saw that four oocytes in HTFM and three oocytes in SSM were degenerated. 2E: 2 cell embryo, MR: Morula, MII: Metaphase II, DO: Degenerated Oocyte, C: Compaction. Magnification: x100.

Table 1. The lifetime of oocytes in AMM, CM, SSM, HTFM in 0th, 3rd, 6th, 9th and 11th days.

The Number of Oocyte	AMM	SSM	HTFM	CM
0th DAY	14	15	16	17
3th DAY	11	13	15	16
6th DAY	9	10	11	14
9th DAY	1	4	3	9
11th DAY	0	1	0	3

DISCUSSION

In the current study, we investigated the effects of CM of BMSCs on the oocyte lifetime, germ cells cleavage, embryo quality, and degeneration rates, comparing the other conventional media, Alpha-MEM Media (AMM), Human Tubal Fluid Media (HTFM), and Single Step Media (SSM). We performed the evaluation of parameters morphologically in the culture conditions. We ascertained that the CM showed a positive effect on the development of reproductive cells and embryos.

With the discovery of stem cells, experimental or clinical researches on their use in the treatment of many diseases have increased. The stem cell itself has been used in many previous studies, and then many studies have been carried out with the conditioned medium from stem cells due to its ability to secrete many

factors. It has been established that the CM contained many growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), macrophage stimulating protein (MSP), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), transforming growth factors (TGFs) and interleukins (ILs), also microvesicles (Ma et al., 2014; Ullah et al., 2015; Bhardwaj et al., 2016; Noverina et al., 2019; de Oliver Bezerra et al., 2019; Yang et al., 2019). Nowadays, infertility is a major subject that causes people not to have children and gives important problems about having a good quality of life and peace. One of the most important problems with infertility is that oocytes and sperm which not enough maturation can create a fertilization problem (Mirnamniha et al., 2019). Despite some successful IVF trials, the success rate is still around 30% (Wasilewski et al. 2020). For this reason; some researcher has been trying to create new medium and they have been providing critical achievement.

In the previous studies, the BMSC-conditioned media was used to evaluate the development of follicles and oocyte maturation (Ling et al., 2008; Feng et al., 2009; de Olivera Bezerra et al., 2019). According to an experiment, 48 h of CM of BMSCs was compared with HTFM, AMM, Dulbecco's modified Eagle's medium (DMEM) using 120 oocytes of mice for each medium. CM was exhibited two times efficiency than other media which was included 4 times tubal fluid. More importantly, embryos development until blastocyst were increased between 2 and 8 times by CM (Ling et al., 2008). Similar to previous study, Feng et al., (2009) stated the effects of CM of BMSCs on development of oocytes and meiosis in mice. The CM has been found to support parthenogenetic development of oocytes. It is thought that not only CM can play an important role in oocyte activation, but also it can affect pronuclear formation by using Ca²⁺ (Feng et al., 2009). Likewise, CM of Wharton's jelly-derived mesenchymal stem cells exhibited the similar effect on the growth of ovine oocytes morphologically. Though, mitochondrial activity of oocytes was enhanced in the AMM and AMM+CM conditions, whereas the level of reactive oxygen species (ROS) was decreased with the AMM+CM and CM treatments (de Olivera Bezerra et al., 2019). Microvesicles, one of the agents secreted by mesenchymal stem cells, have been observed to trigger follicle development and oocyte maturation in mice with premature ovarian insufficiency (Yang et al., 2019). The findings we obtained in our

study were in parallel with these studies.

In a clinical study, the CM of human umbilical cord mesenchymal stem cells (hUCMSc) was indicated that it triggered the maturation of oocytes which harvested from infertile women, and it was compared with AMM. Together, the maturation effect of these two media on vitrified and non-vitrified oocytes was evaluated. It has been demonstrated that the 48 h of CM has a greater maturation effect (85.18%) on non-vitrified oocytes than the AMM (79.24%). The parthenogenesis induced with ionomycin was enhanced in the presence of CM in comparison with AMM. In the AMM, there was no seen the development of blastocyst, whereas 5% blastocyst development was reported in the CM in the non-vitrified oocytes (Akbari et al., 2017). In another clinical study, the effects of CM of ADSCs on sperm motility and viability were searched. Oxidative stress-treated and non-treated sperm were incubated with 24, 48 and 72 h of CM for 24 h, and the parameters of motility, vacuolization and DNA fragmentation were ameliorated in the presence of 24 h of CM. Especially, vacuolization and DNA fragmentation of sperm were diminished significantly. This clinical study contains promising results in the case of male infertility (Bader et al., 2018). We only analyzed the effect of CM on oocyte and fertilization, and we found that CM supported the oocyte maturation and quality. At the 9th day, we detected the morula stage in the CM. However, it was seen that CM delayed the oocyte degradation.

CONCLUSIONS

In the other studies and our experiment, the CM was exposed to the oocytes or sperm without isolation of any growth factors. The findings in our study were obtained only as a result of morphological evaluations under the inverted microscope, and they have been supported that the CM from BMSCs may have a beneficial effect in the treatment of infertility. Our study is stated that mesenchymal stem cells and their niche are very important in this process. Because CM has been much more effective than other media in embryonic development which happens until two cells embryo stage to the compaction stage. Although these findings are promising in the future clinical use, the molecular analyzes are needed to fully understand the effects of the CM of mesenchymal stem cells.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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