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INFLUENCE OF HUMAN OVARIAN CELLS ON MOTILITY AND LONGEVITY OF MALE GAMETES

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Abstract: In laboratory practice, the ability of cultured cells to modify the media is used to develop various co-culture systems. This provides the opportunity to investigate the interactions between different cell types and to study the effect of their secretome. In our experimental design, we tested the influence of two types of human ovarian cells (mixed culture of stromal and purified granulosa cells) on the motility and longevity of processed human spermatozoa in a co-culture system. The results point out, that during the first three hours of co-culture, the total motility of the spermatozoa increases in the experimental, as well as in the control groups. After 6 hours, a higher percentage of gametes with retained motility in the co-culture systems has been observed. Both the mixed ovarian and granulosa cell cultures show similar results. Statistically significant increase ($p<0.05$) was detected after 12 hours of incubation with purified granulosa cells. The viability and motility of the spermatozoa were preserved up to 74 hours in the control group, compared to 96 hours for the mixed ovarian cells culture and 110 hours in the granulosa cells. In conclusion, the obtained data suggests that the human ovarian cells modify the culture media by secreting different growth factors and biologically active substances, which have a positive effect on the longevity and motility of the male gametes. This experimental design can be used as a method of evaluation of the functional activity of ovarian cells before and after cryopreservation.

INTRODUCTION

Co-culture systems are widely applied in a number of different analyses in the laboratories engaged in cell biology research. They can be used as a tool for detailed investigation of cell interactions in *in vitro* conditions, to determine the

effect of the physical contact between them, their secretome and its influence on the cell signaling and response (Bogdanowicz and Lu, 2013; Goers *et. al,* 2014). Feeder-layers are implemented in culture of embryonic and induced pluripotent stem cells. They release growth factors into the media, which are not yet fully identified, but facilitate the proliferation of stem cells and prevent their differentiation (Llames *et al,* 2015). In the field of reproductive medicine, the co-culture systems are predominantly used as a method to improve the *in vitro* conditions of preimplantation embryos. It has been established that different somatic cells (endometrial, placental, kidney, fibroblasts, etc.) have a positive influence on the development of the embryos, with a larger number of them reaching to the blastocyst stage (Vithoulkas *et al,* 2017). With the advancement of the assisted reproductive technologies, the interest of scientists is turned towards the ovarian cells. They have a significant importance for the normal function of the ovaries and oocyte maturation (Aghadavodet *et al,* 2015). At the same time, together with the data for the beneficial effect of co-culture with ovarian cells on different biological objects (embryos, oocytes etc.) there are reports that in some cases their use can lead to an accumulation of unwanted growth factors in the medium, which have detrimental consequences (Vithoulkas *et al,* 2017).

In our investigation, we used the co-culture systems as a method to evaluate the functional activity of ovarian cells. In this respect, an appropriate experimental model was the incubation of the analyzed cells with spermatozoa. We examined the influence of human ovarian cells (mixed culture of stromal and granulosa and purified granulosa cells) on the motility and longevity of male gametes.

MATERIALS AND METHODS

Isolation and culture of ovarian cells: Mixed culture of stromal and granulosa cells was isolated from aspirates, obtained during oocyte retrieval in patients undergoing *in vitro* fertilization procedures in In Vitro AG Medical Center “Dimitrov, after receiving an informed consent from the patients After the separation of the cumulus-oocyte complexes the follicular fluid was centrifuged at 2000 rpm for 10 min. The pellet was resuspended and centrifuged again at 200 rpm for 10 min in Ficoll-Paque PLUS (*Pharmacia*) in order to remove the erythrocytes. The cells were then seeded in concentration of 106 cells/ml in flasks and cultured in standard conditions (*Gibko, 2016*). The culture media was DMEM/F-12 (*Lonza*), supplemented with 10% FBS (*Corning*) and penicillin/streptomycin (*Thermo-Ficher Scientific*).

Pure fraction of granulosa cells was obtained after enzymatic digestion (with hyaluronidase) of the cumulus-oocyte complexes, in order to separate the oocyte from its surrounding cells before ICSI.

Preparation of sperm samples: in our investigation we collected 15 ejaculates with normozoospermia in In Vitro AG Medical Center "Dimitrov". To remove the seminal plasma and the leucocytes, we used gradient centrifugation (*SupraSperm System, Medicult*), which delivers a fraction of morphologically normal motile gametes (Bjorndahl L. *et al,* 2010). After flushing with centrifugation with DMEM/F-12, the pellet was resuspended in IVF medium (*Vitrolife*).

Co-culture of ovarian cells with spermatozoa: After the cell cultures have reached confluence, we added the previously processed spermatozoa. Male gametes incubated only in culture media were used as a control. The cells were kept at 37oC, 5.5% CO_2 and the spermatozoa were analyzed on regular intervals with Computer-Assisted Sperm Analysis (CASA).

Sperm motility analysis: the motility of the male gametes was evaluated with Sperm Class Analyser (*Microoptic, Spain*), on regular time intervals (0h, 1h, 3h, 6h, 12h, 24h, 36h, 48h, 72h, 96h, 110h). Standard counting chambers of 10 µl (Leja) were used according to the protocol.

RESULTS AND DISCUSSION

In recent years, ovarian tissue freezing for the so called "fertility preservation" is becoming more frequently recommended for women with diminishing ovarian reserve, oncological patients with impending chemo- or radiotherapy and others (Anderson, *et. al.* 2017). The cryopreservation process can lead to detrimental functional and morphological changes in biological objects. In this aspect, it is quite important to study the influence of the freezing procedure on the biological properties of ovarian cells, because of their crucial role for the normal follicular development and oocyte maturation in the subsequent transplantation or *in vitro* culture of the thawed tissue.

In our experiments, during the primary culture of mixed ovarian cells we have observed both epithelial and fibroblast morphology. That heterogeneity is due to the fact that different somatic cells – stromal, epithelial, granulosa, theca (Lai *et al,* 2015) are presented in the follicular fluid. The granulosa cells are characterized by epithelial-like shape. During long-term culture, they dedifferentiate and that process leads to the acquisition of a fibroblast morphology (**Fig. 1**). Their cytoplasm is rich in granules, which is most likely due to increased secretory function. In the culture conditions that we used during the current investigation, the ovarian cells tended to form colonies and possessed average proliferative activity. Twenty four hours after their seeding, we were able to observe their adhesion to the culture dish. Confluent monolayer was detected after 12-14 days in both types of cultured cells. After subculture, the time for reaching confluency was decreased to 8-10 days.

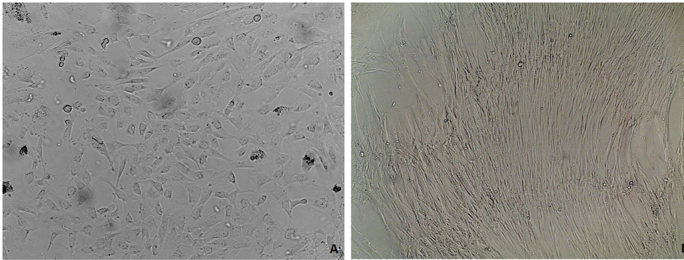

Fig. 1 Culture of ovarian cells.
A: mixed culture (10x), day 3; B: pure granulosa cells (10x), day 9.

The results from the co-culture study of ovarian cells with processed spermatozoa showed increased motility and biological longevity of the gametes. After the first hour of incubation, we were able to note a rise in their total motility both in the experimental and control groups (**Table 1**). That observation can be explained by the spermatozoa hyperactivation, which is evident up to the third hour and is due to the presence of protein molecules in the culture medium. On the sixth hour, we were able to distinguish a tendency for better motility preservation of the gametes in both experimental groups compared to the control. Statistically significant difference was detected after the 12th hour of incubation. The spermatozoa retained their motility up to 72 hours in the control group, whereas with ovarian cells (mixed culture) that period was extended to 96 hours. In co-culture with purified granulosa cells single mobile gametes were observed until the 110th hour. The analysis of the spermatozoa's motility was performed with CASA and a representative sample is presented on fig. 2. The kinetic parameter curvilinear velocity (VCL) is the average speed of the sperm head through its real path, and the average path velocity (VAP) is its mean speed through its average trajectory. A possible explanation for that effect of the ovarian cells is the fact that they modify the medium by secreting growths factors, biologically active molecules, etc. which have a positive influence on the longevity and motility of the spermatozoa.

Table 1 Co-culture system of ovarian cells (mixed culture and purified granulosa cells) with male gametes

	Motility (%)										
	0h	1h	3h	6h	12h	24h	36h	48h	72h	96h	110h
Co-culture (mixed culture)	88,1±1,1	88,9±1,4	89,3±1,1	84,4±2,1	72,3±1,9*	70,5±1,5**	62,9±2,2***	48,2±1,9***	18,1±1,4***	single	0
Co-culture (purified granulosa	88,7±0,7	89,2±1,3	89,5±1,4	86,3±1,8	85,9±2,3**	81,9±2,0**	73,6±1,9***	59,3±1,2***	28,2±1,6***	15,1±2,0***	single
Control	88,4±1,3	88,5±2,2	88,6±0,8	81,2±1,0	69,1±1,2	55,2±2,1	39,8±1,1	21,2±0,9	10,5±1,3	0	0

(***p<0.001; **p<0.01; *p<0.05).

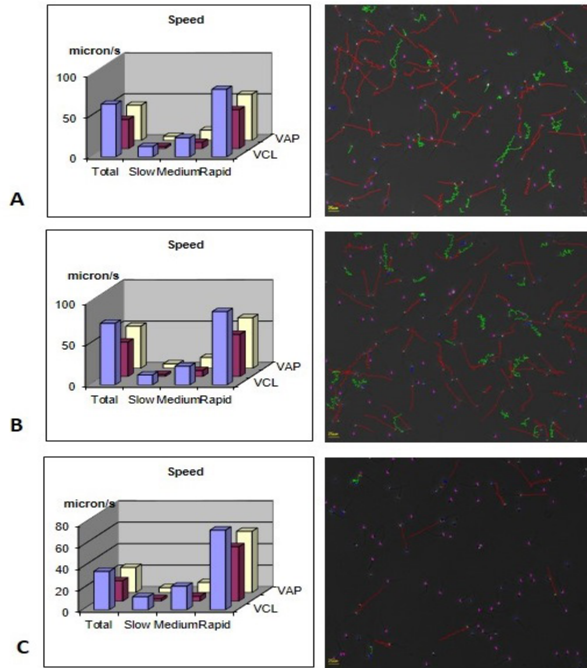

Fig. 2 Co-culture of ovarian cells with spermatozoa after 24 hours:
A: with mixed ovarian cells; **B:** with purified granulosa cells;
C: control group. VCL – curvilinear velocity; VAP – average path velocity.

The purpose of the current investigation was to evaluate to what extend the cell cultures modify the medium and how that affects the longevity and motility of the gametes. Kalthur et al. (2009) have performed similar experiments and point out that the cumulus cells have a positive impact on the spermatozoa, reporting good overall motility (14%) on the 72th hour compared to none in the control group. In addition, the same study showed that hyperactivation and acrosomal reaction can be induced in human male gametes after 6 to 24 hours of co-culture with different types of cells (epithelial from the oviduct, epididimal, endometrial) as well as with follicular or peritoneal fluid. An interesting issue is the interaction between the spermatozoa and the ovarian cells. There are data that during joint culture of spermatozoa and oviduct cells, the gametes adhere to their apical surface (Dobrinski *et al,* 1996). Information exists, that during that period the cilia encompass the tail of the gametes. In our experiments, we were able to visually notice the attachment of a part of them to the monolayer, but the contact was short-lasting. About 10% of them were adherent to the ovarian cells and that proportion remained the same during the whole period of the investigation. Probably, those observations represented weak interactions between the gametes and the cells, because the adherent spermatozoa were relatively easily detached

by pipetting of the medium. It is important to be noted, that the cells cultured in our study did not possess cilia, which makes the interpretation of those results more difficult.

According to published data, the usage of co-culture systems with cumulus cells with human embryos leads to overcoming of the block of division and reduction in the fragmentation in the early stages of embryogenesis (Vithoulkas *et al,* 2017), which helps to increase the percentage of successful pregnancies in patients undergoing IVF procedures (Bhadarka *et. al.,* 2017). It is suggested, that this effect is due to the biologically active molecules – epidermal growth factor, vascular endothelial growth factor A and C, fibroblast growth factor, insulin-like growth factor I and II, different cytokines (interleukin 6,8,1B,24, leukaemia inhibitory factor), prostaglandins and progesterone secreted by the cells (Vithoulkas *et. al,* 2017; Assou *et al,* 2015; Gómez-Torres *et. al,* 2015). Virant-Klun *et. al* (2018) reported that feeder-layers of granulosa cells, isolated from cumulus-oocyte complexes of mature oocytes improve the in vitro development of immature ones. Co-culture systems of this nature may allow more extensive investigation of the mechanisms of oocyte maturation, which is important in infertility treatment of women with polycystic ovarian syndrome, diminishing ovarian reserve, cancers and others (Siristatidis *et al.,* 2015; Revelli *et al.,* 2012). The obtained results during the current investigation, show the functional activity of the ovarian cells and may be used in other co-culture systems, as well as the evaluation of the biological properties of the cells after cryopreservation.

CONCLUSION

The obtained data suggests that the human ovarian cells modify the culture media by secreting different growth factors and biologically active substances, which have a positive effect on the longevity and motility of the male gametes. This experimental design can be used as a method of evaluation of the functional activity of ovarian cells before and after cryopreservation.

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CONFLICT OF INTEREST: The authors declare no existing conflict of interest.

AUTHOR CONTRIBUTION STATEMENT: MH designed the experiments. MH, PS, EH and PT conducted the experiments and analyzed the data. MH and EH wrote the manuscript.

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