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DEVELOPING A METHOD FOR SPERM SELECTION TROUGH CUMULUS LIGANDS

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Abstract: This research focuses on the development of a direct technique for isolation and immobilisation of cumulus ligands on a plastic surface. After ovum pick up, cumulus cells samples from 199 patients, undergoing intracytoplasmic sperm injection (ICSI) were collected and stored at -20° C. All samples were thawed, mixed in a pool and stored at -20° C until their next application. Forty sperm samples from normozoospermic men were used during the process of the optimization of the implemented technique. The maximal number and percentage of the attached human spermatozoa were applied as main criteria for successful isolation and immobilization of cumulus ligands. During the optimisation process four enzymes (collagenase type I, accutase, hyaluronidase and trypsin) were tested for isolation of cumulus ligands. The immobilization of cumulus ligands on plastic surface was compared among eight concentrations and three different temperature regimes (-20°C, 4°C and 25°C). The obtained results showed that the optimal conditions were: (1) digestion of cumulus cells with collagenase type I for 20 minutes at 37°C in an incubator, (2) preparation of a pool of samples from donors and dilution in carbonate buffer to a final concentration of 0.008 cumulus samples/sample at Room Temperature (RT), (3) immobilization (placing) 1µl drops of prepared cumulus solution on the plastic surface at RT and (4) storage at 4°C until the addition of human spermatozoa. The described method for utilization of cumulus ligands on plastic surface might be very useful both for the development of new methods for diagnostics and non-invasive selection of human spermatozoa based on their functional characteristics.

INTRODUCTION

About 30% of couples of reproductive age are affected by infertility, with the male factor accounting for up to 50% of reduced fertility (Fleming *et al.,* 1995; Hassan and Killick, 2003). Despite the rapid development of Assisted

Reproductive Technologies (ART) over the last few decades, live birth rates do not exceed 30% (Neri *et al.,* 2004). A little attention is paid to male factor testing and proper sperm selection before intracytoplasmic sperm injection (ICSI). Assisted Reproductive Technologies involve a variety of procedures for separating spermatozoa from seminal plasma in order to select the most viable gametes, capable of fertilizing the eggs. The main purpose of these techniques has long been to gain a population of motile spermatozoa with suitable morphology, although these parameters are insufficient to evaluate the functional activity of male gametes (Henkel and Schill, 2003).

Nowadays, human sperm selection is limited to methods that do not really select the best spermatozoa. The most commonly applied methods are swim-up and density gradient centrifugation (Nadalini *et al.,* 2014). The density gradient centrifugation and the swim-up method are used as standard preparation techniques (Paasch *et al.,* 2007). These procedures are based on sedimentation and migration of spermatozoa, although molecular events such as sperm apoptosis are overlooked, which may negatively impact the final outcomes (Makker *et al.,* 2008). Swim-up is one of the oldest and most commonly used sperm separation method which is easy to perform and very cost-effective. However, this method is restricted to ejaculates with high sperm count and motility and has relatively low yield (Xue *et al.,* 2014). Also, it induces the production of reactive oxygen species (ROS) as a result of higher spermatozoa density, thus decreasing the percentage of normally chromatin-condensed spermatozoa (Fácio *et al.,* 2016). Density gradient centrifugation is relatively easy to perform and eliminates the majority of leucocytes in the ejaculate but it also has disadvantages, such as poor yield and affected DNA integrity (Beydola *et al.,* 2013).

The aim of our study was to develop an effective sperm selection method based on the communication between human spermatozoa and cumulus ligands immobilised on a plastic surface. Here, we described a new method based on mimicking the selection process observed in physiological conditions.

MATERIALS AND METHODS

The cumulus matrixes (CM) used for the experiment were collected from patients at Nadezhda Women's Health Hospital for the period from January 2018 until June 2018.

The protocol of the study and the informed consent forms have been approved by the IRB committee of the Nadezhda University Hospital and conforms to the ethical principles of the Declaration of Helsinki for medical research involving human subjects.

For each of the embodiments related to optimizing the steps of the method (enzyme treatment of the CM, working concentration, shape of the immobilized area, CM storage conditions and immobilization procedure), the binding activity

of the sperm to the resulting ligands, after a different type of treatment of the ejaculate were compared in 5 to 15 patients/replicates.

Semen samples were obtained from male patients by masturbation after 3-5 days of sexual abstinence.

Isolation of cumulus-oocyte complex

After ovum pick up, the cumulus-oocyte complexes were incubated for 3-5 hours in a culture medium Global for fertilization (LifeGlobal®, USA) under paraffin. They were stored under controlled conditions (37°C, 5.0% CO_2). Separation of the cumulus from the cumulus-oocyte complex was performed mechanically with a glass needle. The cumulus matrix was then placed in a new culture medium Global for fertilization (LifeGlobal®, USA).

Enzyme treatment of the CM

To determine the decomposition of the cumulus complex to cumulus matrix and cumulus cells 4 enzymes treatments were compared. Namely: accutase (MDL MFCD01632905), collagenase type I (EC 3.4.24.3), trypsin (EC 3.4.21.4) and hyaluronidase (EC 3.2.1.35) (Sigma-Aldrich Corporation, S. Louis).

Separation of the CM from the cumulus cells was performed by centrifugation (600G/5min), and then the remaining lumps of cumulus cells were discarded (**Fig. 1**).

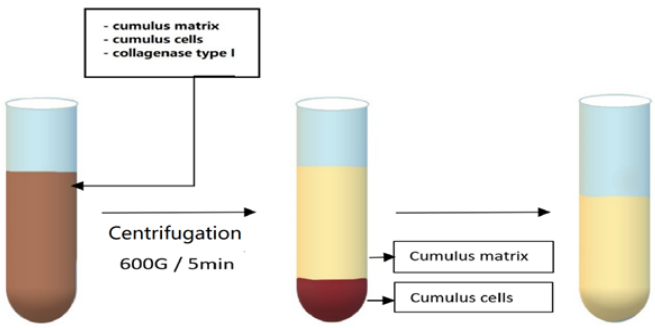

Fig. 1. Procedure for cumulus matrix isolation.

Determination of the protein concentration and working cumulus concentration

The protein concentration in a pool of 199 CM was determined by a colorimetric assay (Bradford *et al.,* 1976).

The optimal ligand concentration for the sperm-adhesion test was determined by comparing the quantity and the distribution of the adhered spermatozoa on

serial dilutions of the CM ligands in the range of 2 to 4096 folds.

The experiment was performed in a 96-well polystyrene plate. After complete immersion of the immobilized ligands at 37°C, sperm were placed on them at a concentration of 1 mg/ml in DMEM F12 medium + 10% HSA treated by the swim-up method (**Fig. 2**).

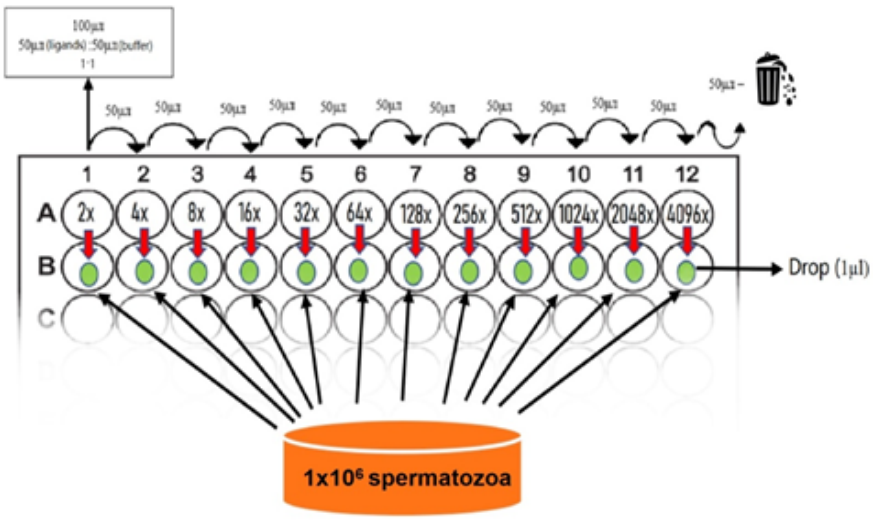

Fig. 2. A diagram illustrating how to perform serial dilutions and determination of the optimal dilution of the cumulus ligands in carbonate buffer.

Comparison of the applied sperm preparation procedures on the cumulus-binding activity

The cumulus-binding activity was compared among 1x106/ml of: native spermatozoa, swim-up treated spermatozoa, density gradient centrifugated spermatozoa and centrifugated & washed spermatozoa (**Fig. 3**). The sperm binding capacity was measured as mean number of attached spermatozoa per 100 µm² cumulus ligands surfaces (CLS).

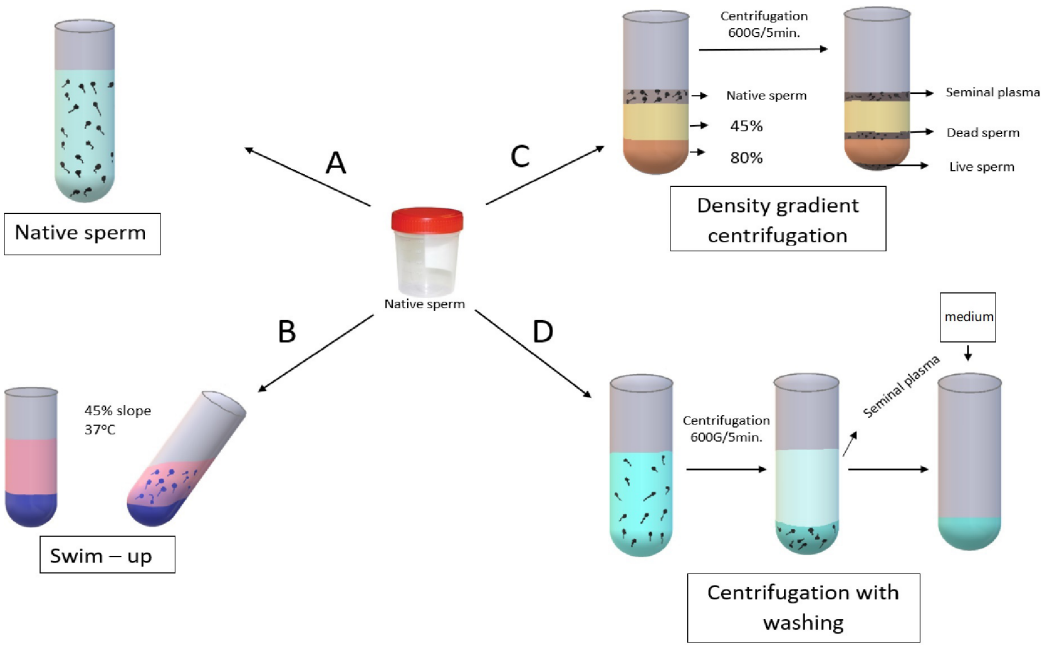

Fig. 3. Description of the compared sperm preparation procedures
A. Native sperm; **B.** Swim-up; **C.** Density Gradient Centrifugation and **D.** Centrifugation with washing.

Determination of ligand storage and immobilization conditions

To determine the best conditions for immobilizing the ligands on the selected surface, droplets with concentration - 0.022 mg cumulus protein/ml were plated onto plastic plates and were allowed to dry under different temperatures (4°C, 37°C and room temperature) for 12 hours.

The optimal storage conditions for the obtained cumulus ligands were compared among 4°C, -20°C and room temperature for 3 days.

Sperm analysis

Spermatozoa characteristics were examined under bright field microscope.

Swim-up

The semen was centrifuged at 600 g for 5 minutes in a conical tube and the pellet was overlaid with DMEM + 10% HSA. After 30 minutes at 37°C the upper layer of the solution, containing the motile spermatozoa, was transferred into a new tube.

Density Gradient Centrifugation

Human semen sample was placed on two-layer gradient solution (80% and 45%) (LifeGlobal, ALLGRAD®), followed by centrifugation at 600 g for 5 minutes.

Selection by immobilized cumulus ligands

The native ejaculate was centrifuged at 600 g for 5 minutes and the pellet was resuspended in the same volume DMEM + 10% HSA. The obtained

spermatozoa were brought to a concentration of $5x10^6$/ml and placed on a plate with immobilized cumulus ligands. The immotile and dead sperm were removed after 30 minutes. The attached spermatozoa were mechanically collected and counted using a MAKLER counting chamber.

RESULTS AND DISCUSSION

Enzyme treatment of the CM

Among the studied four enzymes, collagenase type I appeared to be the most suitable for performing the assay (**Fig. 5**). The mean number of attached spermatozoa was significantly higher (2134 / μm² CLS, $p<0.05$) compared to those attached after treatment with the other three enzymes. The most effective treatment time was 20 minutes. During trypsin treatment of the cumulus (**Fig 4, A**), a complete degradation of the cumulus to cumulus cells and matrix was observed after a period of 40 minutes. Dissociation with accutase (**Fig. 4 B**) and hyaluronidase (**Fig. 4 C**) resulted in final decomposition of the cumulus after 30 minutes.

Accutase and hyaluronidase are often used in cumulus cell isolation procedures by other authors (Leese *et al.,* 1985; Joyce *et al.,* 1999; Latham *et al.,* 1999; Ferrari *et al.,* 2010; Yamatoya *et al.,* 2011). Collagenase type I was isolated from Clostridium histolyticums and exhibits collagenase, caseinase, clostripain and tryptic activities. Collagenase type I is a protease that cleaves the bond between a neutral amino acid (X) and glycine in the Pro-X-Gly-Pro sequence and the peptide bonds of native and triple-helical collagen.

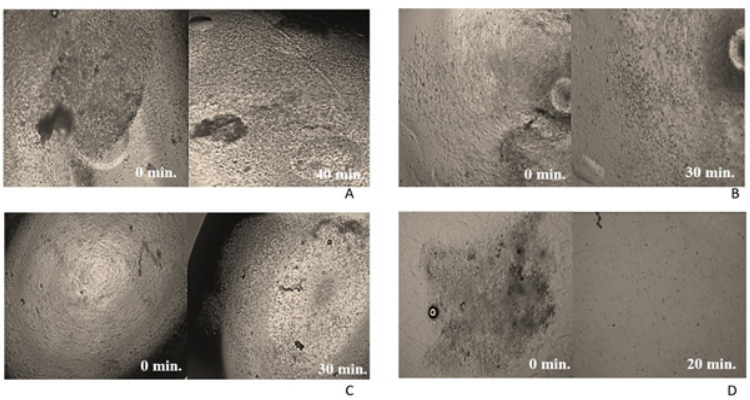

Fig. 4. Bright field microscopic images of CM decomposition after:
A. Treatment with trypsin **B.** Treatment with Accutase **C.** Treatment with hyaluronidase **D.** Treatment with collagenase type **I.** Magnification 40x

The most optimized CM decomposition was achieved after treatment with collagenase type I (**Fig. 4D**). In this experimental setting complete degradation of the cumulus was observed after 20 minutes at 37°C.

The choice of collagenase type I was also confirmed by the results from the conducted sperm binding assay (**Fig. 5**).

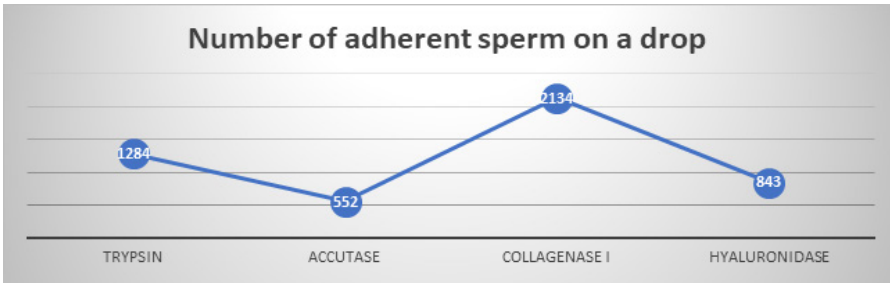

Fig. 5. Number of attached spermatozoa on a differently treated CM.

Protein content and determination of working cumulus ligands concentration

The protein concentration of the CM pool was determined as 2.89 mg/ml (Table 1).

Table 1 Protein concentration of the cumulus samples, measured by Bradford method.

Number of cumulus samples	Protein concentration (mg/ml)	Amount of protein in a cumulus matrix (µg)
199	2.89	14

In determination of the working concentration of the cumulus ligands the number of the attached spermatozoa was taken into account. The optimal dilution of the cumulus ligands in coating buffer was 128 folds, which corresponds to 0.022 mg/ml protein (**Fig. 6**).

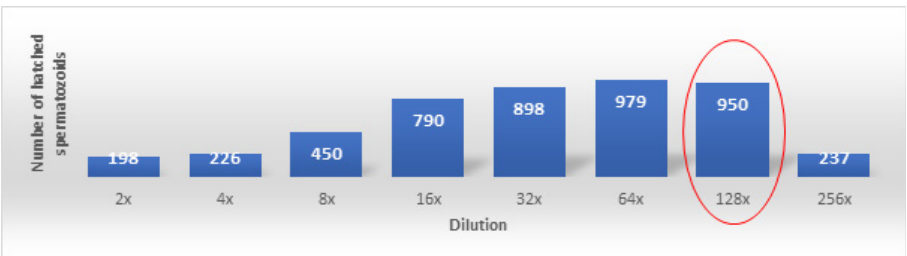

Fig. 6. Number of attached (hatched) spermatozoa on serial dilutions of the cumulus ligands.

Comparison of the applied sperm preparation procedures on the cumulus-binding activity

The applied treatment procedures resulted in different mean number of attached spermatozoa on the immobilized cumulus ligands (Table 2). Untreated spermatozoa showed less adhesion in comparison to the applied three preparation methods (34 / 100 μm² CLS in a drop). In addition, both centrifugation procedures led to significantly lower number of attached spermatozoa compared with the swim-up isolated cells (3214 vs. 213 and 378 spermatozoa / 100 μm² CLS respectively). The observed lower spermatozoa adhesion abilities after centrifugation could be due to the sperm cell damage during the process of centrifugation (Zini *et al.,* 2000) or the lack of ready-to-use receptors to capture the cumulus-matrix ligands (Lin *et al.,* 1994; Kim *et al.,* 2005). Our results revealed that the swim-up sperm separation procedure ensures not only motile but functionally active spermatozoa that are able to attach to the cumulus matrix ligands as in vivo conditions (**Fig. 7**).

Table 2 Number of attached spermatozoa on 100 μm2 cumulus ligands surface (CLS) after different type of sperm treatment

Type of processing	**Number of attached spermatozoa / 100 μm² cumulus ligands surface**
Native sperm	34
Density gradient centrifugation	378
Swim-up	3214
Centrifugation with washing	231

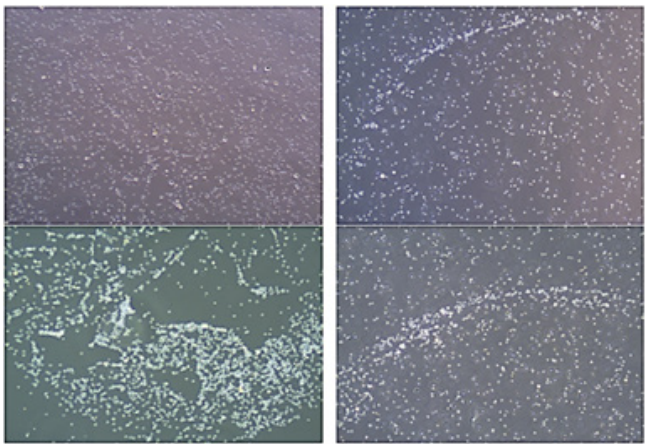

Fig. 7. Adhesion of human spermatozoa after different types of treatment: **A.** Native spermatozoa **B.** Density centrifugation gradient **C.** Swim-up method **D.** Centrifugation with washing. Magnification 100x

An optimal sperm processing technique aims to obtain vivid and functional spermatozoa (Henkel *et al.,* 2003). The centrifugation of the semen induces production of harmful reactive oxygen species (Zalata *et al.,* 1995; Aitken *et al.,* 1988). Therefore, more gentle sperm selection techniques such as density gradient centrifugation and swim-up could be a better choice (Mortimer *et al.,* 1991; Aitken *et al.,* 1995; Oehninger *et al.,* 1995), especially in combination with the following cumulus binding procedures.

Storage conditions

In this study the storage of the cumulus at room temperature resulted in the lowest number of attached spermatozoa – 112 / 100 µm² CLS (Table 3). A slightly higher result was observed after storage of the ligands at 4°C whereas storage at -20°C leads to ten folds more attached spermatozoa (1204 / 100 µm² CLS) (Fig. 8).The extracted cumulus matrix proteins were allowed to interact interfering with sperm-binding cites at room temperature and at 4°C. The collagenase type I optimal temperature was 37°C but it was also active at room temperature and at 4°C. Its action could also impair the sperm-binding cites of the cumulus derived ligands.

Table 3. Number of attached spermatozoa / 100 µm² cumulus ligands surface (CLS), stored at RT, 4°C and 20°C.

Storage conditions			
Conditions	**Room temperature, RT**	**4°C**	**-20°C**
Number of attached spermatozoa / 100 µm² CLS	112	202	1204

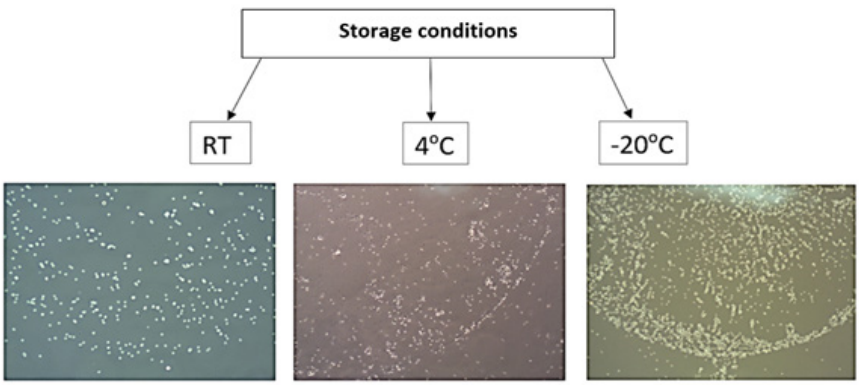

Fig. 8. Distribution of the attached spermatozoa / spermatozoa / 100 µm² cumulus ligands surface (CLS) stored at room temperature (RT), 4°C and 20°C. Magnification 100x

Optimization of immobilization condition

At this stage of the study the immobilization was performed on a polystyrene plate by pipetting 1 µl droplets at final concentration - 0.022 mg cumulus protein/ml. Plates were kept at 4°C for 2-10 days (**Fig. 9**). The lowest number of attached spermatozoa to the cumulus ligands was observed on immobilized cumulus ligands at room temperature (Table 4) and stored at 4°C.

The obtained results showing the optimal immobilization procedure steps for assessment of human spermatozoa cumulus binding capacity are presented on Figure 10.

Table 4 Number of attached spermatozoa spermatozoa / 100 µm² cumulus ligands surface (CLS) in different conditions of immobilization

Conditions of immobilization			
Conditions	**4°C**	**37°C**	**Room temperature**
Number of attached spermatozoa / 100 µm2 CLS	1324	432	84

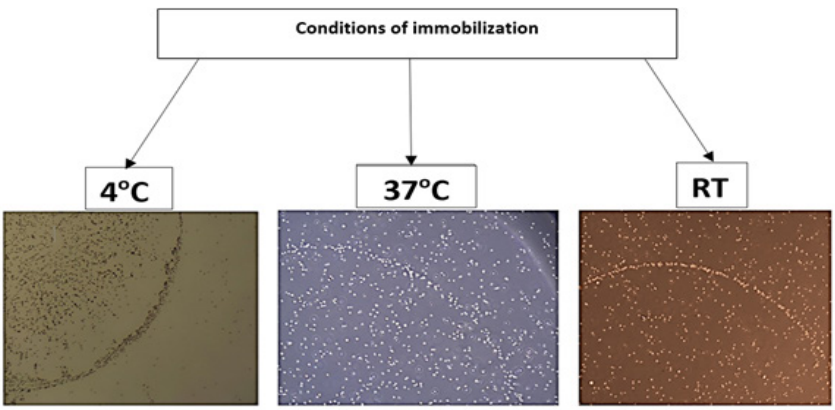

Fig. 9. Number of attached spermatozoa / 100 µm² cumulus ligands surface (CLS) after the applied conditions of immobilization. Magnification 100x

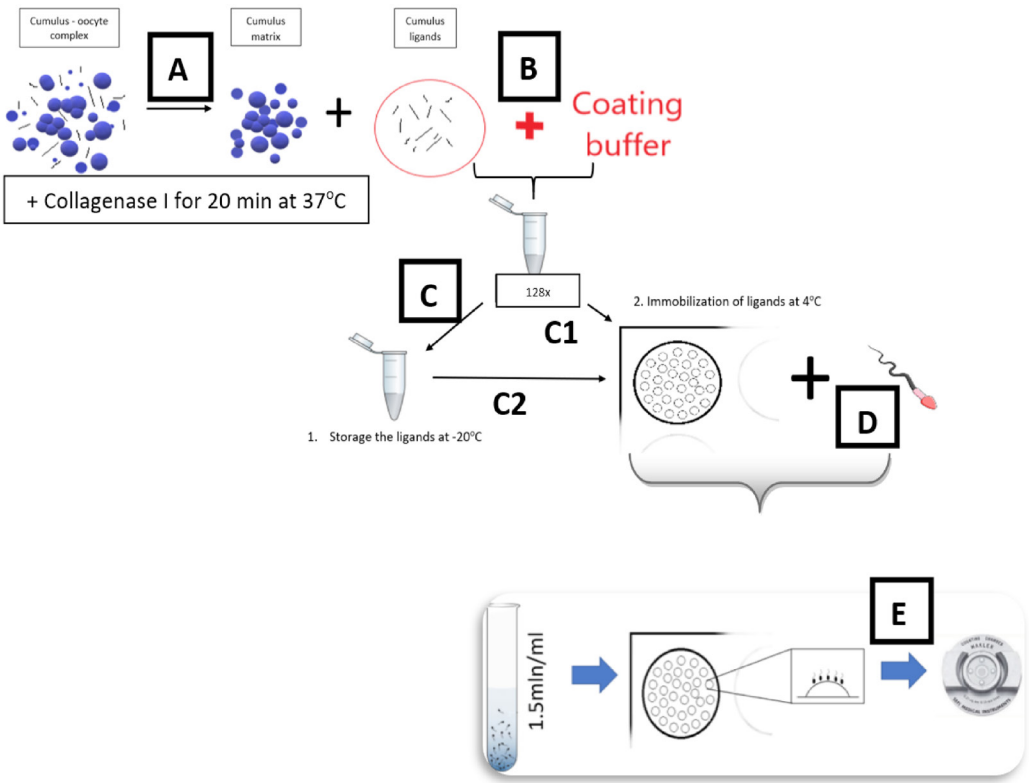

Fig. 10. Schematic representation of the developed method for sperm selection trough cumulus ligands. **A.** Treatment with collagenase type I (Dissociation of the cumulus complex). **B.** Dilution of cumulus ligands in coating buffer (0.022 mg cumulus protein/ml, 128x) **C.** Storage of the ligands. C1-C2. Immobilization of ligands **D.** Adding 1x106/ml spermatozoa in the well. **E.** Detachment and counting of the spermatozoa using MAKLER counting chamber.

CONCLUSION

The described method for utilization of cumulus ligands on plastic surface is simple technique that provides an accurate non-invasive selection of human spermatozoa based on their functional characteristics.

AUTHOR CONTRIBUTIONS

M.G., G.S. and T.C. conceived the experiment; M.G., R.G., K.N., F.S. and T.C. conducted the experiment; M.G., T.C. and D.P. analyzed the results. M.G., R.G, D.P and T.C. wrote the main manuscript text and prepared tables and figures. T.C., G.S. and D.P. edited the manuscript and made its final revision. All authors critically reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Aitken, R.J., Buckingham, D.W., Brindle, J., Gomez, E., Baker, H.W., Irvine, M. 1995. Analysis of sperm movement in relation to the oxidative stress created by leukocytes in washed sperm preparations and seminal plasma. *Human Reproduction*, 10 (8): 2061-2071.
2. Lourenço, B., Sousa, A.P., Almeida-Santos, T., Ramalho-Santos, J. 2014. Relation of Cumulus Cell Status with Single Oocyte Maturity, Fertilization Capability and Patient Age. *Journal of Reprod Infertil*, 15(1): 15–21.
3. Beydola, T., Sharma, R.K., Lee, W., Agarwal, A. 2013. Sperm preparation and selection techniques. In: Rizk, B., Aziz, N., Agarwal, A., editors. Male Infertility Practice. New Delhi: Jaypee Brothers Medical Publishers, 2013. pp. 244–251.
4. Fácio, L., Previato, F.L., Machado-Paula, L.A., Matheus, C.P., Araújo, E.A. 2016. Comparison of two sperm processing techniques for low complexity assisted fertilization: sperm washing followed by swim-up and discontinuous density gradient centrifugation. Cássio. *JBRA Assist Reprod*, 20(4): 206–211.
5. Ferrari, S., Lattuada, D., Paffoni, A., Brevini, T.A.L., Scarduelli, C., Bolis, G., Ragni, G., Gandolfi, F. 2010. Procedure for rapid oocyte selection based on quantitative analysis of cumulus cell gene expression. *J Assist Reprod Genet*, 27(7): 429–434.
6. Fleming, S., Green, S., Hall, J. 1995. Analysis and alleviation of male infertility. *Micros Ann*, 35:37–39
7. Hassan, M. and Killick S.R. 2003. Effect of male age on fertility: Evidence for the decline in male fertility with increasing age. *Fertil Steril*, 79:Suppl 3:1520-1527.
8. Henkel, R.R., Schill, W.B. 2003. Sperm preparation for ART. *Reproductive Biology and Endocrinology,* 1:1-108.
9. Joyce, I.M., Pendola, F.L., Wigglesworth, K., Eppig, J.J.. 1999. Oocyte regulation of kit ligand expression in mouse ovarian follicles, *Devolopmental biology,* 214(2):342-53.
10. Kim, E., Baba, D., Kimura, M., Yamashita, M., Kashiwabara, S., Baba, T. 2005. Identification of a hyaluronidase, Hyal5, involved in penetration of mouse sperm through cumulus mass. *Proc Natl Acad Sci U S A.,* 102(50):18028–18033.
11. Latham, E.K., Dale, F., Bautista, M., Hirao, Y., O'Brien, M., Eppig, J.J. 1999. Comparison of Protein Synthesis Patterns in Mouse Cumulus Cells and Mural Granulosa Cells: Effects of Follicle-Stimulating Hormone and Insulin on Granulosa Cell Differentiation In Vitro. *Biology of Reproduction*, Pages 482–492.
12. Lin, Y., Mahan, K., Lathrop, WF., Myles, DG., Primakoff, P. 1994. A hyaluronidase activity of the sperm plasma membraneprotein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *J Cell Biol.,* 125(5):1157–1163.
13. Makker, K., Agarwal, A., Sharma, R.K., Indian, J. 2008. Magnetic activated cell sorting (MACS): utility in assisted reproduction, *Exp Biol,* 46(7):491-7.
14. Mortimer, D. 1991. Sperm preparation techniques and iatrogenic failures of in-vitro fertilization, *Human Reproduction* vol.6 no 2 pp.173-176.

15. Nadalini, M., Tarozzi, N., Di Santo, M., Borini, A. 2014. Annexin V magnetic-activated cell sorting versus swim-up for the selection of human sperm in ART: is the new approach better then the traditional one? *J Assist Reprod Genet*, 31:1045-51.
16. Neri, Q.V., Tanaka, N., Wang, A., Katagiri, Y., Takeuchi, T., Rosenwaks Z. 2004. Intracytoplasmic sperm injection. Accomplishments and qualms. *Minerva Ginecol,* 56(3):189-96.
17. Oehninger, S., Blackmore, P., Mahony, M., Hodgen, G. 1995. Effects of hydrogen peroxide on human spermatozoa. *Assist Reprod Genet*, 12(1):41-7.
18. Paasch, U., Grunewald, S., Glander, HJ. 2007. Sperm selection in assisted reproductive techniques. *Soc Reprod Fertil Suppl*, 65:515-25.
19. Xue, X., Wang, WS., Shi, JZ., Zhao, WQ, Guo, BZ, Qin, Z. 2014. Efficacy of swim-up versus density gradient centrifugation in improving sperm deformity rate and DNA fragmentation index in semen samples from teratozoospermic patients. J *Assist Reprod Genet,* 31(9):1161-6.
20. Yamatoya, K., Ito, C., Araki, M., Furuse, R., Toshimori, K. 2011. One-step collagenase method for zona pellucida removal in unfertilized eggs: easy and gentle method for large-scale preparation. *Reprod Med Biol.,* 10(2):97-103.
21. Zalata, A., Hafez, T., Comhaire, F. 1995. Evaluation of the role of reactive oxygen species in male infertility. *Hum Reprod,* 10(6):1444-51.