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REPRODUCTIVE BIOLOGY: ORIGINAL ARTICLE

Assessing the Impact of the Novel Sperm Selection Technique 'Annexin-V Coated Polystyrene Bead Technique' on Mouse Assisted Reproductive Techniques Outcomes: Preliminary Findings

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Abstract

ICSI is one of the most commonly used techniques to treat infertility. The sperm selection for the procedure is done 'randomly' by the embryologist according to the motility and morphology parameters which is known not to reflect the potential of a sperm for fertilization, pregnancy and a healthy childbearing. Since the apoptosis rate is higher in sperm cells of infertile patients, it is more likely to choose an apoptotic sperm by the 'random selection method'. We recently introduced a novel sperm selection technique namely 'Annexin-V coated polystrene bead technique'(APB-Tech), for the selection of non-apoptotic sperm cells. The principal of the technique is based on the binding affinity of an apoptotic sperm to 'Annexin-V covered beads' enabling to distinguish a viable and a healthy sperm by light microscopy. The aim of the present study was to observe the effects of this technique on ICSI outcomes in mice. Sibling-oocyte trial was conducted and the outcome measures were compared with the results of traditional sperm selection method. Embryo and blastocyst qualities and blastocyst development rates were significantly increased in APB-Tech group, while the other parameters were not affected. Promising results obtained from the technique reflect its promising potential as a new and powerful tool for sperm selection and thus infertility techniques.

Keywords Sperm selection · ICSI · Infertility · APB-Tech · Embryo

Introduction

Infertility is a worlwide significantly increasing health problem affecting more than 15% of the couples planning to conceive [1]. Intracytoplasmic sperm injection (ICSI) is the most frequently used assisted reproduction technique accounting for 70-80% of the cycles [2] although the success rate cannot exceed 40% [3]. During natural conception, the healthiest sperm and oocyte are naturally selected to obtain the healtiest offspring. However, if the pregnancy is achieved via ICSI, the offspring may face certain health risks possibly as a result of bypassing these mechanisms. A recent meta-analysis reported an increased overall risk of chromosomal abnormalities in ICSI compared to both standard IVF (OR 1.42 (95% CI 1.09-1.85)) and natural conception (OR 2.46 (95% CI 1.52-3.99)) and an increased risk of de novo anomalies in ICSI compared to natural conception (OR 2.62 (95% CI 2.07-3.31)) in the newborn [4].

Apoptosis is an important physiological process that eliminate damaged or abnormal cells of body including the gametes [5]. Approximately 75% of the spermatogonia die via apoptosis during spermatogenesis [6, 7]. Sperm cells are more prone to DNA damage because of their limited repairing capacity [8, 9] which trigger apoptosis to prevent the offspring. As a result, DNA damages are observed more frequently in sperm cells than in oocytes [10]. However, if apoptosis is triggered in a cell, no visible and detectable markers are represented until the end of the process,which makes it impossible to visually recognise or distinguish [11].

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Since the fate of ICSI is directly correlated with the quality of gametes among many other contributors, it is of great importance to select most compotent gametes without any type of abnormality [12]. However, gamete selection can only be applied to sperm cells because of the limited number of oocytes (~15) [13].

The most common sperm selection methods are based on choosing the "best looking sperm" randomly by the embryologist with the best morphology and motility which is not enough to reflect the potential of a sperm for healthy fertilization, pregnancy and childbearing [14]. Besides, since sperm DNA fragmentation rate is known to be higher in infertile patients [12], it is more likely to choose an apoptotic sperm with fragmented DNA by this selection method. A meta-analysis reported a 7.3 times higher pregnancy rate when sperm DNA fragmentation index (DFI) is less than 30% [15]. In addition, sperm freezing, which is a commonly used auxiliary method to preserve fertility, is known to cause apoptosis and DNA breaks due to the cryodamage [16, 17].

In the light of these data, it is of great importance to be able to choose the healtiest sperm, which is more likely to have the highest potential for a healthy pregnancy and offspring during ICSI.

Various techniques such as swim-up (SU), density gradient seperation (DGS), microfluidics [18], magnetic cell separation (MACS) [19], ultra-high magnification (IMSI) [20], hyaluronic acid binding [21], electrophoresis [22] and artificial intelligence applications [23] are available for sperm selection in the IVF laboratory, which have various advantages and disadvantages over each other. Most of these techniques only consider morphology and motility parameters of sperm, ignoring the DNA fragmentation parameter [24], which is associated with embryo quality and miscarriage rates [14]. Among these, the most commonly used techniques are SU and DGS [25], which are reported not to provide a sperm population with an acceptable level of intact DNA [26–28]. Hyaluronan binding assay tests the maturity of the sperm cells [21] while microfluidics applications [18] and artificial intelligence [23] evaluate the morphology and motility parameters. Among a few technique developed for apoptotic sperm selection [19, 22], none of them could find place in IVF labs because of their need for extra and expensive equipments, training, and high costs. In all of these techniques, a sperm population that is more likely to be healthy is obtained and sperm selection is done randomly from this population and none of them ensure to select a 'single' and a 'viable' sperm cell for microinjection.

Recently, our group developed a novel technique to eliminate apoptotic sperm cells namely 'Annexin-V coated polystrene bead technique'(APB-Tech) which is patented by Turkish patent and trademark institute with an access number of E-39616753-110-220554024 (28.07.2022).

The technique's principal is based on the binding ability of Annexin-V covered polystrene beads to the phosphatidylserine that is externalized at the outer leaflet of an apoptotic sperm's plasma membrane. The technique is optimized, and tested for validity and toxicity which were reported previously [29].

The aim of the present study was to analyse the effects of this novel technique on ICSI outcomes in mice. It will be faving the pathway for human use and thus will help to increase the outcome parameters and success rates of ICSI.

Materials and Methods

Ethical Statement

The present study was conducted in accordance with the ethical standards of the Declaration of Helsinki and national guidelines. The Ethics Committee of Istanbul Medipol University (38828770e604.01.01 E.55888, October 9, 2019) approved the study.

Study Design

A randomized sibling-oocyte trial was conducted in order to evaluate the effect of APB-Tech solution when compared with traditionally selected spermatozoa. Randomization was performed during oocyte pick-up and the cumulus oocyte complexes (COCs) were collected to different dishes. Oocyte denudation were performed with hyaluronidase and pipetting.

Animals

12 female and 12 male BALB/c mice obtained from Medipol University Experimental Animal Research and Application Center (MEDİTAM), were used for the study. Ovarian stimulation were applied to female mice and sperm retrieval to male mice. All mice were kept in an environment with a temperature of 22 ± 2 °C, a room humidity of 50 ± 2% and a light/dark cycle of 12/12 h and were fed Ad libitum with a standard diet. Weight of all mice were recorded on the first day of the study. Female mice who gained ≥ 10% weight, were discarded in order to eliminate mice with ovarian hyperstimulation syndrome (OHSS).

Sperm Retrieval

Sperm retrieval were performed from male mice as previosly described [29]. Mice were euthanized by CO_2 inhalation. After cleaning the genital area with 70 % alcohol, a scrotal incision of 1 cm is cut via a lancet followed by the testis removal (Fig. 1A). Testicles were placed in a culture dish

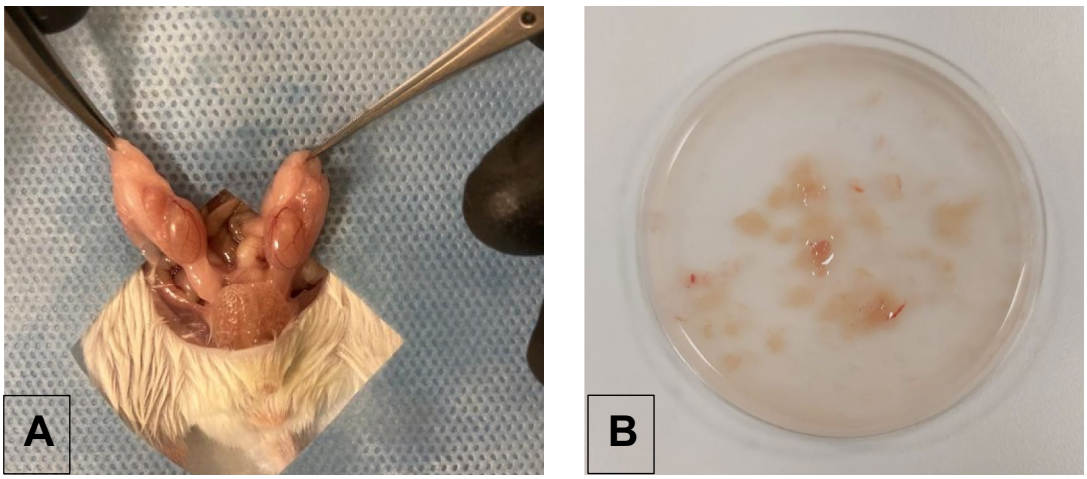


Fig. 1 Sperm retrieval procedures are demonstrated **A**. Testis removal **B**. Testicular tissue cut into pieces are demonstrated

filled with pre-warmed (37°C) HEPES buffered G-Gamete (Vitrolife, Sweden) (60 mm , Falcon 353004, BD, USA) and cut into pieces mechanically by an insulin syringe (Fig. 1B). All surgical procedures were performed under sterile conditions. The tissue extracts were collected in a conical based test tube (Falcon 352095, BD, USA), centrifuged for 10 min (300 G), and suspended in 2 ml of the same media. Sperm parameters were evaluated by light microscopy (Nikon E400 ; Niko n Instrument Group, USA) and samples were kept in 37 °C incubator (J.T.Baker, 7722-84-1, Thermo Fisher Scientific, Oslo, Norway) until use.

Preparation of APB solution

APB solution is prepared as previously described [29]. Briefly, 200 µl (1 mg) of bead suspension, (streptavidin polystyrene, 3.11 µm, Spherotec Inc, USA) were washed according to the manufacturer's instructions. The solution is suspended in 400 µl of binding buffer (PBS + % 0.05 BSA + CaCl2, pH 7.4) and centrifuged at 1.5K for 20 min X 4. Five µg biotinylated Annexin-V (Annexin A5, Sino Biological Inc. Eschborn, Germany), per mg of beads is mixed with beads and incubated at room temperature for 30 min. The solution is then centrifuged and washed four times in PBS/ BSA and is resuspended with PBS/ BSA. The solution is stored at 4 °C.

Sperm Preperation and Selection

Neat semen were first preparaed by density gradient centrifugation to eliminate dead, non-motile sperm and seminal plasma as described previously [29]. Shortly, after washing, sperm concentration was adjusted to 5 ml/mL. For the study group, 10 µl of sperm solution was mixed with 10 µl of annexin-V coated bead solution (APB sol) and incubated for 30 minutes at 37°C heated stage. After the incubation, sperm cells with no bead attached on any part of them were collected with a microinjection needle. The bound and unbound sperm images are demonstrated in Fig. 2. The sperm were washed sequentially 3 times in different medium drops (10 µl) and finally transferred to a 5 µl of polyvinylpolypyrrolidone (PVP) drop to be immobilised. For the control group, 3 µl of sperm sample is added onto a 5 µl drop of PVP (ICSI, Vitrolife) solution for immobilisation in a microinjection dish (Falcon 1006, BD, New York, USA) (Fig. 3).

Ovarian Stimulation and Oocyte Collection

Female mice were given subcutaneous injections of PMSG (10 IU) on 2 consecutive days, following an 30 IU of hCG administration on day 3 to induce ovulation. Mice were sacrificed 26 h post-hCG injection for oocyte collection. The ovaries and fallopian tubes were removed and washed with prewarmed G-MOPS media (G-MOPS, Vitrolife, Sweden). Ampulla regions of both side were isolated and the oocytes were collected by making a small incision in the ampulla (Fig. 4A,B,C,D) and by dissecting the ovaries under a stereo microscope. Retrieved oocytes were collected in a prewarmed (37°C) MOPS buffered media (G-MOPS, Vitrolife, Sweden) under mineral oil (Ovoil, Vitrolife), (Fig. 4E and F).

Two hours after the oocyte pickup (OPU) procedure, the oocytes were denuded by pipetting the COCs in 40 IU hyaluronidase (HYASE, Vitrolife, Sweden) via 2 different sized denudation pipettes (170–140 µm resp.). Oocytes were then washed 3 times in G-MOPS and placed in a culture dish containing 25 µl of G-IVF drops (Vitrolife, Sweden). The culture dishes were put in a CO_2, O_2, N_2

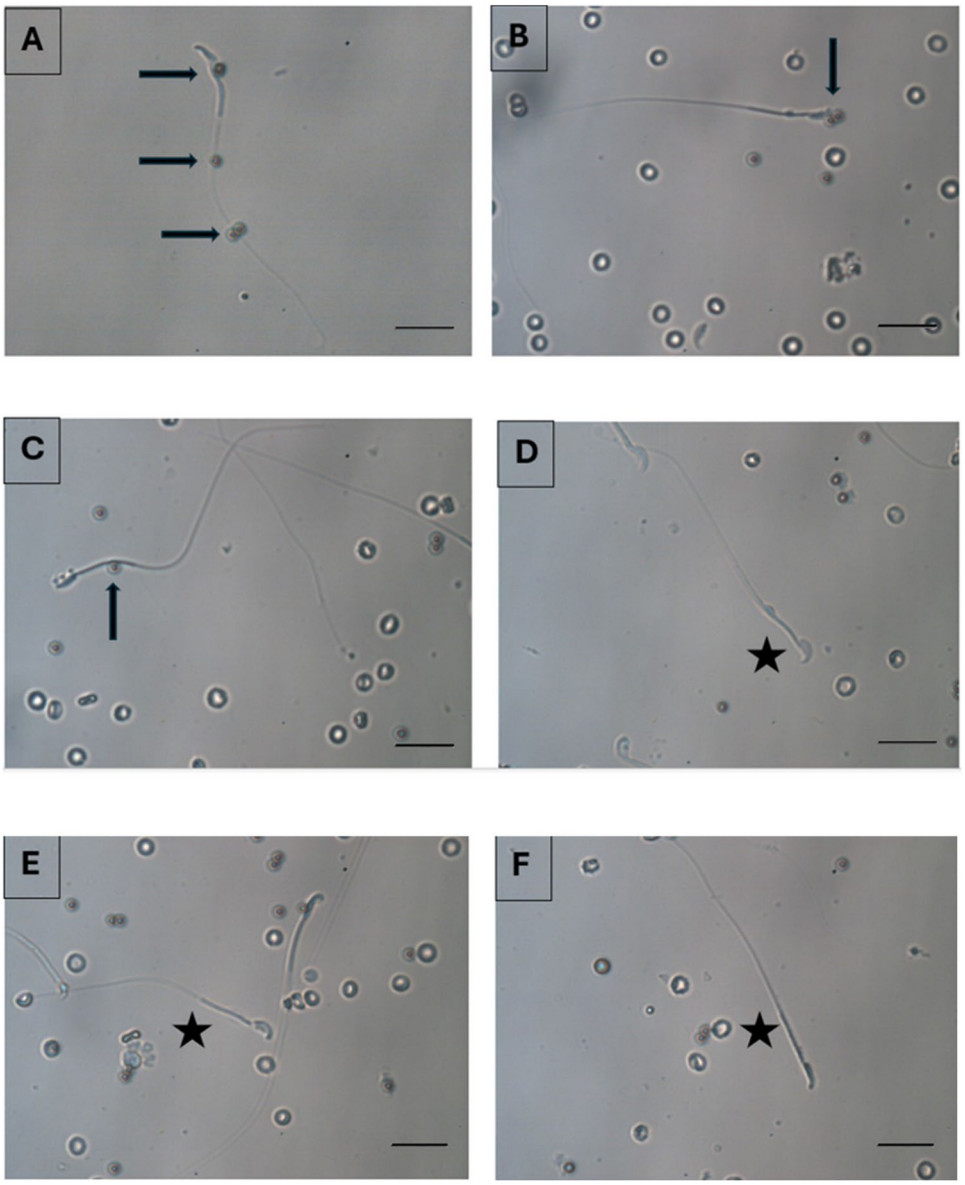


Fig. 2 Pictures after the sperm suspension is exposed to the APB solution including the beads. Arrows (➡) show bead bounded sperm cells with DNA fragmentation, asterix (★) show unbound sperm cells with intact DNA (X40)

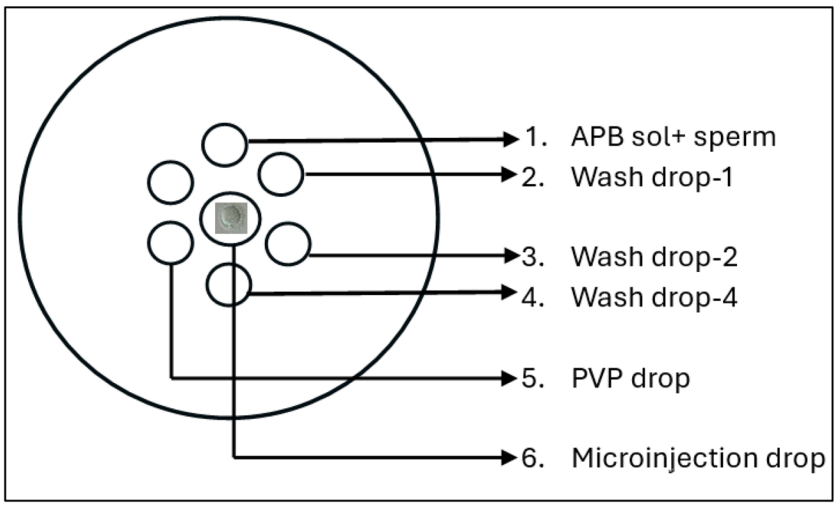


Fig. 3 Sperm selection steps via APB-tech were demonstrated on a microinjection dish for the microinjection (ICSI) procedure

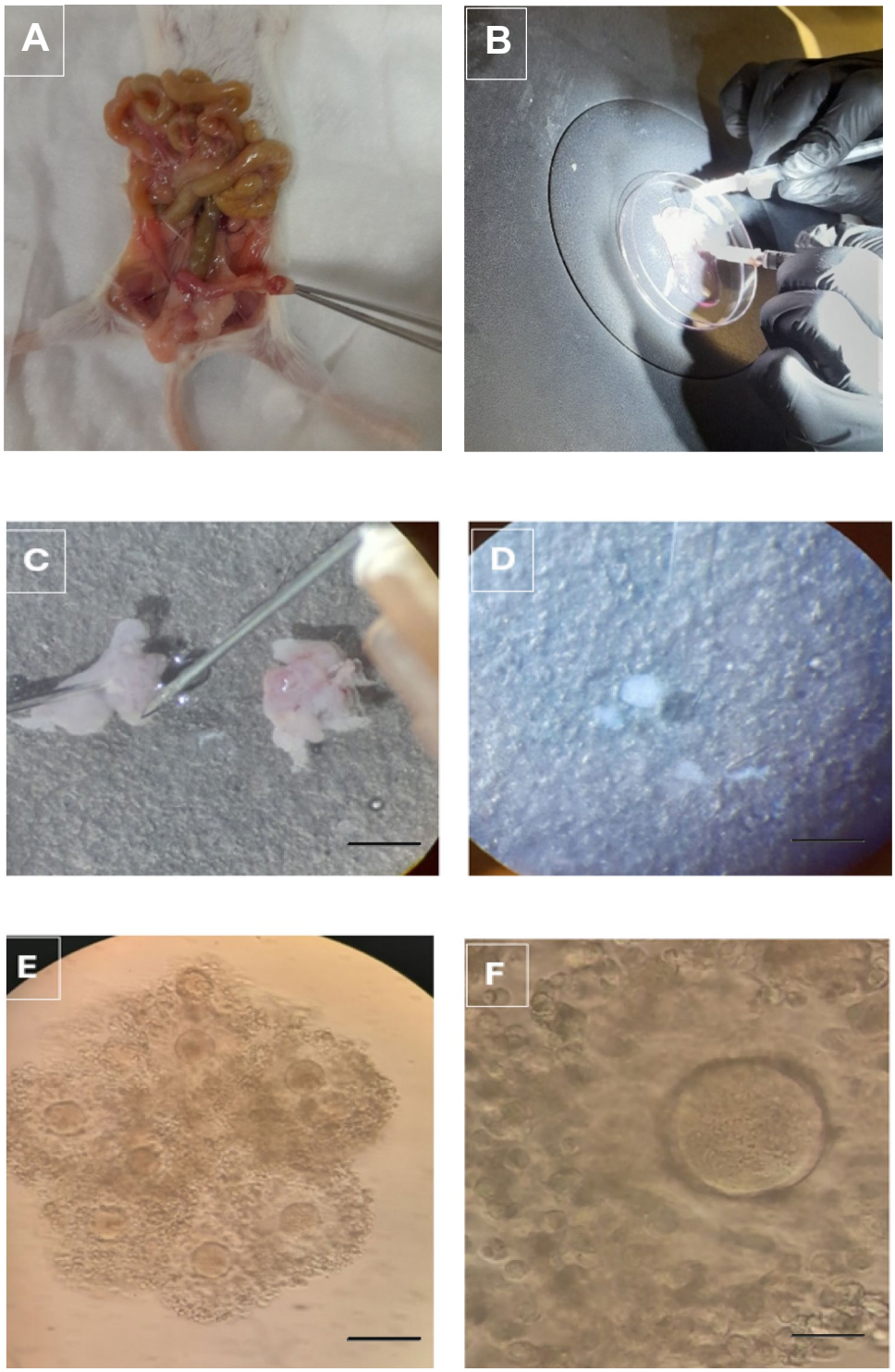


Fig. 4 The ovary and Fallopian tubes are demonstrated after mouse decapitation (**A**). Ovary and Fallopian tubes are isolated and dissected under a stereo microscope (**B**). Ampulla region (**C**) and the contents of the ampulla shed after the rupture of the ampulla region (**D**). Cumulus-oocyte complexes (COC) are demonstrated (**E**). Photographs of an oocyte cell surrounded with corona radiata cells are demonstrated (X40)

controlled and humidified incubator for culturing. Nuclear maturation of the oocytes was assessed with an inverted microscope (X40). Oocytes with a polar body (PB) and oocytes that extrude their polar body within 4 h after denudation were defined as a mature oocyte. Oocytes with no polar body or with a germinal vesicle were defined as immature oocytes (Fig. 5) [30].

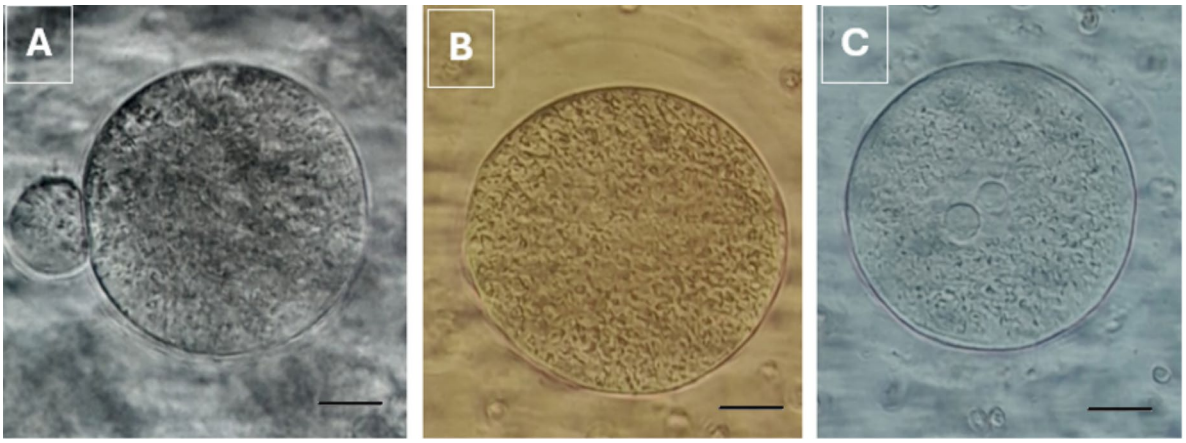


Fig. 5 Oocyte cells after denudation are demonstrated at different stages of maturity. A mature oocyte in the Metaphase 2 (MII stage) (Polar Body is shown by the arrow) (**A**), an immature (immature) oocyte in the Metaphase 1 (MI stage) where the polar body is not observed (**B**), and an immature oocyte in the germinal vesicle (GV stage) shown by the arrow (X40) (**C**)

Microinjection (ICSI)

Mature oocytes were microinjected with a sperm as described previously by Van Steirteghem et al [31]. Shortly, after adjusting the polar body location to clock 12:00 position, and stabilizing the oocyte by aspirating with a holding pipette, an immobilised sperm is injected to the oocyte cytoplasm with a spiked microinjection pipette, a piece of cytoplasm is absorbed and the sperm together with the cytoplasm is released back to the oocyte cytoplasm (Fig. 6).

Embryo Culture and Evaluation

Further culture of injected oocytes was performed in 25 mL microdrops of culture medium (IVF, Vitrolife, Sweden) under light paraffin oil (OVOIL, Vitrolife, Sweden). All embryos were cultured for 5 days in a 37°C humidified incubator. Fertilization was evaluated 18 h after microinjection. Presence of two pronucleus and two PBs were accepted as signs of normal fertilization (Fig. 7). Fertilization rates were calculated as the number of fertilized oocytes / the number of mature oocytes ×100. Oocytes showing signs of degeneration (dark colour, shrinkaged cytoplasm) were considered as degenerated oocytes (Fig. 8).

Fertilized oocytes were transferred to fresh drops of culture medium (G1) covered with mineral oil. On day 2, 3, and 5, morphologic evaluation were performed. Embryos with less than 10% fragmentation and 2-4 evensized blastomeres without any vacuolization, granulation, and multinucleation on Day 2, seven to eight evensized blastomeres on day 3 and a visible intact outer cell mass with an intact, homogeneous inner cell mass on day 5 were considered as top quality embryos and blastocysts respectively and the rest as bad quality embryos and blastocyts respectively (Fig. 9). The percentage of top quality embryos were calculated as the number of top quality embryos on day 2,3,5 / the number of embryos transferred ×100.

Statistical Analysis

SPSS statistical program (Version 22) was used to analyze the data. Data were expressed as mean ± standard deviation (SD). Differences between the groups were evaluated by t-test. All tests were interpreted with a significance level of 95% ($p < 0.05$).

Results

The results obtained for 8 parameters among 17 indicators of ART laboratory indicators accepted by ESHRE Special Interest Group of Embryology, (Vienna 2017) and additional parameters analysing the embryo qualities on 2 nd and 3 rd day were demonstrated in Table 1 and 2 respectively.

Good quality embryo (Total, Day 2 and Day 3) rates (p=0.045, 0.047, 0.038 resp), good quality blastocyst rates (p=0.047) and blastocyst development rates (p=0.036) were significantly higher in APB-Tech group (Fig. 10), while there were no difference for abnormal fertilization (1 ronucleus) rate (p=0.238), oocyte degeneration rate (p=0.453), normal fertilization rate (p=0.569), cleavage rates (p= 0.651), 2 nd and 3 rd day embryo development rates (p=0.752, p=0.644).

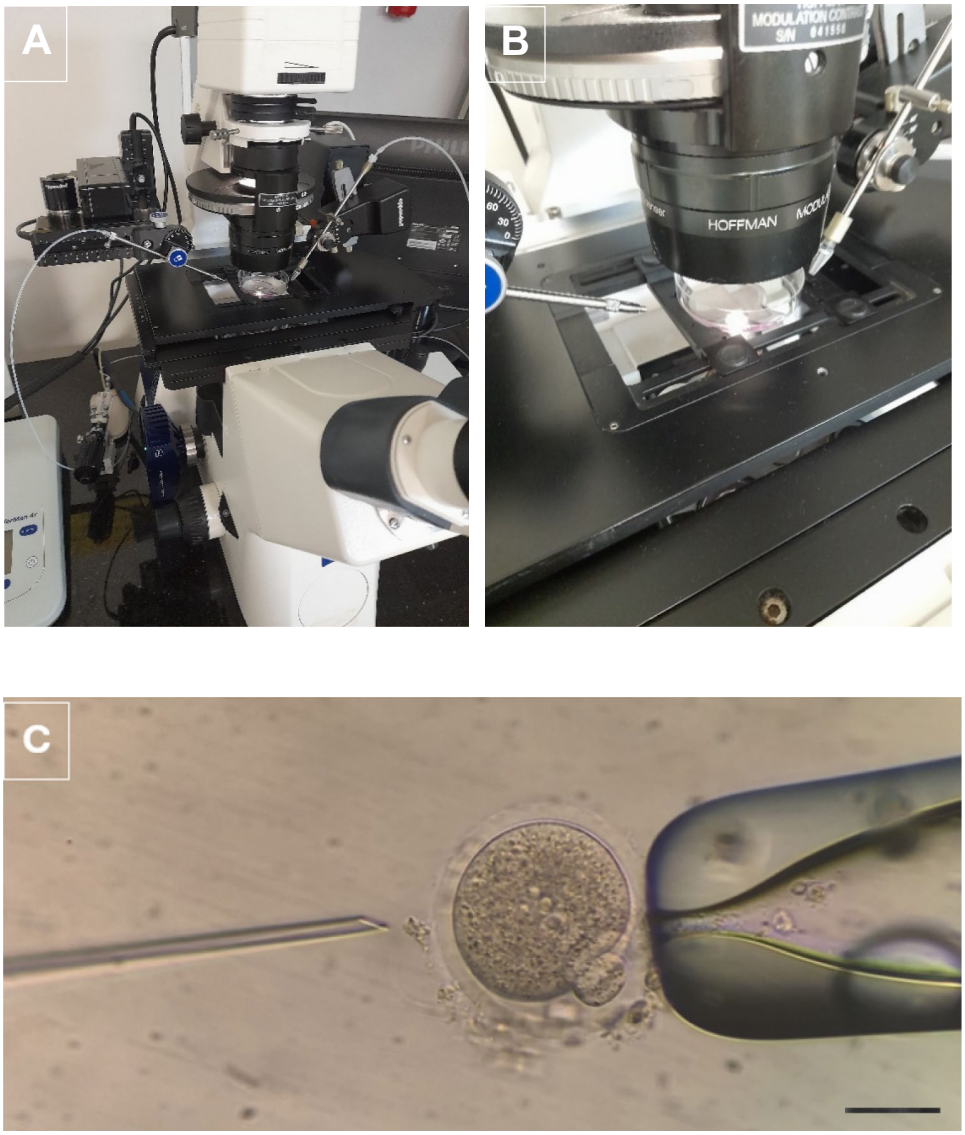


Fig. 6 The micromanipulator that the microinjection process is performed (**A**), the dish in which the oocytes are placed during the microinjection process (**B**), and the microinjection process (**C**) are demonstrated (X20)

Discussion

Contribution of the sperm quality on IVF success lead to a need for developing novel techniques to choose better sperm cells with the highest 'competency'. Routinely used sperm selection techniques in IVF labs suggest to provide a healthier 'sperm population' that are based only on the motility and morphology patterns of the sperm, which is reported not to reflect the overall fertility potential of sperm cells [32, 33]. Sperm DNA fragmentation, a marker of apoptosis, is reported to be significantly associated with, embryo quality [34], pregnancy and miscarriage rates [35, 36] and ICSI outcome [37]. It has been reported that, the two most commonly used sperm selection techniques (swim-up and DGS) do not decrease the rate of DNA fragmentation level of a sperm population to an acceptable level [33].

There are more advanced techniques for apoptosis detection such as electrophoresis [22] and MACS [19] which can only be used for diagnostic purposes or other techniques such as IMSI [20] and hyaluronan binding [21] which do not offer to select apoptotic sperm. Diagnostic methods include fixation, etc., which restrict the usage of the sperm for ICSI procedure. A similar method, magnetic activated cell sorter (MACS), provides a sperm suspension with a relatively reduced rate of apoptosis instead of a single sperm [19]. In addition, the effect of the magnetic field on sperm cells and the stress that sperm cells are exposed to during these processes may be detrimental [38]. All these techniques are rarely used in IVF laboratories because of high costs, and the need for extra equipment and training while none of those ensure the selection of a 'single, and a 'viable' sperm cell devoid of apoptotis which 'can' be used for microinjection.

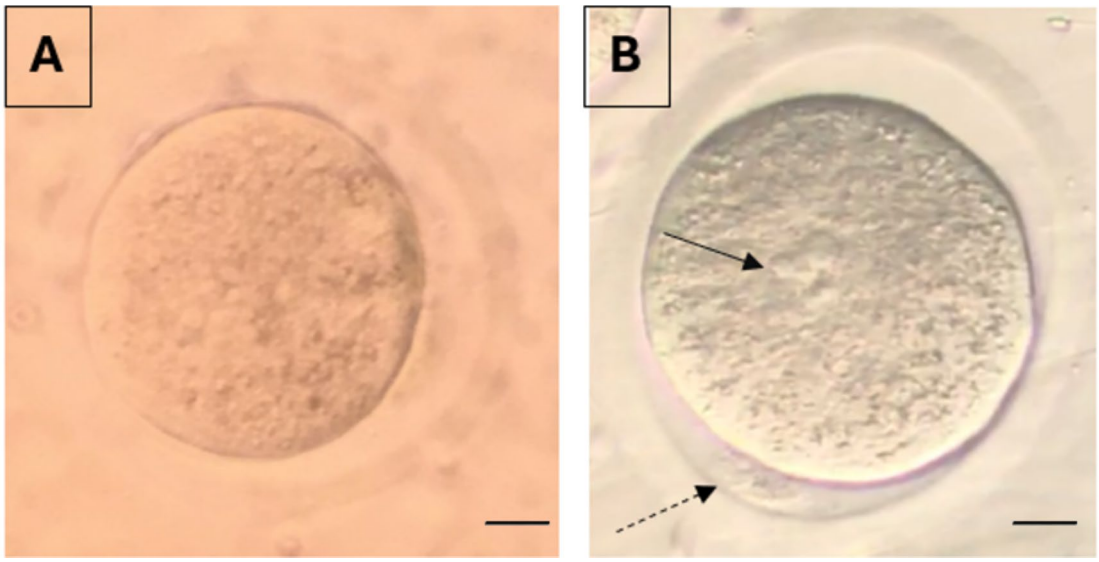


Fig. 7 A non-fertilized (**A**) and a fertilized oocyte (**B**) with 2 pronuclei (arrows) and 2 polar bodies (dashed arrows) are demonstrated (X40)

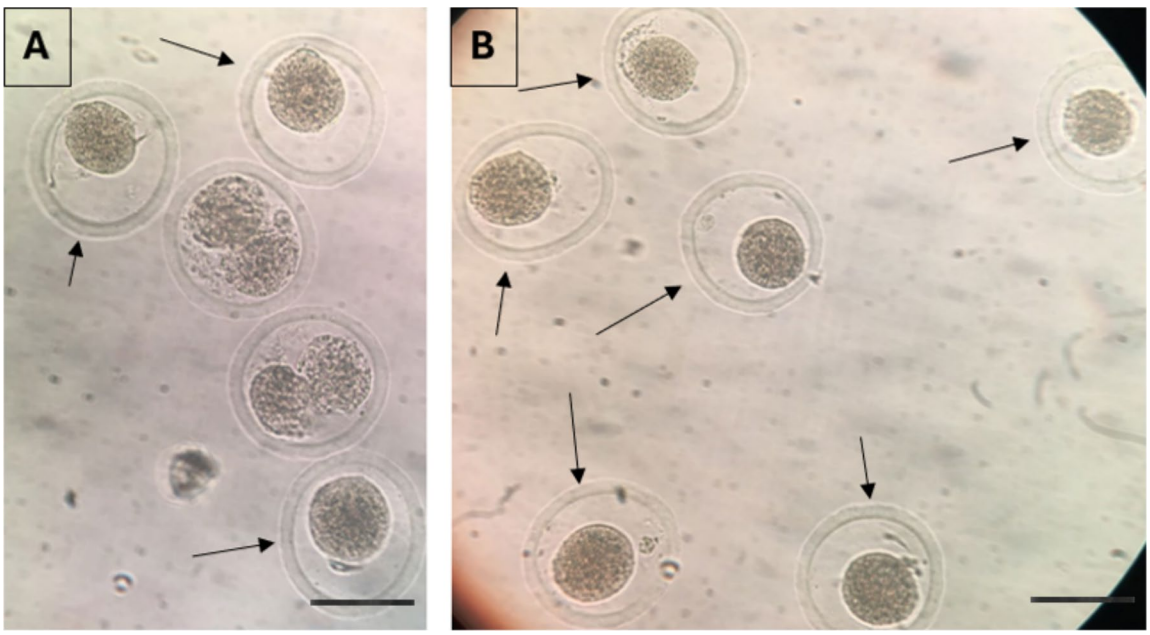


Fig. 8 Degenerated oocytes (**A**, **B**) (cytoplasms are dark colored and retracted) are demonstrated (X20)

Recently, we introduced a new, easy and a reliable technique, APB-Tech, which enable IVF professionals to diagnose and select 'a non-apoptotic and a viable sperm for microinjection' via light microscopy [29]. This technique is based on the principle that Annexin-V can bind to phosphatidylserine (PS) located on the outer leaflet of the plasma membrane in apoptotic cells as an early apoptotic marker [29]. The technique can be used either for diagnostic purposes or interventional, and is user-friendly allowing users without any need for training or extra equipments. Besides, sperm cells are prevented against different kinds of extrinsic stress that can be triggered in various processes and environments.

To test the accuracy and reliability of the APB tech we compared the DNA fragmentation levels of APB-Tech with two reliable and well-known methods (SCSA, TUNEL), and found statistically similar results (13.51% for APB, 15.56% for SCSA and 17.27% for TUNEL, $p>0.05$) in our previous

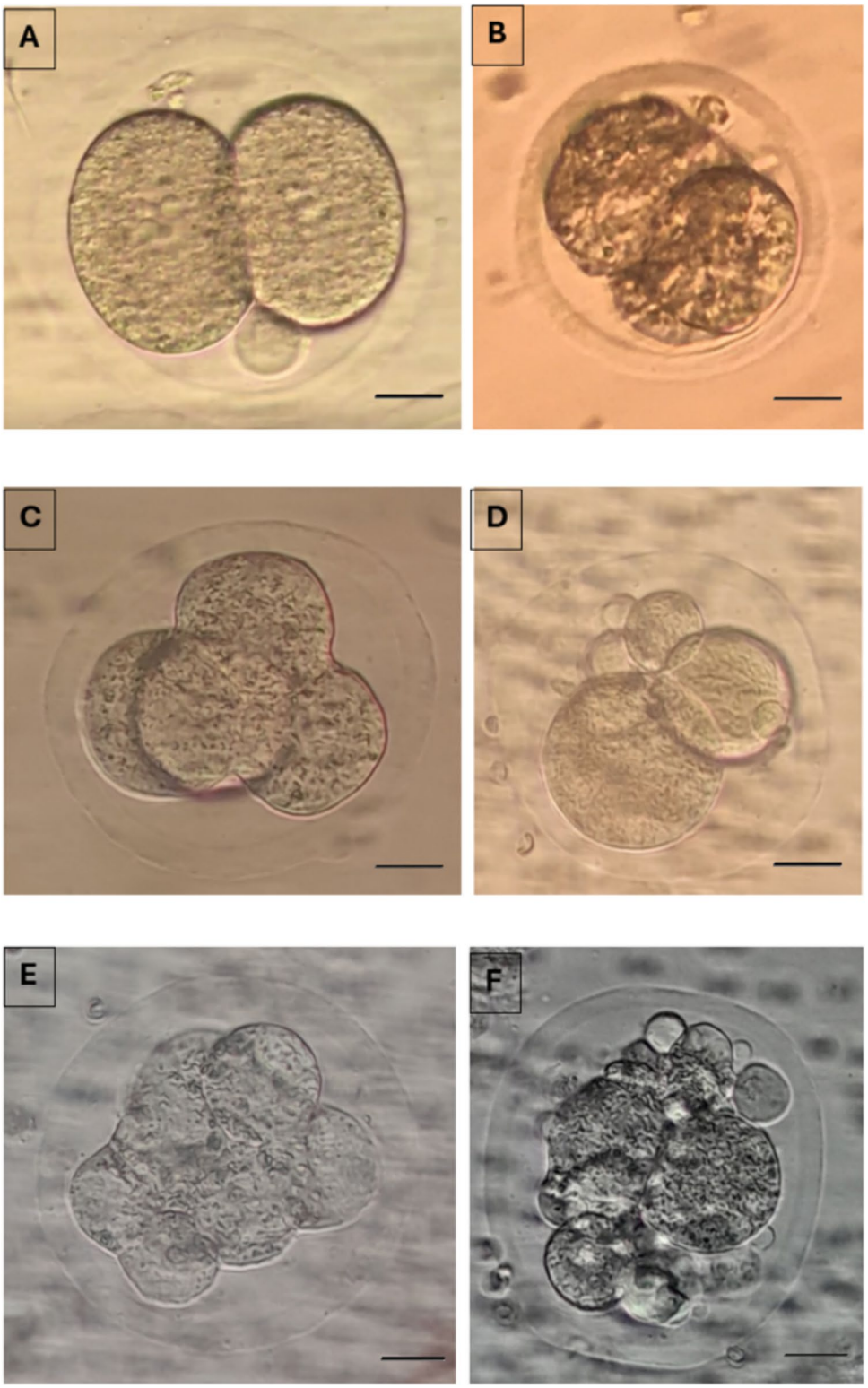


Fig. 9 2-cell good quality (**A**) and poor quality embryo (**B**) at 24 hours; 4-cell good quality (**C**) and poor quality embryo (**D**) at 48 hours (Day 2); 8-cell good quality (**E**) and poor quality embryo (**F**) at 72 hours (Day 3) and, compacting embryos (**G**) and compact embryo (**H**) at the 96th hour (Day 4); Cavitating embryo (**I**; **J**) at 100 hours; Good quality (**K**) and poor quality blastocyst stage embryos (**L**) at 120 hours and, a hatching embryo (**M**) at 130 hours is demonstrated (X40)

study [29]. We then performed sperm survival test (SST) for toxicity testing which is one of the most commonly used technique to test the toxicity of any solution, medium and consumables to be used in in vitro fertilization [39]. Sperm survival rates (total motility and motility subgroups rates) for all tested time intervals (2, 4, 6, 8, 24, 48 hours) were statistically similar with the control group proving the reliability of the technique without an adverse effect [29].

In the recent study, as the next step, we tested the technique's effect on IVF outcome which was the main goal of

Fig. 9 (continued)

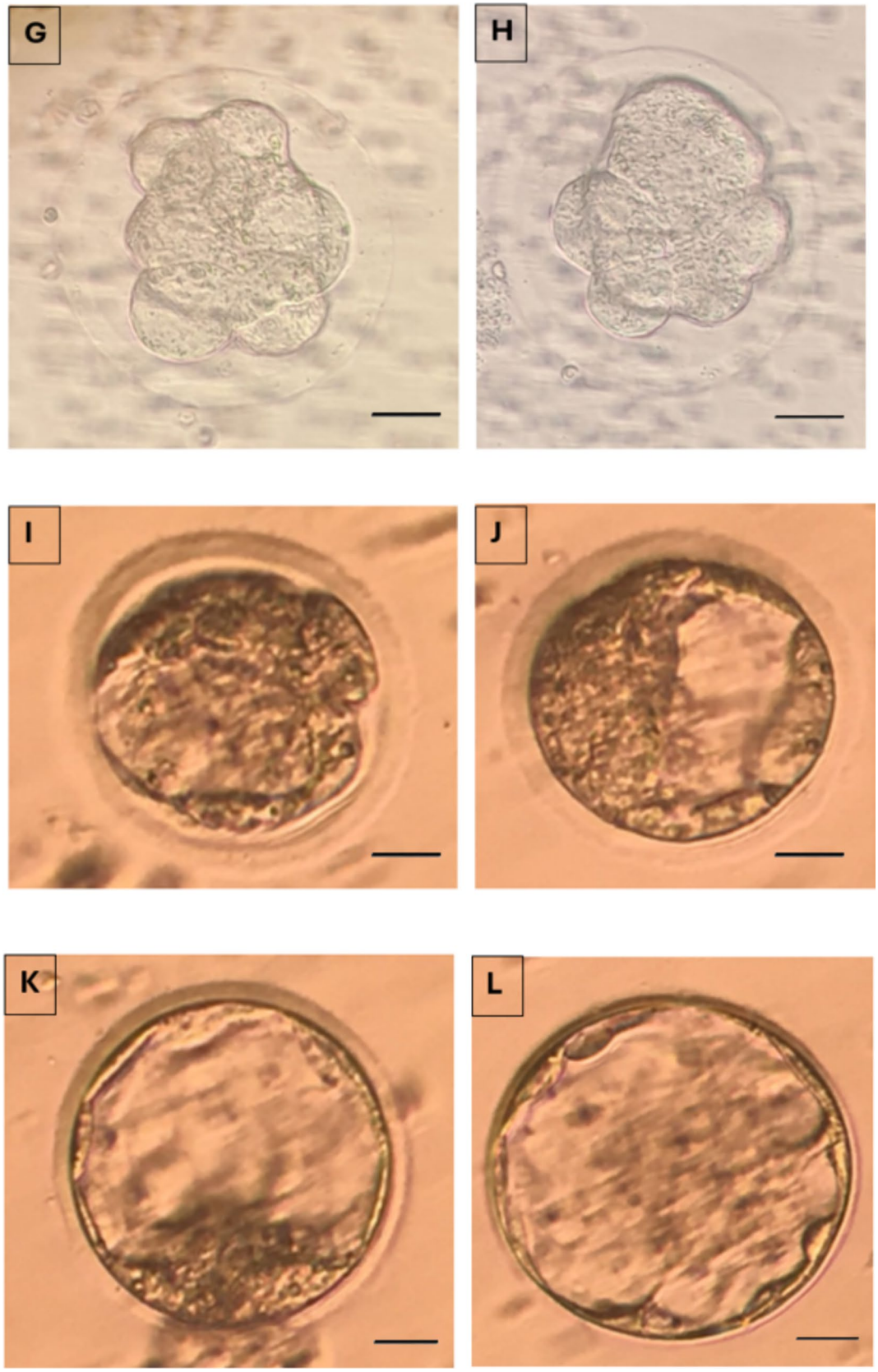


Fig. 9 (continued)



Table 1 17 indicators (8 of which our study included) accepted as ART (in vitro fertilization) performance laboratory indicators by ESHRE Special Interest Group of Embryology, Vienna 2017. MII: Metaphase 2; MI: Metaphase 1

PI (Performance indicators) and KPI (Key performance indicators)	Competency (%)	Control (97 oocyte)	APB-Tech (112 oocyte)	*p*-value
Degeneration rate (%) (Number of damaged or degenerated oocyte / Number of injected MII oocytes x100)	≤10	6	6.4	0.453
Normal fertilization rate (%) (Number of fertilized oocyte rate / Number of injected MII oocytes x100)	≥65	73.4	79.1	0.569
1 PN rate (%) (Number of 1 PN oocyte / Number of injected MII oocytes x100)	<3	1.2	1.3	0.238
Cleavage rate (%) (Number of cleaved embryo on Day 2 / Number of fertilized oocyte on Day 1 x100)	≥95	96.3	98.2	0.651
2 nd day embryo development rate (Number of 4-cell embryo on Day 2 / Number of fertilized oocyte on Day 1 x100)	≥50	78.4	81.2	0.752
3 rd day embryo development rate (Number of 8-cell embryo on Day 3 / Number of fertilized oocyte on Day 1 x100)	≥45	72.8	78.5	0.644
Good quality embryo development rate (%) (Number of good quality blastocyst number on Day 5/ Number of fertilized oocyte on Day 1x100)	≥30	39.2	46.6	0.045
Blastocyst development rate (%) (Number of blastocyst on Day 5/ Number of fertilized oocyte on Day 1 x100)	≥40	46.5	54.4	0.036
Good quality blastocyst rate (%) (Number of top-quality blastocysts on Day 5/ Number of fertilized oocyte on Day 1 x100)		11.6	23.4	0.047

Table 2 Clevage stage embryo qualities among groups.

Embryo quality	Control (97 oocyte)	APB-Tech (112 oocyte)	*p*-value
Day 2 (% top-quality embryos/2-PN)	29.0 ± 1.8	37.9 ± 2.0	0.047
Day 3 (% top-quality embryos/2-PN)	31.1 ± 1.9	38.5 ± 1.9	0.038

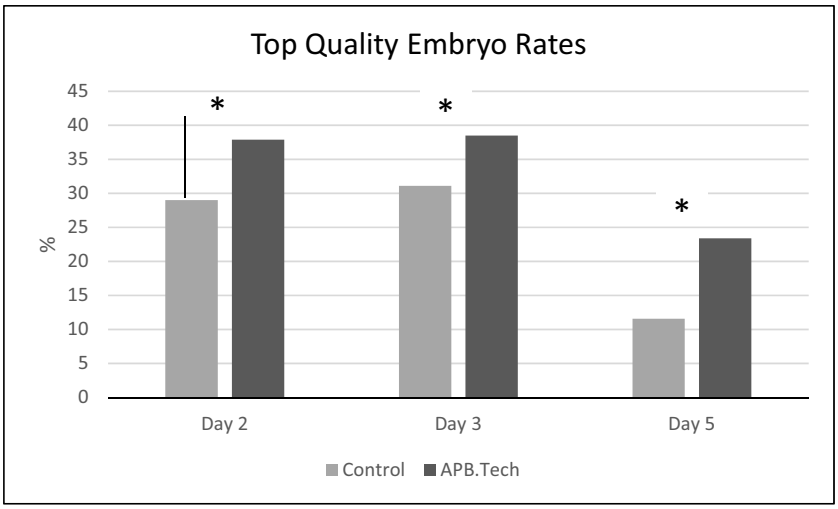


Fig. 10 Top quality embryo rates on Day 2, 3 and 5

the technique. The outcome of cycles in which APB-Tech is used, were compared with the results of classic cycles without the technique.

Eight parameters among 'ART laboratory success indicators' accepted by ESHRE Special Interest Group of Embryology, Vienna 2017, were assessed including degeneration, normal fertilization, abnormal fertilization (1PN), 2 and 3rd day embryo development, good quality embryo, blastocyst development and good quality blastocyst rates. Among those parameters we observed a significant increase in good quality embryo, good quality blastocyst and blastocyst development rates in the cycles where APB-Tech is used although other parameters were not significantly different.

According to our results, the basic effect of the technique is detected on the quality and development rates of the embryo and blastocysts. This finding supports the findings of studies which report that embryo and blastocyst quality and development rates are affected by sperm DNA fragmentation [40–42]. The referenced studies together with some others report no effect of sperm DNA on the fertilization rates verifying our results obtained for the fertilization rates [43]. Our results that embryo and blastocyst qualities and development rates are affected while fertilization rates are not, is compatible with and supports the results of the data that sperm genome is activated on the 3rd day of embryonic development [44, 45]. Since blastocyst formation occurs in 5 th day of embryonic development, it is comprehensible to observe the expected outcome on this stage. The results obtained for degeneration rates seem not to be affected by the technique as expected, but it provide evidence about its nontoxic nature during IVF procedures.

The promising results observed for these important parameters of IVF is an indicator for its potential to achieve the expected positive effects. As this study is conducted on mice, it is expected to have a much higher impact in human studies due to the genetic similarity and low DNA fragmentation rates of mice, as well as being fertile.

The developed method is expected to positively affect ICSI parameters and treatment outcome and minimize neonatal health risks by enabling professionals to select sperm that is more likely to be chosen by natural selection mechanisms.

Further studies including pregnany, ongoing pregnancy, miscarriage and live-birth rates are needed to investigate the overall effects of the technique on IVF outcome before implementing the technique on human IVF studies.

Code Availability (software application or custom code) Not applicable

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Data Availability The data is available upon request.

Declarations

Ethical Approval Ethical approval is provided within the text.

Consent to Participate Not applicable.

Conflict of Interest The authors declare no conflict of interest

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References

1. Mehra BL, Skandhan KP, Prasad BS, Pawankumar G, Singh G, Jaya V. Male infertility rate: a retrospective study. Urologia. 2018;85(1):22–4.
2. Rubino P, Viganò P, Luddi A, Piomboni P. The ICSI procedure from past to future: a systematic review of the more controversial aspects. Hum Reprod Update. 2016 Mar-Apr;22(2):194–227.
3. Henkel R. Sperm preparation: state-of-the-art--physiological aspects and application of advanced sperm preparation methods. Asian J Androl. 2012;14(2):260–9.
4. Berntsen S, Laivuori H, la Cour FN, Loft A, Söderström-Anttila V. B Oldereid N, Romundstad LB, Magnusson Å, Petzold M, Bergh C, Pinborg A. A systematic review and meta-analysis on the association between ICSI and chromosome abnormalities. Hum Reprod Update. 2021 Aug 20;27(5):801–47.
5. Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. In: Programmed Cell Death (Apoptosis). 4th ed. New York: Garland Science; 2002. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26873/.
6. Print CG, Loveland KL. Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioassays. 2000;22:423–30.
7. Martincic DS, Virant Klun I, Zorn B, Vrtovec HM. Germ cell apoptosis in the human testis. Pflugers Arch. 2001;442:59–60.
8. Steven WW, Coffey DS. DNA Packaging and Organization in Mammalian Spermatozoa: Comparison with Somatic Cell. Biol Reprod. 1991;44:569–74.
9. Baarends W, Van Der Laan R, Grootegoed J. DNA Repair Mechanisms and Gametogenesis. Reproduction. 2001;121:31–9.
10. González-Marín C, Gosálvez J, Roy R. Types, Causes, Detection and Repair of DNA Fragmentation in Animal and Human Sperm Cells. Ijms. 2012;13:14026–52.
11. Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Dondero F, Lenzi A. Study of apoptotic DNA fragmentation in human spermatozoa. Hum Reprod. 2000;15:830–9.
12. Sakkas D, Ramalingam M, Garrido N, Barratt CL. Sperm selection in natural conception: what can we learn from Mother Nature to improve assisted reproduction outcomes? Hum Reprod Update. 2015;21(6):711–26.
13. Magnusson Å, Källen K, Thurin-Kjellberg A, Bergh C. The number of oocytes retrieved during IVF: a balance between efficacy and safety. Human Reprod. January 2018;33(1):58–64.
14. Wang C, Swerdloff RS. Limitations of semen analysis as a test of male fertility and anticipated needs from newer tests. Fertil Steril. 2014;102(6):1502–7.
15. Evenson D, Wixon R. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. Reprod Biomed Online. 2006;12(4):466–72.
16. Le MT, Nguyen TTT, Nguyen TT, Nguyen TV, Nguyen TAT, Nguyen QHV, Cao TN. Does conventional freezing affect sperm DNA fragmentation? Clin Exp Reprod Med. 2019 Jun;46(2):67–75.
17. Lusignan MF, Herrero X, Li B, Delbes G, Chan PTK. Effects of different cryopreservation methods on DNA integrity and sperm chromatin quality in men. Andrology. 2018;6(6):829–35.
18. Huang J, Chen H, Li N, Zhao Y. Emerging microfluidic technologies for sperm sorting. Eng Regen. 2023;4(2):161–9.
19. Pacheco A, Blanco A, Bronet F, Cruz M, García-Fernández J, García-Velasco JA. Magnetic-Activated Cell Sorting (MACS): A Useful Sperm-Selection Technique in Cases of High Levels of Sperm DNA Fragmentation. J Clin Med. 2020;9(12):3976.
20. De Vos A, Polyzos NP, Verheyen G, Tournaye H. Intracytoplasmic morphologically selected sperm injection (IMSI): a critical and evidence-based review. Basic Clin Androl. 2013;23:10.
21. Witt KD, Beresford L, Bhattacharya S, Brian K, Coomarasamy A, Cutting R, Hooper R, Kirkman-Brown J, Khalaf Y, Lewis SE, Pacey A, Pavitt S, West R, Miller D, Cutting R. Hyaluronic Acid Binding Sperm Selection for assisted reproduction treatment (HABSelect): study protocol for a multicentre randomised controlled trial. BMJ Open. 2016;6(10):e012609.
22. Simon L, Murphy K, Aston KI, Emery BR, Hotaling JM, Carrell DT. Micro-electrophoresis: a noninvasive method of sperm selection based on membrane charge. Fertil Steril. 2015;103(2):361–6.
23. Cherouveim P, Velmahos C, Bormann CL. Artificial intelligence for sperm selection-a systematic review. Fertil Steril. 2023;120(1):24–31.
24. Avendaño C, Oehninger S. DNA fragmentation in morphologically normal spermatozoa: how much should we be concerned in the ICSI era? J Androl. 2011;32(4):356–63.
25. Henkel R, Schill W-B. Sperm preparation for ART. Reprod Biol Endocrinol. 2003;1:108.
26. Vaughan DA, Sakkas D. Sperm selection methods in the 21st century. Biol Reprod. 2019;101(6):1076–82.
27. De Martin H, Miranda EP, Cocuzza MS, Monteleone PAA. Density gradient centrifugation and swim-up for ICSI: useful, unsafe, or just unsuitable? J Assist Reprod Genet. 2019;36(12):2421–3.
28. Zhang XD, Chen MY, Gao Y, Han W, Liu DY, Huang GN. The effects of different sperm preparation methods and incubation time on the sperm DNA fragmentation. Hum Fertil (Camb). 2011;14(3):187–91.
29. Karabulut S, Camcı İY, Atambay C, Yiğit P, Keskin İ. Sperm selection with Annexin-V coated polystrene bead technique (APB-Tech): A novel and reliable method for the microscopic selection of viable and non-apoptotic sperm to be used for intracytoplasmic sperm injection. Theriogenology. 2022 Dec;194:92–103.
30. Karabulut S, Aksunger O, Korkmaz O, Eren Gozel H, Keskin I. Intracytoplasmic morphologically selected sperm injection, but for whom? Zygote. 2019;27(5):299–304.
31. Van Steirteghem A, Liu J, Nagy Z, et al. Use of assisted fertilization. Hum Reprod. 1993;8(11):1784–5.
32. Said TM, Land JA. Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. Hum Reprod Update. 2011;17:719–33.
33. Oseguera-López I, Ruiz-Díaz S, Ramos-Ibeas P, Pérez-Cerezales S. Novel Techniques of Sperm Selection for Improving IVF and ICSI Outcomes. Front Cell Dev Biol. 2019;7:298.
34. Morris ID, Ilott S, Dixon L, Brison DR. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Hum Reprod. 2002;17:990–8.
35. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod. 2012;27:2908–17.
36. Ribas-Maynou J, García-Peiró A, Fernandez-Encinas A, Amengual MJ, Prada E, et al. Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. PLoS One. 2012;7:e44679.

37. Yılmaz S, Demiroglu Zergero A, Yılmaz E, Sofuoglu K, Delikara N, Kutlu P. Effects of Sperm DNA Fragmentation on Semen Parameters and ICSI Outcome Determined by an Improved SCD Test, Halosperm. Int J Fertil Steril. 2010;4(2):73–8.
38. Galaktionova GV, Mastryukova M, Strzhizhovsky AD. Sensitivity of mammalian tissues to prolonged effects of constant magnetic fields of high strength. Kosm Biol Aviakosm Med. 1985;19:78–81.
39. De Jonge CJ, Centola GM, Reed ML, Shabanowitz RB, Simon SD, Quinn P. Human sperm survival assay as a bioassay for the assisted reproductive technologies laboratory. J Androl. 2003;24(1):16–8.
40. Borges E Jr, Zanetti BF, Setti AS, Braga DP, Provenza RR, Iaconelli A Jr. Sperm DNA fragmentation is correlated with poor embryo development, lower implantation rate, and higher miscarriage rate in reproductive cycles of non–male factor infertility. Fert and Steril. 2019;112:483–90.
41. Mateo-Otero Y, Llavanera M, Recuero S, et al. Sperm DNA damage compromises embryo development, but not oocyte fertilisation in pigs. Biol Res. 2022;55:15.
42. Alvarez Sedó C, Bilinski M, Lorenzi D, Uriondo H, Noblía F, Longobucco V, Lagar EV, Nodar F. Effect of sperm DNA fragmentation on embryo development: clinical and biological aspects. JBRA Assist Reprod. 2017 Dec 1;21(4):343–50.
43. Li F, Duan X, Li M, et al. Sperm DNA fragmentation index affect pregnancy outcomes and offspring safety in assisted reproductive technology. Sci Rep. 2024;14:356.
44. Tesarik J. Paternal effects on cell division in the human preimplantation embryo. Reprod Biomed Online. 2005;10:370–5.
45. Janny L, Menezo YJ. Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. Mol Reprod Dev. 1994;38:36–42.

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