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TECHNOLOGICAL INNOVATIONS

Effects of the microfluidic chip technique in sperm selection for intracytoplasmic sperm injection for unexplained infertility: a prospective, randomized controlled trial

Selçuk Yetkinel[1] · Esra Bulgan Kilicdag[1] · Pinar Caglar Aytac[1] · Bülent Haydardedeoglu[1] · Erhan Simsek[1] · Tayfun Cok[1]

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Abstract

Purpose The new-generation spermatozoon selection method, microfluidic technique called Fertile Chip® gives the chance to select spermatozoa with lower DNA fragmentation indexes. We aimed to determine the effect of microfluidic techniques for spermatozoon selection in ICSI treatment in patients with unexplained infertility.

Methods This prospective randomized controlled study was conducted at a university hospital. One hundred twenty-two couples with unexplained infertility were included, in which 61 of them were treated with conventional swim-up techniques (control group) and another 61 with the microfluidic technique (study group) for spermatozoon selection in IVF treatment. The fertilization rates and the quality of embryos were the primary outcomes, and clinical pregnancy (CPR) and live birth rates (LBR) were the secondary outcomes of our study.

Results CPR in the study group and control group were 48.3% and 44.8% ($p = 0.35$) and LBR were 38.3% and 36.2% ($p = 0.48$), respectively. The fertilization rates were similar (63.6% and 57.4%, $p = 0.098$). A total number of grade 1 embryos were significantly higher in microfluidic technique group than in control group (1.45 ± 1.62 vs. 0.83 ± 1.03, $p = 0.01$). There were more surplus top quality embryos leftover to freeze in the study group (0.71 ± 1.48 vs. 0.22 ± 0.69, $p = 0.02$).

Conclusion Our study showed that the microfluidic technique does not change fertilization, CPR, and LBR during IVF treatment for couples with unexplained infertility. Despite the fact that the total number of grade 1 embryos after ICSI treatment and the surplus number of grade 1 embryos after embryo transfer were higher in the microfluidic technique group, the study was not powered to detect this difference.

Trial registration NCT02488434

Keywords Microfluidic technique · Unexplained infertility · Embryo · ICSI

Introduction

Selecting healthy spermatozoa is requisite for intracytoplasmic sperm injection (ICSI) to achieve higher fertilization rates and to obtain higher quality embryos and live birth rates, which is the goal of in vitro fertilization (IVF). Spermatozoon selection usually depends on conventional density gradient centrifugation or swim-up techniques that require chemical and mechanical processes which may increase oxygen radical levels in spermatozoa [1]. Elevated oxidative stress in spermatozoa induces DNA base oxidation, increased DNA fragmentation, and eventually cell death [2]. Therefore, new spermatozoon selection methods have been introduced to obtain higher quality spermatozoa to increase fertilized embryo quality. Combined with the selection of spermatozoa with normal morphology, such methods allow selection of spermatozoa with reduced DNA injury and fragmentation rates and higher DNA integrity [3–8]. One new spermatozoon selection method is the microfluidic chip technique called Fertile Chip® (Koek Biotechnology, Izmir, Turkey). Although the microfluidic technique was shown to select spermatozoa with a lower DNA fragmentation index, no clinical studies have examined its effect upon embryo quality and pregnancy rates [1, 9]. In contrast to the chemical and centrifuge stages involved in the classical swim-up process for sperm selection, the microfluidic

✉ Selçuk Yetkinel
syetkinel@hotmail.com

[1] Division of Reproductive Endocrinology and IVF Unit, Department of Obstetrics and Gynaecology, Faculty of Medicine, Baskent University, Adana, Turkey

technique mimics natural sperm selection routes in the female reproductive system. The selection of sperm using the Fertile Chip® leads to less oxygen radical formation and DNA fragmentation and eventually the collection of spermatozoa with higher DNA integrity.

Unexplained infertility is a diagnosis of exclusion and accounts for 10 to 30% of all infertility cases [10]. One of these excluding tests is the sperm analysis defined by the World Health Organization (WHO) in 2010. Although this sperm analysis defines the quantity, motility, and morphology of spermatozoa, it only provides limited information about DNA integrity. Zandieh et al. showed that sperm from couples with unexplained infertility had elevated reactive oxygen species and DNA fragmentation indexes compared with healthy fertile men [11].

The aim of this randomized controlled study was to investigate fertilization rates, embryo quality, and pregnancy rates in the treatment of couples with unexplained infertility by ICSI using spermatozoa selected by the microfluidic technique. To the best of our knowledge, this is the first prospective randomized clinical investigation into the effect of microfluidic spermatozoon selection on embryo quality, and fertilization, pregnancy, or live birth rates in the treatment of unexplained infertility using ICSI, in comparison with the alternative conventional swim-up technique.

Materials and methods

Study population

This prospective randomized controlled study was conducted between 27 June 2015 and 13 April 2016. A total of 122 couples were included in the study with 61 couples per group, for a power analysis to increase the fertilization rate from 65 to 80%, with a 10% error margin and 95% reliability. In the study group, the microfluidic technique was used for spermatozoon selection before ICSI. In the control group, the conventional swim-up technique was used for selection of spermatozoa for ICSI. Patient selection was performed by sealed envelope randomization techniques, during the day of human chorionic gonadotropin (hCG) administration at the end of ovarian hyperstimulation. Informed consent was taken from all couples in the study. The study protocol was approved by the Clinical Research and Ethics Committee (Project No: KA15/132) and from the Turkish Drug and Medical Device Institution of the Turkish Ministry of Health. Approval was obtained from *ClinicalTrials.gov* with the NCT02488434 approval number.

The inclusion criteria were as follows: women aged between 20 and 37 years, male partners aged between 20 and 40 years, patients who were within the first or second IVF cycles and who had unexplained infertility. Since unexplained infertility is a diagnosis of exclusion, the identification of normal tubal patency, uterine cavity and ovulation function tests, absence of poor ovarian reserve according to Bologna criteria [12], presence of normal semen analysis results according to WHO criteria [13], and absence of endometrioma were taken into account to diagnose couples with unexplained infertility. The patient selection flowchart is summarized in Fig. 1.

Ovarian hyperstimulation

The controlled ovarian stimulation protocols were based on patients' age, weight, antral follicle count, anti-Müllerian hormone level, and previous cycle characteristics. Ovarian hyperstimulation was performed according to either the agonist or antagonist protocol. At the end of ovarian hyperstimulation, oocyte retrieval was performed 36 h after hCG administration. Oocyte pickup was performed with the guidance of a transvaginal ultrasonography probe with a 17-gauge single-lumen needle and the patient under sedoanalgesia. Oocyte-corona complexes were initially denuded using 80 IU/ml hyaluronidase (Hyase-10x, Vitrolife, Sweden) and then further denuded mechanically via IVF Pasteur pipettes (Origio, Denmark) for 30 s, followed by washing with modified Human Tubal Fluid medium (HTF HEPES, Irvine Scientific, USA). Denuded oocytes were transferred to a single-step medium (Sage, Origio, Denmark) and incubated for 2–3 h. Only metaphase II (MII) oocytes were prepared for ICSI. Routine ICSI was performed after a 2–2.5-h incubation as described previously by Palermo et al. [14]. After 16–18 h, embryos were checked for fertilization and then for cleavage every day up to the transfer day. Embryos were graded according to the system of the ALPHA Scientists Special Interest Group [15]. One or two embryos were transferred on days 3, 4, and 5 according to embryo morphology. Three to five days after ICSI, embryos were transferred with an embryo transfer catheter (Cook Medical Inc., USA). The day after oocyte pickup, luteal support was initiated by vaginal application of a 200-μg progesterone capsule (Progestan, Kocak, Turkey) three times a day or a daily progesterone gel (Crinone 8%, Merck Serono, Italy) until the day of the hCG test.

In frozen-thawed ICSI cycles, endometrium preparation involved a daily oral 6-mg estradiol tablet (Estrofem, Novo Nordisk, Australia). Estradiol treatment started at the third or fourth day of menstruation after no ovarian cysts were observed transvaginally. After 7 or 8 days of estradiol treatment, endometrium thickness was examined, and if > 8 mm, luteal support was started using a 200-μg progesterone capsule three times a day vaginally or progesterone gel once per day vaginally. Embryos were transferred on the fourth or fifth day of luteal support.

Clinical pregnancy was defined as the presence of an embryo with cardiac activity detectable by transvaginal ultrasonography. Live birth rate was defined as the number of

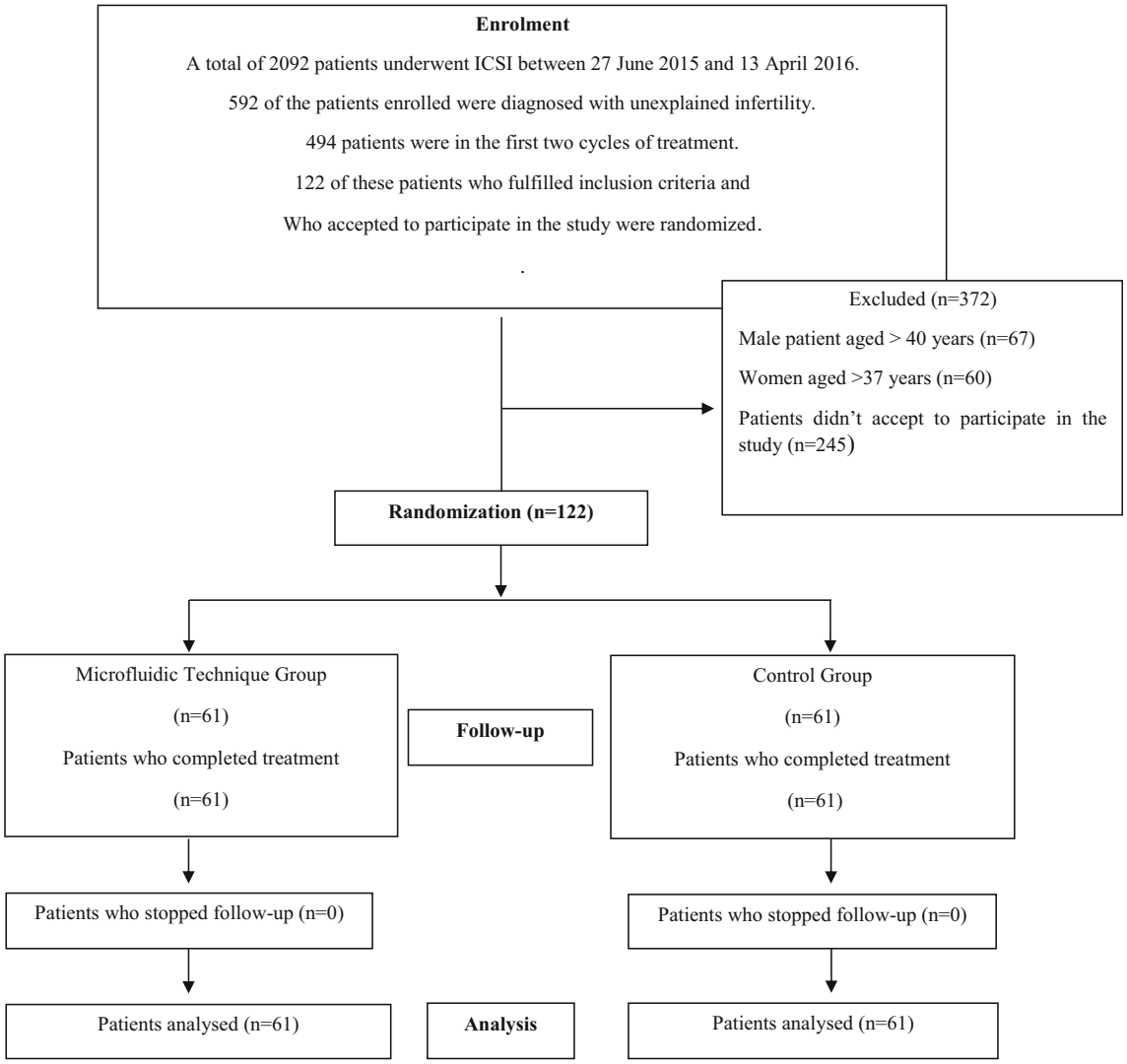

Fig. 1 The patient selection flowchart

deliveries that resulted in a live born neonate, expressed per 100 embryo transfers.

Conventional swim-up technique

In the conventional swim-up technique, all semen samples were liquefied in a 37 °C incubator for 1 h. The liquefied semen sample was then diluted 1:1 with culture medium and centrifuged for 10 min at 1500 rpm. Thereafter, the supernatant was discarded and 1 ml of fresh culture medium was layered above the pellet. The tube was inclined at 45° and incubated for 1 h (37 °C, 6% CO_2). After incubation, the supernatant was ready for ICSI and transferred into an empty tube.

Microfluidic technique

Fertile Chip® contains microfluidic channels created with polymethylmethacrylate and double-sided adhesive film. Inlet and outlet ports were created by cutting holes through the polymethylmethacrylate (Fig. 2). For sperm sorting, the microfluidic channel was first filled with medium and the outlet port was filled with medium followed by a thin layer of mineral oil to avoid medium evaporation. After liquefaction of the sperm sample, 1 ml was added to the channel inlet, and the microfluidic chip was then placed into an incubator at 37 °C for 30 min. Healthy spermatozoa in the sample swam through the microchannels from the inlet up to the outlet and were collected for ICSI as described [16].

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) software. Categorical variables were expressed as numbers and percentages, whereas continuous variables were expressed as mean and standard deviation (min–max, where applicable). Comparison of

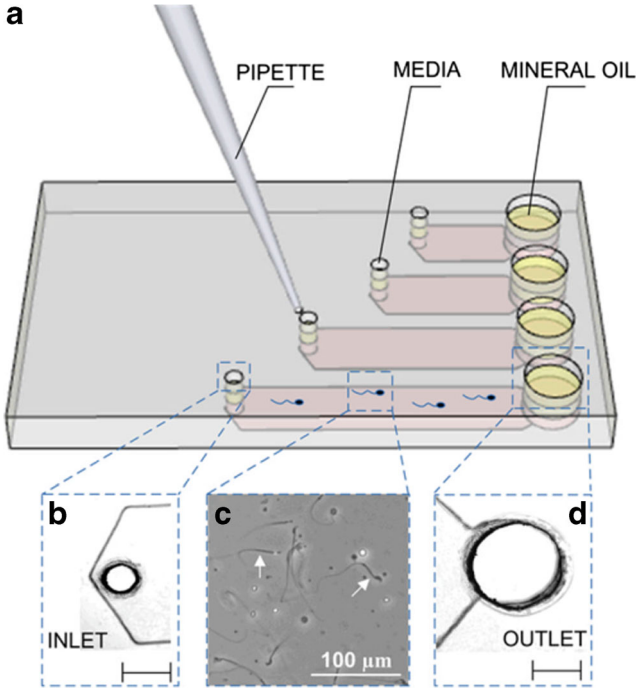

Fig. 2 (a) schematic illustration of fertile chip channel systems. (b) Microscopic image of channel inlet under a 2X objective. (c) Image of swimming sperm cells inside a microchannel under a 10X objective. (d) Microscopic image of channel outlet under a 2X objective [16]

continuous variables between groups was made, and Student's *t* test was used when the assumption for the precondition of a parametric distribution was met, whereas the Mann-Whitney *U* test was used when the parametric assumption was not met. The chi-square or Fisher's exact test was used for comparison of categorical variables. To determine risk factors affecting pregnancy, multivariate logistic regression analysis was used to evaluate variables found to be statistically significant by univariate analysis. A p value < 0.05 was considered statistically significant.

The fertilization rate and availability of top quality embryos on the day of embryo transfer were considered the primary outcomes of the study, whereas clinical pregnancy and live birth rates were considered secondary outcomes.

Results

No difference was found between demographic characteristics of the patients, as summarized in Table 1.

Characteristics of the controlled ovarian hyperstimulation cycle

Baseline mean FSH dose, induction duration, and the total FSH doses were similar in the two groups. The estradiol and progesterone levels measured on the day of hCG administration were both found to be significantly higher in the microfluidic technique group ($p = 0.040$ and $p = 0.032$). The number of collected oocytes and metaphase II oocytes was similar in the two groups ($p = 0.41$ and $p = 0.36$) (Table 2).

The mean fertilization rate was 57.4% and 63.6% in the control and microfluidic technique groups, respectively. No statistically significant difference was found in fertilization rate ($p = 0.098$) (Table 3).

The mean total number of embryos obtained after ICSI was similar between the two groups ($p = 0.409$). In contrast, the total number of grade 1 embryos obtained after ICSI was significantly higher in the microfluidic group compared with that in the control group (1.45 ± 1.62 vs. 0.83 ± 1.03, $p = 0.01$). After the embryo transfer in fresh IVF cycles, surplus embryos were frozen for future frozen-thawed cycles. Evaluation of the quality of these surplus embryos demonstrated that the number of grade 1 embryos was significantly higher ($p = 0.02$) in the microfluidic group compared with that in the control group but the study was not powered to detect this difference. These findings are summarized in Table 3.

Embryo transfer for five couples was canceled in the control group because of the absence of fertilization, cleavage arrest, genetic diagnosis of preimplantation, or ovarian hyperstimulation and premature luteinization. Seven couples were canceled in the microfluidic technique group because of elevated estradiol and progesterone levels, cleavage arrest, and thin endometrium. Embryos of six couples in the microfluidic group and two couples in the control group were frozen that were the continuation of the previous fresh cycles preserved to be used during the next frozen-thawed cycles.

Of the 122 patients who underwent treatment, the pregnancy rate per cycle was equivalent in the microfluidic technique and control groups (57.4% and 54.1%, respectively, $p = 0.42$). The clinical pregnancy rate per cycle was also similar in the microfluidic technique and control groups ($p = 0.35$). The live birth rate per cycle was equivalent in the microfluidic technique and control groups (37.7% and 34.4%, respectively, $p = 0.42$). In addition, the pregnancy rate per embryo transfer and clinical pregnancy rate per embryo transfer were similar in the two groups ($p = 0.50$ and $p = 0.39$, respectively). Evaluation of all patients enrolled in the study demonstrated that the pregnancy, clinical pregnancy, and live birth rates per patient were similar between two groups ($p = 0.51$, $p = 0.35$, and $p = 0.48$, respectively). The findings are summarized in Table 4.

Regression analysis was performed because of the difference in estradiol and progesterone levels between the two groups on the day of hCG administration. Regression analysis demonstrated that these two variables did not affect pregnancy rates ($p = 0.949$ and $p = 0.586$).

Table 1 Main patient characteristic properties

	Microfluidic technique ($n = 61$)	Control group ($n = 61$)	p
Female age	28.61 ± 2.96	28.21 ± 3.31	0.49
Male age	32.74 ± 3.72	32.82 ± 3.73	0.90
Duration of infertility (years)	5.23 ± 3.49	4.36 ± 2.94	0.14
BMI (kg/m^2)	24.19 ± 3.55	24.33 ± 4.37	0.86
Sperm count (× 10^6/ml)	66 ± 37	58 ± 41	0.27
A + B motility (%)	57.08 ± 14.84	54.70 ± 14.37	0.37
Antral follicle count (n)	7.97 ± 2.02	7.72 ± 2.18	0.52

Discussion

In this randomized controlled trial, comparison of spermatozoon selection by the microfluidic and conventional swim-up techniques demonstrated that the fertilization, pregnancy, and live birth rates of the couples were similar in both groups. The embryo quality is overall affected by multiple factors in addition to the possible effects by the processing and selection methodologies of sperm. Here, we changed the sperm processing method and demonstrated a statistically significant difference in the study group compared to the control. The total number of grade 1 embryos after ICSI treatment and the surplus number of grade 1 embryos after embryo transfer were higher in the microfluidic technique group. Furthermore, the rate of embryo freezing (for extra embryos) after embryo transfer was higher in the microfluidic group. Despite all, the study was not powered to detect this difference.

This is the first clinical study that shows potential effects of the microfluidic technique in the selection of spermatozoa for ICSI. The primary outcomes of our study are fertilization rate and embryo quality. Few studies have investigated new-generation sperm selection techniques using embryo quality. Gianaroli et al. suggested that sperm selected with an inverted microscope produced higher embryo quality and ongoing pregnancy rates compared with classical ICSI, but fertilization rates were similar [4]. In contrast, Balaban et al. found no difference in the quality of embryos or live birth rates for IVF treatment using intracytoplasmic morphologically selected sperm injection (IMSI) compared with classical ICSI [17]. In our study, we found higher number of grade 1 embryos in the study group but we did not find any significant differences in terms of fertilization, pregnancy, and live birth rates between the microfluidic techniques compared with classical ICSI. In the meta-analysis of magnetic-activated cell sorting (MACS) (another new-generation sperm selection method) compared with classical ICSI, authors suggested that the pregnancy rate was higher in the MACS group (RR = 1.50, 95% CI 1.14–1.98). However, this study did not include data about the quality of embryos or the fertilization rate [18].

Although the levels of estradiol and progesterone on the day of hCG were statistically different in our study, we did not find any significant difference in regression analysis of these parameters. The days of embryo transfer were one of the third, fourth, or fifth days. Although different days of embryo transfer might affect the pregnancy and live birth rates, the transfer days were homogeneously distributed in the two groups.

Spermatozoon selection by the microfluidic technique was made objectively and successfully with lower cost and without the human margin of error of the conventional swim-up technique. It is an easy, less time-consuming procedure with high clinical applicability and repeatability. Instead of the chemical and centrifugation stages involved in the conventional swim-up technique which impairs sperm DNA integrity by increasing oxygen radical levels in the spermatozoa, the microfluidic selection technique mimics the natural routes that selects healthy spermatozoa traveling through the cervix,

Table 2 Characteristic properties of the ICSI cycle

	Microfluidic technique ($n = 61$)	Control group ($n = 61$)	p
FSH baseline dose (IU)	217.01 ± 58.92	209.79 ± 64.65	0.519
Induction duration (days)	9.05 ± 1.44	8.82 ± 1.83	0.439
Total GnRHa dose (IU)	2605.90 ± 1391.47	2086.87 ± 778.79	0.849
hCG day serum estradiol level (pg/ml)*	2605.90 ± 1391.47	2125.20 ± 1137.82	0.040*
hCG day serum progesterone (ng/ml)*	1.14 ± 0.65	0.92 ± 0.43	0.032*
hCG day endometrium (mm)	10.82 ± 1.88	10.54 ± 1.92	0.403
Number of follicles after induction (n)	13.30 ± 4.97	13.43 ± 5.47	0.885
Oocytes picked up (n)	16.62 ± 7.30	15.45 ± 8.43	0.415
Metaphase II oocyte number (n)	12.79 ± 5.73	11.70 ± 7.29	0.363

*$p < 0.05$

Table 3 Embryology data following ICSI

	Microfluidic technique	Control group	*p*
Pronucleus (*n*)	7.90 ± 4.15	6.62 ± 4.90	0.122
Cleavage embryo (*n*)	7.51 ± 4.27	6.50 ± 4.86	0.227
Fertilization rate (%)	63.64 ± 19.11	57.42 ± 21.90	0.098
Total number of embryo after ICSI (*n*)	7.49 ± 4.31	6.48 ± 4.87	0.409
Total number of grade 1 embryo after ICSI (*n*)	1.45 ± 1.62	0.83 ± 1.03	0.01*
Total number of grade 2 embryo after ICSI (*n*)	4.08 ± 2.74	3.53 ± 2.84	0.28
Total number of grade 3 embryo after ICSI (*n*)	1.82 ± 2.55	2.22 ± 3.23	0.45
Number of transferred embryos (*n*)	0.98 ± 0.46	1.11 ± 0.52	0.095
Transferred grade1 embryo (*n*)	0.74 ± 0.44	0.62 ± 0.61	0.237
Transferred grade2 embryo (*n*)	0.36 ± 0.61	0.54 ± 0.65	0.115
Transfer day			
Third day (*n*)	20 (32.8%)	24 (39.3%)	0.942
Fourth day (*n*)	3 (4.9%)	2 (3.3%)	
Fifth day (*n*)	32 (50.8%)	29 (49.2%)	
Number of patients with frozen embryo after transfer (*n*)*	44/54 (81.5%)	36/56 (64.3%)	0.04*
Total number of leftover grade 1 embryo after transfer (*n*)*	0.71 ± 1.48	0.22 ± 0.69	0.02*
Total number of leftover grade 2 embryo after transfer (*n*)	3.73 ± 2.85	2.98 ± 2.77	0.15
Total number of leftover grade 3 embryo after transfer (*n*)	1.82 ± 2.55	2.22 ± 3.23	0.47

*$p < 0.05$

uterine cavity, and fallopian tubes. The microchannels of the microfluidic chip technique resemble the tubal channels, and the most progressive motile spermatozoa reach to the end of the microchannel, allowing the selection of spermatozoa with lower DNA fragmentation rate for ICSI.

We did not find any difference in fertilization rate with selected spermatozoa via microfluidic chip. Although we found better embryo quality in the study group, pregnancy and live birth rates of both groups were similar. After embryo transfer of the fresh IVF cycles, there were more surplus grade 1 embryos in the microfluidic technique group resulting in higher freezing rates of remaining embryos. These surplus embryos will be used in future frozen-thawed cycles, providing opportunity to obtain cumulative pregnancy rates compared to the control group. Finally, even if we do not get a single-cycle LBR difference, cumulative pregnancy rates need to be further studied with larger number of patients as we continue our work.

Its randomized controlled nature to investigate the success rate of the microfluidic technique is one of the pros of this study. Another strength of it is that patients were followed to the end of pregnancy to determine live birth rates, which is the goal of IVF. On the other hand, its small study population is a limitation of the study. Other one is that, although more embryos were frozen in the microfluidic technique compared to the control group, cumulative pregnancy rates per patients could not be determined until all frozen embryos were used.

In conclusion, our study indicates that the microfluidic technique does not change fertilization, live birth, and clinical pregnancy rates during IVF treatment for couples with

Table 4 Pregnancy outcome

	Microfluidic technique	Control group	*p*
Pregnancy rate (per patient %) (*n*)	57.4 (35/61)	54.1 (33/61)	0.42
Clinical pregnancy rate (per patient %) (*n*)	47.5 (29/61)	42.6 (26/61)	0.35
Live birth rate (per patient %) (*n*)	37.7 (23/61)	34.4 (21/61)	0.42
Pregnancy rate (per fresh ET %) (*n*)	53.7 (29/54)	55.4 (31/56)	0.50
Clinical pregnancy (per fresh ET %) (*n*)	46.3 (25/54)	44.6 (25/56)	0.39
Live birth rate (per fresh ET %) (*n*)	40.7 (22/54)	37.5 (21/56)	0.43
Pregnancy rate (per frozen + fresh ET %) (*n*)	58.3 (35/60)	56.9 (33/58)	0.51
Clinical pregnancy (per frozen + fresh ET %) (*n*)	48.3 (29/60)	44.8 (26/58)	0.35
Live birth rate (per frozen + fresh ET %) (*n*)	38.3 (23/60)	36.2 (21/58)	0.48

ET embryo transfer

unexplained infertility. Embryo quality after ICSI was one primary outcome of our study, and the higher number of grade 1 embryos supports the need for further studies of the microfluidic technique. Already we have planned larger scale studies dealing with couples with abnormal sperm morphological parameters, such as the male factor, or groups with high DNA fragmentation rates to further investigate the clinical effects of the microfluidic selection method.

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Compliance with ethical standards The study protocol was approved by the Clinical Research and Ethics Committee (Project No: KA15/132) and from the Turkish Drug and Medical Device Institution of the Turkish Ministry of Health.

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