ORİJİNAL ARAŞTIRMA ORIGINAL RESEARCH

DOI: 10.24074/tjrms.2020-80471

Which to Choose for Sperm Sorting? Swim up Versus Density Gradient: A Sibling Oocyte Study

Sperm Elde Etmek için Hangisini Seçmeliyiz? Kardeş Oosit Çalışması: Yüzdürme Yöntemine Karşı Yoğunluk Gradyan

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ABSTRACT

Objective: The efficiency of different sperm sorting methods for retrieving higher quality sperm cells during infertility treatment has been under investigation for many years. The aim of this study was to compare the effects of two commonly used sperm sorting methods on laboratory and clinical outcomes by using sibling oocytes. **Materials and Methods:** 72 female patients having a total of 1075 mature oocytes and their partners were included in this study. All semen samples were divided into two aliquots and prepared either by pellet swim up or single layer centrifugation before intracytoplasmic sperm injection. Mature oocytes of each patient were split into two groups; and each group was assigned to one of the sperm sorting methods. **Results and Conclusion:** The main outcome measure of the study was total blastocyst development rate whereas other outcome measures (fertilization rate, blastocyst development rate, embryo quality, pregnancy and implantation rate) were also compared between the groups as the secondary outcomes. Although pellet swim-up method demonstrated a higher blastocyst yield (total blastocyst development and blastocyst utilization rate) compared to single layer centrifugation in our study, the difference did not reach a statistical significance level ($p>0.05$). Moreover, no significant differences were found between the groups in terms of fertilization, embryo development, embryo quality on day 2, pregnancy and implantation rates ($p>0.05$). Our data suggest that both methods have comparable effects in terms of blastocyst utilization and implantation rates; thus, may be used alternately according to the conditions and needs of different laboratories.

Keywords: Blastocyst; implantation; pregnancy; sperm sorting

ÖZET

Amaç: İnfertilite tedavisi sırasında daha kaliteli sperm hücrelerini elde etmek için farklı sperm elde etme yöntemlerinin etkinliği uzun yıllardır araştırılmaktadır. Bu çalışmanın amacı, yaygın olarak kullanılan iki sperm ayıklama yönteminin, kardeş oositleri kullanarak laboratuvar ve klinik sonuçlar üzerindeki etkilerini karşılaştırmaktır. **Gereç ve Yöntemler:** Çalışmaya 72 hastadan elde edilen toplam 1075 olgun oosit dahil edildi. Tüm semen numuneleri iki parçaya bölündü ve intrasitoplazmik sperm enjeksiyonundan önce pellet yüzerek veya tek katmanlı santrifüj ile hazırlandı. Her hastanın olgun oositleri iki gruba ayrıldı ve her gruba sperm elde etme yöntemlerinden birisi ile işlem yapıldı. **Bulgular ve Sonuç:** Çalışmanın ana sonuç ölçütü toplam blastosist gelişme oranı iken, diğer sonuç ölçütleri (fertilizasyon oranı, blastosist gelişme hızı, embriyo kalitesi, gebelik ve implantasyon oranı) ikincil sonuçlar olarak gruplar arasında karşılaştırıldı. Pelet yüzdürme yöntemi, çalışmamızda tek katmanlı santrifüjlemeye göre daha yüksek blastosist edinimi (toplam blastosist gelişimi ve blastosist kullanım oranı) göstermesine rağmen, aradaki fark istatistiksel anlamlılık göstermemiştir ($p>0.05$). Ayrıca fertilizasyon, embriyo gelişimi, 2. gün embriyo kalitesi, gebelik ve implantasyon oranları açısından gruplar arasında anlamlı farklılık bulunmadı ($p>0.05$). Verilerimiz, her iki yöntemin de blastosist kullanımı ve implantasyon oranları açısından karşılaştırılabilir etkilere sahip olduğunu göstermektedir; bu nedenle farklı laboratuvarların koşullarına ve ihtiyaçlarına göre dönüşümlü olarak kullanılabilirler.

Anahtar Kelimeler: Blastosist; implantasyon; gebelik; sperm ayırma

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Peer review under responsibility of Turkish Journal of Reproductive Medicine and Surgery.

Received: 07 Dec 2020 ***Accepted:*** 03 Jan 2021 ***Available online:*** 03 Feb 2021

Unlike oocytes, it is more likely to make a selection among the sperm cells during assisted reproductive treatment (ART) due to their relatively higher number. Today, there are several techniques used in assisted reproduction laboratories for semen processing including particularly swim-up and density gradient methods.[1] Although it has been investigated in many studies for several years, there is still no clear evidence on which sperm preparation technique can yield better clinical outcomes that are the most important criteria for success in ART.

Numerous reports in the literature have confirmed that both techniques are effective in eliminating abnormal spermatozoa in the semen samples.[2-4] However; the study by Hammadeh et al. reported that density gradient centrifugation (DGC) yielded a significantly higher proportion of morphologically normal sperm than swim up in a group of infertile patients whereas no significant difference was found by means of morphologically normal spermatozoa between both techniques in the study by Borges et al.[5,6]

Recent studies have shown that degree of deoxyribonucleic acid (DNA) integrity can be used as a diagnostic tool to assess sperm quality; however, clinical results comparing both techniques in terms of this parameter are still limited and controversial. In terms of the effect of both semen processing methods on sperm DNA integrity, some authors found that swim up technique allowed better selection of spermatozoa with lower fragmentation rate compared to DGC.[7,8] On the contrary, DGC was found to be associated with lower DNA fragmentation index (DFI) compared to swim up in the study by Xia et al.[9]

This lack of consensus has led us to design this study aiming to compare the efficiency of two sperm processing techniques in terms of laboratory and clinical outcomes of intracytoplasmic sperm injection (ICSI) cycles. To our knowledge, this is the first study comparing swim-up and a gradient-based method in a sibling oocyte setting in human ICSI cycles.

MATERIALS AND METHODS

STUDY DESIGN AND PATIENTS

This study was carried out in an assisted reproduction unit of a private hospital in Turkey between 05.25.2018 and 01.01.2020. It was designed as a prospective cohort study using sibling oocytes of patients who underwent an ART cycle in this hospital between the indicated dates.

Inclusion criteria of the study were having at least 5 metaphase II (MII) oocytes per ICSI cycle (irrespective of total oocyte number) and having an age of ≤37 years old for the female participants and having a diagnosis of normozoospermia based on World Health Organization (WHO) criteria for the male partners (irrespective of age). Exlusion criteria were determined as having an etiology of repeated implantation failures with more than two failed cycles, varicocele, any chromosomal abnormalities in the karyotype analyses of both partners, endometriosis and polycystic ovary syndrome. Preimplantation genetic diagnosis cycles were also excluded from the study. All couples were informed about the study and provided written informed consent. Ethics committee approval for the study was given by Kocaeli University ethics committee for non-interventional clinical research, under the approval number of KIA 2018/225 on May 8, 2018. The study was conducted in accordance with the Helsinki Declaration Principles.

SPERM SORTING BY PELLET SWIM UP (PSU) AND SINGLE LAYER CENRIFUGATION (SLC)

Semen samples were left to liquefy at least for 20 minutes at room temperature and divided into two aliquots. Semen analysis was carried out according to WHO criteria by the same technician.

In SLC procedure, 1 ml of semen was put on 50% gradient medium which was prepared by diluting stock solution (Puresperm 100, Nidacon, Sweden) by 1/1 with sperm rinse solution (Vitrolife, Sweden), and centrifuged for 10 minutes at 1600 rpm. In PSU, 1 ml of semen was mixed with 1 ml of sperm washing media (SpermRinse, Vitrolife, Sweden) and centrifuged for 10 minutes at 1600 rpm. Then, both pellets were mixed with 2 ml of sperm washing media (PureWash, Nidacon, Sweden) and centrifuged at 1600 rpm for 5 minutes. Whole supernatants were removed and the remaining pellets were left for resuspension in 1 ml of media. Both groups were centrifuged for a total of 15 minutes in order to

eliminate the possible differences between groups based on centrifugation time.

CONTROLLED OVARIAN HYPERSTIMULATION AND ICSI

Ovarian stimulation and oocyte collection were performed as previously described by twi et. al.[10] The oocyte-cumulus complexes (COCs) were incubated in GIVF media (Vitrolife, Sweden) supplemented with 5% human serum albumin (HSA) until ICSI under the conditions of 37°C, 7% CO_2 and 7% O_2. After two to three hours, all oocytes were denuded and patients having at least five MII oocytes were enrolled in the study.

The mature (MII) oocytes of each patient were divided into two groups for microinjection with spermatozoa sorted either by SLC or PSU technique. All ICSI procedures were performed by the same embryologist. Spermatozoa were sorted at least one hour before ICSI.

ASSESSMENT OF FERTILIZATION AND EMBRYO CLEAVAGE

Fertilization check was done after 18-22 hours of ICSI and fertilized oocytes were cultured up to day 5/6. Embryo cleavage was checked only for once on day 2 in order to minimize the external stress created on the embryos. Embryos which had more than 2 and equal size of blastomeres having ≤15% fragmentation were accepted as good quality embryos on day 2. Any patients having less than three good quality embryos underwent day 3 transfer and thus, they were withdrawn from the study.

Blastocyst transfer was planned for each patient in the study who had at least three good quality embryos on day 2. All embryos were incubated under the same conditions of 37°C, 7% CO_2 and 7% O_2 until transfer. All fresh embryo transfers were performed on day 5. The assignment of embryo transfer to groups was done consecutively according to their oocyte pick up times. For double embryo transfers, in case that there were not two good quality blastocysts in the assigned group, two blastocysts were chosen from the other group for transfer or two blastocysts were transferred from either group. Therefore, these patients were excluded from the analysis of clinical outcomes since implantation of each blastocyst was not clear in the circumstance that two embryos from different groups were resulted in one clinical sac. Besides, some patients did not undergo a fresh embryo transfer due to the factors such as ovarian hyperstimulation (OHSS) risk, thin endometrium or high level of progesterone on transfer day; and they were only included in the analysis of laboratory outcomes due to total freezing of embryos at that cycle. All good quality blastocysts except the ones transferred, were vitrified on days 5 and 6. Blastocyst scoring was done according to the classification system of Gardner & Schoolcraft.[11]

A ß-hCG value of ≥50 mIU/mL after 10 days following embryo transfer indicated a positive pregnancy. Biochemical pregnancy was defined as positive hCG without a gestational sac; clinical pregnancy rate was defined as intrauterine pregnancy with fetal heart beat and implantation rate was defined as the number of gestational sacs per total number of embryos transferred.

STATISTICAL ANALYSIS

All the statistical analyses in the study were performed by SPSS statistical software (v 20.0; IBM Corp., Chicago, IL). Power analysis showed that it was required to include at least 523 MII oocytes per group for a power of 80% at an alpha level of 0.05 in order to detect a possible difference of 10% in blastocyst utilization rate. As a result, a total of 72 patients having a total of 1075 mature oocytes were included in the study in which 537 were assigned for SLC and 538 for PSU group.

Mann-Whitney U test was used to compare numerical variables showing nonparametric distribution such as as the number of MII oocytes, fertilized oocytes, good quality day 2 embryos, total blastocyst and top quality blastocysts.

Clinical outcomes were calculated as the secondary findings of the study. They were analyzed retrospectively since randomization failed due to the absence of good blastocysts in the assigned group. Accordingly, a total of 47 embryo transfers were analyzed due to the exclusion of 25 cases who underwent total embryo freezing. Among these, there were 10 double and two single embryo transfers in PSU group, 16 double and 6 single embryo transfers in SLC group and 13

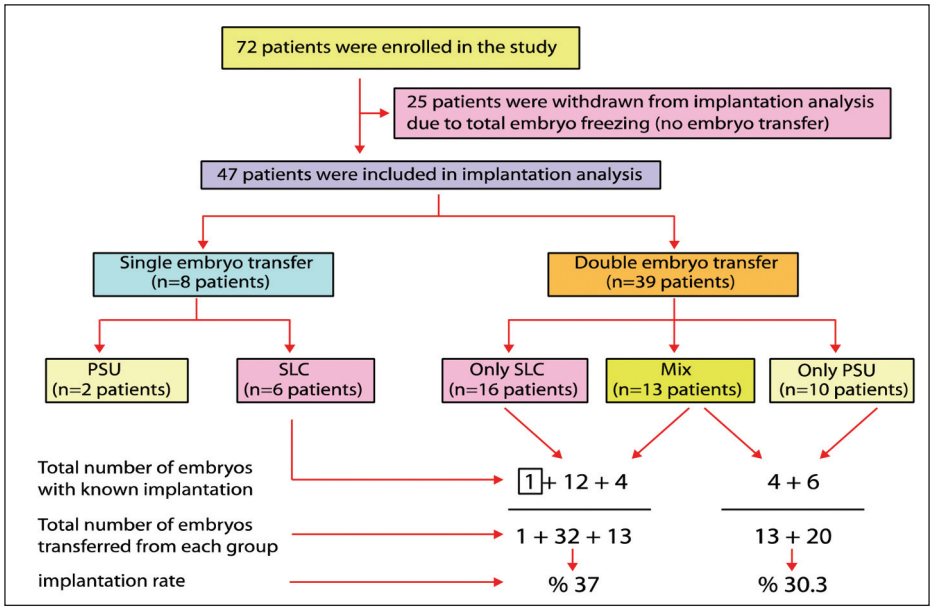

FIGURE 1: Patient enrollment in implantation analysis.
PSU: Pellet swim-up; SLC: Single layer centrifugation.

patients were transferred two embryos from either group (shown as mix group in Figure 1).

Implantation rates were calculated by dividing total number of blastocysts with known implantation to the total number of blastocysts transferred in all groups. In addition, clinical outcomes were compared between groups by using Chi-Square test. A value of $p<0.05$ was considered as statistically significant for all analyses.

RESULTS

When we analyzed demographic characteristics of 72 patients included in the study, we found a mean maternal age of 34.3±3.1 years old and a paternal age of 36.1±3.6 years old. In terms of semen characteristics of the male patients included in the study, mean semen volume was found to be 3.6±1.2 ml; mean sperm concentration was 52±21x10^6 million/ml; total motility was 63±24% and progressive motility was 37±15%.

Laboratory outcomes of both groups were given in Table 1. The groups were found to be comparable in terms of all laboratory key performance indicators indicated in the table (p>0.05 for each). Although PSU method demonstrated a higher blastocyst yield (total blastocyst development and blastocyst utilization rate) compared to SLC in our study, the differences did not reach a statistical significance level (p>0.05).

Patient enrollment for implantation analysis was shown in detail in Figure 1. Implantation rates were found to be 30.3% vs 37% for the embryos transferred from PSU and SLC groups, respectively. Although there was an increase in the SLC group in terms of implantation, the difference again did not reach a statistical significance (p=0.058).

The comparison of the clinical outcomes was given in Table 2. There were 6 pregnancies (including 4 singletons, one twin and one biochemical) in PSU group whereas 12 pregnancies (including 7 singletons, one twin and 4 biochemicals) were present in

TABLE 1: Comparison of laboratory performance indicators between SLC and PSU groups.

Variables	PSU (n=72)	SLC (n=72)	p value
Number of mature (MII) oocytes (n)	7.47±3.8	7.6±3.8	0.85
Number of fertilized (2PN) oocytes (n)	6.1±2.3	6.2±3.1	0.8
Number of good quality embryos on day 2 (n)	4.74±2.7	4.93±2.9	0.68
Total blastocyst development rate (%)	61.1±24.3	55.8±25.2	0.21
Blastocyst utilization rate (%)	42.1±13.1	36.4±12.7	0.3

Values are mean ± SD unless otherwise stated. PSU=Pellet swim-up technique; SLC=single layer centrifugation technique.

TABLE 2: Comparison of clinical outcomes between SLC and PSU groups.

Variables	PSU (n=12)	SLC (n=22)	p value
Pregnancy rate (n/%)	6/50	12/55	0.25
Clinical pregnancy rate (n/%)	5/42	9/41	0.07
Implantation rate (n/%)	10/30	17/37	0.09

SLC group. The differences between groups were not statistically significant in terms of all clinical parameters analyzed ($p>0.05$ for all).

DISCUSSION

It has been well known that quality of the gametes has a direct influence on the embryonic development and the success of the IVF cycle. It is more likely to select an abnormal sperm cell from a cell population having semen parameters which are poorer than normal values because any possible damage on the spermatozoa may not be recognized by visual inspection during the ICSI procedure. Therefore; especially in the laboratories using predominantly ICSI technique, semen preparation for ICSI gains more importance to have higher fertilization rates and better outcomes. Although most of them are based on similar protocols, several techniques with slight modifications are introduced in routine clinical applications of IVF laboratories recently. Selection of the suitable technique depends basically on different requirements which include cost-effectiveness, technical complexity, number of technicians, duration of the procedure, potential capacity of the IVF lab as well as the quality of spermatozoa.

In the present study, we used modified pellet swim-up and single layer density gradient techniques for sperm preparation. It has been reported that serial centrifugations of semen could cause sperm DNA damage through the generation of excessive oxidative stress.[7,12] SLC appears to be a method which is more practical than a two-layer density gradient and may yield more number of spermatozoa by shorter centrifugation periods. The technique was found to yield comparable sperm recovery rates and cause less oxidative stress; therefore is suggested to be a good alternative for sperm preparation.[13-16] Pellet swim-up technique was also analyzed and was found to be the technique that causes the lowest DNA fragmentation rate in the study of Volpes *et al*. (2016), and is suggested to be the best option in terms of low cost and reduced time.[17]

Paternal contribution to embryonic development has been shown to start from day 2 until the blastocyst stage during the early embryonic development.[18] Therefore, whether any of the methods create a difference in blastocyst development comprised the question of this study. According to the present data, we may suggest that both sperm sorting methods seem to have comparable effects on the blastocyst development rate and blastocyst quality although there was a slight increase in the PSU group which should be further analyzed by larger patient populations.

Sibling oocyte study design has an important advantage which allows comparing the embryonic development of the oocytes from the same maternal origin. In a similar study using sibling oocytes, two sperm preparation techniques were compared in terms of some key performance indicators in the IVF laboratory; and have reported significantly better results in favor of direct micro swim-up compared to density gradient. However, they used no centrifugation for micro swim-up technique; thus, suggested that the adverse effects obtained in density gradient technique are due to the centrifugation process.[19] To eliminate any possible adverse effect and stress that may be caused by the difference in centrifugation times, we shortened the duration of centrifugation in SLC and equalized the duration of centrifugation in both methods. Another study including this design compared the laboratory and clinical outcomes of swim up versus a microfluidic sperm sorting chip; and concluded that such a chip did not improve the laboratory and clinical performance indicators significantly compared to conventional swim up method.[20]

Although having a higher blastocyst utilization rate, our results demonstrated a lower implantation rate in the PSU group suggesting that the increase in blastocyst development rate may not reflect the implantation potential of the blastocysts. This result seems to be parallel with the results of Capalbo *et al*. who reported that common indicators of blastocyst evaluation were not enough to select the embryos with higher implantation potential among euploid embryos.[21]

Evaluation of clinical outcomes was the main limitation of our study. These secondary outcomes could be analyzed retrospectively in this study since blastocyst transfers could be performed based on final morphology in some cases rather than the assignment made in the beginning of the study. Since a mix group occurred due to the incompliance to this assignment in some double embryo transfers, the number of patients within both groups remained very limited to make a powerful analysis. Besides, the analysis of live birth rates will be noteworthy to observe the contribution of paternal genome to a healthy newborn.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Ender Yalçınkaya Kalyan;* ***Design:*** *Seren Can Çelik, Ender Yalçınkaya Kalyan;* ***Control/Supervision:*** *Eray Çalışkan;* ***Data Collection and/or Processing: S****eren Can Çelik, Dilara Akgöl, Özlem Okan, Seçkin Yalçınkaya, Ender Yalçınkaya Kalyan;* ***Analysis and/or Interpretation:*** *Eray Çalışkan, Gözde Kaya;* ***Literature Review:*** *Ender Yalçınkaya Kalyan;* ***Writing the Article:*** *Ender Yalçınkaya Kalyan;* ***Critical Review:*** *Gözde Kaya;* ***References and Fundings:*** *Eray Çalışkan;* ***Materials:*** *Eray Çalışkan.*

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