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Journal of Assisted Reproduction and Genetics
Official Publication of ALPHA, Scientists in Reproductive Medicine

ISSN 1058-0468
Volume 30
Number 6

J Assist Reprod Genet (2013) 30:849-854
DOI 10.1007/s10815-013-9989-x

Springer

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TECHNOLOGICAL INNOVATIONS

Intracytoplasmic morphologically selected sperm injection outcomes: the role of sperm preparation techniques

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Received: 14 December 2012 / Accepted: 20 March 2013 / Published online: 2 April 2013

Abstract
Purpose To compare the results of intracytoplasmic morphologically selected sperm injection (IMSI) between cycles in which the swim-up (SUP) or the density gradient centrifugation (DGC) techniques were used for sperm preparation.
Methods We evaluated 70 IMSI cycles performed in women with age ≤ 37 years, undergoing IMSI as result of male factor. The couples were divided into two groups: DGC group (n=26) and SUP group (n=44). The groups were compared with regard to IMSI outcomes.
Results There were no significant differences between SUP and DGC groups regarding the number of follicles, oocytes, mature oocytes, oocyte yield and mature oocyte rate. Fertilization rate and high-quality embryos rate on day 5 of development were similar between SUP and DGC groups. Implantation, pregnancy and miscarriage rates were not statistically different between SUP and DGC groups (28.8 vs 33.3 %, 46.2 vs 57.1 % and 8.3 vs 4.2 %, respectively).
Conclusions Both the SUP and the DGC techniques recover improved sperm fractions and result in similar IMSI outcomes. Further randomized trials analyzing both the quality of sperm through MSOME and the IMSI outcomes are needed to elucidate the role of sperm preparation techniques and morphology on IMSI outcomes.

Keywords MSOME · IMSI · Sperm · Swim-up · Density gradient

Capsule Both the swim-up and the density gradient centrifugation techniques recover improved sperm fractions and result in similar IMSI outcomes.

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Introduction

Human ejaculate is composed of a mix of spermatozoids, seminal liquid, epithelial cells, immature and necrotic sperm cells; red and white blood cells and bacteria [21]. Under in vivo conditions, spermatozoa are separated from these detrimental components in the female genital tract by migration through the cervical mucus [16]. Several semen separation techniques have been developed to separate the sperm fraction for use in assisted reproductive techniques. The most commonly used protocols are density–gradient centrifugation (DGC) and swim-up (SUP) [11].

The SUP technique relies on the ability of the motile spermatozoa to "swim up" into the culture medium, while slow and immotile sperm remain behind, along with other components in the semen pellet [2]. The DGC method separates spermatozoa according to their density and favors the isolation of motile and morphologically normal spermatozoa [24].

Several studies addressed whether there was any differences between these two methods regarding sperm motility and concentration after semen preparation and the outcomes of intrauterine insemination [1,6,10,25,35], in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) [9,18–20,22,32,33] However the results are controversial.

A new method for the detailed morphological evaluation of motile spermatozoa in real time, named "motile sperm organellar morphology examination" (MSOME) was developed and allowed the introduction of modified ICSI

procedure, called "intracytoplasmic morphologically selected sperm injection (IMSI). IMSI relies on the selection of morphologically normal spermatozoa, under a magnification of at least 6000 times, to be used for injection. This magnification provides an accurate description of spermatozoa abnormalities, particularly the presence of head vacuoles [3], which is indicative of abnormal chromatin packaging in spermatozoa [13].

One recent study found that the degree of vacuolization of sperm was lower after both gradient centrifugation and swim-up preparation compared with whole semen, suggesting that both methods allow the selection of less vacuolated sperm cells [21]. However, the results of IMSI were never compared between SUP cycles and DGC cycles. Therefore, this was the aim of this study.

Materials and methods

Experimental design, patients and inclusion criteria

We retrospectively evaluated 70 IMSI cycles performed from January 2011 to December 2011. Inclusion criteria were as follows: women with age ≤37 years, undergoing IMSI as result of male factor, with regular menstrual cycles of 25–35 days, normal basal FSH and LH levels, BMI less than 30 kg/m^2, presence of both ovaries and intact uterus, absence of polycystic ovaries, endometriosis, or gynaecological/medical disorders and a negative result in a screening for sexually transmitted diseases. No patient had received any hormone therapy for at least 60 days preceding the study.

The couples were divided into two groups according to the sperm preparation technique: DGC group (n=26) and SUP group (n=44). The groups were compared with regard to IMSI outcomes.

A written informed consent was obtained, in which patients agreed to share the outcomes of their own cycles for research purposes, and the study was approved by the local institutional review board.

Controlled ovarian stimulation

Ovarian stimulation was achieved by the administration of recombinant follicle-stimulating hormone and gonadotropin-releasing hormone antagonist, as previously described [27].

Semen sample collection and preparation

All semen samples were collected by masturbation after 2 to 7 days of ejaculatory abstinence. After liquefaction for 30 min at room temperature, the semen samples were evaluated according to the threshold values established by the WHO in 2010 [34]. The decision of performing DGC or SUP was based on semen sample quality. With a suboptimal quality sample a DGC was performed, which is usually preferred for the greater number of mobile spermatozoa selected from poor characteristics samples (low number, motility and morphology samples) [8]. In addition, the DGC was performed particularly when there was high viscosity semen, elevated leukocytes or high debris contents. For all other sorts of semen samples the method of choice was the swim-up technique.

DGC

Using a sterile pipette 1.0 mL of the "lower layer" (90 % Isolate, Irvine Scientific, Santa Ana, CA, USA) was transferred into a conical centrifuge tube. Using a new sterile pipette 1.0 mL of the "upper layer" (50 % Isolate, Irvine Scientific, Santa Ana, CA, USA) was gently dispensed on top of the lower layer. A liquefied 2.0 mL semen sample was then placed on top of the upper layer and the tube was centrifuged for 20 min at 330 ×g and this process was repeated using additional tubes until the whole ejaculated sample was processed. The upper and lower layers were carefully aspirated without disturbing the pellet. Using a transfer pipette, 1.0 mL of HEPES-buffered human tubal fluid medium (mHTF, Global, LifeGlobal, Connecticut, USA) was added and the re-suspended pellet was centrifuged for 7 min at 330 ×g. The washing procedure was repeated. The supernatant was then removed and the pellet suspended in a volume of 0.5 mL of mHTF. Sperm count and motility were estimated in the recovered fractions.

SUP technique

Using a sterile pipette, 0,5 mL semen was placed in a conical tube and 1 mL culture medium (mHTF, Global, LifeGlobal, Connecticut, USA) was slowly layered on top. The tube was sealed, inclined at 45° and stored at 37 °C for 60 min to allow motile sperm to 'swim up'. After the incubation period, a sterile Pasteur pipette was used to aspirate the supernatant and transfer it to a sterile conic tube. Sperm count and motility were estimated in the recovered fractions.

IMSI technique

Sperm selection for IMSI was examined at high magnification using an inverted Nikon Diaphot microscope equipped with high-power differential interference contrast optics (DIC/Nomarski). The total calculated magnification was x6.600. An aliquot of the sperm cell suspension was transferred to a microdroplet of modified human tubal fluid medium containing 8 % polyvinyl pyrrolidone (PVP; Irvine Scientific, SantaAna,CA) in a sterile glass dish (FluoroDish;

World Precision Instrument, Sarasota, FL). The dish was placed on a microscope stage above a Uplan Apo x 100 oil/1.35 objective lens previously covered by a droplet of immersion oil. The sperm cells exhibiting normally shaped nuclei ([1] smooth, [2] symmetric, and [3] oval configuration) and [4] normal nuclear chromatin content (if it contained no more than one vacuole, which occupies <4 % of the nuclear area) were selected for injection [3].

Fertilization, embryo quality and embryo transfer

Approximately 16 h after IMSI, fertilisation was confirmed by the presence of two pronuclei and the extrusion of the second polar body. Embryos were maintained in a 50 μL drop of culture medium (Global®, LifeGlobal, Connecticut, USA) supplemented with 10 % protein supplement covered with paraffin oil in a humidified atmosphere under 6 % CO2 at 37 °C for 5 days.

High-quality embryos were defined as those showing 8–10 cells on the third day of development, less than 15 % fragmentation, symmetric blastomeres, absence of multinucleation and absence of zona pellucida dysmorphisms.

To evaluate blastocyst-stage morphology, the standard Gardner's grading scale was used [14].

Embryo transfer was performed on day 5 of development by using a soft catheter with transabdominal ultrasound guidance. One to three embryos were transferred per patient.

Clinical follow-up

A pregnancy test was performed 12 days after embryo transfer. All women with a positive test had a transvaginal ultrasound scan 2 weeks after the positive test. A clinical pregnancy was diagnosed when the fetal heartbeat was detected. Pregnancy rates were calculated per transfer. Miscarriage was defined as pregnancy loss before 20 weeks.

Data analysis and statistics

The SUP and DGC groups were compared with regard to: (i) fertilization rate, (ii) percentage of high quality embryos on the third day of development (D3), (iii) percentage of high quality blastocysts on the fifth day of development (D5), (iv) pregnancy, (v) implantation and (vi) miscarriage rates.

Data are expressed as mean ± standard deviation for continuous variables, while percentages were used for categorical variables. Mean values were compared by Student's *t* parametric test or Mann–Whitney non-parametric test. Percentages were compared by the Chi-squared or Fisher exact test, only when expected frequency was five or fewer. Data analysis was conducted using MINITAB 16 Software.

Results

The patient demographic variables, stimulation characteristics and seminal parameters are compared in Table 1. The SUP and DGC groups were similar with respect to female and male ages. The causes of infertility were evenly distributed between the two groups. Total sperm concentration after sperm preparation was significantly higher in the DGC group as compared to the SUP group (6.7±4.7 vs. 2.8±1.9 millions, $p=0.002$), however, a higher percentage of motile sperm after sperm preparation was observed in the SUP group (91.8 vs. 79.5 %, $p=0.013$). There were no significant differences between SUP and DGC groups regarding the number of follicles, oocytes, mature oocytes, oocyte yield and mature oocyte rate.

The comparison of IMSI outcomes between the groups is showed in Table 2. Fertilization rate and high-quality embryos rate on day 3 and 5 of development were similar between SUP and DGC groups. Implantation, pregnancy and miscarriage rates were not statistically different between SUP and DGC groups (28.8 vs. 33.3 %, 46.2 vs. 57.1 % and 8.3 vs. 4.2 %, $p>0.05$, respectively).

Discussion

Several different methods of isolation and concentration of sperm for assisted reproductive techniques have been developed. Evidences suggest that a profound sperm morphological evaluation provide significant prognostic information regarding IVF outcomes [23]. In addition, a positive correlation between morphology and sperm function has been shown [26].

Since the advent of IMSI [3], several articles have been reporting positive outcomes with the use of this technique [3–5,7,12,15,17,28,29]. Therefore, preparing sperm samples with high incidence of morphologically normal cells represent an important step for IMSI.

A recent study investigated whether the SUP or DGC techniques favors the recovery of sperm with lower vacuolization rates. The authors showed that both methods allow the selection of less vacuolated sperm cells and with less DNA fragmentation, however, the SUP recovered sperm with significantly lower vacuolization rate that the DGC technique [21].

Therefore, in this study, we compared the IMSI outcomes associated with these two methods of sperm preparation. Our results showed that the DGC technique yields higher number of sperm than the SUP technique, however, a higher percentage of motile sperm was observed after SUP than after DGC sperm preparation. In addition, our study demonstrated that there are no significant differences between the outcomes of IMSI

Table 1 Demographic variables, stimulation characteristics and seminal parameters in SUP and DGC groups

Variable	SUP group (n=26)	DGC group (n=44)	p value
Female age	32.2±2.7	30.3±3.8	0.112
Male age	36.2±4.2	34.7±4.9	0.379
Male factor (%)	22/44 (50.0)	12/26 (46.2)	0.755
Unexplained infertility (%)	6/44 (13.6)	4/26 (15.4)	0.839
Tubal factor (%)	16/44 (36.4)	10/26 (38.5)	0.860
FSH administered (IU)	2244±512	2028±739	0.317
Estradiol level (pg/mL)	1648±1475	1146±18118	0.380
Number of aspirated follicles	19.6±9.2	20.6±10.0	0.771
Number of retrieved oocytes	13.5±6.6	14.7±5.0	0.594
Recovery rate (%)	70.1±13.6	77.2±17.4	0.192
Number of MII oocytes	10.9±5.8	10.6±4.6	0.913
MII oocyte rate (%)	79.0±10.2	72.8±16.6	0.176
Semen volume (mL)	3.1±1.3	3.4±1.4	0.494
Initial total sperm concentration (million)	147.9±74.9	112.2±96.2	0.231
Sperm motility (%)	60.2±12.8	60.0±13.8	0.953
Sperm morphology (%)	4.8±1.7	4.7±2.0	0.812
Final total sperm concentration (million)	2.8±1.9	6.7±4.7	0.002
Final sperm motility (%)	91.8±14.1	79.5±10.7	0.013

SUP swim-up; *DGC* density gradient centrifugation; *MII* metaphase II

cycles in which the SUP or the DGC techniques were used for sperm preparation.

Several studies have investigated the efficacy of these methods with regards to the recovery of reduced percentage of sperm cells with fragmented DNA, however the results are still controversial [30,31,36,37]. While one study showed that the DGC is more effective than SUP in reducing the percentage of sperm with DNA damage [25], another one, in contrast, demonstrated that SUP recovers lower percentage of sperm with fragmented DNA as compared to DGC [37]. Enciso et al. [11] showed that SUP and DGC are equally efficient in eliminating spermatozoa containing double-strand DNA damage and sperm with highly damaged DNA, as characterized by the presence of both single- and double-strand DNA breaks. However, DGC was more efficient than SUP in selecting spermatozoa that are free from single-strand DNA damage.

So far, a single study has investigated the relationship between MSOME and sperm preparation techniques [21]. Monqaut et al. [21] observed that both techniques were efficient in recovering lower percentage of sperm cells with vacuolization. Unfortunately, the findings of the aforementioned study were not investigated in our study since we did not classify the sperm cells after sperm preparation. In addition, the main drawback of our study is the fact that we did not evaluate sperm morphology after semen preparation.

It is important to highlight that the "best-looking spermatozoon is selected for the IMSI procedure and therefore the IMSI could have surpassed the influences of the separation methods. Nevertheless, the fact that IMSI would become easier and faster if one preparation approach resulted in more "best-looking" spermatozoa could not be overlooked.

Conclusions

Adding our findings to those obtained in Monqaut's study, we could suggest that both techniques recover improved

Table 2 IMSI outcomes in SUP and DGC groups

Variable	SUP group (n=26)	DGC group (n=44)	p value
Fertilization rate (%)	72.3	68.9	0.631
High-quality embryos rate on D3	56.7±27.7	47.6±27.5	0.355
High-quality embryos rate on D5	32.5±11.7	30.7±11.3	0.698
Transferred embryos	2.2±0.8	2.1±1.0	0.770
Implantation rate	28.8	33.3	0.734
Pregnancy rate (%)	12/26 (46.2)	24/42 (57.1)	0.378
Miscarriage rate (%)	1/12 (8.3)	1 /24 (4.2)	1.000

SUP swim-up; *DGC* density gradient centrifugation

sperm fractions and result in similar IMSI outcomes. In addition, MSOME may be a surrogate tool for selecting sperm with better physiological status. Further randomized trials analyzing both the quality of sperm through MSOME and the IMSI outcomes are needed to elucidate the role of sperm preparation techniques and morphology on IMSI outcomes.

References

1. Allamaneni SS, Agarwal A, Rama S, Ranganathan P, Sharma RK. Comparative study on density gradients and swim-up preparation techniques utilizing neat and cryopreserved spermatozoa. Asian J Androl. 2005;7:86–92.
2. Alvarez JG, Lasso JL, Blasco L, Nunez RC, Heyner S, Caballero PP, et al. Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile lifetime. Hum Reprod. 1993;8:1087–92.
3. Bartoov B, Berkovitz A, Eltes F, Kogosowski A, Menezo Y, Barak Y. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. J Androl. 2002;23:1–8.
4. Berkovitz A, Eltes F, Lederman H, Peer S, Ellenbogen A, Feldberg B, et al. How to improve IVF-ICSI outcome by sperm selection. Reprod Biomed Online. 2006;12:634–8.
5. Berkovitz A, Eltes F, Yaari S, Katz N, Barr I, Fishman A, et al. The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. Hum Reprod. 2005;20:185–90.
6. Boomsma CM, Heineman MJ, Cohlen BJ, Farquhar C. Semen preparation techniques for intrauterine insemination. Cochrane Database Syst Rev. 2004;CD004507.
7. de Almeida Ferreira Braga DP, Setti AS, Figueira RC, Nichi M, Martinhago CD, Iaconelli Jr. A, Borges Jr. E, Sperm organelle morphologic abnormalities: contributing factors and effects on intracytoplasmic sperm injection cycles outcomes. Urology. 2011; 78:786–91.
8. Canale D, Giorgi PM, Gasperini M, Pucci E, Barletta D, Gasperi M, et al. Inter and intra-individual variability of sperm morphology after selection with three different techniques: layering, swimup from pellet and percoll. J Endocrinol Invest. 1994;17:729–32.
9. De Vos A, Van De Velde H, Joris H, Verheyen G, Devroey P, Van Steirteghem A. Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. Fertil Steril. 2003;79:42–8.
10. Dodson WC, Moessner J, Miller J, Legro RS, Gnatuk CL. A randomized comparison of the methods of sperm preparation for intrauterine insemination. Fertil Steril. 1998;70:574–5.
11. Enciso M, Iglesias M, Galan I, Sarasa J, Gosalvez A, Gosalvez J. The ability of sperm selection techniques to remove single- or double-strand DNA damage. Asian J Androl. 2011;13:764–8.
12. Figueira Rde C, Braga DP, Setti AS, Iaconelli Jr A, Borges Jr E. Morphological nuclear integrity of sperm cells is associated with preimplantation genetic aneuploidy screening cycle outcomes. Fertil Steril. 2011;95:990–3.
13. Franco Jr JG, Mauri AL, Petersen CG, Massaro FC, Silva LF, Felipe V, et al. Large nuclear vacuoles are indicative of abnormal chromatin packaging in human spermatozoa. Int J Androl. 2012;35:46–51.
14. Gardner DK, Schoolcraft WB, eds. In vitro culture of human blastocysts. In: Toward Reproductive Certainty: Fertility and Genetics Beyond. London: Parthenon Publishing London; 1999.
15. Garolla A, Fortini D, Menegazzo M, De Toni L, Nicoletti V, Moretti A, et al. High-power microscopy for selecting spermatozoa for ICSI by physiological status. Reprod Biomed Online. 2008;17:610–6.
16. Henkel R. Sperm preparation: state-of-the-art–physiological aspects and application of advanced sperm preparation methods. Asian J Androl. 2012;14:260–9.
17. Knez K, Tomazevic T, Zorn B, Vrtacnik-Bokal E, Virant-Klun I. Intracytoplasmic morphologically selected sperm injection improves development and quality of preimplantation embryos in teratozoospermia patients. Reprod Biomed Online. 2012.
18. Kupker W, al-Hasani S, Schulze W, Kuhnel W, Schill T, Felberbaum R, et al. Morphology in intracytoplasmic sperm injection: preliminary results. J Assist Reprod Genet. 1995;12:620–6.
19. Lundin K, Soderlund B, Hamberger L. The relationship between sperm morphology and rates of fertilization, pregnancy and spontaneous abortion in an in-vitro fertilization/intracytoplasmic sperm injection programme. Hum Reprod. 1997;12:2676–81.
20. Mansour RT, Aboulghar MA, Serour GI, Amin YM, Ramzi AM. The effect of sperm parameters on the outcome of intracytoplasmic sperm injection. Fertil Steril. 1995;64:982–6.
21. Monqaut AL, Zavaleta C, Lopez G, Lafuente R, Brassesco M. Use of high-magnification microscopy for the assessment of sperm recovered after two different sperm processing methods. Fertil Steril. 2011;95:277–80.
22. Nagy ZP, Liu J, Joris H, Verheyen G, Tournaye H, Camus M, et al. The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. Hum Reprod. 1995;10:1123–9.
23. Oehninger S, Acosta AA, Kruger T, Veeck LL, Flood J, Jones Jr HW. Failure of fertilization in in vitro fertilization: the "occult" male factor. J In Vitro Fert Embryo Transf. 1988;5:181–7.
24. Pousette A, Akerlof E, Rosenborg L, Fredricsson B. Increase in progressive motility and improved morphology of human spermatozoa following their migration through Percoll gradients. Int J Androl. 1986;9:1–13.
25. Sakkas D, Manicardi GC, Tomlinson M, Mandrioli M, Bizzaro D, Bianchi PG, et al. The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. Hum Reprod. 2000;15:1112–6.
26. Seracchioli R, Porcu E, Flamigni C. The diagnosis of male infertility by semen quality. Sperm morphology is not the only criterion of male infertility. Hum Reprod. 1995;10:1039–41.
27. Setti AS, Cortezzi SS, Figueira Rde C, Martinhago CD, Braga DP, Iaconelli Jr A, et al. A chromosome 19 locus positively influences the number of retrieved oocytes during stimulated cycles in Brazilian women. J Assist Reprod Genet. 2012;29:443–9.
28. Setti AS, Figueira Rde C, Braga DP, Iaconelli Jr A, Borges Jr E. Intracytoplasmic morphologically selected sperm injection benefits for patients with oligoasthenozoospermia according to the 2010 World Health Organization reference values. Fertil Steril. 2011;95:2711–4.
29. Souza Setti A, Ferreira RC, Paes de Almeida Ferreira Braga D, de Cassia Savio Figueira R, Iaconelli Jr A, Borges Jr E. Intracytoplasmic sperm injection outcome versus intracytoplasmic morphologically selected sperm injection outcome: a meta-analysis. Reprod Biomed Online. 2010;21:450–5.
30. Spano M, Cordelli E, Leter G, Lombardo F, Lenzi A, Gandini L. Nuclear chromatin variations in human spermatozoa undergoing swim-up and cryopreservation evaluated by the flow cytometric sperm chromatin structure assay. Mol Hum Reprod. 1999;5:29–37.
31. Stevanato J, Bertolla RP, Barradas V, Spaine DM, Cedenho AP, Ortiz V. Semen processing by density gradient centrifugation does

not improve sperm apoptotic deoxyribonucleic acid fragmentation rates. Fertil Steril. 2008;90:889–90.
32. Sukcharoen N, Sithipravej T, Promviengchai S, Chinpilas V, Boonkasemsanti W. Sperm morphology evaluated by computer (IVOS) cannot predict the fertilization rate in vitro after intracytoplasmic sperm injection. Fertil Steril. 1998;69:564–8.
33. Svalander P, Jakobsson AH, Forsberg AS, Bengtsson AC, Wikland M. The outcome of intracytoplasmic sperm injection is unrelated to 'strict criteria' sperm morphology. Hum Reprod. 1996;11:1019–22.
34. rWHO, eds. WHO laboratory manual for the examination and processing of human semen. Geneva: World Health Organization; 2010.
35. Xu L, Lu RK, Chen L, Zheng YL. Comparative study on efficacy of three sperm-separation techniques. Asian J Androl. 2000;2:131–4.
36. Younglai EV, Holt D, Brown P, Jurisicova A, Casper RF. Sperm swim-up techniques and DNA fragmentation. Hum Reprod. 2001;16:1950–3.
37. Zini A, Finelli A, Phang D, Jarvi K. Influence of semen processing technique on human sperm DNA integrity. Urology. 2000;56:1081–4.