Annexin V-MACS in infertile couples as method for separation of sperm without DNA fragmentation

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ABSTRACT

Objective: To determine the effect of using MACS technology on clinical pregnancy, as a method for separation of damaged sperm in infertile patients.

Methods: 136 infertile men having normal semen parameters in accordance with WHO 2010 criterion, undergoing ICSI cycle were enrolled during the course of the study. The patients were prospectively randomized and enrolled after oocyte retrieval and were assigned to the ICSI group, PICSI group or MACS group. Embryo development and clinical pregnancy were assessed. In 17 randomized MACS patients, sperm DNA fragmentation was tested in the presumptive apoptotic and no apoptotic spermatozoa fractions.

Results: Similar results were obtained between groups for the following parameters: fertilization rates of 78.97% (95% confidence interval [CI]:74.37-83.57), 70.15% (95% CI:63.98-76.33) and 80.28% (95% CI:73.74-86.81) for ICSI, PICSI and MACS group, respectively; Number of Day-3 embryos was 5.04 (95% CI:4.09-5.98), 5.17 (95% CI:4.24-6.10) and 5.59 (95% CI:4.31-6.87) for ICSI, PICSI and MACS group, respectively; number of freezing embryos in blastocyst stage was 0.78 (95% CI:0.25-1.31), 0.70 (95% CI:0.27-1.14) and 1 (95% CI:0.37-1.6) for ICSI, PICSI and MACS group, respectively. However, clinical pregnancy rates of 58.1% for MACS group versus 40.4% and 27.3% for PICSI and ICSI group, respectively, were showed statistical difference ($P=0.019$). DNA fragmentation index for the two sperm MACS fraction showed statistical differences ($P=0.000$), MACS reduced the D.F.I of the sperm sample.

Conclusions: The use of MACS technology improves the clinical pregnancy on infertile couples and can be applied as a method for sperm separation, discriminating sperm with high DNA fragmentation.

Keywords: MACS, ICSI, DNA fragmentation, PICSI, Morphology.

INTRODUCTION

Selection of human spermatozoa prior to intracytoplasmic sperm injection (ICSI) technique is currently based on criteria as viability, motility and morphology. Double density gradient centrifugation (DGC) and the swim-up procedure are currently used as standard preparation techniques. However, new insights in the development of molecular selection strategies have been introduced: Namely, the hyaluronic acid (HA) mediated sperm selection know as Physiologic ICSI (PICSI) and the annexin V magnetic activated cell separation (MACS) are ones of the most recently methods introduced (Paasch *et al.*, 2007).

MACS is a new tool to optimize sperm selection in assisted reproduction. The technique is based on the binding of superparamagnetic Annexin-microbeads to externalized phosphatidylserine (PS) at the outer leaflet of the plasma membrane of sperm with activated apoptosis signaling or membrane damage (Grunewald & Paasch, 2013; Sheikhi *et*

al., 2013). There are already some reports comparing ICSI with MACS. However the positive effect of MACS sperm selection is still to be confirmed in larger-scale studies; in the meantime, our study compare the clinical pregnancy and embryo development in MACS, with PICSI and conventional ICSI.

MATERIAL AND METHODS

Design

It was a unicentric, prospective and randomized study at the Centro de Fertilidad y Reproduccion Asistida (CEFRA), Lima- Peru. The main endpoint of this study was clinical pregnancy after ICSI, with and without use of MACS, PICSI or conventional sperm selection method.

Patient

From January 2013 to July 2014 patients with infertility having normal sperm concentration parameter in accordance with WHO 2010 criterion, undergoing ICSI cycle were enrolled during the course of the study. 136 patients were prospectively randomized after oocyte retrieval and were assigned to either the ICSI group (for which sperm selection for injection was only based on visual assessment), PICSI group, or to MACS group. The endometriosis was the unique exclusion criteria used in women.

Procedure

Oocyte cumulus complexes (OCC's) were retrieved in buffered media (GlobalW/Hepes, Life global, USA) supplemented with 5% of Human Seroalbumin (HSA) and transferred to Global Total for Fertilization (Life Global, USA) for culture at 37°C, 5% O₂ and 6% CO₂ for 3 to 4h. Meanwhile, the semen sample (obtained by masturbation from the husband) was subjected to double density gradient centrifugation (All Grade, Life Global, USA) at 300 g for 10 min. The resulting pellet was washed (Global W/Hepes, Life Global, USA) and allocated to any of the groups of sperm selection.

OCC's were denuded mechanically using a glass pipette after brief exposure to hyaluronidase (Hyaluronidase, Life Global, USA) for 30s. Mature oocytes were injected with sperm according to the standard ICSI protocol of our center. In the ICSI group, a 5µl polyvinyl pyrrolidone drop (PVP, Life Global, USA) under oil (Lite Oil, LifeGlobal, USA), was used to select spermatozoa with normal morphology for subsequent injection. In the PICSI group, SpermSlow™ (MediCult-Origio) was applied in order to get the most viable sperm, the dish for sperm injection was prepared according to the protocol used by Parmegiani *et al.*, 2012. In the MACS group, the pellet was resuspended in 2-5 ml with 1X binding buffer (BB; Miltenyi Biotec, Germany), centrifuged at 300g for 5 min and the supernatant removed. The sample was then incubated with annexin V conjugated microbeads (Miltenyi Biotec) for 30min at 25°C during continuous agitation. After rinsing the column with BB, the sperm-bead suspension was added on top with BB and, immediately after that, the annexin V-neg-

ative fraction was obtained. To obtain the annexin V-positive fraction, the column was taken out of the magnet and 1 ml BB was added to rinse it using a plunger provided by the manufacturer (MiniMACS; Miltenyi Biotec). The fraction of presumptive no apoptotic fraction with intact membranes were used for ICSI. Both fractions were stored in order to evaluate sperm DNA fragmentation.

In order to evaluate the MACS system we performed Sperm chromatin dispersion test (SCDt) with the Halosperm kit (Halotech DNA, Spain) in both fraction accordance to the MACS system: apoptotic fraction and no apoptotic fraction in 17 patients chosen at random. DNA Fragmentation Index (D.F.I) was recorder in order to compare in both fractions (Fernandez *et al.*, 2003)

Fertilization was checked the next morning at 16–18h post insemination checking the presence of 2PN; embryos were then prolonged cultured in 50 ul drops of Global Total Medium (Life Global, USA) covered with mineral oil (Lite oil, Life global, USA) from day 1 to 3 and from day 3 to 5. Embryo transferred was performed on Day-3 or Day-5 post injection depending of the embryologist scoring/decision, and extra embryos were vitrified on Day-5 if blastocyst was found.

Fertilization rate was calculated as the ratio of number of 2PN embryos over the total number of MII oocytes injected, and expressed as percentage.

A clinical pregnancy was defined as presence of one or more gestational sacs with heart beat on ultrasound 2 to 3 weeks after positive β hCG. Ectopic pregnancies were not counted as clinical pregnancies. Pregnancy loss rate was defined as the number of clinical pregnancies lost before 20 weeks of gestation divided by the total number of clinical pregnancies.

Statistical Analysis

Data were checked for normality before statistical analysis. To compare the various groups and to make the comparisons normally distributed continuous variables were compared using Post hoc test Benforrini, while Kruskal – Wallis tests were performed to evaluate differences in the median for those variables that were not normally distributed. Categorical variables were analyzed using the chi square test. t-student was applied for related samples. For all statistical tests, a *P*-value lower than 0.05 was considered statistically significant.

RESULTS

Out of 136 patients that were enrolled and randomized for the study, 3 cases from the MACS group were excluded from the pregnancy rate analysis since they did not have a embryo transfer instead all embryos were frozen. A total of 55 cases were analyzed in the ICSI group, 47 cases were analyzed in the PICS group and 34 cases were analyzed in the MACS group. There were no significant differences in female age, male age, BMI, semen volume, sperm concentration and motility sperm (Table 1).

The number of oocytes and MII's retrieved, injected, the number of Day-3 embryos, the number of embryos frozen and the number of embryos transferred between the three groups showed no significance differences (Table 2). Similarly, no differences in the number of 2PN were observed; however, the fertilization was reduced in 8% in the PICS group compared to others (*P*= 0.036), and no differences between other groups were observed. The number of Day-3 embryos, number of freezing embryos was similar in all the groups and no significant differences were found; the number of embryos transferred was similar in ICSI, PICS and MACS group (2.18, 2.26 and 1.94, respectively). The clinical pregnancy rate was statistical significantly higher in the MACS group compared to ICSI and PICS groups (58.1%, 27.3 % and 40.4 % respectively; *P*= 0.019). Biochemical pregnancy and pregnancy loss rate was not significantly different between the three groups (Table 2).

To further validate the efficiency of MACS technique for sperm separation, we performed sperm DNA fragmentation analyses in 17 patients. There was a significant reduction of sperm with fragmented DNA after MACS system (*P*= 0.000). Furthermore, from the 17 patients 10 who got pregnant, the D.F.I was less than 14% in the samples used for ICSI (Table 3).

DISCUSSION

The selection of ideal spermatozoa before ICSI could help to optimize the outcome of the treatment, because the injection of a sperm based on viability and normal morphology does not eliminate the chance for damaged DNA or immature spermatozoa and may account for a considerable percentage of failed embryo development post-ICSI. Furthermore, infertile men with spermatozoa with apparently normal morphology may have

Table 1. Descriptive characteristics of the female and male in the ICSI, PICS and MACS group.

Characteristic		ICSI group (55)	PICS group (47)	MACS group (33)	P
Female					
	Age (Y)	37.07 [36.04 - 38.11, 0.52]	36.53 [35.38-37.69, 0.57]	36.94 [35.29 - 38.58, 0.81]	N.S
	BMI (kg/m2)	24.71 [23.91 - 25.52, 0.40]	25.6 [24.60 - 26.60, 0.49]	25.14 [24.06 - 26.22, 0.53]	N.S
Males					
	Age (Y)	38.85 [37.57 - 40.13, 0.64]	38.65 [37.01 - 40.29, 0.81]	37.29 [35.55- 39.03, 0.85]	N.S
	Semen Volume (ml)	2.39 [2.03 - 2.76, 0.18]	2.5 [2.20 - 2.87, 0.17]	2.5 [2.05 - 2.96, 0.22]	N.S
	Sperm Concentration (millions/ml)	62.48 [52.32 - 72.63, 5.06]	53.36 [43.05 - 63.67, 5.12]	49.76 [39.19 - 60.34, 5.19]	N.S
	Sperm Motility (%)	48.5 [44.47 - 52.53, 2.01]	43.53 [38.72 - 48.34, 2.39]	47.59 [41.96 - 53.21, 2.76]	N.S

Note: unless otherwise indicated, values are means [95% CI:SD]

N.S. = not significant

Table 2. Comparison result in the embryo quality ICSI, PICSi and MACS group.

Parameters	ICSI group (55)	PICSi group (47)	MACS group (33)	P
No. Of MII oocytes injected	7.09 [5.99 - 8.19, 0.55]	8.32 [7.25 - 9.38, 0.53]	7.68 [6.29 - 9.07, 0.68]	N.S
No. 2 PN embryos	5.53 [4.59 - 6.46, 0.47]	5.87 [4.87 - 6.87, 0.49]	6.32 [5.01 - 7.64, 0.65]	N.S
Fertilization rate (%)	78.97 [74.37 - 83.57, 2.29]	70.15 [63.98 - 76.33, 3.07]	80.28 [73.74 - 86.81, 3.21]	*
No. Of 3-day embryos	5.04 [4.09 - 5.98, 0.47]	5.17 [4.24 - 6.10, 0.46]	5.59 [4.31 - 6.87, 0.63]	N.S
No. Embryos transferred	2.18 [1.99 - 2.38, 0.09]	2.26 [2.11 - 2.39, 0.07]	1.94 [1.65 - 2.22, 0.14]	N.S
No. Freezing embryos	0.78 [0.25 - 1.31, 0.26]	0.70 [0.27 - 1.14, 0.22]	1 [0.37 - 1.6, 0.31]	N.S
Clinical Pregnancy rate (%)	27.3 (15/55)	40.4 (19/47)	58.1 (18/31)	*
Biochemical pregnancy (%)	10.9 (6/55)	8.5 (4/47)	3.2 (1 /31)	N.S
Pregnancy loss rate (%)	13.3 (2/15)	5.3 (1/19)	5.5 (1/18)	N.S

Note: unless otherwise indicated, values are means [95% CI:SD]

N.S. = not significant

* = Significant difference (P-value <0.05)

2 PN = 2 pro nucleus

Table 3. Descriptive characteristics of male and DNA sperm fragmentation in no apoptotic fraction.

Patient	Male Age	Apoptotic cell reduction (%) in the MACS fractions	D.F.I (%) no apoptotic fraction	Clinical Pregnancy result
1	33	22.6	9.37	No
2	41	6.34	6.44	No
3	37	10.17	12.56	Yes
4	45	13.67	11.21	Yes
5	45	16.48	20.51	No
6	28	14.18	7.25	Yes
7	41	5.1	0.73	Yes
8	29	38.59	15.75	No
9	35	17.76	0.94	No
10	32	-2.88	7.68	Yes
11	38	31.95	9.62	Yes
12	36	24.19	0.39	Yes
13	38	21.38	7.83	Yes
14	42	21.31	15.20	No
15	37	-0.03	4.48	Yes
16	36	6.3	1.11	No
17	39	10.11	12.75	Yes

ND.F.I = DNA fragmentation index

DNA fragmentation (Avendaño & Oehninger, 2011).

Different laboratories have introduced novel procedures to replace traditional sperm selection methods, however, the efficiency of these techniques is still debatable and questionable (Nasr-Esfahani *et al.*, 2012).

The use of magnetic cell sorting using annexin V-conjugated micro beads, at molecular level can mark and isolate sperm with PS translocation to the membrane, specifically targets spermatozoa with damaged membranes, which is a manifestation of apoptosis. However, there is not enough

information concluding the benefits of these methods. In fact, Romany *et al.* (2014) applying MACS technology in unselected males did not find an improvement in the reproductive outcome of ICSI in ovum donation cycles. In our case we decided to test the benefit of MACS in infertile couples because we hypothesize the benefit of avoiding oocyte quality bias could have a disadvantage, since the oocyte can repair the DNA strand breaks before the initiation of the first cleavage division. It is well known that recognition of DNA lesions and repair in the paternal chroma-

tin after fertilization by the oocytes (Derijck et al., 2008; Barton et al., 2007; Ashwood-Smith & Edwards, 1996) and the capability to DNA repair of the oocytes are age dependent. In fact, we found significant differences in clinical pregnancy compared to ICSI group (58.1% vs 27.3 %, respectively). Biochemical pregnancy and pregnancy loss are reduced in MACS groups, however they were not statistical significant (Table 2).

Favoring selection of spermatozoa with intact DNA and normal nucleus, HA may be another alternative to optimize ICSI outcome, in fact some studies have shown significant differences (Parmegiani, 2010) while others did not find differences in clinical pregnancy rate or embryo quality (Ménézo & Nicollet, 2004; Majumdar & Majumdar, 2013). We decided to compare the two new methods for sperm selection, patients of PICS group show clinical pregnancy rate benefits compared to ICSI group (40.4% vs 27.3 %, respectively), but its benefit was inferior to the MACS group (58.1%). It was remarkable to observe a reduced pregnancy loss in both method of sperm selection, PICS and MACS (Table 2).

We can conclude that ICSI could be improved applying another sperm selection method, MACS would be an alternative because it can limit the amount of selected sperm (for ICSI) with damaged DNA ultimately leading to improved clinical pregnancy rates.

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CONFLICT OF INTERESTS

No conflict of interest have been declared.

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