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Research Article

High Pregnancy and Implantation Rates Can Be Obtained Using Magnetic-Activated Cell Sorting (MACS) to Selection Spermatozoa in Patients with High Levels of Spermatic DNA Fragmentation

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Abstract

Purpose: DNA fragmentation in spermatozoa has been associated with poor semen quality, low fertilization rates, impaired preimplantation development, and high miscarriage rates. The objective of this study was to evaluate the effects of the use of non-apoptotic MACS-selected spermatozoa in ICSI cycles, compared to those ICSI cycles in patients with normal sperm DNA fragmentation.

Methods: A total of 134 cycles of ICSI were included in the study. The study group consisted of patients with high DNA fragmentation and their spermatozoa were selected by MACS (n=57); and the control group, of patients with normal DNA fragmentation and their spermatozoa were selected by classic morphological characteristics. The fertilization rate, cleavage rate, embryo quality, pregnancy rate (PR), implantation rate (IR) and miscarriage rate (MR) were compared between groups.

Results: There was no difference in the fertilization rate (74.5 and 76.5%), cleavage rate and good embryo quality at Day 3 (98.3 and 89.1%; 88.4 and 83.7%) or blastocyst formation rate (50.8 and 41.1%) for the study and control groups, respectively. PRs, IRs and MRs were similar for the study group compared to the control group (PR: 63.2 versus 45.5%; IR: 37.4 versus 28.1%; MR: 8.3 versus 17.1). The women were distributed into three groups: <35 years, 35-39 years and ≥ 40 years. Pregnancy, implantation and miscarriage rates were similar in the three evaluated groups (*P*: NS).

Conclusions: By selecting non-apoptotic sperm by MACS, we can achieve very acceptable pregnancy and implantation rates; being a good option for couples with high sperm DNA fragmentation and repeated assisted reproduction failures.

Keywords: Sperm DNA fragmentation; SCD test; ICSI; Pregnancy rate; Implantation rate

Introduction

Semen quality is frequently used as an indirect measure of male infertility. Ejaculate volume, sperm concentration, motility and morphology, determined according to World Health Organisation (WHO) standards, are the most important parameters evaluated in infertility centers as a part of routine semen analysis. However, these traditional criteria provide little indication of possible nuclear DNA damage.

Normal sperm genetic material is required for a successful fertilization, as well as for further embryo and fetal development in order to produce healthy offspring. Sperm DNA contributes half of the offspring´s genomic material and abnormal DNA can lead to derangements in the reproductive process. There is now good evidence that shows that sperm DNA and chromatin damage are associated with male infertility and reduced natural conception rates [1-3].

In the last years, the integrity of sperm DNA is being recognized as a new parameter of semen quality and a marker of male infertility [4,5]. Nevertheless, DNA integrity assessment is not being carried out as a routine part of semen analysis in the clinical laboratory [6]. Sperm DNA fragmentation can be caused by apoptosis in the seminiferous tubule epithelium, defects in chromatin remodeling during the process of spermiogenesis, oxygen radical-induced DNA damage during sperm migration from the seminiferous tubules to the epididymis, the activation of sperm caspases and endonucleases, damage induced by chemotherapy and radiotherapy, and the effect of environmental toxicants [7]. In humans, high levels of sperm nuclear DNA damage have been related to low fertility potential, failure to obtain blastocysts, blockage in embryo development after embryo implantation, increased risk of recurrent miscarriages, reduced chances of successful implantation, and negative effects on the health of the offspring [8-11].

Magnetic-Activated Cell Sorting (MACS) is an excellent tool for selecting the desired cells or sperms out of a mixed cell population on membrane surface markers [12]. Externalization of phosphatidylserine (PS) to the outer membrane of sperm is considered early sign of apoptosis. Annexin V is a protein with a molecular weight of 35 KDa that has high affinity to PS in the presence of physiological concentrations of Ca^{+2} and is unable to pass through intact sperm membranes; thus, annexin V binding by a sperm cell indicates that its membrane integrity has been compromised [13,14]. MACS using annexin V conjugated with magnetic microspheres, which are exposed to a magnetic field in an affinity column, can separate apoptotic from non-apoptotic sperm.

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Several studies reported an improvement in fertilization rates [15,16] and embryo quality [17-19] because the best sperms were selected using MACS compared to standard selection methods. On the other hand, Gil et al. [20] showed that the implantation and miscarriage rated did not vary between MACS or standard sperm selection methods; however, they did observe an improvement in pregnancy rates.

In this study, we evaluated the effects of using MACS as a sperm selection technique in patients with high DNA fragmentation compared with patients with normal DNA fragmentation and determine the efficacy of such technique in the hopes of improving ART success rates.

Materials and Methods

Patients

In this study, we analyzed 134 cycles of ICSI that were done between March 2012 and April 2014 at FERTILAB Laboratory of Assisted Reproduction (Lima, Peru), all approved by the Institutional Review Board (IRB) and the corresponding Ethics Committee. Written consent was obtained from all patients and their partners included in this study. The study group was made up of patients with high sperm DNA fragmentation (n = 57) and their wife's oocytes were injected with non-apoptotic spermatozoa selected using MACS and; the control group, of patients with normal sperm DNA fragmentation (n = 77) and their wife's oocytes were injected with spermatozoa selected by classic morphological characteristics.

Sperm DNA fragmentation assessment

Prior to the hormonal stimulation, sperm DNA fragmentation values were evaluated with the Sperm Chromatin Dispersion (SCD) test [21] using Halosperm® Kit (Halotech Dna, Spain). Briefly, sperm samples from each patient, containing after dilution or concentration not <5 million and not >10 million spermatozoa per milliliter, were used. The kit contains aliquots of agarose gel in Eppendorf tubes. Each semen sample was processed after the agarose gelled (from immersion in a water bath at 90ºC for 5 min). When the Eppendorf tubes reached a temperature of 37ºC (5 min at 37ºC in a dry atmosphere), 25 µL of sperm were added and gently mixed. Twenty microliters of this mixture were placed on precoated slides and covered with 22 x 22-mm coverslide. The slides were maintained at 4ºC for 5 minutes to produce a microgel containing embedded spermatozoa. The coverslides were gently removed, and the slides were immersed in a previously prepared acid solution (80 µL of HCl added to 10 mL of distilled water) for 7 minutes. After removal from this solution, the slides were incubated for 25 minutes in 10 mL of lysing solution (provided in the Halosperm kit). After rinsing in distilled water, the slides were dehydrated for 2 minutes in three concentrations of alcohol (70%, 90% and 100% vol) for 2 minutes each and either were stored (storage was possible several months in optimal conditions) or were processed immediately with staining solution for 10 minutes with continuous airflow. Staining was performed with 1:1 (vol/vol) by using Wright's solution (Merck, Darmstadt, Germany) and phosphate-buffered saline solution (Merck). The slides were rinsed in tap water, allowed to dry at room temperature, processed for upright or inverted bright-field microscopy at x100, and covered with 22 x 22 coverslide. Operators scored ≥500 spermatozoa for each patient according to the patterns established by Fernández et al. [21]. Strong staining is preferred to visualize the dispersed DNA loop halos. Removal of sperm nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loops. The sperm tails remain preserved. The acid treatment produces DNA unwinding that is restricted in those nuclei with high levels of DNA strand breakage. After the subsequent lysis, sperm nuclei with fragmented DNA produce very small or no halos of dispersed DNA. However, nuclei without DNA fragmentation released their DNA loops to form large halos.

Ovarian stimulation and oocyte collection

The menstrual cycles of patients were stimulated using recombinant FSH (Gonal®, Merck Serono laboratories, Peru) and HMG (Menopure®, Ferring Pharmaceutical, Peru) according to the stimulation protocols previously established and starting on day 2 of the menstrual cycle until when at least three follicles reached ~18 mm in diameter. The oocyte pickup was performed by vaginal ultrasound 36 h after the intramuscular application of Human Chorionic Gonadotropin, hCG (Pregnyl®, Ferring Pharmaceutical, Peru). For the procedure, the patient was under general anesthesia with 200 mg of Propofol iv (Diprivan® 1% P/V; AstraZeneca Laboratories, UK).

During the follicular aspiration procedure, the oocytes were recovered in Global®-HEPES-buffered medium (IV Fonline, Canada) supplemented with 10% vol/vol Serum Substitute Supplement (SSS; Irvine Scientific, USA). After retrieval, cumulus-oocyte complexes were manually trimmed of excess cumulus cells and cultured in ~200 µL drops of Global®-Fertilization medium (IV Fonline, Canada) plus 10% SSS under oil at 37°C and an atmosphere containing 6% CO_2, 5% O_2 and 89% N_2 for 5 hours before the ICSI procedure.

Seminal samples and sperm non-apoptotic selection by MACS

Study group sperm cells were prepared by density gradient centrifugation after MACS of non-apoptotic spermatozoa. Control group sperm cells were prepared by density gradient centrifugation without magnetic sorting. On the day of the ICSI procedure, all patients' partners collected the semen samples by masturbation in aseptic conditions into sterile cups after 3-5 days of sexual abstinence. Concentration, progressive motility and morphology from spermatozoa were assessed after semen liquefaction for 30 min at room temperature according to World Health Organization criteria (2010). Motile spermatozoa were separated from the seminal plasma by centrifugation through 1.0 mL 95% and 45% Isolate gradients (Irvine Scientific, USA). The pellet was washed once by centrifugation for 5 min, and was resuspended in HEPES-buffered Global medium + 10% SSS for ICSI.

For magnetic selection, spermatozoa were incubated with annexin V-conjugated microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) for 15 min at room temperature. One hundred microliters of microbeads were used for each 10 million separated cells. The sperm/microbead suspension was loaded in a separation column containing iron globes, which was fitted in a magnetic field (Mini MACS; Miltenyi Biotec). The fraction composed of apoptotic spermatozoa was retained in the separation column, whereas the fraction with intact membranes was eluted through the column and was collected as non-apoptotic spermatozoa.

ICSI, fertilization and embryo culture

In every patient, all oocytes in metaphase II were injected 5 hours after aspiration according to methods previously described [22]. After the ICSI procedure (day 0), all injected oocytes were cultured at 37°C in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2.

The fertilization was evaluated 16-18 hours post injection by presence of two pronuclei (day 1). The zygotes were individually cultured under mineral oil, in 10-µL droplets of Global® medium (IVF online, Canada) supplemented with 10% vol/vol SSS from day 1 to day 3. On day 3, the embryos were moved to fresh 10-µL droplets of Global® medium + 10% SSS and cultured 2 days more up to the transfer day in blastocyst stage.

On day 3 the embryos were evaluated for cell number, fragmentation, and multinucleation. Good quality day 3 embryos were defined as those with 6-8 cells and ≤ 10% of fragmentation. Good quality blastocysts were defined as having an inner cell mass (ICM) and trophoectoderm type A or B [23]. The ICM score was evaluated as follow: type A = compact area, many cells present; type B = cells are loosely grouped. The trophoectoderm was scored as follows: type A = many cells forming a tight epithelial network of cells; type B = few cells forming a loose network of cells.

Embryos were transferred on day 5 in all patients using an Emtrac embryo transfer catheter (Gynétics Medical Products, Lommel, Belgium) that had been previously washed with culture medium. The catheter was completely filled with culture medium and the blastocysts filled in the last 10 μL of the catheter. All transfers were performed according to the methods previously described by Mansour [24]. The blastocysts that were not transferred were cryopreserved or discarded according to their morphology.

Pregnancy determinations

The biochemical pregnancy was assessed 14 days after the embryo transfer by measuring the Human Chorionic Gonadotropin beta subunit (hCG-b) in blood. The clinical pregnancy was determined by transvaginal ultrasonography to detect gestational sacs and fetal heartbeats at approximately 21 and 28 days after transfer, respectively.

Statistical analysis

Statistical analysis was carried out using the statistic package Stata 10 (StataCorp, College Station, TX). Data are represented as Mean ± SD. Group comparisons were made using the χ^2 test and Student's t-test. It was considered a statistical significant difference when P <0.05.

The normal fertilization rate was calculated from the number of zygotes with two pronuclei of ICSI and divided by the number of oocytes injected by 100. The cleavage rate was calculated from the number of embryo with ≥ 6 cells at day 3 and divided by the total number of zygotes by 100. The rate of implantation was calculated dividing the number of gestational sacs observed by ultrasound at the 21 st day post transfer divided by the total number of embryos transferred by 100. The rate of clinic pregnancy was calculated from the number of patients with at least one gestational sac divided by the total embryo transfers by 100. The miscarriage rate was defined as the number of pregnancies with total loss of gestational sacs before the 20 weeks of gestation between the numbers of pregnancies by 100.

Results

The mean days of stimulation were similar in the study and control groups (8.10 ± 1.08 versus 8.02 ± 1.32 days; P: NS). The women of the study group compared with the control group had significantly lower mean rFSH treatment (1292.21 ± 291.71 versus 1386.19 ± 313.21; IU/l; data not shown). Women and their partners from the study group were significantly older than that those from the control group (P <0.05; Table 1). Furthermore, the study group had higher percentages of DNA fragmentation compared to the control group (P <0.05). Both evaluated groups had similar sperm concentration, progressive motility and morphology (P: NS; Table 1).

Results of laboratory and clinical outcomes obtained from the study group (high DNA Fragmentation) and the control group (normal DNA fragmentation) are shown in (Table 2). A total of 588 and 672 oocytes were collected from women of study and control groups, respectively. Four hundred and eighty-six and five hundred and seventy-eight oocytes from de study and control groups, respectively, were inseminated. The normal fertilization (2PN) was similar in both evaluated groups (study group: 74.5% versus control group: 76.5%). There was no difference in the cleavage rate and good embryo quality at day 3 between groups. Blastocyst development rates were similar (50.8% and 41.1%, respectively) for the study and control groups. In addition, embryos reaching the blastocyst stage were morphologically similar in both groups. In the study group, a total of 107 embryos were transferred to 57 patients with a mean of 1.88 embryos. In the control group, a total of 146 embryos were transferred to 77 patients with a mean of 1.90 embryos. There was no significant difference in the clinical PR per transfer in the evaluated groups: 63.2% in the study group and 45.5% in the control group. Implantation and miscarriage rates were similar in both groups (Figure 1). The percentages of single and twin pregnancies were similar between both studied groups.

	Study Group	Control Group
No. cycles	57	77
Female age (years) (Mean ± SD)	34.64 ± 4.55*	32.76 ± 4.21
Male age (years) (Mean ± SD)	42.24 ± 7.76*	38.38 ± 5.79
Sperm DNA fragmentation (%)	38.21 ± 11.33*	18.76 ± 9.07
Sperm concentration (x 10^6/mL)	77.65 ± 41.29	67.53 ± 35.57
Progressive motility (%)	23.63 ± 11.22	21.60 ± 11.58
Sperm morphology (%)	5.01 ± 4.85	6.67 ± 6.84

*$P<0.05$ compared to the control group

Table 1: Comparison of seminal results in the study and control groups.

	Study Group	Control Group
Cycles	57	77
Total number of oocytes	588	672
Total number of injected oocytes	486	578
Total number of fertilized oocytes (2PN) (%)	362 (74.5)	442 (76.5)
Cleavage rate of embryo at day 3 (%)	98.3	89.1
Number of cells at day 3 (Mean ± SD)	7.19 ± 0.88	7.24 ± 0.87
Good quality embryos at day 3 (%)	88.4	83.7
Blastocyst development (%)	50.8	41.1
Good quality blastocysts (%)	71.9	69.3
Full blastocyst (%)	35.3	43.5
Expanded blastocyst (%)	60.9	54.2
Hatching blastocyst (%)	3.8	2.3
Total number of embryo transferred/patient (Mean ± SD)	107	146
	(1.88 ± 0.33)	(1.90 ± 0.38)
Pregnancy rate (%)	63.2	45.5
Implantation rate (%)	37.4	28.1
Single pregnancies (%)	88.9	82.9
Twin pregnancies (%)	11.1	17.1
Miscarriages (%)	8.3	17.1
Biochemical pregnancy rate (%)	0	0

P: NS

Table 2: Comparison of laboratory results and clinical outcomes between both evaluated groups.

The total number of cycles was allocated to three age groups: <35; 35-39 and ≥ 40 years old (Table 3). The pregnancy, implantation and miscarriage rates were similar in each age group in both the high DNA fragmentation (study group) and normal DNA fragmentation (control group). Our data showed that pregnancy and implantation rate is similar with advancing age in the study group.

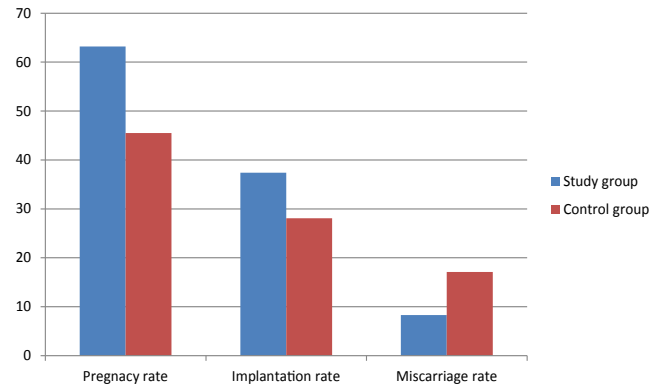

Figure 1: Pregnancy, Implantation and Miscarriage rates

	Study Group				Control Group			
Age (years)	n	PR (%)	IM (%)	MR (%)	n	PR (%)	IR (%)	MR (%)
<35	36	66.7	38.9	8.3	53	49.1	31	19.2
35-39	15	53.3	29.6	0	19	36.8	21.1	14.3
≥ 40	6	66.7	50	25	5	40	25	50

PR: Pregnancy rate; IM: Implantation rate; MR: Miscarriage rate

P: NS

Table 3: Clinical outcomes in the study and the control group according the age of patients.

Discussion

Sperm chromatin is a well-organized, compact, crystalline structure, consisting of haploid DNA and heterogeneous proteins. Its highly condensed and insoluble nature plays a protective role during the transfer of the paternal genetic information through the male and female reproductive tracts, adjusting to the extremely limited volume of the sperm nucleus [25,26].

Apoptosis is an ongoing physiological phenomenon that maintains the number of germ cells within the supportive capacity of the Sertoli cells [27]. This process includes a cascade of events such as interruption of membrane phospholipid asymmetry, condensation and destruction of the chromatin, compaction of cytoplasmic organelles, reduced mitochondrial transmembrane potential, mitochondrial release of cytochrome c, production of reactive oxygen species, expansion of the endoplasmic reticulum, and a diminishing in cell volume [28]. Among these events, translocation of phosphatidylserine (PS), as an early event of the execution phase of apoptosis, is considered one of the signals for specific recognition and removal of apoptotic cells by phagocytosis [29]. In men, increased rates of externalized PS are associated with decreased sperm motility, morphology or concentration in ejaculated semen [30], and its occurrence is reported to be higher in semen samples from infertile men compared with fertile men [31]. Based on PS externalization to the outer membrane, and using magnetic cell sorting using annexin V (used as an apoptotic marker) conjugated with microbeads, apoptotic sperm can be separated from non-apoptotic sperm [32].

Sperm DNA fragmentation may exert its effect at different stages of the reproductive procedure, beginning from the pre-implantation development of the embryo to achievement and sustaining of pregnancy and finally the creation of healthy offspring. Several studies demonstrated the impact of sperm DNA damage and its correlation with clinical endpoints including fertilization rates, embryonic development, implantation, pregnancy and miscarriage rates [33-35].

Negative correlation has been associated between fertilization results with the presence of high levels of sperm DNA fragmentation [9,36-39]. However, if the type and extent of DNA damage can be balanced by the reparative ability of the oocyte, it is possible to achieve fertilization even in the presence of elevated sperm DNA fragmentation rates [10,40,41]. Additionally, in ICSI, the natural selection barriers are bypassed entirely and fertilization with highly DNA-fragmented sperm is possible. Although this damage may also be repaired in the oocyte, excessive damage may potentially result in early reproductive failures [42]. During the 4 to 8 cell stage, when the paternal genome is switched on, development of the embryo is definitely affected by sperm DNA integrity. Then, apoptosis and fragmentation will be present within the embryo and subsequently there is some difficulty in reaching to blastocyst stage [26,43]. An inverse relationship has been reported between the likelihood of achieving pregnancy either by natural intercourse, intrauterine insemination or by application of ART and the presence of high sperm DNA fragmentation levels [11,40,44,45].

In the present study, MACS was utilized for the selection of non-apoptotic sperm in men with high DNA fragmentation levels, and these selected spermatozoa were used in an ICSI procedure. The results have shown the feasibility and efficiency of MACS to select non-apoptotic spermatozoa. The data obtained demonstrated similar fertilization rates, preimplantation embryo development and clinical outcomes in those patients with high sperm DNA fragmentation treated with MACS compared with those patients with normal sperm DNA fragmentation. These findings are very important because they show that the utilization of selected spermatozoa favors normal pregnancy and implantation rates. Furthermore, the use of this technique is a good choice for couples with repeated assisted reproduction failures in which sperm apoptosis is present.

There are multiple applications for MACS technology in the male reproduction. MACS, in combination with anti-CD45 microbeads, have been used repeatedly with great success to eliminate leukocytes from seminal fluid [46,47]. Also, non-apoptotic sperm obtained through MACS can be used for insemination in infertile men couples with autoimmune male infertility [48,49]. Additionally, MACS has been employed to facilitate the analysis of distinctive homogeneous spermatogenic cell populations by overcoming the heterogeneity of somatic and germ cells within the testicular tissue [50,51]; and to select a high proportion of sperm with normal morphology and significantly lower sperm deformity index [52].

In regards to the application of MACS in ART, our study showed an improvement in the fertilization, embryo quality, pregnancy and implantation rates in those patients with high levels of fragmented spermatic DNA. Several authors previously reported similar results in fertilization rates [15,16,53], embryo quality [17,18], pregnancy rates [18,20,53-55], and additionally healthy infants who are born with normal neonatal assessments [53,56-58].

Cleavage rate, cleavage stage embryo morphology, cytoplasmic fragmentation and multinucleation have been shown to be important markers of embryo quality and viability that may be observed over time during *in vitro* culture. After the advent of extended embryo culturing, higher IRs have been reported because of better embryo selection compared with earlier developmental stages and because of better synchronization between the embryo developmental stage and uterine environment.

Our observations demonstrate that MACS is a flexible, fast and simple cell sorting system for separation of cells; and employing annexin V microbeads can effectively remove apoptotic sperm.

Hence, sperm cells prepared by MACS have high motility, viability, morphology, and display reduced apoptosis manifestations, including DNA fragmentation. Additionally, by selecting non-apoptotic sperm by MACS, we can achieve satisfactory pregnancy and implantation rates, rendering the procedure a good option for couples with high sperm DNA fragmentation and repeated assisted reproduction failures.

Finally, our data demonstrated the importance of adequate diagnosis and sperm selection pre ART when high levels of sperm DNA damage are observed. Consequently, we suggest that further research and well-designed prospective studies are carried out, in which all variables are controlled, in order to reveal more advantages regarding the utilization of MACS in ART.

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