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Variation of DNA Fragmentation Levels During Density Gradient Sperm Selection for Assisted Reproduction Techniques

A Possible New Male Predictive Parameter of Pregnancy?

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Abstract: Predicting the outcome of in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) is one main goal of the present research on assisted reproduction. To understand whether density gradient centrifugation (DGC), used to select sperm, can affect sperm DNA integrity and impact pregnancy rate (PR), we prospectively evaluated sperm DNA fragmentation (sDF) by TUNEL/PI, before and after DGC. sDF was studied in a cohort of 90 infertile couples the same day of IVF/ICSI treatment. After DGC, sDF increased in 41 samples (Group A, median sDF value: 29.25% [interquartile range, IQR: 16.01–41.63] in pre- and 60.40% [IQR: 32.92–93.53] in post-DGC) and decreased in 49 (Group B, median sDF value: 18.84% [IQR: 13.70–35.47] in pre- and 8.98% [IQR: 6.24–15.58] in post-DGC). PR was 17.1% and 34.4% in Group A and B, respectively (odds ratio [OR]: 2.58, 95% confidence interval [CI]: 0.95–7.04, $P=0.056$). After adjustment for female factor, female and male age and female BMI, the estimated OR increased to 3.12 (95% CI: 1.05–9.27, $P=0.041$). According to the subgroup analysis for presence/absence of female factor, heterogeneity in the association between the Group A and B and PR emerged (OR: 4.22, 95% CI: 1.16–15.30 and OR: 1.53, 95% CI: 0.23–10.40, respectively, for couples without, $n=59$, and with, $n=31$, female factor).

This study provides the first evidence that the DGC procedure produces an increase in sDF in about half of the subjects undergoing IVF/ICSI, who then show a much lower probability of pregnancy, raising concerns about the safety of this selection procedure. Evaluation of sDF before and after DGC configures as a possible new prognostic parameter of pregnancy outcome in IVF/ICSI. Alternative sperm selection strategies are recommended for those subjects who undergo the damage after DGC.

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Abbreviations: ARTs = assisted reproductive techniques, BMI = body mass index, DGC = density gradient centrifugation, ICSI = intracytoplasmic sperm injection, IQR = interquartile range, IVF = in vitro fertilization, OR = odd ratio, PI = propidium iodide, PR = pregnancy rate, sDF = sperm DNA fragmentation, TdT = terminal deoxynucleotidyl transferase.

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INTRODUCTION

In developed countries 1.7% to 4% of births derive from the application of assisted reproductive techniques (ARTs),[1] which represent the main medical treatment for most infertile couples. Despite ARTs success having improved greatly in the last decades, the current pregnancy rate (PR) in European countries remains low (about 30%).[2]

Many factors are believed to influence the in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) outcome, including the fact that a clinical laboratory setting cannot recreate the conditions of natural conception. In particular, whereas in natural conditions selection of the male gametes occurs during transit in the female genital tracts, in the ART laboratory, spermatozoa are selected with routine separation techniques such as density gradient centrifugation (DGC). The latter procedure selects spermatozoa with better morphology, motility,[3] and chromatin maturity[4] and blunts the amount of immature germ cells and leukocytes,[5] considered responsible for high levels of oxidative stress in semen.[6,7] However, proper sperm selection is not sufficient to guarantee successful fertilization, embryo development, establishment of pregnancy, and delivery of live babies.[8,9] The integrity of sperm chromatin is a mandatory trait, especially in the case of aged women whose oocytes may have a limited ability to repair the DNA damage brought by the spermatozoon.[10,11]

Among the different types of DNA damage, sperm DNA fragmentation (sDF) has been extensively investigated and many studies have demonstrated its negative impact on ART outcome[12–16] and on the health of offspring in animal models.[17] Whether DGC increases or decreases sDF is currently unclear. Indeed, although several studies have indicated that DGC

improves the yield of DNA-intact spermatozoa,[18] others have reported no change or even a worsening of DNA quality in DGC-selected spermatozoa.[19–23] In addition, and most importantly, whether an eventual effect of DGC procedure on DNA damage impacts pregnancy achievement by ART is presently unknown.

We report here the effect of DGC on sDF levels, as assessed in the same samples utilized for IVF/ICSI treatments and the impact of such an effect on PR after ART.

MATERIAL AND METHODS

Patients

Infertile couples undergoing ART cycles were prospectively recruited at Tecnobios Procreazione (Bologna and Udine, Italy) from January 2012 to December 2014. Of the 103 recruited couples, 13 were excluded from the study because embryos were frozen and embryo transfer postponed (n = 10) or because it was not possible to determine sDF in both unselected and DGC selected sperm (n = 3). In the remaining 90 couples (79 treated by IVF and 11 by ICSI), the infertility diagnosis was: 49% female factor (including diminished ovarian reserve, uterine factors, endometriosis and tubal factors), 8% male factor, 9% male and female factor in combination, and 35% unexplained. As in ARTs the tubal factor is completely overcome by embryo transfer, this diagnosis (n = 20) was not considered as a female factor in the statistical analyses. The obtainment of an informed written consent was the only criterion for inclusion in the study. The study initially planned to evaluate the impact of sDF levels in pre- and post-DGC semen samples on PR after IVF/ICSI treatment of infertile couples and was approved as such by the ethical committee of Azienda Ospedaliera Universitaria Careggi (protocol no. 54/10). The different effect of DGC on sDF levels and the impact of the increase/decrease of sDF during DGC on PR were observed and further investigated during data analysis. Clinical data, standard semen parameters, and flow cytometric measures of sDF were centralized in an electronic database at the Unit of Sexual Medicine and Andrology of the University of Florence.

Sperm Collection and Preparation

Semen samples were collected by masturbation and analyzed for sperm number, concentration, motility, and morphology according to WHO procedures.[24] Sperm selection for IVF/ICSI treatment was performed by discontinuous PureSperm (Nidacon, Gothemberg, Sweden) gradient.[12] Briefly, semen samples were layered upon a 40:80% PureSperm density gradient, processed by centrifugation at 600*g* for 15 minutes and resuspended in 1 mL of sperm culture medium (PureSperm wash, Nidacon, Gothemberg, Sweden). After DGC, evaluation of concentration, total and progressive motility and morphology was repeated. All semen analyses were conducted on the same day of IVF procedure before evaluation of sDF.

Ovarian Stimulation, IVF, ICSI, and Embryo Development

Ovarian stimulation was achieved by recombinant follicle-stimulating hormone (Gonal F, Serono, Rome, Italy; Puregon, Organon, Rome, Italy) and monitored by endovaginal echography and plasma estradiol evaluation.[12] Thirty-six hours before oocyte retrieval, 10,000 IU of hCG (Gonasi, Amsa, Rome, Italy) was administered.[12] Oocyte retrieval was carried out under general anesthesia by a vaginal ultrasonography-guided aspiration.[12] At 16 to 18 hours after insemination or microinjection, as previously described,[25,26] oocytes were assessed for 2 pro-nuclei presence. Forty-eight hours after oocyte retrieval, embryos were classified according to their morphology and then transferred into the uterus.[12] Clinical pregnancy was determined by ultrasound detection of gestational sac.[12]

TUNEL/PI Coupled to Flow Cytometry

sDF was determined in spermatozoa before and after DGC on the day of oocytes retrieval. After washing twice with Sperm Wash Medium, semen samples ($3-10 \times 10^6$ sperm) were fixed with 200 μL of paraformaldehyde (4% in phosphate-buffered saline [PBS], pH 7.4) for 30 minutes at room temperature. For labeling DNA breaks, the In Situ Cell Death Detection Kit, fluorescein, (Roche Molecular Biochemicals, Milan, Italy) was used, as previously described.[27] Briefly, semen samples were washed twice with 200 μL of PBS/1% bovine serum albumin, and spermatozoa were permeabilized with 100 μL of 0.1% sodium citrate/0.1% Triton X-100 (4 minutes in ice), and labeled with 50 μL of labeling solution (supplied by the kit) containing the terminal deoxynucleotidyl transferase (TdT) enzyme (1 hour at 37°C in the dark). Samples were then washed twice, resuspended in 500 μL of PBS and shipped at 4°C[27] to the Unit of Sexual Medicine and Andrology of the University of Florence. For detection of sDF, samples were stained with PI (0.6 μg/mL, 10 minutes at room temperature in the dark) and acquired using a flow cytometer FACScan (Becton Dickinson, Mountain View, CA) equipped with a 15-mW argon-ion laser for excitation. For each test sample, 3 further sperm suspensions were prepared for instrumental setting, fluorescence compensation, and data analysis: by omitting both PI staining and TdT; by omitting only TdT (negative control); and by omitting only PI staining. Green fluorescence of TUNEL labeling was revealed by an FL-1 detector (515–555 nm wavelength band) and red fluorescence of PI was detected by an FL-2 detector (563–607 nm wavelength band). For each sample, 8000 events were recorded within the flame-shaped region (FR) characteristic of spermatozoa[27] in the forward light scatter/side light scatter dot plot. This region excludes debris and large cells (such as somatic ones) and includes spermatozoa and semen apoptotic bodies.[28,29] The latter can be excluded from the analysis of sDF subsequently, by gating the nucleated events (ie, the events labeled with PI) within FR.[27]This strategy guarantees that TUNEL fluorescence is analyzed in a population formed by only and all spermatozoa present in the analyzed semen sample.[28–30] As shown in Figure 1 A and B, reporting pre- and post-DGC TUNEL/PI dot plots in representative samples of Groups A and B (see below), respectively, for determination of the percentages of sDF, a vertical marker is established in the TUNEL axis of the dot plot of negative control, including 99% of events. Such a marker is translated in the corresponding test sample and all the events beyond the marker are considered as TUNEL positive. TUNEL/PI detects sDF in the total and in 2 different sperm populations, brighter and dimmer.[28] Dimmer sperm (Figure 1 A, B in blue) are all dead (besides DNA fragmented)[31,32] and thus they are almost completely blunted during DGC (Figure 1A, B), provoking per se a decrease of sDF in postselection samples. This decrease owing to the blunting of dead dimmer sperm can mask the eventual increase in brighter ones if sDF is evaluated in the total sperm population. Conversely, evaluation of the variation of sDF during DGC in the brighter population (Figure 1A, B in red) is more sensitive in

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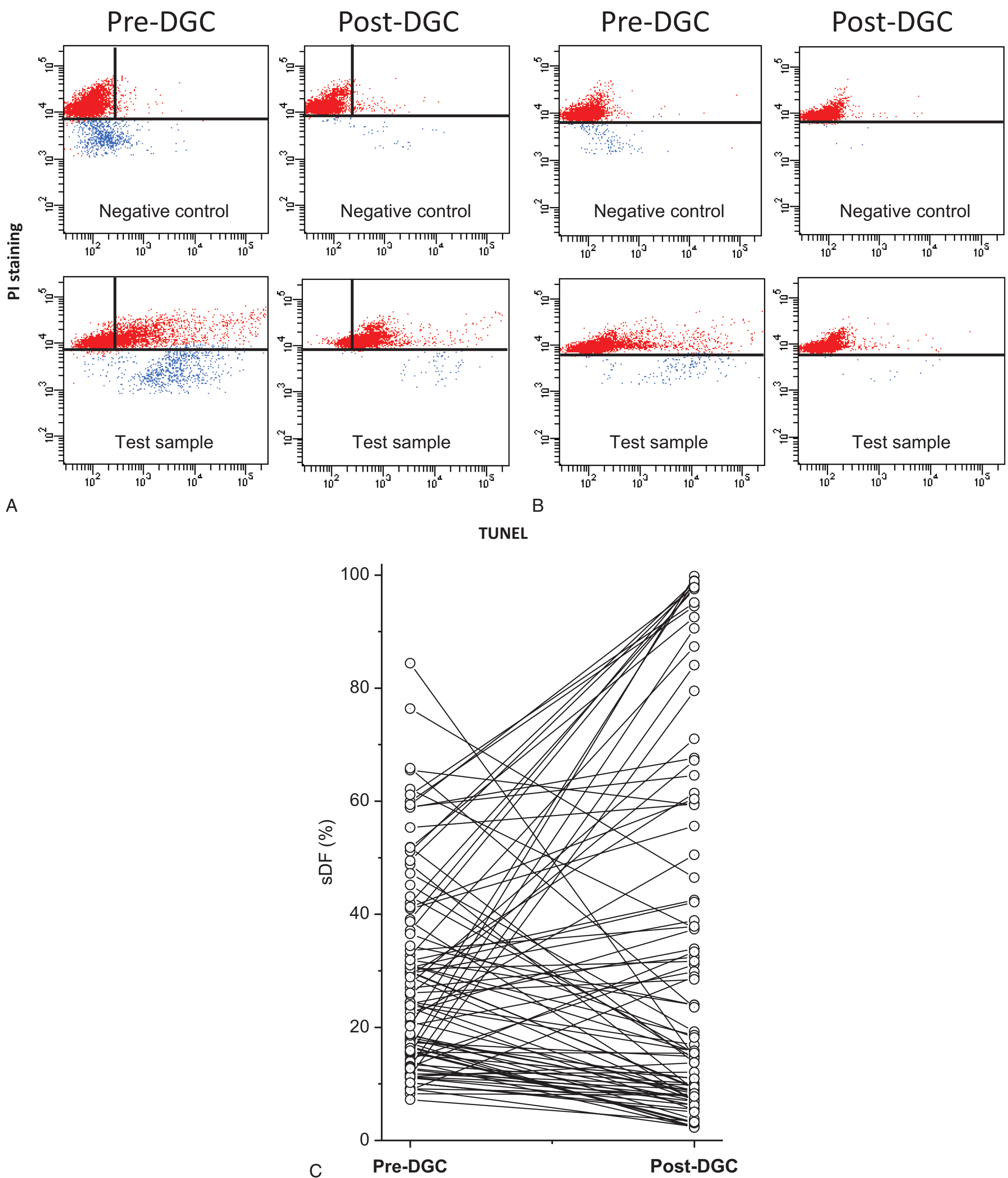

FIGURE 1. Typical PI/TUNEL dot plots of pre- and post-DGC samples in subjects wherein selection induces an increase (A) or a decrease (B) of sperm DNA fragmentation (sDF). Note the presence on y axis of 2 sperm populations, differing for the intensity of PI staining (brighter in blue and dimmer in red) and that the dimmer one is virtually blunt in post-DGC samples. Measures of sDF before and after DGC refers to the brighter population (see also Material and Methods section). Negative control, samples prepared by omitting TdT enzyme. (C) Pre- and post-DGC sDF levels in the 90 subjects included in the study. DGC = density gradient centrifugation.

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detecting eventual increases of sDF. Hence, to investigate the effect of selection on sperm DNA damage, we calculated sDF before and after DGC in only the brighter population (hereafter termed sDF).

Statistical Analysis

The study was designed to explore the impact of sDF levels in pre- and post-DGC semen samples on PR, without any prespecified hypothesis; the number of required couples was not calculated. All continuous variables were assessed for normal distribution with the Kolmogorov-Smirnov test and results were expressed as median (IQR) or as both median (IQR) and mean (±SD). Female body mass index (BMI) was analyzed as a continuous variable after single imputation of missing values in 13 cases. The Mann–Whitney *U* test was used for comparing sDF in pregnant and nonpregnant couples. In some cases, a multifactor analysis of variance was also used. Comparisons of proportions were performed with the χ^2 test for heterogeneity. The variation of the parameters evaluated before and after DGC was tested by means of the Wilcoxon signed ranks test.

sDF was considered increased (Group A) or decreased (Group B) after DGC when the coefficient of variation (CV) between pre- and post-DGC measures was >5% (intra-assay CV of the TUNEL/PI technique).[27] Samples showing CVs <5% (n = 4) were considered as increased. Belonging to Group A or B is hereafter termed A/B variable (categorical variable).

To assess the ability of total sperm number and concentration to predict the outcome of DGC, receiver-operating characteristic (ROC) curves were built and the area under the curve (AUC) calculated.

The association between the clinical parameters, including the A/B variable and PR (number of clinical pregnancies/number of treated couples), was studied in a binary logistic model, both in univariate and multivariate settings. Subgroup analyses were performed by means of an interaction test to determine the consistency of the association between A/B variable and PR according to key baseline characteristics. The likelihood ratio test was used to test the linear hypotheses about the regression coefficients.

All statistical tests were 2-sided, and *P* values of ≤0.05 were considered to indicate statistical significance. Data were analyzed with SAS Statistical Software, version 9.2 (SAS Institute Inc, Cary, NC).

RESULTS

Age of male and female partners, pre- and post-DGC sperm parameters, and sDF levels of the subjects included in the study are reported in Tables 1 and 2. As shown, median values of sDF were not affected by selection (Table 2); however, when individual samples were considered, we found that in 41 of 90 (46%) of them (Group A), sDF increased, even dramatically in some cases (Figure 1C). In the remaining 49 of 90 (54%) samples (Group B), sDF, after selection, decreased (Figure 1C). The 2 groups of subjects differed in pre-DGC total sperm number and concentration (Table 1) and post-DGC sDF values (Table 2). Of note, DGC selection resulted in the expected increase in sperm motility, in both groups (Table 2). Considering that pre-DGC sperm number and concentration differ between the 2 groups (Table 1), we evaluated whether the 2 parameters were able to predict the outcome of DGC by ROC curve analysis. Both parameters predicted DGC

TABLE 1. Male and Female Age, Presence of Female and Male Factor and Semen Parameters in All Recruited Subjects and in Group A and B

Variable	Total (n = 90)	Group A (n = 41)	Group B (n = 49)	P
Female age	34.77 ± 3.17	34.54 ± 3.34	34.96 ± 3.00	0.825*
	35.00 (33.00–37.00)	32.50 (35.00–37.50)	35.00 (33.00–37.00)	
Female factor	31/90 (34.4%)	13/41 (31.7%)	18/49 (36.7%)	0.617†
Female BMI	21.65 ± 4.07	21.33 ± 3.31	21.91 ± 4.63	0.4316*
	20.80 (19.13–23.00)	20.50 (19.00–22.20)	21.10 (19.70–23.00)	
Male age	37.37 ± 4.22	36.73 ± 4.30	37.90 ± 4.11	0.135*
	37.00 (35.00–40.00)	36.50 (34.00–39.75)	38.00 (36.00–40.50)	
Male factor	14/90 (15.5%)	6/41 (14.6%)	8/49 (16.3%)	0.825†
Male abstinence, days	3.74 ± 2.37	3.95 ± 2.34	3.57 ± 2.40	0.1968*
	3.00 (3.00–4.00)	3.000 (3.00–4.00)	3.00 (3.00–3.00)	
Sperm concentration, millions/mL	46.91 ± 32.01	39.24 ± 29.85	53.32 ± 32.64	0.010*
	40.00 (23.00–65.75)	31.00 (20.00–60.00)	42.00 (32.50–71.50)	
Sperm Number (millions/ejaculate)	103.23 ± 86.28	83.63 ± 50.68	119.63 ± 105.16	0.016*
	86.35 (60.00–23.30)	67.20 (43.70–117.90)	100.00 (71.75–131.00)	
Total Motility (%)	43.72 ± 8.60	43.41 ± 8.325	43.98 ± 8.90	0.913*
	40.00 (40.00–50.00)	40.00 (40.00–50.00)	40.00 (40.00–50.00)	
Progressive Motility (%)	37.21 ± 8.11	37.44 ± 8.597	37.02 ± 7.77	0.703*
	35.00 (34.75–41.25)	35.00 (30.00–45.00)	35.00 (35.00–40.00)	
Normal Morphology (%)	25.03 ± 6.68	24.59 ± 8.11	25.41 ± 5.26	0.671*
	28.00 (20.00–28.00)	28.00 (20.00–28.00)	28.00 (20.00–28.00)	

Data are represented as mean ± SD and median (IQR). BMI = body mass index.
*Group A vs B; Mann–Whitney *U* test.
†Group A vs B; χ^2 test for heterogeneity.

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TABLE 2. Pre and Post-DGC Values of Total and Progressive Motility, and of sDF in Total Recruited Subjects and in Group A and B

Variable	Total (n = 90)	Group A (n = 41)	Group B (n = 49)	P^*
Total motility (%)				
Pre-DGC	43.72 ± 8.597 40 (40.00–50.00)	43.41 ± 8.325 40 (40.00–50.00)	43.98 ± 8.896 40 (40.00–50.00)	0.913
Post-DGC	89.67 ± 4.23 90 (90.00–90.00)	89.39 ± 4.90 90 (90.00–90.00)	89.90 ± 3.61 90 (85.00–92.50)	0.975
$P^†$	<0.001	<0.001	<0.001	
Progressive motility (%)				
Pre-DGC	37.21 ± 8.115 35 (34.75–41.25)	37.44 ± 8.60 35 (30.00–45.00)	37.02 ± 7.77 35 (35.00–40.00)	0.703
Post-DGC	84.00 ± 5.31 85 (80.00–85.00)	83.54 ± 6.91 85 (82.50–85.00)	84.39 ± 3.48 85 (80.00–85.00)	0.814
$P^†$	<0.001	<0.001	<0.001	
sDF (%)				
Pre-DGC	29.20 ± 17.70 24.45 (15.37–39.54)	31.21 ± 16.27 29.25 (16.01–41.63)	27.53 ± 18.82 18.84 (13.70–35.47)	0.162
Post-DGC	33.06 ± 31.43 17.87 (8.17–53.00)	58.06 ± 29.71 54.6 (31.58–91.25)	12.39 ± 11.03 8.98 (6.24–15.58)	<0.001
$P^†$	0.746	<0.001	<0.001	

Data are represented as mean ± SD and median (IQR). DGC = density gradient centrifugation, sDF = sperm DNA fragmentation.
*Group A vs B; Mann–Whitney *U* test.
†Pre- vs post-DGC; Wilcoxon signed ranks test.

outcome with AUC values of 0.657 (95% confidence interval [CI]: 0.543–0.771, $P = 0.010$) and 0.648 (95% CI: 0.531–0.766, $P = 0.016$), respectively, for sperm concentration and number. Male as well as female factor was similarly distributed in the 2 groups (Table 1).

Table 3 reports PR in the 2 groups. As shown, PR was 34.7% in group B and 17.1% in group A (odds ratio [OR] = 2.58, 95% CI: 0.95–7.04; $P = 0.056$) (Figure 2). Table 3 also reports the results of univariate analysis of the association between A/B variable, presence of female factor, female age, male age, and female BMI and PR. As can be observed, women's age represents another factor approaching statistical significance in affecting PR; in particular, for every year of aging, the chance of pregnancy decreases by 13%. Consistently, the PR was 35% and 18% in women with an age, respectively, <35 and ≥35 years (median value of the cohort of this study). Standard semen parameters as well as the presence of male factor did not differ between couples achieving or not pregnancy (data not shown).

After adjustment for the presence of female factor, female age, male age, and female BMI, the probability of achieving pregnancy for Group B further increased with an OR value of 3.12 (95% CI: 1.05–9.27, $P = 0.041$) (Table 4). Another possible confounder is basal sperm concentration, which differs between Groups A and B (Table 1). However, this parameter does not impact PR (OR = 1.00; 95% CI: 0.99–1.01, $P = 0.480$) and thus cannot be considered a confounding factor in this study population. Consistently, if sperm concentration is added as a

TABLE 3. Univariate Analysis of the Association Between Evaluated Clinical Parameters and Pregnancy Rate

Variable	No. of Couples (N = 90)	No. of Pregnancies (N = 34)	P^*	OR (95% CI)	$P^†$
Group					
Subjects A	41	7 (17.1%)	0.060	1 (ref.)	0.056
Subjects B	49	17 (34.7%)		2.58 (0.95–7.04)	
Female factor					
Absent	59	18 (30.5%)	0.256	1 (ref.)	0.247
Present	31	6 (19.4%)		0.55 (0.19–1.56)	
Female age, y (cont.)	—	—	—	0.87 (0.74–1.01)	0.056
Male age, y (cont.)	—	—	—	0.95 (0.85–1.06)	0.365
BMI	—	—	—	1.07 (0.96–1.19)	0.243

BMI = body mass index, CI = confidence interval, OR = odds ratio.
*χ^2 test for heterogeneity.
†Likelihood ratio test.

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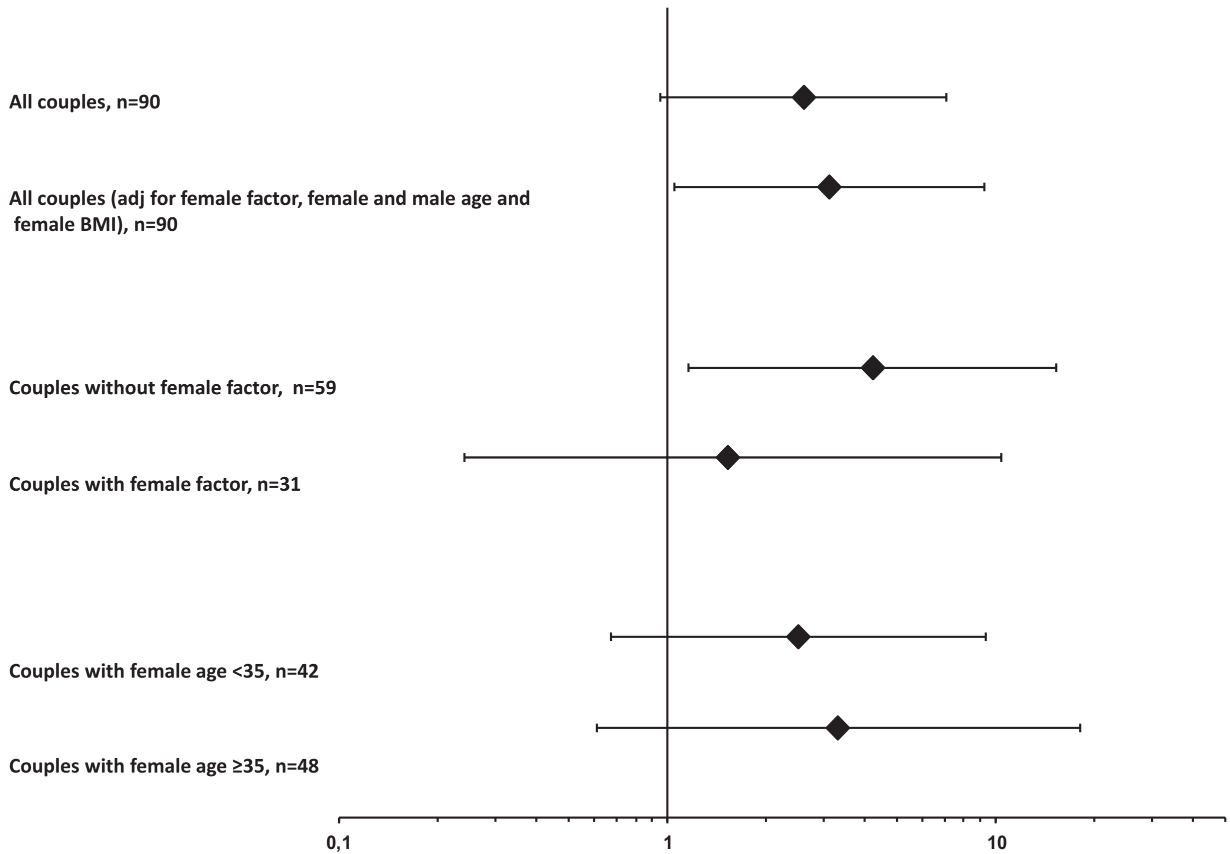

FIGURE 2. Forest plot of ORs in Group B in the total cohort in univariate, after adjustment for confounding factors and in subgroup analysis. OR = odd ratios.

confounder in the multivariate analysis, no substantial change in OR for pregnancy in Group B is observed (not shown). Since no pregnancy was obtained in the 11 couples treated with ICSI (vs 30.4% in IVF), we evaluated the distribution of the type of treatment in the 2 groups. ICSI and IVF were equally distributed in Group A (6/49, 12.24%) and B (5/41, 12.20%), $P = 0.998$.

TABLE 4. Multivariate Analysis of the Association Between Evaluated Clinical Parameters and Incidence of Pregnancy (N = 90)

Variable	OR (95% CI)	P^*
Group		
Subjects A	1 (ref.)	0.041
Subjects B	3.12 (1.05–9.27)	
Female factor		
Absent	1 (ref.)	0.198
Present	0.48 (0.15–1.48)	
Female age, y (cont.)	0.84 (0.70–1.01)	0.067
Male age, y (cont.)	0.99 (0.86–1.13)	0.864
BMI	1.08 (0.96–1.21)	0.226

BMI = body mass index, CI = confidence interval, OR = odds ratio.
*Likelihood ratio test.

To further verify whether the association between the A/B variable and PR was affected by the presence of a female factor, a subgroup analysis was performed. We found that in the 59 couples without female factor, PR was 41.9% in Group B as compared to 17.8% in Group A (OR = 4.22, 95% CI: 1.16–15.3, Figure 2). Conversely, within the 31 couples with a female factor, PR in Group B was 22.0% with respect to 15.4% in Group A (OR = 1.53, 95% CI: 0.23–10.4; test for interaction $P = 0.396$; Figure 2).

Finally, no significant interaction between the A/B variable and female age (<35 vs ≥35 years) was present (Figure 2). Indeed, in the 42 women younger than 35 years, PR was 25% and 45%, respectively, in Groups A and B, whereas in the 48 women aged 35 years or older, PR was 9% and 26%, respectively, in Groups A and B, indicating that the relative probability to achieve pregnancy in Group A versus B was not affected by female age (Figure 2).

Pre- and post-DGC sDF values were not different between couples achieving or not achieving pregnancy (26.75% [12.76%–40.63%] vs 24.34% [15.37%–39.54%], $P = 0.920$ in pre- and 11.48% [6.45%–60.13%] vs 26.21% [9.18%–56.48%], $P = 0.177$ in post-DGC samples). After adjusting for the presence of female factor and male and female age in a multivariate model, the difference of post-DGC sDF levels between couples achieving or not achieving pregnancy approached the statistical significance level ($P = 0.071$).

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DISCUSSION

Predicting successful pregnancy in IVF/ICSI is one of the goals of present research in the field of assisted reproduction. This study shows, for the first time, that DGC selection of sperm for ARTs may induce sDF and, most importantly, when such an event occurs, the couples have about a 50% lower chance to achieve pregnancy. As such, the variation of sDF after DGC (A/B variable) configures as a new predictive parameter of pregnancy outcome in ARTs.

Our study demonstrates that DGC selection is not devoid of risk for sperm DNA integrity. Indeed, we found that besides the subjects in which DGC selection produces a decrease in sDF, mainly owing to deletion of mostly DNA fragmented moribund/dead cells,[33] in about 50% of subjects, post-DGC sDF levels are higher as compared to pre-DGC values, suggesting the induction of a de novo DNA damage during the procedure. The causes of this damage are unclear. Although initial studies suggested that the shearing forces generated during centrifugation can damage sperm DNA through generation of reactive oxygen species,[21,34] recent data demonstrated that contamination of commercially available colloidal silicon gradients by transition metals is the main cause of oxidative damage and breakage to sperm DNA during DGC.[22] However, the fact that not all the samples undergo an increase of sDF during selection indicates that metal contamination of gradients is not sufficient to induce the damage. We hypothesize that the concomitant presence of intrinsic features of chromatin (such as defects in sperm chromatin maturation) or of other sperm abnormalities (such as lower sperm defenses to oxidative attack or high levels of ROS in semen) render the sample more susceptible to the noxious agents. Whatever is the nature of such abnormalities, it does not appear to be associated with the presence of male factor infertility, which, in our study, was equally distributed among the subjects of Groups A and B. Moreover, although sperm concentration and number, among pre-DGC parameters, were different between the 2 groups of patients, ROC analysis demonstrated that they are poorly predictive of the DGC outcome. Thus, at present, only evaluation of pre- and post-DGC sDF can assign patients to one of the 2 groups. Such evaluation should be performed determining sDF with a method, such as the TUNEL/PI used in our study, that excludes non-sperm elements that may be present in variable amounts in selected and unselected samples.[28,30] Only in this case, a direct comparison of sDF levels between pre- and post-DGC samples is possible. As mentioned before, subjects whose semen samples underwent DNA damage during DGC selection had a lower probability of achieving pregnancy. The strength of this association further increases after adjustment for potential confounding factors such as female factor infertility, female age[35] (and present study), female BMI,[36–37] and male age,[38] which may also affect ART outcomes. Among such confounders, female factor appears to be an effect modifier of the association between the A/B variable and PR. In fact, the subgroup analysis showed that in couples without female factor, the OR for pregnancy of group B is similar to the adjusted one. Conversely, despite the fact that the probability for pregnancy in aged women was lower in both Groups A and B, the relative probability of pregnancy of Group B with respect to A is similar in women with an age <35 and >35 years.

Many studies reported the impact of sDF on natural and assisted reproduction showing a link to several fertility check points (from fertilization to embryo quality and PR). The presence of breaks in the male genome may be repaired by the oocyte,[10] but the ability of the oocyte to repair the damage depends on several factors, including its quality, age of the woman, and iatrogenic factors. It is also possible that the oocyte repair machinery is not sufficient to repair all the DNA damage present in the male genome or that mutations and epimutations may be introduced because of partial oocyte repair[11]; in such cases, the embryo may fail to develop or implant in the uterus or may be miscarried at a later stage. In our cohort, pre-DGC sDF values are not different between couples achieving or not achieving pregnancy, whereas the difference in post-DGC sDF levels is more evident, approaching significance after adjustment for the presence of female factor and male and female age. It is conceivable that statistical significance will be reached with an enlarged number of couples. Finding that post-DGC sDF levels better discriminate between couples achieving or not achieving pregnancy than pre-DGC ones was expected considering that DGC-selected sperm are those used for IVF/ICSI procedures. However, in previous studies comparing pre- and post-selection sDF levels, sDF in selected sperm did not discriminate[39] or discriminated less[40–41] between pregnant and nonpregnant couples compared to native semen. In the study by Bungum et al,[39] sperm chromatin structure assay was used to reveal sDF. Interestingly, this method appears to be unable to detect the DNA breakage induced by DGC selection,[22] providing a possible explanation for the lack of impact of selected sDF values on pregnancy after ART.[37]

Our study should be considered as preliminary owing to the post-hoc nature of some analyses and, in some circumstances, to the low power level of the statistical tests. Whereas the results on the effect of DGC on sDF levels are solid (performed in 90 samples), it is necessary to confirm the impact of the A/B variable on PR in an enlarged number of recruited couples to consolidate it as a new predictive parameter for ARTs outcome. Another limitation of the study is the lack of data on male BMI. Although recent meta-analyses[42,43] could not univocally demonstrate the impact of this parameter on ART outcome, some studies[44,45] have reported that BMI may affect clinical pregnancy and live birth rate.

In conclusion, our study demonstrates that DGC selection of sperm for ART may be dangerous for DNA integrity in approximately 50% of subjects, who then show a much lower probability of pregnancy. This finding indicates that current gradient preparation procedures to select sperm for ARTs may negatively impact PR, evidencing the need to utilize alternative strategies for sperm selection for those subjects in which DGC produces the damage. Finally, our results show that the A/B variable after DGC is a promising predictor of PR in ARTs, independently of age and female factor. If, as expected, future studies show that the A/B variable is a stable condition over time in one individual, this semen trait could be useful for counseling in the couple infertility workup.

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