Sperm Genomic Integrity by TUNEL Varies throughout the Male Genital Tract

Philip Xie, Derek Keating, Alessandra Parrella, Stephanie Cheung, Zev Rosenwaks, Marc Goldstein and Gianpiero D. Palermo*

From the Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine and Department of Urology (MG), Weill Cornell Medicine, New York, New York

Abbreviations and Acronyms

ART = assisted reproductive technology

CP = clinical pregnancy

ICSI = intracytoplasmic sperm injection

IUI = intrauterine insemination

SCF = sperm chromatin fragmentation

SR = surgically retrieved

TUNEL = terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling

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* Correspondence: Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine, Weill Cornell Medicine, 1305 York Ave., 6th Floor, New York, New York 10021 (telephone: 646-962-8448; FAX: 646-962-0344; e-mail: gdpalerm@med.cornell.edu).

Purpose: We assessed sperm chromatin fragmentation at different levels of the male genital tract.

Materials and Methods: Ejaculated specimens from consenting male partners were screened for sperm chromatin fragmentation by TUNEL (terminal deoxynucleotidyl deoxyuridine triphosphate nick end labeling). Men with intracytoplasmic sperm injection failure and high ejaculated sperm chromatin fragmentation underwent surgery to retrieve spermatozoa from different levels of the male genital tract, which were then reassessed for sperm chromatin fragmentation. Approximately 500 or more spermatozoa were assessed per patient with a 15% threshold. Intracytoplasmic sperm injection results of cycles using spermatozoa from different levels of the male genital tract were compared.

Results: Topographical assessment of the male genital tract showed a mean ± SD of 20.4% ± 10% sperm chromatin fragmentation in the vas deferens, 15.8% ± 8% in the epididymis and 11.4% ± 6% in the testis. All values were lower than in ejaculated controls (mean 32.9% ± 20%, $p < 0.05$). A total of 25 couples who underwent intracytoplasmic sperm injection with surgically retrieved spermatozoa had lower sperm chromatin fragmentation ($p < 0.001$), and higher implantation, clinical pregnancy and delivery rates ($p < 0.01$). A total of 45 couples with a history of intracytoplasmic sperm injection failure with ejaculate performed elsewhere were treated solely with surgically retrieved spermatozoa at our center. Compared to historical cycles, surgically retrieved spermatozoa had a lower fertilization rate (65%, $p < 0.05$) but enhanced rates of implantation (19.1%), clinical pregnancy (40.0%) and delivery (34.3%) (each $p < 0.01$).

Conclusions: To our knowledge we report for the first time that sperm chromatin fragmentation increases progressively from the testicle to the epididymis and the vas deferens, and is highest in the ejaculate. Men with high ejaculated sperm chromatin fragmentation can benefit from using surgically retrieved sperm for in vitro fertilization and/or intracytoplasmic sperm injection.

Key Words: testis; spermatozoa; chromatin; sperm injections, intracytoplasmic; in situ nick-end labeling

Approximately 30% of infertility cases are due to combined factors while the remainder stem equally from the female or male partner.[1] However, there are situations in which the etiology of the inability of a couple to procreate remains unexplained despite a young female partner with a negative infertility workup and a male partner with adequate semen parameters.[2] Previous studies have suggested that abnormal capacitation develops due to sperm membrane dysfunction which impairs the acrosome reaction.[3,4] However, this

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is not the only male gametal abnormality which is undetectable by standard semen analysis. Indeed, SCF may be present in men with normal or even optimal semen parameters.[5]

During spermiogenesis the process of chromatin packaging involves the substitution of somatic nucleosomal histones by protamines, incorporating an elevation in histone acetylation, ubiquitin activity and alteration in DNA topology.[6–8] This process is repaired by deoxyribonuclease-ligase mechanisms, allowing for nucleic acid supercoiling during nuclear compaction.[9] In addition, as spermatozoa pass through the epididymis, chromatin is stabilized by disulfide bonds.[10] If this fails, abnormal spermatozoa are picked up by the epididymis and phagocytized. If a defective epididymis neglects to recognize these impaired gametes, ejaculated spermatozoa with high SCF may appear.[11,12]

Since excess SCF can hinder procreation by natural conception, timed intercourse or IUI,[13] we developed a treatment algorithm based on the SCF level[14] of couples with poor IUI outcomes despite normal semen parameters. According to our algorithm couples with repeat IUI failure and normal SCF should undergo in vitro insemination, moving on to ICSI if this fails. Those with abnormal SCF, which is responsible for impaired embryonic development and a higher pregnancy loss rate, should undergo ICSI directly.[15,16] Since an inverse relationship between SCF and sperm motility was previously established,[17] the advantage of ICSI in these cases is its ability to select the most motile spermatozoon since the first sign of apoptosis in spermatozoa is a loss of motility.[18]

Even with ICSI the ooplasm unravels the male genome from the protamine and replaces eventual chromatin breaks during prefertilization steps.[19] This requires a healthy ooplasm, as confirmed in studies using donor oocytes.[20] In these cases retrieving gametes with better genomic integrity from the epididymis or the seminiferous tubules was proposed.[21] Enhanced genomic integrity of SR spermatozoa appears to be related to their lack of exposure to reactive oxygen species, which pervade the male genital tract.[22]

In this study the hypothesis was that spermatozoa undergo certain DNA damage as they progress through the male genital tract and, therefore, using surgically retrieved spermatozoa would enable a superior clinical outcome. We identified men with elevated ejaculated SCF and compared their ICSI outcomes to those in men with normal SCF. Men who failed to achieve pregnancy with their partner after undergoing cycles using ejaculated spermatozoa were invited to participate in a study in which spermatozoa retrieved from different levels of the genital tract would be assessed for SCF and subsequently used for ICSI.

MATERIALS AND METHODS

Study Design

Ejaculates from consenting men were screened for SCF using a TUNEL assay (IRB No. 1006011085). ICSI outcomes were compared in couples in whom the male partner had compromised or normal ejaculated SCF. We then assessed the SCF of spermatozoa retrieved from different levels of the genital tract of men with recurrent ART failure. ICSI outcomes were compared in cycles using SR spermatozoa vs those using ejaculated spermatozoa (IRB No. 1705018205).

Study Population

This study included 306 couples classified with normal (142) and abnormal (162) ejaculated SCF. Of the 162 men with abnormal ejaculated SCF 79 elected not to proceed with surgical sperm extraction while 83 accepted surgical sperm retrieval with TUNEL assessment. Of these 83 men 13 underwent surgical sperm retrieval but only ejaculated spermatozoa were used for ICSI. In the remaining 70 couples ICSI outcomes were compared according to whether ejaculated or SR spermatozoa were used. A total of 25 couples underwent cycles using ejaculated and SR spermatozoa at our center while 45 underwent ICSI cycles with ejaculated spermatozoa elsewhere before undergoing SR cycles at our center (fig. 1). Couples with an abnormal body mass index, a smoking history, recreational drug use or excessive drinking were excluded from study. Men with severely impaired spermatogenesis, such as nonobstructive azoospermia or cryptozoospermia, were also excluded.

Sperm Collection

Ejaculated specimens were collected by masturbation. Surgical sperm retrieval from the testes was performed as previously described.[23] The seminiferous tubules were dissected, transferred into a suspension and then through a 24 gauge angiocatheter to be assessed under a phase contrast microscope at 200× magnification. If spermatozoa were not identified, further extraction was done from the same testis and eventually from the contralateral testis. Testicular tissue was digested with collagenase when no spermatozoa were identified. Epididymal sperm aspiration was performed by dissecting and puncturing the epididymal tubule using a microknife.[24] Vasal spermatozoa were retrieved microsurgically via hemivasotomy,[25] which was closed microsurgically to prevent stricture or obstruction at the vasotomy site.

Sperm Chromatin Fragmentation Assessment

Ejaculated and SR SCF was assessed by TUNEL using the In Situ Cell Death Detection Kit (Roche Diagnostics, Rotkreuz, Switzerland) as previously described.[14] Briefly, 5 μl of raw semen specimens were smeared on a glass slide and fixed using 4% paraformaldehyde. Permeabilization was achieved by exposure to 0.1% Triton™ X-100 in phosphate buffered saline for 2 minutes at 4C. The reagents provided in the kit were applied to the slides, which were then left to incubate in 92% humidity at 37C. At least 500 spermatozoa were assessed per sample under a fluorescence microscope with a normal threshold of 15%.[26]

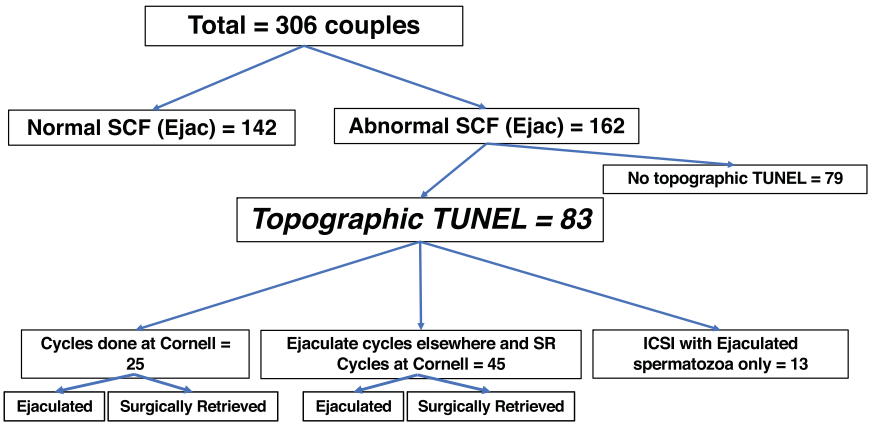

Figure 1. Ejaculate SCF level was assessed in 306 couples. Those with abnormal SCF and those in whom ICSI cycles with ejaculated (*Ejac*) spermatozoa failed underwent surgical retrieval sampling of different areas of male genital tract. Those specimens were eventually used for subsequent ICSI. Clinical outcome was compared in couples who underwent ICSI with ejaculated vs SR spermatozoa at our center. Couples treated with ICSI cycles using ejaculated spermatozoa elsewhere underwent ICSI with SR spermatozoa at our center.

Stimulation Protocols, Oocyte Retrieval and Embryo Transfer

The stimulation protocol and the treatment plan were determined carefully, considering patient age, weight, antral follicular count, serum antimüllerian hormone level and the response to prior stimulation protocols. Patients were administered daily gonadotropins, including Menopur®, Gonal-F (EMD Serono, Geneva, Switzerland) and/or Follistim®. A gonadotropin releasing hormone antagonist, including Cetrotide® or Ganirelix Acetate (Merck, Kenilworth, New Jersey), or a gonadotropin releasing hormone agonist (leuprolide acetate) was administered to suppress pituitary gland function. When the 2 leading follicles reached a diameter of 17 mm or greater, human chorionic gonadotropin (Ovidrel®) was administered to trigger the patient for retrieval. Transvaginal oocyte retrieval was performed 35 to 37 hours after human chorionic gonadotropin administration. After ICSI the embryos were cultured until transfer, which was done 3 to 5 days after insemination.

Statistical Analysis

Statistical analysis was performed of study population data and respective clinical outcomes. The Student t-test was used to analyze continuous variables, which are shown as the mean ± SD. Categorical variables were analyzed by the chi-square test with ANOVA used for more than 2 comparisons. Statistical significance was considered at $p < 0.05$.

RESULTS

ICSI outcomes using ejaculated spermatozoa were initially compared between 162 couples who presented with increased SCF (mean 23.6% ± 10%) and 142 who presented with normal SCF (9.2% ± 3%) (see table). While maternal age was comparable in these 2 groups, the male partner was significantly older in the group with increased SCF ($p < 0.01$). Fertilization and CP rates were lower in couples with high ejaculated SCF but the differences were not statistically significant. However, implantation and delivery rates were significantly impaired in couples who presented with abnormal ejaculated SCF ($p < 0.05$).

Men with high ejaculated SCF and ICSI failure underwent surgical retrieval of spermatozoa from the vas deferens, the epididymis and the testis. We assessed the SCF at different topographic areas of the male genital tract and observed a SCF of 20.4% ± 10% in the vas deferens ($p < 0.05$), 15.8% ± 8% in the epididymis ($p < 0.00001$) and 11.4% ± 6% in the testis ($p < 0.00001$, fig. 2). These values were noticeably lower than in the ejaculated counterpart (32.9% ± 20.0%). In the 7 men who provided a vas deferens sperm specimen the range was 18.8% to 59.0% in the ejaculate, 5.8% to 35.0% in the vas deferens, 5.3% to 31.0% in the epididymis and 1.5% to 23.1% in the testis.

We compared ICSI outcomes in 25 couples with abnormal ejaculated SCF who were treated at our center using ejaculated and SR spermatozoa subsequently. While fertilization rates did not significantly differ, the rates of implantation ($p < 0.05$), CP ($p < 0.01$) and delivery ($p < 0.01$) were superior in cycles in which SR spermatozoa were used (see table). Pregnancy loss trended higher in cycles in which ejaculated spermatozoa were used, although this was not significant. We then assessed the performance of testicular and epididymal spermatozoa individually. While fertilization and pregnancy loss did not differ between the groups, rates of implantation ($p < 0.01$), CP ($p < 0.01$) and delivery ($p < 0.01$) were significantly higher in ICSI cycles using epididymal spermatozoa (see table).

A total of 45 couples with a history of 1 or more failed ICSI cycles performed elsewhere in whom failure was due to high ejaculated SCF were treated at our center by ICSI cycles using SR spermatozoa.

ICSI outcomes

	No. Pts/No. Cycles	Mean ± SD Yrs SCF	Mean ± SD Yrs Age		No./Total No. (%)				
			Maternal	Paternal	Fertilization	Implantation	Clinical Pregnancy	Pregnancy Loss	Delivery
ICSI outcomes									
Ejaculated SCF:*									
Normal (15% or less)	142/313	9.2 ± 3	37.5 ± 5	39.6 ± 6	2,010/2,688 (74.8)	86/746 (11.5)	67/313 (21.4)	12/67 (17.9)	55/313 (17.6)
Abnormal (greater than 15%)	162/289	24.3 ± 10	38.0 ± 5	42.4 ± 8	1,609/2,222 (72.4)	54/694 (7.8)	43/273 (15.8)	11/43 (25.6)	32/273 (11.7)
p Value	—	<0.00001	Not significant	<0.0001	Not significant	<0.05	Not significant	Not significant	<0.05
High ejaculate SCF + SR sperm ICSI:†	25/—								
Ejaculated	—/57	36.9 ± 12	38.3 ± 5	48.2 ± 11	303/458 (66.2)	3/100 (3.0)	3/49 (6.1)	1/3 (33.3)	2/49 (4.1)
Surgically received	—/44	12.8 ± 6	38.3 ± 4	50.2 ± 12	251/395 (61.1)	14/109 (12.8)	12/41 (29.3)	3/12 (25.0)	9/41 (22.0)
p Value	—	<0.001	Not significant		Not significant	<0.05	<0.01	Not significant	<0.01
High ejaculate SCF + epididymal/testicular sperm:‡									
Ejaculate	25/57	34.7 ± 15	38.3 ± 5	48.2 ± 11	303/458 (66.2)	3/100 (3.0)	3/49 (6.1)	1/3 (33.0)	2/49 (4.1)
Epididymal	3/7	19.2 ± 4	40.7 ± 2	55.4 ± 6	50/74 (67.6)	5/24 (20.8)	4/7 (57.1)	1/4 (25.0)	3/7 (42.9)
Testicular	22/37	12.6 ± 6	37.8 ± 4	47.3 ± 10	204/331 (61.6)	9/85 (10.6)	8/34 (23.5)	2/8 (25.0)	6/34 (17.6)
p Value	—	<0.0001	Not significant		Not significant	<0.01	<0.01	Not significant	<0.01
Clinical outcomes									
High ejaculate SCF + surgical sperm retrieval vs couple historical cycles:§	45/—							—	
Ejaculate	—/92	36.2 ± 15	38.6 ± 5	45.8 ± 9	479/680 (70.4)	13/174 (7.5)	10/75 (13.3)		9/75 (12.0)
Surgical retrieval	—/81	11.9 ± 6	37.8 ± 4	46.5 ± 11	504/774 (65.1)	32/168 (19.1)	28/70 (40.0)		24/70 (34.3)
p Value	—	<0.00001	Not significant		<0.05	<0.01	<0.001		<0.01
High ejaculate SCF, epididymal + testicular sperma ICSI vs couple historical cycles:¶								—	
Ejaculate	45/92	36.2 ± 15	38.6 ± 5	45.8 ± 9	479/680 (70.4)	13/174 (7.5)	10/75 (13.3)		9/75 (12.0)
Epididymal	14/25	12.4 ± 7	38.7 ± 5	49.5 ± 13	166/232 (71.6)	15/55 (27.3)	13/21 (61.9)		12/21 (57.1)
Testicular	33/56	11.7 ± 5	37.4 ± 4	45.2 ± 10	338/542 (62.4)	17/113 (15.0)	15/49 (30.6)		12/49 (24.5)
p Value	—	<0.00001	Not significant		<0.01	<0.001	<0.0001		<0.0001

* Higher chromatin fragmentation seemed to be more common in older men and it affected implantation and delivery rates.
† Compared to subsequent cycles when ICSI was done with surgically retrieved sperm.
‡ Chromatin fragmentation decreased from ejaculate to epididymal to testicular.
§ Vs cycles done elsewhere using ejaculate and lower SCF for surgically retrieved spermatozoa.
¶ Clinical outcomes of couples who used epididymal and testicular sperm.

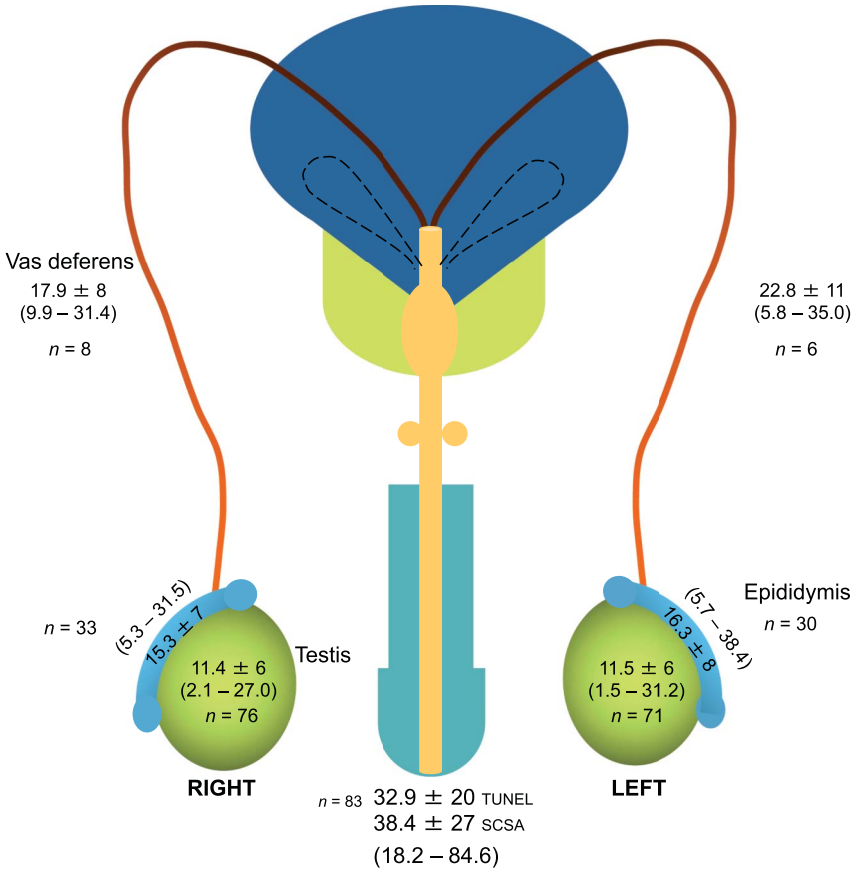

Figure 2. SCF mean ± SD and range of ejaculated and SR spermatozoa extracted from different areas of male genital tract. SCF improved as specimen was isolated proximally from 32.9% ± 20% in ejaculate, falling to 20.4% ± 10% in vas deferens, 15.8% ± 8% in epididymis and below threshold at 11.4% ± 6% in testis. *SCSA*, sperm chromatin structure assay.

The average SCF of SR spermatozoa was significantly lower than that of ejaculated counterparts ($p < 0.00001$). Although cycles using SR spermatozoa yielded a lower fertilization rate ($p < 0.05$), they yielded superior rates of implantation ($p < 0.01$), CP ($p < 0.001$) and delivery ($p < 0.01$, see table). Furthermore, cycles in which epididymal spermatozoa were used yielded the highest fertilization rate of 71.6% ($p < 0.01$), implantation rate of 27.3% ($p < 0.001$), CP rate of 61.9% ($p < 0.0001$) and delivery rate of 57.1% ($p < 0.0001$) compared to cycles in which testicular and ejaculated spermatozoa were used.

DISCUSSION

ICSI can increase the chance of conception in most couples in whom the male partner has elevated SCF.[14] However, in men with persistently elevated SCF in whom ICSI intervention fails it remains pertinent to explore other approaches, such as surgical sperm retrieval, to enhance the chance of fathering a child. This option has enabled clinicians to use these less mature gametes to treat couples with high ejaculated SCF.[27]

We compared SCF at different areas of the male genital tract in men with high ejaculated SCF who were unable to conceive. We established that the genomic integrity of spermatozoa progressively ameliorates when spermatozoa were retrieved proximally from the vas deferens, epididymis and testis. A mean SCF of 32.9% ± 20% in the ejaculate was observed while testicular spermatozoa had an average SCF within the normal range. This unique process gave us better insight into the loss of sperm chromatin integrity as spermatozoa progressed through the genital tract or were exposed to a flawed epididymis. Even in patients with severely elevated SCF the spermatozoa with an intact genome could be retrieved directly from the seminiferous tubules.

This novel and valuable information has enabled us to counsel patients who experienced ART failure and offer them a more efficient treatment option, such as using SR spermatozoa for ART cycles.[14] A total of 162 men a mean of 42.4 ± 8 years old had elevated SCF (range 15.3% to 65.4%) with impaired implantation ($p < 0.05$), concordant with previous studies showing a decline in the implantation rate

(86 of 746 cases or 11.5% vs 54 of 694 or 7.8%).[16] Similarly the delivery rate significantly decreased (55 of 313 cases or 17.6% vs 32 of 272 or 11.8%, $p < 0.05$, see table) when the SCF was above a certain threshold. Inability of the oocyte to overcome and repair severe sperm chromatin damage has also been postulated.[28]

After counseling we offered repeat ICSI cycles using SR spermatozoa to 25 couples in whom ICSI intervention failed at our center using ejaculated spermatozoa with elevated SCF. TUNEL assessment showed that the mean SCF dropped dramatically from 36.9% ± 12% in the ejaculate to 12.8% ± 6% in the SR spermatozoa ($p < 0.001$). SR spermatozoa yielded superior rates of implantation ($p < 0.05$), CP ($p < 0.01$) and delivery ($p < 0.01$, see table). These findings have been corroborated in studies which established that SR spermatozoa, specifically those retrieved directly from the testis, were superior to ejaculated spermatozoa presenting with high SCF in regard to the clinical outcome.[29,30]

To further delineate the topographical relevance of the spermatozoa we compared clinical outcomes among cycles using an ejaculated, testicular or epididymal source (see table). SCF decreased from 34.7% ± 15% in the ejaculate to 19.2% ± 4% in the epididymis and 12.6% ± 6% in the testis ($p < 0.0001$). Although testicular spermatozoa retained the highest chromatin integrity, cycles using epididymal spermatozoa resulted in significantly greater implantation, CP and delivery rates ($p < 0.01$).

Encouraged by these findings, we directly offered ICSI with SR spermatozoa to 45 couples with a history of poor ART outcomes using ejaculated spermatozoa at a different center. The SR specimen showed lower mean SCF of 11.9% ± 6% compared to 36.2% ± 15% in the ejaculate ($p < 0.00001$). Although somewhat lower fertilization was reported ($p < 0.05$), the rates of implantation, CP and delivery were significantly enhanced ($p < 0.01$), providing further evidence to support our earlier findings (see table).

An additional subanalysis of clinical outcomes was done in patients who underwent surgical spermatozoa retrieval divided into testicular or epididymal and compared to the ejaculate. We found that cycles using epididymal spermatozoa yielded higher rates of fertilization ($p < 0.01$), implantation ($p < 0.001$), CP and delivery ($p < 0.0001$) than cycles in which ejaculated and even testicular spermatozoa were used (see table).

While we cannot undoubtedly exclude the role of confounding factors in this study, stimulation protocols were consistent, particularly in cycles performed at our center. In addition, ethnicity, smoking habits, recreational drug use and drinking habits were controlled for as described.

The observation that SR spermatozoa yielded higher pregnancy and delivery rates than ejaculated spermatozoa indicates that they represent a good source of spermatozoa with an integral genome. Epididymal spermatozoa may represent a compromise between the maturity of the spermatozoa and superior chromatin integrity. To our knowledge this is the first study to describe spermatozoa retrieved from different levels of the male genital tract assessed for SCF and used for ICSI, which showed better genomic integrity as we progressed proximally through to the testis. Finally, the performance of these spermatozoa has been confirmed in terms of the ability to support embryo development and consequent reproductive outcomes compared to ejaculated spermatozoa with persistently elevated SCF.

CONCLUSIONS

We provide evidence that progressively less impaired spermatozoa may be obtained in the upstream male genital tract. Using these gametes yielded improved ICSI outcomes. This study represents the need for diligent SCF assessment, especially in male partners of couples with an inconspicuous reason for recurrent ART failure. Our findings may guide reproductive physicians to offer surgical sperm extraction as an optimal treatment plan on an individual basis, minimizing the number of unnecessary cycles by ensuring that the most competent spermatozoa are used for ICSI.

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