Increased count, motility, and total motile sperm cells collected across three consecutive ejaculations within 24 h of oocyte retrieval: implications for management of men presenting with low numbers of motile sperm for assisted reproduction

Al-Hasen Said¹ · Michael L. Reed¹

Received: 25 February 2015 / Accepted: 2 June 2015 / Published online: 17 June 2015
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Abstract

Purpose The purpose of this study was to quantitate changes in seminal volume, sperm count, motility, qualitative forward progression, and total motile sperm cells per ejaculate, across three consecutive ejaculates collected from individuals within 24 h preceding an IVF cycle.

Methods Men presenting with oligoasthenozoospermia or asthenozoospermia attempted three ejaculates within 24 h preceding IVF. Ejaculate 1 was produced the afternoon prior to oocyte retrieval, and ejaculates 2 and 3 were produced the morning of oocyte retrieval with 2–3 h between collections. Ejaculates 1 and 2 were extended 1:1 v/v with room temperature rTYBS. Test tubes were placed into a beaker of room temperature water, then placed at 4 °C for gradual cooling. Ejaculate 3 was not extended, but pooled with ejaculates 1 and 2 and processed for intracytoplasmic sperm injection (ICSI).

Results Out of 109 oocyte retrievals, 28 men were asked to attempt multiple consecutive ejaculations. Among this population, 25/28 (89.3 %) were successful, and 3/28 men (10.7 %) could only produce two ejaculates. Mean volumes for ejaculates 1, 2, and 3 were significantly different from each other

($p<0.01$); the volume decreased for each ejaculate. Mean sperm counts, motility, qualitative forward progression, and total motile cells per ejaculate for the ejaculates 1, 2, and 3 demonstrated the following: ejaculates 2 and 3 were not significantly different, but counts, motility, and total motile sperm were improved over ejaculate 1 ($p<0.01$).

Conclusions Pooling three consecutive ejaculates within 24 h increased the numbers of available motile sperm in this population by 8-fold compared to the first ejaculate alone, facilitating avoidance of sperm cryopreservation and additional centrifugation steps that could affect sperm viability and/or function.

Keywords IVF · ICSI · Sexual abstinence · Oligoasthenozoospermia · Fertility preservation

Introduction

In our laboratory, men presenting with oligozoospermia, asthenozoospermia, and oligoasthenozoospermia [1] are asked to attempt three consecutive ejaculates within 24 h of the scheduled oocyte retrieval: one ejaculate to be attempted mid-afternoon prior to the retrieval and two ejaculates attempted the morning of the retrieval, within a period of 2 to 3 h. The three consecutive ejaculates are pooled prior to processing for IVF. The goals of this novel strategy were to alleviate stress-induced anejaculation on the day of oocyte retrieval [2, 3] and to maximize quality and numbers of motile sperm available by eliminating potential iatrogenic cryopreservation-induced damage, further loss of already-diminished numbers of motile sperm following cryopreservation, and eliminating unpredictability of between- and within-patient post-thaw sperm viability [4–7]. And, while we stipulate that in most, if not all cases, there would likely have been sufficient sperm in a single ejaculate from these men for ICSI,

Capsule Pooling consecutive ejaculates produced within 24 h significantly increased the numbers of available motile sperm for IVF in asthenozoospermic and oligoasthenozoospermic men by 8-fold over a single ejaculate alone.

✉ Michael L. Reed
mleroyreed@gmail.com
Al-Hasen Said
Alh.said@gmail.com

¹ Center for Reproductive Medicine of New Mexico, 201 Cedar Street SE, Suite S1-20, Albuquerque, NM 87106, USA

we considered it very important to avoid potentially damaging effects of centrifuge-mediated sperm pelleting, e.g., generation of reactive oxygen species, by being able to process sperm by gradient separation [8–11].

Over time, we noted, but failed to quantify, a pattern of increased sperm numbers and/or motility in the second and/or third ejaculations of most men. This phenomenon is not widely documented [12–14], but it has been discussed anecdotally between laboratory professionals where it has been a routine practice to ask for a second specimen, spontaneously, in the event of a poor first specimen.

In context to the semen analysis parameters for the patient population described in this study, oligozoospermic men may benefit from multiple consecutive, or more frequent ejaculations compared to normozoospermic men [12, 15, 16]. Also, Koscinski et al. [17] trialed a multiple specimen collection strategy in an effort to avoid sending cryptozoospermic patients for testicular surgery—two ejaculates were collected approximately 2 h apart on the day prior to IVF, the specimens were pooled, washed, and briefly incubated in microdrops, after which motile sperm were captured using an injection pipette and moved to a separate microdrop. The motile sperm that survived overnight incubation were then used in some of the men for IVF with ICSI.

The goal of this observational, retrospective study was to quantitate and document changes in basic semen analysis parameters (volume, sperm count, motility, and qualitative forward progression) and calculated total motile sperm per ejaculate, across three ejaculates within individual patients, collected within approximately 24 h of planned oocyte retrieval. The time frame designated for this study was one calendar year, and the null hypothesis was that frequent ejaculations over a short time frame (three ejaculations within 24 h) would not alter semen analysis parameters from ejaculate to ejaculate.

Materials and methods

A deidentified data base was examined retrospectively as a basis for the study, and therefore, it does not contain clinical or other identifiers that might disclose the identity of the patients. The methodology for semen collection, extension, and processing was standard operating procedure for our laboratory and not experimental; therefore, patient consent and Institutional Review Board approval were not solicited.

All men presenting for IVF are instructed to collect a semen specimen between 8:00 am and 9:00 am the morning of the scheduled oocyte retrieval procedure, regardless of whether they are assigned to the single ejaculate or multiple ejaculate protocol, with planned sexual abstinence of 3 to 4 days prior to scheduled oocyte retrieval. The semen is extended as described, cooled, and held at 4 °C until processing.

Men presenting for IVF with oligozoospermia, asthenozoospermia, or oligoasthenozoospermia, as determined by prior semen analysis in our laboratory [1], were asked to attempt three ejaculates within 24 h of scheduled oocyte retrieval. The first ejaculate was to be produced after 3 to 4 days of abstinence, ideally mid-afternoon the day prior to oocyte retrieval, followed by two ejaculates the next morning at approximately 8:00 am and again after 2 to 3 h.

The first ejaculate was allowed to undergo liquefaction at room temperature, after which a brief manual semen analysis was performed, consisting of volume (ml), sperm count (m/ml), percent motility (%), and qualitative forward progression (1=twitch only, to 4=very rapid, nonlinear progression). After the analysis, the ejaculate was extended 1:1 v/v with Refrigeration Medium TEST Yolk Buffer (rTYBS; Irvine Scientific, Irvine, CA) in a conical 15-ml test tube labeled with patient-specific identifiers. The semen and rTYBS were gently mixed by repeated inversion of the test tube, which was then placed into a 150-ml beaker with 100-ml room temperature water, where the volume of water was sufficient to ensure that up to 10 ml of extended semen was below the water level. The beaker was then moved to the refrigerator; the room temperature water acted as a thermal insulator, allowing gradual cooling of the extended semen to 4 °C [18].

The second ejaculate, collected the morning of the oocyte retrieval, was processed in the same manner as the first, where after the brief semen analysis, the semen was extended 1:1 v/v with rTYBS, after which the test tube was placed into room temperature water, and allowed to cool in the refrigerator as previously described.

The third ejaculate was allowed to liquefy prior to undergoing a brief semen analysis. This semen was not extended with rTYBS, but combined with the two previous extended ejaculates within 30 min of collection and prior to centrifugation over a single layer gradient (PureCeption 80 %, SAGE, Trumbull, CT). Pooled specimens were centrifuged for 20 min at 500g, and then, the supernatant was removed, and the pellet was washed with 10 ml of Hepes-buffered medium (Quinn's Sperm Washing Medium, SAGE, Trumbull, CT). Specimens were then resuspended in 0.5 ml of the same medium and held at room temperature until the ICSI procedure. No postprocessing analysis was performed. In all cases, sperm were available for selection for ICSI, and no further processing, e.g., concentration, swim-out, or treatment, was required.

Between-ejaculate comparisons, within patient, and IVF cycle outcomes for the two sperm collection protocols were analyzed using analysis of variance, where $p < 0.01$ was considered significant (OpenStat, 2008). Paired comparisons were made between ejaculate 1 and ejaculate 2, ejaculate 1 and ejaculate 3, and between ejaculate 2 and ejaculate 3. Comparisons included the following: ejaculate volume, sperm count, percent motility, qualitative forward progression, and

total motile cells per ejaculate. Total motile cells were calculated by multiplication of ejaculate volume, sperm count, and percent motility (as a fraction) and included in the data analysis, as the number of sperm per ejaculate since this convention has been reported as a more appropriate measure of sperm production within a defined time period [19, 20]. Sperm cell morphology was not addressed by this study, as the patient population for this time period, and to the date of this writing, are predominantly undergoing genetic testing of embryos requiring sperm injection as the mechanism for fertilization.

Statistical analysis was performed only on the 25 individuals completing all three ejaculates. Mean and standard deviation were calculated for ejaculate volume, sperm count, percent motility, total motile cells per ejaculate, qualitative forward progression score. The mean and standard deviation for the time interval, in minutes, between ejaculations were also calculated. For comparison, mean and standard deviation were calculated for semen analysis results of 81 men presenting a single ejaculate on the morning of oocyte retrieval; these data were not included in the statistical analysis. Sperm morphology was not evaluated on any of the sperm used for these IVF cycles. IVF cycle outcome metrics were evaluated using Chi-square, $p < 0.01$.

Results

The predetermined period of study was 1 year. For calendar year 2014, there were 109 oocyte retrievals that utilized patient ejaculate sperm, 13 oocyte retrievals utilizing patient surgically retrieved sperm, 9 oocyte retrievals utilizing anonymous donor sperm, and 3 oocyte banking cycles. For oocyte retrievals with patient ejaculated sperm, 28/109 (25.8 %) of the men were asked to attempt three ejaculates within 24 h of the oocyte retrieval, based on screening semen analysis results performed prior to IVF; of the men included in this study, 6/28 men were preclassified as oligozoospermic, and 22/28 men were preclassified as oligoasthenozoospermic. In terms of the severity of male factor, only one of these men was classified as severe, e.g., cryptozoospermic, where only rare numbers of sperm, motile or otherwise, could be found after concentration of sperm by centrifugation; this individual was not able to achieve three ejaculations and was not included in the statistical analysis.

Of the 28 men assigned to the multiple collection protocol, 25 (89.3 %) were successful in producing all three ejaculates, and 3/28 (10.7 %) were only able to achieve two ejaculates, one the day prior, and one the morning of the oocyte retrieval.

Semen analysis results for semen analysis, for men collecting three multiple ejaculations across approximately 24 h, and for men collecting a single specimen on the morning of the oocyte retrieval, are presented in Table 1.

The relative ejaculatory abstinence intervals were calculated for the first and second ejaculates and the second to third ejaculates; the data represent only the men that were able to provide three ejaculates. The mean ejaculatory interval, in minutes, between the first and second ejaculates was 1084.6 min, or approximately 18 h, while the mean interval between the second and third ejaculations was 141.7 min, or approximately 2.4 h.

Mean ejaculate volumes for first, second, and third ejaculates were 3.0, 2.3, and 1.5 ml, respectively, and all three ejaculate volumes were significantly different from each other ($p < 0.01$). Mean sperm counts for the first, second, and third ejaculates were 10.6, 19.8, and 18.0 m/ml, respectively; the second and third ejaculate sperm counts were not significantly different; however, both were significantly higher than the sperm count for the first ejaculate ($p < 0.01$). Mean sperm motility (total percent motility) for the first, second, and third ejaculates was 15.7, 43.0, and 49.3 %, respectively; the second and third ejaculate percent motilities were not significantly different; however, both were significantly higher than the percent motility for the first ejaculate ($p < 0.01$). Mean total motile cells for each of the first, second, and third ejaculates were 4.6 million, 18.8 million, and 13.9 million motile sperm, respectively; the second and third ejaculate total motile cells were not significantly different; however, both were significantly higher than the number of total motile sperm compared to the first ejaculate ($p < 0.01$). Mean qualitative forward progression scores (QFPS) for each of the first, second, and third ejaculates were 1.4, 1.7, and 1.8, respectively; the second and third ejaculate QFPS were not significantly different; however, both were significantly higher than the QFPS for the first ejaculate ($p < 0.01$). For comparison, semen analysis data for men collecting a single ejaculate for IVF on the morning of oocyte retrieval were means of 3.0 m/ml volume, 65.1 m/ml sperm count, 50.3 % motility, 100.0 million motile sperm, and 2.0 QFPS.

With regard to the effect of combining ejaculates on the total number of motile sperm available for an IVF case, or any other assisted reproduction procedure, combining only the first and second ejaculates would yield an approximate 5-fold increase in the numbers of total motile sperm available for processing, while combining all three ejaculates would yield an approximate 8-fold increase in the numbers of total motile sperm available, compared to having only the first ejaculate available.

IVF cycle outcome statistics were not significantly different for mean (sd), representing patient age, oocytes numbers retrieved, mature oocytes for ICSI, fertilization, cleavage, and development to blastocyst stage by day 5 and/or day 6, for multiple ejaculates and single ejaculate: 33.8 (4.7) and 36.0 (5.0), 12.3 (7.7) and 13.6 (6.5), 10.8 (7.3) and 11.1 (5.5), 9.1 (6.5) and 9.3 (5.0), 9.3 (6.5) and 8.7 (5.3), and 5.0 (3.6) and 5.2 (3.9), respectively (Table 2).

Table 1 Semen analysis results (mean±1 sd) for 25 male factor patients across three consecutive ejaculations per patient within approximately 24 h of oocyte retrieval, and 81 patients with a single ejaculate collected on the morning of oocyte retrieval

Ejaculates	Volume (ml) [range]	Count (m/ml) [range]	Motility (%) [range]	Total motile sperm (millions) [range]	Qualitative forward progression [range]	Minutes between ejaculates* [range]
Multiple (<i>n</i> =25 men)						
1st ejaculate	3.0±1.2 ^a [0.25–5.0]	10.6±9.8 ^a [0.5–31.4]	15.7±12.2 ^a [3.3–54.9]	4.6±6.2 ^a [0.1–86.2]	1.4±0.3 ^a [1.25–2.0]	**
2nd ejaculate	2.3±0.9 ^b [0.75–4.0]	19.8±23.1 ^b [1.0–74.4]	43.0±23.0 ^b [5.0–88.6]	18.8±25.6 ^b [0.1–84.9]	1.7±0.3 ^b [1.25–2.5]	1084.6±122.3 [970–1460]
3rd ejaculate	1.5±0.7 ^c [0.5–3.0]	18.0±21.7 ^b [0.3–73.4]	49.3±18.7 ^b [7.9–77.4]	13.9±18.4 ^b [0.1–71.4]	1.8±0.4 ^b [1.25–2.5]	141.7±32.9 [110–245]
Calculated combined total motile sperm cells				37.3±46.3 [1.5–172.2]		
Single (<i>n</i> =81 men)						
1st ejaculate	3.0±1.5 [0.5–7.0]	65.1±40.8 [12.1–238.0]	50.3±13.0 [16.1–78.6]	100.0±90.2 [5.7–434.3]	2.0±0.4 [1.5–3.0]	**

Different superscripts within columns are significantly different at $p<0.01$, for $n=25$ male factor patients

*24 h=1440 min

**Planned 3 to 4 days abstinence prior to first ejaculation

Discussion

As with some protocols in embryology and andrology, procedural practices that are considered common, or routine, may not be evaluated with scientific rigor on a regular basis, rather these practices may be established and used without further consideration unless a problem arises. In our laboratory, men with low sperm counts and/or low motility are routinely asked to provide multiple ejaculates within a short time frame, to allow processing of sperm for ICSI by gradient separation; while there was a perception of benefit, we determined that for quality management, a strict evaluation of the multiple collection protocol, in a defined period of time, was in order. Approximately 25 % of the sampled population across 1 year qualified for multiple specimens; we would expect this percent to fluctuate year to year.

A benefit to using the ICSI procedure is that it only requires one sperm cell for each mature oocyte, and data provided in Table 1 demonstrates that there would likely have been more

than enough sperm for each cycle, had only the first ejaculate been used. We have historically elected to attempt gradient separation for sperm when possible to reduce potential deleterious iatrogenic damage to the sperm [4–11]. In the sampling for this single year, only one of the 29 men who qualified for multiple specimens would require pelleting of sperm; for all others, there were sufficient sperm for gradient separation; note that this is not always the case in this laboratory, where in previous years, we have had to rely on other sperm preparation techniques to acquire enough sperm for ICSI.

Combining multiple ejaculations collected within 24 h of oocyte retrieval significantly increased the number of total motile sperm available and did not compromise IVF cycle outcomes with respect to fertilization and embryo development. Of the basic semen analysis parameters evaluated, only ejaculate volume decreased from ejaculate to ejaculate; sperm count and percent motility were significantly higher for either of the two subsequent ejaculates compared to the first, on average. Our data is supported by findings of previous studies

Table 2 IVF ICSI parameters (mean and sd) for cycles utilizing sperm from either pooled multiple ejaculations, or single ejaculations for processing

	# Cycles	Patient age	# Oocytes retrieved	# Mature oocytes	# Fertilized ova	# Cleaving embryos day 3	# Blastocysts day 5/6
Multiple	25	33.8 (4.7)	12.3 (7.7)	10.8 (7.3)	9.1 (6.5)	9.3 (6.5)	5.0 (3.6)
Single	81	36.0 (5.0)	13.6 (6.5)	11.1 (5.5)	9.3 (6.5)	8.7 (5.3)	5.2 (3.9)

Data within columns were not statistically different $p<0.01$

[12–14]; in particular, the work of Tur-Kaspa et al. (1990) found that in normospermic men, the combined median total motile sperm increased by 144 %, and importantly, in oligospermic men, the combined median total motile sperm increased by 329 %, across two ejaculates collected within a short time period of 1 to 4 h between ejaculates. Our data demonstrated an 8-fold increase in total motile sperm cells comparing the mean total motile cells of the first ejaculate to the calculated, combined mean total motile cells from three ejaculates.

As a follow-up, we anticipate quantifying changes in all multiple specimen collection patients, no matter the severity of male factor. We also recognize the importance of a brief postcombined, postprocessing analysis to clarify the actual benefit of combining the three samples, rather than relying on assumptions of benefit as discussed in the paragraphs immediately prior to this. Additional questions remain; if these results do not represent only a quantitative improvement, are there other physiological metrics that change as well between frequent ejaculates, for example, sperm morphology, sperm binding, DNA fragmentation, and postfertilization events?

The key to facilitating combining three consecutive, multiple ejaculations in our laboratory was the use of a commercial semen extender, rTYBS. Historically, the use of rTYBS has been standard operating procedure for all semen specimens presented to our laboratory for IVF, for over 15 years; all men presenting for IVF are instructed to collect a semen specimen between 8:00 am and 9:00 am the morning of the scheduled IVF procedure. This specimen is extended with rTYBS, and cooled as described, allowing flexibility for the laboratory staff as to timing of sperm processing, regardless of the time of the oocyte retrieval. The benefit to expanding the semen collection to the day prior to oocyte retrieval is that semen may be extended, cooled, and held overnight without harm [18].

Transport or storage of liquid human semen overnight, cooled or at room temperature after extension in one of several buffers, including those formulated with hen's egg yolk, protects sperm motility and function [18, 21–28]. According to Manjunath [29], the protective effect of hen's egg yolk or milk-based extenders appears to be due to low density lipoprotein (LDL) components in the buffer that competitively bind with and scavenge a family of proteins known, in hoof stock, as binder of sperm (BSP) proteins, reducing the sensitivity of the sperm membrane to cooling, and protecting/replacing cholesterol within the sperm membrane.

In functional terms, the use of this multiple ejaculation protocol did not negatively affect embryological metrics (Table 2) for fertilization, embryo cleavage, and development to blastocyst stage. Clinical outcomes were not summarized, as the majority of ICSI cycles at this clinic include genetic testing of embryos, with cryopreservation of all biopsied embryos instead of transfer during the same cycle. However, it is clear that sperm selected for ICSI from pooled multiple

ejaculates performed as well as sperm selected from single ejaculates. This novel approach to management of male factor patients required more disposable materials per cycle compared to cycles utilizing only single ejaculate specimens; but first and foremost, it facilitated use of centrifuge-mediated gradient separation rather than other methods of sperm preparation that might compromise sperm quality [8–11].

Based on the findings of this study, combining multiple specimen over a very short time period could be expanded for use with other assisted reproductive interventions. Ejaculatory interval and individual variation in semen analysis parameters both impact quantity and quality of sperm. In the context of ejaculatory abstinence and clinical assisted reproductive interventions, Jurema [30] demonstrated improved insemination cycle pregnancy rates when ejaculatory abstinence was equal to or less than 3 days, and Marshburn [31], stated that despite lower numbers of motile sperm available for insemination, abstinence of 2 days or less was associated with higher pregnancy rates.

In many studies [32–36], the predominant positive predictor of pregnancy was postwash motility and/or total motile sperm cells. Recommendations from these studies regarding minimum numbers of motile sperm required for a successful insemination cycle varied between 1×10^6 and 5×10^6 motile cells. Using Table 1 data and a conservative percent motile sperm recovery of approximately 16.5 % for this laboratory (data not shown), men with very low motile sperm counts might benefit significantly from the multiple ejaculate collection protocol, facilitating insemination with numbers of sperm reaching the recommended motile sperm concentrations.

In conclusion, the results of this study were positive, supporting continuance of the multiple ejaculation protocol for oligozoospermia, asthenozoospermia, and oligoasthenozoospermic men for IVF as a mechanism to facilitate routine and/or routine with alternate sperm processing techniques, without having to resort to cryopreservation of the patient's sperm. Additionally, the results were encouraging with regard to expanding this practice to this same patient population undergoing insemination cycles or fertility preservation.

Acknowledgments The authors would like to thank and acknowledge Michael T. Zavy, Ph.D., for his critical review of this manuscript, and for his insights and suggestions. The authors declare that there were no funding sources, grants, gifts, or other financial incentives for this study. Study data were derived by retrospective data mining using a deidentified data base. The manuscript does not contain clinical or other identifiers that might disclose the identity of the patients. The methodology for semen collection, extension, and processing was standard operating procedure for this laboratory and not experimental; therefore, patient consent and Institutional Review Board approval were not solicited.

Compliance with ethical standards The authors declare that there were no conflicts of interest: including financial, personal, or other relationships with people or organizations that could inappropriately

influence, or be perceived to influence this work. The research represented in this manuscript was not funded by any grant, award, gift, or any other form of financial support. Further, the manuscript has been reviewed and approved by all authors, stipulating that the manuscript and data herein have not been previously published, and is not being considered for publication by another journal. Study data were derived by retrospective data mining using a deidentified data base. The manuscript does not contain clinical or other identifiers that might disclose the identity of the patients. The methodology for semen collection, extension, and processing was standard operating procedure for this laboratory and not experimental; therefore, patient consent and Institutional Review Board approval were not solicited.

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Funding agencies The research represented in this manuscript was not funded by any grant, award, gift, or any other form of financial support. The authors declare that there are no conflicts of interest, including any financial, personal, or other relationships with people or organizations that could inappropriately influence, or be perceived to influence this work.

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