ORIGINAL PAPER

The Effectiveness of Sperm Preparation Using Density Mini-Gradient and Single-Layer Centrifugation for Oligospermia Samples

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ABSTRACT

Background: Sperm preparation is an important step during assisted reproduction, and different assisted reproductive techniques have different sperm quality requirements. For intrauterine insemination (IUI), the total motile sperm count is a predictor of a patient's fertility. **Objective:** The aim of this study was to compare the sperm recovery rate and DNA fragmentation index (DFI) outcomes following density mini-gradient and single-layer centrifugation in preparation for intrauterine insemination (IUI). **Methods:** A total of 30 semen samples with concentrations under 15 million cells/ml were obtained, and each sample was divided into 3 aliquots, with each aliquot subjected to 1 of 3 separation methods: mini-gradient, single-layer using a 90% density layer (single 90-layer), and single-layer using a 45% density layer (single 45-layer). Total sperm motility and sperm recovery rates were compared before and after preparation using each method. **Results:** The sperm concentration obtained using single 45-layer was higher than the other groups ($p<0.05$), but sperm motility was higher using the mini-gradient and single 90-layer methods higher than the single 45-layer method ($p<0.05$). The recovered sperm motility rates for the mini-gradient, single 90-layer, and single 45-layer methods were 57.6% ± 20.6%, 62.8% ± 18.5%, and 78.7% ± 12.4%, respectively, indicating a better outcome for the single 45-layer method than for the other methods. **Conclusion:** All of these methods can be applied to sperm preparation for IUI, and the optimal method can be selected based on initial sperm quality to collect sperm with good motility and DNA integrity to achieve a satisfactory pregnancy rate.

Keywords: Sperm preparation, IUI, mini-gradient, single-layer centrifugation.

1. BACKGROUND

Sperm preparation is an important step during assisted reproduction, and different assisted reproductive techniques have different sperm quality requirements. For intrauterine insemination (IUI), the total motile sperm count is a predictor of a patient's fertility (1, 2).

Current commonly used methods for sperm preparation include discontinuous density gradient centrifugation and the swim-up method, and the percentage of normal chromosome-concentrated sperm obtained using the discontinuous density gradient centrifugation method tends to be higher than that using the swim-up method (3).

For oligospermia samples, the discontinuous density gradient centrifugation method remains the preferred method for sample preparation. However, the standard discontinuous density gradient centrifugation method is not always able to obtain an effective motile sperm count for artificial insemination. To improve sperm recovery from oligospermia samples, our center now uses improved gradient centrifugation techniques, such as density mini-gradient or single-layer centrifugation (90% or 45% density).

However, no studies have assessed the true effectiveness of these 3 methods on oligospermia samples. Our study evaluated the ability to recover sperm and remove sperm with fragmented DNA using these 3 methods.

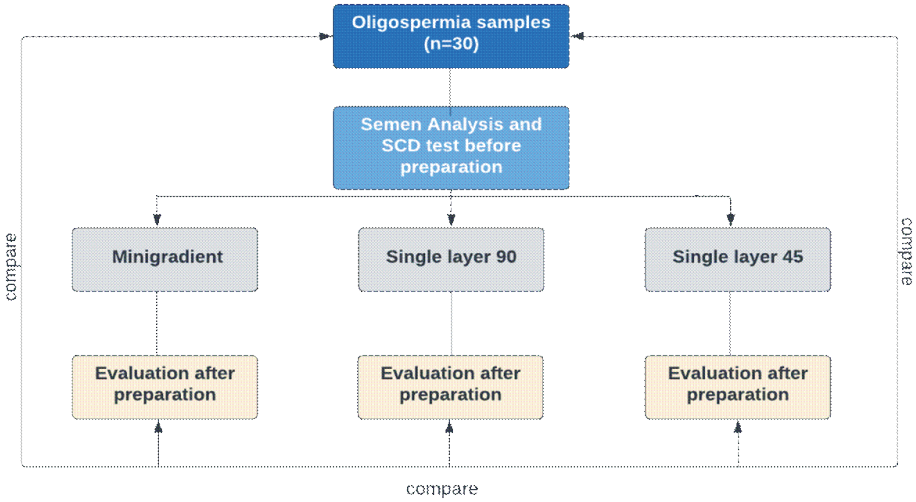

Figure 1. Research model

2. OBJECTIVE

The aim of this study was to compare the sperm recovery rate and DNA fragmentation index (DFI) outcomes following density mini-gradient and single-layer centrifugation in preparation for intrauterine insemination (IUI).

3. PATIENTS AND METHODS

3.1. Patient selection criteria

Semen samples (2 mL) with concentrations <15 million sperm/mL were collected from 30 patients. Sperm samples retrieved from the epididymis or the testicles were excluded from this study.

3.2. Methods

This study was performed as a laboratory experiment. The study design is described in Figure 1

Preparing sperm samples

Each patient provided a semen sample elicited by masturbation and ejaculated into a specimen container. After the semen was liquefied, the sperm concentration was assessed using a Makler counting chamber. Semen samples with a density <15 million cells/mL were collected. Semen quality was evaluated by assessing sperm concentration, motility, morphology, and the presence of round cells. The initial sperm chromatin dispersion (SCD) was assessed.

Sperm sample preparation

Each sample was divided into three 0.5-ml aliquots in Sil-Select upper 45/lower 90 filter medium and washed with Ferticult washing medium (Fertipro, Belgium).

In the mini-gradient centrifugation group, 0.5 mL liquefied semen was loaded onto a 45% and 90% discontinuous gradient (each 0.5 mL) and centrifuged at 1500 rpm for 10 minutes at room temperature. Sufficient supernatant was removed to obtain a sample volume of 0.2 ml, and 2 ml Ferticult was added and mixed. The sample was again centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded, leaving a 0.2 ml volume of medium containing spermatozoa, which was mixed well before use.

In the single-layer centrifugation at 90% density (single 90-layer) group, 0.5 mL liquified semen was layered over 0.5 mL Sil-Select Lower 90 and centrifuged at 1500 rpm for 10 minutes at room temperature. Sufficient supernatant was removed to obtain a sample volume of 0.2 ml, and 2 ml Ferticult was added and mixed. The sample was again centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded, leaving a 0.2 ml volume of medium containing spermatozoa, which was mixed well before use.

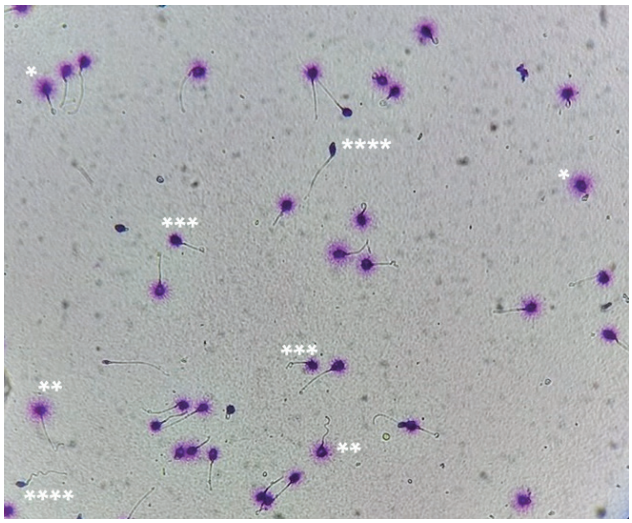

Figure 2. Assessment of DNA fragmentation in sperm cells*, ** indicate sperm without DNA damage; *, **** indicate sperm with DNA damage**

In the single-layer centrifugation at 450% density (single 45-layer) group, 0.5 mL liquified semen was layered over 0.5 mL Sil-Select Upper 45 and centrifuged at 1500 rpm for 10 minutes at room temperature. Sufficient supernatant was removed to obtain a sample volume of 0.2 ml, and 2 ml Ferticult was added and mixed. The sample was again centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded, leaving a 0.2 ml volume of medium containing spermatozoa, which was mixed well before use.

3.3. Assessment of DNA fragmentation sperm (SCD test)

The LensHooke® SCD Sperm DNA Fragmentation Kit (Bonraybio, Germany) was used. Sperm were denatured with HCl and lysed with dithiothreitol to obtain DNA, which was stained with Wright-Giemsa stain. After staining, at least 500 spermatozoa/slide were evaluated

by microscope under 40× magnification.

3.4. Classification

Sperm with a halo larger than at least 1/3 of the smallest sperm head diameter was classified as integral sperm (Figure 2). Sperm with a halo equal to or smaller than 1/3 the diameter of the sperm head, with no halo, or presented with an irregularly colored or pale head were classified as fragmentary sperm (Figure 2)

3.5. Assessed Variables and Indices

The following variables were assessed to evaluate sperm quality, as recommended by the World Health Organization guidelines published in 2010: sperm concentration (millions of cells/mL); progressive motility (PR, %); sperm motility (PR + non-progressive motility [NP], %); vitality (%); and morphology (% normal sperm).

The sperm recovery rate (%) was calculated as

$$\text{Sperm recovery rate} = \frac{\text{Total obtained sperm count}}{\text{Total initial sperm count}} \times 100\%$$

The motile sperm recovery rate (%) was calculated as

$$\text{Motile sperm recovery rate} = \frac{\text{Total obtained motile sperm count}}{\text{Total initial motile sperm count}} \times 100\%$$

The progressive motile sperm recovery rate (%) was calculated as

$$\text{Progressive motile sperm recovery rate} = \frac{\text{Total obtained progressive motile sperm count}}{\text{Total initial progressive motile sperm count}} \times 100\%$$

The DNA fragmentation index (DFI, %) was calculated as

$$\text{DFI} = \frac{\text{Number of sperm with DNA damage}}{\text{Number of sperm assessed}} \times 100\%$$

A DNA fragmentation rate of 26.1% was considered an indicator of male infertility, according to Wiweko et al (4).

3.6. Study setting

The study was conducted at the Center of IVF and Tissue Engineering, Hanoi Medical University Hospital, between April 2018 and October 2020.

3.7. Statistical analysis

All statistical analyses were performed using SPSS software (version 20.0, SPSS Inc). All numeric data are presented as the mean ± standard deviation. The T-test was applied to compare median values between 2 groups before and after preparation. Differences between values were considered significant when $p < 0.05$.

3.8. Research Ethics

The study of collected semen did not affect the patient's test results. The samples used in this study were not used for any other purposes. The goal of this study was to improve treatment efficiency for patients.

4. RESULTS

4.1. Characteristics of the study population

Characteristics of the samples after preparation

Table 1 displays the values of conventional semen parameters and DNA fragmentation rates of the 30 study samples with densities below 15 million cells/mL

The semen samples included in this study had an average sperm concentration of 9.9 ± 3.2 million cells/mL. Motility abnormalities were detected in 4 samples (13.3%), and morphological samples were detected in 26 samples (86.7%). Survival rates below 58% were evaluated in 8 samples (26.7%). The average DNA fragmentation rate was 23.7% ± 10.9%, and 11 samples (36.7%) had high percentages of fragmented DNA.

Table 2 displays the characteristics of the semen samples following preparation using the 3 tested methods.

In the same row, values with different symbols are significantly different. [a] vs. [b] $p < 0.001$; [a] vs [c], $p < 0.05$. PR, progressive motility; NP, non-progressive motility; SD, standard deviation.

No significant differences were detected between semen prepared using the mini-gradient and single 90-layer techniques ($p = 0.191$). The concentration of semen prepared using the single 45-layer method was significantly higher than those prepared using the other 2 methods ($p < 0.0001$). However, the percentages of motile and progressive motile spermatozoa obtained using the mini-gradient and single 90-layer techniques were significantly higher than those obtained using the single 45-layer method ($p < 0.05$).

4.2. Comparing the effectiveness of the three tested methods

Total obtained sperm count

When sperm is being prepared for use in IUI, the total obtained sperm count, especially motile sperm, is important. Sperm counts and motile spermatozoa were assessed before and after the 3 preparation methods (Figure 3). After preparation, the total sperm counts in the mini-gradient, single 90-layer, and single 45-layer groups were $2.15 \pm 0.96 \times 10^6$, $2.39 \pm 1.10 \times 10^6$, and $3.84 \pm 1.60 \times 10^6$ cells, respectively. The total numbers of obtained sperms were significantly reduced after preparation compared with before preparation. The total number of sperm cells

Variables	N	Mean ± SD	Median (Min–Max)	Normal (n, %)
Concentration (10^6 cells/ml)	30	9.9 ± 3.2	9 (4 –14)	
Progressive motility (PR, %)	30	46.5 ± 13.1	47.5 (17.0 – 69.0)	25 (86.7 %)
Total motility (PR + NP, %)	30	56.8 ± 13.7	56.0 (28.0 – 83.0)	26 (83.3 %)
Normal morphology (%)	30	2.3 ± 1.1	2 (1–5)	4 (13.3%)
Vitality (%)	30	63.3 ± 13.7	64.5 (35.0 – 91.0)	22 (73.3%)
DNA fragmentation rate	30	23.7 ± 10.9	21.3 (6.0 – 47.6)	19 (63.3%)

Table 1. Characteristics of the study population. Abbreviations: PR, progressive motility; NP, non-progressive motility; SD, standard deviation.

Variable	Mini-gradient Mean ± SD	Single 90-layer Mean ± SD	Single 45-layer Mean ± SD
Concentration (10^6 cells/mL)	10.8 ± 4.8[a]	12.0 ± 5.5[a]	19.2 ± 8.0[b]
Progressive motility (PR, %)	61.3 ± 21.8[a]	61.7 ± 18.5[a]	47.8 ± 15.8[c]
Total motility (PR + NP, %)	72.3 ± 15.4[a]	72.8± 15.1[a]	58.7 ± 13.0[b]
Vitality (%)	81.4 ± 11.6[a]	80.1 ± 12.0[a]	67.6 ± 11.5[b]

Table 2. Characteristics of the samples after preparation using the 3 tested methods

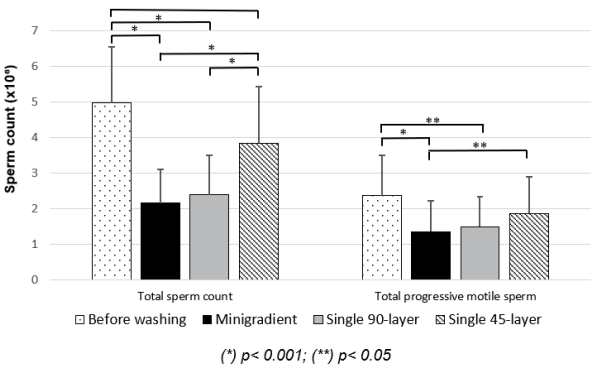

Figure 3. Comparison of sperm counts and motile sperm counts before and after preparation using the 3 tested methods: mini-gradient, single 90-layer, and single 45-layer. *, p < 0.0001; **, p < 0.0001; data represent the mean ± SD

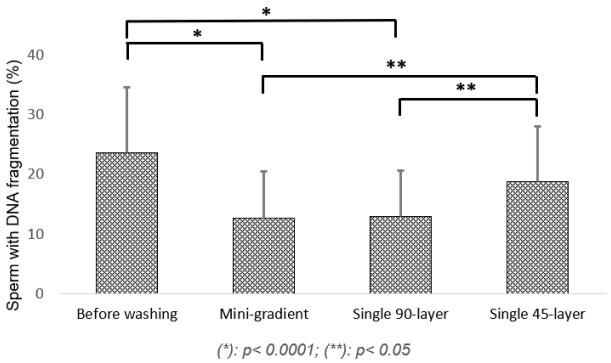

Figure 4. Comparison of percentages of sperm with DNA fragmentation before and after preparation using the 3 tested methods: mini-gradient, single 90-layer, and single 45-layer. *, p < 0.0001; **, p < 0.05; data represent the mean ± SD.

obtained following single 45-layer preparation was higher than the numbers using the other methods ($p < 0.001$).

The total progressive motile sperm count before preparation was 2.36 ± 1.13 × $10^{6,}$ and after preparation, these values for the mini-gradient, single 90-layer, and single 45-layer groups were 1.35 ± 0.87 × 10^6, 1.49 ± 0.85 × 10^6, and 1.86 ± 1.03 × 10^6 cells, respectively. The total progressive motile sperm counts obtained in the single 45-layer group were the highest among the preparation techniques, with no significant difference observed before and after preparation ($p > 0.05$). The total progressive motile sperm counts obtained using the mini-gradient and single 90-layer methods decreased significantly after preparation ($p < 0.05$).

Variable	Mini-gradient Mean ± SD	Single 90-layer Mean ± SD	Single 45-layer Mean ±SD
Sperm recovery rate (%)	44.3 ± 14.1[a]	48.8 ± 15.3[a]	76.2 ± 15.4[b]
Motile sperm recovery rate (%)	58.2 ± 24.6[a]	64.2 ± 19.5[a]	78.1 ± 17.6[c]
Progressive motile sperm recovery rate (%)	57.6 ± 20.6[a]	62.8± 18.5[a]	78.7 ± 12.4[b]

Table 3. Comparison of sperm recovery abilities among the 3 methods. In the same row, values with different symbols are significantly different. [a] vs. [b], p < 0.0001; [a] vs. [c] p < 0.05

The ability to recover sperm using the three tested methods

Each preparation method was associated with a different ability to recover sperm, as described in Table 3.

Among the 3 methods, the single 45-layer approach resulted in the highest recovery percentages for total, motile, and progressive motile spermatozoa (76.2% ± 15.4%, 78.1% ± 17.6%, and 78.7% ± 12.4%, respectively), which were significantly higher than the percentages obtained using the other methods ($p < 0.05$).

The rate of DNA fragmentation among sperm after preparation using the three tested methods

To evaluate the abilities of the 3 tested methods to remove sperm with DNA fragmentation, we used the SCD test. After mini-gradient, single 90-layer, and single 45-layer preparation, the percentages of sperm with fragmented DNA were 12.6% ± 7.9%, 12.9% ± 7.8%, and 18.8% ± 9.2%, respectively. The DNA fragmentation rate of the single 45-layer method was not significantly different from the rate before preparation and was significantly higher than the rates obtained using the other 2 methods - $p < 0.05$ (Figure 4).

5. DISCUSSION

Sperm preparation using density gradient centrifugation is considered an effective technique for artificial insemination. When performing IUI, progressive motile and total motile sperm counts are the factors most closely related to a patient's fertility (5). Studies have shown that total motile sperm counts before preparation ranges from 5 million, and some studies have defined an impact threshold of 10 million motile sperm as necessary for effective insemination using IUI (1). A total motile sperm count greater than 1 million after preparation is recommended, and increases in total motile sperm counts greater than 4 million after preparation are associated with stable increases in pregnancy rates (1).

Although sperm preparation by density gradient centrifugation is considered to result in better sperm recovery than the swim-up method (6, 7), for oligospermia samples, the standard density gradient centrifugation method may not yield a sufficient number of sperm for effective fertilization. However, spermatozoa with poor endurance may not survive the passage through a long density layer, affecting the ability to recover motile sperm after centrifugation. Therefore, the mini-gradient method was developed for the preparation of oligospermia samples (8). Recently, an improved density gradient centrifugation technique using a single density layer has been developed to obtain high-quality sperm. Obtaining sperm with chromosomal integrity is also important for successful fertilization (9).

In our study, the single 45-layer method resulted in the best sperm recovery at 76.2% ± 15.4% (Table 3). The single 45-layer method resulted in comparable outcomes for total sperm recovery, motile sperm recovery, and progressive motile sperm recovery (76.2% ± 15.4%, 78.1% ± 17.6%, and 78.7% ± 12.4%, respectively (Table 3). The single 45-layer technique appears to separate seminal plasma and foreign cells from spermatozoa while allowing abnormal or dead spermatozoa to cross the density surface along with motile sperm under centrifugal

force. In addition, we also found the DNA fragmentation rate of the single 45-layer group to be the highest and not different from that before preparation. The study by Yu et al (2020) suggested that a monolayer is an effective method for rejecting infectious factors (9). In addition, single-layer centrifugation was able to remove epithelial cells and blood cells, reducing the formation of free radicals that might affect the IUI fertilization process (10). Therefore, single 45-layer remains a useful approach for IUI sperm preparation when the motile sperm input is low. Single 45-layer results in high sperm recovery rates and has advantages over other preparation methods because it is able to remove most of the extra-sperm factors that can adversely affect fertilization, such as non-sperm cells or cytoplasmic debris.

No significant differences were observed between the mini-gradient and single 90-layer techniques in terms of progressive motile sperm recovery (57.6% ± 20.6% and 62.8% ± 18.5%, respectively, p = 0.308 (Table 3), and both methods were able to remove sperms with DNA fragmentation, resulting in fragmented DNA percentages of 12.6% ± 7.9% and 12.9% ± 7.8%, respectively, which represent significant reductions compared with prior to preparation (23.7% ± 10.9% (Figure 4). The single 90-layer eliminates dead and chromosomally abnormal sperm, resulting in sperm samples with better DNA quality.

However, this study has some limitations. First, the sample size of our study was quite small, and studies with larger sample sizes remain necessary to determine whether these methods can be used to obtain better sperm quality and quantity. In the near future, we will conduct research using additional functional tests, including sperm viability testing and sperm capacitation testing, to fully evaluate the efficiency of these preparation methods.

6. CONCLUSION

All 3 tested sperm preparation methods have certain advantages. The single 45-layer method results in the best motile sperm recovery rate. Single 90-layer and mini-gradient are equally capable of recovering motile sperm and removing DNA-damaged spermatozoa. We can select the most appropriate method according to the sperm quality.

- **Ethical approval:** This study was approved by the Ethics Committee of Hanoi Medical University.
- **Author's contribution:** Nguyen Thanh Hoa and Nguyen Khang Son contributed equally to this article. Nguyen Thanh Hoa and Nguyen Khang Son gave a substantial contribution to the acquisition, analysis, and data interpretation. Nguyen Minh Duc and Nguyen Manh Ha had a part in preparing the article for drafting and revising it critically for important intellectual content. Each author gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
- **Conflicts of interest:** There are no conflicts of interest to declare.
- **Financial support and sponsorship:** Nil.

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