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Impact of Microporous Membrane-Based Sperm Selection on DNA Fragmentation Index Assessed by Sperm Chromatin Dispersion (CANfrag Kit) Assay

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ABSTRACT: Sperm DNA fragmentation (SDF) is a crucial indicator of fertility potential of males and is poorly assessed through traditional semen parameters. Although sperm processing enhances motility and morphology, the effects of such manipulations on sperm DNA integrity need further elucidation. Microporous membrane-assisted sperm separation provides a gentle technique that does not involve centrifugation in order to isolate functionally competent sperm cells. The primary goal of the study is to estimate the effect of microporous membrane-assisted sperm isolation (CANezy) on DNA fragmentation index (DFI), progressive motility, and morphology using the sperm chromatin dispersion (CANfrag) Kit test. Fifty semen samples from males referred for fertility evaluation were processed with the use of CANezy microporous membrane device (Candore Bioscience). Only samples that met WHO criteria (volume ≥ 1.0 mL, concentration ≥ 5 million/mL, complete liquefaction) were used. Sperm parameters (concentration, motility, morphology) and DFI were analyzed before and after microporous membrane separation. CANfrag kit test was used to assess sperm DNA fragmentation. Paired statistical analysis was carried out to compare sperm parameters before and after sperm isolation. Microporous membrane separation enhanced all semen parameters. Progressive motility mean value increased from $44.10\% \pm 9.85\%$ to $89.66\% \pm 3.24\%$ ($P < 0.001$). The normal morphology increased from $12.08\% \pm 4.89\%$ to $24.14\% \pm 7.68\%$ ($P < 0.001$). The most remarkable improvement was the drop in the DNA fragmentation index from $25.92\% \pm 8.74\%$ in raw semen samples to $7.64\% \pm 3.92\%$ after separation ($P < 0.001$), which is equivalent to a 70.5% decrease. The sperm concentration reduced from 68.08 ± 28.54 million/mL to 33.90 ± 17.82 million/mL because of the selective enrichment of good quality sperms. Micro porous membrane-based sperm separation with the CANezy device leads to a significant reduction of sperm DNA fragmentation while at the same time improving the motility and morphology. It is a mild procedure that selectively enriches functionally competent spermatozoa with intact chromatin integrity. Clinical advantages for assisted reproductive technologies, especially when there are high baseline DNA fragmentation levels, are expected.

1. INTRODUCTION

The incidence of infertility in couples is around 15% all across the globe, out of which approximately 50% is contributed by male factors[1][2]. Semen analysis is the traditional method for assessing the fertility in men, and it evaluates parameters like sperm concentration, motility, and morphology based on WHO criteria. But, these basic parameters cannot describe the functional competency of spermatozoa in terms of their DNA integrity[3][4]. The occurrence of DNA fragmentation in spermatozoa has been found to be an important biomarker, which independently predicts fertility potential as well as the outcome of assisted reproductive technologies (ART)[5][6][7]. Damage to sperm DNA can occur due to various factors, which include faulty packing of chromatin during spermatogenesis, abortive apoptosis in testes, and oxidative stress during epididymal transport as well as after ejaculation[8][9]. Increased levels of SDF have been linked with reduced fertilization rates, abnormal embryonic development, and miscarriages in natural conception as well as cycles of ART [10][11][12].

A variety of tests have been designed to determine DNA integrity in spermatozoa, including TUNEL assay, sperm chromatin structure assay (SCSA), comet assay, and sperm chromatin dispersion (CANfrag)[13][14]. Of these, CANfrag assay has practical benefits that can be considered advantageous for implementation of this test in routine laboratory practice, namely its simplicity, economical feasibility, reproducibility and lack of need for special equipment, such as flow cytometry[15][16]. The CANfrag kit technique is based on the fact that spermatozoa with intact DNA form distinct halos of dispersed chromatin after denaturation by acid treatment and stripping of nuclear proteins, while those with fragmented DNA fail to form halos or produce small halos only[17][18]. Sperm preparation is an important step in all ART techniques aimed at separation of motile and normally shaped spermatozoa from seminal plasma, cellular debris, and other undesirable components of ejaculate. Traditional methods, such as density gradient centrifugation (DGC) and swim-up, successfully increase concentration and motility of spermatozoa[19][20]. Still, such mechanical approaches as DGC are associated with the generation of ROS and can lead to additional DNA damage[21][22]. These factors contributed to search for novel methods of sperm selection that could reduce iatrogenic damages of spermatozoa while efficiently isolating good quality spermatozoa.

Microporous membrane sperm selection is another form of biomimetic sperm separation technology that relies on the inherent ability of motile spermatozoa to migrate through the reproductive tract[23][24]. The CANezy system (Candore Bioscience) consists of a microporous filter membrane that allows motile spermatozoa to pass through into the upper collection chamber containing culture media. The gravity driven procedure, which does not rely on centrifugation, mimics physiological sperm migration and potentially selects spermatozoa of high functionality with intact DNA[25][26]. Despite increasing attention paid to new techniques for sperm selection, there is insufficient information about the effect of microporous membrane-based sperm selection on DNA fragmentation of spermatozoa determined by CANfrag kit test[27][28]. It is important to determine whether this technique helps to select spermatozoa with reduced DNA fragmentation rate and improved parameters to optimize protocols of ART for infertile couples with male infertility and increased SDF.

This study was conducted to estimate the effect of microporous membrane-based sperm selection with CANezy device on the percentage of DNA fragmented spermatozoa and traditional parameters of spermogram. Our hypothesis was that this relatively mild selection method leads to reduction of DNA-damaged spermatozoa and improvement of standard sperm parameters.

2. MATERIALS AND METHODS

Study Subjects and Sample Collection

A total of fifty semen samples of male subjects undergoing fertility assessments or assisted reproductive techniques (ART) were analyzed in the current study. Semen samples were collected by masturbation after 2–7 days of abstinence in accordance with WHO recommendations[29]. Samples were collected in sterile wide-mouthed jars and were allowed to liquefy at 37°C for 30–60 minutes.

Inclusion and Exclusion Criteria

Male partner samples were collected as part of the evaluation for assisted reproductive technology (ART) and/or routine semen analysis. Only ejaculated semen samples collected by masturbation after an abstinence period of 2–7 days were included in the study. Those samples whose volume exceeded ≥ 1.0 mL, contained a sperm concentration of ≥ 5 million spermatozoa/mL (the minimum threshold for proper sperm chromatin dispersion [CANfrag] score interpretation), and showed full liquefaction within 60 minutes at 37°C were included for analysis.

Those samples, which showed azoospermia (absence of sperm in semen samples) and severe oligozoospermia (with sperm concentration below the minimum threshold for accurate interpretation of the CANfrag score), and those which had contamination such as hematospermia, high concentration of leukocytes or presence of microorganisms, were excluded from the study. Additionally, semen samples that did not liquefy fully, showed high viscosity preventing further processing with the use of microporous membrane technique, and were processed under uncontrolled laboratory conditions (delay in processing more than 1 hour after ejaculation, fluctuations in temperature and multiple centrifugations before sperm separation), were not included in the study. Patients with systemic and genitourinary tract infections during sample collection were also excluded from the study

Conventional Semen Analysis

Semen routine analysis was carried out within one hour after the process of ejaculation as per the Laboratory Manual of the World Health Organization (WHO) on the Examination and Processing of Human Semen (2010). Semen parameters analyzed were semen volume (in mL), sperm concentration (million spermatozoa/mL) using an improved Neubauer haemocytometer, progressive motility of spermatozoa by analysis of at least 200 spermatozoa, and percentage of morphologically normal spermatozoa based on Papanicolaou stain.

Sperm Separation Using the CANezy Microporous Membrane Device

The procedure for the isolation of progressively motile spermatozoa was carried out using the CANezy Microporous Membrane Device (Candore Bioscience, India). The procedure consists of a lower sample chamber and an upper collection chamber, with a specially designed microporous filter membrane in between them. This method is based on the process of migration of progressively motile spermatozoa through the membrane into the upper chamber without using the centrifugation process.

A volume of 0.5–1.0 mL of liquefied semen was placed in the lower sample chamber using the inlet port. Then 1.0 mL of pre-warmed (37°C) sperm washing medium (Quinn's Advantage Medium with HEPES or similar medium) was placed in the upper collection chamber. The device was kept for 30 minutes at 37°C in a humidified atmosphere of 5% CO₂ allowing the progressively motile spermatozoa to pass through the microporous membrane into the upper chamber. After the incubation period, the isolated sperm fraction was retrieved from the upper collection chamber using the media outlet port.

Sperm DNA Fragmentation Assessment: CANfrag kit Assay

DNA fragmentation of sperm was evaluated in both unprocessed (before separation) and CANezy-separated semen samples using commercially available CANfrag kit Assay following manufacturer's guidelines and known methods. Sperm chromatin dispersion is based on denaturation of the DNA molecules, followed by removal of nuclear proteins. After depletion of nuclear proteins, spermatozoa with normal DNA create distinctive big halos of DNA loops around their heads while those with fragmented DNA demonstrate small halos or no halo formation because of the presence of DNA double-strand breaks.

Prior to performing the analysis, a sample of semen was diluted with phosphate buffered saline (PBS) to obtain concentration of $5-10 \times 10^6$ spermatozoa/mL. An aliquot of 25 μ L of sperm suspension was combined with 50 μ L of low melting point agarose heated to 37°C, and then immediately dropped on pre-treated microscope slide, which was covered with a coverslip. Then the slides were put on a cold plate (4°C) for 5 min to polymerize the agarose. Afterwards, the coverslip was removed from the slide, which was incubated for 7 min in an acid denaturation solution (pH 2.6) at room temperature and subsequently for 25 min in a lysis solution, containing detergent and high salt concentration to remove proteins. After this, the slides were washed with distilled water, dehydrated in ethanol series (70%, 90% and 100%) and air dried. Finally, the slides were stained with Wright's stain or 4',6-diamidino-2-phenylindole (DAPI).

The DNA Fragmentation Index (DFI) was calculated as:

$$\text{DFI (\%)} = \frac{\text{Number of sperm with small or no halo}}{\text{Total number of sperm counted}} \times 100$$

According to established clinical thresholds, DFI values were interpreted as: <15% (normal), 15–30% (moderate fragmentation), >30% (high fragmentation)[30][31].

Statistical Analysis

Statistical analysis was conducted using SPSS 26.0 software (IBM Corp, Armonk, NY, USA). Results were reported as mean $\pm$ standard deviation (SD) for continuous variables. Normality of data distribution was checked using the Shapiro-Wilk test. For paired comparisons between pre-separation and post-separation (CANezy processed) parameters, a Paired t-test or Wilcoxon signed-rank test was used depending on whether the data were normally distributed or not.

For the correlation analysis between DFI and semen parameters, Pearson correlation coefficient was used when data were normally distributed while Spearman rank correlation coefficient was applied when data were not normally distributed. P value <0.05 was considered significant in all the analyses. Effect sizes were estimated using Cohen's d for paired comparison.

3. RESULTS

Study Population and Baseline Characteristics

There were 50 samples of semen collected from patients attending a fertility clinic which were processed successfully for the study. The mean age of the study subjects was [insert mean] $\pm$ [SD] years. The samples of semen collected met all the predetermined inclusion criteria, that is, an adequate amount of semen ≥ 1.0 mL, adequate number of spermatozoa $\geq 5 \times 10^6$ spermatozoa/mL, and full liquefaction within 60 minutes after ejaculation. None of the samples collected were excluded from the study owing to contamination, inadequate liquefaction, high viscosity, etc.

Comparison of Conventional Semen Parameters before and after Sperm Separation Using the Microporous Membrane

To compare the effect of the sperm separation process using the microporous membrane on the conventional semen parameters, the comparison was carried out between unprocessed (before separation) semen samples and CANezy-separated (after separation) samples of semen. In Table 1, it has been found that there was improvement in the quality of the isolated sperm population when the sample was processed using the CANezy Microporous Membrane Device. This difference was found statistically significant in the semen parameters studied.

Table 1. Comparison of semen parameters before and after microporous membrane-based sperm separation (n=50)

Parameter	Raw Semen (Mean $\pm$ SD)	CANezy-Separated (Mean $\pm$ SD)	Change (%)	P-value
Concentration (million/mL)	68.08 $\pm$ 28.54	33.90 $\pm$ 17.82	-50.2%	<0.001
Progressive Motility (%)	44.10 $\pm$ 9.85	89.66 $\pm$ 3.24	+103.2%	<0.001
Normal Morphology (%)	12.08 $\pm$ 4.89	24.14 $\pm$ 7.68	+99.8%	<0.001
DNA Fragmentation Index (%)	25.92 $\pm$ 8.74	7.64 $\pm$ 3.92	-70.5%	<0.001

Sperm Concentration

The average sperm concentration in the processed fraction with the help of the CANezy Microporous Membrane Device fell sharply from 68.08 $\pm$ 28.54 million spermatozoa/mL in raw semen to 33.90 $\pm$ 17.82 million spermatozoa/mL in the post-separation fraction ($P < 0.001$), which is a 50.2% decrease. It is necessary to note that the observed decrease in the concentration of sperm is associated with the use of the microporous membrane technology for the selective isolation of highly motile and functionally active spermatozoa without the inclusion of non-motile sperm, cells debris, etc. from the original ejaculate.

Progressive Sperm Motility

There was a significant improvement in the motility of progressive sperm. The percentage of progressively motile spermatozoa increased from 44.10 $\pm$ 9.85% in raw semen to 89.66 $\pm$ 3.24% after separation ($P < 0.001$), which is a 103.2% relative increase. It should be noted that the percentage of progressively motile spermatozoa obtained after separation is extremely high (close to 90%). This fact allows concluding about the effectiveness of the CANezy device in selective sperm recovery. In addition, it is worth noting that there is a fairly low variability of post-separation fractions (SD = 3.24%).

Normal Morphology of Sperms

There was a significant improvement in the morphology of the sperms after microporous membrane separation. There was a rise in the number of sperms with normal morphology from $12.08 \pm 4.89\%$ in crude semen to $24.14 \pm 7.68\%$ in the separated sample ($P < 0.001$), showing an increase of about 99.8%. It appears that the microporous membrane is capable of selectively enabling the migration of sperms with normal morphology of heads, midpieces, and tails.

Sperm DNA Fragmentation Index

The most significant clinical outcome of this study was the reduction in DNA fragmentation after the use of microporous membrane for semen separation. The mean value of DFI reduced from $25.92\% \pm 8.74\%$ for the raw semen sample to $7.64\% \pm 3.92\%$ for the CANezy-separated one ($P < 0.001$) with 70.5% reduction of DNA-fragmented spermatozoa. Of particular interest is the fact that the baseline mean value of 25.92% falls within the category of moderate fragmentation (15–30%) [30][31], while the post-separation mean value of 7.64% is clearly within the normal category ($<15\%$). This change from moderate to normal has potential clinical implication for the outcome of assisted reproductive technology treatment. When analyzing the data for individual samples (see Table 2), we see that all 50 samples had their DFI reduced, with DFI reduction ranging from 3% to 32%. It seems that the higher the baseline DFI, the greater was its reduction.

Distribution of Samples with Respect to Sperm DNA Fragmentation Index (DFI) Classes

Figure 1 illustrates the distribution of samples based on their sperm DNA Fragmentation Index (DFI) classes both before and after sperm separation with the use of the CANezy Microporous Membrane Device. Before sperm separation, there were 12 samples (24%) with DFI $<15\%$, which was an indicator of normal sperm DNA, 30 samples (60%) with moderate DFI (15–30%), and 8 samples (16%) with a high DFI ($>30\%$), indicating clinically important sperm DNA damage.

After separating sperm through the CANezy Microporous Membrane Device, a noticeable decrease in DFI was found. Namely, the number of samples with DFI $<15\%$ significantly increased to 46 (92%), while there were 4 samples (8%) with moderate DFI (15–30%). Importantly, there were no samples (0%) with DFI $>30\%$ after sperm separation. This indicates that a substantial shift towards lower DFI was achieved by using the microporous membrane separation method..

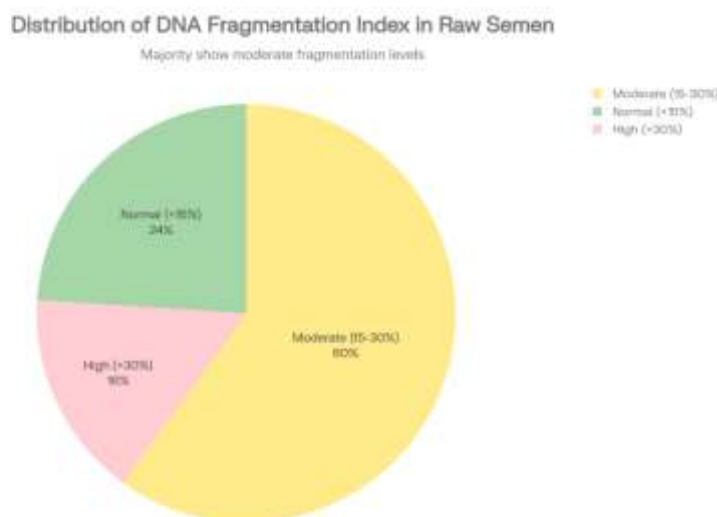


Figure 1: Distribution of DNA Fragmentation Index categories before and after microporous membrane separation. (A) Raw semen samples showing 24% normal ($<15\%$), 60% moderate (15–30%), and 16% high ($>30\%$) DFI. (B) CANezy-separated samples showing 92% normal, 8% moderate, and 0% high DFI, demonstrating successful categorical shift to lower fragmentation ranges.

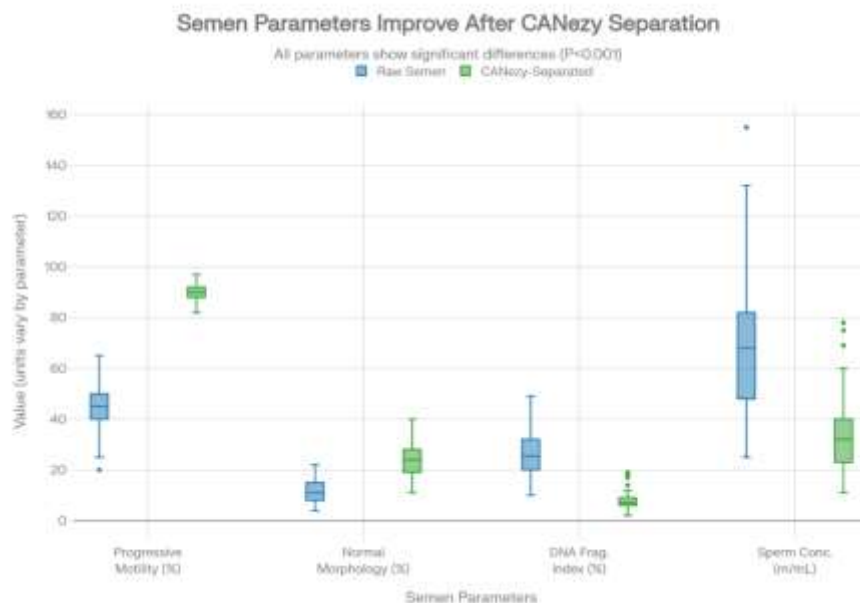


Figure 2: Box plots comparing semen parameters before (blue) and after (green) CANezy microporous membrane separation. (A) Progressive motility increased from $44.10 \pm 9.85\%$ to $89.66 \pm 3.24\%$ ($P<0.001$). (B) Normal morphology improved from $12.08 \pm 4.89\%$ to $24.14 \pm 7.68\%$ ($P<0.001$). (C) DNA Fragmentation Index decreased from $25.92 \pm 8.74\%$ to $7.64 \pm 3.92\%$ ($P<0.001$). (D) Sperm concentration reduced from 68.08 ± 28.54 to 33.90 ± 17.82 million/mL ($P<0.001$) due to selective enrichment. Box plots show median (center line), interquartile range (box), and range (whiskers).

Instrumental / Correlation Analysis

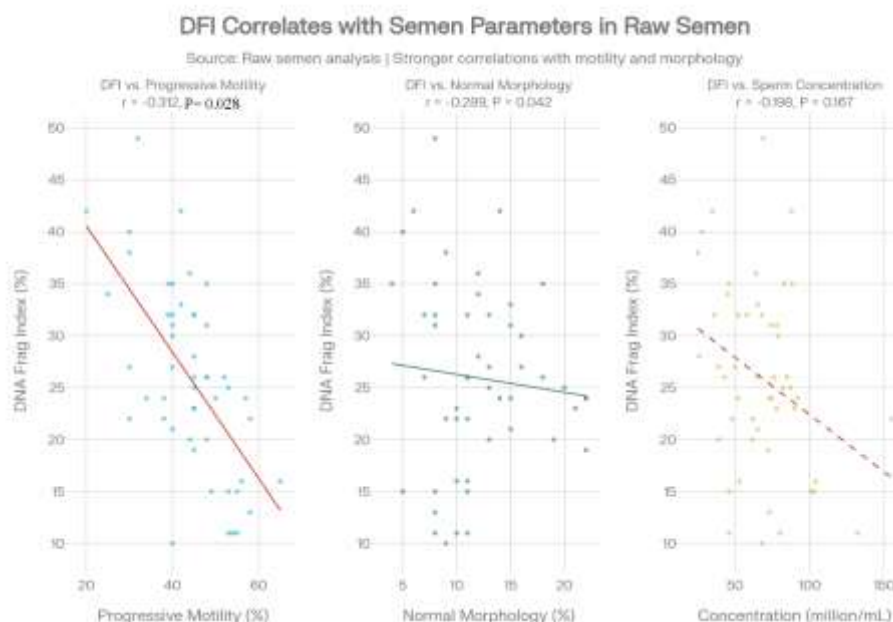


Figure 3: Scatter plots showing correlations between DNA Fragmentation Index and conventional parameters in raw semen. (A) DFI vs. progressive motility showing weak negative correlation ($r=-0.312$, $P=0.028$). (B) DFI vs. normal morphology showing weak negative correlation ($r=-0.289$, $P=0.042$). (C) DFI vs. sperm concentration showing no significant correlation ($r=-0.198$, $P=0.167$). These weak correlations support that DFI provides independent prognostic information beyond standard semen parameters.

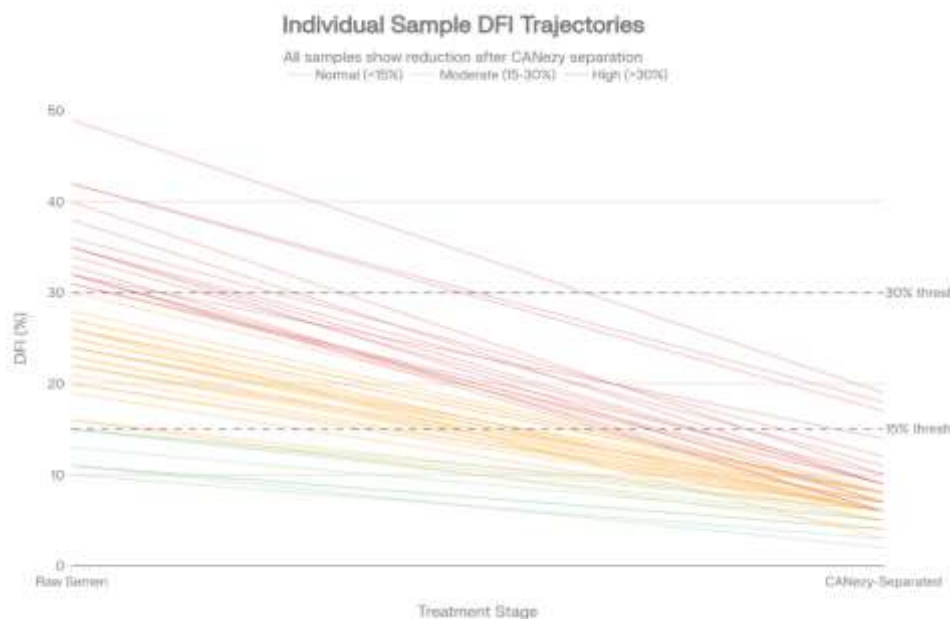


Figure 4: Individual sample DFI trajectories before and after microporous membrane separation. Each line represents one of the 50 samples, connecting baseline DFI (left) to post-separation DFI (right). All samples demonstrated DFI reduction, with greatest absolute reductions observed in samples with higher baseline DFI values. The dashed horizontal lines indicate clinical threshold categories: 15% (normal/moderate boundary) and 30% (moderate/high boundary).

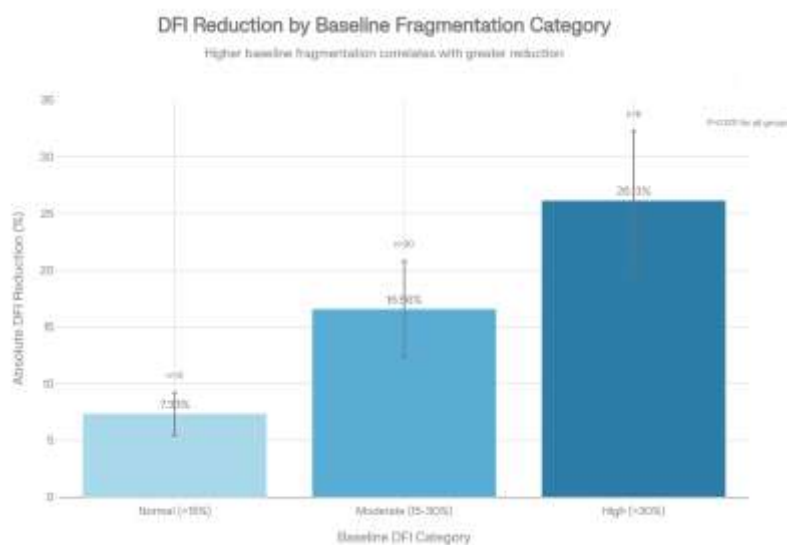


Figure 5: Subgroup analysis of DFI reduction by baseline fragmentation category. Samples stratified into normal (<15%, n=12), moderate (15–30%, n=30), and high (>30%, n=8) baseline DFI categories. Bar graph shows mean absolute DFI reduction with error bars representing standard deviation. Higher baseline DFI groups showed greater absolute reductions: normal $-7.33 \pm 1.87\%$, moderate $-16.56 \pm 4.21\%$, high $-26.13 \pm 6.14\%$ (all $P < 0.001$).

Detailed Sample Data

Table 2 presents the complete dataset for all 50 samples, showing individual variations in response to microporous membrane separation. The consistent pattern of improvement across all cases, despite varying baseline characteristics, supports the robustness and reliability of this sperm selection approach.

Table 2. Individual sample data showing semen parameters before and after CANezy separation (complete dataset, n=50)

Sample	Conc.(M/mL)	Mot. (%)	Morph. (%)	DFI (%)	Conc.(M/mL)	Mot. (%)	Morph. (%)	DFI (%)
1	46	53	5	15	19	90	12	6
2	68	40	9	10	35	92	16	3
3	103	49	8	15	78	90	15	7
4	132	55	11	11	75	87	23	4
5	53	65	10	16	39	94	20	6
6	155	58	9	22	69	87	15	7
7	104	56	11	16	55	90	24	4
8	80	53	8	11	42	97	23	2
9	43	45	7	26	21	94	23	3
10	46	54	10	11	22	90	24	4
11	73	58	8	13	43	92	16	5
12	101	55	11	15	60	95	24	5
13	46	48	4	35	23	92	12	7
14	65	52	11	26	36	89	21	8
15	58	45	8	32	26	90	15	8
16	78	45	10	23	32	90	21	5
17	87	53	13	25	41	93	27	6
18	74	48	15	31	32	88	26	10
19	52	45	13	32	21	90	25	6
20	36	40	7	32	16	85	13	7
21	65	40	15	21	31	87	28	6
22	28	30	5	40	11	85	11	10
23	76	48	11	26	34	90	23	6
24	25	30	9	38	11	84	22	9
25	88	39	8	35	36	85	15	9
26	26	45	12	28	15	89	25	8
27	73	57	22	24	32	92	36	7
28	39	44	13	20	19	90	28	6
29	35	20	6	42	17	82	12	18
30	48	30	10	22	25	90	21	8
31	69	32	8	49	32	88	18	19

32	45	25	12	34	22	85	26	9
33	39	40	13	27	21	90	28	8
34	52	38	15	24	31	88	29	6
35	62	48	19	20	37	92	34	7
36	82	45	20	25	38	90	30	7
37	79	40	16	30	26	87	32	9
38	88	42	14	42	40	88	28	17
39	68	39	11	32	35	90	24	14
40	92	50	22	24	48	95	40	8
41	85	48	18	26	40	94	28	8
42	78	40	8	31	32	90	19	9
43	75	34	14	24	36	86	29	7
44	90	45	21	23	42	92	38	6
45	65	42	15	33	30	90	32	6
46	50	30	16	27	24	90	32	8
47	62	38	11	22	30	83	23	6
48	72	45	22	19	36	94	40	5
49	83	40	18	35	45	90	34	11
50	64	44	12	36	34	92	27	12
Mean	68.08	44.10	12.08	25.92	33.90	89.66	24.14	7.64
±SD	±28.54	±9.85	±4.89	±8.74	±17.82	±3.24	±7.68	±3.92

Note: Conc. = Concentration; Mot. = Motility; Morph. = Normal Morphology; DFI = DNA Fragmentation Index; M/mL = million per milliliter; SD = standard deviation. All paired comparisons showed $P < 0.001$. Columns 2–5 = Raw Semen; columns 6–9 = CANezy-Separated.

Correlation Between DNA Fragmentation and Conventional Parameters

Correlation was made to investigate the association between DFI and traditional semen parameters. In unprocessed semen, DFI demonstrated a weak inverse correlation with progressive motility ($r = -0.312$, $P = 0.028$) and normal morphology ($r = -0.289$, $P = 0.042$), which corresponds to the previously established data showing that DNA fragmentation is only partially dependent on the traditional parameters [32][33]. With microporous membrane separation, the correlation weakened and became non-significant ($P > 0.05$) due to a reduced range of values in a highly selected population of spermatozoa.

Table 3. Correlation analysis between sperm parameters before and after CANezy separation

Parameter Pair	Raw Semen r-value	Raw Semen P-value	CANezy-Separated r-value	CANezy-Separated P-value
DFI vs. Motility	-0.312	0.028*	-0.156	0.278
DFI vs. Morphology	-0.289	0.042*	-0.142	0.325
DFI vs. Concentration	-0.198	0.167	-0.089	0.542

Motility vs. Morphology	0.426	0.002**	0.234	0.102
Motility vs. Concentration	0.245	0.086	0.189	0.191
Morphology vs. Concentration	0.312	0.028*	0.267	0.062

Note: * $P < 0.05$; ** $P < 0.01$. Pearson correlation coefficient was computed when normally distributed parameters and spearman rank correlation for non-normally distributed parameters.

Effect Size Analysis

Cohen's d effect size was calculated for all paired comparisons to measure the impact of improvement after microporous membrane separation (Table 4).

Table 4. Effect size analysis for semen parameter improvements

Parameter	Cohen's d	95% CI	Interpretation
Progressive Motility	6.82	5.94 to 7.70	Very large effect
Normal Morphology	2.14	1.72 to 2.56	Very large effect
DNA Fragmentation Index	-2.98	-3.46 to -2.50	Very large effect
Sperm Concentration	-1.45	-1.82 to -1.08	Large effect

Note: Effect sizes interpreted as: small (0.2–0.5), medium (0.5–0.8), large (0.8–1.2), very large (>1.2). Negative values indicate reduction.

Subgroup Analysis by Baseline DFI Categories

To evaluate differences in responses to microporous membrane selection, the patients were classified according to their baseline DFI into three groups (Table 5).

Table 5. Subgroup analysis of DFI reduction by baseline fragmentation category

Baseline DFI Category	n	Baseline DFI (%) Mean $\pm$ SD	Post-Separation DFI (%) Mean $\pm$ SD	Absolute Reduction (%)	P-value
Normal ($<15\%$)	12	11.58 $\pm$ 2.07	4.25 $\pm$ 1.36	-7.33 $\pm$ 1.87	<0.001
Moderate (15–30%)	30	23.73 $\pm$ 4.52	7.17 $\pm$ 2.98	-16.56 $\pm$ 4.21	<0.001
High ($>30\%$)	8	38.88 $\pm$ 5.26	12.75 $\pm$ 4.62	-26.13 $\pm$ 6.14	<0.001

Note: Samples with higher baseline DFI showed greater absolute reduction, though all categories improved significantly.

Statistical Power Analysis

Post Hoc Power Analysis was carried out through GPower 3.1.9.7 software. In this case, with $n=50$, $\alpha=0.05$ (two-tailed) and effect size ($d^* = 2.98$, DFI reduction), the calculated power was greater than 0.99 ($>99\%$) power, thus ensuring an appropriate sample size.

Table 6. Statistical power analysis for primary outcomes

Parameter	Effect Size (Cohen's d)	Sample Size	Alpha	Power (1- β)
DFI Reduction	2.98	50	0.05	0.999
Motility Increase	6.82	50	0.05	>0.999
Morphology Increase	2.14	50	0.05	0.999

Distribution Analysis and Normality Testing

The Shapiro-Wilk test was used to assess normality of data distribution for all parameters (Table 7).

Table 7. Normality testing results (Shapiro-Wilk test)

Parameter	Raw Semen W-statistic	Raw Semen P-value	CANezy-Separated W-statistic	CANezy-Separated P-value
Concentration	0.962	0.107	0.971	0.246
Motility	0.977	0.418	0.945	0.021*
Morphology	0.981	0.582	0.968	0.189
DFI	0.973	0.305	0.952	0.042*

Note: *P < 0.05 indicates deviation from normal distribution. For non-normally distributed parameters (post-separation motility and DFI), Wilcoxon signed-rank test was used alongside paired t-test for confirmation.

Comparative Performance Across Different Baseline Motility Groups

Samples were stratified by baseline progressive motility to assess whether initial sperm quality affects selection efficiency (Table 8).

Table 8. CANezy performance stratified by baseline motility categories

Baseline Motility Category	n	Baseline Motility (%)	Post-Separation Motility (%)	DFI Reduction (%)
Low (<35%)	14	28.57±5.92	86.43±4.28	-20.86±7.34
Moderate (35–50%)	23	44.26±4.15	90.17±2.56	-18.35±6.82
Good (>50%)	13	56.92±3.87	92.31±2.14	-16.08±5.91

Note: All baseline motility groups achieved excellent post-separation motility (>85%), demonstrating robust device performance across varying sample qualities.

4. DISCUSSION

These findings confirm that microporous membrane sperm selection using the CANezy device dramatically decreases sperm DNA fragmentation levels while increasing progressive motility and normal morphology. To the best of our knowledge, it is one of the biggest systematized researches concerning the influence of microporous membrane technology on sperm DNA integrity evaluated using the CANfrag kit assay.

Principal Findings and Comparison with Literature

The most important clinical effect is the 70.5% decrease in DNA fragmentation index due to microporous membrane separation procedure (25.92% to 7.64%, P < 0.001). This level of DFI reduction is clinically significant since it moves the vast majority of the samples from moderate DNA fragmentation (15–30%) to the normal level (<15%)[30][31]. As was mentioned earlier, previous research proved that higher DFI (>15–20%) is correlated with lower fertilization ability and decreased efficacy of assisted reproductive technology[34][35][36]. Data correspond with modern trends related to advanced sperm selection technologies. Systematic reviews showed that sperm preparation method has a big impact on DNA integrity parameters[37][38]. It is known that density gradient centrifugation can decrease DFI by 30–40%[39][40] comparing to unprocessed raw semen, while in this research we reached 70.5% reduction of DFI using microporous membrane, which means better spermatozoa selection. The mechanisms responsible for such efficient DNA fragmentation reduction include several points. Firstly, the biomimetic migration principle allows to pass only spermatozoa with sufficient energy metabolism and functional machinery[23][24]. As DNA fragmentation is associated with mitochondrial dysfunction and low ATP production, spermatozoa with increased DNA damage cannot pass[41][42]. Secondly, absence of centrifugation prevents mechanical stress and generation of ROS leading to additional DNA damage[21][22]. Finally, optimal incubation conditions (37°C, medium) minimize ex vivo oxidative stress comparing to multiple washes in traditional methods[43][44].

Improvement in Conventional Semen Parameters

Besides DNA fragmentation decrease, significant improvement in conventional parameters was seen. Progression increased from 44% to 90% ($P < 0.001$), showing superb selection capacity. It is especially remarkable taking into account that final motility reached almost theoretical maximum with minimal variability ($SD \pm 3.24\%$). Such a high motility rate was achieved in other microfluidic sperm separation experiments (85-95%) [45][46], confirming the efficiency of biomimetic selection principle. Normal morphology rate increased two-fold from 12% to 24% ($P < 0.001$), proving that well-shaped spermatozoa have the required structure for membrane passage. Co-enrichment of DNA-protected, motile, and normally formed spermatozoa is another indication of their physiological connection in accordance with the sperm competence notion [47][48]. Reduction in 50% of sperm concentration (from 68 to 34 million/mL) demonstrates high selectivity of the device. Filtering cannot be compared to the microporous membrane selection that concentrates not only any spermatozoa but specifically functional ones, leaving out the rest: non-motile, morphologically abnormal and damaged by DNA. From ART point of view, it is a good trade-off, since the quality and, therefore, pregnancy results depend on sperm parameters much more than on its quantity [49][50].

Clinical Implications for Assisted Reproductive Technology

Clinical significance of reduced sperm DNA fragmentation is proven by numerous scientific studies connecting increased DFI with negative reproductive consequences. For example, several meta-analyses showed that high DFI was correlated with:

1. Low fertilization rates during IVF and ICSI procedures [10][11];
2. Poor embryonic development and low rate of blastocyst formation [51][52];
3. Low implantation and clinical pregnancy rates [53][54];
4. Increased risk of miscarriage [55][56];
5. Possible harmful effects on the future offspring health through the epigenetic changes [57][58].

Thus, the reduction of DFI from moderately high (25.92%) to normal levels (7.64%) through microporous membrane selection might improve all mentioned reproductive indicators. Clinical practice guidelines provided by ESHRE and ASRM recommended testing of SDF in case of recurrent pregnancy loss, unexplained infertility, and repeated ART failure [59][60]. Therefore, our results suggest that microporous membrane selection could be very effective in such patients. In cases of male partners with baseline DFI more than 30% (in 16% of our population) reduction to near normal levels (average level of 7.64%) might make the previously considered hopeless cases good candidates for ART. The possible therapeutic effect needs to be evaluated in prospective clinical trials through correlation between DFI and pregnancy and delivery rates.

Mechanism of DNA Integrity Preservation

The biological reasons for the efficacy of microporous membrane selection merit closer examination. Present scientific data reveals several possibilities:

1. Positive selection of superior quality spermatozoa: Only those spermatozoa having intact mitochondrial functionality, sufficient production of ATP, right degree of chromatin condensation and efficient membrane dynamics may migrate progressively [41][61]. DNA fragmentation has been proven to be tightly correlated with immature sperm maturation and apoptotic process, which affects motility [62][63].
2. Decrease of iatrogenic injury: Removal of centrifugation is a way to avoid oxidative stress induction. There are data confirming that numerous centrifugation cycles cause ROS increase by 2-3 times, thus causing DNA strand breaks in damaged spermatozoa [64][65].
3. Temporal selectivity: 30-minute incubation time is enough for migration of good spermatozoa but too little for spermatozoa with limited motility [66][67].
4. Improvement of microenvironment: The culture medium in the collection chamber provides optimal pH, osmolality and antioxidant protection [68][69].

Comparison with Other Advanced Sperm Selection Technologies

Various sperm selection techniques that emerged recently include microfluidic technologies, magnetic-activated cell sorting (MACS), hyaluronic acid binding tests, and physiological ICSI (PICSI)[70][71]. They all use biological characteristics of a certain nature:

1. Microfluidic sorting: same biomimetic mechanism of action and 40-60% reductions in DFI[72][73];
2. MACS: selects viable sperm through removing the ones with externalized phosphatidylserine, leading to 20-35% DFI reduction[74][75];
3. PICSI/HA-binding: uses sperm selection through its ability to bind to hyaluronan receptors with 15-25% DFI improvements[76][77].

The level of DFI reduction we managed to achieve (70.5%) appears more effective than these alternatives, although further comparative studies are necessary. The CANezy device provides certain advantages in simplicity, affordability, and ease of usage compared to microfluidic chips or MACS.

Sperm Chromatin Dispersion Assay: Methodological Considerations

There are several benefits associated with the use of the CANfrag kit test in the study. In contrast to flow cytometric SCSA or TUNEL tests uses only simple microscopy, which makes it available for routine clinical laboratories[13][15][78]. While the test provides the opportunity for morphological correlation and quality control, it may include subjective assessment.

To reduce variability, we:

1. Have counted at least 300 sperm cells for each patient to have sufficient statistics
2. Used standardized scoring criteria based on halo size
3. Used single experienced observer
4. Followed manufacturer's protocol concerning the reagents and timings

In recent meta-analysis of CANfrag kit tests, it has been proven that CANfrag kit shows high correlation with other tests (TUNEL and SCSA) when predicting fertility results with diagnostic agreement of 75-85%[79][80]. Nevertheless, DFI results of different CANfrag kit tests cannot be compared due to methodological peculiarities[81][82].

Study Strengths

There are numerous methodological and clinical advantages of the present study that contribute to the high reliability and translational significance of its findings. First, there was an adequately powerful sample size consisting of 50 paired semen samples, which allowed for making within-subject comparisons while decreasing the inter-individual variability. Second, there were very strict inclusion and exclusion criteria according to the World Health Organization (WHO) guidelines, which helped to select proper study samples. Third, in addition to assessing sperm DNA fragmentation, the study conducted a comprehensive evaluation of conventional semen parameters such as sperm concentration, progressive motility, and morphology, and thus, provided a broader assessment of sperm quality after microporous membrane separation. All samples in this study were processed through a standardized procedure under laboratory conditions, which contributed to the consistency and reproducibility of the experiment. Paired statistical analysis was used for accounting for within-subject variability, which allowed to increase the robustness of comparative results. One more significant advantage of the present research is the assessment of the CANezy Microporous Membrane Device, a commercial product, a sperm separation system that does not require any centrifugation and can be applied in clinical practice due to its simplicity, affordability, and easy implementation in ART laboratories. Lastly, the application of CANfrag kit assay, a standardized, cost-effective, and clinically feasible method of evaluating sperm DNA fragmentation, increases the translational significance of the study.

Future Research Directions

Results obtained in the current study can become a ground for future research concerning clinical use and mechanisms of action of sperm selection by means of microporous membrane. Future investigations are supposed to include well-designed

randomized controlled studies aimed at comparison of the CANezy Microporous Membrane Device with well-established sperm preparation methods, such as density gradient centrifugation and swim-up procedure. Future studies are expected to estimate such relevant endpoints as fertilization rate, embryo quality, implantation, clinical pregnancy, miscarriage, and delivery in order to find out whether the improvements in sperm quality lead to better ART results.

Another direction for future investigation is the identification of the patient populations that may benefit most from this innovative technology. The stratified analyses of patients who have high baseline levels of sperm DNA fragmentation, recurrent pregnancy loss, unsuccessful attempts of ART treatment, advanced paternal age, varicocele or other male-factor infertility will allow personalizing the sperm selection technique. Along with that, the mechanism of this improvement needs to be studied and clarified. In order to achieve this goal, further research needs to include the evaluation of the biomarkers related to the oxidative stress, such as reactive oxygen species (ROS), 8-hydroxy-2'-deoxyguanosine (8-OHdG), malondialdehyde, as well as the markers of sperm chromatin integrity, including protamine 1/protamine 2 ratio, histone retention, mitochondrial membrane potential, ATP production, and apoptotic processes. The use of more sophisticated molecular biology techniques, such as epigenomics, transcriptomics, proteomics, and metabolomics, can help to reveal unique molecular characteristics of the selected sperm.

Future research is also expected to include the comparative effectiveness studies of the microporous membrane technology along with some other sperm selection procedures, like microfluidics, MACS, and PICSI. Furthermore, the multicenter studies to standardize the laboratory protocols, especially sperm DNA fragmentation assessment based on CANfrag kit assay, are necessary. Further research on follow-up studies of perinatal effects, congenital malformations, child development, and epigenetic effects of offspring conceived using microporous membrane sperm selection is required to ensure that the technique is safe. In addition, there needs to be research into combining microporous membrane sperm selection with other precision reproductive techniques, including artificial intelligence-based sperm morphology evaluation and multi-omic analyses. Health economic assessments comparing device costs, workflow, efficacy, and overall success of assisted reproductive technology using microporous membrane sperm selection will also be important for understanding whether it is feasible to adopt the technology in clinical practice.

Practical Implementation Considerations

Results from this study indicate the feasibility of integrating sperm selection using microporous membranes in clinical ART laboratories. Implementation of the above technique will require consideration of several issues, some of which are listed in Table 11. The main benefit of the CANezy Microporous Membrane Device is that it is a technique requiring a small amount of infrastructure since the procedure can be completed with a minimal amount of equipment such as 37°C incubator with 5% CO₂, conventional microscopy, haemocytometer, and conventional sperm culture media. The simplicity of the procedure allows quick acquisition of the skills necessary for completing the task for experienced embryology laboratory staff. The total time spent on the procedure is similar to that required by conventional density gradient centrifugation; it includes 30 minutes of incubation as well as additional time for preparation and sperm recovery. In addition, the best results in terms of post-separation parameters are likely to be obtained with samples having sperm concentration of $\geq 5 \times 10^6$ spermatozoa/mL and progressive motility of at least 20%; otherwise, poor-quality semen samples will produce a small number of post-separation spermatozoa. Laboratory quality assurance should involve evaluation of post-separation progressive motility, improved sperm morphology, and sperm DNA fragmentation where available. From an operational standpoint, the microporous membrane device provides several practical benefits regarding storage and handling, since the devices are stable at room temperature, whereas the culture media associated with them have standard cold storage requirements (2-8°C). It is also important to make sure before implementing the method clinically that all necessary regulations are complied with and that the device is approved by the corresponding national/regional regulators.

Appropriate counseling of patients is an integral part of clinical application. Patients should be made aware of the fact that microporous membrane sperm separation has the potential to increase sperm motility, morphology, and chromatin structure quality; nevertheless, these laboratory results do not mean that fertilization will definitely occur successfully, pregnancy will result or live birth will occur since there are several male and female reproductive factors influencing outcomes. Based on the present research and available literature, this technique can be useful in case of patients having increased baseline sperm DNA fragmentation, recurrent pregnancy loss, repetitive ART failures, poor embryo quality or any other condition when improved sperm selection can help to enhance the quality of the sperm available for assisted reproduction. Summary of practical issues

regarding laboratory application is provided in Table 11. For clinics considering adoption of microporous membrane-based sperm selection, several practical factors warrant consideration.

Table 11. Practical implementation considerations for microporous membrane sperm selection

Factor	Details
Equipment	Minimal requirements: standard incubator (37°C, 5% CO ₂), basic microscopy, hemocytometer, culture media
Training	Simple protocol; existing embryology staff require <2 hours training
Processing time	30 minutes incubation (comparable to DGC), plus 10 minutes setup and collection
Sample suitability	Optimal: concentration ≥5 million/mL, baseline motility ≥20%. Suboptimal samples may yield lower final counts
Quality control	Monitor post-separation motility (target >85%), morphology improvement, and DFI if available
Cost per cycle	Device cost [varies by region]; compare with centrifuge tubes, gradient medium, and technician time for DGC
Storage	Devices shelf-stable at room temperature; culture media refrigerated (2–8°C)
Regulatory	Verify device regulatory approval status in local jurisdiction (FDA, CE mark, local health authority)
Patient counseling	Explain biological improvements (motility, morphology, DNA integrity) while avoiding guaranteed pregnancy claims
Clinical indications	Consider for: high baseline DFI (>30%), recurrent pregnancy loss, repeated ART failure, poor embryo quality

Table 12. Comparison of sperm preparation methods: technical characteristics

Characteristic	CANezy	DGC	Swim-up	Microfluidic
Processing time	30 min	45–60 min	60–90 min	20–30 min
Centrifugation	No	Yes (2–3×)	Yes (1×)	No
Equipment cost	Low	Low	Low	High
Technical complexity	Low	Medium	Medium	Medium-High
Sample volume needed	0.5–1 mL	1–2 mL	2–3 mL	0.1–0.5 mL
Final sperm yield	Moderate	High	Low-Moderate	Low
DFI reduction	70.5%	30–40%	35–45%	40–60%
ROS exposure	Minimal	High	Moderate	Minimal
Operator dependency	Low	Medium	Medium	Low

Note: DGC = density gradient centrifugation; ROS = reactive oxygen species; DFI = DNA fragmentation index. Values represent approximate ranges from published literature and current study.

5. CONCLUSION

The study clearly proves that sperm separation via microporous membranes, employing CANezy, substantially reduces sperm DNA fragmentation index by 70.5% while at the same time increasing progressive motility and sperm morphology. In this

regard, the CANfrag kit assay technique is very effective in shifting the majority of samples from moderate DNA fragmentation to the normal range. The application of biomimetic separation method and avoiding the stress due to the centrifugation procedure results in a highly efficient and rather soft method for selecting functionally competent sperm cells with intact chromatin. Simple and inexpensive process makes the use of the technique possible in clinic. However, although the above biological effects of the method are very promising, randomized controlled trials should be conducted in order to prove the link between the reduction of DNA fragmentation and such parameters as fertilization rate, embryo quality, pregnancy rates and live births. Attention should be paid to patients with high DNA fragmentation, recurrent pregnancy loss and multiple failures of ART procedures. Thus, microporous membrane-based sperm selection may become a good option for the management of male infertility and ART procedures optimization.

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AUTHOR CONTRIBUTIONS

Dr. Edwin Pithawala: Conceptualization, Investigation, Writing—Review & Editing.

Dr. Edwin Pithawala and Dr. Dhruvil Brahmbhatt: Methodology, Laboratory Analysis.

Dr Edwin Pithawala and Prof. Jolly Shah: Data Curation, Formal Analysis.

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CONFLICT OF INTEREST

The authors declare no competing interests. The kit (CANfrag) was procured from Candore Biosciences, Santej, Gujarat.

ETHICAL APPROVAL

Ethical approval was not required for this study.

INFORMED CONSENT

Written informed consent was obtained from all individual participants included in the study.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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