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Sequencing Matters: Comparative Effectiveness of Density-Gradient Centrifugation and MACS for Enhancing Sperm Quality and DNA Integrity

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Abstract

This systematic review of 25 original studies examined the efficacy of density gradient centrifugation (DGC) combined with magnetic-activated cell sorting using Annexin V (MACS) for improving sperm quality in infertile men undergoing ICSI. The evidence consistently demonstrates that DGC-MACS significantly reduces sperm DNA fragmentation, with reported reductions ranging from 2.82% to 21.9% in absolute terms, and relative reductions of 39%-83%. The combination of DGC followed MACS achieved superior outcomes compared to either technique alone, reducing DNA fragmentation index (DFI) 4.1 to ± 1.3% compared with 8.1 ± 4.1% for DGC alone and 7.4 ± 3.9% for MACS alone. The treatment improved sperm motility, membrane integrity and overall spermatozoa health by reducing protamine deficiency and chromosomal abnormalities. Clinical results in ICSI cycles showed that although fertilization rates were similar between the treated and control groups, DGC followed by subsequent MACS treatment significantly improved embryo quality (72.5% vs. 51.47% top-quality day-3 embryos), blastocyst formation rate (69.69% vs. 48%), pregnancy rates (60.7% vs. 51.5%, p=0.014), and live birth rates (47.4% vs. 31.2%, p=0.001) with a reduced miscarriage rate (14.7% vs. 20.6%, p=0.034). The technique proved most beneficial in patients with high baseline DNA fragmentation (≥30%) and in those with asthenozoospermia or asthenoteratozoospermia. Studies suggest, and consistently supported by our study results, that performing DGC before MACS (DGC-MACS) may yield superior results compared to the reverse sequence. This is because DGC leads to a primary separation of sperm based on density, motility, and morphology and thus producing a high-density fractions enriched in morphologically normal sperms. MACS by specifically binding to phosphatidylserine (PS) residues on sperm membranes selectively removes PS-positive/apoptotic sperm in a subsequent purification step. Overall, the literature strongly supports the use of combined DGC-MACS as an effective sperm preparation technique for ICSI in infertile men with elevated sperm DNA fragmentation.

Keywords: semen analysis; male infertility; diagnostic; ART

Methods and Literature Research

We conducted a literature search to identify studies evaluating sperm DNA fragmentation with both pre- and post-treatment measurements. Particular attention was given to differences in the timing and sequence of density-gradient centrifugation (DGC) and use of a MACS Annexin V separation column, and their resulting impact on sperm quality. Randomized controlled trials, controlled trials, cohort studies, before–after studies, and cross-sectional studies in humans published in English were screened and included.

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The aim was to determine which sequence protocol provided superior outcomes when comparing the different approaches, including DGC alone, MACS alone, sequential combinations (DGC followed by MACS or MACS followed by DGC), and protocols incorporating swim-up. The variables investigated included total sperm count (TSC) or concentration, total motile sperm count (TMSC), progressive motility (%), normal morphology, vitality, semen volume, and other baseline semen parameters—particularly baseline sperm DNA fragmentation (SDF/DFI) values (reported as mean ± SD, median, or range)—which were compared with post-treatment values. The analysis included statistical tests (e.g., paired t-test and Wilcoxon signed-rank test), P values for primary comparisons, power analyses or sample size calculations when reported, and corrections for multiple comparisons when applicable.

For each sample, total sperm count (TSC), total motile sperm count (TMSC), and SDF before and after treatment were evaluated blindly. Finally, fertility outcomes obtained using different procedural approaches were compared. Unpublished findings from our own dataset were evaluated alongside previously published data.

Statistical Analysis:

Data were extracted on statistical methodology and significance testing, including the statistical tests used (e.g., paired t-test, Wilcoxon signed-rank test), P-values for primary comparisons, confidence intervals when reported, and effect sizes or the magnitude of observed differences.

Overview of Sperm Selection Techniques to Reduce DNA Fragmentation

Sperm DNA fragmentation (SDF) is currently regarded as one of the most critical parameters in the evaluation of male infertility, as elevated SDF has been associated with reduced fertilization rates, impaired embryo development, increased miscarriage risk, and lower live birth rates in ART cycles. Therefore, optimization of sperm selection protocols with respect to DNA fragmentation is crucial for improving ART outcomes. A range of techniques is currently employed for this purpose and can be summarized as follows:

A. Density Gradient Centrifugation (DGC) Protocol and Efficacy

The usual DGC method includes layering semen on top of a discontinuous density gradient (often 40% and 80%), then spinning it at 300–500g for 15–20 minutes, which separates motile, normally shaped sperm from debris and dead cells. (1) Although DGC consistently reduces sperm DNA fragmentation relative to unprocessed semen, some samples paradoxically exhibit increased SDF after processing, particularly in men with high baseline SDF or poor semen quality. Thus, DGC reliably improves motility and morphology; however, its ability to reduce SDF is lower than that of the swim-up method in samples with <30% SDF. (1)When combined with MACS, the reduction in DNA fragmentation becomes significantly greater than when the technique is used alone. (2-5)

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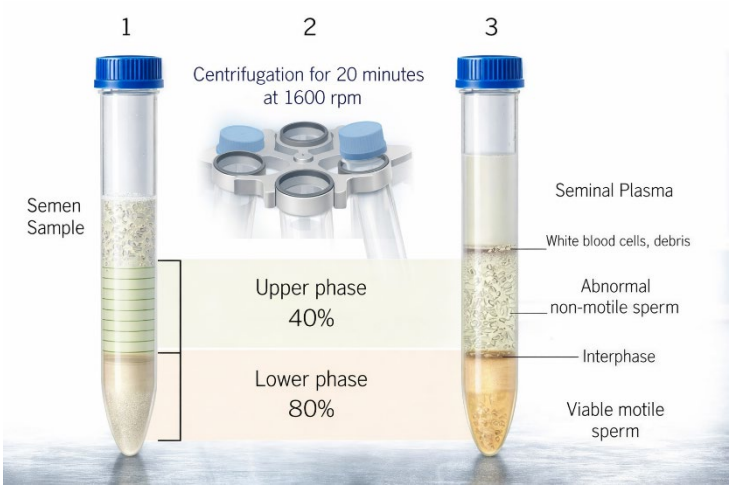

Figure 1. Sperm separation technique by DCG after centrifugation at 1600 rpm (400 g) [picture refined by AI-generated app using Google AI Studio Gemini 3, Nano Banana Pro: 28.12.2025].

B. Swim-Up Technique Protocol and Efficacy

The swim-up procedure is performed by overlaying liquefied semen with culture medium and then incubating it for about 30–60 minutes at 37 °C. Sperm motility allows spermatozoa to swim into the medium, which is then collected. (1, 6, 7)The swim-up method has been reported to yield a higher proportion of motile sperm than DGC, with healthier and less fragmented DNA when applied to samples with a DFI below 30%. (1, 8) However, the percentage that survives after 24 hours is lower in comparison with DGC. (1)Optimized protocols, such as the one-step swim-up/ICSI approach, which reduces, and in some cases even completely eliminates the need for centrifugation, can select a sperm population with near-zero SDF for ICSI. Therefore, such procedure can outperform conventional swim-up technique and in some instances even achieve better results than MACS. (7)

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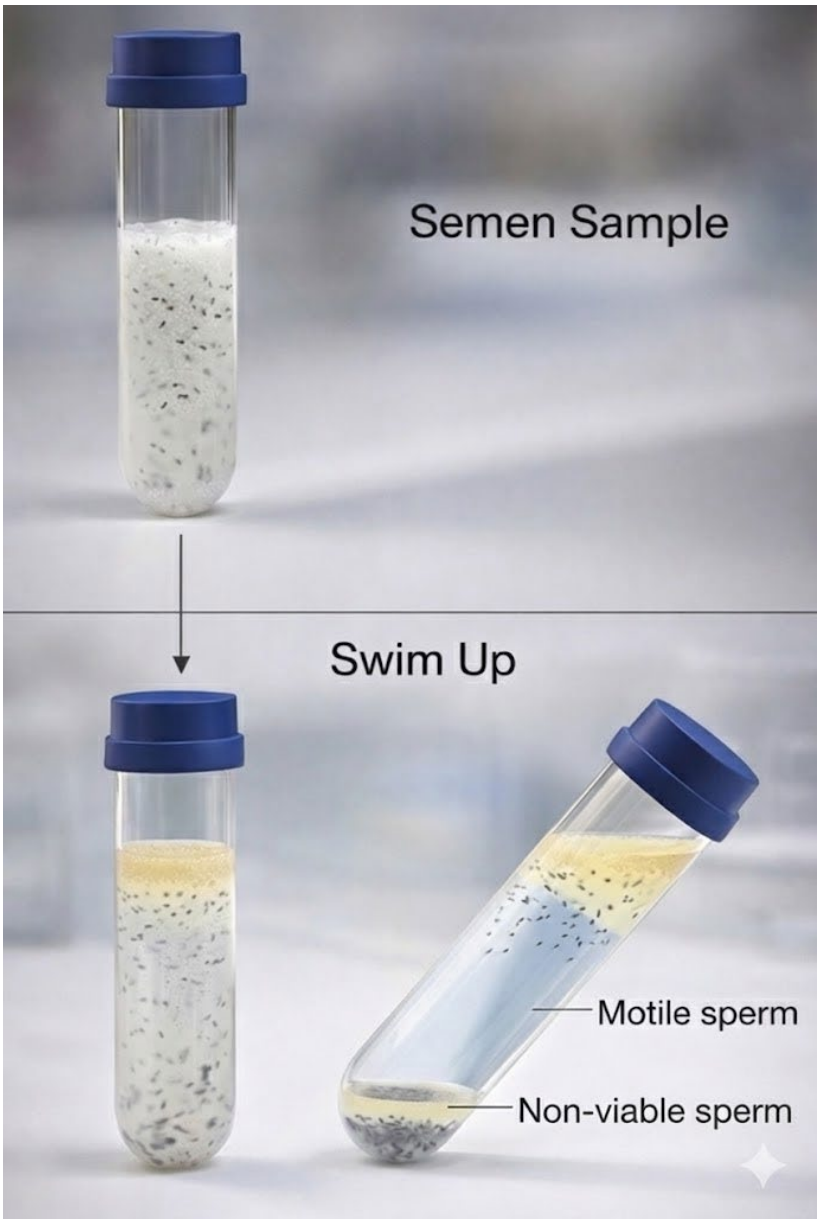

Figure 2. Demonstration for the 45° slope Swim-up technique for semen clearance. [picture refined by AI-generated app using Google AI Studio Gemini 3, Nano Banana Pro: 28.12.2025].

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C. Magnetic-Activated Cell Sorting (MACS) Protocol and Efficacy

MACS uses annexin V-conjugated magnetic beads for the depletion of apoptotic sperm by binding phosphatidylserine-externalizing cells, thereby enriching the final preparation with non-apoptotic, DNA-intact sperm. (2, 9)MACS is most effective in patients with high SDF, and when combined with DGC or swim-up it can further reduce DNA fragmentation beyond the levels achieved by conventional methods. (2, 9)According to some studies MACS is considered most effective in patients with high SDF, and when combined with DGC or swim-up it can further reduce DNA fragmentation beyond the levels achieved by conventional methods. (2, 9)They found that the protocol with greatest reduction in DNA fragmentation is the MACS-DGC-SU sequence, in which MACS is applied to raw semen, followed by DGC and then swim-up. (4)Although this approach provides the most substantial decrease in SDF, it may compromise sperm vitality and motility in samples with poor baseline parameters. (4)Nevertheless, the optimized protocol remains under experts review.

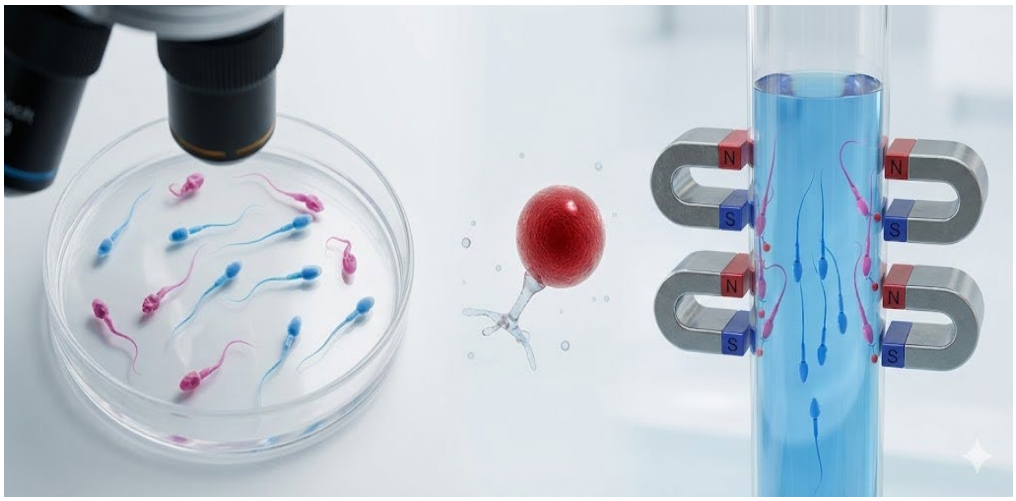

Figure 3. Principle for Magnetic-Activated Cell Sorting (MACS) technique.

Results

Characteristics of Included Studies

This review identified 25 primary studies examining the use of DGC combined with MACS for sperm selection in assisted reproductive techniques as shown in Table 1. Studies varied considerably in design, population characteristics, and outcome measures. The included studies span from 2006 to 2023, with sample sizes ranging from 15 to 724 treatment cycles. Patient ages, where reported, ranged from 24 to 66 years, with male patients typically aged 34-45 years. The specified duration of infertility ranged from 1 to 12 years. Study populations were heterogeneous, including patients with varicocele, oligoasthenoteratozoospermia, teratozoospermia, unexplained infertility, and elevated DNA fragmentation indices.

Table 1. Included studies and their specifications.

Study	Reference	Study Type	Sample Size	Population	Primary Diagnosis
Tavalacee et al., 2012	(5)	Primary study	15 infertile men	Male infertility	Not specified
Degheidy et al., 2015	(10)	Primary study	36 patients	Varicocele patients	Varicocele

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Study	Reference	Study Type	Sample Size	Population	Primary Diagnosis
Sanchez-Martin et al., 2017	(11)	Retrospective cohort	305 couples	High SDF (≥30%)	High DNA fragmentation
Toishibekov et al., 2021	(12)	Primary study	63 patients	Primary infertility	Oligoasthenoteratozoospermia
Novin et al., 2021	(13)	Primary study	30 couples	Male factor infertility	High DFI (>30%)
Zhang et al., 2018	(3)	Primary study	16 patients	Male factor infertility	Asthenozoospermia
Bucar et al., 2014	(4)	Primary study	100 semen samples	Various diagnoses	Teratozoospermia, asthenozoospermia
Chi et al., 2016	(14)	Primary study	458 semen samples	Various diagnoses	Asthenozoospermia, teratozoospermia
Yang, S et al., 2023	(15)	Primary study	16 patients	Poor sperm quality	Asthenozoospermia, teratozoospermia
Pacheco et al., 2020	(2)	Retrospective study	724 cycles	High SDF (>20%)	High DNA fragmentation
Lee et al., 2010	(16)	Primary study	60 couples	Unexplained 12infertility	Unexplained infertility with IUI failures
Delbes et al., 2013	(17)	Primary study	42 patients	Va13rious diagnoses	Normozoospermic, asthenoteratozoospermic, teratozoo…
Fang et al., 2018	(18)	Primary study	Not specified	IVF patients	Various
Mei et al., 2021	(19)	Primary study	86 patients	High DFI (≥30%)	High DNA fragmentation
Notrica et al., 2013	(20)	Primary study	74 couples	Male factor infertility	Teratozoospermia with high DNA fragmentation
Tezcan et al., 2020	(21)	Primary study	17 couples	Unexplained infertility	Unexplained infertility
Esbert et al., 2017	(22)	Prospective study	16 males	Abnormal FISH	Chromosomal abnormalities
Berteli et al., 2017	(23)	Primary study	Not specified	Not specified	Various
Said et al., 2006	(24)	In vitro model	35 samples	Not specified	Various
Ziarati et al., 2019	(25)	Prospective RCT	62 semen samples	ICSI candidates	Male infertility
Çakar et al., 2016	(26)	Primary study	20 donors	Normozoospermic, oligozoospermic	Oligozoospermia
Bibi et al., 2023	(27)	Primary study	385 couples	Isolated teratozoospermia	Teratozoospermia
Merino-Ruiz et al., 2019	(28)	Experimental study	92 couples	IVF/ICSI patients	Various factors
El Fekih et al., 2022	(29)	Experimental study	6 men (cryopreserved sperm samples)	infertile male	Not mentioned, high sperm DNA fragmentation

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Study	Reference	Study Type	Sample Size	Population	Primary Diagnosis
Bibi R et al., 2023	(27)	Prospective study	385 couples	Infertile couples undergoing ART/ICSI;	isolated TZS (allocation to 4 sperm prep methods)
Falquet Guillem M et al., 2025	(9)	Systematic review & meta-analysis	41 studies	comparing MACS vs conventional sperm selection in MAR	Male infertility / high sperm DNA fragmentation

Table 1 Notes: The included studies span from 2006 to 2023, with sample sizes ranging from 15 to 724 treatment cycles. Patient ages, where reported, ranged from 24 to 66 years, with male patients typically aged 34-45 years. The duration of infertility ranged from 1 to 12 years. Study populations were heterogeneous, including patients with varicocele, oligoasthenoteratozoospermia, teratozoospermia , unexplained infertility, and elevated DNA fragmentation indices. The study (9) is a SRMA, the (12) presented as a congress proceeding and (21) available only in non-English language, evaluated teratozoospermia (TZS).

Treatment Protocols

Studies employed various combinations and sequences of DGC and MACS procedures, as shown in Table 2.

Table 2. Treatment protocols.

Protocol Type	Studies	Key Details
DGC-MACS (DGC first)	Degheidy et al.(10); Sánchez-Martín et al.(11); Toishibekov et al.(12); Zhang et al.(3); Chi et al.(14); Pacheco et al.(2); Lee et al.(16); Delbes et al.(17); Esbert et al.(22); Said et al.(24); Bibi et al.(27); Merino-Ruiz et al. (28)	DGC followed by MACS selection
MACS-DGC (MACS first)	Tavalacee et al.(5); Novin et al.(13); Fang et al.(18); Ziarati et al.(25); Tezcan et al. (21)	MACS before DGC
Combined with swim-up	Bucar et al.(4); Mei et al. (19)	DGC-MACS-SU or MACS-DGC-SU sequences
Comparative protocols	Berteli et al.(23); Çakar et al. (26)	Multiple protocol comparisons

Different DNA Fragmentation Assessment Methods were applied across the studies, including the TUNEL assay(2, 4, 17, 20, 21, 26), the SCSA (Sperm Chromatin Structure Assay)(12), the SCD (Sperm Chromatin Dispersion) technique (13) (13, 27)(27), and the Halosperm method(14).

Results

All included studies demonstrated a significant reduction in DFI. The primary outcome across the literature was the extent of sperm DNA fragmentation reduction achieved through DGC-MACS or MACS-DGC treatment, as outlined in Table 3. The data demonstrate consistent reductions in DNA fragmentation following combined DGC-MACS treatment. The magnitude of reduction varied substantially across studies, ranging from modest improvements of 2.82% to substantial reductions exceeding 20 percentage points. Among studies reporting percentage change, reductions in DNA-fragmented spermatozoa ranged from 39.12% to 83.3%.

Chi et al. reported that while DGC alone reduced DFI from 11.5% to 8.1% and MACS alone reduced it to 7.4%, the combination of DGC and MACS achieved the lowest sperm DFI at 4.1%. (14) This synergistic effect was statistically significant ($p < 0.05$).

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Notably, the sequence of procedures appeared to influence outcomes. Bucar et al. demonstrated that the MACS-DGC-and swim-up (SU) protocol achieved the highest reduction rate (83.3%), while DGC-SU-MACS showed the lowest efficacy (53.8%). (4)

DFI was evaluated in 100 semen samples using various sequential processing protocols, and an overall reduction was observed, as follows: DGC-SU (73.4%), DGC-MACS-SU (78.9%), DGC-SU-MACS (53.8%) and MACS-SU (73.5%). A significant decrease in sperm DNA fragmentation was observed, but the highest reduction rate was obtained with MACS-DGC-SU protocol with (83.3%).(4) This finding was supported by Tavalaee et al. in his study from fifteen infertile men who were divided into three separate fractions: control, DGC, and MACS. (5)They proposed MACS-DGC rather than DGC-MACS for clinical implementation based on superior separation of active caspase-positive cells. (5)In fact, during capacitation spermatozoa can externalize PS without being apoptotic and without caspase activation. This PS exposure is physiological, transient, and does not indicate apoptosis.

Table 3. Effects on DFI in different sequence protocols for DGC and MACS or SU treatments. percentage points reported as [pp] for absolute reduction calculation, whereas the relative reduction reported in %. Teratozoospermia (TZS); Sperm chromatin dispersion (SCD).

Study (arm)	DFI assay	Pre-treatment DFI (%)	Post-treatment DFI (%)	Absolute Reduction (pp)	% Reduction	Statistical Significance	Notes
Tavalaee et al., 2012—DGC	TUNEL	29.72±3.41	21.27±3.47,	8,45	28,4%	p<0.05 vs control	N= 15 semen sample, grouped in OAT (n=3), OA (n=2), asthenozoospermic (n=3) and normozoospermic (n=7)
Tavalaee et al., 2012—MACS	TUNEL	29.72±3.41	21.72±3.41	8,00	26,9%	p<0.05 vs control	
Tavalaee et al., 2012—DGC→MACS	TUNEL	29.72±3.41	17.63±3.72	12,09	40,7%	p<0.05 vs control	
Tavalaee et al., 2012—MACS→DGC	TUNEL	29.72±3.41	15.27±3.49	14,45	48,6%	p<0.05 vs control	
Degheidy et al., 2014—DGC→MACS	TUNEL	12,43	9,61	2,82	22,7%	p<0.05	Pre = post-DGC aliquot before MACS; Post = after MACS.
Toishibekov et al., 2021—Annexin− fraction	SCSA (DFI)	32,40	10,50	21,90	67,6%	p<0.01 (vs original)	Pre = raw semen; Post = Annexin V− after processing (double DGC + MACS).
Chi et al., 2016—DGC	SCD (Halosperm)	11,50	8,10	3,40	29,6%	p<0.05 vs control	Pre = raw semen control.
Chi et al., 2016—MACS (Annexin−)	SCD (Halosperm)	11,50	7,40	4,10	35,7%	p<0.05 vs control	Pre = raw semen control.
Chi et al., 2016—DGC+MACS	SCD (Halosperm)	11,50	4,10	7,40	64,3%	p<0.05 vs control; lower than DGC or MACS alone	Pre = raw semen control.

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Study (arm)	DFI assay	Pre-treatment DFI (%)	Post-treatment DFI (%)	Absolute Reduction (pp)	% Reduction	Statistical Significance	Notes
Zhang et al., 2017—DGC	TUNEL	9,56	5,25	4,31	45,1%	p<0.05 vs control	Pre = unprocessed control.
Zhang et al., 2017—DGC+MACS	TUNEL	9,56	2,75	6,81	71,2%	p<0.05 vs control and vs DGC; overall p<0.01	Pre = unprocessed control.
Tezcan et al., 2020—DG→MACS+DG	TUNEL	80,12	41,00	39,12	48,8%	p<0.01	Both values are post-processing (DG vs MACS+DG); raw baseline not reported.
Berteli et al., 2017—DGC	TUNEL	24,00	10,00	14,00	58,3%	processed groups differ (p<0.05)	Values are medians; baseline not part of processed-group comparison
Berteli et al., 2017—DGC→MACS	TUNEL	24,00	6,00	18,00	75,0%	processed groups differ (p<0.05)	Values are medians; baseline not part of processed-group comparison
Berteli et al., 2017—MACS→DGC	TUNEL	24,00	4,00	20,00	83,3%	processed groups differ (p<0.05)	Values are medians; baseline not part of processed-group comparison
Berteli et al., 2017—MACS	TUNEL	24,00	8,00	16,00	66,7%	processed groups differ (p<0.05)	Values are medians; baseline not part of processed-group comparison
Bucar et al., 2014—DGC→SU	TUNEL	4,30	1,10	3,20	74,4%	T0 vs T1: p<0.05	T0=raw semen; T1=after DGC+SU; n=20;
Bucar et al., 2014—DGC→MACS→SU	TUNEL	5,00	1,00	4,00	80,0%	T0 vs T1: p<0.05	T0=raw semen; T1=after DGC+MACS+SU; n=20;
Bucar et al., 2014—DGC→SU→MACS	TUNEL	8,20	4,20	4,00	48,8%	T0 vs T1: p<0.05; less efficient vs other	T0=raw semen; T1=after DGC+SU+MACS; n=20;

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Study (arm)	DFI assay	Pre-treatment DFI (%)	Post-treatment DFI (%)	Absolute Reduction (pp)	% Reduction	Statistical Significance	Notes
						groups: p<0.05	
Bucar et al., 2014—MACS→DGC→SU	TUNEL	5,50	1,10	4,40	80,0%	T0 vs T1: p<0.05	T0=raw semen; T1=after MACS+DGC+SU; n=20;
Bucar et al., 2014—MACS→SU	TUNEL	4,30	1,20	3,10	72,1%	T0 vs T1: p<0.05	T0=raw semen; T1=after MACS+SU; n=20;
Bibi et al., 2023—DGC	SCD	20,90	14,70	6,20	29,7%	Post-prep comparison p=0.01;	TZS Baseline SDF comparable across groups (p=0.68) DGC-MACS lower than DGC & SU
Bibi et al., 2023—SU	SCD	23,10	14,50	8,60	37,2%	Post-prep comparison p=0.01;	DGC-MACS lower than DGC & SU
Bibi et al., 2023—DGC-SU	SCD	25,15	14,20	10,95	43,5%	Post-prep comparison p=0.01;	DGC-MACS lower than DGC & SU
Bibi et al., 2023—DGC-MACS	SCD	25,30	12,30	13,00	51,4%	(ANOVA p=0.01; Tukey)	Lower than DGC & SU after preparation

Aside direct DFI changes in sperms—see Table 3 -additional effects after treatments have been investigated for several semen parameters, including motility or the concentration of live spermatozoa, as shown in Table 4.

The DGC-MACS procedure consistently improved sperm motility and viability parameters. Toishibekov et al. demonstrated a significant increase in motility from 32.7% to 47.2% in the annexin-negative fraction ($P < 0.003$). (12)Zhang et al. showed that DGC-MACS could select viable spermatozoa from completely immotile populations, increasing the proportion of live spermatozoa from 65.88% to 85.81%. (3)

However, some studies reported potential trade-offs. Çakar et al. observed a significant loss in total and rapid progressive spermatozoa when an additional MACS procedure was applied, raising concerns about sperm recovery rates, particularly in oligozoospermic patients. (26)The authors included only a small number of semen samples obtained from normozoospermic (n=10) and oligozoospermic (n=10) men. Each man's single semen sample was per protocol split into four aliquots and processed in parallel as: SU, DG, SU+MACS, and DG+MACS. The subsequent analyses investigated the outcome for sperm motility, morphology, DNA integrity. As expected, sperm processing reduced sperm concentration. In normozoospermic samples, total sperm concentration decreased by 50.9%–80.7%, while in oligozoospermic samples it decreased by 35.4%–74.0% (relative to fresh). Similarly, rapid progressive sperm concentration declined across all methods, ranging from −52.6% to −87.2% in normozoospermic samples and −37.5% to −99.2% in oligozoospermic samples. Overall, the addition of MACS did not demonstrate a consistent benefit and was associated with depletion of total/rapid progressive sperm; the only reported SU+MACS vs SU significance was a reduction in normal morphology in the oligozoospermic group. Yet, because the added MACS step

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after SU reached statistical significance only in the oligozoospermic group, its clinical usefulness in this setting is debatable. Likewise, DG alone produced a more pronounced decline in post-treatment motility in this cohort than the DG + MACS protocol. As a result, the cost–benefit balance of incorporating MACS into routine practice was called into question, and its use was recommended primarily for samples with higher sperm concentrations.

Table 4. Effects on Sperm Parameters.

Study	Parameter		Pre-treatment	Post-treatment	Change
Toishibekov et al., 2021	Motility		32.7 ± 5.9%	47.2 ± 6.3%	+14.5%
Zhang et al., 2018	Live spermatozoa		65.88 ± 12.77%	85.81 ± 5.2%	+19.93%
Zhang et al., 2018	Membrane integrity		52.5 ± 12.21%	81.81 ± 5.29%	+29.31%
Esbert et al., 2017	Progressive motility		Baseline	Sign. increased	P < 0.001
Salehi Novin et al., 2023	Progressive motility		Not reported	Sign. improved	Sign. increase
Salehi Novin et al., 2023	Normal morphology		Not reported	Sign. improved	Sign. increase
Cakar Zeynep at al,. 2016	Sperm concentration in NZS	SU	43.0±21.3	21.1±11.9	−50.9%
		SU+MACS		8.3±6.1	−80.7%
		DGC		20.0±11.2	−53.5%
		DGC+MACS		12.1±7.6	−71.9%
	Sperm concentration in OZS	SU	9.6±3.8	3.8±2.9	−60.4%
		SU+MACS		2.5±2.1	−74.0%
		DGC		6.2±2.8	−35.4%
		DGC+MACS		3.5±3.4	−63.5%
Cakar Zeynep at al,. 2016	Rap. prog. motility in NZS	SU	19.6±14.3	9.3±6.9	−52.6%
		SU+MACS		4.1±2.7	−79.1%
		DGC		6.0±4.5	−69.4%
		DGC+MACS		2.5±1.8	−87.2%
	Rap. prog. motility in OZS	SU	2.4±0.8	1.4±1.0	−41.7%
		SU+MACS		0.02±0.04	−99.2%
		DGC		0.9±0.8	−62.5%
		DG+MACS		1.5±2.5	−37.5%

Legend: Normozoospermia (NZS); Oligozoospermia (OZS), density gradient centrifugation sperm selection (DCG); MACS: magnetic-activated cell sorting (Annexin V–based sperm selection); swim up (SU).

Effects on Protamine Deficiency and Chromatin Structure

Protamines replace most histones in late spermiogenesis and compact sperm DNA into highly condensed toroidal structures, achieving much tighter chromatin packaging than in somatic cells. (30)Conversely, protamine deficiency impairs proper chromatin condensation and is associated with abnormal sperm morphology, reduced motility, and increased DNA fragmentation—findings typically observed in samples with poorer semen quality. (31, 32)Many clinical studies report higher proportions of protamine-deficient sperm in subfertile or infertile men, and meta-analyses indicate a strong association between protamine deficiency, increased sperm DNA damage, and male infertility. (32)

Therefore, strategies that can reduce these alterations may offer clinical benefits in the treatment of male infertility, and the most effective processing technique has to be established.

Chi et al. reported that the combination of DGC and MACS significantly reduced protamine deficiency rates from baseline to 1.6 ± 1.1% compared with 4.4 ± 3.2% after DGC alone and 3.4 ± 2.2% after MACS alone. (14)This is consistent with the observed reduction in DFI, which shows a

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significant negative correlation with sperm motility ($r = -0.347$, $p < 0.001$) and morphology ($r = -0.114$, $p < 0.05$). Delbes et al. observed that DGC selected for more mature spermatozoa with high DNA compaction, and Annexin-V MACS allowed enrichment of spermatozoa with good chromatin quality as measured by TUNEL and SCSA. (17)Raw asthenoteratozoospermic and teratozoospermic samples had higher proportions of spermatozoa containing DNA breaks compared to normozoospermic samples underscoring the benefit of implementation by such techniques, particularly for those men with more severe infertility.

Indeed, our observations are supporting these findings. Spermatozoa exhibiting protamine deficiency and enriched in histones—as indicated by the aniline blue test—are not eliminated as effectively as fragmented spermatozoa, since they are not are not apoptotic and can therfore"escape" MACS selection. The feature of MACS consists in eliminating those sperms with higher externalization of PS (early/late apoptosis) which are typically the ones with high DNA fragmentation and high rates of numerical chromosomal abnormalities. This discrepancy arises because the MACS kit does not target their removal unless these protamine-deficient spermatozoa also present apoptotic and fragmented features, rendering them detectable by annexin V. Consequently, this leads to a greater persistence of these cells in the inseminating sample.

Since MACS mainly affects apoptotic/fragmented sperms, rather than non-apoptotic protamine-deficient sperms, any observed decrease in aniline blue–positive cells or chromatin immaturity in adapted DGC-MACS protocol is likely due to a selection effect of DGC (and any additional swim-up), rather than the MACS step itself. Therefore, the observed improvement in chromatin quality is likely attributable solely to the density gradient effect, supporting the use of initial DGC treatment and, in some cases, additional MACS. Microfluidic methods can effectively lower both DNA fragmentation and protamine deficiency.

Consequently, in the authors' opinion, MACS pretreatment alone may be suitable for TESE or microTESE samples to decrease DFI levels, as it offers good recovery rates and can also be applied to immotile spermatozoa. This conceptual protocol has implications in the direct postinterventional handling of testicular retrieved samples, as explained below.

Effects on Chromosomal Abnormalities

Chromosomal abnormalities become more frequent as semen quality worsens, ranging from about 1% (0.5–2%) in infertile men with normal semen to ~6–7% and ~10–20% chromosomal abnormalities for mild oligozoospermia (≈5–15 million/mL) and severe oligozoospermia (<5 million/mL), correspondingly. (33, 34)

The highest risk of chromosomal aberrations was seen in men with more severe infertility, particularly those with non-obstructive azoospermia(34, 35), these abnormalities can significantly impair spermatogenesis, increase the risk of miscarriage, and be transmitted to offspring. (36)Many of these genetic abnormalities can be identified during the initial pre-analytical workup through blood-based testing, which supports appropriate counselling of the couple. Nevertheless, the key concern is genetic alterations within sperm DNA, and the goal remains to minimize the transmission of genetic abnormalities or aneuploidies in both spontaneous and ART-assisted pregnancies.

Both magnetic-activated cell sorting (MACS) and density gradient centrifugation (DGC) can reduce the number of chromosomal abnormalities in semen. DGC alone is effective in decreasing sperm aneuploidy and chromosomal imbalance, including in carriers of chromosomal rearrangements. Compared with unprocessed semen, DGC is associated with a significant reduction in disomy rates for autosomes and sex chromosomes.(37, 38)

In 2013, Brahem et al. compared native semen with samples after processing trough PureSperm density gradient fractions in 15 fertile men and 30 infertile men with teratozoospermia. (37)Consistent to earlier studies, the teratozoospermic group showed, as expected, a significantly higher rate of chromosomal abnormalities; however, gradient processing reduced aneuploidy frequencies, with a significant reduction in disomy rates for an autosome and for each sex chromosome ($P < 0.001$). (37)

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By using fluorescence in situ hybridization to establish the chromosome segregation spermatozoa pattern Rouen A et al. demonstrated in pre-post analysis of 21 men that DGC technique significantly decreased the proportion of unbalanced spermatozoa in all but 1 of the 21 chromosomal rearrangement carriers ($P < 0.05$).(38). Additionally, MACS, especially in patients with elevated sperm DNA fragmentation, can enhance the selection of spermatozoa with reduced DNA damage and fewer chromosomal imbalances, as apoptotic sperm fractions typically exhibit a higher incidence of chromosomal abnormalities. (9, 22, 29)Esbert et al. demonstrated that MACS columns selectively retained spermatozoa carrying chromosomal abnormalities. (22)Frequencies of aneuploidies in the eluded fraction were significantly lower than in the retained fraction (0.59% vs. 0.75%; $p = 0.010$). This finding suggests an additional benefit of MACS beyond DNA fragmentation reduction. The optimal algorithm for applying these two techniques remains unclear, including whether they should be used in combination and, if so, in which sequence.

Clinical Outcomes

Clinical outcomes showed patterns that were quite similar across studies. While fertilization rates mostly recorded no significant difference between DGC alone and DGC-MACS, downstream outcomes including embryo quality, pregnancy rates as well as live birth rates favored the MACS treated groups.

The largest retrospective analysis (724 cycles) was performed by Pacheco et al. and it brought to light significantly improved pregnancy rates (60.7% vs. 51.5%, $p = 0.014$) with reduced miscarriage rates (14.7% vs. 20.6%, $p = 0.034$) and even higher live birth rates (47.4% vs. 31.2%, $p = 0.001$) in the MACS group. (2)As has been reported by Sánchez-Martín et al., there is no evidence of miscarriages in any cohort of patients following MACS treatment. (11)Mei et al. reported that clinical pregnancy and implantation rates in the first embryo-transfer cycles did not differ significantly between the DGC and swim-up only group (serving as the internal control) and the group in which sperm were prepared using initially MACS followed by DGC and swim-up. However, the MACS group required fewer embryos transferred per oocyte-retrieval cycle (1.7 ± 0.7 vs 2.3 ± 1.6) and fewer transfer cycles per retrieval cycle (1.2 ± 0.5 vs 1.6 ± 0.8) compared with controls. (19)

Table 5. Clinical outcome for different variables.

Study	Outcome Measure	DGC Only	DGC-MACS	Statistical Significance
Salehi Novin et al., 2023	Fertilization rate	73.11%	72.07%	N.s.
	Top-quality embryos (Day 3)	51.47%	72.5%	$P < 0.05$
	Blastocyst rate	48%	69.69%	Significant
Novin et al., 2021	Fertilization rate	73.11%	72.07%	N.s.
	Day 3 good grade embryos	51.47%	72.5%	$P < 0.05$
Pacheco et al., 2020	Fertilization rate	73.3%	75.1%	$P = 0.13$
	Pregnancy rate	51.5%	60.7%	$P = 0.014$
	Miscarriage rate	20.6%	14.7%	$P = 0.034$
	Live birth rate	31.2%	47.4%	$P = 0.001$
Ziarati et al., 2019	Fertilization rate	Control	No significant difference	N.s.
	High-quality embryos	Control	Significantly higher	Significant
	Pregnancy rate	Control	Significantly higher	Significant
Mei et al., 2021	Live birth rate (first cycle)	53.9%	63.2%	Trend toward improvement
	Cumulative live birth rate	70.7%	79.5%	Trend toward improvement

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	Transfer cycles per retrieval	1.6 ± 0.8	1.2 ± 0.5	Significant reduction
Notrica et al., 2013	Fertilization rate (ICSI)	84.2 ± 4.4%	77.4 ± 3.0%	P = 0.4
	Pregnancy rate (ICSI)	42.9%	39.0%	P = 1
Bibi et al., 2023	Pregnancy rate	Lower in other methods	Higher	P < 0.01

IVF Versus ICSI Outcomes

Fang et al. reported differential effects based on the fertilization method (18) In IVF cycles, no significant differences were observed in fertilization rate, embryo quality, implantation rate, pregnancy rate, or live-birth rate between MACS-DGC and DGC alone groups. However, in ICSI cycles, the percentage of high-quality embryos, pregnancy rate, implantation rate, and live-birth rate were all significantly higher in the MACS-DGC group. This suggests that MACS may be particularly beneficial when sperm selection bypasses natural fertilization barriers. The benefit in decreasing chromosomal abnormalities by reduction disomy rates for autosomes and sex chromosomes after DCG or MACS was demonstrated in several studies and could play an important factor to consider for improving clinical outcomes. (22, 37, 38)Conversely, Tezcan et al. concluded that MACS may not be necessary for ICSI protocols, where natural sperm selection does not occur, suggesting that it may be more effective in IUI and conventional IVF applications. (21)

Factors Associated with Treatment Response

Several studies have identified factors predicting response to DGC-MACS treatment, as summarized in Table 5. However, there is conflicting data on the most effective framework and technical settings for achieving the highest fertilization and pregnancy rates, and these findings only partially translate into improved fertilization and ICSI outcomes. Bucar et al. (2015) compared five sperm-processing sequences and found that all protocols significantly reduced TUNEL-measured sperm DNA fragmentation. (4)The greatest mean reduction occurred with MACS–DGC–SU (83.3 ± 15.4%), whereas DGC–SU–MACS was least effective (53.8 ± 24.1%). In the MACS–DGC–SU group, the magnitude of reduction was inversely associated with vitality (r = −0.842), membrane integrity/ HOST (r = −0.799), and progressive motility (r = −0.528), suggesting this sequence may be particularly advantageous in poorer-quality samples. (4)Notably, in the same study, teratozoospermic patients tended to have lower SDF reduction rates than asthenozoospermic and asthenoteratozoospermic patients. (4)Berteli T.S. advocated starting with MACS to minimize handling of damaged sperm and reduce the risk of iatrogenic increases in SDF during later centrifugation steps, which can elevate ROS and promote DNA fragmentation during DGC. (23)Taken together, this sequence therefore combines complementary mechanisms—early removal of apoptotic (annexin V+) sperm with compromised membranes and higher ROS burden, followed by density- and morphology-based enrichment—thereby maximizing the recovery of motile sperm with lower SDF. (23)Said et al. demonstrated that the oocyte penetration rate was negatively correlated with apoptotic marker expression, while sperm chromatin decondensation following ICSI was associated only with apoptosis in sperm with damaged membranes. (24)

Societies guidelines do not endorse any specific order or routine for DGC vs MACS use and there is no study investigating in detail the sequence DGC followed by MACS. Nevertheless, as indirect study results, Tavalaee et al. directly compared DGC-MACS and MACS-DGC and found that combining MACS with DGC improved DNA integrity (TUNEL) and chromatin maturity (CMA3) compared with either method alone; they ultimately favored MACS-DGC, primarily because it yielded a lower proportion of caspase-positive sperm. They further noted that PS externalization may occur during capacitation independently of apoptosis/caspase activation, raising the possibility that initiating selection with MACS before DGC could mitigate depletion of capacitated sperm that might otherwise be lost with a DGC-first approach; however, fertilization outcomes were not evaluated in this study. (5)However, in a retrospective cohort of ICSI cycles involving men with high SDF (>20%

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by TUNEL), Pacheco et al. reported that DGC followed by MACS (n = 366) was associated with higher pregnancy and live-birth rates and a lower miscarriage rate than DGC alone (n = 358), while fertilization rates were similar (75.1% vs 73.3%; p = 0.133). Specifically, the pregnancy rate was higher in the group with DGC followed by MACS (60.7% vs 51.5%; p = 0.014), the miscarriage rate was lower (14.7% vs 20.6%; p = 0.034), and the live-birth rate was higher (47.4% vs 31.2%; p = 0.001)(2). Again, Pacheco et al. did not perform a head-to-head comparison of MACS followed by DGC versus DGC followed by MACS; they compared DGC followed by MACS with DGC alone. In the literature there is one other randomized, prospective study comparing MACS-DGC vs DGC and reporting fertilization, although in this study the fertilization was not significantly different. (25)These findings were similarly reported in a clinical study comparing MACS-DGC vs DGC alone, whereby again there was no difference in the fertilization rate.(39)

To date, no larger randomized head-to-head trial has directly compared DGC followed by MACS with MACS followed by DGC and demonstrated superiority of either sequence for fertilization outcomes.

Table 6. Factors influencing treatment outcomes.

Factor	Findings	Study
Initial sperm quality	Samples with low progressive motility, vitality, and membrane integrity showed best SDF reduction	Bucar et al., 2014; Ventura Bucar et al., 2014
Semen diagnosis	Teratozoospermic patients showed lower SDF reduction rates than asthenozoospermic or asthenoteratozoospermic patients	Bucar et al., 2014
Baseline DFI	Higher baseline DFI associated with greater absolute reduction	Toishibekov et al., 2021
PLCζ expression	Higher PLCz1 expression associated with better treatment outcomes	Salehi Novin et al., 2023
Apoptotic markers	High motility, low caspase-3 activation, MMP integrity predicted good response	Said et al., 2006
Morphology baseline	Patients with values above reference for rapid progressive motility showed higher SDF reduction	Ventura Bucar et al., 2014

Own Study Algorithm and Results

The research was conducted as a prospective, blind observational study. It included a cohort of 67 male patients undergoing evaluation for infertility, with a mean age of 43.16 ± 7.4 years (range: 30-66 years). The primary inclusion criterion for participation was the presence of a pathological level of sperm DNA fragmentation, defined arbitrarily as a baseline value of ≥30% A standardized, two-step sperm preparation technique was applied to all semen samples, as shown in the graph 1. Initially, all samples were processed using a standard DGC technique to separate motile sperm from seminal plasma, debris, and non-motile cells.

The samples were stratified on a discontinuous density gradient (80% and 40%), centrifuged at 400g for 20 min, the supernatant discarded and the sperm pellet washed by centrifugation for 5 min at 500g.

As this step was followed by a MACS preparation, the post-gradient sample was washed with 2 ml of specific binding buffer for MACS. The binding buffer used in the Annexin V MACS kit (Miltenyi Biotech™) is calcium-free because Annexin V requires calcium to bind to PS residues on the sperm membrane. Keeping the buffer devoid of calcium prevents premature or nonspecific binding of Annexin V to PS. Calcium is added only at the appropriate step, ensuring controlled activation of the Annexin V–PS interaction and allowing a more precise removal of PS-positive/apoptotic sperm. Then sperm pellet was resuspended in 100 µl of Annexin V conjugated microbead reagent -allowing a maximal concentration of 10^7 spermatozoa—and 400 µl of buffer were

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added making a final volume of 500ul. Annexin V represents the limiting reagent, and the 100-µL working volume is suitable for staining up to 10^7 spermatozoa. The suspension, incubated for 15 min at room temperature, was finally loaded on a separation column, previously rinsed with 1 ml of binding buffer. The calcium required for Annexin V–PS interaction is provided directly within the Annexin V–conjugated MicroBeads, ensuring that binding occurs only during the controlled incubation step. This design improves the specificity and efficiency of removing PS-positive/apoptotic sperm during magnetic separation. Immature germ cells can also expose PS and therefore bind Annexin V. When present in high numbers, these cells may compete with apoptotic sperm for Annexin V MicroBeads, reducing the availability of binding sites and potentially decreasing the efficiency of the MACS separation.

For this reason, we believe that the sequential use of DGC followed by MACS could be more effective. When placed in a magnetic field, apoptotic sperm with externalized PS bind to the beads are retained, allowing the non-apoptotic fraction to be collected. A calcium-free medium such as the buffer provided in the Miltenyi MACS kit can temporarily reduce the observed progressive motility of the eluted spermatozoa. Calcium is essential for optimal flagellar activation, so progressive motility may appear diminished while the cells remain in a calcium-free environment. This effect is reversible once the sperm are transferred back into a physiological medium containing calcium.

For this reason, the eluted fraction was washed with 2 ml of HTF medium, centrifuged at 500 g for 5 minutes, and the resulting pellet was resuspended in fresh medium prior to analysis. For each patient, sperm DNA fragmentation (SDF) was evaluated using the Halosperm assay (Halotech™) before and after MACS treatment to allow direct comparison. A repeated semen analysis was performed according to the WHO 6th edition at both time points—prior to and following the dual sequential treatment. All assessments were conducted blindly to prevent observational bias.

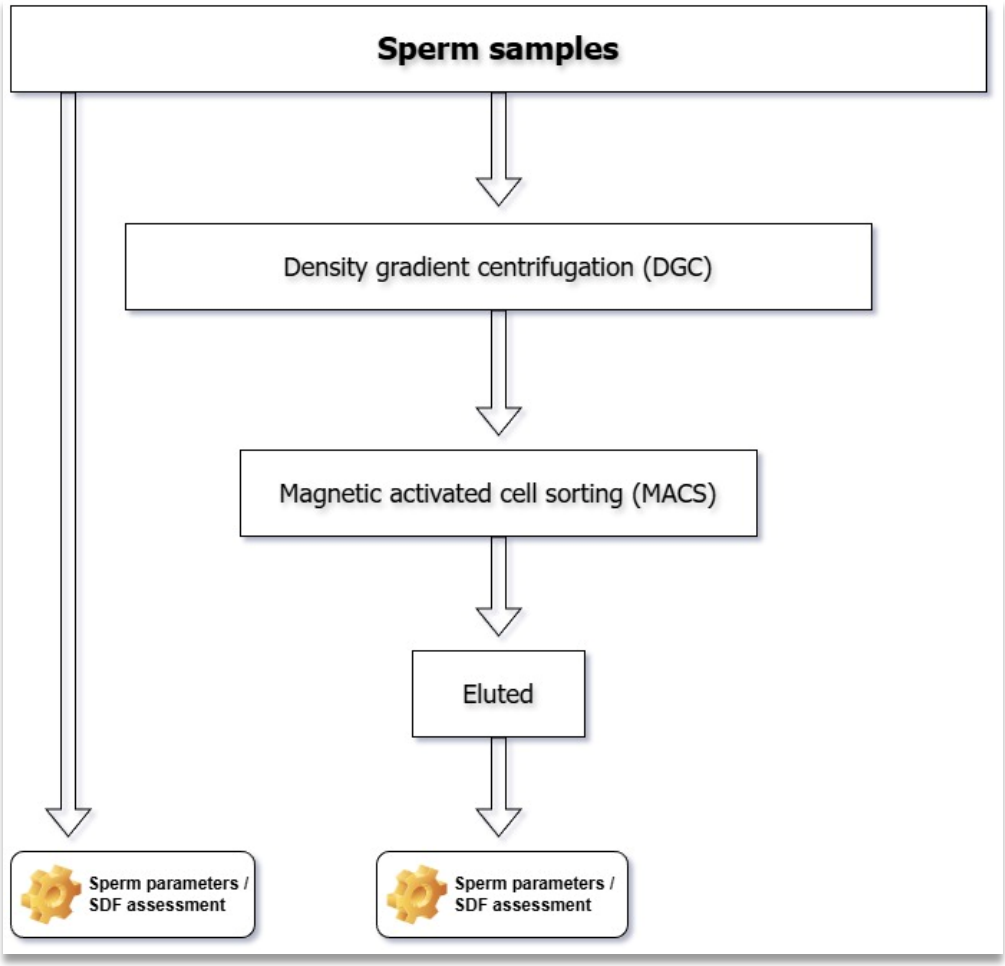

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Figure 4. Study algorithm.

The primary outcome measure was the change in SDF following the combined DGC-MACS treatment demonstrating a significant reduction in sperm DNA damage following the combined DGC-MACS protocol (P<0.0001), as shown in Table 7

Table 7. Sperm DNA Fragmentation (SDF) Before and After DGC-MACS Treatment (n=67).

Parameter	Mean ± S.D.	Range	Median
Native Sperm SDF	41.61 ± 11.2%	30-78%	38%
Post-Treatment Difference	19.42 ± 9.4%	4-43%	19%
Statistical Significance	*P*<0.0001		

As detailed in Table 7 the mean SDF in the native semen samples was 41.61%. After treatment, the mean absolute reduction in damaged cells was 19.42 ± 9.4%. A statistical analysis using the Wilcoxon-paired rank test confirmed that this decrease was highly significant (P<0.0001), underscoring the potent effect of the DGC-MACS intervention.

While the overall effect was positive, the study revealed significant variability in individual patient outcomes. The cohort could be stratified into distinct response groups:

Adequate Responders: The majority of patients (85.07%; 57 out of 67) responded favorably to the treatment. In this group, the DGC-MACS procedure successfully reduced SDF levels to below the 30% cut-off as pathological threshold. The mean SDF difference for this subgroup of responder was substantial with -20.65%± 9.45 (median 20%; range 4-43).

Inadequate Responders: A smaller subgroup of 10 patients (14.92%) did not achieve the desired outcome. Although their absolute SDF levels decreased, the reduction was inadequate, and their post-treatment SDF remained still above the 30% threshold after the dual sequential treatment procedure. This group exhibited a lower mean SDF difference of -12.4%± 5.4 (median 13%; range 5-19).

Exceptional Responders: Interestingly, the study noted that a few cases (6 patients; 8,95%) experienced an almost total elimination of fragmented sperm cells, demonstrating the protocol's potential for profound impact in certain individuals.

To determine if baseline semen quality predicted the degree of SDF improvement, a Linear Regression analysis was performed. The results showed no correlation between the reduction in SDF and the native total sperm count or total motile sperm count. (P>0.1; n.s.). This is an expected finding and consistent with established literature(40, 41), where it has demonstrated that infertile men with normospermia can still exhibit pathologically high levels of SDF. This reinforces SDF as an independent and crucial measure of sperm quality.

In summary, this study reported a mean absolute reduction in SDF of -19.42%. This result aligns robustly with the existing literature. A systematic review of 25 studies found that the absolute reduction in SDF ranged from 2.82% to 21.9%. In relative terms, this represents a 39% to 83% reduction in the number of sperm with damaged DNA. Therefore, the efficacy in SDF reduction after dual procedures—beginning with DGC and followed by MACS—observed in the present study falls comfortably within this established range.

Combining DGC with subsequent MACS significantly improves sperm quality by creating a synergistic selection process that targets different markers of sperm health. While DGC selects for mature spermatozoa with high DNA compaction, MACS utilizes Annexin V-conjugated microbeads to remove apoptotic (dying) sperm cells that exhibit PS externalization. Overall, the mean percentage reduction is approximately 47%—a 19.42% decrease from the baseline value of 41.61%. This estimate aligns well with the broader literature, which reports reductions ranging from 39% to 83%, providing an additional layer of validation. Indeed, the literature also reinforces the value of the combined DGC-MACS approach. Chi et al. similar demonstrated the synergistic effect of this two-step protocol. (14)While DGC alone reduced SDF from a baseline of 11.5% to 8.1% and MACS alone reduced it to

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7.4%, the combined DGC-MACS technique achieved a greater reduction, lowering SDF to just 4.1%. This shows that the combined approach is more effective than either method alone.

Nonetheless, the existing literature is not entirely consistent, with some studies reporting contradictory results. Studies comparing MACS-DGC versus DGC-MACS sequences revealed that MACS performed before DGC (MACS- DGC) may achieve superior outcomes. Tavalaee et al. proposed MACS-DGC for clinical implementation based on higher efficiency in separating active caspase-positive sperm. Bucar et al. demonstrated that MACS-DGC-SU achieved the highest SDF reduction rate (83.3%) compared to DGC-SU-MACS (53.8%). (5) Berteli et al. confirmed that MACS-DGC led to significantly higher percentages of spermatozoa with progressive motility and normal morphology than DGC-MACS.(23) Notably, in this Berteli et al. study, the "calcium-free Miltenyi MACS buffer reduces motility" explanation is unlikely, since the Annexin V binding requires Ca^{2+} in the kit system and thus can temporarily reduce the observed progressive motility of the eluted spermatozoa. Calcium is essential for optimal flagellar activation, so progressive motility may appear diminished while the cells remain in a calcium-free environment. This effect is reversible once the sperm are transferred back into a physiological medium containing calcium. However, the paper does not clearly describe a standardized post-MACS wash/resuspension prior to motility scoring, and the results strongly track with which step was performed last (DGC last = high motility; MACS last = low motility. Therefore, the reported motility advantage of MACS-DGC over DGC-MACS may partially depend on buffer/handling (including how completely binding buffer was removed and what medium sperm were in at the time of assessment), not only on the biological "DGC-induced PS exposure removes functional sperm" mechanism. The more assumable mechanistic explanation for this observation relates to the fact that DGC may trigger membrane changes linked to capacitation/acrosome-related remodeling, which can expose PS on otherwise viable sperm. Since MACS targets PS-positive (annexin V–binding) cells, performing MACS after DGC could inadvertently remove capacitated, functional sperm in addition to apoptotic sperm.

Although this study focused on the laboratory outcome of SDF reduction and did not report on clinical results, the systematic review provides strong evidence of the likely downstream benefits for patients undergoing ART. The literature consistently shows that while fertilization rates are often comparable between DGC-MACS or MACS-DGC treated and control groups, the primary impact of the technique is observed in post-fertilization development. Indeed, clinical improvements had been reported in DGC-MACS sequencing protocol across multiple studies, whereby a significant increase in top-quality day-3 embryos (e.g., 72.5% vs. 51.47%), higher rates of successful development to the blastocyst stage (e.g., 69.69% vs. 48%), higher pregnancy and live birth rates had been shown (e.g., 60.7% vs. 51.5%, p=0.014 and 47.4% vs. 31.2%, p=0.001, respectively). Overall, a significant reduction in the rate of pregnancy loss (e.g., 14.7% vs. 20.6%, p=0.034) was additionally reported. Regarding benefit for boosting sperm motility and DFI, Toishibekov et al. reported that unprocessed semen had motility of 32.7 ± 5.9% and DFI of 32.4 ± 5.9%; after MACS, the annexin-negative fraction showed higher motility (47.2 ± 6.3%) and lower DFI (10.5 ± 3.8%) than the annexin-positive fraction (motility 3.5 ± 2.3%; DFI 67.8 ± 5.9%; $P < 0.003$ for both comparisons). MACS also reduced DFI from 32.4% in the original sample to 10.5% in the annexin-negative fraction ($P < 0.01$), (12)This was considered beneficial for IVF and ICSI cycles when selecting the best spermatozoa.

Population-Specific Considerations

The evidence suggests that DGC-MACS benefits are most pronounced in specific populations:

(A) Patients with baseline high DFI (≥30%): Multiple studies specifically enrolled patients meeting this criterion and demonstrated significant improvements in embryo quality and pregnancy outcomes.

(B) Patients with asthenozoospermia or asthenoteratozoospermia: These populations showed better SDF reduction compared to isolated teratozoospermia.

(C) Patients with immotile but viable sperm: Zhang et al. demonstrated that DGC-MACS could effectively select viable spermatozoa from completely immotile populations.

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(D) Patients requiring cryopreservation: The combination of DGC-MACS prior to cryopreservation enhanced post- thaw sperm quality and reduced DNA fragmentation.

Limitations Affecting Generalizability

Cost-benefit considerations were raised by Çakar et al., who noted significant sperm loss during MACS processing and questioned routine application in all cases. The technique may be more appropriate for patients with adequate sperm concentrations rather than those with severe oligozoospermia.

The lack of standardized protocols across studies—including variations in gradient concentrations, incubation times, and the sequence of procedures—limits direct comparisons.

Summary

The evidence from **25** studies consistently supports the efficacy of combined DGC-MACS treatment for reducing sperm DNA fragmentation and improving sperm quality parameters. However, heterogeneity exists in the magnitude of effects and clinical outcomes across studies.

Our study protocol yielded excellent results when performing a DGC-MACS sequencing process in terms of reduction of SDF and improving semen parameters. This is conciliable when DGC is considered as a coarse sieve that filters out debris and immature cells based on their weight and density, while MACS is like a precision magnet that specifically pulls out “bad apples” (apoptotic cells) that look healthy on the outside but are chemically flagged for disposal. Combining them ensures you have the heaviest, most mature, and chemically healthiest “fruit” for the final selection. Nevertheless, the literature is reporting conflicting data about the best diagnostic approach to improve sperm quality and there are no clear guidelines for the best sequencing algorithm in treating infertile men.

Future developments will focus on optimizing the selection sequence and identifying which subgroups of infertile men benefit most from choosing the appropriate algorithm within these frameworks, with the aim of improving outcomes by reducing the use of spermatozoa exhibiting major alterations or chromosomal aberrations. One interesting research step is focusing on the introduction of additional fluorescence-based microscope when performing MACS or alternatively a flow cytometry alongside MACS; this would require a multiparametric sperm-sorting methodology that combines apoptosis markers with chromatin/epigenetic markers, rather than relying only on annexin V. Technically, this implies either using a fluorescence microscope for “manual” selection or, more powerfully, a flow cytometer/sorter capable of detecting multiple fluorescent probes simultaneously and isolating the desired population in real time. For practical implementation, semen would first be prepared using density gradient centrifugation (DGC), with optional addition of MACS. An aliquot of the processed sample would then be stained using a rapid fluorescent chromatin assay (e.g., CMA3 or an SCSA-derived HDS marker) and examined under an epifluorescence microscope. The resulting chromatin pattern would guide selection, during ICSI, of spermatozoa that are morphologically normal and negative (or only weakly positive) for chromatin immaturity. Conceptually, this approach resembles MSOME, but it relies on fluorescence-based assessment rather than high magnification alone. When available, fluorescence-based flow cytometry equipped with sorting capability and multiple laser/filter configurations for various fluorochromes can rapidly quantify and discriminate populations differing in DNA fragmentation, chromatin maturity, aneuploidy, and apoptosis markers within the same sample, and can be coupled with cell sorting to physically recover the optimal subpopulation.

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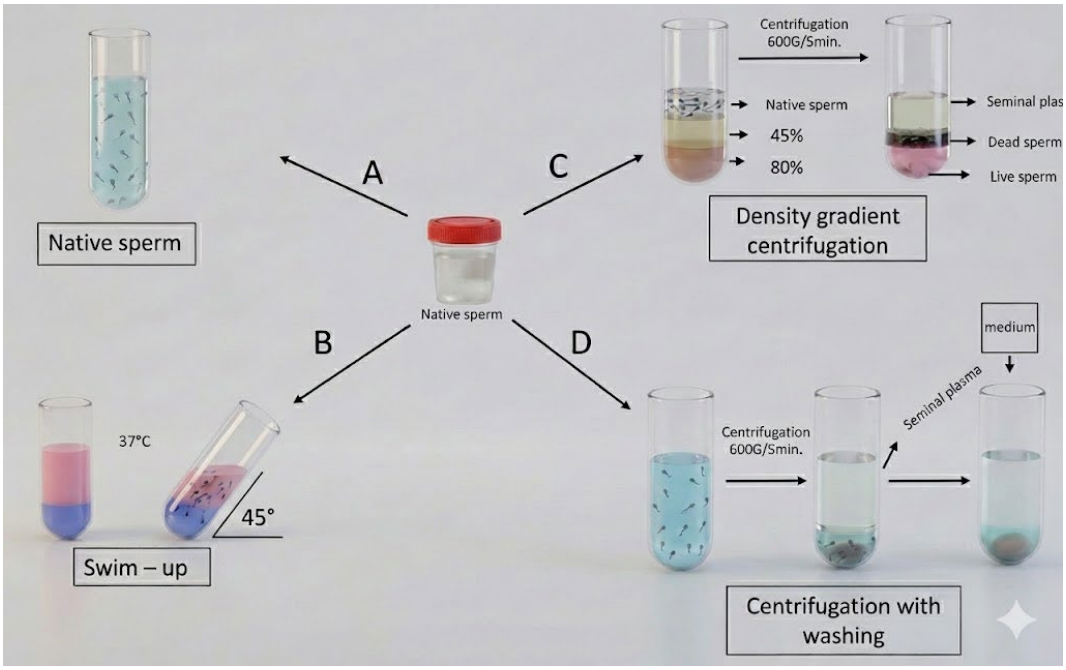

Figure 5. Potential therapeutic options for improving sperm quality.

Author Contributions: GMP: EP GMP: 1) Have made a substantial contribution to the concept and design of the article in writing the original draft, interpretation of data for the article. **2**) Drafted the article critically for important intellectual content; AND. **3**) Approved the version to be published; AND. **4**) 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. **V**EP and EB have made a substantial contribution in reviewing and correcting the manuscript and approved 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.

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Ethical: considerations: **E**thical approval was not required for this study, as it primarily comprises a systematic review of the literature and an analysis of previously collected laboratory data, for which no personal or directly identifiable information was used. No additional study-specific data were collected, and no investigations or procedures were conducted beyond those required for routine clinical care. **F**or all cases in which data were available and included in the analysis, informed consent was obtained from participants for the use of their data in fully anonymized form, without any personal identifiers. Consent was sought through a comprehensive information process, and participation was entirely voluntary.

Consent for Publication: All authors and co-authors have read and carefully reviewed the manuscript and approved its content and the final version for publication.

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