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SYSTEMATIC REVIEW

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Methods and efficacy of processing testicular sperm samples in obstructive and non-obstructive azoospermia: a systematic review

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Abstract

Intracytoplasmic sperm injection (ICSI) is a cornerstone in managing male infertility, especially in obstructive azoospermia (OA) and non-obstructive azoospermia (NOA), necessitating sperm retrieval via testicular sperm extraction (TESE) or microdissection TESE (mTESE). However, the varied post-sperm extraction processing methods pose uncertainty regarding optimal approaches. To address this, a systematic review following preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) guidelines was conducted, identifying 16 relevant studies. These studies exhibited significant heterogeneity in methodologies and outcomes, with mechanical preparation and enzymatic digestion being the primary techniques investigated. Mechanical methods, including shredding, mincing, vortexing and crushing, yielded varying sperm counts per 100 mg of tissue, with mincing showing promise in NOA cases. Enzymatic digestion, particularly with collagenase type IV, also showed effectiveness, though inconsistently. Additionally, techniques such as microfluidics and magnetic levitation showed potential for improving sperm retrieval efficiency. However, the lack of standardization in outcomes and reporting impedes the establishment of best practice protocols. While collagenase type IV with elastase seemed promising for OA samples and microfluidics for NOA cases, further studies with standardized methodologies and outcomes are necessary. Assessment of DNA damage and comparisons of ICSI success rates between processing methods are crucial for informed clinical practice. In conclusion, optimizing sperm quantity and quality for ICSI necessitates standardized methodologies and outcomes, with microfluidics and collagenase type IV with elastase showing promise pending further validation through well-designed studies.

Keywords

Azoospermia; Testicular sperm extraction; Laboratory processing

1. Introduction

For patients with obstructive azoospermia (OA) and non-obstructive azoospermia (NOA), invitro fertilization/intracytoplasmic sperm injection (ICSI) remains important in managing male infertility, with spermatozoa obtained by conventional or microdissection testicular sperm extraction (TESE and mTESE) [1]. Given the low number of spermatozoa extracted from testicular biopsy samples, especially in NOA, laboratories process samples via various methods to increase the sperm yield, lower stress and improve quality (assessed by sperm DNA fragmentation assays) for ICSI [2]. This is especially important because patients invest considerable financial resources into infertility treatments [3, 4], which often leads to financial strain, as some spend nearly 20% of their annual household income on out-of-pocket expenses [3]. Also, male infertility is associated with greater anxiety for patients—overall [5] and around the time of diagnosis and treatment [6]. The psychological stress of male infertility intensifies surrounding treatment failures, when patients experience increased anxiety, persistent sadness, and even depression [6, 7]. However, protocols vary between laboratories, and the optimal method to maximize spermatozoa yield post-sperm extraction processing is not established. In this systematic review, we evaluate the quality and efficacy of published protocols for processing testicular biopsy samples.

2. Materials and methods

The protocol for this systematic review was based on the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P). A comprehensive literature review was performed by systematically searching MEDLINE, EMBASE the Cochrane Library, and ClinicalTrials.gov from

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database inception through April 2024 for all studies reporting protocols for post-sperm extraction processing from database inception. Search strategy was as follows: (TESE OR "testicular sperm extraction" OR microTESE OR mTESE OR "testicular sperm" OR "testicular tissue" OR "testicular biopsy") AND (Purification OR digestion OR mechanical OR density gradient OR enzymatic OR collagenase). A study was included if it assessed sperm processing after sperm retrieval surgery (Fig. 1). All study types except case reports were included.

Three trained investigators (SR, TPK and MFA) independently abstracted the following information using a standardized form. Discrepancies were resolved by discussion and adjudication of a third reviewer (ASH). The review process was registered and published in Prospective Register of Systematic Reviews (PROSPERO) (CRD-register@york.ac.uk) website with registration number "CRD42024556240".

3. Results

Our search identified 611 articles of which 160 were duplicate. The remaining 451 article abstracts were screened, and 410 records were excluded. The full texts of the remaining 41 articles were assessed for eligibility, with 28 articles subsequently excluded due to not meeting inclusion criteria, yielding 16 articles for analysis. Table 1 summarizes the included studies. Overall, the studies were prone to (1) bias due to the lack of laboratory protocol and reporting standards and (2) infrequent use of adequate control cohorts. Given the vast heterogeneity and lack of comparison groups, a meta-analysis was not possible.

3.1 Mechanical preparation

Four studies described the results of sperm extraction processing via mechanical preparation. The efficacy of four mechanical techniques—shredding (the control), fine mincing, vortexing and crushing—was examined [2]. Seventeen patients, of which 14 had OA, underwent TESE with their samples fractioned into four parts. The control specimen was shredded with two glass slides until the seminiferous tubules were broken and unraveled. This manipulation was always carried out as an initial step for the three other procedures. The second fraction was finely minced with two forceps into 1 mm^3 tissue pieces. In the third fraction, shredded samples were vortexed at 2500 rpm for 5 minutes. The fourth fraction was slightly crushed using an electrical potter at 250 rpm for 10 seconds. All fractions were centrifuged at 300 G for 20 minutes in a two-layer Percoll gradient for purification. Post Percoll centrifugation, the mincing technique yielded 35,500 spermatozoa, significantly more than did shredding (24,000), vortexing (26,000) and crushing (electrical potter) (14,500) per 100 mg of tissue.

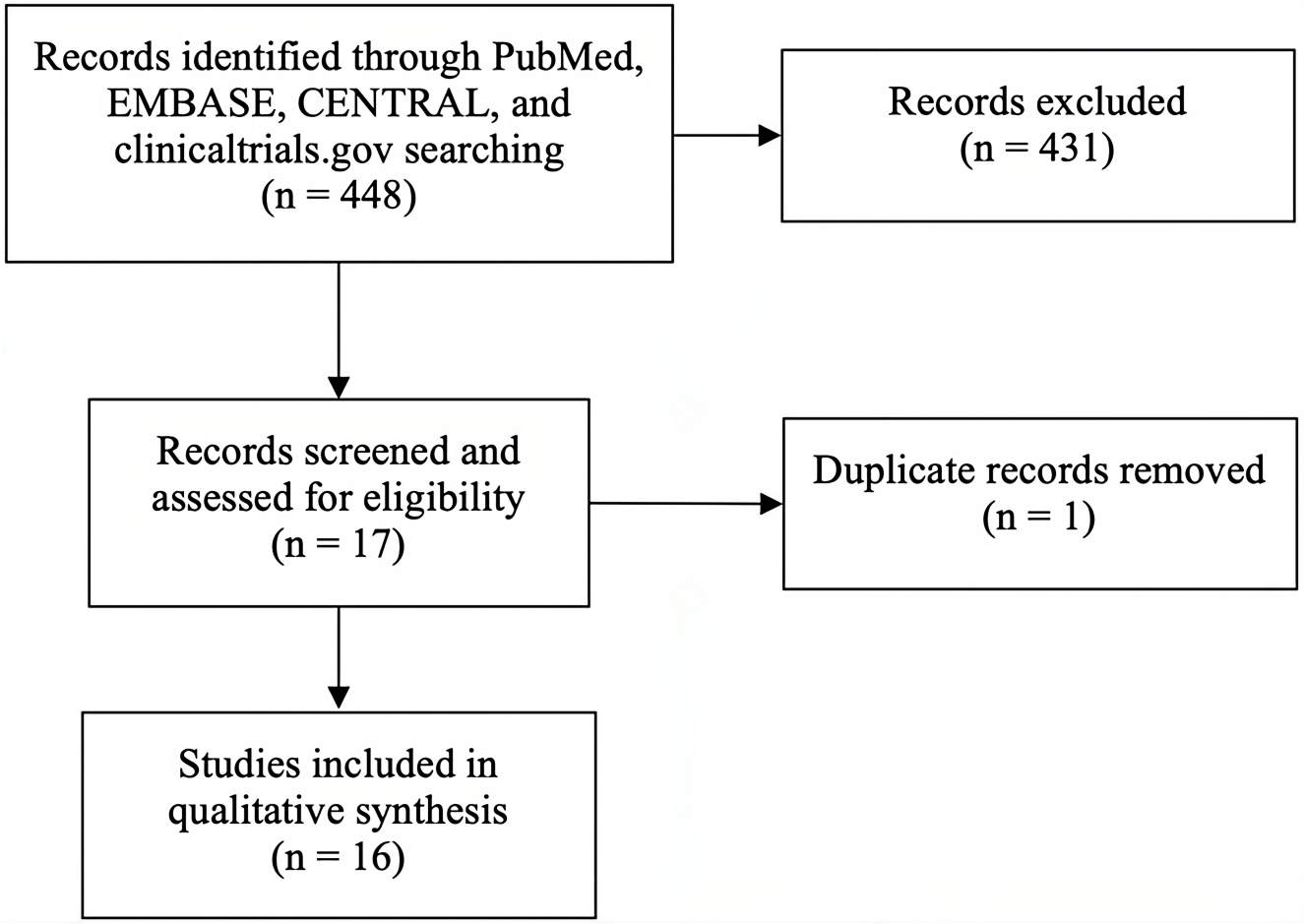

FIGURE 1. PRISMA flow diagram. Studies were included if they were (1) published abstracts or peer-reviewed published studies and (2) included protocols for processing of testicular biopsy samples. Studies were excluded if the study was a case-report.

TABLE 1. Summary of studies reporting processing techniques of testicular sperm samples and reported outcomes.

Study Year	Outcome[1]	Comparison Groups				Study Population[2]		Reference number
		Group 1	Group 2	Group 3	Group 4	OA	NOA	
2023	Spermatozoa Retrieval Rate	Mechanical maceration	Enzymatic digestion (if mechanical maceration failed)	–	–	–	X	[8]
		23.7% (28 of 118)	37.2% (44 of 118)					
2021	Spermatozoa Retrieval Rate	3-D printed Microfluidics	–	–	–	–	X	[9]
		>96% spermatozoa recovery rate in 5 minutes						
	Mean spermatozoa count per milliliter[3] (mean ± SD)	Erythrocyte lysis buffer	–	–	–	Not described		[10]
		$34.4 \pm 22.0 \times 10^6$						
2018	Proof of Concept	Magnetic Levitation	–	–	–	Not described		[11]
		Spermatozoa can be sorted						
2017	Spermatozoa Retrieval Rate (Relative)	Microfluidics	Manual disruption with enzymatic digestion	–	–	–	X	[12]
		6.8× more spermatozoa in each half hour	Reference group					
2014	Mean number of spermatozoa removed	Mechanical maceration	Mechanical maceration and residual tissue removal	–	–	–	X	[13]
		39.4 ± 24.8	60.2 ± 28.2					
	Spermatozoa Retrieval Rate	Mechanical processing	Enzymatic digestion with density gradient centrifugation	–	–	X	X	[14]
		71.6% (63 of 88)	Not described			Results not stratified by NOA *vs.* OA		

TABLE 1. Continued.

Study Year	Outcome[1]	Comparison Groups				Study Population[2]		Reference number
		Group 1	Group 2	Group 3	Group 4	OA	NOA	
2011	Spermatozoa Retrieval Rate	Mechanical maceration and angiocatheter 52% (553 of 1054)	–	–	–	–	X	[15]
2009	Spermatozoa Retrieval Rate	Mechanical maceration 78 of 146 (53%)	–	–	–	X Results not stratified by NOA *vs.* OA	X	[1]
2005	Spermatozoa Retrieval Rate	Mechanical maceration 65 of 177 (36%)	Enzymatic digestion (if mechanical maceration failed) 37 of 112 (33%)	–	–	–	X	[16]
2002	Spermatozoa Retrieval Rate	Mechanical maceration 213 of 319 (66.8%)	Enzymatic digestion 308 of 520 (59.2%)	–		X Results not stratified by NOA *vs.* OA	X	[17]
	Spermatozoa Retrieval Rate	Mechanical maceration 14 of 41 (34%)	Enzymatic digestion (if mechanical maceration failed) 8 of 27 (30%)	–	–	–	X	[18]
1998	Spermatozoa Concentration	Mechanical maceration 83,000 sperm/mL	Mechanical maceration and angio-catheter 390,000 sperm/mL	–	–	–	X	[19]
	Primary spermatocyte cell count per 100 mg of testicular tissue (mean ± SD)	VSUG with DPC OA: 71,000 ± 6000 NOA: 61,000 ± 5000	Separation with FACS OA: 79,000 ± 12,000 NOA: 71,000 ± 10,000	–	–	X	X	[20]

TABLE 1. Continued.

Study Year	Outcome[1]	Comparison Groups				Study Population[2]		Reference number
		Group 1	Group 2	Group 3	Group 4	OA	NOA	
1997	Spermatozoa count per 100 mg testicular tissue (mean ± SEM)	Collagenase 1A	Collagenase IV	Collagenase 1A and elastase	Collagenase IV and elastase	X	–	[21]
		22,000 ± 4000	34,000 ± 7000	21,000 ± 6000	40,000 ± 15,000			
1995	Total spermatozoa count per 100 mg testicular tissue	Shredding	Mincing	Vortexing	Crushing	X	–	[2]
		24,000	35,000	26,000	14,500			

[1] *Unless otherwise noted, numbers reported next to rates refer to number of samples.*
[2] *The symbol "X" denotes the population included in the study; the symbol "–"denotes that the population was not included in the study.*
[3] *This study (Year 2021, [16]) included four comparison groups, but only one group used testicular sperm. The other three groups used sperm from ejaculate, which were outside the scope of this review of testicular sperm samples and were therefore excluded from this table. Among the various clinical outcomes reported, sperm count was selected for display because it was the most comparable to the outcomes of other studies.*
Abbreviations: OA, obstructive azoospermia; NOA, non-obstructive azoospermia; SD, standard deviation; VSUG, velocity sedimentation under unit gravity; DPC, discontinuous Percoll centrifugation; FACS, fluorescent-activated cell sorter; SEM, standard error of the mean.

A technical modification of the mincing process was used with 146 men with azoospermia, who underwent TESE, to investigate the predictive value of supernatant sperm [1]. Their specimens were placed in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) medium for 10 minutes. The specimens were transferred to another plate for mechanical shredding with fine needles. The remaining supernatant medium was centrifuged at 1800g for 5 minutes, and the resultant pellet was re-suspended in 20 μL HEPES medium. The shredded specimen was centrifuged and re-suspended. The presence of spermatozoa was assessed microscopically. 79 cases (53%) demonstrated successful sperm recovery, defined by the identification of one or more spermatozoa in the biopsy. In all cases of successful spermatozoa recovery, spermatozoa were also observed in the supernatant; in unsuccessful recoveries, they were not seen in the supernatant. This study did not have any comparison methods.

Mechanical mincing was compared to mincing with subsequent passage through a 24-gauge catheter [19]. Biopsy specimens of 81 men with NOA who underwent TESE were minced using fine-tipped scissors. Forty-seven patients (58%) had spermatozoa in their specimen. Twenty patients also had testicular tissue passed through a 24-gauge angiocatheter post-mincing. This dispersion increased the average number of spermatozoa retrieved from 83,000 sperm/mL to 390,000 sperm/mL ($p = 0.005$).

The effects of removing residual tissue after mechanical maceration were examined in 20 men with NOA who underwent TESE [13]. The retrieved specimens were mechanically dissected in sperm wash media and separated into two groups before gradient centrifugation. In the first group, the minced material, along with the sperm wash media, was centrifuged. In the second group, only the sperm wash media was centrifuged. On average, 39.4 $\pm$ 24.8 and 60.2 $\pm$ 28.2 cells were recovered ($p < 0.001$) in the first and second groups, respectively. Since the non-functional residual tissue prevents spermatozoa from migrating through the centrifuge's gradient, using only sperm wash media increases the number of available mature spermatozoa.

3.2 Enzymatic digestion

Six studies investigated enzymatic digestion, often used with mechanical preparation. Twenty patients, primarily with OA, underwent testicular sperm extraction, and the specimens were minced. Collagenase type IV and IA were investigated with and without elastase. The specimens were exposed to enzyme preparations for 1 hour at 37 °C, and the cells were isolated after centrifugation [21]. The number of spermatozoa per 100 mg of tissue was calculated, but sperm quality was not assessed. Collagenase type IV resulted in a higher yield of spermatozoa than collagenase type IA (340,000 $\pm$ 70,000, *vs.* 220,000 $\pm$ 40,000; $p = 0.017$; n = 17). This difference did not remain statistically significant with the addition of elastase ($p = 0.119$). A follow-up study investigated the efficacy of enzymatic digestion in 41 patients with NOA who underwent testicular sperm extraction [18]. Specimens were shredded with two glass slides, minced with fine forceps, and digested in erythrocyte lysing buffer. Fourteen (34%) of the 41 samples yielded spermatozoa. The 27 failures after mincing underwent enzymatic digestion with collagenase type IV for 1 hour. Eight samples (30%) had spermatozoa.

A multicenter study compared mechanical preparation with enzymatic digestion in fresh or cryopreserved samples from men with both OA and NOA [17]. When comparing fresh to cryopreserved samples, the authors found no significant difference in the proportion of cases with motile spermatozoa. However, mechanical preparation yielded a significantly higher proportion of cases with motile spermatozoa compared to enzymatic preparation (66.8% *vs.* 59.2%; $p = 0.03$).

In a prospective study, men with NOA underwent mTESE and mechanical preparation of samples with fine needles and incubation in erythrocyte-lysing buffer when needed [16]. Of 177 patients, 65 (36%) had visible spermatozoa after mechanical preparation. Of 112 patients in which no spermatozoa were visualized by mincing and erythrocyte-lysing buffer, 37 (33%) had recoverable spermatozoa after enzymatic digestion in an incubation medium containing 25 μg/mL DNAse (Sigma DN25) and 1000 IU/mL of collagenase type IV (Sigma C5128) for one hour at 37 °C. The study concluded that out of 177 NOA cases, the conventional mincing method combined with enzymatic treatment successfully recovered sperm for ICSI in 102 cases (57%).

A large retrospective study of 1054 men with NOA used a combination of mechanical preparation and enzymatic digestion to process samples [15]. After mTESE, samples were minced with scissors and passed through a 24-gauge angiocatheter multiple times. Spermatozoa was identified intraoperatively in 553 (52.4%) samples. If spermatozoa were not visible, the samples were digested in type IV collagenase and DNase I at 37 °C for one hour. The samples were centrifuged at 500g for 5 minutes, and if spermatozoa were identified, again at 1500–3000g for 5 minutes. Of 501 samples that underwent further enzymatic processing, spermatozoa was identified in 35 (7%; 95% confidence interval 2.24–9.61).

A retrospective single-center cohort study included patients with NOA who underwent their initial TESE via open multiple-biopsy between 2004 and 2022 [8]. The primary objective was to assess the sperm retrieval rate following the mincing and/or enzymatic treatment of testicular biopsies in NOA. Following mechanical mincing, the biopsies were examined for 30 minutes; in cases where no or insufficient spermatozoa were detected, enzymatic treatment using collagenase type IV was administered. A total of 118 patients were included in the analysis, among whom 72 (61.0%) ultimately achieved successful sperm retrieval. Spermatozoa were retrieved through mechanical mincing alone in 28 patients (23.7% of 118) and following additional enzymatic digestion in another 44 patients (37.2% of 118). Therefore, among the 90 patients who required enzymatic digestion, sperm retrieval was successful in 44 individuals (48.9%).

A prospective study investigated enzymatic digestion combined with density gradient centrifugation [14]. Eighty-eight men underwent TESE, and undescribed mechanical processing was used to screen for spermatozoa. Samples from 63 men had spermatozoa intraoperatively and were further processed with enzymatic digestion in type IV collagenase. Then, the samples were centrifuged with a density gradient of silica

particle layers. As only samples in which spermatozoa were identified intra-operatively were included, the efficacy of the technique was not discussed.

3.3 Other techniques

A study compared (1) velocity sedimentation under unit gravity (VSUG) combined with discontinuous Percoll centrifugation and (2) separation in a fluorescence-activated cell sorter (FACS) for obtaining purified mixed spermatogenic cells after testicular tissue enzymatic digestion [20]. Fifty-seven men with OA and NOA case underwent TESE. The biopsies underwent enzymatic digestion with Trypsin and DNase I. After, the spermatocytes were isolated from the suspension using either the VSUG with discontinuous Percoll centrifugation (8 OA and 23 NOA cases) or FACS separation (9 OA and 17 NOA cases). With VSUG, cells were separated into 115 fractions after sedimenting in a STA-PUT chamber. Afterwards, discontinuous Percoll centrifugation was used to isolate the spermatogenic cells from the contaminants by resuspending and diluting the pelleted cells in Percoll solution. For the FACS method, cells were sorted into fractions by their forward-scatter signals by FACS. Total cell count was assessed in a haemocytometer, and after separation each fraction was visualized with Normarski differential interference microscopy. In the OA samples, the difference in number of primary spermatocytes isolated from each technique was not significant while in NOA, FACS separation produced a higher mean cell count of primary spermatocytes than VSUG with discontinuous Percoll centrifugation ($0.71 \pm 0.10 \times 10^6$ *vs.* $0.61 \pm 0.05 \times 10^6$, respectively, $p < 0.001$). However, isolation of primary spermatocytes and spermatids using VSUG with discontinuous Percoll centrifugation and FACS cannot be generalized to identification of spermatozoa as spermatocytes and spermatids are different stages of maturation to spermatozoa.

Isolating spermatozoa from TESE samples using a magnetic levitation-based method was attempted [11]. The authors placed samples in a paramagnetic medium and applied a magnetic field to a platform made of polymethyl methacrylate, permanent neodymium magnets and aluminum coated mirrors. The TESE sample levitated, and the cells from it flowed and were sorted according to their unique levitation properties. The equilibrium height of the cells was captured and measured with an image analysis software developed at the authors' institution. Mature spermatozoa were purified successfully.

A two-part system using microfluidics to enhance sperm recovery and decrease sperm isolation time in mTESE samples was explored [12]. Part A (traditional method) separated spermatozoa from the other cells in the sample, while Part B enriched and concentrated the aforementioned spermatozoa. For Part A, five testicular biopsy samples from NOA patients underwent manual disruption and enzymatic digestion with collagenase for 1–2 hours. In Part B, part of the sample was diluted and processed with sequential microfluid enrichment modules to concentrate and enrich the spermatozoa. Each process was compared by spermatozoa identified per time period. Using microfluids produced ~7 times more spermatozoa in each half hour over the traditional method.

A 3-D-printed inertial microfluidic spermatozooa separation device was developed for potential use in patients with NOA [9]. The device enabled >96% sperm recovery in five minutes, with each spermatozoa spending less than 0.25 seconds in the chip. The flow rate 1.1 mL/min was most optimal for spermatozooa separation, trading a slightly lower percentage of sperm in the target outlet for fewer background cells (red blood, white blood and epithelial cells), compared to the 1.2 mL/min flow rate. The microfluidic device did not alter sperm motility, normal morphology, vitality, or sperm DNA fragmentation after four microfluidic separation runs compared to the unprocessed sample.

In a randomized controlled trial, 302 patients with abnormal sperm DNA fragmentation who underwent ICSI were randomized into 4 sperm selection technique groups: testicular sperm (n = 73) and 3 other groups that utilized sperm from ejaculate [10]. Testicular sperm was extracted via mTESE or testicular sperm aspiration and washed with erythrocyte lysis buffer before sperm selection. This review focuses on testicular sperm (*vs.* ejaculated sperm) and thus will report only the results of the testicular sperm group to allow comparison to other studies. Mean sperm count (in 10^6/mL) was 34.4 ± 22.0 for testicular sperm (Table 1).

4. Discussion

This systematic review reveals the inconsistency in outcomes/metrics reported in post-sperm extraction sample processing protocol studies. Not only are results often not stratified by the patient populations studied (*i.e.*, OA *vs.* NOA *vs.* other), the outcomes/metrics reported are not standardized. Therefore, it remains unclear if testicular sperm processing methods are equally effective in both patients with OA and NOA. Moreover, the wide variation in outcomes reporting makes it extremely difficult for laboratories and fertility specialists to establish best practice protocols that maximize sperm yield among the over 30 years of data generated in this field.

Based on our review of studies that included mostly patients with OA, for patients with OA, collagenase type IV and elastase offered the best yield of spermatozoa at approximately $40{,}000 \pm 15{,}000$ spermatozoa per 100 mg of testicular tissue [21] compared to other methods [2]. Collagenase type IV only gave the next highest yield [21]. We were unable to utilize studies that included both patients with OA and NOA, since they did not stratify their results by treatment indication (OA *vs.* NOA) [1, 14, 17], and the one study that had stratification did not report on spermatozoa yield [20]. For patients with NOA, the best protocol was also challenging to define, for protocols switched from reporting total spermatozoa count per 100 mg of testicular tissue to percentage of spermatozoa recovery. Nevertheless, microfluidics seems to have the best chance of extraction in terms of recovery rate, efficiency, and spermatozoa count. The novel 3-D printed micro fluidics device had the best recovery rate at 96% in 5 minutes [9], making it possibly the most efficient. Microfluidics produced ~7 times more spermatozoa versus the traditional manual disruption and enzymatic digestion with collagenase [12].

However, our recommendations are limited by the variation in study population and outcome type reported in various pro-

tocols through the last two decades. More studies are needed with standardized outcomes. In the future, studies should stratify results by population (NOA *vs.* OA *vs.* other) and report both spermatozoa recovery rate and mean spermatozoa count recovered for a comprehensive characterization of the methods evaluated. To facilitate easy comparison to past literature, studies should also report mean spermatozoa count per both units of volume (mL) and mass (mg of testicular tissue). Future studies should include more randomized controlled trials that compare processing techniques of only testicular sperm and include larger sample sizes so that multiple comparisons across the processing techniques can be statistically powered.

In addition, the studies included in this systematic review by Durmus *et al.* [11], Jenkins *et al.* [12], Vasilescu *et al.* [9], and Baukloh *et al.* [17] commented on potential DNA damage and DNA fragmentation. while the remaining studies focused on the number of spermatozoa yielded. DNA damage can be assessed via sperm DNA fragmentation and is essential to determine the optimal sample processing technique. This is a major limitation that prevents proper interpretation of these studies, as all these methods must be scrutinized for their effect on embryo quality. Also, studies comparing the efficiency and success rates of ICSI between processing methods and cost-effectiveness and scalability of processing methods are critical in choosing the optimal processing method. Additionally, the studies often do not account for the quality of the microscope, the number and experience level of andrology technicians, and the total time spent identifying spermatozoa. These factors can significantly impact the success rates and outcomes of sperm recovery procedures. Considering the financial and emotional investment of patients undergoing ICSI, the best approach for post-biopsy processing should be developed just as it has for the surgical approach for obtaining testicular samples.

5. Conclusions

Post-sperm extraction sample processing is necessary to increase sperm quantity and quality before ICSI, especially when considering the financial and psychological burden associated with male infertility and its treatments. Microfluidics seems to be the best method for spermatic extraction for NOA samples, while collagenase type IV and elastase works best for OA samples in retrieving spermatozoa. However, the literature surrounding efficacy of post-sperm extraction sample processing is plagued by samples of heterozygous nature, high risk of bias due to lack of laboratory protocol standardization, and inconsistent outcomes reporting. This makes it difficult to definitively compare processing protocols and their outcomes. Therefore, further well-designed studies are essential and urgently needed to confirm the optimal processing methodology. By establishing expert consensus and eventually standardization of outcome reporting, fertility specialists can offer their patients the best chance of sperm retrieval.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

AUTHOR CONTRIBUTIONS

SR, TPK and ASH—designed the research study. SR, TPK and MFA—abstracted data. ASH—resolved any discrepancies in the article inclusion process. TCT, SR, TPK, MFA, MM and MHZ—analyzed data and wrote the manuscript. AKG—provided critical review. All authors contributed to the editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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