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Development of a Simplified Swim-up Method for Sperm Processing

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Abstract

Established sperm preparation techniques have been under the spotlight in support of the affordable assisted reproduction drive. Affordability and safety are particularly relevant in developing countries, with restricted access to basic infertility care due to limited resources. This study investigated a modified sperm swim-up method in comparison to a commercial sperm preparation kit. Spermatozoa were processed using three different volume disposable syringes: 5 ml (SW-5), 10 ml (SW-10), and 20 ml (SW-20), with respect to concentration and motility. Hereafter, the syringe method that resulted in the highest sperm yield was matched against a commercially available device (SEP-D kit) for the evaluation of sperm motility, concentration, vitality, morphology, and deoxyribonucleic acid (DNA) fragmentation. Semen processed using the SW-10 method resulted in a higher total motile sperm count (6.62 $x10^6$), in comparison to the SW-5 and SW-20 methods. When compared to the commercial device, spermatozoa harvested with the SW-10 method presented with significantly improved total motility (75.35% vs 87.05%) and concentration (14.35 $x10^6$/ml vs 17.10 $x10^6$/ml, $p<0.0001$). Furthermore, there was a significant increase in spermatozoa viability after processing using the SW-10 (79.47% vs 70.05 for the hypo-osmotic swelling test, 82.31% vs 72.00% for eosin and nigrosin test, $p<0.001$), and fewer spermatozoa with DNA damage (13.70% vs 23.20%, $p<0.0001$). This modified swim-up method can therefore be integrated into a cost-effective intrauterine insemination treatment for selected patients in a low-resource setting.

Keywords: Developing countries, Infertility, Artificial insemination, Reproduction, Spermatozoa, Swim-up, Syringes

Introduction

Assisted reproductive technologies (ART) in developing countries are mostly restricted to private settings, and only accessible to those with financial resources (1, 2, 3). Approaches to providing cost-affordable diagnostic and accessible therapeutic infertility treatment in these countries have been discussed in the literature, leading to technological developments such as alternative sperm preparation methods, low-cost laboratory supplies, and a simplified embryo culture system (4-11).

Intrauterine insemination (IUI) which involves the insemination of a processed sperm sample into the uterus (12), is used as the first-line treatment for infertility due to the relative simplicity and cost-effectiveness of the procedure. It is suitable for couples with unexplained infertility as well as for those with mild male factor sub-fertility (13, 9, 14, 15). Semen processing methods are performed before the IUI to separate sperm cells (with improved motility and morphology) from the seminal plasma products such as leukocytes, bacteria, and dead spermatozoa which can compromise fertilization ability (16). Different sperm processing techniques have been modified following the considerably increased need for affordable ART over the last twenty years (17, 18). An ideal sperm preparation should minimize damage to sperm cells, as well as maximize the recovery of a high number of functional and morphologically normal spermatozoa (19), with the elimination of non-sperm cells.

Three semen processing methods i.e: simple washing, direct swim-up, and density gradient centrifugation (DGC) are commonly used for the examination and processing of human semen (20). The direct swim-up method and the DGC are the most used techniques, depending on the semen sample characteristics (21). The swim-up method selects sperm according to their motility, however, density gradient centrifugation is a procedure that separates spermatozoa according to their density or specific gravity, consequently allowing motile sperm cells with high density to actively form a pellet (22). A study by Raad *et al*., (2021) indicated an increase in the better spermatozoa in terms of DNA integrity, reactive oxygen species levels, acrosome status, and mitochondrial activity following the direct swim-up method in comparison to DGC (23). The latter research confirmed the previous investigation by Zini *et al*., (2000) who found a decrease in the number of spermatozoa with damaged DNA after processing using the swim-up method (24). Furthermore, high levels of reactive oxygen species (ROS) that induce damage to sperm DNA were reported following the DGC method (22). In contrast, another study indicated a decrease in DNA fragmentation (25) and ROS in sperm harvested after DGC (26).

Additionally, the SEP-D kit, which is commercially available in a set of five syringes filled with a Hepes buffered medium, can also be used for the preparation of semen samples for IUI. The SEP-D kit has been reported to be less time-consuming and simpler when compared to the standard direct swim-up and the DGC methods (27). The study reported a significant increase in pregnancy rates after IUI when semen was processed using the SEP-D, rather than the standard swim-up method. However, no significant differences were found

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with regards to sperm motility and sperm morphology parameters (27). No statistical differences between the swim-up and the DGC methods regarding pregnancy outcome after IUI have been indicated in a Cochrane Review (16).

Several sperm processing techniques can be used for ART, the challenge lies in the development of an efficient, cost-affordable, and simple sperm purification method, with minimal procedural steps. This study was aimed at simplifying the direct swim-up method used for sperm preparation to potentially provide an alternate cost-effective IUI treatment to meet the rising need for infertility management in low-resource settings.

Materials and Methods

This study was approved by the University of Pretoria's Ethics Committee and Steve Biko Academic Hospital (protocol number: 54/2014).

Study population

Semen samples (n=45) were obtained from human immunodeficiency virus (HIV) negative and non-smoking medical students at the University of Pretoria and patients participating in the Assisted Reproduction Programme at Steve Biko Academic Hospital, an informed consent form was obtained from each participant. The sample inclusion criteria were a minimum concentration of 15 $x10^6$ sperm/ml, total sperm motility of 40% or more, and a minimum semen volume of 1.5 ml, as per the requirements stipulated by the World Health Organization (WHO) guidelines (2010). Sperm morphology was in accordance with the Tygerberg Strict criteria for sperm morphology (20; 28).

Modified sperm swim-up method using various syringes (Study i)

Subsequent to liquefaction, semen samples (n=25) were prepared using three disposable syringes of different volumes: 5 ml (SW-5) and 20 ml (SW-20) from Promex Health and Medical and Surgical™ (3 Dock Road, Cape Town, South Africa) and 10 ml (SW-10) from Kendall monoject™ (Massachusetts, United States, www.vitalitymedical.com) (Figure 1).

Figure 1: Disposable Promex™ (5 and 20 ml) and 10 ml from Kendall monoject™ syringes used for the current study

The comparison between the disposable syringes of different volumes is illustrated in Figure 2. Semen samples were divided into three equal aliquots and each sample was processed similarly to the direct swim-up method described by the WHO (2010). A volume of 1 ml PureSperm Wash® (Nidacon International, Sweden, www.nidaconinternational.com) was aspirated into each syringe, followed by a 0.5 ml semen sample. The syringes were subsequently incubated for 60 minutes at 37ºC at a 45º angle. After incubation, the semen was expelled gently and the remaining medium (0.3 ml) was used to evaluate sperm motility and concentration through computer-aided sperm analysis (CASA) (medeaLab CASA; MTG-GmbH, Altdorf, Germany) at 200x magnification using an Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany).

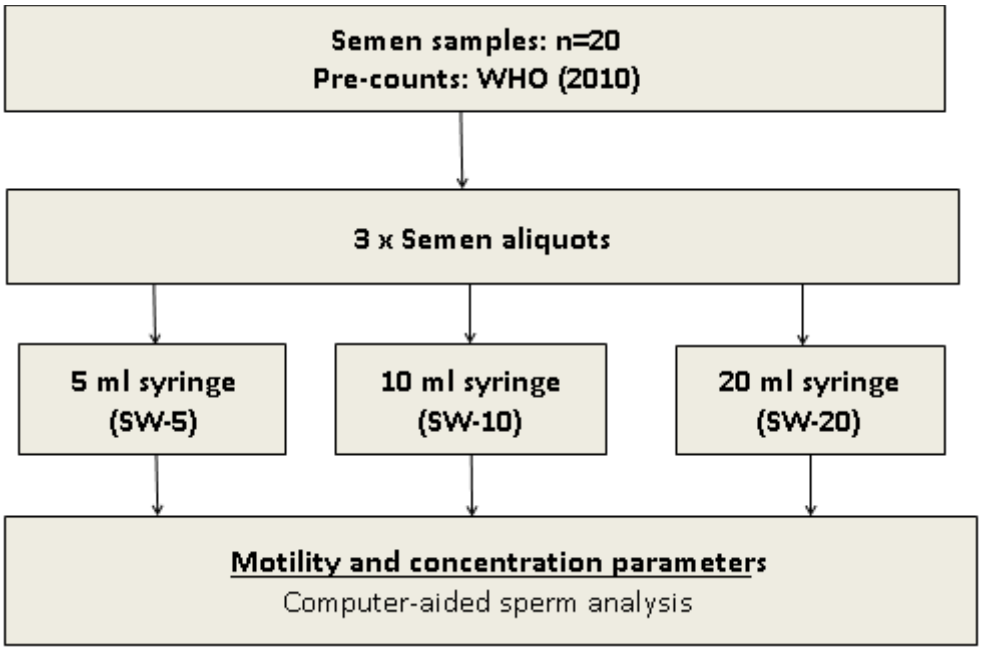

Figure 2: Diagram of the simple sperm swim-up experimentation method using syringes of different volumes

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For quality control purposes, two separate chambers of a Leja slide (Two chambers slides, 20 µm deep; Leja®, Nieuw-Vennep, Netherlands) were loaded with the same processed sample, and video recordings of at least 10 random representative fields per chamber were evaluated for 30 seconds. A minimum of 200 cells per sample were evaluated. Internal quality control for CASA calibration was performed by an experienced spermatologist before experimentation. The total motile count (TMC) after sperm preparation was obtained by multiplying the concentration ($x10^6$/ml) by the progressive motility/100 by the insemination volume (0.3 ml) (29; 30).

The modified swim-up vs the commercial SEP-D method (Study ii)

Semen samples (n=20) were split into equal aliquots that were processed in parallel using the SEP-D kit (SureLife, Singapore, www.surelifeivf.com) (Figure 3) and the modified swim-up method using the syringe that yielded the highest TMC (SW-10 method) in study i. Figure 4 illustrates the comparison between the simplified sperm swim-up (SW-10) method and the SEP-D kit. For the SEP-D method, approximately 1 ml of unprocessed semen was aspirated slowly into the SEP-D syringe and incubated at 37ºC for 60 minutes according to the manufacturer's guidelines (www.surelifeivf.com). After incubation, the semen was gently expelled until 0.3 ml of culture medium remained. Sperm samples recovered by these preparation techniques were then assessed for motility, concentration, viability, morphology, and DNA fragmentation as previously described.

Sperm motility and concentration: computer-aided analysis

Sperm motility and concentration were assessed using CASA, and for quality control, all samples were subjected to manual count using the Improved Neubauer® (Marienfeld, Germany, http://www.marienfeld-superior.com) and Makler® counting chamber (Sefi-medical instrument, Israel, http://www.sefimedical.com) at 200X magnification.

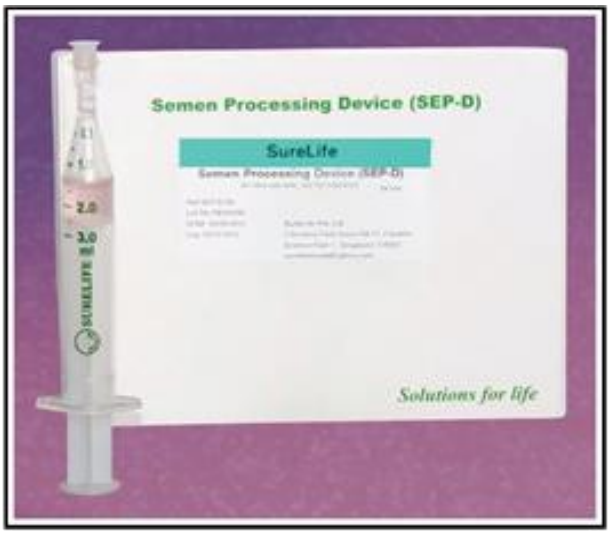

Figure 3: SEP-D kit used for semen processing in the current study

Sperm viability: one-step eosin-nigrosin and hypo-osmotic swelling test

Sperm viability was evaluated through the one-step eosin-nigrosin vitalscreen kit (FertiPro, Belgium, www.fertipro.com) and the hypo-osmotic swelling (HOS) test according to standard operating procedures of the Reproductive Biology Laboratory, Steve Biko Academic Hospital, Pretoria, South Africa. The eosin-nigrosin kit contains two solutions: 0.67% eosin Y (red solution) and 10% igrosine (black solution). Sperm vitality was assessed 30 minutes after semen collection by adding two drops of eosin Y solution to 50 µL of semen for 30 seconds, followed by 3 drops of igrosine for 30 seconds.

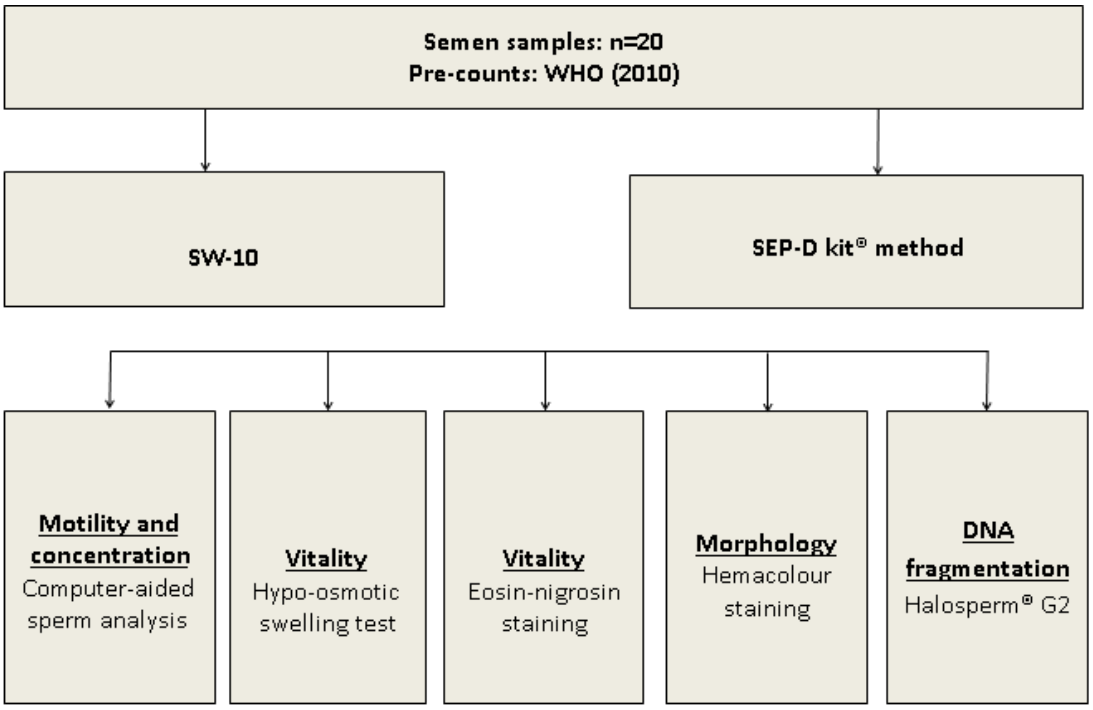

Figure 4: Flow diagram illustrating the comparison between the simplified sperm swim-up (SW-10) method and the SEP-D kit

One drop of the mixture was transferred to a microscope slide and a smear was made. A minimum of 200 spermatozoa were evaluated under 100x oil immersion using the Axiostar plus microscope (Carl Zeiss, Göttingen, Germany, www.micro-shop.zeiss.com).

The HOS solution was prepared following the method firstly described by Jayendran *et al*., (1984). The protocol consisted of dissolving 0.735 g of sodium citrate dehydrate and 1.351 g of D-fructose in 100 ml of purified water. The solution was incubated at 37ºC before the addition of the semen sample. A volume of 100 µL of semen was transferred into the swelling solution and incubated at 37ºC for 30 minutes. Subsequently, 10 µL of spermatozoa was placed on a clean slide and covered with a 22 mm x 22 mm coverslip. The slide was analyzed using the Axioskop 40, phase-contrast microscope (Carl Zeiss, Göttingen, Germany, www.micro-shop.zeiss.com) at 200x magnification. A total of 200 spermatozoa were evaluated, whereby the number of unswollen (dead) and swollen (live with intact plasma membrane) spermatozoa were determined. A positive control, for both tests, was performed by exposing spermatozoa to a very low temperature (4ºC) for 15 minutes.

Sperm morphology analysis

Sperm morphology staining was performed using the commercially available Hemacolor® kit (Merck, Darmstadt, Germany) and evaluation was done according to the Tygerberg strict criterion system, originally described by Kruger *et al*., (1987). This technique involved the preparation of semen smears, fixation, and staining before visualization. The semen smears procedure consisted of pipetting 10 µL on a slide and performing the feathering technique (WHO, 2010). Hereafter, the slides were immersed in methanol for 15 seconds. Following fixation, the slides were treated with an eosin solution for 25 seconds and then placed in haematoxylin for 20 seconds. Hereafter, the slide was rinsed with distilled water to remove excess haematoxylin. The slide was then mounted using Entalin® before being left to dry overnight. Normal and abnormal spermatozoa were examined under immersion oil, with light microscopy using the Axiostar plus (Carl Zeiss, Göttingen, Germany, www.micro-shop.zeiss.com) at 1000x magnification.

DNA fragmentation evaluation: Halosperm® G2 assay

The DNA fragmentation was analyzed using the Halosperm® G2 assay, following the method recommended by the manufacturer's guidelines (Halotech, Spain, www.halotechdna.com). The Halosperm® G2 protocol has been described in detail by Fernadez *et al*., 2005. This method involves the immersion of unfixed spermatozoa on a slide in an agarose microgel, followed by DNA denaturation in those sperm with fragmented DNA, using an acid solution. Nuclear proteins are then removed by a lysis solution. Nucleiods from sperm with fragmented DNA will result in minimal or no dispersion halos, while less DNA denaturation will form large halos of spreading DNA (Manufacturer's guidelines, www.halotechdna.com). Sperm cells were analyzed using bright field microscopy (Axioskop 40, Carl Zeiss, Göttingen, Germany) and a minimum of 300 spermatozoa were evaluated at 400x magnification.

Data analysis

Statistical analyses were performed using the Wilcoxon signed ranks tests. The values were expressed as mean and standard deviation (±SD). A random effect, together with the generalized least squares were calculated. The significance level was set at $P<0.05$ using Stata Release 11 (www.xlstat.com, 2014).

Results

Descriptive statistics for study (i) and study (ii) are summarized in Table 1. The Raw semen samples (n=25 for study I and n=20 for study ii) had an average volume of 2.4 ml and 2.1 ml for each study section respectively, with an average pH of 7.5. Sperm concentration and normal morphology of semen samples were 41 $x10^6$/ml (± 8.72) with 8% (±7.42) of normal sperm for the modification of the direct swim-up method, and 38 $x10^6$/ml (±8.86) with 7% (±7.83) normal morphology for the comparison between the simplified swim-up and the SEP-D processing.

Table 1: Summary statistics for study (i) and Study (ii)

	Study (i)	Study (ii)
Sample size (n)	25	20
Semen Volume (mL)	2.4	2.1
Semen pH	7.5	7.5
Concentration ($x10^6$/ml)	44	38
Normal Morphology (%)	8	7

Modification of the direct sperm swim-up method (Study i)

Sperm concentration, progressive motility, and TMC obtained after processing with the 5 ml (SW-5), 10 ml (SW-10), and 20 ml (SW-20) syringes are indicated in Table 1. The SW-10 method provided significantly ($p<0.05$) improved sperm concentration than sperm harvested with the SW-5 and the SW-20. Spermatozoa processed using the SW-10 procedure also resulted in significantly ($p<0.05$) higher progressive motility when compared to that yielded by the SW-5 and the SW-20 method. Consequently, the TMC of sperm was found to be significantly ($p<0.001$) higher when using the SW-10 method. Despite no significant differences noted in the TMC of the harvested sperm samples processed by the SW-5 and the SW-20 methods ($p<0.479$), statistical differences ($p<0.015$) were observed in progressive sperm motility and concentration.

Comparing the simplified swim-up method (SW-10) and the SEP-D kit (Study ii)

Spermatozoa harvested with the SW-10 procedure produced better quality post-processed sperm parameters (motility and concentration) in comparison to SW-5 and SW-20, consequently, the SW-10 was compared to the SEP-D kit. Sperm parameters (concentration, motility, morphology, viability, and DNA fragmentation) resulting from the comparison between the SW-10 processing and the SEP-D methods are depicted in Table 2. Spermatozoa obtained using the SW-10 method displayed significantly ($p<0.001$) higher total motility with a concurrent higher average sperm concentration than that harvested using the commercial SEP-D kit. The SW-10 method yielded sperm samples with slightly higher morphologically normal spermatozoa, compared to the SEP-D kit ($p=0.42$). Viability assessments of harvested spermatozoa indicated that the SW-10 method resulted in more sperm cells with intact plasma membranes. The HOS test results indicated that 79.47% (±6.31) of sperm cells harvested with the SW-10 displayed an intact plasma membrane and 70.05% (± 9.98) for the SEP-D samples ($p<0.001$). Similar results were observed using the dye exclusion test (eosin-nigrosin) where spermatozoa obtained with the SW-10 method were yielded more viable than the SEP-D procedure ($p<0.001$).

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Table 1: Summary of concentrations and motility parameters of sperm processed with the 5 ml, 10 ml and 20 ml syringes (n=25)

Sperm parameter	5 ml (a) (SW-5)	10 ml (b) (SW-10)	20 ml(c) (SW-20)	p-value (95% CI)		
	Mean (SD)			(a *vs* b)	(a *vs* c)	(b *vs* c)
Concentration (10^6/ml)	**15.97 (8.04)**	**28.47 (8.83)**	**19.16 (9.12)**	**<0.001** (0.173;0.312)	**0.012** (0.022;0.165)	**<0.001** (0.105;0.157)
Rapid progressive motility (%)	**50.62 (10.58)**	**77.54 (9.02)**	**46.52 (10.27)**	**<0.001** (0.151;0.351)	**0.014** (0.0261;0.043)	**<0.001** (0.243;0.441)
Total motility (%)	**74.17 (12.40)**	**83.12 (10.43)**	**67.03 (12.87)**	**0.014** (0.016;0.143)	**0.018** (-0.146;-0.012)	**<0.001** (0.092;0.131)
TMC ($x10^6$)	**2.42 (14.05)**	**6.62 (12.46)**	**2.67 (14.88)**	**<0.001** (0.244;0.401)	**0.479** (-0.049;0.106)	**<0.001** (0.115;0.171)

*a= 5 ml syringe
*b=10 ml syringe
*c=20 ml syringe

Table 2: Summary of sperm parameters (motility, concentration, morphology, vitality and DNA fragmentation) using the SW-10 and the SEP-D kit

Sperm Parameters	Motility (%)	Concentration (10^6/ml)	Vitality tests (%)		Morphology (%)	DNA fragmentation (%)
			HOS	Eosin-Nigrosin		
SW-10 Mean (SD)	**87.05 (4.18)**	**17.10 (5.95)**	**79.47 (6.31)**	**82.31 (5.15)**	**9.75 (2.83)**	**13.70 (3.85)**
SEP-D kit Mean (SD)	**75.35 (4.86)**	**14.35 (4.35)**	**70.05 (9.98)**	**72.00 (8.56)**	**8.10 (1.66)**	**23.20 (6.77)**
P-value (95% CI)	**p<0.0001** (0.111;0.163)	**p<0.0001** (0.123;0.177)	**p<0.0001** (0.114;0.172)	**p<0.0001** (0.125;0.176)	**p=0.42** (0.116;0.164)	**p<0.0001**(0.098;0.162)

A larger number of spermatozoa showed significant DNA fragmentation in the SEP-D group when compared to the SW-10 group (p<0.001).

Discussion

The present study was designed to simplify the direct swim-up method, using a commercial syringe, in order to obtain motile and viable sperm. The swim-up method using the 10 ml syringe (SW-10) yielded significantly higher sperm concentration and progressive motility compared to the 5 ml and 20 ml syringe volumes. Consequently, a significantly higher total motile count (TMC) was found in harvested sperm samples processed using the SW-10 method. Keel & Webster (1990) reported an increase in sperm TMC due to a larger surface area for sperm migration during the swim-up method. Surface area can possibly explain the difference in TMC obtained after using the SW-5 and the SW-10 methods in the present study, due to the 10 ml syringe providing the most efficient surface area for sperm to migrate. Difficulties were experienced in the course of the experimentation using the SW-20 method, with the highest surface area, during the aspiration of such a small volume of semen (1 ml). The 10 ml volume syringe was the easiest and most practical to use. A meta-analysis by van Weert *et al.* (2004) suggested that a post-wash TMC between 0.8-5 $x10^6$ of sperm can be a good predictor of a successful IUI (31). In addition, Ombelet *et al.* (2003) reported that a minimum inseminating motile sperm count (IMC) of $1x10^6$ with >5% normal spermatozoa could predict IUI success (32). Based on the preliminary results of the current study (i) the SW-10 was then selected and compared to the commercially available SEP-D kit.

The comparison between the SW-10 and the SEP-D kit indicated significant differences, with regards to all sperm parameters evaluated except morphology. Higher concentration, progressive motility and viability were obtained in the SW-10 group compared to the SEP-D kit. Sperm motility and morphology were reported to have a significant effect on IUI success (33; 34). A study performed by Sun *et al.* (2012) showed an increase in pregnancy rates following IUI in patients with high percentage of morphologically normal spermatozoa (35).

According to the findings of this study, an increase in a number of spermatozoa with fragmented DNA was recovered using the SEP-D method, while the SW-10 method provided a sample with higher sperm motility and less DNA damage. Tandara *et al*. (2013) also indicated a negative correlation between motility parameters and the percentage of sperm with DNA fragmentation (36). In the current study, we found no significant difference in the percentage of morphologically normal spermatozoa between the SW-10 and the SEP-D kit. Similar results were reported by Gentis *et al.* (2012) who found no significant differences in sperm morphology between the SEP-D kit and the swim-up method (27).

In conclusion, processing semen samples using the SW-10 method yielded spermatozoa with increased DNA conformation and plasma membrane integrity. Furthermore, processing a single semen sample using the SW-10 procedure protocol costs R229 (€13.63), which is more affordable than the SEP-D device (R347.47; €20.68)[2]. This modification of the direct swim-up sperm processing method demonstrates the potential to simplify conventional techniques while retaining effectivity to provide more cost-affordable reproduction treatment. The simplified method could be integrated into a resource-poor healthcare system, where basic infertility diagnoses/treatments are not available, to establish an affordable IUI program to address limited accessibility. The present research reinforces initiatives to encourage research and advance service delivery, advocacy and networking to achieve global access to infertility care.

Ethical issue

Authors are aware of and comply with, best practices in publication ethics specifically about authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests, and compliance with policies on research ethics. Authors adhere to publication requirements that submitted work is original and has not been published elsewhere in any language. Also, all procedures performed in studies involving human participants were under the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All procedures performed in this study involving animals were following the ethical standards of the institution or practice at which the studies were conducted. The study was conducted in line with the Declaration of Helsinki for medical research and institutional approval was granted (Faculty of Health Sciences Research Ethics Committee, University of Pretoria, no: 54/2014) for the study. The authors declare that there is no conflict of interest.

Competing interests

The authors declare that no conflict of interest would prejudice the impartiality of this scientific work.

Authors' contribution

All authors of this study have a complete contribution to data collection, data analyses, and manuscript writing.

[2] All costs were obtained in ZAR, with an exchange rate of € 1 to ZAR 16, 80 (14/09/2021). All calculations include 14% Value Added Tax (VAT).

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