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Original Research

A Comparison of Triple and Double Sperm Washing for Density Gradient Preparation in Intrauterine Insemination Cycles when Overnight Incubation of Specimens Occurred: A Retrospective Cohort

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Abstract

Background: The number of sperm washes to maximize outcomes for intra-uterine insemination has not been well investigated. Therefore, we undertook to compare the pregnancy and live birth rates of triple sperm washing and double sperm washing for density gradient preparation for intrauterine insemination (IUI) cycles. **Methods**: A retrospective cohort study including 279 couples (136 couples with triple sperm washing and 143 couples with double sperm washing) with a diagnosis of unexplained infertility and mild male subfertility who had IUI cycles between April 2015 and April 2017 were evaluated. After overnight incubation of the sperm, subjects underwent either traditional double sperm washing or Triple sperm washing which consists of use of a third gradient and spinning procedure to the conventional double gradient sperm washing in order to obtain a higher quantity of motile sperm. **Results**: Total sperm count after triple washing was higher than double sperm washing (98.25 $\pm$ 62.06 *vs*. 81.08 $\pm$ 31.57; $p = 0.003$). Positive β-hCG test and live birth per cycle were higher in triple sperm washing (25.8% *vs*. 13.3%, $p = 0.009$; 18.4% *vs*. 9.8%, $p = 0.039$; respectively) than in the double sperm washing group. **Conclusions**: The use of motile sperms obtained from triple sperm washing may increase the rates of pregnancy and live birth in IUI cycles of women with unexplained and mild male factor infertility. A prospective randomized study should be undertaken to confirm the results.

Keywords: intrauterine insemination; live birth; pregnancy; infertility

1. Introduction

Intrauterine insemination (IUI) is a relatively inexpensive and easily performed common assisted reproductive technique, which is cost-effective when compared to *in-vitro* fertilization (IVF) [1]. However, there is no consensus on how to prepare sperm for insemination. Pregnancy outcomes of IUI cycles in the literature demonstrate wide variations in success rates with published outcomes varying from 4% to 24% per cycle [1–6]. The reasons for this are related to the patients selected for this treatment, medications used to stimulate the ovary, and potentially the laboratory techniques used to prepare the sperm. IUI is often the preferred first-line treatment in unexplained infertility, cervical factor infertility, and mild to moderate male factor infertility [7].

Clinical pregnancy rates may be influenced by the method of ovarian stimulation, type and duration of infertility, sperm parameters, ovarian stimulation response, and female age [5,8]. Preparation of sperm for insemination is a crucial step for favorable clinical outcomes. Semen samples contain cellular debris, leucocytes, prostaglandins, bacteria, and immotile and dead sperm cells. Some of these compositional agents increase oxygen free radicals and may harm the viable spermatozoa [9]. Sperm processing concentrates motile sperm for insemination in a relatively small volume while removing unnecessary and possibly damaging components of the ejaculate. Thus, at the end of the sperm gradient, 0.5 mL–1 mL volume remains for IUI [10]. An important part of the process is washing the sperm, which is traditionally performed twice prior to insemination. Washing twice was chosen based on the concept that it was better than washing once to remove the non-desirable products and debris. However, little effort has been put into determining the ideal number of washes needed for sperm preparation for IUI. Therefore, this study was performed to determine if washing sperm three times is more effective than washing twice in terms of preparing the semen specimen for IUI. It was hypothesized that washing a third time may remove a greater amount of the harmful components than washing twice and result in viable sperm with greater potential for insemination. This would demonstrate itself through an increase in the pregnancy outcomes. Based on

our investigation, this is likely the first study in the medical literature to compare washing the sperm two and three times prior to insemination.

2. Materials and Methods

This retrospective cohort study was conducted at the Assisted Reproductive Center of Medicana Samsun International Hospital, Samsun, Turkey, between April 2015 and April 2017. All couples had primary infertility due to unexplained infertility with or without male sub-fertility. Written informed consent for scientific study participation was obtained from all couples. This study was approved by the Institutional Review Board of Medicana Samsun International Hospital, Turkey.

All 349 unique couples who underwent IUI during the protocol study dates were investigated. Seventy files of couples with other diagnoses of infertility, including endometriosis, tubal factor, anovulation or oligo-ovulation, polycystic ovary syndrome, decreased ovarian reserve, BMI greater than 35 kg/m^2, untreated uterine fibroids or polyps, presence of uterine abnormalities, untreated thyroid or prolactin abnormalities, moderate or severe male factor infertility (defined as total motile sperm counts less than 10 million sperm or strict morphology of less than 4% of normal forms on two semen analyses), and the presence of an ovarian cysts were excluded from the study group. Of the remaining patients who fit the inclusion criteria and underwent care during the study period, 279 were included in the analysis; 136 files were treated with triple sperm washing for IUI (Group 1) and 143 files were treated with conventional double sperm washing for IUI (Group 2). None of the males in the study were proscribed dietary supplements.

Each participating couple had only one attempt of IUI included during this study, which was their first IUI ever performed. All subjects had two patent fallopian tubes on laparoscopy for hysterosalpingography. All couples had basal hormonal tests, semen analysis with at least 10 million total motile sperm count and strict morphology greater than 4%, a normal hysterosalpingography without tubal or uterine factors (no polyps or sub-mucosal fibroids), a day 2–5 trans-vaginal ultrasound evaluation with baseline follicle count greater than 10 and lack of intramural fibroids or ovarian cysts, body mass index (BMI) $<$35 kg/m^2, and antral follicle counts (AFC). All subjects had normal serum thyroid and prolactin levels on at least one of two specimens. These results were recorded and compared as baseline data.

Patient characteristics, type and time of infertility, semen parameters, hormonal parameters, total progressive motile sperm counts, gonadotropins doses, number of follicles, endometrial thickness, and clinical outcomes were collected.

Double sperm washing (conventional method): The first step is to prepare the gradient. To obtain 90% gradient, 9 mL PureSperm (Medicult, Queen's PureSperm, Cooper Surgical, Trumbull, CT, USA) and 1 mL SpermWash (Medicult, Queen's SpermWash 100%, Cooper Surgical, Trumbull, CT, USA) were mixed. A Falcon round-bottom 14 mL tube was used for storage. To obtain 45% gradient, 4.5 mL PureSperm (Medicult, Queen's PureSperm Cooper Surgical, Trumbull, CT, USA) and 5.5 mL SpermWash (Medicult, Queen's SpermWash 100%, Cooper Surgical, Trumbull, CT, USA) were mixed. A Falcon round-bottom 14 mL tube was used for storage.

The male and female patients' first names and surnames were written on both a 15 mL conical Falcon tube and a 5 mL Falcon round-bottom tube for tracking purposes. For patients with a sperm count less than 10 million/mL, 1 mL of the 90% gradient was put into the bottom of a 15 mL conical Falcon tube, and 0.5 mL of the 45% gradient was carefully added over the 90% gradient without mixing the two layers in order to obtain the best sperm quality. For patients with a sperm count over 10 million/mL, 1 mL of the 90% gradient was put into the bottom of a 15 mL conical Falcon tube, and 1 mL of the 45% gradient was carefully added over the 90% gradient without mixing the two layers in order to obtain the best sperm quality. Liquified semen sample was poured on the 45% gradient without mixing, and the prepared gradient was placed into the centrifuge (Eppendorf, Hamburg, Germany) at a rate of 2500–3000 rpm for 20 minutes. Subsequently, the pellet at the bottom of the tube (below 90% of the gradient) was removed using a sterile glass Falcon pasteur pipette. The withdrawn pellet was subsequently placed into a 5 mL Falcon round-bottom tube and, 4 mL of G-IVF solution (Vitrolife, Göteborg, Sweden) was added. The specimen and the tube were then incubated overnight at 37 °C in an atmosphere of 6.8% CO_2 and 5% O_2. The following morning, the Falcon tube containing the sperm and the solution was centrifuged at a rate of 3000–3500 rpm for 10 minutes. Then, the 4–4.5 mL solution at the top of tube was removed and discarded. The remaining 0.5–1 mL of solution containing motile sperm was collected and used for insemination.

In the triple sperm washing technique, all steps are the same as the double sperm washing method except that here, the obtained 0.5–1 mL of pellet of motile sperm were put into a 5 mL round-bottom Falcon tube with 4–4.5 mL of G-IVF (Vitrolife, Göteborg, Sweden) incubated overnight at 37 °C in an atmosphere of 6.8% CO_2 and 5% O_2. The tube was subsequently closed, and it was centrifuged (Eppendorf, Hamburg, Germany) at a rotation rate of 3000–3500 rpm for 10 minutes. Then, the 4.2–4.6 mL of liquid at the top of tube was removed, and the remaining 0.4–0.8 mL of motile sperm were removed from the tube using an insulin syringe, readying the sperm for insemination.

Semen samples with the diagnosis of mild sub-fertility due to mild asthenospermia with $<$20% progressive motile sperm were allocated to triple sperm washing, while samples with progressive motile sperm $>$20% were allocated to double sperm washing for comparison.

Table 1. Baseline characteristics and serum hormone levels of participant.

	Double wash (n = 143)	Triple wash (n = 136)	*p* values
Age (years)	30.04 ± 5.66	29.97 ± 5.09	0.913
Partners age (years)	32.87 ± 6.76	32.05 ± 5.03	0.251
Infertility duration (years)	6.18 ± 4.12	6.08 ± 3.94	0.247
Basal serum FSH (mIU/mL)	6.27 ± 1.94	5.97 ± 1.88	0.787
Basal serum LH (mIU/mL)	6.37 ± 2.06	6.44 ± 1.96	0.102
Basal serum E_2 (ng/m)	47.43 ± 16.08	51.66 ± 17.03	0.642
Basal serum prolactin (ng/mL)	14.02 ± 5.51	13.72 ± 5.57	0.366
Basal serum TSH (uIU/mL)	2.40 ± 0.79	2.54 ± 0.89	0.245

*$p < 0.05$ is significant.

2.1 Ovarian Stimulation, IUI Luteal Phase Support and Pregnancy Confirmation by B-hCG Test

All participating couples had a diagnosis of unexplained infertility and mild male subfertility. Recombinant gonadotropins 75 international units (IU) (Gonal-f, Merck Serono, Darmstadt, Germany) subcutaneous (SC) daily were started on day three of the patients' menstrual cycles, and female patients were monitored for follicular response. Patients were re-monitored on days seven and ten of their menstrual cycles and subsequently at two-day intervals as required. Gonadotropin stimulation continued until the leading follicle reached 17 mm and endometrial thickness was at least 8 mm, at which point a single dose of recombinant hCG 250 μg (Merck Serono, Darmstadt, Germany) was injected subcutaneously 36-hours before IUI.

For IUI, a sterile speculum was used to visualize the uterine cervix, which was cleansed with isotonic solution (Baxter, Eczacıbaşı, Turkey). An IUI catheter (Techno Cath IUI Catheter, Tekservis, Ankara, Turkey) was inserted through the cervical canal, and the prepared sperm was injected over 60 seconds. Oral progesterone 100 mg (Progestan Tablet, Koçak Farma, Istanbul, Turkey) to be taken three times a day was started after insemination as luteal support. B-hCG blood levels were assessed 15 days after the IUI procedure to evaluate pregnancy.

2.2 Statistical Analysis

The data in the study were analyzed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp, Armonk, NY, USA). In the tables, the quantitative data are presented as the mean ± SD, and the categorical data as number (n) and percentage (%). The Student's *t*-test was used to compare the independent groups, and Pearson's chi-square test to compare the categorical variables. Data were determined at a 95% confidence level, and a *p* value of <0.05 was accepted as statistically significant.

3. Results

During the study period, a total of 279 women who fit the inclusion criteria were treated with their first IUI due to infertility at the study center. There was no difference in demographic and basal hormone levels between the study and control groups. Demographic characteristics and baseline hormonal values of all participants included in the analysis are summarized in Table 1.

Total sperm count and total motile sperm count (TMSC; total sperm count in semen volume x % motile sperm concentration/100) before washing were similar in both groups. Interestingly, after washing in the triple wash group, the total number of sperm was higher (81.08 million *vs*. 98.25 million, $p = 0.003$). However, the TMSC after washing was only slightly higher in the triple wash group and not statistically so (Table 2).

Importantly, the pregnancy rate was higher in the triple wash group compared to the double wash group. In the triple wash group, 25.8% of the women achieved pregnancy per cycle, while the rate of achieving pregnancy in the double wash group was 13.3% per cycle. The live birth rate per cycle was also higher in the triple wash group as compared to the double wash group ($p = 0.039$). There was no difference in the miscarriage rate (Table 3).

4. Discussion

A contemporaneous topic in infertility is sperm DNA fragmentation or damage. Studies have suggested that the resultant level of DNA damage may depend on the sperm preparation method used. Xue *et al*. [10] demonstrated that both the swim-up and density gradient centrifugation yielded a significantly lower sperm deformity rate and DNA fragmentation index in comparison to unprocessed whole semen. However, the density gradient centrifugation was better than the swim-up technique at lowering the DNA fragmentation index [11]. In a prospective randomized study comparing the swim-up technique and a density gradient performed in couples with unexplained infertility, the density gradient resulted in higher clinical pregnancy and ongoing pregnancy rates [12]. This study suggested that density gradients result in better outcomes than swim-up sperm preparation for IUI [12]. It has been argued that the centrifugation used in the density gradient may cause sperm DNA damage. However, Karamahmutoglu *et al*. [11] demonstrated better ongoing pregnancy rates with den-

Table 2. Sperm parameters for the double versus the triple sperm washing procedure.

	Double washing (n = 143)	Triple washing (n = 136)	*p* values
Total sperm count before sperm washing (million)	226.70 ± 148.22	202.04 ± 133.17	0.137
Total motile sperm count before washing (million)	78.64 ± 35.82	79.19 ± 42.83	0.875
Total sperm count after washing (million)	81.08 ± 31.57	98.25 ± 62.06	0.003*
Total motile sperm count (million)	64.15 ± 19.29	69.86 ± 18.54	0.247

*$p < 0.05$ is significant.

Table 3. Pregnancy outcomes for the double versus the triple sperm washing procedure.

	Doublewash (n = 143)	Triplewash (n = 136)	*p* values
Positve β-hCG test per cycle start	19 (13.3%)	35 (25.8%)	0.009*
Live birth per cycle start	14 (9.8%)	25 (18.4%)	0.039*
Spontaneous abortion rate per cycle	5 (3.5%)	10 (7.4%)	0.153

*$p < 0.05$ is significant.

sity gradient rather than swim-up techniques, which argues against greater DNA fragmentation occurring with the density gradient. It should also be noted that many sperm preparations for IUI and *in-vitro* fertilization (IVF) are density gradient based. However, Erdem *et al*. [13] studied DNA fragmentation caused by sperm preparation techniques in sub-fertile patients and concluded that sperm DNA damage is more frequent in density gradient preparation methods than with the swim-up technique. They reported that centrifugation may increase the aneuploidy and miscarriage rates possibly due to sperm DNA damage, with a resultant decrease in IUI success [13].

Studies have recommended routine use of sperm DNA fragmentation testing as part of the male infertility workup [13]. However, this is controversial [14], would add significant cost and goes against current guidelines. The role of DNA fragmentation testing is yet to be well established. Simon *et al*. [15] previously reported on the negative impact of increased sperm DNA damage on live birth rates after IVF. Wright *et al*. [16] reported on the relationship between reactive oxygen species and sperm DNA damage. They found higher sperm DNA fragmentation in the presence of varicoceles, smoking, elevated organophosphorus levels, lead, bisphenol A, increased testicular heat, mobile phone use, ambient radiation, obesity, leucocytes in the seminal fluid, advancing male age, and xenobiotics use [16]. Therefore, many factors are involved in the generation of sperm DNA fragmentation. However, sperm preparation for IUI may have the ability to decrease the level of damaged sperm placed in the reproductive tract [11,12].

The aim of this study was to determine if outcomes differed based on two or three sperm washes when preparing sperm for IUI. When washing the sperm three times, we increased the total centrifugation process to three episodes. This also resulted in a slight increase in the total time spent in centrifugation. It is possible that the increased time in centrifugation resulted in an increase in sperm quality to be inseminated and affected the outcomes as compared to the washing procedure alone. A future study would be useful to compare longer and shorter sperm centrifugation times for IUI sperm preparation.

The commonly used sperm preparation method for IUI is the density gradient with double sperm wash and centrifugation. In this study, in addition to the conventional double sperm wash, a third sperm wash and low spin rate centrifugation for 10 more minutes were performed. With this method, it was observed that the total spin rate and total time for centrifugation were slightly increased. Whether it was the additional washing, the longer duration of centrifugation, or a combination of both, that improved outcomes cannot be determined.

In the case of this study, the sperm was incubated overnight with inseminations being performed 12 to 13 hours after ejaculation. Although sperm can live for more than 24 hours in the warming bath, the role of this incubation on outcomes should be considered. It is possible that had the sperm not undergone this incubation and been inseminated 3 to 4 hours after ejaculation that the results of the study may have been different. It is even possible that this prolonged incubation may have in some way increased oxygen radicals in the seminal fluid, which is why the third wash may have resulted in better outcomes.

Being a retrospective study, the results are susceptible to an allocation bias. This is a small- to moderate-sized study, which may have impacted outcomes. However, being the first study to evaluate a third sperm wash and having demonstrated improvement in the live birth rate the results are novel and indicate that a prospective randomized study would be performed. None of the males in the study were proscribed dietary supplements to affect sperm quality. Although an improvement in sperm quality is controversial with these supplements, this may have impacted results [17]. Male obesity was also not considered which may have impacted results as well [18].

The substantial increase in pregnancy rates seen with three as opposed to two sperm washes is surprising. As such the results should be considered as evidence to perform a large prospective study and not to initiate triple sperm washing at this time.

In conclusion, when compared to the double sperm washing method, the pregnancy rates and live birth rates obtained with the triple sperm washing method were found to be higher. This may be due to the overnight incubation performed on the sperm or may be due to the procedure of sperm washing itself. The retrospective nature and the small number of participants are limitations of this study, which may have affected the results. Further prospective randomized controlled studies are warranted to clarify the impact of three *vs*. two sperm washes on IUI outcomes. Such a study may be the only way to limit bias and understand the value of this additional sperm wash.

5. Conclusions

In conclusions, the use of motile sperms obtained from triple sperm washing may increase the rates of pregnancy and live birth in IUI cycles of women with unexplained and mild male factor infertility. A prospective randomized study should be undertaken to confirm the results.

Author Contributions

CSC, SH conceived of this study. CSC, KH, SC, AB, ESH collected the data, and relevant articles and analyzed the data. CSC, SH, MHD wrote the article. CSC, KH, SC, AB, ESH, SH, MHD edited the article.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board of Medicana Samsun International Hospital, Turkey (No. karar Sayisi Tuek 37-2019-BADK/8-69). All subjects gave their informed consent for inclusion before they participated in the study.

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Conflict of Interest

The authors declare no conflict of interest. MHD is serving as one of the editorial board members and guest editors of this journal. We declare that MHD had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to SM.

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