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## Single or double sperm wash processing by density gradient centrifugation: effect on clomiphene citrate induced intrauterine insemination cycle outcomes

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**Aim:** To compare the motility yields of single or double wash after density gradient centrifugation of the ejaculate and their effects on pregnancy rates in clomiphene citrate induced intrauterine insemination (IUI) cycles.

**Materials and methods:** This prospective randomized controlled study included 341 IUI cycles. Sperm specimens processed by density gradient centrifugation either with single wash (the single wash group, n = 170) or double wash treatment (the double wash group, n = 171). Sperm parameters before and after the preparation and the cycle outcomes were evaluated. The main outcome measure was the pregnancy rate.

**Results:** The prewash sperm concentrations, the total motile sperm count, sperm motility and the mean percentage of normal sperm morphology of the groups were similar (P > 0.05). Post-wash sperm concentrations and inseminated motile sperm concentrations were similar in both groups (P > 0.05). Post-wash fast progressive motility (+4 motility) was significantly higher in the double wash group compared to the single wash group (P = 0.017). A total of 62 (18%) clinic pregnancies were obtained in 341 treatment cycles. Clinical pregnancy rate per insemination cycle was 11.8% for the single wash group and 24.6% for the double wash group (P = 0.003).

**Conclusion:** Double wash treatment after density gradient centrifugation of the ejaculate yields a better post-wash fast progressive sperm motility and higher IUI pregnancy rate after ovarian stimulation with clomiphene citrate.

**Key words:** Intrauterine insemination, single sperm wash, double sperm wash, density gradient centrifugation, sperm motility

### Dansite gradyan santrifügasyon yöntemi ile tek ve çift sperm yıkamanın klomifen sitrat ile indüklenen intrauterin inseminasyon sikluslarına etkisi

**Amaç:** Klomifen sitrat kullanılan intrauterin inseminasyon (İÜİ) sikluslarında, sperm örneklerinin dansite gradyan santrifügasyon yöntemi sonrası tek veya çift yıkanmasının sperm motilitesi ve gebelik sonuçları üzerine etkisini görmeyi amaçladık.

**Yöntem ve gereç:** Bu prospektif çalışma 341 İÜİ siklusunu içermektedir. Sperm örneklerine dansite gradyan santrifügasyon sonrası tek yıkama (Tek yıkama grubu, n = 170) veya çift yıkama (Çift yıkama grubu, n = 171) yöntemi uygulandı. Yıkama öncesi ve yıkama sonrası sperm parametreleri ile siklus sonuçları değerlendirildi.

**Bulgular:** Grupların yıkama öncesi sperm konsantrasyonu, total motil sperm sayısı, sperm hareketliliği ve normal sperm morfolojisi oranları benzerdi (P > 0,05). İki grubun yıkama sonrası sperm konsantrasyonu ve insemine edilen motil sperm konsantrasyonu benzerdi (P > 0,05). Yıkama sonrası hızlı ileri hareketli sperm motilitesi (+ 4) çift yıkama grubunda anlamlı olarak yüksek bulundu (P = 0,017). Üç yüz kırkbir siklusta 62 (% 18) klinik gebelik elde edildi. Siklus başına klinik gebelik oranı tek yıkama grubunda % 11,8, çift yıkama grubunda % 24,6 olarak saptandı (P = 0,003).

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**Sonuç:** Klomifen sitrat kullanılan İÜİ sikluslarında, dansite gradyan santrifügasyon yöntemi sonrası çift yıkama yapılan sperm örneklerinde, yıkama sonrası daha fazla ileri hızlı sperm hareketliliği ve daha yüksek gebelik oranları elde edilmiştir.

**Anahtar sözcükler:** İntrauterin inseminasyon, çift sperm yıkama, dansite gradyan santrifügasyon, sperm hareketliliği

## Introduction

Intrauterine insemination (IUI) is frequently used as a first line strategy in the treatment of a high proportion of infertile couples because of its relatively low cost and simplicity. Studies have confirmed its efficiency for specific indications like unexplained infertility, endometriosis, male subfertility and ovulatory disorders (1,2). Pregnancy rates after IUI differ from one study to another depending on the patient selection criteria, the presence of various infertility factors, the methods of ovarian stimulation, the numbers of cycles performed, the different sperm parameters and technique of preparation (3,4).

Predictive sperm parameters and threshold values with respect to the semen characteristics for successful IUI have been controversial. A positive correlation between the pregnancy rate and the total number of motile sperm count inseminated has been challenged in some studies (5,6), but others have reported no such correlation (7,8). Some investigators have found a positive correlation between post-wash sperm motility and pregnancy success (5,9-10).

Many different methods of isolation and concentration of sperm for IUI have been developed. The separation of spermatozoa from seminal plasma to allow capacitation and expression of their intrinsic fertilizing ability is a fundamental prerequisite for assisted reproduction technology. Density gradient centrifugation is a one of the main sperm separation methods that selects spermatozoa on the basis of their density; therefore highly motile spermatozoa with optimal morphology can be selected (11,12). The technique has also been shown to yield sperm populations with better DNA integrity and chromatin packaging (13,14).

The method of processing semen used in the present study includes density gradient centrifugation with either a single or double wash. The literature contains many reports on the effectiveness of different semen preparation methods; there is no sufficient evidence to recommend any specific sperm

preparation technique. Sperm processing techniques vary from one laboratory to another. Our aim was to compare the motility rates of single or double wash after density gradient centrifugation of the ejaculates and pregnancy outcomes in clomiphene citrate induced IUI cycles.

## Materials and methods

This study included a total of 341 IUI cycles by clomiphene citrate (CC) performed at Fatih University Faculty of Medicine, Department of Obstetrics and Gynecology, Reproductive Endocrinology and Infertility Unit, Ankara, Turkey, from March 2007 to May 2009. Indications for the IUI cycles were male infertility in 62 cycles (18%), unexplained infertility in 197 cycles (58%), and male infertility with anovulation in 82 cycles (24%). Ovarian stimulation was achieved by CC (Klomen, Kocak, Turkey, 50 mg) 50-150 mg daily for 5 days starting from the third day of cycle. Follicular development was monitored by transvaginal ultrasonography examination on daily or alternate days starting on day 11 of the cycle. When leading follicle reached 20-22 mm in diameter, 10,000 IU of urinary hCG (Pregnyl Organon, Holland) or 0.25 µg recombinant hCG (Ovitrelle, Serono, Turkey) was administered. The IUI was performed with a catheter (Gynetics # 4219 Emtrac Plus, Gynetics Medical Products N.V. Hamont-Achel Belgium) 36 h after hCG injection.

Sperm samples were initially analyzed for concentration and motility with a Makler counting chamber using WHO guidelines (15). Morphology was assessed by Kruger strict criteria. Two layer density gradient technique (45%-90%) (Pureception 100% Isotonic, Sage, In-Vitro Fertilization, Inc. Trumbull, CT, USA) was used for sperm preparation. Gradients (45%-90%) were warmed to 37 °C. Using a sterile pipette, 1 mL of liquefied semen sample was placed on top of the layer. Gradients were centrifuged at 300 × g for 15 min at room temperature. The pellet

was resuspended in hepes-HTF supplemented with 5% HSA (Sperm washing medium, Sage, In-Vitro Fertilization, Inc. Trumbull, CT, USA) as sperm washing medium, and then centrifuged at  $300 \times g$  for 10 min.

Sperm specimens processed by density gradient centrifugation were either divided into the single wash treatment (the single wash group,  $n = 170$ ) or double wash treatment (the double wash group,  $n = 171$ ) according to the file numbers of patients (even or odd). The final pellet was resuspended in 0.5 mL of fresh hepes-HTF in the single wash group. For the double wash group, after gradient centrifugation, the sperm washing step was repeated twice, and then the pellet was resuspended in 0.5 mL of fresh hepes-HTF. After preparation, samples were reassessed for concentration, total motility, fast progressive motility (+4), and progressive motility (+3). Total sperm recovered by each method was inseminated in a fixed volume (0.3 mL). Sperm parameters before and after the preparation and the cycle outcomes were evaluated. The main outcome measure was the pregnancy rate.

### Statistical analysis

The statistical analysis of the data was done by using SPSS (version 13). The first part was descriptive in the form of mean  $\pm$  SD and frequency and proportion. The second part was analytic to compare between variables using analysis of variance. For qualitative data (frequency and proportion) the chi-squared test was used. The differences were considered to be statistically significant if P was less than 0.05 at a confidence interval of 95%.

### Results

The clinical characteristics of women in the single wash group and the double wash group are presented in Table 1. The groups were similar with regard to age and duration of infertility. The mean number of dominant follicles and endometrial thicknesses were similar in both groups. Sperm parameters of the 2 groups were presented in Table 2. The prewash sperm concentrations, total motile sperm count (TMSC), prewash sperm motility, and the mean percentage of normal sperm morphology of the groups were similar.

Table 1. Clinical characteristics of the patients.

	Single wash group $n = 170$	Double wash group $n = 171$
Age (years)	$28.7 \pm 4.0$	$28.2 \pm 3.9$
Duration of infertility (years)	$2.8 \pm 1.8$	$2.9 \pm 1.3$
Endometrial thickness (mm)	$8.2 \pm 2.6$	$8.3 \pm 2.9$
Dominant follicle count	$1.4 \pm 1.1$	$1.5 \pm 0.8$

Table 2. Comparison of sperm parameters.

	Single wash group	Double wash group	P value
Sperm morphology (%)	$4.6 \pm 3.3$	$4.3 \pm 3.7$	NS
Prewash motile sperm concentration (million/mL)	$62.1 \pm 57.2$	$48.2 \pm 34.5$	NS
Total Motile Sperm Count	$102.1 \pm 8.44$	$61.7 \pm 6.5$	NS
Prewash sperm motility (%)	$53.2 \pm 20.1$	$52.9 \pm 16.0$	NS
Prewash fast progressive motility (+4 motility) (%)	$9.8 \pm 10.9$	$10.3 \pm 9.1$	NS
Prewash slow progressive motility (+3 motility) (%)	$30.7 \pm 13.8$	$31.07 \pm 12.5$	NS
Postwash sperm concentration (million/mL)	$43.4 \pm 41.3$	$37.9 \pm 29.9$	NS
Inseminated Motile Sperm Count (million/mL)	$11.9 \pm 0.9$	$8.6 \pm 0.9$	NS
Postwash fast progressive motility (+4 motility) (%)	$45.1 \pm 22.6$	$53.96 \pm 21$	0.017
Postwash slow progressive motility (+3 motility) (%)	$53.9 \pm 22.4$	$44.8 \pm 19.9$	0.006

Post-wash sperm concentrations and inseminated motile sperm concentration were also similar in both groups. Post-wash fast progressive motility (+4 motility) was significantly higher in the double wash group than the single wash group ( $P = 0.017$ ).

A total of 62 (18%) clinical pregnancies were obtained in 341 treatment cycles. Clinical pregnancy rate per insemination cycle was 11.8% (20/170) in the single wash group and 24.6% (42/171) in the double wash group ( $P = 0.003$ ).

In logistic regression analysis including the prewash sperm concentrations, the prewash sperm motility, the percentage of normal sperm morphology, the post-wash sperm concentrations, the post-wash forward progressive motility, dominant follicle count, and endometrial thickness the only statistically significant determinant for pregnancy was the sperm preparation technique that was double wash after density gradient centrifugation.

## Discussion

Intrauterine insemination has been used with variable success rates for the treatment of numerous indications in infertile couples (16,17). Sperm preparation procedures are necessary to remove prostaglandins, infectious agents, and antigenic proteins. Another advantage of these techniques is removal of the nonmotile spermatozoa, leukocytes, or immature germ cells. This may be an important factor for improving sperm quality by decreasing the release of lymphokines and/or cytokines and reducing the formation of free oxygen radicals after sperm preparation (18). In the present study, we assessed the efficacy of double wash treatment after density gradient centrifugation for the post-wash sperm motility and pregnancy outcomes on CC induced IUI cycles.

Brasch et al. (19) reported an increased pregnancy rate with increasing total motile sperm count. They found a significantly higher chance for pregnancy when total motile sperm count for IUI exceeded  $20 \times 10^6$ . Huang et al. (6) found a similar trend toward an increased success rate with an increased total motile sperm count. In their study, 939 couples undergoing 1375 cycles of IUI were reviewed, and they found that total motile sperm count was significantly higher in pregnant versus nonpregnant cycles when total motile

sperm count exceeded  $5 \times 10^6$ . In contrast, Zhao et al. (20) reported that sperm concentration and total motile sperm count, either before or after processing, did not correlate with IUI outcome. In our study, TMSC was similar in both groups and we could not find a correlation between pre- and post-processed specimen TMSC and pregnancy outcomes.

There is no consensus on the impact of sperm morphology on IUI outcome. Some investigators reported significant effects of sperm morphology on IUI outcome (21,22) whereas others found no influence on IUI outcome (23,24). Ombelet et al. (22) evaluated the prognostic value of the inseminated motile count (IMC) and sperm morphology (using strict criteria) on success rates after homologous IUI combined with CC stimulation. They showed that IUI combined with CC-hCG can be offered as a very safe and non-expensive first-line treatment, at least with an IMC of  $>1 \times 10^6$  spermatozoa. In cases with  $<1 \times 10^6$  spermatozoa, CC-IUI remains important as a first-choice therapy provided the morphology score is  $\geq 4\%$  (22).

Burr et al. (21) suggested that sperm morphology rather than motility is a sensitive guide to IUI outcome and they found no correlation between the number of spermatozoa inseminated and pregnancy. In our study, we have shown that there were no statistically significant differences between the 2 groups with regard to sperm morphology.

Hendin et al. (25) reported that sperm motility was associated with successful IUI outcome; this study referred to post-wash motility rather than motility in the original semen specimen. Zhao et al. (20) found that forward progression score of +3 to +4 (after processing) in the final inseminated specimen is a major factor associated with pregnancy. Progression 1 to 2 (non-motile, non-progressive) did not result in any pregnancy, indicating that sperm motion at moderate to rapid progression is necessary for oocyte fertilization and pregnancy in patients undergoing IUI. Sperm preparation technique in their study was also density gradient centrifugation as in our study, but with the difference of double wash treatment of semen after density gradient centrifugation. In our study, post-wash fast progressive motility (+4 motility) was significantly higher in the double wash group compared to the single wash group.

The efficiency of the double sperm wash processing is assessed in some studies. A higher motile sperm recovery with double wash method compared with other well-known sperm wash methods in cryopreserved and fresh donor sperms was reported (28). In addition, sperm kinematic parameters were also shown to be enhanced after the double method wash. However, the processed sperm in that study were not used for insemination and data on pregnancy outcomes were lacking. Su et al. (29) also compared the sperm kinematic parameters after the double method wash with percoll or after the centrifuge wash method. An almost 2-fold increase was seen in the pregnancy rate with the double method compared with the centrifuge method wash. In this study, the double method wash, not to be confused with double washes whereby a specimen washed twice using same method, was clinically shown to enhance the motility and velocity of sperm when compared with the centrifuge wash method.

In the literature, pregnancy rates per cycle vary from 8% to 22% (3,4) but very low 4% and high 40% pregnancy rates have also been reported (26,27). In our study a total of 62 (18%) pregnancies were obtained in 341 treatment cycles. Clinical pregnancy

rate per insemination cycle was significantly higher in the double wash group (24.6%) than in the single wash group (11.8%).

Although the number of samples in each group might not be considered enough to allow a decision about the superiority of the double wash technique, in this preliminary study sperm preparation by density gradient centrifugation of the ejaculate yielded higher fast progressive scores (+4 motility) and as a result higher pregnancy rates in CC induced IUI cycles.

Until enough evidence from large randomized clinical trials comes for recommendation for the routine use of double wash in intrauterine insemination cycles our results show that double wash improved pregnancy results.

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