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ORIGINAL RESEARCH

Effectiveness of sperm washing by discontinuous density gradient centrifugation to remove antibodies bound to the sperm membrane

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OBJECTIVE: We evaluated the effectiveness of sperm washing using the discontinuous two-layer density gradient centrifugation method to remove antisperm antibodies attached to the sperm surface.

METHOD: We prospectively enrolled sixty-six men with unexplained infertility who were seeking evaluation. Each patient delivered one semen specimen for the study. We determined antisperm antibody levels using the direct immunobeads test. Specimens were classified into two groups according to the pre-washing levels of antibody-bound spermatozoa: group 1 (low antisperm antibodies levels, immunobeads test $<20\%$; $n = 54$), and group 2 (high antisperm antibodies levels, immunobeads test $\geq 20\%$; $n = 12$). Sperm washing was carried out using the discontinuous two-layer colloidal density gradient centrifugation method. Pre- and post-wash levels of antisperm antibodies were compared in the groups.

RESULTS: The pre- and post-wash percentage of spermatozoa with antisperm antibodies attached to their surface was 11% and 6.5% in group 1 (mean difference $= 40\%$; $p < 0.01$), and 30% and 19.5% in group 2 (mean difference $= 36.8\%$, $p = 0.02$), respectively. The effectiveness of density gradient centrifugation in removing antisperm antibodies was not different between the groups, but individual variations from -52.3% to -3.9% were observed.

CONCLUSIONS: Sperm washing by density gradient centrifugation is an overall effective method to remove antibodies bound to sperm membranes, regardless of the levels of antisperm antibodies in the neat semen. Due to an inter-individual variation in the effectiveness of the method, we recommend that each patient be tested before applying sperm washing by density gradient centrifugation in intrauterine insemination.

KEYWORDS: Antibodies; sperm; sperm washing; immunobead test; male infertility.

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INTRODUCTION

Antigens are expressed on developing spermatocytes and spermatids during spermatogenesis.[1] Sperm production takes place as a developmental syncytium protected from recognition by the blood-testis barrier.[1] Rupture of the blood-testis barrier and exposure of sperm antigens to the immune system may occur in cases of obstructive, inflammatory, or traumatic injury of the male genital tract. Exposure of such antigens to immunocompetent cells leads to the formation of antisperm antibodies (ASA).[2]

Antisperm antibodies have been associated with sperm agglutination and immobilization, which impair the progressive movement of the male gamete through the female genital tract.[2] ASA can also affect sperm-oocyte interaction by impairing sperm capacitation and acrosome reaction.[3,4] Embryo arrest at the cleavage state has also been observed when autoimmunity is activated against sperm antigens.[5]

Several strategies have been attempted to minimize the deleterious effects of ASA-mediated infertility. Medication,[6,7] sperm washing combined with intrauterine insemination (IUI),[8] *in vitro* fertilization (IVF),[9–11] and intracytoplasmic sperm injection (ICSI)[12–14] have been reported with varying success rates. Studies focusing on the effectiveness of sperm washing to remove ASA have been performed in the past with conflicting results.[8,15,16] Sperm washing techniques using more efficient and less toxic colloidal gradients, however, have been introduced over the last years, aiming to optimize the effectiveness of the method to select the best fraction of spermatozoa for assisted reproductive techniques.[17]

In this study, we evaluated the effectiveness of sperm washing by discontinuous two-layer silica-coated colloidal density gradient centrifugation in removing antisperm antibodies from the sperm surface.

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METHOD

Patient selection criteria

In this prospective study, we enrolled sixty-six men with unexplained infertility seeking evaluation. All individuals agreed to deliver a single semen specimen by masturbation after a period of ejaculatory abstinence of 2-3 days. Institutional review board approval was obtained prior to conducting the research.

Direct Immunobeads Binding Test (IBT)

Determination of antisperm antibodies in the semen, both before and after sperm washing, was performed by the direct Immunobeads Binding Test (IBT, H + L, Biorad-Irvine Scientific, Santa Ana, USA).[18] Immunobeads are polyacrylamide microspheres coated by human anti-immunoglobulins of classes IgA, IgG and IgM combined. The beads have an equal affinity for human immunoglobulins irrespective of the class. Immunobeads adhere to light or heavy antibody-chains. The test is termed 'direct' when it investigates the presence of ASA on the surface of motile spermatozoa.[2] An aliquot containing 8-10 million motile sperm was removed from the neat semen and the post-wash sperm suspension to test for ASA. Specimens containing spermatozoa to be tested as well as the immunobead suspension were diluted with phosphate buffer saline (PBS, Irvine Scientific, USA, 1:2 v/v) supplemented with 0.3% bovine albumin (Irvine Scientific, USA), and washed by centrifugation at 600*g* for 20 minutes. Both sperm and immunobead pellets were resuspended in 100 μL and 50 μl of PBS supplemented with 5% BSA, respectively. Then, aliquots of 8 μl of both the final sperm suspension and immunobeads were placed on a glass microscope slide and mixed together. A cover glass was placed onto it, and the slides, which were prepared in duplicate, were left for incubation in a humid chamber for 8 minutes at room temperature. Lastly, slides were analyzed under phase-contrast microscopy at X400 magnification to check for the presence of beads bound to the sperm surface,[18,19,20] as illustrated in Fig. 1. Only motile spermatozoa were evaluated, and at least 400 cells were analyzed. The same observer (CF) performed all readings. Results were expressed as the percentage of spermatozoa with beads bound to their membrane, irrespective of the site of binding. Immunoglobulin subtyping was not performed.

Specimens were classified into two groups according to the pre-washing levels of antibody-bound spermatozoa:

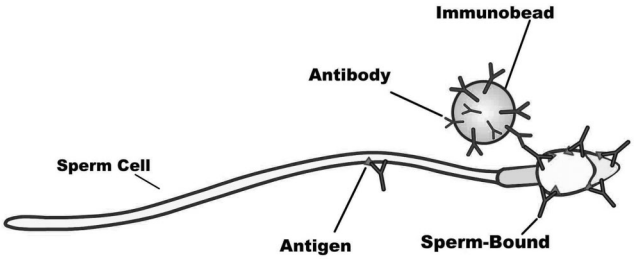

Figure 1 - Illustration depicting the Immunobead Binding Test (IBT). Human anti-immunoglobulin coated-beads are mixed with motile spermatozoa. Anti-immunoglobulins (antibodies) combine with the immunoglobulins (sperm-bound) present on the sperm surface. The presence of beads bounding to the sperm surface is examined under phase-contrast microscopy.

group 1 (low ASA levels, IBT < 20%; n = 54) and group 2 (high ASA levels, IBT ≥ 20%; n = 12).

Sperm washing

A two-layer colloidal gradient (Isolate, Irvine Scientific, USA) was prepared by first transferring 1 mL of the lower phase gradient into a conical bottom disposable centrifuge tube. Then, 1 mL of the upper phase was slowly layered on top of the lower phase using a transfer pipet. A distinct line separating the two layers was observed, and the preparation was kept at room temperature until use. The colloidal suspensions (upper phase: 47%; lower phase: 90%) are composed of silica particles stabilized with covalently bonded hydrophilic silane supplied in hydroxyethyl piperazineethanesulfonic acid (HEPES).

Upon liquefaction and after taking an aliquot for IBT, semen specimens were layered on top of the two-layer colloidal gradient. The preparation was centrifuged at 300*g* for 25 minutes. The supernatant was discarded and the lower phase layer was subsequently diluted with HEPES-buffered culture medium (modified HTF, Irvine Scientific, USA; 1:2, v/v), and washed again by centrifugation at 300*g* for 10 minutes. The pellet was resuspended in 200 microliters of a HEPES-buffered culture media and kept at 37°C. An aliquot was removed for ASA determination by IBT.

Statistical Analysis

Statistical differences in ASA between the groups with low and high ASA levels before and after sperm washing were assessed by the Wilcoxon signed rank test. Differences in semen parameters between the groups were analyzed by Mann-Whitney test. Data were expressed as median and 95% confidence interval. Significance was considered at $p < 0.05$. Analyses were performed using R version 2.14.2 (Free Software Foundation, Boston, MA, USA).

RESULTS

Semen parameters of patients enrolled in groups 1 and 2 are shown in Table 1. The groups were comparable for semen volume, sperm count, sperm motility and sperm morphology results (Table 1).

The pre- and post-wash percentages of spermatozoa with antibodies bound to their surface in groups 1 and 2 are presented in Table 2. The proportion of spermatozoa wih antibodies bound was significantly lower after sperm washing by density gradient centrifugation in both groups. Overall, density gradient centrifugation effectively decreased the percentage of spermatozoa with ASA in 79.6% (43/54) and 75% (9/12) of the cases in groups 1 and 2, respectively. The results did not differ between the groups. Absolute individual differences between pre- and post-wash ASA results ranged from −57.1% to −16.6% in group 1, and −52.3% to −3.9% in group 2.

Individual pre- and post-wash ASA data of group 2 patients is presented in Fig. 2. Post-wash ASA levels remained above the cut-off value of 20% in half (6/12) of the patients.

DISCUSSION

Our findings indicate that sperm washing by density gradient centrifugation is an effective method to remove antisperm antibodies. Overall, the proportion of

Table 1 - Semen parameters of sixty-six men with immunobead test (IBT) results below and above the cut-off point of 20%

	IBT $<$20% (n = 54)	IBT $\geq$20% (n = 12)	P value
Volume (mL)	3.6 (2.5; 4.4)	3.2 (2.7; 4.0)	0.77
Count (X10^6/mL)	65.7 (29.7; 99.1)	82.9 (46.9; 125.6)	0.16
Motility (%)	78.0 (68.0; 82.0)	75.5 (71.5; 79.5)	0.95
Progressive motility (%)	62.0 (56.0; 73.0)	65.5 (61.0; 70.0)	0.65
Vitality (%)	79.0 (74.0; 85.0)	77.0 (75.0; 82.5)	0.76
Strict morphology (%)	8.0 (5.0; 10.0)	9.0 (5.0; 12.0)	0.13

Data reported as median and 95% confidence interval. Mann-Whitney test used to compare semen parameters between the groups.

antibody-bound spermatozoa was 40% lower after the sperm washing procedure. The method's effectiveness was unrelated to the percentage of antibody-bound spermatozoa in a given specimen, since removal occurred to a similar extent in specimens with either low or high proportions of antibody-bound spermatozoa.

Density gradient centrifugation, usually applied as a sperm washing method to select the best motile sperm fraction for assisted reproductive techniques, can also be used to decrease the proportion of ASA in the semen. In our patient population, the method was effective to decrease ASA in three out of four individuals. Despite being effective to decrease ASA in most individuals, the method was unable to decrease the proportion of antibody-bounded spermatozoa to values below the cut-off point of 20% in half of the patients with high ASA levels. Our findings also indicate that a high inter-individual variability exists in the ability of density gradient centrifugation to elute ASA, as shown by the large 95% confidence intervals. We therefore suggest that each patient should be tested prior to applying density gradient centrifugation to elute ASA for intrauterine insemination.

Our study depended primarily on a discontinuous two-layer colloidal gradient in which colloids were composed of silane-coated silica particles in a buffered culture medium. The reason we selected this method was because this is simple and easy to perform in the laboratory, and the reagents are endotoxin-tested and supplied ready-to-use by the manufacturer. The aforementioned discontinuous two-layer colloidal density centrifugation has been used in our assisted reproductive techniques (ART) program as the method of choice for sperm washing for the last ten years. While we were successful overall in removing ASA by density centrifugation, other methods have been applied to obtain ASA-free sperm pools. Simple dilution with culture media was attempted in the past, but results were poor.[21,22] As normal antigen-antibody reactions usually have affinity constants between 10^7 and 10^9 L/mol, a simple washing step was shown to be insufficient for removing antibodies from sperm surface.[23] The swim-up technique has been used as well, with overall disappointing results. In one study, Adeghe evaluated nine ejaculates with ASA. Each ejaculate was split into three portions; one was washed as soon as ejaculation occurred, another was washed 2 h later, and the remaining portion was washed by swim-up. The authors observed that the percentage of spermatozoa with ASA significantly increased with incubation in native semen, but neither simple washing nor swim-up could decrease ASA levels.[24] Windt et al. tested eleven semen samples before and after a wash/swim-up procedure and did not find significant differences in the proportion of ASA-positive sperm as assessed by direct immunobead test.[25] Jeulin et al., studying 51 men, observed variable success of the swim-up method in eluting ASA bound to spermatozoa.[26] Lastly, Almagor et al.[28] used a discontinuous colloidal gradient that resulted in a significant reduction in the antibody loading on the sperm head, but did not decrease antibody binding to the sperm tail. The authors of the aforementioned study used non-coated silica particles (Percoll) in their washing method, which could explain the differences in effectiveness as compared with our findings.

The clinical implication of eluting ASA prior to therapeutic procedures, such as intrauterine insemination, would be to increase pregnancy rates. The possibility of achieving a better recovery of non-immunologically compromised spermatozoa by sperm washing is compelling, since inhibition of sperm motility, as well as fertilization and acrosome reaction by ASA have been well documented.[3,4,27] The advantage of applying intrauterine insemination to infertile couples in which ASA is contributory to the infertility status relies on the fact that IUI is a simple and relatively inexpensive modality of ART, which yields good results in cases of unexplained infertility.[28] Other therapeutic approaches to overcome ASA-mediated male infertility include oral administration of corticosteroids, in vitro fertilization and intracytoplasmic sperm injection. Therapeutic protocols based on immunossupressive steroids have been discontinued[6,21] due to reports of major side effects with long-term use.[6,29] Conventional in vitro fertilization (IVF) usually results in lower fertilization and cleavage rates in cases involving ASA-mediated infertility.[10,11,21,30] Intracytoplasmic sperm injection, on the other hand, has been

Table 2 - Effect of density gradient centrifugation on ASA levels before and after sperm washing in patients with low (IBT $<$ 20%) and high (IBT $\geq$20%) ASA

% Antisperm antibodies	Group 1 (IBT $<$20%)	Group 2 (IBT $\geq$20%)	P value
Pre-wash	11.0 (7.0; 15.0)	30.0 (24.0; 32.5)	$<$0.001
Post-wash	6.5 (4.0; 11.0)	19.5 (13.0; 27.5)	$<$0.001
Percent change	$-$40.0 ($-$57.1; $-$16.6)	$-$36.8 ($-$52.3; $-$3.9)	0.75
P value	$<$0.01	0.02	

Data reported as median and 95% confidence interval. Percent change calculated as median of individual changes. Wilcoxon rank sum test used to compare pre- and post-wash ASA levels, as well as ASA levels between the groups.

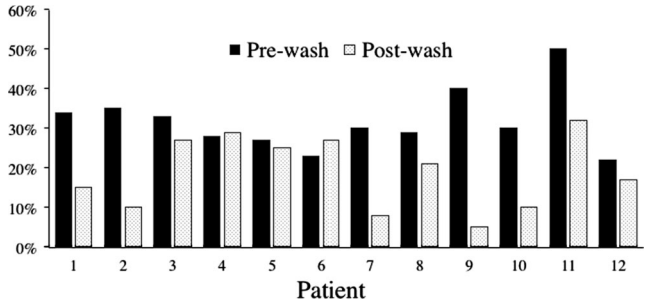

Figure 2 - Distribution of individual ASA results in group 2 (IBT ≥20%) before and after sperm washing by density gradient centrifugation.

shown to successfully overcome any deleterious effects of ASA. ICSI has yielded excellent results irrespective of the ASA levels, and results in fertilization rates, embryo development, and pregnancy rates similar to that which has been observed in other categories of male infertility.[12–14] Despite being successful, ICSI carries a high economic burden for patients.[28]

Although we demonstrated that sperm washing by discontinuous two-layer density gradient centrifugation was useful to remove ASA in the semen, our study has shortcomings. We did not compare density gradient centrifugation with other methods of washing, or with multi-layer density centrifugation. Moreover, we did not test the effectiveness of the post-wash sperm pools in intrauterine insemination. Therefore, caution should be applied to the interpretation of our findings until well-designed clinical trials confirm the effectiveness of density gradient centrifugation for eluting ASA and increasing the chances for conception in couples with unexplained infertility.

CONCLUSIONS

Our data indicate that the sperm washing by discontinuous two-layer density gradient centrifugation is an effective method to decrease ASA load in the semen. The method has similar effectiveness in specimens with low and high ASA levels in the native semen. However, due to large inter-individual variation in ASA removal, we recommend that each patient be tested before applying sperm washing by density gradient centrifugation with intrauterine insemination.

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