Induction effectiveness of acrosome reaction in prepared human spermatozoa assessed using the CD46 surface antigen

Grzegorz Bakalczuk[1], Artur Wdowiak[2], Magdalena Lewicka[1], Magdalena Sulima[1], Sava Perovic[3], Juric Anton[3], Miroslaw A. Kielak[4]

[1] Department of Obstetrics, Gynaecology and Obstetric-Gynaecological Nursing, Faculty of Health Sciences, Medical University, Lublin, Poland

[2] Diagnostic Techniques Unit, Faculty of Health Sciences, Medical University, Staszica 4-6, 20-081 Lublin, (Collegium Maximum), Poland

[3] Department of Urology, University Children's Hospital, School of Medicine, University of Belgrade, Serbia

[4] Healthpoint Medical Group-Tampa, Florida, USA

European Journal of Medical Technologies
2016; 1(10): 44-53

www. medical-technologies.eu
Published online 22.03.2016
7.00 scores MNiSW

Corresponding address:
Artur Wdowiak
Diagnostic Techniques Unit, Faculty of Health Sciences, Medical University in Lublin (Collegium Maximum), Poland, 20-081 Lublin, Staszica street 4-6

Abstract

The semen used for artificial insemination must be subject to appropriate preparation, which involves separating sperm from seminal plasma and selecting those having the greatest reproductive capacity. One way of preparation was the centrifugation of semen by means of Percoll gradient.

Our study was part of a larger research program, in which we examined the properties of the different techniques of sperm preparation. The aim of this study was to investigate whether the technique used for the preparation of semen on the basis of Percoll gradients affects the quality and the occurrence of

Key words:

acrosome reaction, Percoll, flow cytometry, semen preparation, CD46 antigen

acrosome reaction in human sperm. The study included 30 ejaculates obtained from patients ranging 21-53 of age. The samples of ejaculate were divided into two equal volumes. The first group was subject to the preparation with different Percoll gradients and the second group was without preparation. The induction of acrosome reaction with the calcium ionophore was performed on both group. Preparation of sperm with a gradient of Percoll significantly improved semen parameters such as motility, density and percentage of morphologically normal spermatozoa, but negatively affected the occurrence of the human sperm acrosome reaction. Preparation of the Percoll gradient was not practical in clinical use. Viability (VIAB) was the only practical parameter in the case of usefulness of classical semen analysis in predicting the ability of sperm to achieve the state of capacitation.

Introduction

Conjugal infertility affects an increasing number of married couples in the Polish society, and the problem of treatment of this condition is becoming an increasingly important challenge for medicine. Infertility treatment centers receive couples who are offered to undergo intrauterine insemination as the initial method of treatment. This procedure consists in directly placing the sperm in the uterine cavity. The condition for carrying out these procedures are unobstructed fallopian tubes and adequate sperm quality.

The semen used for artificial insemination must be subject to appropriate preparation, which involves separating sperm from seminal plasma and selecting those having the greatest reproductive capacity. One way of preparation was the centrifugation of semen by means of Percoll gradient. Sperm placed in the woman's reproductive tract undergo a process of capacitation, during which they are prepared for the fertilization of the ovum [1,2]. The course of capacitation is affected by substances contained in the fluid in which sperm are suspended. The fluid contains secretions of testicular tissues and epididymis, extra male glands: seminal vesicles, prostate and bulbourethral glands, as well as the secretion of the uterus and vesicular fluid [1]. In the case of insemination process, capacitation may also be affect by substances, such as Percoll among other, used in the preparation of semen which sperm are transferred into [3].

During capacitation, the sperm becomes hyperactive, which means increased motility due to increase in frequency and force of impact of the tails, leading to changes of the movement path of the male gamete. Hyperactive sperm move along a curved track, so that they can penetrate the zona pellucida and the fertilized egg. When moving rectilinearly in the seminal fluid, sperm do not have the ability to fertilize the oocyte [4].

Sperm, which may have the necessary motility parameters and being able to achieve the state of capacitation necessary to cross the oocyte barriers, should bind to the zona pellucida and then complete the acrosome reaction, so that its action can bring about pregnancy. Acrosome reaction is the last step of activating the male gamete and is essential to the subsequent penetration of the zona pellucida by sperm, with the possibility of it being mediated by acrosome enzymes released during this process. Acrosome reaction is also preparing the sperm to merge with the cell membrane and can be induced by follicular fluid, progesterone, oocytes and chemicals such as the Ca^{2+} ionophore [5,6,7]. In the literature, we can find reports indicating that only 15-45% of correctly motile sperm used in IVF are capable of acrosome reaction in *in vitro* conditions [8,9,10,11]. The ability to predict whether the sperm have the ability to complete the process of fertilization would be of great importance in the diagnosis and treatment of the so-called idiopathic infertility cases, but also in making decisions on how to fertilize the ovum *in vitro*.

This creates a need to search for new predictive factors of male fertility, which may include specific hypoglycosylated isoform of the regulator complement of the membrane cofactor protein (MCP – mebrane cofactor protein, CD46), being located on the inner membrane of the sperm acrosome. Human membrane cofactor protein (MCP, CD46) is a ubiquitously expressed protein known to protect cells from complement attack. This membrane is exposed after the acrosome reaction and is subject to the phenomenon of exocytosis occuring after contact with the zona pellucida of the ovum. MCP plays an important role in the reproduction, and its expression in human spermatozoa is strictly limited to the inner membrane of the acrosome [9,10,11]. Changes in the expression of MCP on spermatozoa have been associated with infertility [12,13] because the MCP can be a target for antisperm antibodies [14]. It is also believed that MCP is also linked with the flow of calcium ions through the inner membrane of acrosome [9]. The subsequent hypothesis concerns the complement regulatory function of MCP, thanks to which it can protect the spermatozoon already undergoing the acrosome reaction in the final stages of fertilization.

Flow cytometry is used to study mammalian acrosome reaction [17], and, in conjunction with lectins related with FITC (fluorescein isothiocyanate), to research human acrosome reaction [18]. The CD46 antibody [19,20,21] associates antigens present on the inside of the acrosome of the sperm membrane [22]. Use of CD46 antibodies in association with the flow cytometry could become a rapid test to routinely assess the acrosome reaction [22].

Percoll was previously used in assisted reproductive technology to select sperm from semen by density gradient centrifugation, for use in techniques as in vitro fertilization or intrauterine insemination. However, in 1996, Pharmacia sent out a letter to laboratories stating that Percoll should be used for research purposes only, not clinical. Pharmacia had not marketed it as a sperm preparation product; it had been repackaged and sold as such by third-party manufacturers. The FDA Warning Letter was due to Concerns that PVP might cause damage to sperm (an unknown issue), and also that some batches of Percoll contained high levels of endotoxin (a known issue). The latter concern also applies to the use of Percoll with any other cells that might be injected back into the patient, since endotoxin can cause severe inflammation and fever. Since then, it has been replaced with other colloids in the ART industry [3].

Our study was part of a larger research program, in which we examined the properties of the different techniques of sperm preparation. The aim of this study was to investigate whether the technique used for the preparation of semen on the basis of Percoll gradients affects the quality and the occurrence of acrosome reaction in human sperm.

Materials and methods

The study included 30 ejaculates obtained from patients ranging 21-53 of age (mean: 32.6 ± 7.2 years old, median: 30 years years old) coming for routine examination during the diagnosis of male infertility. Material consisted of semen samples obtained in the seminological laboratory of the Fertility and Andrology Clinic of Medical University of Lublin.

All samples have been obtained by means of masturbation after 48-72 hours of abstinence and put in sterile containers, having been analyzed according to the WHO protocol in 1999 [32]. We excluded patients whose MAR test result was positive.

Examination technique

The samples of ejaculate were divided into two equal volumes. The first(study) group was subject to the preparation with different Percoll gradients and the second (control) group was without preparation.

Preparation technique with various gradients of Percoll was carried out according to the following protocol. Percoll solutions (0.5 ml of 95% and 47.5%) were gently stacked to a sterile 5 ml Falcon tube (Becton Dickinson, USA); 0.3-0.5 ml of semen was gently stacked on surfaces of the gradient, and the mixture was centrifuged at 350 g for 20 minutes at room temperature. Percoll layers were then aspirated and the remaining semen sediment was collected and diluted

in 2 ml of F10 (medium of cultured sperm) (Sera and Vaccines Manufacturing, Lublin). It was then centrifuged at 300 g for 10 minutes at room temperature, the supernatant was aspirated and the sediment was diluted in 1 ml of F10.

The induction of acrosome reaction with the calcium ionophore was performed on both group (after preparation with Percoll) and the control group (without preparation).

We added 10 l calcium ionophores to both tubes (A 23187 USA Sigma). They were closed and incubated at 37°C for 45 minutes. After incubation, 5 ml of F10 was added to each tube, and then they were centrifuged at 300 g for 10 min. The supernatant was aspirated and 0.5 ml of F10 was added to the remaining sediment to each tube.

The resulting suspension was diluted with 2 ml of PBS (0.9% buffered physiological saline solution – Label Sera and Vaccines, Lublin) and centrifuged at 500g for 5 minutes. We added 5 l of human monoclonal anti-CD46 associated with FITC (fluorescein isothiocyanate) to both test tubes, and then mixed with the sediment by slightly shaking. The suspension was then incubated in the dark at room temperature for 15 minutes, and then rinsed twice in PBS. The final sediment was diluted in 1 ml of PBS.

Data acquisition and analysis was performed using a BD FACSCalibur flow cytometer (FlowCytometry-CoreFacility) (Becton Dickinson, USA) equipped with a 488 nm argon laser and the CellQuest computer software (Becton Dickinson, USA) [26].

For each test sample, we performed an acquisition of 2x104 cells based on the gate formed in the FSC/SSC coordinate system (forwardscatter/sidescatter). Both cells exhibiting positive fluorescence, as well as mean fluorescence intensity (MFI – mean fluorescence intensity), were assessed, which indirectly determines the amount of antigen for the examined cell. The MFI value of the inspected antigen was calculated after subtracting the MFI isotype control. In order to optimize the settings of the flow cytometer, we used the Calibrite calibration kit (Becton Dickinson, USA).

The results were statistically analyzed. The values of the analyzed parameters measured on the ratio scale were characterized by means of the mean value, standard deviation, median, 25th and 75th percentile indicating the range of variation.

To assess the compatibility of decomposition of tested parameters with normal distribution, we used the Shapiro Wilk test. Because of the skewed distribution of the tested parameters, we used the Wilcoxon test to compare results before and after treatment. In contrast, to investigate the existence of the relationship between the analyzed parameters, we used the Spearman rank correlation coefficient.

Spearman's rank correlation coefficient (rho), as a measure of the direction and strength of the relationship, may assume numerical values within the range of <−1, +1>. In case of positive value, this means a directly proportional relationship, while a negative value – an inversely proportional relationship. The closer the absolute value to 1, the stronger the relationship.

The inference error was set at 5% and the associated $p<0.05$ significance level shows the existence of statistically significant differences or correlations.

Statistical analyses were performed based on the STATISTICA software v.6.0 (StatSoft, Poland).

Results

When comparing the average percentage of CD46+ sperm between the groups, it was found that higher values are more likely to occur in the group not subject to preparation than in the control one, with the difference being statistically significant ($Z = 3.07$; $p = 0.002$). Statistical analysis showed no statistically significant differences in mean fluorescence intensity of the CD46+ sperm of control group compared to the values observed in the study group ($Z = 0.85$; $p = 0.39$) (Fig. 1).

When comparing the average sperm density in both groups, it was shown that it has higher values in the study group, but these relations were not statistically significant ($Z = 1.78$; $p = 0.07$). In the case of the percentage of sperm pathology of the test samples involved, higher values were found in the control group, with these differences being statistically significant ($Z = 3.89$; $p<0.005$) (Fig. 2). During the evaluation of sperm motility, it was shown that

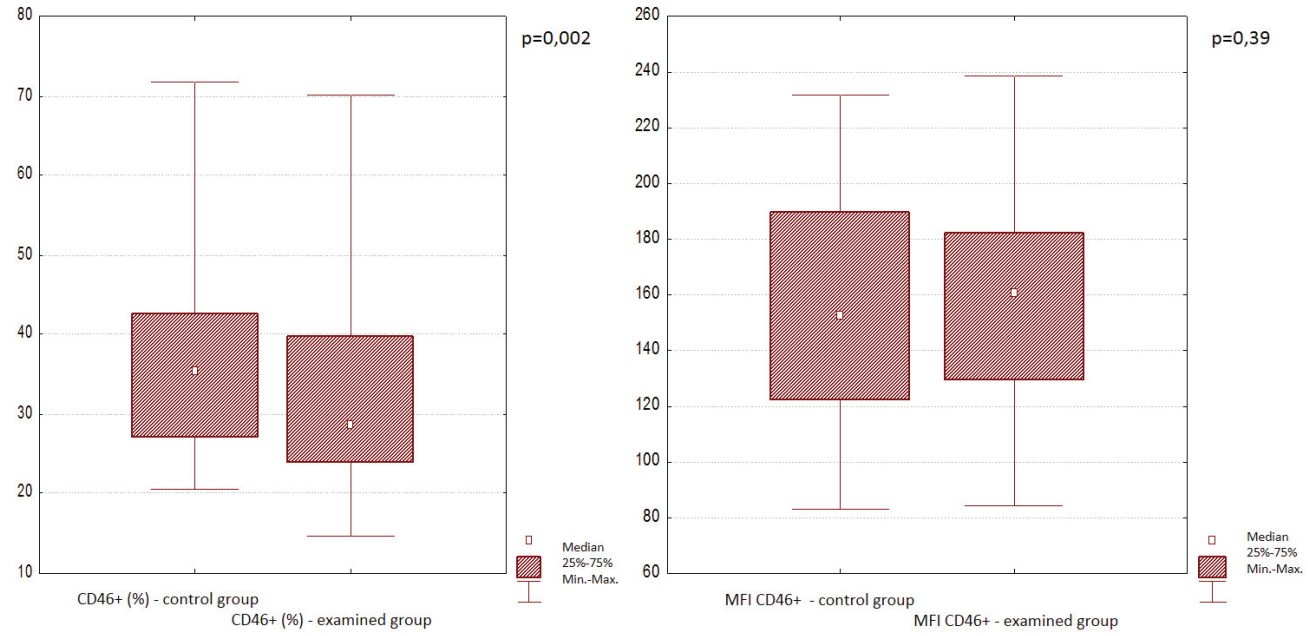

Fig. 1.

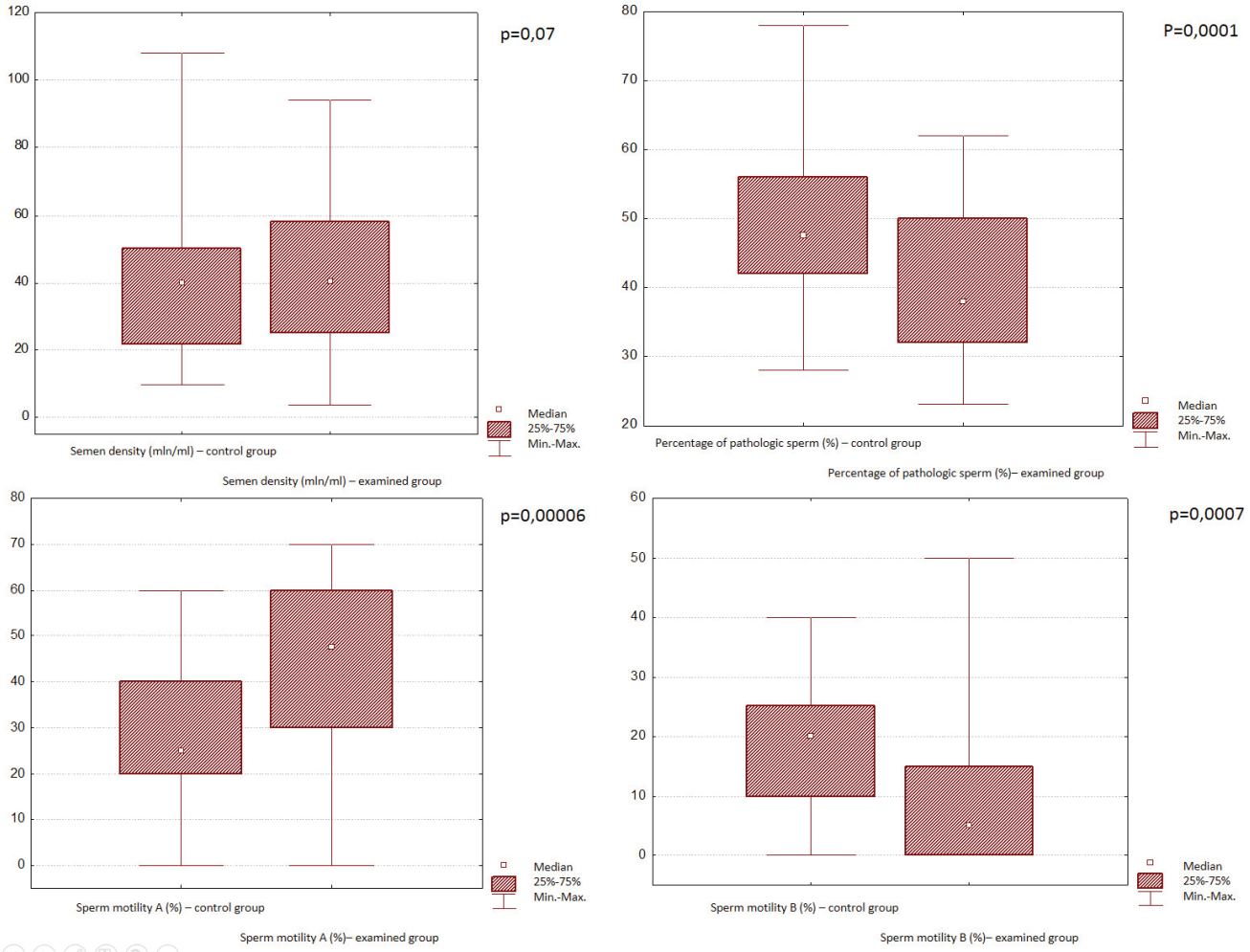

Fig. 2.

significantly lower values of the average mobility of type A are in the control group when compared with the value of the test group (Z = 4.01; p<0.005). In the case of motility values of types B and C, they were significantly higher in the control group compared to the test one (Z = 3.4, Z = 3.47; p<0.005). Parameters such as vitality, HOST and pH did not differ significantly between the two groups.

In the control group, the average higher values of MFI CD46+ were accompanied by higher VIAB values, with this relationship being statistically significant (r = 0.59; p = 0.0006). There were no statistically significant correlations between the percentage of sperm cells CD46+ of the control group and VIAB (r = −0.36; p = 0.047). In the study group, we found close significances of the correlations between the average percentage of CD46+ and viability (R = −0.35; p = 0.055), while in the case of MFI CD46+ this relationship was not statistically significant (R = 0.45; p = 0.01) (Fig. 3).

In the control and the study groups, we found no statistically significant correlations between the mean fluorescence intensity of CD46+ sperm and HOST, pH, density of sperm, percentage of pathological sperm count and motility A, B, C.

Discussion

The acrosome reaction of human sperm induced by calcium ionophore test method described in this paper is a simple, fast and accurate technique that can be helpful in the study of the causes of male infertility [7]. Detection of acrosome reaction in induced human sperm by means of a flow cytometer, monoclonal antibodies [33] and binding lectins with acrosome matrix [34,35] has already been described by many authors and considered the most accurate method of assessing the acrosome reaction [17].

In this research, we used the method for determining sperm acrosome reaction using the CD46 monoclonal antibodies. In their work, Liu et al. and have shown that there is a correlation between the acrosome reaction in living sperm and the acrosome reaction occurring throughout the sample of sperm after incubation with calcium ionophore [36]. They showed that there are no significant differences in the results of the studied acrosome reaction, regardless of whether the whole ejaculate was subject to the examination or only the living sperm obtained after the preparation of semen.

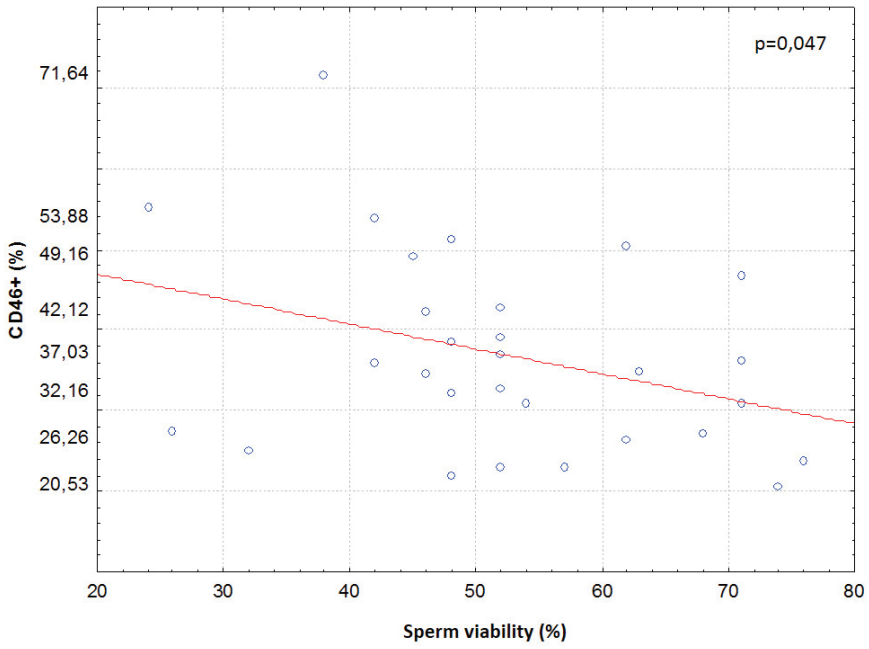

Fig. 3.

Our audit was limited to semen samples obtained from patients with normal sperm parameters. The previously described by Bohring C. et al. possibility of antisperm antibodies occurring in normal donors and the acrosome reaction, induced by the calcium ionophore, may be inhibited by sperm antibodies [35]. Therefore, we included only the patients with negative MAR test in the study.

Many researchers who focused on the sequence of changes associated with the acrosome reaction [38] determined that the first phase of the acrosome reaction which can be visualized by means of fluorescence microscopy is associated with the presence of sperm with an intact acrosome during dyeing.

Calcium ionophore-induced acrosome reaction is not a physiological process. However, the ARIC test (acrosome reaction to ionophore challenge) is recommended as a practical test in the diagnosis of infertility, as correlations were found between human sperm response to calcium ionophore and their ability to fertilize [39].

Flow cytometry is a method that allows not only the detection of sperm subpopulations where acrosome reaction occurred or not occurred, as well as a quantitative assessment of their morphology, functions and metabolic parameters. Brook et al demonstrated the existence of two subpopulations of human sperm with a different intracellular pH obtained upon stimulation of acrosome reaction by means of calcium ionophore [41]. Differences in the levels of intracellular calcium levels are described in two subpopulations of cells after the induction of acrosome reaction of bull and boar sperm [42,43]. It is obvious that flow cytometry can be of great use in the study of acrosome reaction mechanisms and its abnormalities, which can affect fertility.

In the present study, we assessed the total acrosome reaction before (control group) and after (study group) preparation by means of the difference of the concentration gradients using Percoll. Accordingly, calcium ionophore was utilized as the inductor of the acrosome reaction, and the monoclonal antibody anti-CD46 – as a marker of acrosome reaction enabling assessment by means of flow cytometer.

In this study, we have observed statistically significant lower values of pathological sperm in the study group (40.9%) compared to the control group (48.5%) and almost statistically significant increase in the number of sperm in the study group (41.8 million) compared to the control group (39.2 million). These results are consistent with results obtained by Nolan J.P. et al. and Carver-Ward J.A. et al. and prove that preparation of semen was properly conducted [43,44].

The test results obtained using a flow cytometer include: the percentage of sperm capable of inducing acrosome reaction amounted to 36.89% in the control group and 31.57% in the test group. The CD46+ values of the control group were significantly higher than in the study group ($Z = 3.07$; $p = 0.02$). In contrast, the CD46+ MFI values in the control group was 155.08 and 151.94 in the study group, where no statistically significant differences were shown in the test group in comparison to the control group ($Z = 0.85$; $p = 0.39$). In this work, we achieved significantly higher statistical sperm values associated with the CD46 antibody control group (36.89%) compared to the corresponding values in the study group (31.57%). This phenomenon can be explained through the damaging effect of Percoll to the membrane of the acrosome. It can manifest itself in the decreased number of sperm entering the acrosome reaction while improving other basic semen parameters such as percentage of morphologically normal sperm, motility and density.

The present study examined the effects of human sperm preparation by means of the difference between concentration gradients of Percoll to call induced acrosome reaction of sperm. Semen quality can be improved during preparation by means of: removal of immotile sperm, improved morphological and biological parameters and a statistically significant increase in sperm motility may be the cause of the decline of induced acrosome reaction after preparation. Removal of sperm with abnormal morphological and biological parameters reduces the likelihood of premature acrosome reaction. From the standpoint of predicting the chances of fertilization, the amounts of both normal sperm parameters,

motility, and their ability to acrosome reaction are crucial.

The results obtained from our research confirm the rightness of the Pharmacia concern's decision to ban Percoll in the case of assisted reproduction techniques and explain one of the harmful mechanisms of this preparation [46,47,48].

Conclusions

- Preparation of sperm with a gradient of Percoll significantly improves semen parameters such as motility, density and percentage of morphologically normal spermatozoa, but negatively affects the occurrence of the human sperm acrosome reaction.
- Preparation of the Percoll gradient is not practical in clinical use.
- VIAB is the only practical parameter in the case of usefulness of classical semen analysis in predicting the ability of sperm to achieve the state of capacitation.

References

1. Wassarman PM. The biology and chemistry of fertilization. Science 1987; 235: 553-560
2. Wassarman PM. The zona pellucida: a coat of many colors. BioEssays 1987; 6: 161-166
3. Mortimer D. Sperm preparation methods. Journal of Andrology 2000; 21(3): 357–66. PMID 10819443. Retrieved 5 May 2010
4. Takahashi K, Wetzels AM, Goverde HJ, Bastaans BA, Janssen HJ, Rolland R. The kinetics of the acrosome reaction of human spermatozoa and its correlation with in vitro fertilization. Fertil Steril 1992; 57: 889-894
5. De Jonge CJ, Mack SR, Zaneveld LJD. Synchronous assay for human sperm capacitation and the acrosome reaction. J Androl 1989; 10: 232-239
6. Gianesello L, Ferlin A, Menegazzo M, Pepe A, Foresta C. RXFP1 is expressed on the sperm acrosome, and relaxin stimulates the acrosomal reaction of human spermatozoa. Ann N Y AcadSci 2009; 1160192-3
7. Cross NL, Morales P, Overstreet JW, Hanson FW: Two simple methods for detecting acrosome-reacted human sperm. Gamete Res 1986; 15: 213-226
8. Lee TH, Liu CH, Shih YT, Tsao HM, Huang CC, Chen HH, Lee MS. Magnetic-activated cell sorting for sperm preparation reduces spermatozoa with apoptotic markers and improves the acrosome reaction in couples with unexplained infertility. Hum Reprod 2010; 25(4): 839-46
9. Falzone N, Huyser C, Franken DR. Comparison between propidium iodide and 7-amino-actinomycin-D for viability assessment during flow cytometric analyses of the human sperm acrosome. Andrologia 2010; 42(1): 20-6
10. Riley-Vargas RC, Atkinson JP. Expression of membrane cofactor protein (MCP; CD46) on spermatozoa: just a complement inhibitor? Modern Aspects of Immunobiology 2003; 3: 75-78
11. Yudin AI, Vandevoort CA, Li MW, Overstreet JW. PH-20 but not acrosin is involved in sperm penetration of the macaque zona pellucida. Mol Reprod Dev 1999; 53(3): 350-62
12. Anderson DJ, Michaelson JS, Johnson PM: Trophoblast/leukocyte-common antigen is expressed by human testicular germ cells and appears on the surface of acrosome-racted sperm. Biol Reprod 1989; 41: 285-293
13. Fenichel P, Dohr G, Grivaux C, Cervoni F, Donzeau M, His BL. Localization and characterization of the acrosomal antigen recognized by GB24 on human spermatozoa. Mol Reprod Dev 1990; 27: 173-178
14. Kitamura M, Matsumija K, Yamanaka M, Takahara S, Hara T, Matsomoto M et al. Possible association of infertility with sperm-specific abnormality of CD46. J Reprod Immunol 1997; 33: 83-88
15. Nomura M, Kitamura M, Matsumiya K, Tsujimura A, Okuyama A, Matsumoto M et al. Genomic analysis of idiopathic infertile patients with sperm-specific depletion of CD46. Exp Clin Immunogenet 2001; 18: 42-50
16. Jiang H, Pillai S. Complement regulatory proteins on the sperm surface: relevance to sperm motility. Am J Reprod Immunol 1998; 39: 243-248
17. Anderson DJ, Abbot AF, Jack RM: The role of complement component C3b and its receptors in sperm-oocyte interaction. Proc Natl Acad SCI USA 1993; 90: 10051-10055

18. Morales P, Vigil P, Franken DR, Kaskar K, Coetzee K, Kruger TF. Sperm oocyte interactions: studies on the kinetics of zona pellucida binding and acrosome reaction of human spermatozoa. Andrologia 1994; 26: 131-137
19. Stock CE, Fraser LR. The acrosom reaction of human sperm from men of proven fertility. Hum Reprod 1987; 2: 109-119
20. Garrido N, Meseguer M, Remohi J, Pellicer A, Simon C. Flow cytometry in human reproductive biology. GynecolEndocrinol. 2002;16(6):505-21
21. Purvis K, Rui H, Scholberg A, Hesla S et al. Application of flow cytometry to studies on the human acrosome. J Androl 1990; 11: 361-366
22. Ballard LL, Seya T, Teckman J, Lublin DM, Atkinson JP. A polymorphism of the complement regulatory protein MCP (membrane cofactor protein or gp 45-70). J Immunol 1987; 138: 3850-3855
23. Ballard LL, Bora NS, Yu GH, Atkinson JP. Biochemical characterization of membrane cofactor protein of the complement system. J Immunol 1988; 141: 3923-3929
24. Liszewski MK, Post TW, Atkinson JP. Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. Annu Rev Immunol 1991; 9: 431-455
25. D'Cruz OJ, Haas GG. Flow cytometric quantitation of the expression of membrane cofactor protein as a marker for the human sperm acrosome reaction. Fertil Steril 1992; 58: 633-636
26. Riley-Vargas RC, Lanzendorf S, Atkinson JP. Targeted and restricted complement activation on acrosome-reacted spermatozoa. J Clin Invest 2005; 115(5): 1241-9
27. Lampiao F, du Plessis SS. Insulin and leptin enhance human sperm motility, acrosome reaction and nitric oxide production. Asian J Androl 2008; 10(5): 799-807
28. Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M, Herzenberg LA. The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford Clin Chem 2002; 48: 1819-1827
29. Watson JV. The early fluidic and optical physics of cytometry. Cytometry 1999; 38: 2-14
30. Melamed MR, Lindmo T, Mendelsohn M. Flow cytometry and Sorting. Wiley-Liss New York 1990
31. Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M, Herzenberg LA. The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. Clin Chem 2002; 48: 1819-1827
32. World Health Organization (1999) WHO Laboratory Manual for the Examination of Human Semen and sperm-cervical mucus interaction, 4th edn.: World Health Organization Herzenberg L.A.: FACS innovation: a view from Stanford. Clin Invest Med 2004; 5: 240-25
33. Roliński J. Monitoring minimal residual disease by the flow cytometry methods. Appl Biol Commun 1996; 6: 135-143
34. Bohring C, Skrzypek J, Krause W. Influence of antisperm antibodies on the acrosome reaction as determined by flow cytometry. FertilSteril 2001; 76(2): 275-80
35. Henley N, Baron C, Roberts KD. Flow cytometric evaluauon of the acrosome reaction of human spermatozoa: a new method using a photoactivatedsupravital stain. Int J Androl 1994; 17: 78-84
36. The Use of Anti-VDAC2 Antibody for the Combined Assessment of Human Sperm Acrosome Integrity and Ionophore A23187-Induced Acrosome Reaction Bianjiang Liu, Peng Wang, Zengjun Wang, Wei Zhang, PLoS One 2011; 6(2): e16985
37. Martin G, Sabido O, Durand P, Levy R. Phosphatidylserine externalization in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. Hum Reprod 2005; 20(12): 3459-68
38. Benoff S, Cooper GW, Hurley I, Mandel FS, Rosenfeld DL. Antisperm antibody binding to human sperm inhibits capacitation induced changes in the levels of plasma membrane sterols. Am J Reprod Immunol 1993; 30: 113-130
39. Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J Androl 1988; 9: 367-376
40. Cummins JM, Pember SM, Jequier AM, Yovich JL, Hartmann PE. A test of the human sperm acrosome reaction following ionophore challenge. J Androl 1991; 12: 98-103
41. Brook PF, Lawry J, Cooke ID, Barratt CL. Measurement of intracellular pH in human spermatozoa by flow cytometry with the benzo[c]xanthene dye SNAFL-1: a novel, single excitation, dual emission, molecular probe. Mol Hum Reprod 1996; 2: 18-25

42. Harrison RAP, Mairet B, Miller NG. Flow cytometric studies of bicarbonate-medium Ca2+ influx in boar sperm populations. Mol Reprod Dev 1993; 35: 197-208
43. Nolan JP, Graham JK, Hammerstedt RH. Artificial induction of exocytosis in bull sperm. Arch Blochem Biophys 1992; 292: 311-322
44. Carver-Ward JA, Jaroudi KA, Einspenner M, Parthar RS, Al-Sedairy ST, Sheth KV. Pentoxifylline potentiates ionophore (A23187) mediated acrosome reaction in human sperm: flow cytometric analysis using CD46 antibody. Hum Reprod 1994; 9: 71-76
45. Cooper TG, Yeung CH. A flow cytometric technique using peanut agglutinin for evaluating acrosomal loss from human spermatozoa. J Androl 1998; 5: 542-550
46. Rüssmann L, Jung A, Heidrich HG (1982). The use of percoll gradients, elutriator rotor elution, and mithramycin staining for the isolation and identification of intraerythrocytic stages of plasmodium berghei. Z Parasitenkd 66(3): 273-280. doi:10.1007/BF00925344. PMID 6177116
47. Pertoft H, Laurent TC, Laas T, Kagedal L. Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll). Anal Biochem 1978; 88(1): 271-282. doi:10.1016/0003-2697(78)90419-0
48. Mortimer D. Sperm preparation methods. Journal of Andrology 2000; 21(3): 357-66. PMID 10819443. Retrieved 5 May 2010