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Male Infertility

Relationship Between Human Sperm Morphology and Acrosomal Function

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***Purpose*:** In this study, we investigated the relationship between functionality of the acrosome and sperm morphology.
***Methods*:** Acrosome reaction (AR) was separately determined in live and dead sperm and in those with normal, small, and large sized acrosomes by means of the triple stain. Morphology was analyzed according to strict criteria after Papanicolaou stain.
***Results*:** AR and morphology correlated regarding detection of large and small sized acrosomes, but not for normal sized acrosomes. Spontaneous AR was significantly influenced by acrosomal size. Sperm with large (11.4%) and normal (9.2%) acrosomes exhibited a significantly higher percentage of life spontaneously acrosome-reacted sperm than those with small acrosomes (4.5%). Sperm with small acrosomes were associated with a higher percentage of cell death.
***Conclusion*:** The results indicate that sperm with small acrosomes are more susceptible to cell death and nonphysiological acrosomal loss. Acrosome size reflects the physiological capability of sperm function and therefore male fertility potential.

KEY WORDS: Acrosome morphology; acrosome reaction; sperm morphology; strict criteria; triple staining.

INTRODUCTION

The acrosome reaction (AR) is a receptor-mediated exocytotic cellular process involving fusion of the sperm plasma membrane and the outer acrosomal membrane that results in the release of the acrosomal enzymes (1). This process can be induced by different physiological substances like follicular fluid (2), progesterone (3), zona pellucida protein three (ZP_3) (4) and nonphysiological substances such as calcium ionophore A23187 (5) and low temperature (6–8). Since only acrosome-reacted spermatozoa can penetrate the zona pellucida (9), the AR is an essential requirement for mammalian fertilization and the occurrence thereof has repeatedly been shown to be predictive for fertilization in vitro (7,10). Normal acrosome reaction of spermatozoa is therefore an essential requirement for normal mammalian fertilization, and patients showing aberrations in the capability of their spermatozoa to undergo the AR may present a lowered fertility potential (11).

Sperm morphology as evaluated by strict criteria (12), one of the important parameters of the standard semen analysis, is another good predictor for fertilization in vivo (13) and assisted reproduction (14–16). In contrast to the evaluation of the acrosome reaction, morphology is a simple and cost-effective method that can be performed in every andrological and IVF laboratory, after thorough training (17). Sperm morphology also correlates significantly with the sperm cell's ability to bind to the zona pellucida (18,19). In

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addition, Liu and Baker (20) and Menkveld *et al*. (21) demonstrated that normal sperm acrosomal morphology correlated significantly with sperm binding to the zona pellucida while Franken *et al*. (4) showed that a strong relationship exists between normal sperm morphology and the inducibility of the acrosome reaction. However, only little is known about the relationship between acrosomal size and acrosomal functionality. Therefore, the aim of this study was to investigate the relationship between acrosomal functionality in terms of the occurrence of spontaneous acrosome reaction and the inducibility of the acrosome reaction, with acrosomal size of normal sized spermatozoa in order to improve the sperm morphology evaluation process as a diagnostic tool to help in the prediction of a male's fertilization potential for in vivo conception and assisted reproductive procedures.

MATERIALS AND METHODS

Semen samples were obtained from 50 randomly selected patients with 3–5 days of abstinence who attended the andrological outpatient clinic at the Department of Dermatology and Andrology, Giessen, Germany. The acrosomal status of spermatozoa was determined using the triple stain technique (22) before and after induction of the acrosome reaction by means of the low temperature method (6,8). In brief, sperm samples were washed with human tubal fluid medium containing 1% serum albumin (Centeon Pharma, Marburg, Germany) (HTF-HSA), centrifuged for 10 min at 400 $\times$ *g* and the pellet resuspended in fresh HTF-HSA and split into 2 aliquots of 250 μL each for test and control. While the control sample was processed immediately, the test sample was incubated at 4°C for 16 h to induce the acrosome reaction. Afterwards, the triple stain technique for evaluation of acrosomal status was performed. On each slide, 600 spermatozoa were evaluated and the percentage of acrosome-reacted sperm in live and dead sperm was separately categorized in sperm cells that showed normal sized, small, and large acrosomes. Acrosomal size was regarded as small if the area of the acrosome occupied <40% of the sperm head, as normal if the area of the acrosome was between 40 and 70% of the sperm head, and as large if it was more than 70% of the sperm head.

In addition, smears were made from control and test samples of the same patients, stained according to the Papanicolaou method and sperm morphology was evaluated in 100 spermatozoa according to strict criteria (12). In addition, the complete acrosome index (AI) (21) was also included in the evaluation using the following five categories: morphological normal acrosomes, small acrosomes, large acrosomes, normal acrosomes but with staining defects and other defects. Spermatozoa were placed in one of these classes irrespective of the morphological appearance of the rest of the spermatozoon. If an acrosome could not be placed in any of the previous four classes, mostly because of very severe morphological aberrations of the sperm head itself, or if the spermatozoa were too large or too small, it was placed under the fifth category of other defects. Spermatozoa classified as having normal acrosomal morphology include normal sized sperm heads, normal form, normal acrosomal size, and normal staining pattern. An exception was that spermatozoa with normal acrosomes but with slight abnormalities of the postacrosomal end were also classified as normal. Classification of small sized acrosomes after Papanicolaou staining was thus only based on acrosomal size, i.e., acrosomal size smaller than 40% of the sperm head and regarded as large if the acrosomal size was bigger than 70% of the sperm head. The category "acrosomal staining defects" consisted of normal acrosome sized spermatozoa (acrosome size between 40 and 70% of the sperm head) having any staining defects of the acrosome.

Sperm morphological and acrosomal data were then correlated to functional (spontaneous acrosome reaction and acrosome reaction inducibility) data of the acrosome. According to the distribution of the data, normal or not normal, parametric or nonparametric tests were applied. To predict functionality of the acrosome by morphological features i.e. percentage of morphological normal spermatozoa and normal acrosomes, or acrosome index, of the sperm and vice versa, respectively, ROC curve analyses were performed. Values used were minimum acrosome inducibility at 7.5% and minimum of 13% acrosome-reacted sperm after induction (7). For normal sperm morphology a cutoff point of 4% (normal) was used (16,23). By performing an ROC curve analysis, cutoff and predictive values for a set parameter in relation to another parameter can be calculated. This is important to assess the clinical value of an assay. All statistical calculations were performed by means of the MedCalc program, version 6.14, MedCalc Software, Mariakerke, Belgium.

RESULTS

Relationship Between Staining Method and Acrosome Characteristics/Size

The mean values for morphologically normal spermatozoa of $4.9 \pm 3.9\%$ and the acrosome index (AI) of $12.9 \pm 8.4\%$ normal acrosomes as observed with Papanicolaou staining was significantly lower ($P < 0.0001$) as the mean of $71.2 \pm 9.0\%$ normal acrosomes as obtained with the triple staining technique. No correlation could be found between the percentages of normal sized acrosomes as determined by Triple stain and the percentage of Papanicolaou stained morphologically normal spermatozoa ($r = 0.213$; $P = 0.1361$) or the acrosome index ($r = 0.198$; $P = 0.1653$). The results of the Papanicolaou and the triple staining methods correlated highly significant for the detection of large ($r = 0.529$; $P < 0.0002$) and small acrosomes ($r = 0.629$; $P < 0.0001$). However, the mean percentage of large acrosomes detected by the Papanicolaou staining of $35.5 \pm 20.9\%$ was significantly higher than that of the triple staining technique of $12.1 \pm 7.6\%$ ($P < 0.0001$). On the other hand, for small sized acrosomes no difference ($P = 0.0923$) could be observed between the Papanicolaou ($19.1 \pm 12.1\%$) and triple staining ($16.8 \pm 10.9\%$) techniques.

Acrosome Size and Inducibility of the Acrosome Reaction

With Papanicolaou staining no significant relationship was found between normal sperm morphology ($4.9 \pm 3.9\%$), and the overall inducibility of the acrosome reaction. However, normal sperm morphology as detected by Papanicolaou staining correlated significantly with the inducibility ($5.1 \pm 5.8\%$) of the acrosome reaction of sperm heads with normal sized acrosomes as seen with the triple stain technique ($r = 0.282$; $P = 0.048$). The percentage of spontaneously acrosome-reacted sperm was also significantly influenced by acrosomal size as determined by the triple stain technique. In the original semen samples spermatozoa with large- ($8.7 \pm 10.8\%$) and normal sized ($9.2 \pm 6.7\%$) acrosomes exhibited a significantly ($P = 0.0176$ and $P < 0.0001$, respectively) higher percentage of live acrosome-reacted spermatozoa than those with small acrosomes ($4.5 \pm 3.20\%$) (Fig. 1(A)). After the induction of the acrosome reaction, spermatozoa with normal-sized acrosomes ($12.2 \pm 8.0\%$) and with large acrosomes ($11.4 \pm 13.4\%$) showed

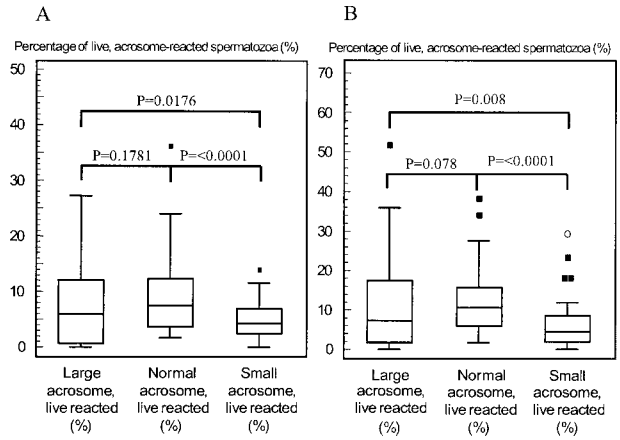

Fig. 1. Comparison of the percentage of live acrosome-reacted spermatozoa with large, normal, and small acrosomes before (A) and after induction of acrosome reaction (B). Spermatozoa with small sized acrosomes exhibit a significantly lower spontaneous acrosome reaction ($4.5 \pm 3.2\%$) than those with normal sized ($9.2 \pm 6.7\%$) or large acrosomes ($8.7 \pm 10.8\%$). After induction of acrosome reaction (B), spermatozoa with large acrosomes exhibit $11.4 \pm 13.4\%$, spermatozoa with normal acrosomes $12.2 \pm 8.0\%$, and spermatozoa with small acrosomes $6.2 \pm 6.1\%$ live, reacted sperm, respectively.

a significantly ($P < 0.0001$ and $P = 0.0083$, respectively) higher percentage of acrosome-reacted spermatozoa than spermatozoa with and small acrosomes ($6.2 \pm 6.1\%$) (Fig. 1(B)). No difference in the percentage of acrosome-reacted spermatozoa could be observed between spermatozoa with large and normal acrosomes (P=0.0781) (Fig. 1(B)).

Dead spermatozoa with small acrosomes exhibited a significantly higher ($P < 0.0001$) incidence of spontaneous-reacted acrosomes ($52.6 \pm 20.9\%$) as compared to those spermatozoa with large ($30.6 \pm 16.8\%$) and normal acrosomes ($30.9 \pm 10.3\%$), which was not statistically significant different ($P = 0.5855$) from each other (Fig. 2). Moreover, spermatozoa with small acrosomes were also associated with a significantly ($P < 0.0001$) higher percentage of cell death as compared to those spermatozoa with normal or large acrosomes (Fig. 3). Cold treatment for the introduction of the acrosome reaction did not show any effect in these groups as no difference could be observed in the mean acrosome reacted values before and after induction of the acrosome reaction (Table I) (Fig. 3).

ROC Curve Analysis

By applying the functional acrosomal parameters cutoff value of 7.5% for the inducibility of acrosome

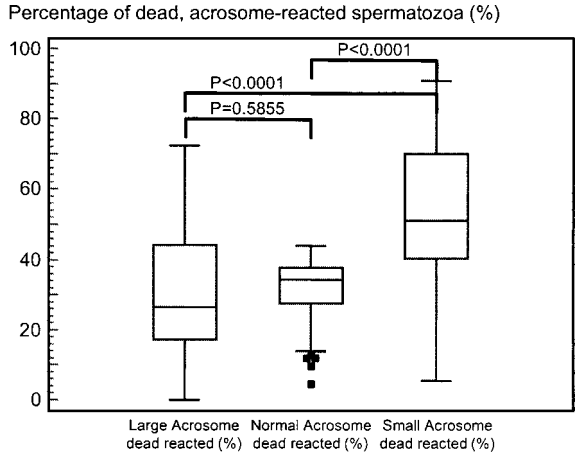

Fig. 2. Comparison of the percentages of acrosome-reacted sperm among dead sperm with large, normal, and small acrosomes. Spermatozoa with small acrosomes show a significantly higher percentage of dead acrosome-reacted sperm ($52.6 \pm 20.9\%$) than those with large ($30.6 \pm 16.8\%$) and normal sized acrosomes ($30.9 \pm 10.3\%$).

reaction and the 13% cutoff value for the minimum percentage of acrosome-reacted sperm after induction of acrosome reaction in an ROC curve analysis both functional values provided a cutoff value of 4% morphological normal spermatozoa. The statistical parameters for this prediction were good—sensitivity: 75.0%, specificity: 63.2%, positive predictive value: 39.1%, negative predictive value: 88.9%;

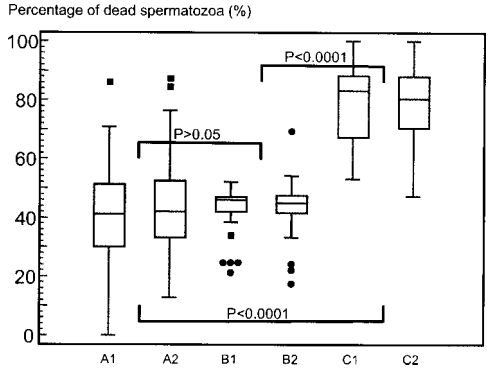

Fig. 3. Percentage of dead cells among spermatozoa with large, normal, and small sized acrosomes. Spermatozoa with small acrosomes show a significantly higher incidence of cell death than the other sperm. No difference could be observed for all three groups before and after induction of acrosome reaction. A: Large Acrosome; A1: before induction of AR; A2: after induction of AR B: Normal Acrosome; B1: before induction of AR; B2: after induction of AR C: Small Acrosome; C1: before induction of AR; C2: after induction of AR.

Table I. Percentage of Dead Spermatozoa with Small, Normal, and Large Sized Acrosomes Before and After Induction of Acrosome Reaction

Size of acrosome	Dead sperm before induction of AR (%)	Dead sperm after induction of AR (%)	Significance
Small	78.3 ± 13.8	79.1 ± 12.9	ns
Normal	43.5 ± 6.8	43.8 ± 8.1	ns
Large	40.4 ± 18.1	44.4 ± 16.3	ns

Note. The mean values before and after induction of acrosome reaction do not differ (Mean $\pm SD$; $N = 50$).

and (sensitivity: 70.6.%, specificity: 66.7%, positive predictive value: 52.2%, negative predictive value: 81.5%, respectively.

For the acrosome index (AI) the respective AI cutoff values were at 8 and 16% normal acrosomes, for acrosome-reaction inducibility (sensitivity: 91.7%; specificity: 42.1%; positive predictive value: 33.3%; negative predictive value: 94.1%) and minimum percentage of acrosome-reacted spermatozoa (sensitivity: 29.4%; specificity: 81.8%; positive predictive value: 45.5%; negative predictive value: 69.2%), respectively. When the ROC curve analysis was done in the opposite way to predict acrosomal function by means of morphology (at 4% normal) a cutoff value of 17.1% for the percentage of acrosome-reacted spermatozoa after induction of acrosome reaction was found (sensitivity: 39.1%; specificity: 96.3%; positive predictive value: 90.0%; negative predictive value: 65.0%) and for the inducibility a cutoff value of 6.4% (sensitivity: 52.2%; specificity: 88.9%; positive predictive value: 80.0%; negative predictive value: 68.6%).

DISCUSSION

Conventional semen analysis still remains the cornerstone of andrological management in assisted reproductive programs. Notwithstanding, a series of advanced diagnostic methods have been described (24–26) to improve the andrological diagnosis process. These advanced diagnostic methods are, however, more expensive and time-consuming and not always available in routine laboratories. Therefore, there is a continuous need to improve existing standard laboratory procedures in order to bring about better diagnostic and prognostic abilities of the standard semen analysis. In our effort to investigate possible additional diagnostic capabilities of the standard semen analysis, we focused on the role between sperm

morphology and functionality with emphasis on the role of acrosomal morphology as an alternative to the investigation of acrosomal functionality by means of functional tests. This was done because a standard sperm morphological evaluation is part of every semen analysis.

The comparison of the morphological evaluation of human spermatozoa by means of strict criteria on the Papanicolaou stained smears and the triple stain technique correlated for the identification of large and small acrosomes but with a significantly by higher percentage of large acrosomes by means of the standard morphology evaluation of the Papanicolaou stained smears. Although the classification criteria for the acrosome size were the same, the observed discrepancy can be attributed to different appearance of the acrosomes with the different staining methods. The explanation why no correlation could be found between normal acrosomes as determined by triple stain and morphologically normal sperm by Papanicolaou stain might also be due to the different staining methods used. Another reason may also be the fact that for the AI evaluation five classes are used against the three for the triple staining technique. This problem seems not to exist for the small acrosomes, possibly because a small acrosome is a much better definable entity.

When focusing on the acrosome reaction in normally shaped sperm heads, a significant correlation between normal sperm morphology and inducibility of acrosome reaction could be observed and it reflects the close relationship between the morphological structure and its functionality that Franken *et al*. (4) already pointed out. This strong relationship between sperm morphological structure and sperm function has, inter alia, also been demonstrated in the literature with regard to sperm binding to the zona pellucida (18,20) and the ability to undergo the acrosome reaction (4), for which functional progesterone receptors are needed that may be dependent on the stability of the plasma membrane and/or normal sperm morphology (27).

Carrell *et al*. (28) reported that morphological normal spermatozoa, as seen with triple staining, undergo the acrosome reaction faster than other types of abnormal spermatozoa such as tapered, large, and small headed spermatozoa. Heywinkel *et al*. (29) reported that normozoospermic semen samples and samples with mainly (>40%) elongated spermatozoa had the same rate of acrosomal reaction. However, in semen samples with mainly (>40%) acrosomal-defect spermatozoa, only few acrosome-reacted spermatozoa were observed. Thus, it appears that spermatozoal form and acrosomal morphology are strongly functionally orientated. Our results also demonstrated that spermatozoa with small sized acrosomes are ineffective for fertilization because these cells show not only a significantly higher percentage of cell death, but also an increased nonphysiological acrosomal loss, which reflects the existence of different subpopulations of sperm cells, those being able to undergo acrosome reaction and those not being able to a physiologic response (27,30). This might even be influenced by the normal process of sperm selection through cervical mucus (12,27) and the cumulus cells (28) as this process selects morphologically normal spermatozoa.

Therefore, by just using smears from the ejaculate for either standard sperm morphology evaluation or evaluation of the acrosome reaction, the relationship between morphology and acrosomal functionality could clearly be demonstrated for the normal sized acrosomes and confirms the correlation between the AI and acrosomal function as reported by Menkveld *et al*. (21). Thus, it is mandatory to take only spermatozoa with normal sized acrosomes into account when assessing the acrosome-reacted state in a given sample. This is also important for the evaluation of normal sperm morphology where attention should be focused on the size of the sperm head, especially the size of the acrosome (20,21,29), which in essence is done with the determination of the AI. Although different criteria may have been used for assessment of sperm morphology and acrosome reaction, prediction of acrosomal functionality from standard sperm morphology evaluation can provide a strong indication of acrosomal function. Therefore, it seems recommendable to include the assessment of the AI as an indication of acrosomal function in a complete andrological diagnostic work up because this may give some additional information on human sperm function.

With reference to the ROC curve analysis, the cutoff value of 4% morphologically normal sperm obtained by both functional acrosomal parameters versus the inducibility and minimum percentage of induced acrosome-reacted spermatozoa (7) is in good agreement with the cutoff value for normal morphology needed for in vivo conception (13), in vitro fertilization (31) and IUI (16,23). The cutoff value of 8% for the AI as obtained with an acrosome inducibility of 7.5% is also in agreement with the value of 8% reported by Menkveld *et al*. (31,32) for in vitro fertilization as well as for the prediction of a fertile population, respectively.

In conclusion, our results clearly demonstrate that acrosomal size, thus morphological appearance, reflects the physiological capability of sperm function and therefore male fertility potential. It indicates that spermatozoa with small acrosomes are more susceptible to cell death and a nonphysiological acrosomal loss. The molecular reasons and connections for this functional disability of small-sized acrosomes, however, are still unknown and will be subject of future work.

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