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·*Original Article*·

Assessment of released acrosin activity as a measurement of the sperm acrosome reaction

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Abstract

Aim: To develop a method for assessing sperm function by measuring released acrosin activity during the acrosome reaction (AR). **Methods**: Human semen samples were obtained from 24 healthy donors with proven fertility after 3–7 days of sexual abstinence. After collection, samples were liquefied for 30 min at room temperature. Standard semen parameters were evaluated according to World Health Organization (WHO) criteria. Calcium ionophore A23187 and progesterone (P4) were used to stimulate the sperm to undergo AR. After treatment, sperm were incubated with the supravital dye Hoechst 33258, fixed in a glutaraldehyde-phosphate-buffered saline solution, and the acrosomal status was determined by fluorescence microscopy with fluorescein isothiocyanate-labeled *Pisum sativum* agglutinin (FITC-PSA). The percentage of sperm undergoing AR (AR%) was compared to sperm acrosin activities as assessed by spectrocolorimetry. The correlation between AR% and acrosin activity was determined by statistical analysis. **Results**: The AR% and released acrosin activity were both markedly increased with A23187 and P4 stimulation. Sperm motility and viability were significantly higher after stimulation with P4 versus stimulation with A23187 ($P < 0.001$). There was a significant positive correlation between released acrosin activity and AR% determined by FITC-PSA staining ($r = 0.916$, $P < 0.001$). **Conclusion**: Spectrocolorimetric measurement of released acrosin activity might serve as a reasonable alternative method to evaluate AR. *(Asian J Androl 2008 Mar; 10: 236–242)*

Keywords: human sperm; released acrosin activity; sperm function

1 Introduction

The acrosome reaction (AR) is an essential step for the fertilization of ova by spermatozoa. The AR can occur only in capacitated spermatozoa, and is a calcium dependent, exocytotic event, resulting in the release of

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hydrolytic enzymes. The AR facilitates the penetration of the zona pellucida by spermatozoa and the subsequent fusion of the sperm plasma membrane with the oocyte's oolemma. Reference studies have shown that an abnormal AR can be one of the causes of unexplained infertility [1].

Recently, the AR assay has become an important method for evaluating sperm function and investigating human reproduction. Since Barros *et al.* [2] verified the AR by electron microscopy, a variety of methods for detecting the AR have been introduced using triple or double staining or staining of acrosome with fluorescein-isothiocyanate *Pisum sativum* agglutinin (FITC-PSA). The most frequently used techniques for evaluating the AR are based on light microscopy, in which the acrosomal content is labeled either by a triple staining technique or with fluorescent lectins, such as FITC-PSA. However, these methods are either complex or have difficulty in sperm sample conservation. Flow cytometric analysis using monoclonal antibodies against the spermatozoa membrane cofactor protein CD46 has also been introduced to assess the AR. However, the limited availability of these antibodies and their high cost precludes their routine application. Electron microscopy is the "gold standard" method for assessing the AR [3]. However, the equipment is expensive and the process is complex.

Because the acrosome of human spermatozoa is small, it is the difficult to detect the AR. Moreover, all methods developed so far have some limitations, therefore they cannot be used widely in clinical practice. All these methods detect only acrosome-reacted spermatozoa, and do not assess released acrosin activity, which is characteristic of the AR. For example, when AR occurs, there are differences in the quantity and activity of released acrosin between large head sperm and small head sperm. As a result, sperm AR is also different. Accurate assessment of the level of acrosome-reacted sperm in a sperm population is of great importance for both basic research of human reproduction and the clinical evaluation of male fertility. Therefore, it is important to develop a technique for detecting the sperm AR function to elucidate the mechanism of the AR and fertilization.

The purpose of this work was to develop a method for assessing human sperm AR function by detecting released acrosin activity during the sperm AR. AR is required to obtain released acrosin. Physiologically, the AR in human spermatozoa takes place only after their binding to the zona pellucida. However, gaining zona pellucida is not easy. Progesterone (P4) is used more frequently as a near-physiological agent. However, it is debatable whether P4 can promote the acrosome reaction at physiological concentrations.

In the current study, several comparative analyses were carried out to clarify whether released acrosin activity of sperm AR can assess sperm function or not.

2 Materials and methods

2.1 Collection and analysis of semen sample

Human semen samples were collected from 24 healthy donors (age 26–34 years). All donors had proven fertility and samples were taken after sexual abstinence for 3–7 days. Semen samples from infertile patients were provided by the First Hospital, Jilin University. Samples were liquefied for 30 min at 37ºC. Completely liquefied samples were analyzed for standard semen parameters according to the World Health Organization (WHO) criteria [4]. A simplified discontinuous Percoll gradient centrifugation was applied to collect motile sperm. The sperm viability was analyzed using eosin-Y staining.

In addition, semen samples were collected from 39 infertile subjects, including oligozoospermic (11), asthenozoospermic (15) and teratozoospermic (13) semen. Percentage of acrosome-reacted sperm and AR released acrosin activity were detected.

2.2 Sperm capacitation

The concentration of the motile spermatozoa was adjusted to 10×10^6/mL. The sperm suspension was then incubated for 4 h under an atmosphere of 5% CO_2 at 37ºC.

2.3 Measure of AR released acrosin activity

P4 (Sigma, St. Louis, MO, USA) and calcium ionophore A23187 (Sigma, St. Louis, MO, USA) were dissolved in DMSO (Sigma, St. Louis, MO, USA). Stock solutions of P4 and A23187 were kept at –20ºC. Working solutions were prepared by diluting the thawed stock solutions 1:10 in BWW 30 min before adding them to the sperm suspension. Capacitated sperm were treated with 10 μmol/L A23187, or with concentrations of P4 ranging from 1 μg/mL–40 μg/mL. Tubes were loosely capped and incubated at 37ºC for 30 min. The supernatant was collected after centrifugation in five different Eppendof tubes, then sperm acrosin activities were ana-

lyzed by spectroco-lorimety according to the method of Cui *et al.* [5].

2.4 Acrosomal staining with FITC-PSA

After treatment with A23187 or P4, the sperm solution was centrifuged (5 min). Glutaraldehyde- phosphate-buffered saline (PBS) solution (1 mL, 3%) was added to the sediment for fixation (10 min). Then, spermatozoa were washed twice in PBS solution. Sperm suspensions (10 μL) were smeared onto glass slides and air-dried. Staining of acrosome with FITC-PSA was performed according to Mendoza *et al.* [6]. The FITC-PSA solution was washed out by dipping the slides into PBS solution.

2.5 Quality control

All experiments included a positive control. To avoid a false measurement because of dead sperm or sperm with degenerative acrosomal loss, double-staining of sperm was performed using FITC-PSA and Hochest33258. Every treated sperm sample was smeared onto two slides. By means of a fluorescence microscope, at least 200 spermatozoa were evaluated blindly according to the fluorescence pattern of their acrosomes. Two investigators evaluated each slide, and the error between their data was less than 15%. The average of two independent values was recorded as the AR.

2.6 Statistical analysis

SPSS 11.5 software (SPSS, Chicago, IL, USA) was used for the statistical calculations. The values reported in the text and tables are mean ± SD (range). The significance of the difference between the treatments was assessed using unpaired *t*-test. Differences were considered to be statistically significant when $P < 0.05$.

3 Results

3.1 Undetected acrosomal contents revealed

After glutaraldehyde-PBS fixation agglutinin (FITCPSA) Staining, undetected acrosomal contents were revealed (Figure 1).

3.2 Comparison of the percentage of acrosome-reacted sperm and AR-released acrosin activity after stimulation with calcium ionosphore A23187 and P4

The percentage of acrosome-reacted sperm and AR-released acrosin activity were both markedly increased ($P < 0.05$), when compared to control (capacitation 4 h) (Table 1).

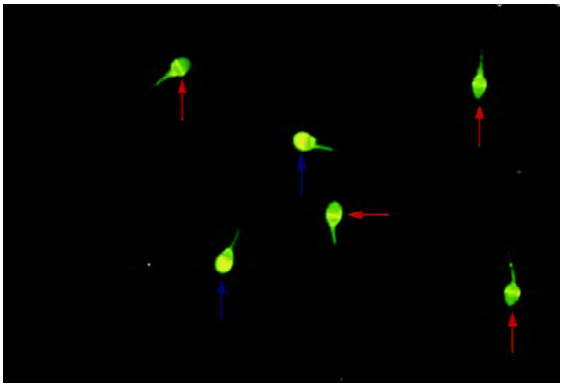

Figure 1. After acrosome stained with fluorescein isothiocyanate-labeled *Pisum sativum* agglutinin, absence of fluorescently-labeled acrosome indicate sperm structure is not intact, that is, it develops acrosome reaction (AR) (red arrows indicate "acrosome reacted sperm"). Fluorescently-labeled acrosome indicate sperm structure is intact, that is, it does not develop AR (blue arrows indicate "sperm of intact acrosome").

Table 1. Comparison of the percentage of acrosome-reacted sperm and released acrosin activity when stimulating with calcium ionosphore A23187 and progesterone (for sperm capacitation, the sperm suspension was incubated for 4 h. Capacitated sperm were treated with A23187 or progesterone of corresponding concentrations. Then incubation time was 30 min) (means ± SD, $n = 24$). $^bP < 0.05$, compared with controls. P4, progesterone.

Group	Acrosome reaction released acrosin activity (μIU/10^6 spermaozoa)	Percentage of acrosome reacted sperm (%)
Capacitation (0 h)	0.78 ± 0.83	1.21 ± 0.42
Control (4 h)	8.86 ± 2.12	9.66 ± 1.53
A23187 (10 μmol/L, 4 h)	31.47 ± 4.69^b	39.02 ± 6.15^b
P4 (10 μg/mL, 4 h)	24.86 ± 2.71^b	31.63 ± 4.78^b

Table 2. Effect of P4 concentration on the percentage of acrosome-reacted sperm, sperm motility and viability (mean ± SD, n = 24). [b]$P < 0.05$, compared to controls; [c]$P < 0.001$, compared to A23187 positive control. P4, progesterone.

Group	Percentage of acrosome-reacted sperm (%)	Sperm motility (%)	Sperm viability (%)
Control	12.24 ± 2.17	78.41 ± 6.63	75.25 ± 3.56
A23187 control	35.18 ± 4.52	12.26 ± 2.43[b]	21.94 ± 2.0[b]
P4 (μg/mL)			
1	14.49 ± 2.76	75.07 ± 5.24[c]	73.33 ± 3.45[c]
5	18.42 ± 3.24[b]	70.47 ± 5.61[c]	74.58 ± 3.63[c]
10	30.91 ± 5.25[b]	70.16 ± 4.07[c]	71.63 ± 5.87[c]
20	32.59 ± 4.62[b]	73.56 ± 4.62[c]	70.85 ± 4.91[c]
40	34.54 ± 5.06[b]	71.39 ± 5.06[c]	73.45 ± 4.57[c]

Table 3. Effect of P4 concentration on the percentage of acrosome-reacted sperm and released acrosin activity (means ± SD, n = 24). [b]$P < 0.05$, compared to 1 μg/mL and 5 μg/mL group. AR, acrosome reaction; P4, progesterone.

P4 concentration (μg /mL)	AR released acrosin activity (μIU/10^6)	Percentage of acrosome-reacted sperm (%)
1	12.64 ± 2.13	14.49 ± 2.76
5	14.67 ± 3.46	18.42 ± 3.24
10	25.92 ± 3.64[b]	30.91 ± 5.25[b]
20	24.63 ± 4.91[b]	32.59 ± 4.62[b]
40	27.05 ± 5.04[b]	34.54 ± 5.06[b]

Table 4. Comparison of the percentage of acrosome-reacted sperm and released acrosin activity between fertile group and infertile groups (mean ± SD). [b]$P < 0.05$, compared with fertile group. AR, acrosome reaction.

Group (number)	Percentage of acrosome-reacted sperm (%)	AR released acrosin activity (μIU/10^6)
Fertile group (16)	29.63 ± 6.17	26.92 ± 4.12
Infertile groups		
Oligozoospermic (11)	21.44 ± 4.61[b]	16.73 ± 4.28[b]
Asthenozoospermic (15)	19.83 ± 3.95[b]	15.86 ± 3.97[b]
Teratozoospermic (13)	16.71 ± 3.82[b]	13.68 ± 4.47[b]

3.3 Effect of P4 concentration on the percentage of acrosome-reacted sperm, sperm motility and viability

At final concentrations of P4 between 10 μg/mL and 40 μg/mL, the acrosome reaction of the sperm was markedly increased. The differences were statistically significant when compared to the control group ($P < 0.05$). At a final P4 concentration of 1 μg/mL, the difference was not statistically significant ($P > 0.05$). There was no difference in the percentage of acrosome reacted sperm when compared to the A23187 positive control ($P > 0.05$). There was also no difference in sperm motility and viability when compared with control ($P > 0.05$). However, the differences of sperm motility and viability were statistically significant when compared to the A23187 control group ($P < 0.001$) (Table 2).

3.4 Relationship of the percentage of acrosome-reacted sperm and released acrosin activity, when using P4 at different concentrations to induce AR

At concentration of 10 μg/mL, the percentage of acrosome reacted sperm and released acrosin activity were both markedly increased when compared to doses of 1 μg/mL and 5 μg/mL ($P < 0.05$) (Tables 2 and 3).

In addition, Spearman correlation coefficients were calculated for the values obtained by labeling with FITC-PSA and released acrosin activity during the AR. There was a significant positive correlation between released acrosin activity during the AR and the percentage of acrosome-reacted sperm ($r = 0.916$, $P < 0.001$).

3.5 Comparison of the percentage of acrosome-reacted sperm and released acrosin activity of infertile group with those of the fertile group

The percentage of acrosome-reacted sperm and released acrosin activity in the oligozoospermic, asthenozoospermic and teratozoospermic groups were all lower than those of the fertile group ($P < 0.05$). The percentage of acrosome-reacted sperm and released acrosin ac-

tivity in the teratozoospermic group was the lowest of the three infertile groups. However, there was no difference between the result for the oligozoospermic, asthenozoospermic or teratozoospermic groups ($P > 0.05$) (Table 4).

Spearman correlation coefficients were calculated for the values obtained by labeling with FITC-PSA and released acrosin activity during the AR. There was a significant positive correlation between released acrosin activity during the AR and the percentage of acrosome-reacted sperm ($r = 0.931$, $P < 0.001$).

4 Discussion

Physiologically, the AR in human spermatozoa takes place only after they bind to the zona pellucida glycoprotein (ZP3). However, because of the paucity of available human ova, the ZP protein is not often used in AR assays. A suitable stimulus is necessary for routine AR assay. The morphological changes of the human sperm acrosome after treatment with calcium ionophore A23187 are similar to those occurring during spontaneous or physiologically-induced AR [7].

However, the spontaneous AR, as well as the AR induced by P4, phorbol myristate ester and follicular fluid, is usually partial. In contrast, the AR induced by A23187 is total (i.e. it includes both partial and complete) [8]. P4 can also be used as a stimulus. Avalos-Rodriguez *et al.* [9] described one phenotypic difference in a study of rabbit sperm. After AR induction with P4, the localization of phosphatidylserine was changed and the Annexin-V binding sites were found only in the acrosomal region, but with a higher number of binding sites in the equatorial area. In contrast, after AR induction with A23187, phosphatidylserine translocation, although predominant over the acrosomal region, was also observed in the post-acrosomal region. However, it is still debatable whether P4 at physiological concentrations promotes AR. In the present study, we used P4 as a near-physiological inducer to induce AR.

From Figure 1, after acrosome stained with FITC-PSA, fluorescently-labeled acrosome indicates whether sperm structure is intact or not. If acrosome is not intact, it indicates that sperm develops AR. Therefore, percentage of acrosome-reacted sperm labeled with FITC-PSA can evaluate sperm AR. The released acrosin activity during AR and the percentage of acrosome-reacted sperm were then evaluated. By comparison, we want to know whether the released acrosin activity during AR can determine sperm AR or not.

Acrosin is an acrosomal protease synthesized as a pro-enzyme and activated into beta-acrosin during the AR. It is presumed to be involved in the recognition and binding of the sperm to the zona pellucida of the ovum and in sperm penetration through the zona pellucida. In 2004, Lax *et al.* [10] described an acrosin activity assay for the evaluation of the mammalian sperm acrosome reaction. Acrosin is a key enzyme in fertilization and its activity directly affects the fertilization rate. Spermatozoa lacking acrosin protein (Acr^-) show a delayed fertilization. Acr^- sperm have a selective disadvantage when they compete with Acr^+ sperm [11]. Langlois *et al.* [12] conclude that the sperm acrosin assay could help to predict sperm fertilizing capacity in *in vitro* fertilization independent of sperm morphology. Chaudhury *et al.* [13] reported that total acrosin activity might be considered as a sensitive biochemical marker for the clinical evaluation of unexplained infertility in men. Therefore, acrosin activity during the AR can reflect the function of the AR.

The percentage of acrosome reacted sperm and released acrosin activity were both markedly increased, when either A23187 or P4 were used to induce AR (Table 1). This indicates that A23187 and P4 can induce human sperm AR, as agreed by Katsuki *et al.* [14], and by Bronson *et al.* [15].

Our results showed that incubation of spermatozoa with 10 μg/mL–40 μg/mL P4 for 30 min induced the sperm AR (Table 2). The differences were statistically significant when compared to control. No significant differences were found between the 10 μg/mL, 20 μg/mL or 40 μg/mL P4-treated group. Some sperm plasma membrane receptors are involved in the P4-initiated AR. If the concentration of P4 is too low, some receptor sites in the heads of the sperm are not active. Therefore, not many cells get stimulated. However, when the concentration of P4 reaches a certain level, the sites of all receptors are activated. At this time, adding more P4 to the sperm suspension cannot further increase activity of P4-receptor.

We found that sperm motility and viability were both markedly decreased with increased A23187 concentration ($P < 0.05$). When using P4 as the AR inducer, there was no significant difference in capacitated sperm motility and viability. However, significant differences were found between P4 and A23187 ($P < 0.001$). This find-

ing implies that P4 would be a near-physiological AR stimulus. Our data are consistent with the principle that there is a clear difference between acrosome reactions induced by P4 and A23287, as reported in the mouse by Brucker *et al.* [16].

According to Bronson *et al.* [15], P4 promotes an acrosome reaction within capacitated spermatozoa. Shoeb *et al.* [17] indicate that P4 induces the formation of horizontal microdomains within the exofacial surfaces of sperm membranes, leading to progressive and independent alterations in molecular dynamics. Pietrobon *et al.* [18] report that the P4-induced AR is driven by activation of G-proteins, which in turn activate PLA_2 and PLC simultaneously, which finally promote acrosomal exocytosis. Therefore, P4-induced AR would be a near-physiological process.

After induction of the AR by P4, the percentage of acrosome reacted sperm and released acrosin activity were both markedly increased (Table 3). There was a significant positive correlation between released acrosin activity during the AR and the percentage of acrosome reacted sperm ($r = 0.916$, $P < 0.001$). This finding indicates that released acrosin activity during the AR is consistent with the values obtained by labeling with FITC-PSA, and suggests that released acrosin activity can indirectly reflect the acrosomal status. The results confirm the finding by Köhn *et al.* [3] that there is a positive correlation between FITC-PSA labeling and studies using transmission electron microscopy.

We compared the percentage of acrosome reacted sperm and released acrosin activity in fertile and infertile men (Table 4). The finding that the acrosin activity of asthenozoospermic men was lower than that of the fertile group is consistent with the conclusion by Nakagawa *et al.* [19]. Previous work has shown that spermatozoa from asthenozoospermic might owe their poor motility and their inability to properly capacitate and develop hyperactivation to impair tyrosine phosphorylation of critical proteins caused by decreased membrane fluidity [20]. Because noncapacitated sperm do not undergo AR, the percentage of acrosome-reacted sperm declines. Released acrosin activity is low too. In addition, the percentage of acrosome-reacted sperm and released acrosin activity of the teratozoospermic group was the lowest among the three infertile groups. Previous work has reported that a defective sperm-zona pellucida interaction was present in 64% of teratozoospermic infertile men: 31% had defective sperm-zona pellucida binding, and 33% low zona pellucida induced acrosome reaction (ZPIAR) [21]. This finding showed that abnormal acrosin activity might be one cause of teratozoospermic infertility.

There was a significant positive correlation between released acrosin activity during the AR and the percentage of acrosome reacted sperm ($r = 0.931$, $P < 0.001$). This finding suggests that the released acrosin activity during the AR is consistent with the values obtained by labeling with FITC-PSA. In previous studies, most of the different methods assess only the acrosomal status without assessing released acrosin activity. Using these methods, one has to make the assumption that the 200 sperm counted are representative of the entire sample. Soderlund *et al.* [22] report that the evaluation of the acrosome index would not accurately predict fertilization. Our study suggests that measuring the released acrosin activity during the AR can assess AR activity independent of the percentage of acrosome reacted sperm.

In conclusion, detecting the sperm AR is an important method for the assessment of sperm function and basic research of human reproduction. Measuring the released acrosin activity during the AR can determine sperm AR function. The procedure is an alternative method for evaluating true AR in whole sperm populations. This method is an objective technique for accurate assessment of AR function and, therefore, the observer's error that is inherent in light microscopic methods is avoided.

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