

Relationship of *in Vitro* Acrosome Reaction to Sperm Function: An Update

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Abstract: Understanding the mechanisms by which the acrosome reaction is regulated is central to models of fertilization. This article reviews the relationship of the acrosome reaction detected *in vitro* to sperm function. Proteolytic enzymes in the acrosome digest through the zona pellucida allowing for sperm-oolemma fusion. When this process is impaired, either by lack of an acrosome or acrosomal dysfunction, fertility can be compromised. Optical microscopy and staining with different fluorescent lectins that bind to acrosomal membranes is the method of choice for acrosomal evaluation. Because acrosomal loss can be a result of sperm death, this test should be used in conjunction with an assay to monitor sperm viability. Different stimulants, such as phosphodiesterase inhibitors, drugs and toxins have been investigated in their ability to affect the sperm ability to undergo *in vitro* acrosome reaction. Sperm acrosomes are also sensitive to the freezing-thawing process and strategies have been described to minimize cryodamage. The assessment of the acrosome has been shown to be a stable parameter of sperm function and a valid tool to predict the fertilizing potential of human spermatozoa. The acrosome reaction following ionophore challenge (ARIC) is an *in vitro* assay with good predictability of the sperm's fertilizing potential for assisted conception techniques including intrauterine insemination and conventional *in vitro* fertilization. The AR determination has been also used as an important biomarker in studies involving drugs and toxins. Recently, novel aspects of sperm-oocyte fusion have been depicted in humans involving glycoproteins present in the zona pellucida and the female reproductive tract.

Keywords: Spermatozoa, acrosome reaction, infertility, male, cryopreservation, phosphodiesterase inhibitors.

INTRODUCTION

Human Sperm Head Formation

The mammalian sperm head is composed of two main structures, the acrosome and the nucleus. The human acrosome is a membrane-bound organelle derived from the Golgi complex. The acrosome formation occurs during spermiogenesis, specifically during the round spermatid stage when accumulation of acrosomal materials and the flattening of acrosome take place [1]. The acrosome is located in the anterior portion of the sperm head and its area varies by species. In humans, the acrosome occupies 40% to 70% of the sperm head [2]. Acrosomal contents are composed of proteases, mainly proacrosin, hyaluronidase and phospholipase A2. These substances are released during the early stages of sperm-egg interaction through an exocytotic process known as the acrosome reaction [3].

Capacitation and Acrosome Reaction

In vivo, ejaculated spermatozoa from all eutherian mammals are unable to fertilize until they have undergone capacitation that allow the acrosome reaction to take place when they approach or contact the oocyte [4]. Capacitation has been recognized as a time-dependent phenomenon, with the absolute time course being species-specific [5]. It prepares the sperm to undergo the acrosome reaction with

the accompanying release of lytic enzymes and exposure of membrane receptors, which are required for sperm penetration through the zona pellucida and for fusion with the oolemma [5]. Sperm transport through the female genital tract can occur quite rapidly (times as short as 15 to 30 minutes have been reported in humans), whereas the capacitation process can take from 3 to 24 hours [5]. It is speculated, therefore, that capacitation is not completed until after the spermatozoa has entered the cumulus oophorus. This delay is physiologically beneficial because the spermatozoa do not respond to acrosome-reaction-inducing signals until they approach the zona pellucida, preventing premature acrosome reactions that lead ultimately to the sperm inability to penetrate the egg vestments [5, 6]. Sperm capacitation is a post-ejaculatory modification of the sperm surface, which involves the mobilization and/or removal of certain surface components from the sperm plasma membrane, such as glycoproteins, decapacitation factor, acrosome-stabilizing factor, and acrosin inhibitor. Sperm capacitation involves major biochemical and biophysical changes in the membrane complex and energy metabolism. The presence of high concentrations of cholesterol in the seminal plasma, which maintains a high cholesterol concentration in sperm membranes, may be the most important factor for the inhibition of capacitation [7]. Capacitation is associated to increased membrane fluidity caused by the removal of cholesterol from sperm plasma membrane via sterol acceptors present in the female tract secretions [8, 9]. A marked change in sperm motility, named hyperactivation, is also associated with capacitation. Hyperactivated spermatozoa exhibit an extremely vigorous but nonprogressive motility pattern, as a result of a Ca^{2+} influx, that causes increased flagellar curva-

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ture [10] and hence extreme lateral movement of the sperm head [8]. Proteasome participates in activating calcium channels that also leads to increased membrane fluidity and permeability [11-14]. These events are followed by or occur simultaneous with [i] a decrease in net surface charge, [ii] devoided area of intra-membrane protein and sterols, and [iii] increased concentrations of anionic phospholipids [14, 15]. Hyperactivated motility is essential for sperm penetration into the intact oocyte-cumulus complexes *in vitro* and *in vivo* [16, 17].

The spermatozoon binds to the zona pellucida (ZP) with its intact plasma membrane after penetrating into the cumulus oophorus. Sperm binding occurs via specific receptors to ZP glycoproteins located over the anterior sperm head [18]. Glycosylation of ZP glycoproteins are important in sperm-ZP interaction. It is believed that human ZP glycoprotein-3 (ZP3) has a central role in initiating the acrosome reaction [19]; however, it has been recently demonstrated that human ZP1 and ZP4 are also implicated in the process [20, 21]. The acrosome reaction is a stimulus-secretion coupled exocytotic event in which the acrosome fuses with the overlying plasma membrane [22, 23]. The multiple fusions between the outer acrosomal membrane and the plasma membrane result in the release of hydrolytic enzymes (mostly acrosin) and in the exposure of new membrane domains, both of which are essential if fertilization is to proceed further. The hydrolytic enzymes released from the acrosome digest the zona pellucida, allowing the spermatozoa to penetrate the oocyte [18]. The acrosome reaction (AR) seems to be physiologically induced by natural stimulants such as the follicular fluid (FF), progesterin, progesterone, and hydroxy progesterone [15]. Follicular fluid and cumulus cells have protein-bound progesterone that has been identified as one of the most important acrosome reaction-inducing agents [23, 24]. Follicular fluid stimulates the acrosome reaction in a dose-dependent manner [24, 25]. A link between locally produced estradiol by ejaculated spermatozoa, acrosome reaction and sperm capacitation has been also described [26]. Moreover, evidence indicates that environmental estrogens can significantly stimulate mammalian sperm capacitation and acrosome reaction [27]. The acrosome reaction is probably initiated when ligands produced by the oocyte bind to the receptors on the spermatozoa. This signal is transduced intracellularly via second messengers, ultimately leading to exocytosis [28]. A number of second messenger pathways have been identified in human spermatozoa, including those that result in the activation of cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), and phospholipid dependent protein kinases [14, 29-31]. These kinases are called, respectively, protein kinase A, protein kinase G, and protein kinase C. It is possible that these pathways interact to assure an optimal response at the correct place and time during the fertilization process. Although the concentration of cyclic GMP in ejaculated human semen is almost 7 times lower than cAMP, it is speculated that both nucleotides have a similar role in the AR since their dependent protein kinases are closely related proteins [32-34]. The AR can also be induced by artificial stimulants that cause and increase in sperm intracellular calcium [14, 35].

Importance of the Acrosome Reaction *in Vivo* and *in Vitro*

Acrosome integrity is crucial for normal fertilization both *in vivo* and *in vitro*. A high proportion of sperm with intact acrosomes is seen in ejaculates of normal men. In such individuals, ~5-20% sperm cells may exhibit spontaneous acrosome reactions that are of no clinical significance [35]. Conversely, several abnormal conditions affecting the sperm may lead to decreased fertilization ability. Acrosomeless round-headed spermatozoa (globozoospermic spermatozoa) are unable to fertilize the oocytes, and increased percentages of morphologically abnormal acrosomes were related to fertilization failure in assisted reproductive technology (ART) using conventional *in vitro* fertilization (IVF) [36]. The AR is a time-dependent phenomenon that cannot take place prematurely or too late [37]. Premature acrosome reaction and the inability of the spermatozoa to release the acrosomal contents in response to proper stimuli (acrosome reaction insufficiency) have been associated with idiopathic male infertility [38]. Although the cause of premature acrosome reaction is unknown, the premature (stimulus-independent) initiation of acrosomal exocytosis seems to be related to a perturbation of the plasma membrane stability. In this situation, the acrosome reaction may not involve a premature activation of the receptor-mediated process, but rather reflect an inherent fragility of the sperm membrane, leading to a receptor-independent acrosomal loss [39]. Antisperm antibodies (ASA) may also adversely affect the ability of sperm to undergo capacitation and acrosome reaction [40]. Chang *et al.* [41] reported reduction in fertilization rate either by IgG directly bound to sperm or IgM present in female serum. The combination of IgG and IgA may have a synergistic negative effect on fertilization [42-45]. Toxic substances to sperm can also impact the AR. High concentrations of dietary phytochemicals, such as genistein isoflavone and β -lapachone, were shown to suppress the AR in a dose and time-dependent manner in the rat model [46]. Inhibition of AR by genistein seems to involve the protein kinase C pathway while β -lapachone has a direct cytotoxic effect on sperm cell membrane. It is suggested that genistein and β -lapachone may impact male fertility via AR suppression in high doses and AR induction in low doses [46]. Calcium channel blockers may also interfere with the AR exocytic event. Sperm incubation with different blockers, such as trifluoperazine (calmodulin inhibitor), verapamil (Ca^{2+} channel inhibitor) and nifedipine (voltage-dependent Ca^{2+} channel inhibitor) significantly reduced the ability of hamster sperm to undergo the acrosome reaction [47].

LABORATORY TESTS TO MEASURE HUMAN SPERM ACROSOME REACTION

The assessment of the sperm acrosome reaction *in vitro* (AR) has been used as a test of sperm function for many years [4, 48]. AR testing has been shown to be useful not only as a tool in andrology for research purposes, such as male contraception [49] and gonadotoxic effects of food and drugs [46] but also to predict fertilization success in assisted conception cycles. The AR testing is based on sperm physio-

logy; it involves capacitation and acrosome reaction [50]. The sperm acrosome can be visualized by phase-contrast light microscopy. However, greater accuracy can be achieved by using either electron and fluorescence microscopy or monoclonal antibodies and simple dyes [50].

Acrosome Reaction Testing Using Fluorescein-Labeled Lectins

Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) or fluorescein isothiocyanate-conjugated pisum sativum (FITC-PSA) may be used to assess the sperm acrosome. FITC-PNA binds specifically to the outer acrosomal membrane (beta-fraction of D-galactose) while FITC-PSA binds to the alpha-methylmannose and labels the acrosome contents [8]. One of the most important aspects involving AR testing is the difficulty in differentiating between pathologic acrosomal loss, secondary to a reduction in cell viability, and a 'true' acrosome loss. We have demonstrated that a discrepancy exists between the proportion of acrosome-reacted spermatozoa in viable and overall sperm specimens [50] (Fig. 1). Dead spermatozoa lose its acrosomal contents by membrane rupture. Consequently, fluorochromes targeted to the acrosome membranes bind to receptors and the AR pattern is observed on fluorescence examination. The differentiation between 'pathological' and 'true acrosomal loss' is particularly important when assessing the acrosome status of cryo-thawed spermatozoa because cryopreservation directly damages sperm membrane resulting in the loss of membrane permeability and subsequent cell death [51]. It is,

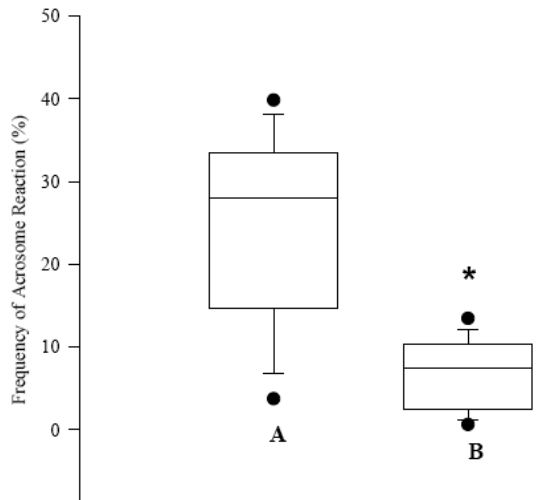


Fig. (1). The frequency of acrosome reaction (AR) in semen specimens of fertile donors. AR was determined by FITC-PNA, and sperm viability by Hoechst-33258 staining. (A) Total acrosome reaction (normal reacted cells plus post mortem degeneration of the acrosomes) and (B) acrosome reaction in live human spermatozoa (* $p < 0.01$). Box covers the middle 50% of the data values, between the lower and upper quartile. The central line is the median and the whiskers extend out to 80% of the data. Bars represent values between the 5th and 95th percentile (Adapted from *International Braz J Urol, Vol. 33, 3, Esteves SC et al. Evaluation of acrosomal status and sperm viability in fresh and cryopreserved specimens by the use of fluorescent peanut agglutinin lectin in conjunction with hypo-osmotic swelling test, page 371, Copyright 2007, with permission from FB Sampaio, editor*).

therefore, recommended that supravital dyes are used in conjunction to fluorochromes to simultaneously monitor viability during AR assessment.

Cross *et al.* described the use of the supravital stain Hoechst-33258 (a fluorescent DNA-binding dye with limited membrane permeability) combined with immuno fluorescence technique for evaluating the acrosome reaction in viable spermatozoa [52]. A simple alternative to evaluate viability of acrosome reacted sperm cells is by using the hypo-osmotic swelling test (HOST) [53]. Sperm specimens are incubated in hyposmotic conditions and smears are prepared on to glass microscope slides. Sperm membranes are permeabilized in ice-cold methanol and then the fixed smears are immersed in a 40- $\mu\text{g}/\text{mL}$ FITC-PNA solution. Each spermatozoon is first examined for tail swelling using phase contrast with halogen illumination. Then, illumination is changed to mercury ultraviolet epi-illumination source for assessing FITC-PNA staining. Spermatozoa are classified as osmotically competent if tail swelling is observed after exposure to the hyposmotic solution. The percentage of sperm exhibiting tail swelling after incubation under hyposmotic conditions are correlated with the percentage of spermatozoa with intact membranes, as it has been shown that HOST can also be used to assess sperm viability in fresh semen [54]. In a recent study, we determined the usefulness of the HOST as a marker of sperm viability for AR testing in fresh and frozen specimens [50]. HOST has potential advantages over

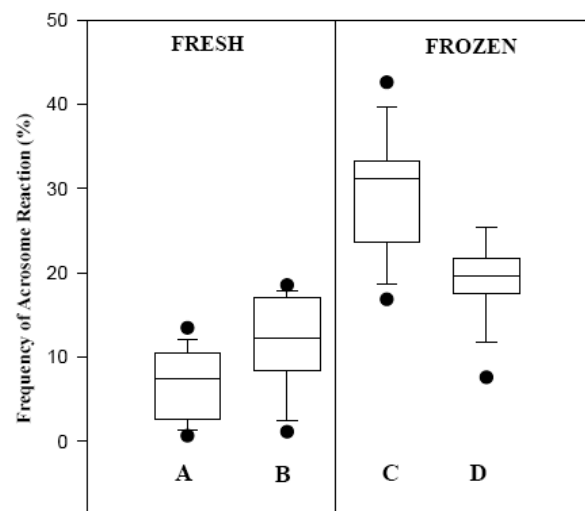


Fig. (2). Frequency of acrosome reaction in live human spermatozoa from 11 samples of fertile donors, determined by (A) FITC-PNA and HOST, and (B) FITC-PNA and Hoechst-33258, in fresh and post-thaw specimens. Similar acrosome reaction rates were detected by both A and B methods in fresh specimens ($P = 0.07$). In frozen specimens, the frequencies of acrosome reaction in viable spermatozoa assessed by A and B methods were significantly different ($p = 0.01$). Box covers the middle 50% of the data values between the lower and upper quartile. The central line is the median and the whiskers extend out to 80% of the data. Bars represent values between the 5th and 95th percentile - (Adapted from *International Braz J Urol, Vol. 33, 3, Esteves SC et al. Evaluation of acrosomal status and sperm viability in fresh and cryopreserved specimens by the use of fluorescent peanut agglutinin lectin in conjunction with hypo-osmotic swelling test, page 372, Copyright 2007, with permission from FB Sampaio, editor*).

protocols involving the use of fluorochrome-dye exclusion techniques. It precludes the use of an additional fluorescence filter cube to simultaneously assess the acrosome and to monitor viability. Our data showed that acrosome reaction determination was accurate by using FITC-PNA in conjunction to HOST to monitor viability in fresh sperm specimens, as validated by Hoescht-33258. However, AR results were not correlated in frozen-thawed spermatozoa (Fig. 2).

We have previously demonstrated the usefulness of the HOST to monitor sperm viability. Fresh and frozen-thawed sperm specimens were incubated with HOST and Hoescht-33258, and viability results were further validated by eosin-nigrosin dyes. The results of HOST and Hoescht-33258, and HOST and eosin-nigrosin, were highly correlated in unprocessed and processed fresh sperm specimens ($r=0.95$), but not in frozen-thawed ones ($r=0.22$) [54] (Fig. 3).

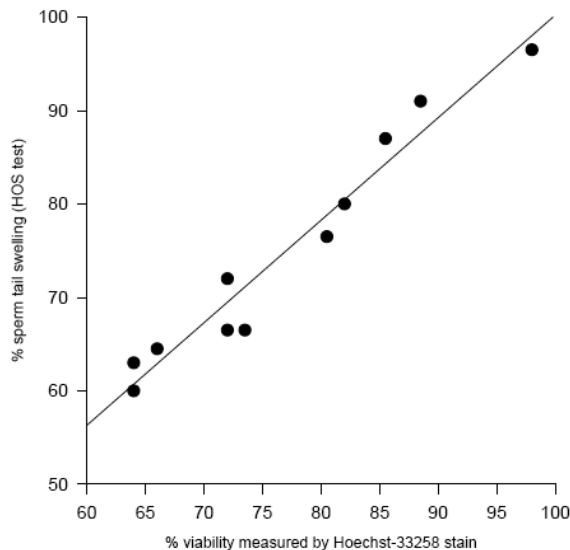


Fig. (3A). Correlation of viability results between sperm tail swelling by HOST and Hoechst 33258 in fresh semen specimens. Strong correlation seen between HOST and Hoechst 33258 ($r = 0.95$, $p < 0.001$). (Adapted from *Fertil Steril*, 1996, Vol. 66, Esteves SC, et al. Suitability of the hypo-osmotic swelling test to assess the viability of cryopreserved sperm, pp. 798-804, 1996).

The reasons for the poor HOST specificity for viability assessment in cryopreserved human sperm are unknown, but we speculate that sperm head and tail membranes have different liabilities to the freeze-thawing process. It may be possible that the determining factor for sperm survival after cryopreservation is the membrane integrity of the sperm head, which is not assessed by HOST. From these observations, we concluded that FITC-PNA labeling in conjunction with the HOST can accurately evaluate the acrosome reaction in viable fresh human spermatozoa, but not in cryopreserved ones. Protocols involving the use of supravital stains are recommended for simultaneous assessment of AR and to monitor viability in frozen-thawed spermatozoa [50]. In these circumstances, we prefer to use FITC-PNA associated to Hoechst-33258. Briefly, an aliquot of the sperm specimens are centrifuged and resuspended in 2 $\mu\text{g/mL}$ Hoechst-33258 solution. The sperm suspension is incubated for 10 minutes in the dark and then spermatozoa are washed

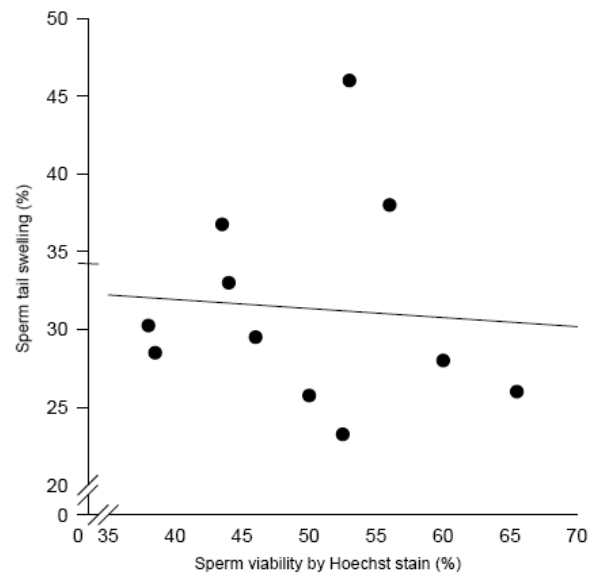


Fig. (3B). Correlation of viability results between sperm tail swelling by HOST and Hoechst 33258 stain in frozen-thawed semen specimens. No correlation was seen between HOST and Hoechst 33258 ($r = 0.11$, $p = 0.70$). (Adapted from *Fertil Steril*, 1996, Vol. 66, Esteves SC, et al. Suitability of the hypo-osmotic swelling test to assess the viability of cryopreserved sperm, pp. 798-804).

to remove excess stain. Smears are prepared on to glass microscope slides and then immersed in ice-cold methanol to permeabilize the sperm membranes. The fixed smears are immersed in a 40- $\mu\text{g/mL}$ FITC-PNA solution, incubated at room temperature, and washed gently in PBS to remove the excess label [50]. A microscope equipped with phase contrast and fluorescence epi-illumination module is used to examine the slides at 1000X magnification. The illumination is selected to mercury ultraviolet epi-illumination source for assessing FITC-PNA and Hoechst-33258 labeling by using appropriate filter cubes. FITC-PNA and Hoechst-33258 fluoresce “apple-green” and bright medium blue, respectively (Fig. 4). Examination of the same spermatozoon for FITC-PNA labeling and for Hoechst-33258 staining is performed by interchanging the fluorescence filter cubes.

Acrosome Reaction Challenge Testing

Acrosome reaction can be induced *in vitro* by ionophores such as A23187 which exchange Ca^{2+} for 2H^{+} . The ionophore-induced acrosome reaction, also termed ‘acrosome reaction to ionophore challenge (ARIC)’ has been extensively validated as a sperm function test with excellent predictive values for outcomes in assisted reproduction [4, 48, 55-58]. Briefly, motile spermatozoa are first capacitated for 3 h by incubation in sperm culture medium at 37°C and then divided into two equal aliquots: one is incubated with 2.5 $\mu\text{mol/L}$ calcium ionophore A23187 solution, and the other is treated with dimethylsulfoxide (10%, v/v) as a control [35, 59, 60]. Acrosome status can be determined by FITC-PNA or FITC-PSA in combination with the nuclear stain bis-benzimide or HOST to monitor viability. The ARIC test score is calculated by subtracting the frequency of AR occurrence following ionophore challenge from the baseline values. Using the acrosome reaction to ionophore

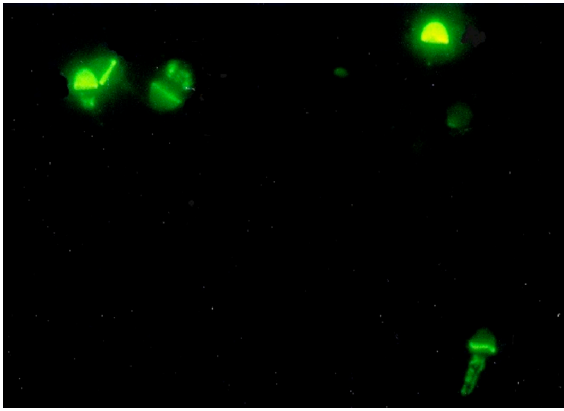


Fig. (4A). Photomicrograph of spermatozoa labeled by FITC-PNA. Spermatozoa were examined under fluorescence epi-illumination at 1,000x magnification. In spermatozoa with intact acrosomes, the acrosomal region of the sperm head exhibited a uniform apple-green fluorescence. In spermatozoa exhibiting acrosome reaction, only the equatorial segment of the acrosome was stained (*Adapted from International Braz J Urol, Vol. 33, 3, Esteves SC et al. Evaluation of acrosomal status and sperm viability in fresh and cryopreserved specimens by the use of fluorescent peanut agglutinin lectin in conjunction with hypo-osmotic swelling test, page 367, Copyright 2007, with permission from FB Sampaio, editor*).

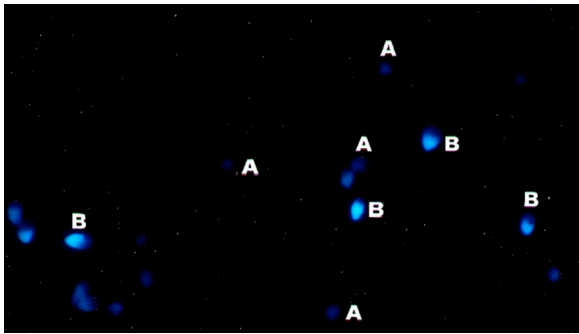


Fig. (4B). Photomicrograph of spermatozoa labeled by Hoechst-33258. Spermatozoa were examined under mercury ultraviolet epi-illumination at 1,000x magnification. Hoechst-33258 stains the nuclei of damaged cells (dead spermatozoa), which show a bright blue-white fluorescence (B) and is excluded from viable cells (live spermatozoa), which show a pale blue fluorescence (A). (*Adapted from International Braz J Urol, Vol. 33, 3, Esteves SC et al. Evaluation of acrosomal status and sperm viability in fresh and cryopreserved specimens by the use of fluorescent peanut agglutinin lectin in conjunction with hypo-osmotic swelling test, page 367, Copyright 2007, with permission from FB Sampaio, editor*).

challenge (ARIC) cut-off of 10%, the sensitivity of the test in predicting subfertility is 54% with a positive predictive value of 64% and specificity of 85% [56]. Patients with ARIC scores above 10% have consistently better fertilization than those with lower scores [4, 48]. The mechanism of the acrosome reaction induced by ionophores differs from the physiological acrosome reaction induced by the zona pellucida [18]. While the physiological acrosome reaction involves activation of several signal transduction pathways, the ionophore-induced acrosome reaction mainly involves a chemical effect on calcium influx that partially reflects the physiological acrosome reaction process [18].

Jaiswal *et al.* [61] compared different acrosome reaction inducers and probes to assess the acrosome reaction. They confirmed that different inducers potentiate different levels of acrosome reaction. Furthermore, their study provided an explanation for these differences by demonstrating that some inducers (e.g. progesterone and follicular fluid) trigger only partial acrosome reaction whereas calcium ionophore A23187 induce both complete and partial acrosome reaction. This appears to be the reason why, in earlier studies, A23187 always yielded higher acrosome reaction levels than progesterone and follicular fluid [62]. It is therefore suggested that inducers such as progesterone and follicular fluid should be used for potentiating partial acrosome reaction, and the assessment should be carried out with probes that target the acrosomal content, e.g. FITC-PSA or FITC-PNA. For inducing a complete acrosome reaction, A23187 is preferable [61].

IMPACT OF SPERM PROCESSING AND *IN VITRO* INCUBATION ON ACROSOME INTEGRITY

In vivo, spermatozoa must be washed free from the seminal plasma before they are capable of undergoing capacitation and acrosome reaction. This process is accomplished under normal physiological conditions when seminal plasma is removed as the spermatozoa traverse the cervical mucus, and capacitation occurs as they are transported across the cervix, uterus and fallopian tubes [5]. Such conditions can be mimetized *in vitro* by sperm processing and incubation with protein-supplemented culture media. We sought to determine whether the swim-up technique selected spermatozoa with better motion characteristics, and how swim-up and subsequent capacitation affected the spontaneous acrosome reaction rate. The motile sperm fraction from normal donors was isolated by the swim-up method from a washed sperm pellet [59]. Then, specimens were capacitated for 3 hours by incubation in modified Biggers-Whitten-Whittingam (BWW) medium supplemented with 3% human serum albumin at 37°C in a 5% CO₂ atmosphere. Sperm motility, viability, and motion characteristics, as assessed by computer-assisted semen analysis (CASA) were significantly increased in both swim-up and capacitated specimens. The percentage of acrosome-reacted spermatozoa remained unchanged (~13%) in both unprocessed and swim-up groups, but was significantly increased by 36% after capacitation. We concluded that swim-up isolates sperm with greater motion characteristics but does not change the acrosome reaction rate [63]. We also reported that *in vitro* capacitation is not an absolute prerequisite for an agonist-induced acrosome reaction using calcium ionophore, although capacitation may significantly optimize the agonist-induced acrosome reaction rate. Our data suggest that artificially-induced *in vitro* acrosome reaction may involve other complex mechanism(s) rather than a physiological change induced by capacitation, as also reported by others [34, 64].

ACROSOME REACTION RESPONSE TO STIMULANTS

Cyclic nucleotide phosphodiesterases (PDE) regulate intracellular levels of cAMP and cGMP by hydrolyzing them to the corresponding 5' monophosphates [65]. Nine different

PDE isoenzymes (PDE₁ to PDE₉) have been described and found to be present at various concentrations in human tissues [66, 67]. Previous studies have shown that mRNA coding for cAMP-specific PDE (PDE_{4A}) isoforms are present in mature rat and mouse germ cells [68], and that the expression of these isoforms is maximal in round spermatids and is maintained in mature spermatozoa [69]. Nitric oxide synthase and PDE types 1, 4 and 5 isoforms are present in human spermatozoa [70, 71]. *In vitro* inhibition of sperm PDE₁, PDE₄ and PDE₅ isoenzymes by specific inhibitors stimulates acrosome reaction and sperm motility [65, 71-73], suggesting that this is not a response specific to only one of the PDE family [74].

The mechanism by which PDE inhibitors exert their pharmacological effects is by inhibiting phosphodiesterase, an enzyme responsible for the degradation of cGMP and cAMP [75]. This creates an environment with increased energy substrates. Increased levels of cAMP and cGMP affect many intracellular functions including calcium transport [76]. This altered level of calcium is known to affect sperm motion and an energy-dependent influx of calcium into the sperm cell is believed to be responsible for initiation of the acrosome reaction [30, 77].

cAMP Phosphodiesterase Inhibitors

Cyclic AMP has a role in sperm kinematics and in the acrosome reaction second-messenger system [30, 78]. Garbers *et al.* [79] made the initial observations that sperm motility could be increased by a variety of putative cyclic nucleotide phosphodiesterase inhibitors (caffeine, theophylline, papaverine). Treatments that increase intra-cellular cAMP concentrations often cause an increase in sperm motility and kinematics as well as in the agonist-induced acrosome reaction [30] and fertilization rates [80, 81]. Pentoxifylline (PF), a phosphodiesterase inhibitor, inhibits cyclic adenosine monophosphate (cAMP) phosphodiesterase, thus increasing intracellular cAMP concentration [80, 82] and tyrosine-phosphorylation at the tail level [83]. Also, it has been postulated that PF has the ability to scavenge reactive oxygen radicals [60, 84-86]. Pentoxifylline treatment increases the capacity of human spermatozoa to undergo to the acrosome reaction in response to both natural (follicular fluid) and artificial (ionophore A23187) stimuli, and it also improves the fertilizing ability in patients with acrosome reaction insufficiency [82, 87]. Significant increase in pregnancy rates were observed following intra-uterine insemination when PF was added to the semen at the time of sperm preparation [88].

In humans, the ideal concentration of PF to be used *in vitro* is a matter of debate. Some authors reported beneficial effects with 3.6 mM on motility and sperm fertilizing ability [82, 89, 90] while others demonstrated a similar response both with lower and higher concentrations [91-94]. A detrimental effect on sperm membrane integrity was reported at the PF concentration of 10 mM [86], but a considerable variable response to PF is also seen in both normozoospermic [92] and oligozoospermic semen specimens [80]. At a concentration of 5 mM PF has oxygen-free radical scavenging capacities by reducing the superoxide release from human spermatozoa following phorbol myristate acetate [85]. Direct addition of PF to sperm specimens was shown to

optimize fertilization rates in assisted conception involving conventional *in vitro* fertilization [80, 95]. According to our data, PF does not alter the frequency of spontaneous acrosome reaction at a 5mM concentration when added directly to the semen of normal and infertile men [35, 60].

cGMP Phosphodiesterase Inhibitors

Cyclic GMP is less extensively studied than cAMP on sperm function. It has been suggested that cGMP regulates lipid metabolism in other vertebrate species [79]. When pig spermatozoa are incubated in a minimal culture medium, added oviduct fluid can cause up to 50-fold increase of cGMP [96]. In the human, dibutyl cGMP induces acrosomal loss in a pathway independent of calcium influx when spermatozoa were first preincubated under capacitating conditions [29]. The concentration of cyclic GMP in ejaculated human semen is < 16 pmol/10⁶ cells, while the corresponding cyclic AMP concentration is 100 pmol/10⁶ cells [32, 33]. Although the concentration of cGMP in ejaculated human sperm is almost 7 times lower than cAMP, it seems reasonable to speculate that both nucleotides may have a similar role, since cyclic AMP- and cGMP-dependent protein kinases, that mediate the actions of these nucleotides in various tissues, are similar in many respects and may be closely related proteins [34].

Sildenafil, a selective type 5 phosphodiesterase inhibitor (PDE₅), acts by increasing intracellular levels of cGMP and cAMP [97]. Taken orally, it causes a marked increase in cGMP concentrations and facilitates erection in men with erectile dysfunction [98]. Acute administration of 100 mg sildenafil promotes a concentration of 0.1-0.3 µmol/L in the ejaculate (Pfizer, ViagraTM data sheet), and this concentration is consistent with a possible inhibitory effect on sperm PDE isoforms [67]. The effects of sildenafil on human sperm function have gained attention in recent years. Cuadra *et al.* [72], studying its effects on sperm motility and acrosome parameters *in vitro*, observed a dose-dependent improvement on sperm motility and an increase in the spontaneous acrosome reaction rate. Aversa *et al.* [99] evaluated the effects of oral sildenafil administration (100 mg) on seminal parameters in young healthy male volunteers. The authors found that sildenafil caused no changes in the seminal parameters when compared to placebo. Conversely, Lefievre *et al.* [100] reported that sildenafil stimulated human sperm motility and capacitation but not acrosome reaction at concentrations of 10, 30, 100, or 200 µM. Recently, Glenn *et al.* [74] reported that sildenafil citrate improved sperm motility and increased the premature acrosome reaction rate *in vitro* at the concentration of 0.67 µM.

We investigated the *in vitro* effects of sildenafil citrate on sperm function of healthy male volunteers with proven fertility. Semen specimens were obtained by masturbation and were divided into four equal aliquots: the first received no treatment (control) and the others were incubated with 0.001µM, 0.01µM and 0.1µM of sildenafil citrate (Pfizer, Sandwich, UK) for 1 hour. After treatment, all aliquots were processed by the swim-up technique and underwent further *in vitro* incubation under capacitating conditions for 3 hours. Overall, motion parameters assessed by CASA were significantly increased after sperm treatment with 0.01 µM

Table 1. Effects of Sperm Treatment with Sildenafil Citrate on Acrosome Integrity and Acrosome Reaction in Response to Ionophore Challenge (ARIC test) in 21 Normozoospermic Men (*Adapted from Fertil Steril 84, Suppl, Esteves et al. Acrosome integrity and agonist-induced acrosome reaction rate after stimulation by varying concentrations of a specific inhibitor of cGMP phosphodiesterase type-5 (Sildenafil) in normozoospermic specimens, page S225, Copyright 2005, with permission from Elsevier*)

Sperm Parameters	Control	Sd1 [0.001 μ Molar]	Sd2 [0.01 μ Molar]	Sd3 [0.1 μ Molar]	P-value*	P value [†]
Frequency of live sperm with intact acrosome (%)	82.0 \pm 8.9 ^a	88.8 \pm 6.8 ^b	84.8 \pm 7.5 ^c	88.5 \pm 7.8 ^d	axb=0.20 axc=0.15 axd=0.25	0.10
Acrosome reaction before ARIC test (%)	7.1 \pm 4.8 ^a	4.1 \pm 3.1 ^b	4.4 \pm 3.2 ^c	3.6 \pm 1.2 ^d	axb=0.02 axc=0.02 axd=0.01	0.26
Acrosome reaction after ARIC test (%)	24.8 \pm 14.7 ^a	30.6 \pm 20.8 ^b	26.6 \pm 14.3 ^c	29.3 \pm 13.1 ^d	axb=0.75 axc=0.24 axd=0.60	0.52
Percent variation (%)	351.6 \pm 346.6 ^a	950.8 \pm 684.1 ^b	633.3 \pm 589.0 ^c	769.2 \pm 401.3 ^d	axb=0.03 axc=0.04 axd=0.04	0.09

* Control *versus* Sildenafil (Sd) at different concentrations; a-d: pairwise comparisons by ANOVA

† Comparisons among different concentrations of sildenafil

P \leq 0.05 was considered significant.

sildenafil. Curvilinear velocity, amplitude of lateral had displacement and hyperactivated motility were also significantly increased at the concentration of 0.1 μ M, but no effects were observed at the sildenafil concentration of 0.001 μ M [101]. Acrosome integrity and acrosome reaction in response to ionophore challenge (ARIC test) was examined by FITC-PNA combined with Hoechst-33258 to monitor sperm viability. Spontaneous acrosome reaction rates were similar in both control and treated groups. However, acrosome reaction rates in response to calcium-ionophore challenge were significantly higher in all sildenafil treated groups compared to control ($p < 0.05$). A dose-dependent effect of type-5 PDE inhibitor on sperm acrosome status was not observed (Table 1) [102].

SPERM CRYOPRESERVATION AND ACROSOME STATUS

The freeze-thawing process directly damages the sperm membranes; as such, loss of membrane permeability and subsequent cell death may occur [51]. Cryopreservation can also cause permanent functional damage (sublethal damage), thus reducing the fertilizing ability of human sperm [35]. Cryopreservation-related impairment in sperm function can be explained partially by the reduction in the percentage of normal intact acrosomes [64]. Although acrosomal damage may be secondary to cell death [51] an increase in the proportion of viable spermatozoa with reacted acrosomes has been reported after cryopreservation [64]. Therefore, adequate assessment of the acrosomal status in cryopreserved sperm [50] should include monitoring of sperm viability, as previously discussed. Cryo-thawed spermatozoa may also have defective sperm function due to elevated levels of reactive oxygen specimens [103]. This may cause peroxidation of unsaturated fatty acids in the acrosomal membrane

and decrease the response to calcium influx signals that trigger the acrosome reaction [35].

Efforts to improve the fertilizing ability of cryopreserved spermatozoa are useful for assisted conception. We have investigated different strategies to minimize the acrosomal loss due to the freeze-thawing process in human sperm. In one study, we examined the impact of freezing sperm after removing the seminal plasma and selecting the motile fraction on the post-thaw acrosome status [59]. It has been shown that prolonged exposure to seminal plasma results in marked decline in both motility and viability, and inhibition of the acrosome reaction [4, 48, 62, 104, 105]. Ejaculated spermatozoa are also damaged by reactive oxygen species emanating from seminal leukocytes and damaged spermatozoa [106]. In assisted reproduction procedures such as intrauterine insemination (IUI) and IVF, ejaculated spermatozoa are separated from the seminal environment as soon as possible. The swim-up procedure accomplishes this, and enables the selection of populations of highly motile spermatozoa for assisted reproduction procedures. To examine if the selection of such a sperm population before freezing minimized the deleterious effects of cryopreservation, we divided semen specimens from normal donors into two equal aliquots: one received no treatment (control) and the other was processed by swim-up from a washed sperm preparation [59]. Both aliquots were cryopreserved by the liquid nitrogen vapor method. Percentage motility and motion characteristics were evaluated by computer-assisted semen analysis. Acrosome integrity as well as spontaneous and calcium ionophore-induced acrosome reactions before freezing and after thawing were assessed by fluorescein isothiocyanate conjugated peanut agglutinin combined with a supra vital dye (Hoechst-33258). We noted that swim-up processing enabled selection of a sperm population with better motion characteristics, percentage motility and viability before freezing ($P < 0.001$), but with no difference in percentage of acrosome-intact spermatozoa ($P = 0.63$). After thawing, the

Table 2. Effects of Pre-Freeze Sperm Processing by Swim-Up on Post-Thaw Calcium Ionophore induced Acrosome Reaction in 15 Normozoospermic Men (Adapted from Hum Reprod 15, 10, Esteves SC et al. Improvement in motion characteristics and acrosome status in cryopreserved human spermatozoa by swim-up processing before freezing, page 2177, Copyright 2000, with permission from Oxford University Press)

Post-Thaw Frequency of Live Spermatozoa with Intact Acrosome (%)	Control group (mean ± SD)	Swim-up group (mean ± SD)	Percentage change ^a (mean ± SD)	P-value ^b
Before ionophore challenge	66.5±9.2	72.1±7.2	10.1±17.5	0.04
After ionophore challenge	55.1±9.9	52.1±10.6	-3.6±21.6	NS
Percent change ^a	-17.1±11.2	-27.6±13.2	-	0.003
P value ^c	<0.001	<0.001	-	-

^a Calculated as mean of individual changes.

^b P < 0.05 was considered significant between control and swim-up groups. (Student's paired t-test).

^c P < 0.05 was considered significant between the frequency of live spermatozoa with intact acrosome before and after ionophore challenge.

NS = not significant.

swim-up specimens exhibited faster velocity and progression than untreated specimens (P < 0.001). They also had higher percentages of spermatozoa with intact acrosomes and spermatozoa able to undergo acrosome reaction in response to calcium ionophore (Table 2). This effect were not explained by higher initial values for acrosome integrity, because pre-freeze acrosome integrity was similar in both sperm preparations. We speculated that the highly motile sperm population selected by swim-up may not be affected by oxygen radicals emanating from damaged leukocytes and from other spermatozoa susceptible to or damaged by lipid peroxidation. In a study using transmission electron microscopy, it was demonstrated [107] that the deleterious effects of cryopreservation on sperm acrosomes were less evident if spermatozoa had been frozen without seminal plasma. We concluded that the swim-up method using a washed sperm preparation before freezing offered the possibility of selecting spermatozoa with better post-thaw motility, acrosome integrity, and ability to undergo AR. Because the yield of spermatozoa obtained by the swim-up procedure is low, oligozoospermic specimens are not suitable to be frozen by this treatment strategy.

In other studies, we investigated the effects of pentoxifylline (PF) added directly to the seminal plasma before freezing on acrosome status of normal and abnormal semen specimens [35, 60]. In these trials, semen specimens were divided into two equal aliquots: one received no treatment (control) while the other was incubated with 5 mM PF (treated). Both aliquots were cryopreserved by the liquid nitrogen vapor method. Acrosome integrity and spontaneous and calcium ionophore-induced acrosome reactions were

assessed with fluorescein isothiocyanate-conjugated peanut agglutinin combined with a supra-vital dye (Hoechst-33258). In normal semen, a PF-induced protective effect on acrosome loss during the freeze-thaw process was observed (Table 3) [35].

In oligoasthenozoospermic specimens, however, a significant decrease in the post-thaw percentage of viable spermatozoa with intact acrosomes in both control and PF-treated specimens was observed [60]. In both normal and abnormal specimens, prefreeze sperm treatment with PF improved the ability of cryo-thawed spermatozoa to undergo the acrosome reaction in response to stimuli (Tables 4 and 5). Interestingly, the ability of cryo-thawed sperm from abnormal specimens to respond to ionophore challenge was lower than that of normal sperm, irrespectively of treatment with PF before freezing. In normal semen, post-thaw acrosome reaction stimulated by calcium ionophore was 34.8% and 53.3% for control and treated specimens, respectively, while values of 11.8% and 30.2% were reported in abnormal semen specimens. In infertile men, ARIC scores were higher than 10% in 50% of the cryo-preserved PF-treated specimens, and in only 19% of the controls. In normal men, ARIC scores higher than 10% were seen in 80% and 60% of the cryo-thawed PF-treated and control specimens, respectively. PF improved ARIC scores in 81.2% and 100% of infertile and normal men, respectively. Our results suggest that normal semen resist cryo-injury better than specimens from oligoasthenozoospermic men. They also indicate that treatment of human sperm with PF may enhance post-thaw sperm fertilizing ability. PF at a 5-mM concentration afford greater cryo-protection in normal semen while a different

Table 3. Effects of Pre-Freeze Sperm Treatment with Pentoxifylline on Spontaneous Acrosome Reaction in 15 Normozoospermic Men before and after Cryopreservation (Adapted from Hum Reprod 13, 12, Esteves SC et al. Cryopreservation of human spermatozoa with pentoxifylline improves the post-thaw agonist-induced acrosome reaction rate, page 3386, Copyright 1998, with permission from Oxford University Press)

Frequency of Spontaneous Acrosome Reaction	Control Median (IQ Range)	Pentoxifylline Median (IQ Range)	P value*
Pre-freeze	10.3 (4.5-15.5)	17.8 (14.7-25.5)	0.0001
Post-thaw	28.2 (26.6-41.7)	32.3 (27.5-53.6)	0.04
Percentage change	219.5 (163.9-495.2)**	127.2 (23.8-212.2)**	0.0003

* < 0.05 was considered significant (Wilcoxon signed-rank test).

** Significant increase after cryo-preservation (P < 0.001).

IQ = interquartile.

Table 4. Effects of Pre-Freeze Sperm Treatment with Pentoxifylline on Post-Thaw Calcium-Ionophore induced Acrosome Reaction in 15 Normozoospermic Men after Cryopreservation (Adapted from Hum Reprod 13, 12, Esteves SC et al. Cryopreservation of human spermatozoa with pentoxifylline improves the post-thaw agonist-induced acrosome reaction rate, page 3386, Copyright 1998, with permission from Oxford University Press)

Frequency of Spontaneous Acrosome Reaction (%)	Control Median (IQ range)	Pentoxifylline Median (IQ range)	P value*
Before ionophore challenge	28.2 (26.6-41.7)	32.3 (27.5-53.6)	0.04
After ionophore challenge	47.9 (34.9-52.5)	58.2 (48.6-64.8)	0.0001
Percentage change	34.8 (10.3-57.8)**	53.3 (22.6-101.7)**	0.03

* < 0.05 was considered significant (Wilcoxon signed-rank test).

** Significant increase after cryopreservation (P < 0.001).

IQ = interquartile

Table 5. Effects of Pre-Freeze Treatment with Pentoxifylline on Post-Thaw Calcium Ionophore-Induced Acrosome Reaction in Sperm from 16 Oligoasthenozoospermic Men (Adapted from Braz J Med Biol Res 2007; 40, 7, Esteves SC et al. Effects of pentoxifylline treatment before freezing on motility, viability and acrosome status of poor quality human spermatozoa cryopreserved by the liquid nitrogen vapor method, page 989, Copyright 2007, with permission from Creative Commons Attribution License)

Post-Thaw Frequency of Live Spermatozoa with Reacted Acrosomes (%)	Control	Pentoxifylline
Before ionophore challenge	43.9 (27.2-54.5)	38.0 (30.1-51.2)
After ionophore challenge	45.6 (25.7-59.1)	52.7 (38.5-67.4)*
Percent change ^a	-11.8 (-32.0-2.9))	-30.2 (-40.8-18.5))*
ARIC score (%)	4.8 (0.5-6.8)	9.7 (6.6-19.7)*
P value ^b	<0.001	<0.001

Data are reported as median with interquartile range in parentheses. Acrosome reaction in response to ionophore challenge (ARIC) score was calculated by subtracting the frequency of acrosome reaction occurrence after ionophore challenge from the frequency of occurrence before it.

^apercent change calculated as median of individual changes;

^bP value comparing frequency of live spermatozoa with reacted acrosomes before and after ionophore challenge (Wilcoxon signed-rank test).

*P < 0.05 compared to control (Wilcoxon signed-rank test).

concentration may be necessary to neutralize excessive reactive oxygenspecies (ROS) emanating from the already compromised spermatozoa from oligoasthenozoospermic individuals.

EXPERT COMMENTARY

The fertilization process is a net result of a series of molecular events enabling sperm to reach, recognize, bind to, and penetrate the oocyte. There has been a dramatic increase in our knowledge of the protein composition of the spermatozoon, its structures and the surrounding fluids (epididymal, vesicular, prostate) contributing to its function. As a result of research involving sperm capacitation and AR, we gained insight into the signalling complexes involved in key sperm physiological processes. Determination of both basal and induced-acrosome reaction may be clinically useful. A high proportion of sperm exhibiting either AR prematurity or stimuli-induced AR deficiency is correlated with decreased the sperm's fertilizing potential *in vivo* and *in vitro*. In assisted conception treatment cycles involving intra-uterine insemination and conventional *in vitro* fertilization, the incubation interval after sperm processing should not exceed 3 hours. Longer post-processing incubation increases the proportion of sperm that undergo untimely acrosome reaction and may negatively impact treatment results.

Although specialized semen tests such as the AR aid in determining specific defects of human sperm physiology, its clinical use is limited nowadays by the success of ICSI for men with male infertility. As such, there is little incentive for infertility clinics to invest in elaborate sperm function tests. Although this policy is prevailing in the present time, ICSI is very expensive, invasive, has limited success, a number of side effects, is not widely available, and we still have little knowledge as to the effects of the use of ICSI on the health of future generations. The increasing demand to treat couples with the least invasive treatment, as posed by some government-funded infertility treatment policies, could encourage a step-back towards the use of prognostic sperm tests for both diagnosis and treatment. Currently, AR testing is rarely used in the clinical setting for men being evaluated after IUI or IVF failure to better discriminate those who may benefit from ICSI. The present focus of the *in vitro* AR testing is andrology research involving male contraception and gonadotoxic effects of food and drugs.

FIVE-YEAR REVIEW

Most studies involving the sperm acrosome reaction were published 20-30 years ago. This boom in research was compelled by the need to understanding the physiology of human fertilization *in vivo* and *in vitro*, as a consequence of the increasing demand of male infertility as an important

health issue. Conventional semen analysis results, despite being an integral step of the male infertility evaluation, do not necessarily correlate with fertility potential because it does not assess sperm function. There are still a significant proportion of patients with normal and abnormal semen analyses showing unexplained infertility and achieving unassisted conception, respectively [108, 109]. In fact, it is not so much the absolute numbers of spermatozoa that determines fertility, but their functional competence. The acrosome reaction testing has been shown to be a stable parameter of sperm function and a valid tool to predict the fertilizing potential of human spermatozoa *in vitro*. In a recent study, calcium ionophore-induced acrosome reaction (ARIC) results have been shown to be a reliable indicator of fertility potential in intrauterine insemination and conventional IVF treatment cycles [110]. The AR threshold values determined using ROC curves for predicting complete fertilization failure and pregnancy in IVF were 21% and 26% respectively, and they were the sole predictors independent of the conventional semen analysis. In addition, no pregnancies were obtained by IUI when ARIC results were below 11%. With ICSI, zona pellucida penetration and oolemmal fusion are bypassed and the role of acrosome reaction is unclear. ARIC results were not predictive of ICSI outcome, and sperm selection from different acrosome-reacted sperm populations appeared to have no impact on fertilization rates [15, 110].

The AR assessment has been also used as an important biomarker in studies involving drugs and toxins. In one report, the frequency of acrosome-reacted spermatozoa was increased by sperm incubation with sildenafil citrate while in another the recreational use of delta-9-tetrahydrocannabinol caused partial inhibition of the sperm ability to undergo stimuli-induced AR [74, 111]. Falzone *et al.* [112], studying the effect of pulsed 900-MHz GSM mobile phone radiation on the AR of human sperm, observed that although radiofrequency electromagnetic fields (RF-EMF) exposure did not adversely affect the AR rate, it had a significant effect on sperm morphometry and in the ability of sperm binding to the hemizona. Mukhopadhyay *et al.* (2010) evaluated the *in vitro* effect of benzo[a]pyrene, a substance present in cigarettes, on sperm hyperactivation and acrosome status of normozoospermic semen specimens. They observed a significant increase in sperm hyperactivation as well as in the premature acrosome reaction rates in presence of benzo[a]pyrene [113].

Recent publications on acrosome reaction focused on the biochemical and functional aspects of sperm-oocyte fusion. It has been demonstrated that in humans, ZP1 in addition to ZP3 and ZP4 binds to capacitated spermatozoa and induces acrosomal exocytosis [20, 21, 114, 115]. The ZP3-induced acrosome reaction involves the activation of T-type voltage-operated calcium channels (VOCCs), whereas ZP1- and ZP4-induced ARs involve both T- and L-type VOCCs. Chiu *et al.* [116] reported that glycodeclin-A, a glycoprotein present in the female reproductive tract, sensitizes spermatozoa to the zona pellucida-induced acrosome reaction in a glycosylation-specific mechanism involving the activation of the adenylyl cyclase/PKA pathway, suppression of extracellular signal-regulated kinase activation and up-regulation of zona pellucida-induced calcium influx. It is therefore suggested

that glycodeclin-A may be important *in vivo* to ensure full responsiveness of human spermatozoa to the zona pellucida.

KEY ISSUES

- Capacitation prepares the sperm to undergo the acrosome reaction with the accompanying release of lytic enzymes and exposure of membrane receptors, which are required for sperm penetration through the zona pellucida and for fusion with the oolema. AR is a time-coordinated phenomenon that cannot occur prematurely or too late.
- When the process of AR is impaired, either by lack of an acrosome or acrosomal dysfunction, fertility can be compromised. Acrosome-reacted sperm lack the ability to bind to the ZP so are essentially infertile sperm.
- *In vitro*, the most widely used methods to assess the sperm acrosome utilize optical microscopy and fluorescent lectins staining that bind to either the outer or inner acrosomal membrane.
- Because acrosomal loss can be a result of sperm death, acrosome testing has to be used in conjunction with viability assays, such as supravital staining techniques and the hyposmotic swelling test, to distinguish non-viable sperm from 'truly' reacted acrosomes.
- HOST is a simple and reliable assay to monitor viability during AR assessment in fresh but not in cryopreserved semen.
- In IUI and conventional IVF, caution should be applied to avoid long post-processing incubation intervals before insemination. The proportion of sperm that undergo spontaneous AR acrosome reaction is increased after 3 hours of incubation.
- Several substances may affect the sperm acrosome, including cyclic AMP and GMP phosphodiesterase inhibitors, calcium ionophore and toxins.
- Strategies to minimize acrosomal loss due to the freeze-thawing process include the freezing of selected sperm populations and the addition of motility stimulants such as pentoxifylline.
- The assessment of the acrosome reaction *in vitro* may predict the sperm's fertilizing potential and is a potential marker to aid in the selection of assisted conception treatment. Currently, with the success of ICSI for male infertility AR testing is rarely used in the clinical setting. The increasing demand to treat couples with the least invasive treatment could encourage a step-back towards the use of such prognostic sperm tests for both diagnosis and treatment.
- *In vitro* AR testing is routinely used in andrology research involving male contraception and gonadotoxic effects of food and drugs.

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CONFLICT OF INTEREST

None Declared.

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