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Original Research Article

Calcium ionophore A23187 versus progesterone: induction of acrosome reaction in thawed and prepared cryopreserved semen samples

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ABSTRACT

Background: Acrosome reaction facilitates fertilization and it can be induced in-vitro using various stimuli like progesterone, calcium ionophore A23187, prostaglandins, etc. We investigated the efficacy of calcium ionophore and progesterone in inducing acrosome reaction in prepared semen samples following cryopreservation, using fluorescein isothiocyanate- labelled *Arachis hypogaea* (peanut) agglutinin (FITC-PNA) as a marker.
Methods: A prospective analysis was carried out on 10 normozoospermic semen samples collected between March and May 2025 at the department of reproductive medicine and andrology, Chettinad Super Speciality Hospital, Chettinad Academy of Research and Education. The collected semen samples were cryopreserved using rapid freezing method and subsequently after thawing, sperm preparation was carried out using direct swim-up method. The obtained post-wash was then subjected to capacitation and in-vitro induction of acrosome reaction using calcium ionophore A23187 and progesterone. The spermatozoa were stained with a fluorescent dye FITC-PNA and by examining under a fluorescent microscope, acrosome reacted and acrosome intact spermatozoa were scored. An independent t-test was performed to determine the statistical significance between the two groups. The statistical significance was set at p value <0.05.
Results: Our study revealed a significant difference between calcium ionophore and progesterone in inducing acrosome reaction in frozen thawed spermatozoa. Calcium ionophore demonstrated a higher acrosome reaction rate when compared to progesterone (p value <0.001).
Conclusions: The in-vitro induced acrosome reaction rate in frozen thawed spermatozoa is significantly higher with calcium ionophore when compared to progesterone.

Keywords: Acrosome reaction, Aqueous progesterone, Calcium ionophore, Cryopreservation, Fertilization failure, Induced acrosome reaction

INTRODUCTION

Acrosome occupies 40-70% of the spermatozoa head and contains hydrolytic enzymes like hyaluronidase, proacrosin, phospholipase A2, etc.[1] The structural integrity and its ability to undergo acrosomal reaction are the key vital aspects for sperm-oocyte interaction and fertilization. The exocytosis of acrosomal contents called the 'acrosome reaction', is facilitated by the fusion of spermatozoa's plasma membrane with the outer acrosomal membrane. This enables sperm penetration through the cumulus cells and zona pellucida during the process of fertilization.[2]

Acrosome reaction in the in-vivo milieu is induced by endogenous factors, notably progesterone, zona pellucida glycoproteins, calcium influx, prostaglandins, and follicular fluid.[3] Any disruptions in the acrosome reaction,

including its premature occurrence or stimuli-induced failure is likely to be associated with idiopathic male factor infertility.[4]

Calcium concentrations play a central regulatory role in acrosomal exocytosis. In patients with complete fertilization failure, a potential underlying defect in spermatozoa's ability to undergo acrosome reaction is suggested to be in association with the lack of increase in calcium concentration affecting oocyte penetration.[5] In such cases, acrosome reaction can be induced in-vitro, using physiological inducers like zona pellucida, follicular fluid or progesterone, as well as artificial inducers like calcium ionophore or heparin.[4]

Calcium ionophore induces in-vitro acrosome reaction by facilitating Calcium (Ca^{2+}) influx, which is coupled with the exchange of intracellular hydrogen ions (H^+).[4] This method is considered reliable in predicting the fertilizing potential of spermatozoa in assisted reproduction procedures.[6]

The acrosome reaction occurred more rapidly and significantly more quickly, when spermatozoa were incubated with calcium ionophore A23187 compared to heparin, at the same time intervals. While heparin was used to capacitate and induce acrosome reactions in cattle, goats and human, calcium ionophore is widely employed in inducing acrosome reactions in humans and mice.[7,8]

The elevated levels of intracellular Ca^{2+} concentration in spermatozoa, in response to progesterone is attributed to its indirect activation of specific calcium ion channels, which is thought to be essential for triggering acrosome reaction.[9,10] Although spermatozoa are capable of detecting low concentrations of progesterone; triggering calcium channel activation altering flagellar beating, and its role in modulating kinase activity, acrosome reaction and spermatozoa motility are totally dose dependent. A nearly three to four-fold increase in acrosome reacted spermatozoa was observed at 30 and 60 minutes of treatment with significant effects seen at 5 and 10 µM progesterone. The lower concentrations of progesterone yielded acrosome reaction rates which were similar to those in the untreated control group.[11]

Research infers that there is a concentration-dependent effect of progesterone on spermatozoa function, and suggests that at low concentrations, (10 nM to 100 nM), it enhances spermatozoa motility, while higher concentrations (1 µM to 10 µM) induces forward vigorous motility, hyperactivation and acrosome reaction.[11,12]

Both progesterone and calcium ionophore A23187 are capable of inducing acrosome reactions, yet they exhibit distinct patterns due to the difference in mechanism and pattern of response. Progesterone, typically triggers a partial acrosome reaction, whereas calcium ionophore results in a more complete reaction. This difference may have an implication on the selection of a functionally competent sperm population, for optimizing ART outcomes.[13]

The changes occurring in the acrosome can be detected by the use of monoclonal antibodies which provide high sensitivity and specificity; however, this approach is expensive and the visualization necessitates the conjugation of a secondary agent, typically a fluorescein-conjugated antibody. Alternatively, the dual staining technique using trypan blue and Giemsa can be employed, which is cost-effective but it limits the ability to accurately differentiate the acrosome status. In contrast, FITC-PNA- a commonly employed fluorescent labelling technique, effectively differentiates the acrosomal status and its reaction in spermatozoa. Because of its high effectiveness, this method has been widely used to assess the acrosome reaction across various species.[14]

Cryopreservation of spermatozoa is a widely used technique in donor insemination programs and in fertility preservation, which can later be used for therapeutic procedures like artificial insemination and Assisted Reproductive Technologies (ART).[15,16] Despite efforts in improvising human semen cryopreservation techniques, literature evidences states that the conception rates with frozen semen samples remain lower when compared to fresh semen samples.[17] This may be attributed to the alterations in acrosomal membrane during the freeze-thaw process, leading to compromised acrosomal integrity, a phenomenon that is previously well documented.[18]

Usually, intracytoplasmic sperm injection (ICSI) is often preferred over conventional in-vitro fertilization (c-IVF) when using cryopreserved spermatozoa, as it helps to reduce the risk of poor or failed fertilization.[19]

Assessing the in-vitro acrosome reaction in frozen thawed semen samples may serve as a valuable tool in predicting the fertility potential of the spermatozoa and subsequently aid us in selecting the suitable population for artificial insemination or c-IVF. Studies have also demonstrated a positive correlation in inducing acrosome reaction and fertilization outcomes, thereby elevating clinical outcomes.[20]

While studies have demonstrated an in-vitro induction of acrosome reaction in spermatozoa using calcium ionophore and progesterone individually, there is a lack of direct comparative outcomes assessing the relative efficacy and specific effects of these stimuli.

Aim

This study aimed to evaluate and compare the effectiveness of calcium ionophore A23187 and progesterone in inducing acrosome reaction in-vitro, in prepared semen samples following cryopreservation human spermatozoa, providing insights into their potential application in fertility treatments.

METHODS

This was a prospective study conducted on 10 semen samples obtained at the department of reproductive medicine and andrology, Chettinad Super Speciality Hospital, Chettinad Academy of Research and Education, between March and May 2025. The study was performed after obtaining approval from the institutional human ethics committee (IHEC-I/3102/24) and was in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments. We included normozoospermic semen samples and excluded those with abnormal sperm parameters during routine semen analysis. Samples collected by methods other than masturbation were also excluded.

Chemicals and reagents

Alexa Fluor 488 PNA (peanut agglutinin) lectin conjugate was obtained from Thermo Fisher Scientific, UK and Calcium Ionophore A23187 was procured from Sigma Aldrich, UK. The aqueous progesterone and lectin PNA conjugate were dissolved in Dulbecco's Phosphate Buffered Saline (DPBS), while calcium ionophore was dissolved in 5% ethanol.

Methodology

The semen sample was initially incubated for 30 minutes to achieve liquefaction. Later, semen analysis was performed as per the latest WHO 6th edition manual and the normozoospermic samples were cryopreserved using rapid freezing method. After thawing, sperm preparation was performed using direct swim-up method to isolate and concentrate the motile spermatozoa. The obtained post-wash was then incubated at 37°C using commercially available sperm wash media (a HEPES buffered media, containing human serum albumin) for a period of three hours to initiate capacitation. The sample was then aliquoted into 2 equal parts measuring 0.5 ml each. To one aliquot, 10 µl of calcium ionophore A23187 (0.5 µg/ml) was added and designated as group I and to the other aliquot, 10 µl aqueous progesterone (15 µg/ml) was added and designated as group II. Both the aliquots were incubated separately at 37°C; group I for 15 minutes and group II for 30 minutes, to induce acrosome reaction. In order to visualize the induced acrosomal changes, 10 µl of semen suspension was smeared onto the glass slide and immersed in 95% ethanol for 30 minutes to permeabilize the spermatozoa membrane and achieve cell fixation. The fixed smears were stained using fluorescein isothiocyanate-labelled *Arachis hypogaea* (peanut) agglutinin (FITC-PNA) solution (40 µg/ml) at 37°C for 30 minutes. The slides were then examined under a fluorescent microscope (×1000 magnification, oil immersion) using a 450-490 nm excitation filter, a 519 nm emission filter and an optimized dichroic mirror.

Scoring was performed based on the 6th edition WHO laboratory manual. Spermatozoa with a fluorescing band at the equatorial segment or no fluorescing stain in the acrosome region is considered acrosome reacted and spermatozoa with more than half the head brightly and uniformly fluorescing is scored as acrosome intact ones.

Statistical analysis

The percentage of acrosome-reacted spermatozoa induced by calcium ionophore A23187 and progesterone was compared and analyzed using IBM SPSS software version 30.0.0.0. An independent t test was performed to determine the statistical significance between the two groups. The statistical significance was set at p value <0.05.

RESULTS

The collected semen samples were obtained from men aged 25 to 45 years. The baseline semen parameters were similar between the groups as the same sample was aliquoted into two groups for the study. The mean values of the semen parameters are summarized in Table 1.

Table 1: Mean values of semen parameters.

Semen parameters	Mean±SD
Volume (ml)	1.78±0.34
Concentration (million/ml)	64.7±14.23
Progressive motility (%)	55.6±11.48
Total motility (%)	74.9±9.56

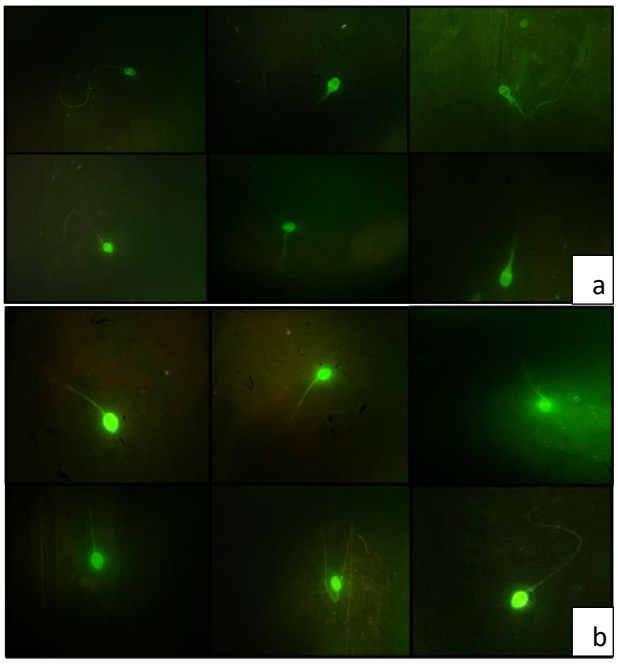

Figure 1:(a) Micrograph showing acrosome reacted spermatozoa with a fluorescing band at the equatorial segment or no fluorescing stain in the acrosome region. (b) Micrograph showing acrosome intact spermatozoa with more than half the head brightly and uniformly fluorescing.

Acrosome-reacted spermatozoa and acrosome intact spermatozoa are illustrated in Figures 1(a) and 1(b) respectively.

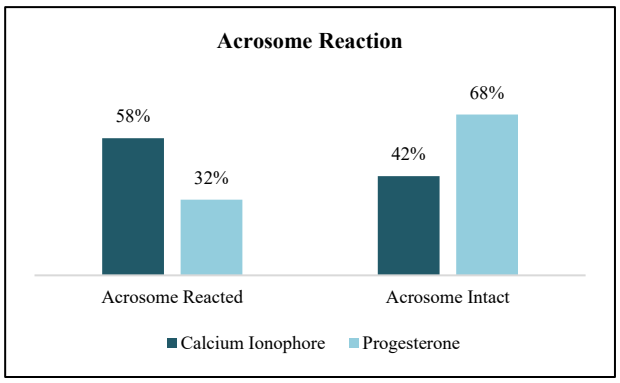

Figure 2: Induced acrosome reaction rate following calcium ionophore and progesterone treatment.

Figure 2 demonstrates the acrosome reacted and acrosome intact spermatozoa rates following treatment with calcium ionophore and progesterone.

Table 2: Comparative analysis of induced acrosome reaction rate.

Group	N	Induced acrosome reaction rate (%)	Statistical analysis
Calcium ionophore	10	57.60	Independent t-test p value<0.001
Progesterone	10	32.30	

As a result, the in-vitro induced acrosome reaction rate was significantly higher with calcium ionophore (57.6%) compared to aqueous progesterone (32.3%), showing a statistical significance (p value <0.001) between the two groups (Table 2).

DISCUSSION

Male factor infertility accounts for approximately 20-30% of infertility cases and contributes to 30-50% of couples who have difficulty in conceiving.[21] In cases of unexplained infertility, impaired acrosome reaction, affecting sperm-oocyte fusion may be an underlying cause for failed fertilisation.[22]

Effective treatment offered in male factor infertility is artificial insemination and assisted reproductive technology (ART) procedures like c-IVF and ICSI.[16,23] The total fertilization failure rate in ICSI is 1.8% whereas it is significantly higher in c-IVF with an incidence of 24%.[24]

A major cause of fertilization failure may be the absence or impairment of acrosome reaction, which is essential for sperm-oocyte interaction. In addition, deficiency or absence of key sperm-oocyte activating factors (SOAFs) like phospholipase Cζ (PLCζ), truncated form of the kit receptor (TR-KIT) and post-acrosomal sheath WW domain-binding protein (PAWP), can also lead to failed fertilisation after ICSI, by affecting oocyte activation.[25] Therefore, performing ICSI in combination with induced acrosome reaction may improve fertilization outcomes in earlier low fertilization cases.[26]

The freeze-thaw process directly disrupts the sperm plasma membranes, resulting in impaired membrane permeability.[27] Cryopreservation-associated sperm dysfunction is partially due to, a decrease in reduced proportion of sperm retaining intact acrosomes.[28] In-vitro induction of acrosome reaction may help overcome fertilization failure, commonly associated with frozen-thawed semen samples. Calcium ionophore A23187 and progesterone are well-known agents used to induce acrosome reaction in human spermatozoa.[4]

The use of calcium ionophore to induce acrosome reaction has been documented in several species, including human, rabbit and bovine.[29] It is a calcium-dependent reaction, requiring only minimal calcium concentrations to elicit a maximal response.[3]

The acrosome reaction ionophore challenge test has been employed to evaluate patients undergoing c-IVF, as reduced acrosome reaction rates have been associated with reduced fertilization outcomes.[30] Calcium ionophore A23187 induces a synchronous acrosome reaction in a significant proportion of spermatozoa and shortens the capacitation period in mammalian spermatozoa.[31]

Progesterone, a key regulator of sperm motility, induces hyperactivation and alters kinematic parameters at physiological concentrations (1-5 µm), while also triggering the acrosome reaction.[13,32] Evidences states that the sperm response to progesterone is a reliable marker in assessing fertilizing ability of human spermatozoa, potentially predicting fertilization rates and IVF outcomes.[14]

Multiple studies have shown that elevated serum progesterone levels can induce distinct changes in spermatozoa motility pattern and aid in inducing the acrosome reaction.[33] Earlier research has revealed that capacitated human spermatozoa typically undergo the acrosome reaction within 30 seconds of progesterone exposure.[34]

The induced acrosome reaction can be visualized using fluorescent markers like FITC-labelled *Pisum sativum* agglutinin (FITC-PSA) and peanut agglutinin (FITC-PNA), which binds specifically to the acrosomal matrix, a method described in the WHO Laboratory Manual for the Examination and Processing of Human Semen.[35]

Our study demonstrated that both calcium ionophore and progesterone can effectively induce acrosome reaction in

human spermatozoa. However, induced acrosome reaction with calcium ionophore is markedly significant, exhibiting its higher efficacy.

These findings suggest that calcium ionophore may be a more potent stimulator of calcium influx, leading to subsequent induction of acrosome reaction. Similarly, our findings are consistent with previous research demonstrating that calcium ionophore effectively triggers complete acrosome reaction.[2]

In contrast, progesterone-induced acrosome reaction was significantly lower, which may be attributed to its indirect activation of calcium channels and variability in the responsiveness of spermatozoa to progesterone.[2]

This approach of employing calcium ionophore in ART procedures may improve the success rates of c-IVF and supports its preference over ICSI, as there is emerging evidence suggesting that there is a higher incidence of epigenetic modifications observed in children born through ICSI due to the increased in-vitro manipulation of gametes employed during ICSI procedure when compared to c-IVF technique.[36] Cryopreserved semen samples are typically used for ICSI. However, if a patient opts/insists for IUI, incorporating acrosome reaction induction into the standard protocol may improve the fertilization potential of frozen-thawed spermatozoa.

A potential limitation of this study is the relatively small sample size. Future studies with larger sample size and additional research are warranted to explore the clinical applications and potential benefits of calcium ionophore in the treatment of male factor infertility.

CONCLUSION

Our study demonstrated that calcium ionophore is significantly more effective than progesterone in inducing the acrosome reaction in frozen thawed human spermatozoa in-vitro. This finding highlights the potential efficacy of calcium ionophore in inducing the acrosome reaction, suggesting that it could be a valuable tool in ART procedures, and its use may lead to novel therapeutic strategies for improving ART outcome in male infertility.

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