INTRODUCTION

The main function of the male reproductive system is to produce spermatozoa and maintain them ready for fertilization. Male gametes produced by testes are immature and they undergo maturation in the epididymidis. The maturation event includes structural and physiological changes aided by addition and removal of many proteins from the spermatozoa. Spermatozoa that emerge from the testis are metabolically and translationally inactive. The highly compact DNA structure of the spermatozoa is proposed to be one of the reason for reduced gene expression efficiency of the growing spermatid (Brewer, Corzett, and Balhorn 2002; Chapman and Michael 2003). Reduction in the cytosolic content of the sperm during spermatogenesis is also considered to be another cause. Therefore the spermatozoa produce proteins that are only vital for its survival for which, it depends on mitochondrial ribosomes (Gur & Breitbart 2008). Hence, they depend on the micro environment for performing most of the biological functions, suggesting that the proteins required for their transport and function are gradually supplemented when they pass through the testis and epididymis and the secretions of seminal vesicles and prostate glands. The spermatozoa that are produced by testes does not possess the ability to fertilize ovum. It has to undergo capacitation to acquire the ability, which is believed to begin in male reproductive tract and completes in the female reproductive tract.

“Capacitation” is a complex process that involves reorganization of membrane proteins (Zaneveld et al. 1991), metabolism of membrane phospholipids (Zanetti et al. 2010), reduction in membrane cholesterol levels (Osheroff et al. 1999), increased Ca^{2+} influx (DasGupta et al. 1993) and hyperactivation (Cancel et al. 2000). These changes, together with the subsequently induced acrosome reaction (AR) (Lee et al. 1987), are essential for a sperm to bind and penetrate the zona pellucida and thereafter fuse with the oocyte plasma membrane (Naz & Dhandapani 2010).
Acrosome is a cap like structure that is present on the anterior half of the mammalian spermatozoa. It contains hyaluronidase (Lin et al. 1994), acrosin (Yamagata et al. 1998) and other proteins that are required for sperm egg recognition and penetration. Acrosome reaction is an important event that is required for the fusion of sperms with oocyte. During acrosome reaction the cell membrane of the spermatozoa fuses with the outer membrane of the acrosome and the contents of the acrosome flow outwardly through the resulting pores. The acrosome reacted spermatozoa thus releases the digestive enzymes which helps in penetrating the zona pellucida of the oocyte thereby resulting in sperm egg fusion (Zaneveld et al. 1991).

Human LYZL proteins although are reported way back in 2005, their function in mammalian reproduction is not reported except for LYZL3/SLLP1 (Mandal et al. 2003). LYZL3 was found to be expressed in the acrosomal region of the spermatozoa and also in Burkit lymphoma Raji cell line. It is expressed in the lymphocytes in case of malignancies and is therefore identified as a useful marker for hematologic malignancies (Wang et al. 2004). Treatment of oocytes with anti-LYZL3 antibody or recombinant LYZL3 protein resulted in inhibition of sperm binding to oolemma (Mandal et al. 2005). Moreover, the interacting partner of LYZL3 was identified to be SAS1B that is expressed on the oocyte membrane. SAS1B knockout female mice showed reduced fertility (Sachdev et al. 2012). LYZL4 was located in intra-acrosomal region of spermatozoa and also in the principal piece of the sperm tail. Treatment of the sperms with anti-LYZL4 antiserum resulted in reduced oocyte fertilization in vitro (Sun et al. 2011). LYZL6 is expressed in testis and epididymis of mouse (Wei et al. 2013). It is also present in the primary spermatocytes, round spermatids, post-acrosomal region and mid piece of the spermatozoa (Wei et al. 2013). The antibacterial activity of LYZL6 also suggests that they may have role in innate immunity in male genital tract. Taken together it is clear that the LYZL proteins are predominantly expressed in male reproductive tract and also on sperm (Zhang et al. 2005), thus hypothesizing that they may
have role in sperm function. Therefore in this part of the work we sought to test the role of LYZL proteins in sperm related function with emphasis on LYZL4 and LYZL6.

MATERIALS AND METHODS

Male Wistar rats aged 90 days were used for this study. They were procured from National Institute of Nutrition, Hyderabad.

Retrieval of spermatozoa from cauda

Adult male Wistar rats were euthanized by cervical dislocation and the cauda was dissected out and placed in a petri plate containing prewarmed capacitation media (M2 media). It was then teased with scalpel to release spermatozoa from the cauda. The resultant spermatozoa were incubated in CO₂ incubator at 37°C for 10 min after which they were counted and resuspended as 5X10⁶ cells/ml. The spermatozoa are then capacitated for 5 h in the presence or absence of antiserum or recombinant proteins.

Treatment of spermatozoa

The possible sperm related functions of LYZL proteins were assessed by two methods, either by treating the spermatozoa with antiserum or recombinant proteins. For antiserum treatment, anti-LYZL4 or anti-LYZL6 serum (1:500) was added to spermatozoa (5X10⁶ cells/ml). The spermatozoa were then allowed to capacitate in CO₂ incubator at 37°C for 5 h. Acrosome reaction was measured to assess the functional ability of the spermatozoa by treating with the ionophore A23187. For recombinant protein treatment, rat spermatozoa (5X10⁶ cells/ml) were incubated with recombinant LYZL4 or LYZL6 protein at the concentration of 50 µg/ml. The spermatozoa are then allowed to capacitate in the presence of recombinant protein in CO₂ incubator at 37°C for 5 h, followed by assessment of capacitation and acrosome reaction.
Assessment of capacitation reaction

Capacitation is a complex process and involves a series of steps that can be studied by various techniques. Figure 3.1 describes the methods used for analysing the processes. The ability of the sperm to undergo hyperactivation was measured using computer assisted sperm analysis (CASA). Calcium and cholesterol dynamics were assessed by fluorescence activated cell sorter (FACS) using calcium binding dye Fluo-3-AM and cholesterol binding dye filipin. Tyrosine phosphorylation was assessed by Western blotting and membrane reorganization was assessed using CTC.

**Figure 3.1:** Processes involved in capacitation and the analytical methods used.

Assessment of capacitation by CTC staining

Assessment of membrane reorganization during capacitation was performed by CTC staining (Ward & Storey 1984). CTC binds to sperm membrane and hence commonly used for assessing the sperm membrane fluidity (Rathi et al. 2001). Spermatozoa obtained from rats
were resuspended in modified Kreb’s Ringer bicarbonate buffer (KBR) pH 7.4 at a density of 5x10^6 cells/ml and treated with recombinant LYZL protein or LYZL antiserum. For CTC staining, a 100 µl sperm suspension was withdrawn at respective time point and mixed with 100 µl CTC stain (0.75mM CTC, 5mM L-cysteine and 150mM NaCl in 20mM Tris buffer pH 7.4) and incubated for 30 sec at room temperature. Thereafter, the sample was fixed with 30 µl of 12.5% glutaraldehyde and a 10µl drop of the fixed spermatozoa suspension was mixed with 5µl of antifade and placed on a glass microscope slide. The droplet was covered with a coverslip. Prepared slides were then stored in the dark until analyses under fluorescence microscope.

**Flow cytometry analysis**

The flow cytometry analysis was carried out using LSR Fortessa cytometer (BD Biosciences). Fluorescence intensity was measured in atleast 10,000 spermatozoa. They were counted at the rate of 200-300 spermatozoa/sec. The fluorescence intensity and the forward scattering (FSC) was collected in logarithmic mode and the side scattering (SSC) was collected in linear mode. Filter options were used based on the dye to be measured.

**Table 9: Details of the dye and the channels used**

<table>
<thead>
<tr>
<th>S. No</th>
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<th>Excitation</th>
<th>Emission</th>
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<td>FITC</td>
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<tr>
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<td>380</td>
<td>510</td>
<td>Amcyan-A</td>
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<tr>
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<td>Fluo 3-AM</td>
<td>506</td>
<td>526</td>
<td>FITC</td>
<td>Logarithmic</td>
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<tr>
<td>4</td>
<td>Propidium iodide</td>
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<td>616</td>
<td>PE-A</td>
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Assessment of cholesterol efflux by filipin staining

Filipin binds to cholesterol with high affinity and its excitation and emission in the UV range can be exploited to assess the cholesterol distribution in the sperm membrane. Caudal spermatozoa of 90 day old Wistar rat was collected in M2 medium and allowed to liquefy for 10 min in 5% CO₂ at 37°C. The spermatozoa were then diluted to 5X10⁶ cells/ml and treated with recombinant LYZL protein or antiserum to LYZL protein for 5 h. At the end of incubation, spermatozoa were washed twice in PBS followed by incubation with 50 µg/ml filipin for 30 min at room temperature to stain the membrane cholesterol. The spermatozoa were then washed with PBS and propidium iodide (10 µg/ml) was added and incubated for 5 min to differentiate live and dead cells. % population (P1) of the stained cells which loses fluorescence intensity was analysed using FACS (Shadan et al. 2004). Appropriate zero hour, preimmune and buffer controls were maintained.

Microscopic assessment of cholesterol efflux

Spermatozoa stained with filipin were smeared on glass slides and observed under fluorescence microscope with excitation and emission wavelength of 380 nm and 510 nm respectively (Barboni et al. 2011). Decreased fluorescence intensity indicates efflux of cholesterol from the sperm membrane. The spermatozoa are compared with uncapacitated zero hour stained spermatozoa.

Assessment of calcium influx by Fluo-3-AM staining

Fluo-3-AM a calcium binding probe, is a acetoxymethyl ester of Fluo 3. The Fluo-3-AM is permeable to plasma membrane and in the cell the ester part is cleaved by the esterase and the resultant is impermeable to plasma membrane. In the absence of calcium the fluorophore is quenched and in the presence of the calcium the quencher moiety of the probe is engaged in
binding to calcium therefore resulting in fluorescence. Thus, Fluo-3-AM can be used to assess the calcium levels inside the cells. Wistar rats (90 days) were dissected and the caudal spermatozoa were collected in M2 media, and allowed to liquefy at 37°C in CO₂ incubator for 5 min. The spermatozoa were then aliquoted (5x10⁶ cells/ml) and treated with recombinant LYZL protein or antiserum to LYZL protein for 5 h according to standard protocol. At the end of incubation 10 μM Fluo-3-AM was added to PBS washed spermatozoa and incubated for 20 min. Mean fluorescence intensity (MFI) of spermatozoa was measured in FITC channel of a LSR Fortessa flow cytometer (Zhang et al. 2010). Spermatozoa treated with preimmune sera and without antibody treatment was used as controls. Propidium iodide was used to stain and eliminate dead cells.

**Computer assisted sperm analysis (CASA)**

Hyperactivation and motility parameters were assessed using CASA, as described earlier (Republic 2008; Vetter et al. 1998). Briefly, spermatozoa diluted in capacitation medium were incubated for 5 h at 37°C. At the end of each hour, 5 μl of spermatozoa were mixed with 30 μl of capacitation medium and observed on a microscopic chamber slide maintained at 37°C. The set up values of the CASA were as follows: frames acquired - 30; frame rate (Hz) - 60; minimum contrast - 100; minimum cell size (pixels) - 7; low average path velocity cut off (μm/sec) - 20; medium average path velocity cut off (μm/sec) - 50; low straight line velocity cut off (μm/sec) - 30; static head intensity limits - 0.14–1.84; static head - size limits - 0.72–8.82; static elongation limits - 0–47; magnification, 1.95 (10x); video frequency (Hz) - 60; bright field - off; slide temperature - 37°C; field selection mode. Based on these kinematic parameters, the non-hyperactivated spermatozoa could be differentiated from the hyperactivated spermatozoa using the SORT facility of the CASA. Spermatozoa with data points ≥15, VCL>300 μm/sec, LIN<40%, ALH>12 μm were sorted as hyperactivated (those exhibiting either circular or helical motility pattern) and spermatozoa with data points ≥15,
VCL<300 µm/sec, LIN>40% and ALH<12 µm were sorted as non-hyperactivated spermatozoa (exhibiting planar motility pattern). The motility parameters such as curvilinear velocity (VCL), linearity (LIN), amplitude of lateral head displacement (ALH) were measured after every hour until 5 h. About 100 spermatozoa were sorted at each time point to assess the hyperactivation.

**Microscopic assessment of acrosome status by PSA-FITC staining**

*Pisum sativum* agglutinin (PSA) binds to the glycoprotein present in the sperm membrane and acrosomal matrix. During acrosome reaction there is loss of acrosomal membrane matrix, leading to reduced fluorescence, which can be used to assess acrosome reaction. To determine the role of LYZL proteins in acrosome reaction, caudal spermatozoa were allowed to capacitate in M2 medium in the presence of LYZL recombinant protein or antiserum, after which acrosome reaction was induced by addition of 20 µM ionophore (A23187) for 20 min. Samples were then washed and treated with PSA-FITC (40 µg/ml), which binds to acrosome specific glycoproteins. Spermatozoa were then smeared on microscopic glass slides, air dried and incubated in absolute methanol for 15 min. Methanol-treated smears were incubated with FITC conjugated PSA (50 µg/ml) for 30 min at room temperature. The slides were then washed with distilled water for 15 min to remove excess unbound probe. After drying, smears were examined immediately using a fluorescence microscope (DasGupta, Mills, and Fraser 1993; Mendoza et al. 1992). Spermatozoa incubated with PBS were used as non-capacitated control.

**FACS analysis of acrosome reaction by PSA-FITC**

Acrosome reaction was induced in spermatozoa (5X10^6 cells/ml) using the ionophore A23287 (20 µM). They were then washed with PBS and stained with PSA-FITC (50 µg/ml) to stain the acrosomal region. The intensity of fluorescence was measured using FACS as means of
acrosome reaction. Decrease in fluorescence intensity indicates acrosome reaction. Fluorescence intensity was measured in a flow cytometer using FITC channel (Jaiswal & Eisenbach 1999). The acrosome reacted population (P2) showing less intensity and the acrosome intact spermatozoa (P3) with high fluorescence intensity were gated and analysed.

**Raising auto antibodies against LYZL6**

![Flow chart for evaluation of LYZL6 as immunocontraceptive](image)

**Figure 3.2:** Flow chart for evaluation of LYZL6 as immunocontraceptive. Numbers represents week. $I_1$, $I_2$, $I_3$, $I_4$, $I_5$ and $I_6$ denoted by the arrow shows the time of immunization. $B_0$ and $B_1$ represent blood collection time points. $M_1$ and $M_2$ represents the time period in which animals were kept for mating.
Male rats aged 90 days were caged in standard day and night conditions and acclimatized. They were immunized with recombinant LYZL6 as per the schedule in figure 3.2. Preimmune serum (B₀) was collected from the tail vein and checked for cross reactivity against recombinant LYZL6 before immunization on day 0. Briefly 100 μg of the protein was mixed with equal volume of Freund’s complete adjuvant and administered intradermally (I₁). The second immunization (I₂) was administered on 3rd day after first immunization in the 1st week by injecting 100 μg of recombinant protein mixed with Freund’s incomplete adjuvant. Third immunization (I₃) was given in 2nd week and subsequent immunizations were administered as shown in figure 3.2. Antibody titer was checked on 6th week by ELISA or dot blot using serum obtained after first blood collection (B₁) of the immunized animals. After confirmation of the presence of antibody against recombinant LYZL6, immunized animals were caged with adult females on 8th and 14th week to allow mating.

**Antibody titre by ELISA**

96 well plates were coated with 40 μg/ml of recombinant protein and incubated overnight at 37°C, followed by incubation at 60°C for 30 min. The plates were then blocked with 1mg/ml BSA for 2 h and then incubated for 3 h with different dilutions of antisera against rat LYZL proteins raised in rabbit. They were then washed 4 times with PBS-T (PBS with 0.1% Tween-20), followed by incubation with HRP conjugated anti-rabbit secondary antibody (1:10000). After thorough washing, O-phenylenediamine (OPD) was added to measure the amount of antibody bound to the protein (Wang et al. 2011). The antibody titre is measured in terms of intensity of the colour developed. Higher the O.D, higher is the titer.

To determine the presence of autoantibodies in the testis and epididymis of immunized rats, ELISA was performed with the tissue fluids (Ellerman et al. 1998). The tissue fluids were obtained in 500 μl of PBS followed by centrifugation at 10,000 rpm for 10 min at 4°C. The
supernatant was quantified for protein concentration and equal quantity of tissue fluid was used for the assay.

**Assessment of fertility**

Immunized rats that had high antibody titer were subjected to natural mating with females of proven fertility (Ellerman et al. 1998). The animals were caged in the ratio of 1 male to 3 females. The females were checked frequently and the pregnant rats were removed and the number of pups produced was noted. In the immunized rats sperm parameters such as calcium influx, cholesterol efflux, tyrosine phosphorylation, hyperactivation and acrosome reaction were analysed in the spermatozoa obtained from immunized animals.
RESULTS

Assessment of capacitation by chlortetracycline (CTC) staining

CTC staining is widely used to assess the capacitation status of the spermatozoa (Lee et al. 1987). Spermatozoa that underwent capacitation in vitro exhibited decreased CTC staining when compared to their uncapacitated counterparts (Figure 3.3). The CTC staining pattern was similar to that of the capacitated control in recombinant LYZL4 or LYZL6 protein treated spermatozoa, implying that addition of protein did not have any effect on sperm capacitation. The staining pattern in LYZL4 or LYZL6 antiserum treated spermatozoa was similar to that in capacitated sperm, indicating that blocking LYZL4 or LYZL6 on sperm surface does not affect membrane reorganization during capacitation. Spermatozoa treated with preimmune serum or buffer control also underwent capacitation (Figure 3.3).

Figure 3.3: Effect of LYZL proteins on capacitation. CTC staining of spermatozoa treated with antiserum or recombinant LYZL proteins. BC-buffer control.
Assessment of capacitation by measuring intracellular Ca\textsuperscript{2+} release

The extent of capacitation was measured in terms of Ca\textsuperscript{2+} release from intracellular stores and is achieved by measuring the fluorescence intensity of calcium binding dye, Fluo-3-AM. Increased fluorescence indicates the occurrence of sperm capacitation. Figure 3.4 shows a typical fluorescence measurement flow cytogram to assess calcium release during sperm capacitation. Spermatozoa suspended in capacitation medium exhibited an increase in Fluo-3-AM fluorescence intensity, indicating the progression of capacitation. The fluorescence peak shifts towards the right in the capacitated spermatozoa due to the binding of Ca\textsuperscript{2+} to Fluo-3-AM. The fluorescence intensity is an indication of extent of Ca\textsuperscript{2+} release. Spermatozoa treated with LYZL4 or LYZL6 recombinant proteins exhibited fluorescence intensity similar to that of capacitated spermatozoa (Figure 3.5A). The buffer control (BC) did not interfere with the measurements. On the other hand, treatment with LYZL4 or LYZL6 antiserum caused a significant decrease in fluorescence intensity when compared to capacitated spermatozoa (Figure 3.5B), suggesting that neutralization of LYZL4 or LYZL6 affects Ca\textsuperscript{2+} release from intracellular stores. Pre-immune serum did not have any effect on Ca\textsuperscript{2+} release.

**Figure 3.4:** FACS analysis of Fluo-3-AM stained spermatozoa. Representative cytogram of measurement of mean fluorescence intensity (MFI) of uncapacitated (A) and capacitated spermatozoa (B).
Figure 3.5: Assessment of capacitation status through calcium influx using Fluo-3-AM. (A)- Mean fluorescence intensity of the spermatozoa incubated with or without recombinant LYZL4 or LYZL6, BC-buffer control. (B)- Mean fluorescence intensity of the capacitated spermatozoa incubated with or without LYZL4 or LYZL6 antiserum. Values shown are mean ± SD. * indicates p<0.05 compared to capacitated spermatozoa.
Assessment of capacitation by measuring cholesterol efflux

During capacitation, cholesterol efflux from the sperm surface is a characteristic feature and these dynamics can be monitored by using filipin, a cholesterol binding dye. Uncapacitated spermatozoa exhibited fluorescence uniformly throughout the head region (Figure 3.6A). As cholesterol is lost during capacitation, the fluorescence intensity due to filipin also decreases (Figure 3.6B). In spermatozoa treated with LYZL4 (Figure 3.6D) or LYZL6 (Figure 3.6E) recombinant protein decreased fluorescence was observed similar to that of capacitated spermatozoa. PBS used as buffer control did not affect capacitation (Figure 3.6C). Similarly, spermatozoa treated with LYZL4 or LYZL6 antibody also showed decreased filipin staining indicating the occurrence of capacitation (Figure 3.6I and 3.6J) Pre-immune serum did not affect cholesterol dynamics during capacitation (Figure 3.6H).

Figure 3.6: Effect of LYZL proteins on cholesterol efflux. Filipin staining of spermatozoa treated with antiserum and recombinant LYZL proteins. BC-buffer control.
Figure 3.7: FACS analysis of filipin stained spermatozoa. Representative cytograms of FACS analysis. Measurement of mean fluorescence intensity (MFI) of uncapacitated (A) and capacitated (B) spermatozoa.

Cholesterol efflux during capacitation was also examined by flow cytometry. Figure 3.7 shows a typical flow cytogram of uncapacitated and capacitated spermatozoa. The population of spermatozoa that did not undergo capacitation (P1) exhibit higher fluorescence intensity, whereas capacitated spermatozoa (P2) exhibit decreased fluorescence intensity due to loss of cholesterol bound filipin. As capacitation progresses, the count of P2 population increases, which is an indicator of the extent of capacitation. The percent of P1 and P2 population was estimated in spermatozoa treated with LYZL proteins or their antiserum.

The P1 population was significantly decreased in spermatozoa subjected to in vitro capacitation (Figure 3.8). Spermatozoa pre-treated with LYZL4 or LYZL6 recombinant protein had the P1 population similar to that of capacitated control (Figure 3.8A). Treatment with LYZL4 or LYZL6 antiserum also had a significantly decreased number of P1 population (Figure 3.8B). These results indicate that neither the LYZL proteins nor their antiserum could influence the capacitation process in vitro.
Figure 3.8: Assessment of capacitation status. (A)- % population of the capacitated spermatozoa after incubation with or without recombinant LYZL4 or LYZL6. (B)- % population of the capacitated spermatozoa (P1) with or without LYZL4 or LYZL6 antibody. BC- buffer control. Values shown are ± SD.
Assessment of capacitation by protein tyrosine phosphorylation

Phosphorylation of proteins, specifically at tyrosine residues is a hallmark of capacitation. The extent of phosphorylation during capacitation was detected by Western blotting using phospho tyrosine antibody. As shown in Figure 3.9A, in proteins obtained from uncapacitated spermatozoa tyrosine phosphorylation was lower. Tyrosine phosphorylation seems to be increased in protein obtained from capacitated spermatozoa (Figure 3.9A). In spermatozoa pre-treated with LYZL4 or LYZL6 proteins, tyrosine phosphorylation was similar to that of capacitated spermatozoa (Figure 3.9A). Protein phosphorylation was evident in spermatozoa treated with LYZL4 or LYZL6 antiserum (Figure 3.9B). Pre-immune serum did not affect protein phosphorylation.

![Figure 3.9: Western blotting for the detection of tyrosine phosphorylation in sperm proteins. Uncap- uncapacitated, Cap- capacitated, BC- buffer control, Preimm- preimmune.](image)

Role of LYZL proteins in acrosome reaction

In conditions that allow acrosome reaction, a gradual decrease of specific glycoproteins are observed in the acrosomal region. Changes in the levels of these specific glycoproteins can be
detected using FITC tagged PSA lectin. In general, decreased fluorescence is observed in the head region of acrosome as the reaction occurs.

Immunocytochemistry was used to track the fluorescence intensity of PSA-FITC as acrosome reaction occurred. Intense staining was observed in acrosome intact spermatozoa (Figure 3.10A). Appreciable decrease in the FITC fluorescence was observed in acrosome reacted spermatozoa (Figure 3.10B). The buffer control (BC) did not affect the progression of acrosome reaction (Figure 3.10C). PSA-FITC staining was similar to that of acrosome reacted spermatozoa in LYZL4 or LYZL6 proteins pre-treated spermatozoa (Figure 3.10D and E). In spermatozoa pre-treated with LYZL4 antiserum, the PSA-FITC staining was found to be similar to that of acrosome reacted spermatozoa (Figure 3.10H) whereas in case of LYZL6 spermatozoa it was similar to acrosome intact spermatozoa (Figure 3.10I).

**Figure 3.10:** Assessment of acrosome reaction by PSA-FITC staining. (A-E) Microscopic observation of rat spermatozoa treated with or without LYZL4 or LYZL6 recombinant proteins (A-E) or with LYZL4 or LYZL6 antiserum (F-J). Acr-acrosome, Preimm-Preimmune, BC-buffer control.
To quantify the percentage of spermatozoa that have undergone acrosome reaction under different conditions, flow cytometric analyses was carried out. Acrosome intact spermatozoa exhibited higher fluorescence intensity whereas acrosome reacted spermatozoa exhibited lower fluorescence intensity due to loss of PSA-FITC binding proteins (Figure 3.11). Hence, both the populations can be estimated by gating (Figure 3.11A). The percentage of acrosome reacted spermatozoa were significantly higher under the *in vitro* conditions tested (Figure 3.11B). The % of spermatozoa that underwent acrosome reaction after treatment with LYZL4 or LYZL6 protein were similar to that of acrosome reacted control (Figure 3.12A). The number of spermatozoa that underwent acrosome reaction after treatment with LYZL4 or LYZL6 antiserum was decreased when compared to acrosome reacted control (Figure 3.12B).

**Figure 3.11:** FACS analysis of PSA FITC stained spermatozoa. (A)- Gating of acrosome reacted (P2- with low fluorescence intensity) and acrosome intact (P3- with high fluorescence intensity) spermatozoa. (B)- Representative cytogram of FACS analysis showing the mean fluorescence intensity (MFI) of acrosome intact and acrosome reacted spermatozoa. Green- acrosome reacted population, blue –acrosome intact population.
Figure 3.12: FACS analysis of acrosome reaction. (A) % population of acrosome reacted spermatozoa after incubation with or without LYZL4 or LYZL6 recombinant proteins. (B) % population of acrosome reacted spermatozoa after incubation with or without LYZL4 or LYZL6 antiserum. Values are the mean± SD. * indicates P<0.05 compared to acrosome reacted control.

Immunococontraceptive potential of LYZL6

The auto antigen model generated by immunizing the rat with recombinant LYZL6 protein was verified by ELISA and the possible function of LYZL6 with regard to male reproduction was assessed.
Enzyme Linked Immunosorbent Assay (ELISA)

Serum was obtained from Wistar rats immunized with recombinant LYZL6 and the antibody titre measured by ELISA. In all the four rats (R1-R4) a dilution dependent antibody titre was detected (Figure 3.13). Preimmune serum did not show any reactivity.

![Graphical representation of serum titres verified by ELISA. Preimmune serum was used as control. R1, R2, R3 and R4 are immunized rats. Values shown are mean ± S.D. *, # and ¥ indicates p <0.05 compared to the preimmune serum.](image-url)

**Figure 3.13:** ELISA with serum obtained from LYZL6 immunized rats. Graphical representation of serum titres verified by ELISA. Preimmune serum was used as control. R1, R2, R3 and R4 are immunized rats. Values shown are mean ± S.D. *, # and ¥ indicates p <0.05 compared to the preimmune serum.
Reproductive tract tissue fluids were tested for presence of LYZL6 antibody to determine whether the antibodies passed the blood testes barrier. The antibody titre was found to be significantly higher in the fluids obtained from the epididymides and testes of immunized rats when compared to the unimmunized controls (Figure 3.14).

Figure 3.14: ELISA for LYZL6 immunized rats. Antibody titres in the tissue fluids of LYZL6 immunized rats. Values shown are mean ± SD. * indicates $p < 0.05$ compared to unimmunized control. Ep-epididymis, T-Testes.
Effect of LYZL6 immunization on sperm count

To determine whether the presence of LYZL6 antibodies in the testicular and epididymal fluids may affect germ cell production, the sperm count was analysed. A significant decrease in the sperm count was observed in LYZL6 immunized rats (Figure 3.15).

**Figure 3.15: Sperm count in LYZL6 immunized rats.** Values shown are mean ± SD. * indicates $p < 0.05$ compared to unimmunized control.
Computer Assisted Sperm Analysis (CASA)

To determine whether the spermatozoa produced by immunized animals have compromised physiological features, CASA was performed. Motility parameters such as path velocity, progressive velocity and track speed were found to be decreased in spermatozoa obtained from immunized rats, when compared to control. These results show that endogenous neutralization of LYZL6 protein results in reduction in motility parameters of spermatozoa.

Figure 3.16: Assessment of sperm parameters by CASA. Spermatozoa were obtained from control and immunized rats and the motility parameters analysed at 1 and 5 h after collection. * and # indicates p<0.05 compared to unimmunized control.
Histopathological evaluation

Cross sections of testes and epididymidis obtained from control and LYZL6 immunized rats were examined under microscope to determine the possible pathomorphological effects. The overall architecture of testis and epididymis were not affected and were similar to the unimmunized control.

Figure 3.17: Morphological assessment of testes and epididymidis. Cross sections of testes and epididymidis obtained from LYZL6 immunized and control rats were stained with hematoxylin and eosin. Magnification 10X. T-testes, Cp-caput, Cd- cauda.
Assessment of tyrosine phosphorylation

Tyrosine phosphorylation of the sperm proteins obtained from control and LYZL6 immunized rats under capacitating conditions was analysed. Extensive protein phosphorylation was observed in spermatozoal proteins that were obtained from control rats (Figure 3.18A). Similar phosphorylation pattern was also observed in spermatozoa obtained from LYZL6 immunized rats.

We next determined whether the antiserum obtained from LYZL6 immunized rats can neutralize the LYZL6 protein on control rat spermatozoa and affect sperm function. Tyrosine phosphorylation was evident in spermatozoa pre-treated with antiserum obtained from LYZL6 immunized rats (Figure 3.18B).

Figure 3.18: Western blotting analysis of LYZL6 auto-antigenic rat spermatozoa and LYZL6 antiserum (rat) treated spermatozoa.
Effect of LYZL6 immunization of calcium influx

Intracellular calcium level was assessed in LYZL6 immunized animals by using the calcium binding dye Fluo-3-AM. In comparison with unimmunized rat spermatozoa, LYZL6 immunized rat spermatozoa showed decreased calcium influx (decreased mean fluorescence intensity) (Figure 3.19A) reiterating similar results obtained in *in vitro* studies. Analysis of the same using antiserum obtained from LYZL6 immunized rats also confirmed that LYZL6 neutralization resulted in decreased calcium influx (Figure 3.19B).

![Figure 3.19](image)

**Figure 3.19:** Evaluation of calcium influx in LYZL6 immunized rat. (A) Mean fluorescence intensity of the spermatozoa obtained from unimmunized and LYZL6 immunized rats. (B) Mean fluorescence intensity of the capacitated spermatozoa incubated with or without LYZL6 rat antiserum. Values shown are mean ± SD. * indicates p<0.05 compared to capacitated spermatozoa.
Effect of LYZL6 immunization on capacitation

Cholesterol efflux of the spermatozoa obtained from LYZL6 immunized rats were analysed to assess the capacitation status using filipin. LYZL6 immunized rat spermatozoa exhibited decreased ability to undergo capacitation (Figure 3.20A). Similar results were obtained on treatment of control spermatozoa with immune sera obtained from LYZL6 immunized rat (Figure 3.20B), suggesting that LYZL6 neutralization may affect capacitation.

Figure 3.20: Assessment of capacitation status. (A)- % population of the capacitated spermatozoa (P1) obtained from LYZL6 immunized rats (B)- % population of the capacitated spermatozoa (P1)treated with LYZL6 rat antiserum. Values shown are ± SD.* indicates p<0.05 compared to uncapacitated control.
Effect of LYZL6 immunization on acrosome reaction

Assessment of acrosome reaction of the spermatozoa obtained from LYZL6 immunized animals was performed by staining the spermatozoa with PSA-FITC and the resultant were analysed by flow cytometry. LYZL6 immunized rat spermatozoa display less ability of undergo acrosome reaction in comparison with unimmunized rat spermatozoa (Figure 3.21A). Control spermatozoa treated with LYZL6 rat antiserum also displayed similar effect suggesting that LYZL6 neutralization is affecting the acrosome reaction (Figure .21B).

**Figure 3.21: FACS analysis of acrosome reaction.** (A) % population of acrosome reacted spermatozoa obtained from LYZL6 immunized rat. (B)- % population of acrosome reacted spermatozoa after incubation with LYZL6 rat antiserum. Values are the mean ± SD. * indicates P<0.05 compared to acrosome reacted control.
Fertility studies

Fertility status of the LYZL6 immunized rats were assessed by mating experiments (Table 12). Each immunized rat was mated with three female rats. The average litter size of the control animals was 11, whereas the same was around 3 in LYZL6 rats.

Table 12: Fertility assessment of LYZL6 immunized animals

<table>
<thead>
<tr>
<th>Immunized male</th>
<th>Females</th>
<th>1st mating</th>
<th>Average litter size</th>
<th>2nd mating</th>
<th>Average litter size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>12</td>
<td></td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10</td>
<td>11</td>
<td>11</td>
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<td></td>
<td>3</td>
<td>11</td>
<td>10</td>
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<tr>
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<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>5.6</td>
<td>0</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Rat 2</td>
<td>1</td>
<td>7</td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>4.3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Rat 3</td>
<td>1</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td>Rat 4</td>
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<td></td>
<td>0</td>
<td></td>
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<tr>
<td></td>
<td>3</td>
<td>0</td>
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</tbody>
</table>

It is surprising to note that some of the females did not have any litter. The number of animals that did not have pups increased after second mating, the time at which the antibody titer is higher than the previous mating. Decreased fertility was observed as the antibody titer increased suggesting that with increase in the antibody titre more LYZL6 is neutralized, thus impairing the fertility.
DISCUSSION

Although there are many proteins that are specifically expressed in male reproductive tract, only some of them have an important role in reproductive function. Examples include CRISP1 (Sivashanmugam et al. 1999), ARP (Cohen et al. 2001), Sept4 (Kissel et al. 2005) and Tektin 3 (Roy et al. 2009) etc. Deciphering the functional role of male reproductive tract specific protein is an active area of interest primarily to develop male contraceptives. Hence, novel proteins are being identified and their role in sperm function analysed. In this part of the study we report the functional characterization of rat LYZL proteins with specific reference to male reproduction.

Neutralization of LYZL4 or LYZL6 by adding immune serum inhibited calcium influx, a vital event during capacitation. SPINKL, a serine protease inhibitor Kazal-type-like protein inhibits capacitation in murine spermatozoa (Tseng et al. 2013). SPINKL acts by blocking extracellular calcium ion influx into the spermatozoa, cholesterol efflux from sperm membrane and increase in intracellular cAMP. It is believed that SPINKL may act as an uncapacitation factor, inorder to prevent precocious capacitation and subsequent acrosome reaction. It is possible that LYZL4 or LYZL6 may also have similar function. On the other hand recombinant LYZL proteins did not exhibit any effect on sperm function and these proteins when added externally may not exhibit the same effect than when bond to sperm surface.

Capacitation leads to cholesterol efflux from the sperm membrane and a change in protein to cholesterol ratio, thereby causing increase in membrane fluidity. It is also possibly related to membrane ion transportation and membrane fusion. BSA which is a component of cell culture media and cellular fluid is present in the capacitation medium and it acts as a inducer for cholesterol efflux (Osheroff et al. 1999). Antibodies to FA-1 present in the semen acts as
Chapter 3

inhibitor for tyrosine phosphorylation (Menge et al. 1999). Cholesterol efflux from spermatozoal membrane was examined in the spermatozoa treated with antisera against LYZL4 and LYZL6 or the recombinant proteins. We observed that addition of LYZL4 or LYZL6 anti-serum (thus neutralizing LYZL4 or LYZL6 on sperm) did not affect cholesterol efflux. Similarly no difference was observed in cholesterol efflux on addition of recombinant LYZL4 or LYZL6 proteins. This indicates that the LYZL proteins may not be involved in events that lead to cholesterol dynamics during sperm capacitation.

Phosphorylation is one of the post-translational regulatory events that occur in many cells inorder to control the signalling cascade. The spermatozoa which emerges from testes is translationally inactive to greater extent and depends on the microenvironment and post translational modifications for its functioning (Kleene 1996). Tyrosine phosphorylation of sperm proteins is an important event that occurs during capacitation (Lewis & Aitken 2001). The effect of neutralizing LYZL4 or LYZL6 by the addition of corresponding antiserum on tyrosine phosphorylation was examined by Western blotting. Pre-incubating spermatozoa with LYZL4 or LYZL6 did not inhibit tyrosine phosphorylation. On the other hand, we did not observe enhanced tyrosine phosphorylation when the spermatozoa were preincubated with recombinant LYZL4 or LYZL6. The role of sperm bound proteins in tyrosine phosphorylation was reported. For example the Sp32, the proacrosin binding protein seems to undergo tyrosine phosphorylation during capacitation (Dubé et al. 2005). Testis specific protein kinase A (PKA) catalytic subunit Ca2 is involved in tyrosine phosphorylation of the proteins (Lefèvre et al. 2002); (Nolan et al. 2004). Spermatozoa lacking functional PKAC are unable to undergo tyrosine phosphorylation and thereby capacitation. As most of the tyrosine phosphorylated proteins are present in tail part of the sperm, inhibition of PKA caused loss of hyperactivation which is hallmark of capacitation (Baker et al. 2009). Treatment of spermatozoa with FA-1 monoclonal antibody also resulted in reduced tyrosine
phosphorylation (Naz & Rajesh 2004). Though LYZL4 or LYZL6 are reported to be localised on the sperm surface, they seem to not have a role in the tyrosine phosphorylation during capacitation.

Exocytosis of acrosome to release its vesicular contents (enzymes) to facilitate penetration of sperm into cumulus layer is referred as acrosome reaction. SABP, seminal plasma secretory actin binding protein inhibits acrosome reaction by binding to actin; and its higher level expression in the seminal plasma correlated with infertility (Capková et al. 2007). In this study we analysed the role of LYZL4 and LYZL6 in acrosome reaction by either treating the spermatozoa with LYZL4 or LYZL6 recombinant protein or by incubating with their corresponding antiserum. Though addition of recombinant proteins did not affect acrosome reaction failure of acrosome reactions was observed when incubated with LYZL6 antiserum. This observation clearly demonstrates that the LYZL6 may be limited during sperm maturation

Treatment of spermatozoa with recombinant LYZL4 or LYZL6 did not influence capacitation and acrosome reaction. However, calcium influx during capacitation and acrosome reaction are inhibited when LYZL6 was neutralized by its antiserum. These results suggest that LYZL6 may have a role in both capacitation and acrosome reaction. Further its role seems to be confined only to calcium dynamics during capacitation, which is an indication of the event specific regulation by sperm protein in a multi-step process such as capacitation.

Blood testes barrier is responsible for the immune evasion of spermatozoa which generally do not allow passage of any immunocompetent cells to the systemic circulation. It is reported that accidental exposure of testicular or epididymal or spermatozoal proteins may cause autoimmune reaction resulting in immune infertility. Examples include YWK-II (Zhuang et al. 2006), TSA-1 (Trivedi & Naz 2002), calpastatin (Liang et al. 1994) and zyxin (Bohring
In vitro neutralization of the protein by adding antibody may not mimic the exact neutralization as the in vivo conditions are different. Therefore the auto antigen model helps in overcoming the drawback of in vitro studies, where antibody against self-protein is raised in the live animal. Presence of antibody against self-protein leads to neutralization of the protein and subsequently its function under in vivo conditions. Therefore such strategies can be used to study function of a protein whose distribution is well known. Similar studies were carried out earlier using this strategy. For example eppin an epididymal protein is found to be suitable for immunocontraception (Rand et al. 2011).

Wistar rats that are immunized with recombinant LYZL6, showed the presence of antibody in the serum and epididymal and testicular tissue fluids suggesting the auto-antigenic nature of LYZL6. Immunization study confirms that LYZL6 antibodies have inhibitory effect on calcium influx, cholesterol efflux, acrosome reaction and hypermotility of spermatozoa similar to YLP12, a peptide from ZP protein, which was reported to inhibit capacitation and acrosome reaction (Naz & Packianathan 2000). Anti-mouse sperm monoclonal antibody A-1 showed inhibitory effect against capacitation, acrosome reaction and calcium influx (Kawai et al. 2000). Treatment of sperm with antibodies against sperm agglutination antigen-1 (SAGA1) also showed similar effect where the motility of the sperms are compromised along with inhibition of penetration of zona free hamster (Diekman et al. 1997). However, morphological appearance of the tissues and the sperm did not show any significant abnormality suggesting that the antibodies did not affect the basic architecture related functions of the male reproductive tract organs. These results suggest that LYZL6 neutralization probably has impact on the earlier stages of capacitation. The results of the fertility assessment study performed by mating the immunized rats with control females showed decrease in fertility of the animal as the antibody titer increases in the animal, which may be due to inability of the spermatozoa to undergo capacitation. This suggests that
LYZL6 protein neutralization has far reaching consequences ultimately affecting the fertility of the animal. EPPIN a epididymal protease inhibitor is shown to have similar effects on immunization and it is trialled as immunocontraceptive in macaques (O’rand et al. 2004). Results of the current study were also similar suggesting to hypothesis that LYZL6 can be used as immunocontraceptive. Although in immunized animals, we could observe the compromise in capacitation parameters and acrosome reaction, *in vitro* fertilization studies would give clear picture on the role of LYZL antibodies in fertilization. Studies on restoration of fertile condition on unexposure to antigen would confirm the lasting effect of auto antibodies, which may further emphasize on LYZL6 as immunocontraceptive. In addition, future studies on silencing or knocking out of LYZL6 gene would help in finding out the exact mechanism of action of LYZL6 in fertilization.
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