Mutual interaction of dog sperm LDHC4, PH-20, actin and tubulin proteins and their immunocontraceptive potential in bitches

Ranjna Sandhey Cheema*, Nisha Vashishat1, Amrit K. Bansal and V.K. Gandotra

Department of Veterinary Gynaecology and Obstetrics,
Guru Angad Dev Veterinary and Animal Science University, Ludhiana-141 004, Punjab, India.
Received: 11-03-2014 Accepted: 24-10-2014 DOI:10.5958/0976-0555.2015.00040.0

ABSTRACT

In this paper, interaction of LDHC4, PH-20, α-actin and β-tubulin on dog’s sperm surface and their immunocontraceptive effect in bitches has been elucidate. Anti spam-1 and anti LDHC4 recognized 46/32 and 36/30/28 kDa proteins in dog sperm extracts on immunoblots. Whereas, proteins of 43, 36 kDa and 62, 46, 30 and 28 kDa were detected on immunoblots with anti-α-actin and anti-β-tubulin. In MALDI-TOF analysis, sequence of 46, 30, 28 and 43, 36 kDa antigens matched twice to β-tubulin and α-actin respectively. Therefore, one sub unit each of LDHC4 and α-actin had same mol wt of 36 kDa and two other sub units each of LDHC4 and β-tubulin shared the same mol wt of 30/28 kDa. Reaction of 46 kDa protein both with anti-spam-1 and β-tubulin indicated similarity in their mol wts. Similarly the reaction of 43 kDa protein both with anti-α-actin and anti- β-tubulin revealed some similarity between both. Sequence analysis revealed 19.49%, 24.28%, 21.42% and 18.75% similarity of LDHC4/α-actin, PH-20/α-actin, LDHC4/β-tubulin and LDHC4/tubulin respectively. Immunofluorescence staining of dog sperm smears also indicated similarity in localization of these four proteins. Localization of PH-20 and β-tubulin was mainly on the entire head region. Whereas, LDHC4 is uniformly distributed on the whole sperm and actin is more concentrated on the post acrosomal cap. Purified native proteins of 46/32 and 36/30/28 kDa were injected to a group of two bitches respectively through i/m route to elucidate their immunocontraceptive potential. Immunization of bitches in both the groups resulted in suppressed heat, and inhibited natural mating. On being subjected to AI, ultrasonography of four immunized and two unimmunized (control) bitches 35 days after insemination confirmed the presence of 0 and 3-4 fetuses respectively. Therefore, it seems that LDHC4/PH-20 and α-actin/ tubulin share their molecular weights or interact with each other or actin/ β-tubulin superimpose LDHC4/PH-20 on sperm surface. In view of observed contraceptive effect, it can be concluded that LDHC4 and PH-20 collectively with α-actin and β-tubulin affect estrus, process of natural mating and ultimately the fertility in bitches.

Key words: Bitches, Immunocontraception, Interaction, Proteins.

INTRODUCTION

Proteins control all biological processes in a cell. Many proteins perform their functions independently, but majority of them interact with others for proper biological activity. Protein-protein interactions can be studied through methods such as co-immunoprecipitation, western blot analysis, mass spectrometry, crosslinking, label transfer etc. Sperm-specific lactate dehydrogenase (LDHC4) is present only in testis and spermatozoa of mammalian and avian species, its function relates to energy metabolism and capacitation of the sperm. LDHC4 is considered as one of the candidates for immunocontraceptive vaccine (Naz, 1999). Spam-1 (PH-20), a glycoprotein associated to the sperm plasma and inner acrosomal membranes possess a hyaluronidase activity and is anchored to glycosyolphosphatidylinositol (Thaler and Cardulo, 1995, Overstreet et al., 1995 and Meyers et al., 1999). Its role is related to cumulus penetration as well as zona binding. Cytoskeletal proteins are localized in sub-acrosomal, para-acrosomal, and postacrosomal regions of the head. In general, cytoskeletal proteins include actin, actin-binding proteins such as spectrin, various tubulins (e.g. α-, β-, γ-tubulin) etc. Extraordinary structural compartmentalization of mature spermatozoa is related to the presence of cytoskeletal proteins (Plessmann et al., 1997 and Palecek et al., 1999), which has a functional role in fertilization and motility.

Immunocontraception is a process of using a reproductive protein to produce a humoral immune response.

*Corresponding author’s e-mail: ranjna.cheema@gmail.com. 1Department of Zoology, Punjab Agricultural University, Ludhiana-141 004, India.
that leads to the infertility in animals for a defined time period. Spermatozoa share both auto- and iso-antigens, and can therefore produce antibodies in both male and female. Immunological interaction with such molecules can cause block of sperm binding to the oocyte and thus fertilization. Interference with fertility could occur at several points: during sperm production in the testis, sperm maturation in the epididymis or interaction with the egg in the female reproductive tract. It has been reported that purified LDHC, or chemically modified LDHC, peptides could induce an immune response in many species. The birthrate of the animals, immunized with LDHC, decreased considerably and a reversible contraception effect was detected (Gupta et al., 1994 and Gupta and Syal, 1997). The role of sperm PH-20 in gamete interaction was first demonstrated in guinea pig spermatozoa, (Primakoff et al., 1985). In spermatozoa, a number of cytoskeletal proteins are implicated in key events such as capacitation and the acrosome reaction (AR) (Howes et al., 2001, Moore 2001, Brener et al., 2003). Actin may play a role in AR (Palecek et al., 1999) and inhibition of actin polymerization by cytochalasin B and D blocked induction of the human acrosome reaction by zona pellucida (Dvorakova et al. 2001). Confocal and electron microscopic studies of boar spermatozoa confirmed that actin, spectrin and tubulin-containing structures are altered after the AR and that cytoskeletal inhibitors influence this event (Peknicova et al. 2001, Dvorakova et al., 2001 Brener et al., 2003). Therefore, keeping in view the above mentioned similarity in their biological activity, the interaction of LDHC, PH-20, α-actin and β-tubulin on dog’s sperm surface was studied and their immunococontraceptive effect in bitches was explored.

MATERIALS AND METHODS
Selection of animals: Five dogs, weighing about 16-20 kg of 2-3 years and six bitches weighing about 10-15 kg of 2-3 years were maintained for semen collection and immunization respectively. Starting 4 weeks prior to experimentation, animals were housed in concrete floored kennels with access to outside runs and fed commercial dog feed (nutripet). The water was available at libitum.

Collection of semen and extraction of sperm membrane proteins: The semen (entire second sperm rich fraction and part of 3rd fraction) was collected by manual manipulation in clean —graduated tube attached to a glass funnel. Ten ejaculates were collected from each dog with a minimum interval of 4 days. Semen was immediately centrifuged to separate out spermatozoa and seminal plasma. Sperm membrane extracts (SDS-SME) were prepared as per our standardized method by suspending the spermatozoa in 2% SDS in 62.5 mM Tris-HCl, pH, 6.8 (Cheema et al., 2011).

Characterization and interaction of LDHC, PH-20, α-actin and β-tubulin in dog sperm Immunoblotting (Towbin et al., 1979): Anti-Spam-1 (PH-20, against human immunogen)/ anti LDHC, (against synthetic peptide), anti-α-actin/ anti-β- tubulin (against human immunogen) were purchased from Sigma Chemicals and Genexbio ltd. Proteins separated by SDS-PAGE under reducing conditions were transferred to nitrocellulose/ PVDF membrane using wet electrophoresis transfer apparatus at 100 V for 2.30 hrs. Transfer quality was checked by 0.2 % ponceau dye and proteins were blocked in 2% BSA as blocking solution for overnight at 4°C. After washing the membrane with PBS+0.05% Tween-20, it was incubated in 1: 1000 diluted anti-spam-1/ LDHC, and 1:2000 diluted α-actin/ β- tubulin for 2.5 hrs. Again washed thrice with PBS+0.05% Tween-20 and incubated with 1:10000 anti rabbit IgG as secondary antibody for 45 min. Washed thrice with PBS+Tween-20 and incubated with substrate (0.05% Diaminobenzidine + 0.015% 4- Chloro Naphtol + 0.06% Hydrogen Peroxide) for 10 min. Gel images were captured on Syngene gel doc using GeneSnap image acquisition software and analyzed by using GeneTools gel analysis software (Syngene).

Immunolocalization of antigenic proteins using FITC labeling (Verdier et al., 2002): Smears of washed dog spermatozoa were prepared on glass slides, air-dried, and fixed in ethanol for 30 minutes. Slides were then covered with PBS containing 1% BSA for 45 minutes to block nonspecific antibody binding. They were then incubated at room temperature in a humidified chamber for 2 hours with 1:1000 diluted anti LDHC/spam-1 antibodies and 1: 2000 diluted anti α-actin and β-tubulin. Slides were then washed and incubated for 1 hour with rabbit anti-dog IgG-FITC-conjugated antibody (Sigma) diluted to 1:100. After 3 washings, slides were mounted with PBS-glycerol (1:1 v/v) and observed on fluorescent microscope (Leica, Germany) and images were captured on Leica digital camera.

Mass spectrometry: Bands of 46, 43, 36, 32, 30 and 28 kDa, detected with Anti-Spam-1, anti LDHC, anti-α-actin and anti-β- tubulin were cut from the CBB stained acrylamide gels and prepared for protein mass fingerprinting by Matrix-assisted laser desorption/ionization-Mass Spectrometry (MALDI-MS) by trypsin in-gel digestion method. Peptide mixture was sent to Central Instrumentation Facility (CIF), Biotech Center, University of Delhi (South Campus) for MALDI-MS analysis. Identified peptide sequences were aligned and compared with the sequences of different proteins.
with already established immunocontraceptive potential using BLAST search engine.

**Purification of LDHC\(_4\) and PH-20, \(\alpha\)-actin and \(\beta\)-tubulin sub units:** After SDS-PAGE, one strip cut from each side of the gel was stained with 0.5% CBB and destained with 7% acetic acid and 10% methanol, rest was kept on glass plates at 4°C. Then stained side strips were lined up along the unstained gel and used as a guide to cut out bands of 46, 43, 36, 32, 30, 28 kDa with a sharp knife. Washed the bands three times (5 minutes each) with 250 mM Tris buffer, pH 7.4 followed by three washings of 5 minutes each with distilled water. Then chopped the gel and added 1.0 ml of extraction buffer i.e. 20 mM Tris-buffer of pH 7.4 and 0.1% SDS, sonicated for 3 minutes (6 X 30 sec) in an ice bath with a 3 mm probe sonicator (Misonix ultrasonic liquid processor CML-4, Coleparmer, USA). Gel containing sample in extraction buffer was centrifuged through mini syringe G-25 sephadex column at 10,000g for 10 minutes. Purified protein in extraction buffer was obtained free of gel matrix. Samples were concentrated by filtering through ultrafiltration units (Millipore). 20 µg protein of each band was re-run by SDS-PAGE under reducing conditions.

**Immunization of bitches:** Keeping in consideration the role of PH-20, LDHC\(_4\) \(\alpha\)-actin and \(\beta\)-tubulin in various aspects of fertilization and their interaction with each other in dog sperm, six bitches were divided into three groups and immunized with 46/32 kDa and 36/30/28 kDa antigens intramuscularly. Three boosters were given at an interval of 15 days as per the following schedule:

Blood was collected on day 45 and 70. Immunoglobulins from blood serum, partially purified at 33 % (NH\(_4\))\(_2\)SO\(_4\) saturation were used for further analysis (Oser, 1965).

**Detection and monitoring of immune response:** Immune response was detected by DID (Oucherlonny, 1949). Ig level in blood serum, ELISA (Crowther, 1995), immunoblotting (Towbin et al., 1979) and immunofluorescence (Verdier et al., 2002). Partially purified Ig against 46/32 kDa and 36/30/28kDa were diluted to 1:200 for immunoblotting and immunofluorescence.

**Effect of immunization on fertility of bitches:** Bitches in the control group as well as the immunized ones were examined for estrus (vaginal cytology/ external genital features, behavior) and were bred either naturally or through artificial insemination. Ultrasound was done after 35 days of breeding to confirm the pregnancy.

**RESULTS AND DISCUSSION**

**Characterization of LDHC\(_4\), PH-20, \(\alpha\)-actin and \(\beta\)-tubulin in dog sperm Immunoblotting:** Anti spam-1 and anti LDHC\(_4\) recognized 46/32 and 36/30/28 kDa proteins in dog sperm extracts on immunoblots (Fig 1). Initially characterized by Primakoff et al., (1985) in guinea pig sperm, PH-20 has been found in many other species including cynomologus macaques, humans and canines (Overstreet et al., 1995, Sabeur et al., 1997 and Sabeur et al., 2002). In the rat, lactate dehydrogenase C also exists in two forms, isoenzymes C4 and A1C3 (Goldberg, 1977). Proteins of 43, 36 kDa and 62, 46, 43, 30 and 28 kDa were detected on immunoblots with anti-actin and anti-tubulin (Fig 1). deLas Heras et al., (1997) recognized single band of 43 kDa with monoclonal anti-actin in ram spermatozoa. In immunoblotting, Dvorakova et al., (2005) also detected actin and tubulin of 42 and 55 kDa in human and rabbit sperm extracts. Similarity in the mol wts of sub units of PH-20, LDHC\(_4\), \(\alpha\)-actin and \(\beta\)-tubulin revealed that one sub unit each of LDHC\(_4\) and \(\alpha\)-actin had same mol wt of 36 kDa and two other sub units each of LDHC\(_4\) and \(\beta\)-tubulin shared the same mol wt of 30, 28 kDa. Reaction of 46 kDa protein both with anti-spam-1 and \(\beta\)-tubulin indicated similarity in their mol wts. Similarly the reaction of 43 kDa protein both with anti- \(\alpha\)-actin and anti-\(\beta\)-tubulin revealed some similarity between both.

**Immunolocalization of PH-20, LDHC, \(\alpha\)-Actin and \(\beta\)-Tubulin:** A very strong signal was detected on the entire head with anti-spam-1 and anti-\(\alpha\)-tubulin. A signal of weak intensity

<table>
<thead>
<tr>
<th>Bitch #</th>
<th>Antigen (kDa)</th>
<th>Protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>46/32 (PH-20)</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>46/32 (PH-20)</td>
<td>2.0</td>
</tr>
</tbody>
</table>

1.0 mg of N-acetyl muramyl-L-alanine-D-isoglutamine hydrate + 100 µl FCA were added as adjuvant in the first injection. In the subsequent boosters, FCA was replaced with 100 µl of IFCA. -

FIG 1: Immunoblotting of anti-LDHC, anti-tubulin and anti-actin with dog sperm extracts.
was also found on the tail with anti-spam-1 (Fig 2, 3). On the other hand localization of PH-20 is reported at the post acrosomal region in guinea pig (Myles and Primakoff, 1984) and stallion (Meyers and Rosenberger, 1999). LDHC₄ was present on the whole sperm as indicated by a signal of uniform intensity with anti LDHC₄. Similar pattern of LDHC₄ is reported in mouse, human, macaque and canine (Overstreet et al., 2005 and Sabeur et al., 2002). Anti actin gave bright staining of postacrosomal region and light staining of tail region (Fig 3). Cytoskeletal proteins are mostly localized to the apical and the equatorial acrosomal region of the sperm head (Dvorakova et al., 2005), while the intensity of staining differs between the sperm types. In human sperm, actin has been identified in the acrosome, post-acrosomal area, neck and principal piece of the tail (Castellani-Ceresa et al., 1993). Therefore, immunofluoresence of dog spermatozoa with anti-spam-1, LDHC₄, α-actin and β-tubulin indicated the localization of PH-20 and tubulin mainly on the entire head region, while that of LDHC₄ on the whole sperm and actin mainly concentrated on the post acrosomal cap.

**Mass Spectrometry:** Peptides of the 46, 36, 32, 30 and 28 kDa proteins (after trypsin digestion) were distinguished and characterized by peptide mass fingerprinting (MALDI-TOF). The resulting peptide peaks were searched against the SWISS-PROT and other NCBI databases using the MASCOT search algorithm. A maximum of 92, 77, 45, 58 and 58 peptides were used for peptide search of 46, 36, 32, 30 and 28 kDa antigens respectively. 14, 8, 5, 6 and 4 peptides were identified by MALDI-TOF along with their calculated mol wt and sequence of amino acids for 46, 43, 36, 32, 30 and 28 kDa antigens respectively (Table 1). Peptides with individual ions scores of > 39 indicated their identity or extensive homology (p<0.05) with other proteins. No hits were found for 46 kDa antigen. The matched peptides of 43, 36 and 32 kDa antigens were identified to have significant matching with the peptides of α-actin., whereas, antigens of 30 and 28 kDa were identified to have matching with peptides of β-tubulin. Peptide sequence of 43; 36, 30, 28 and 32 kDa antigens identified by MALDI-TOF also showed a similarity of about 33.5%; 19.49, 24.28, 21.4 and 18.75% with tubulin; LDHC₄ and PH-20 respectively. It reveals that actin/tubulin, LDHC₄/actin, PH-20/tubulin and LDHC₄/tubulin shares molecular weights of 43, 36, 32 and 30/28 kDa respectively or these antigens interact with each other on dog sperm surface for their common biological activity.

**Interaction of LDHC₄, PH-20, α-actin and β-tubulin in dog sperm:** Protein interactions control most of the cellular processes (Gavin et al., 2006). Therefore, it is essential to identify and characterize protein–protein interactions and their networks for understanding the mechanisms of biological processes on a molecular level. Mammalian fertilization involves highly regulated biochemical interactions i.e. binding of seminal plasma proteins to the sperm surface during ejaculation, interaction of sperm surface proteins with oviductal epithelial cells, sperm capacitation, gamete recognition, primary and secondary binding of the sperm to the ovum, acrosome reaction of sperm, penetration of the sperm through the zona. The plasma membrane of spermatozoa undergoes extensive remodeling when sperm mixes with seminal plasma proteins at ejaculation. Some of seminal plasma proteins become sperm-coating agents. Two types of interactions could be involved in this process: (i) the interaction of proteins with the sperm membrane, (ii) mutual interactions between monomer forms of protein. Reaction of

**FIG 2:** Immunofluoresence staining of dog sperm preparation with anti LDHC4 and PH-20 antibody.

**FIG 3:** Immunofluoresence staining of sperm preparation with anti-actin and tubulin antibody.
465

### TABLE 1: MS analysis of 46, 43, 32, 36, 30 and 28 proteins, detected by MALDI-TOF.

<table>
<thead>
<tr>
<th>Detected on immunoblots</th>
<th>Peptide Sequence detected by MS analysis</th>
<th>Sequence similarity with Matching (%)</th>
<th>No. of peptides searched (Matched)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-20</td>
<td>No hits available</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin/</td>
<td>K.AG FAGDDAPR.A; VFPSIVGRPR.H; K.DSYVGDAEQSR.R; K.YPIERGIYTVNWDDMEK.I; K.IWHHTFYNELV.R; V.R VAEEHVPVLTIEALPNPK.A; R.DLTDYLMK.I; K.SYELPDQGVITIGER.F; R.TTGIVMDSGDTHTPIEYEGYALPHAIL.R.L; R.LDLAGRDLTDYLMK.I; R.GYSFTTTAER.E; K.DLYANTVLSQGTTPMYGIADR.M; K.IAPPERK.Y; K.QEYDEGSQIVHR.K</td>
<td>β-Actin (Arabian camel) 33.5</td>
<td>92 (14)</td>
</tr>
<tr>
<td>LDHC</td>
<td>K.IWHHTFYNELV.R; V.R VAEEHVPVLTIEALPNPK.A; R.TTGIVMDSGDTHTPIEYEGYALPHAIL.R.L; R.LDLAGRDLTDYLMK.I; R.GYSFTTTAER.E; K.SYELPDQGVITIGER.F; R.DLTDYLMK.I</td>
<td>β-Actin (North American Opposum) 19.49</td>
<td>77 (8)</td>
</tr>
<tr>
<td>PH-20</td>
<td>R.TTGIVMDSGDTHTPIEYEGYALPHAIL.R.L; R.LDLAGRDLTDYLMK.I; R.GYSFTTTAER.E; K.SYELPDQGVITIGER.F; R.DLTDYLMK.I</td>
<td>β-Actin (Sheep) 24.28</td>
<td>45 (5)</td>
</tr>
<tr>
<td>LDHC</td>
<td>K.DLYANTVLSQGTTPMYGIADR.M; K.LYLTVAAVFR.G; K.MAVTFGNSTAAIQQELFK.R.I</td>
<td>β-Tubulin (A Laevis) 21.42</td>
<td>58 (6)</td>
</tr>
<tr>
<td>LDHC</td>
<td>R.YLYTFQANSTR.V; R.FPGQLNADLRK.L; R.FPGQLNADLRK.L; K.IREEYPDR.M; R.FPGQLNADLRK.L; R.FPGQLNADLRK.L; R.FPGQLNADLRK.L; R.FPGQLNADLRK.L; R.FPGQLNADLRK.L; R.FPGQLNADLRK.L; R.FPGQLNADLRK.L; R.FPGQLNADLRK.L</td>
<td>β-Tubulin (Sheep) 18.75</td>
<td>58 (4)</td>
</tr>
</tbody>
</table>

43, 36, 30/28 kDa proteins respectively with α-actin/β-tubulin, LDHC/α-actin and LDHC/β-tubulin on immunoblots and similarity in fluorescence pattern of actin and tubulin with LDHC4 and PH-20 indicated that these proteins interact with each other on sperm surface. The reaction of 43, 36, 32, 30 and 28 kDa proteins with anti-actin/tubulin, anti-LDHC4/actin, anti spam-1, anti LDHC4/tubulin on immunoblots further confirmed their interaction. Sequence matching of 43/36/32 to α-actin and that of 30/28 kDa to β-tubulin by MALDI-TOF analysis indicates that LDHC4 and PH-20 are superimposed by actin/tubulin on dog sperm surface. Interaction of LDHC4 with otherproteins e.g. actin, ATP-translocase is well known. Manaskova et al. (2002) also reported mutual specific interactions between protein components of boar seminal plasma. They further revealed that these interactions participate in the formation of aggregated forms of proteins in seminal plasma and probably also in the arrangement and remodeling of protein coating layers of sperm and aggregation of seminal plasma proteins is probably an important phenomenon in the fertilization process. These four proteins have key roles in various aspects of fertilization; therefore their interaction may have some important role in dog’s fertility.

**Response to immunization with 46/32 and 32/30/28 sub units in bitches:** Since PH-20/tubulin, actin/tubulin, LDHC/actin and LDHC/tubulin shared molecular weights of 46, 43, 36 and 30/28 kDa, therefore, proteins of 46/32 kDa and 36/30/28 kDa were pooled to explore their immunocontraceptive potential.

**Immunodiffusion/ Ig level in blood serum:** Single precipitation line as a result of Ag-Ab reaction on the immunodiffusion plates confirmed the production of antibodies against 46/32 kDa and 36/30/28 kDa antigens in the blood serum of bitches immunized with respective antigens (Fig 4). Ig level (mg/ml) in the blood serum of bitch #3, 4, 5 and 6 was 13.63, 19.23, 19.91, 11.46 on 0 DPI, which increased to 32.6, 51.72, 48.96, 46.20 on 45 DPI i.e. 15 days after 3rd booster respectively. It indicated the production of antibodies against 46/32 kDa (bitch #3, 4) and 36/30/28 kDa (bitch # 5, 6) antigens respectively. Ig concentration in blood serum of immunized bitches remained at a higher level even on 70 DPI (Fig 5). It showed efficacy of 46/32 kDa and 36/30/28 kDa sub units as immunocontraceptive antigens, as it has also been reported that higher serum antibody levels achieved after immunization with epididymal specific protein (DE) resulted into higher rate of reduction in the fertility of male rats (Suri, 2005 and Chen et al., 2009).

**FIG 4:** Immunodiffusion reaction of 46/32 (PH-20) and 36/30/28 kDa (LDHC) antigens with partially purified Ig of bitches against the respective antigens.
Ag-Ab titre by ELISA/Immunoblotting: Ag-Ab titre was 6400/3200 and 3200/6400 in the blood serum of bitch # 3/4 and 5/6 on 45 DPI immunized with 46/32 kDa and 36/30/28 kDa antigens, which remained same on 70 DPI. In the similar studies in *Macaca radiata*, Rana et al. (2009) also observed an elevated antibody titre after the first booster which was significantly higher from week 8-12 against human sperm associated antigen 9. Immunization of bulls against spermatozoa resulted in transient rise in the level of IgM antibodies and persistent elevated levels of IgG1 and IgG2 in the serum (Kim et al., 1999). The prevalence of IgG anti-sperm antibodies in serum has been reported in humans and is consistent with a systemic immune response (DeAlmeida et al., 1991).

In immunoblotting, the reaction of 46/32 kDa and 36/30/28 kDa purified antigens with partially purified Ig (35, 70 DPI) of bitch 3, 4 and 5, 6 further confirmed the production of antibodies against 46/32 kDa and 36/30/28 kDa sub units, respectively (Fig 6). Therefore, elevated level of immunoglobulins, antigen-antibody titre in blood serum of immunized dogs and detection of 46/32 kDa and 36/30/28 kDa antigens on immunoblots indicated the production of antibodies in the blood serum of immunized bitches. Naz and Zhu (1997) also opined that an increase in the level of ASA in blood serum of immunized mice has a direct and significant correlation with the reduction in fertility.

Immunolocalization of 46/32 and 36/30/28 kDa antigens: Figure shows localization of 46/32 kDa and 36/30/28 kDa antigens on dog spermatozoa recognized with respective partially purified Ig of bitch # 3, 4 and 5, 6 respectively, visualized by indirect immunofluorescence staining. An intense signal on acrosomal cap, discrete band like structures on the post acrosomal region and a signal of lesser intensity on the tail were observed with anti 46/32 kDa Ig (Fig 7). Whereas a signal of very strong intensity on acrosome and of very weak intensity on post acrosomal surface and tail was observed with anti 36/30/28 kDa Ig. It indicates scattered localization of 46/32 kDa proteins on the entire head surface and that of 36/30/28 kDa proteins mainly on the acrosome (Fig 8). The pattern of immunofluorescence on dog sperm surface, detected with anti 46/32 kDa and 36/30/28 kDa Ig resembles to that of PH-20/ tubulin/actin and LDH C /actin/ tubulin.

Effect of immunization with 46/32 and 36/30/28 kDa antigens on Fertility: The presence of >75 % cornified cells in the vaginal smears, swelling of vulva and acceptance of bitch to the dog (when tried for mating) confirmed the estrus stage of a bitch. These symptoms were well pronounced in bitch # 1 and 2, which were kept as control. Bitches immunized with 46/32 and 36/30/28 kDa antigens were in
estrus, as detected by vaginal cytology, but vulva was not as swollen as in case of control animals. When tried for natural mating, bitches did not react to the dog, and natural mating could not be successful. It indicated that animals were in suppressed heat and PH-20, LDHC$_4$, actin and tubulin sub units were probably affecting the estrus at certain level and hence the process of natural mating. Therefore these bitches were subjected to AI. Fig 9 and 10 shows the ultrasonography of immunized non-pregnant and un-immunized pregnant bitches. Three and four fetuses were clearly visible in the ultrasonographs of bitch #1 and 2 (control) after 35 days of mating (Fig 10). Uterus of immunized bitches 3, 4, 5 and 6 was of normal size (2.4 to 2.6 cm, Fig 9).

**FIG 9:** Ultrasonography of Bitches #3,4 and 5,6 immunized with 46/32 kDa and 36/30/28 kDa antigens respectively after 35 days of insemination.

As PH-20 has its role in cumulus penetration, zona binding and actin/tubulin in capacitation/AR, therefore, it can be interpreted that infertility observed in bitch # 3, 4 immunized with 46/32 kDa antigens may be due to the presence of antibodies against PH-20/actin and tubulin in the oviductal fluid, which inhibited capacitation/acrosome reaction and ultimately sperm-egg binding. In female guinea pigs, it has been proposed that infertility occurs by prevention of sperm–egg binding by PH-20 antibodies (Primakoff et al., 1988). Immunization with affinity purified PH-20 from guinea pig sperm (gpPH20) has been shown to cause complete and reversible infertility in female guinea pigs after a single dose of antigen (Primakoff et al., 1997, Tung et al., 1997). Actin is believed to be involved in cell signalling processes and actin polymerization may represent an important regulatory pathway associated with tyrosine phosphorylation in sperm (Brener et al., 2003, Seligman et al., 2004). Protein tyrosine phosphorylation occurs during capacitation of spermatozoa in the female reproductive tract. In spermatozoa, a number of cytoskeletal proteins are implicated in key events such as capacitation, AR and zona binding (Howes et al., 2001, Moore, 2001, Brener et al., 2003, Liu et al., 2005). Dvorkova et al., (2005) were also of the view that cytoskeletal proteins play an important function at the time of AR to reorganize membrane domains into fusogenic regions. Penciva et al., (2001) also detected a tubulin epitope in the head of boar spermatozoa and indicated change in its distribution after AR. Similarly, infertility observed in bitch # 5, 6 may be due to the combined effect of antibodies against LDHC$_4$, actin and tubulin. Occurrence of antibodies against LDHC$_4$, actin and tubulin in the female reproductive tract may have immobilized the spermatozoa and inhibited the process of capacitation/AR and fertilization. LDHC$_4$ is required for sperm to accomplish its ultimate goal, fertilization (Goldberg et al., 2010). Immunocontraceptive effect of LDHC$_4$ based upon the sperm-derived immunogen is well studied in female baboon (O’Hern et al., 1995), mice/ rabbit (Goldberg 1973, Gupta and Syal, 1997). Synthetic peptides of LDHC$_4$ and DNA vaccines pVAX1-hLDHC and pVAX1-mLDHC have also been noted to reduce fertility in mice (Yong et al., 2008). Therefore, it seems that LDHC$_4$/PH-20 and α-actin/β-tubulin share their molecular weights or interact with each other or actin/β-tubulin superimpose LDHC$_4$/PH-20 on sperm surface. In view of observed contraceptive effect, it can be concluded that LDHC$_4$ and PH-20 collectively with α-actin and β-tubulin affect estrus, process of natural mating and ultimately the fertility in bitches.

**FIG 10:** Ultrasonography of bitch #1,2 (contral) after 35 days of mating.

**REFERENCES**


