

STANDARDIZATION OF A FLOW CYTOMETRIC TECHNIQUE FOR DETECTION
OF ANTI-SPERM ANTIBODIES IN BULLS

by

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Abstract

Presence of anti-sperm antibodies (ASA) is associated with infertility in many species, including bulls but there is no standardized direct technique that allows detection of ASA bound to the sperm surface. The overall objective was to standardize a flow cytometric technique for detection of IgG and IgA directly attached to bovine sperm. The effects of fixation using phosphate buffer solution (PBS) or diluted formalin buffer solution (dFBS), exclusion of dead cells from the analysis, and aliquot variability were assessed using healthy bulls classified as Satisfactory Potential Breeders (SPB, n=9) and bulls with experimentally induced ASA (n=4) (Experiment1). The effect of freezing on the percentage of IgG- and IgA- bound sperm was assessed in samples from immunized bulls (n=4) (Experiment 2). Anti-sperm antibodies on the sperm surface were induced in yearling bulls by intramuscular injection of autologous semen and an adjuvant. Fixation of sperm cells did not affect the percentage of IgG- or IgA-bound sperm in any group of bulls. Exclusion of dead cell from the analysis did not affect the percentage of IgG-bound sperm ($p= 0.0922$ and $p= 0.1525$ for immunized and reproductively normal bulls, respectively). The exclusion of dead cells significantly increased the percentage of IgA-bound sperm in semen samples from immunized bulls ($p= 0.0152$) and significantly decreased the percentage of IgA- bound sperm in semen samples from reproductively normal bulls ($p= 0.0012$). Variability was $< 10\%$ in samples from immunized and reproductively normal bulls for percentage of IgG- and IgA-bound sperm. Freezing did not affect the percentage of IgG- ($p=0.1287$) or IgA-bound sperm ($p=0.4175$). Based on these results, fixation is neither necessary nor detrimental for analysis, and the percentage of antibody-bound cell should be calculated gated on the population of live cells only, especially when evaluating IgA binding. The percentage of ASA-bound sperm can be assessed on frozen-thawed samples. The development of this technique allows for further studies on ASA-bound sperm in populations of normal and abnormal bulls.

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Dedication

To my family, for their unconditional love and support.

Chapter 1 - Introduction

Sperm have long been known to be antigenic. Anti-sperm antibodies (ASA) were first discovered in humans in 1959 and proposed as a factor in male infertility¹. Anti-sperm antibodies have been theorized to negatively impact fertility by affecting sperm motility, cervical mucus penetration, gamete fusion, and potentially even the first steps of embryo development in humans²⁻⁵.

Presence of anti-sperm antibodies is associated with infertility in many species, including bulls. While immune-mediated infertility is frequently diagnosed in men and has been reported in bulls, diagnosis in veterinary medicine has been difficult due to the lack of a standardized method to detect ASA.

Numerous studies have highlighted the impact of ASA on fertility in different species, including bulls. Presence of ASA can inhibit passage of spermatozoa through cervical mucus, prevent membrane fluidity changes needed for capacitation, reduce the ability of spermatozoa to undergo the acrosome reaction, and interfere with binding to the zona pellucida and fertilization⁶. Sub- or infertility in bulls can have profound negative consequences on the cattle industry, and one important undiagnosed cause of reduced fertility may be immune-mediated infertility^{7, 8}. Experimentally induced ASA were shown to affect the ability of bull spermatozoa to fertilize oocytes *in vitro*⁸, while naturally occurring ASA were associated with reduced spermatozoal motility and infertility in two bulls⁹.

Because of the impact of ASA on fertility, several methods are routinely used in andrology laboratories to detect their presence. Sperm agglutination and immobilization tests have been developed in human and veterinary medicine for diagnosis of immune-mediated infertility. However, these tests are generally insensitive, nonspecific and too complex in some cases for routine laboratory analysis of ejaculates. Direct immunofluorescence and enzyme-linked immunosorbent assay (ELISA) techniques have also been used, but these techniques require fixation of spermatozoa. Fixation can result in non-specific binding of antibodies, exposure of intracellular antigens, denaturation of sperm antigens or membrane damage, resulting in false-positive or false-negative results. However, lack of fixation can also lead to patching, capping and shedding of antigens, resulting in false-negative results. Anti-sperm antibodies have been detected in bovine serum or

seminal plasma using ELISA^{8,10}, agglutination tests¹¹, or indirect immunofluorescence¹². Most tests were indirect and involved detection of circulating antibodies. Circulating or seminal plasma antibodies may be of no clinical significance unless bound to spermatozoa^{6,13}. Therefore, direct techniques that allow detection of ASA bound to the sperm surface are preferable. Unfortunately, no standardized direct procedure has been developed in veterinary medicine for diagnosis of immune-mediated infertility.

Flow cytometry has been used extensively in hematology in veterinary medicine, and a method to detect and quantitate ASA with flow cytometry has been developed in human medicine. Flow cytometry allows objective and quantitative estimation of ASA on the surface of living spermatozoa and constitutes a sensitive, specific and repeatable test¹⁴. Flow cytometry has many advantages over agglutination or ELISA tests previously described in bulls and its development will provide a valuable tool for diagnosis of immune-mediated infertility in bulls. However, further work is needed to standardize the technique and make it available as a diagnostic tool. The overall objective of this research was to standardize a flow cytometric technique for detection of antibodies directly attached to sperm. This was performed by induction of anti-sperm antibodies in healthy bulls and identification of anti-sperm IgG and IgA antibodies directly attached to these cells. The use of a fixative solution versus no fixative solution and the inter- and intra-assay variability of the test were evaluated. Due to the availability of frozen semen in the bovine industry, a final trial was performed to assess the effect of freezing on antibody binding on anti-sperm antibody bound in bulls with experimentally induced ASA.

Chapter 2 - Literature Review

Pathophysiology of immune-mediated infertility

Antigenicity of sperm

Sperm are produced in the seminiferous tubules of the testis after the onset of puberty (spermatogenesis). The initial stages of spermatogenesis involve several rounds of mitotic division of sperm precursor cells (called spermatogonia), which remain connected via syncytial bridges. These spermatogonia undergo mitosis and then move between adjacent Sertoli cells to enter the adluminal compartment of the seminiferous tubules where meiotic divisions reduce the chromosome number to haploid and cell differentiation (spermiogenesis) occurs¹⁵. The mature sperm are later on released to the lumen of the seminiferous tubules (spermiation). During the process, a myriad of surface and intracellular proteins is expressed; yet, these new autoantigens are tolerated by the immune system. The immunogenicity of the proteins is not diminished. Rather, is the testis that confers protection⁶. The sperm are then transported through the rete testis to the epididymis where functional maturation takes place and extracellular fluid is reabsorbed, resulting in the concentration of the sperm, which are then stored in the cauda epididymis, vas deferens and ampullae until ejaculated¹⁵.

The spermatozoon is typically divided anatomically into a head and a flagellum (or tail). The head contains the nucleus, overlying acrosome, and a reduced complement of cytosolic elements. The head can be subdivided into an acrosomal region, equatorial segment, post-acrosomal region, and posterior ring, which demarcates the junction between the head and flagellum. The posterior ring is the site of plasma membrane anchoring to the nuclear envelope and is thought to produce a tight seal that separates cytosolic components of the head and flagellum¹⁶. The flagellum can be subdivided into a connecting piece, middle piece (or midpiece), principal piece, and end piece^{17, 18}. The midpiece and tail of the sperm may be considered to form a single functional entity. The tail itself consists of a central axoneme, which, in the region of the midpiece, is sheathed in a helix of mitochondria¹⁶. The acrosome is a

membrane-bound exocytotic organelle that overlies the rostral two thirds of the nucleus, with a fit resembling that of a bathing cap. Anatomically, the membrane is subdivided into an inner acrosomal membrane that is continuous with an outer acrosomal membrane¹⁶.

These various parts of the spermatozoon are surrounded by a common plasma membrane. The composition of the plasma membrane can be subdivided into regional domains that impact its multiple functions, such as sperm-oviductal adhesion, penetration of the cumulus-oophorus matrix; sperm-zona adhesion; the acrosome reaction, acquisition of activated motility and hyper activated motility; and sperm-oocyte adhesion and fusion. Anti-sperm antibodies have been shown to react with each of these major regions of sperm albeit with variable biological effects¹⁵.

The blood-testis barrier (BTB) consists of a continuous layer of Sertoli cells within the seminiferous tubules. These cells separate the basal and adluminal compartments of the seminiferous tubules and are joined by tight junctions called junctional complexes, which form a specialized permeability barrier that prevents large molecular weight materials and immune cells from gaining access to the adluminal compartment. The myoid cells surrounding the seminiferous tubules and the Sertoli cells junctional complexes form the blood-testis barrier¹⁹. The primary purpose of the BTB is to prevent autoimmune reactions from destroying the developing germ cells. The peritubular layer acts as the first barrier against large molecular weight materials. The junctional complexes between Sertoli cells serve as the second barrier against immune cells and immunoglobulins. The most important feature of the BTB is the exclusion of immune cells (macrophages and lymphocytes) and immunoglobulins (antibodies) from the adluminal compartment¹⁹. The BTB is incomplete at the rete testis, a location where immense numbers of spermatozoa with newly adapted surface molecules traverse toward the epididymis, making it a particularly susceptible region for development of autoimmune orchitis. Some other mechanism, besides physical separation, must exist to maintain testicular immune privilege, which requests more robust protection of the tolerogenic environment of the testis⁶. High local testosterone concentrations, characteristic of the testis, seem to play an important role in the maintenance of testicular immune privilege. However, the precise manner in which testosterone mediates its immunosuppressive functions on testicular leukocytes is as yet unknown⁶.

Notwithstanding its immune-privileged status, the testis is clearly capable of mounting normal inflammatory responses, as proven by its effective response to viral and bacterial infection. In pathological circumstances, the misbalance between the tolerogenic and the inflammatory aspects of the testicular immune response can lead to the formation of anti-sperm antibodies and in rare instances, epididymo-orchitis⁶.

The blood-testis barrier provides immunological protection of sperm antigens, and disruption of this barrier by various mechanisms (such as vasectomy, testicular trauma, testicular torsion, infection of the reproductive organs, and testicular surgery) has been associated with the formation of ASA²⁰. The prevention of ASA in the male depends on the sequestration of antigens on germ cells by the presence of the blood-testis barrier. Developmental abnormalities of the formation of the blood-testis barrier, traumatic disruption or unilateral focal cryptic obstructions could lead to ASA formation. Also gastrointestinal exposure to sperm has been associated with the development of ASA both in animal experiments as well as in homosexual men^{21, 22}. Natural causes of testicular disease, including trauma and infection, can break down the blood-testis barrier and lead to exposure of sperm to leukocytes, with the subsequent formation of anti-sperm antibodies. The presence of anti-sperm antibodies in seminal plasma has been documented in cases of blood-testis barrier disruption resulting from testicular injury²³. The occlusion of the ductus deferens results in epithelial rupture, formation of sperm microgranulomas, exposure of sperm antigens to the immune system, and generation of anti-sperm antibodies²⁴. Typically, high levels of anti-sperm antibodies are found in men with a history of testicular torsion, testicular surgery, vasectomy, and epididymo-orchitis²⁵. A high percentage of these individuals develop epididymal sperm granulomas and testicular degeneration associated with the formation of ASA^{26, 27}.

Both acute and chronic infection and/or inflammation, especially of the epididymis, may cause partial or complete obstruction of sperm transport leads to oligozoospermia or azoospermia. Also, pressure-induced tears of the distal segments of the epididymal duct or efferent ducts may occur²⁸. The immunological defense will then be activated and production of anti-sperm antibodies initiated²⁹⁻³¹. First, IgM antibodies will be produced, but these are not secreted into the genital tract because their size is too large to pass the epithelial barrier. Shortly afterwards, antibodies of the IgG class appear, and these can enter the genital tract. The anti-sperm antibodies of the IgG class come into contact with the spermatozoa and attach to these³². In some cases –and more commonly indeed during infection- secretory IgA anti-sperm

antibodies are produced locally in the genital tract (probably the epididymis)³³. Antibodies of the IgA class can then be detected on the ejaculated spermatozoa, but not in serum, and this is associated with an additional reduction of their fertilizing capacity³⁴.

A humoral and cellular immune response is mounted that results in destruction of all tubular components, except for Sertoli cells and spermatogonia³⁵. Subsequently, infertility can result from antibodies directly binding sperm, or from aspermatogenesis due to allergic orchitis²⁵.

As stated before, an immune privileged environment does not mean that effective inflammatory or immune-responses cannot occur: the testis appears to be no more susceptible to infection or tumors than other tissues. In fact, infections of the testis appear to be considerably less common than in the epididymis or remainder of the genital tract³⁶. The specific suppression of adaptive immunity necessary for immune privilege in the testis, therefore, implies a greater reliance upon innate immune mechanisms. Innate immunity is initiated through specific pattern recognition receptors expressed principally on macrophages and epithelial cells^{37, 38}, as opposed to the heterologous and polymorphic antigen-recognition receptors that mediate T cell and antibody-mediated responses. Innate immunity involves rapid mobilization of macrophages, polymorphonuclear cells and NK cells, in particular, as well as secretion of specific anti-microbial products, such as the interferons and defensins^{39, 40}. Several recent studies have shown that testicular macrophages and Sertoli cells express Toll-like receptors (TLR), which are pattern recognition receptors necessary for recognition of a range of viral and bacterial infections⁴¹⁻⁴⁵. These cells respond to TLR activation by initiating inflammation, although the inflammatory responses produced have unique features that appear to be more consistent with immune privilege^{41, 44, 46}. Inflammation exerts inhibitory effects on testicular steroidogenesis and spermatogenesis, due to that fact that inflammatory cytokines, reactive oxygen species and even glucocorticoids produced in response to inflammation, have predominantly negative effects on the hypothalamic–pituitary–Leydig cell axis and seminiferous epithelium⁴⁷⁻⁵⁰. However, most men and experimental animals recover with only minor effects on continuing fertility following an inflammatory or infectious episode. Clearly, an additional precipitating event, which leads to the breaking of tolerance and loss of immune protection, is required for autoimmunity to develop. In experimental models, the critical event leading to autoimmunity is the development of activated T cell responses either to testicular autoantigens or to graft allo- or xenoantigens⁵¹⁻⁵³. In certain animal models, autoimmune orchitis has also been observed following physical

disruption of the reproductive tract^{54, 55} or as a sequelae of infectious orchitis^{56, 57}. Infection-mediated autoimmunity is believed to be due to molecular mimicry, bystander immune activation, and presentation of autoantigens by activated antigen-presenting cells during infection⁵⁸⁻⁶⁰. How failure of immune-privilege might arise in humans, species in which it has been mostly studied, to cause infertility, is a more speculative issue. The data linking infections with autoimmune orchitis is sparse at best and vasectomy rarely causes orchitis, even though sperm antibody reactions are common^{61, 62}. However, it is to be expected that, as in other autoimmune diseases, genetic factors play a role. The possibility that epigenetic influences may also contribute to testicular autoimmunity has yet to be considered. Other possibilities include alterations in testicular macrophage or dendritic cell immune-regulatory functions or antigen burden overload due to disruption of the blood–testis barrier as a result of extended chronic inflammation⁶³.

Overall Sertoli cell function is maintained by pituitary follicle-stimulating hormone (FSH) and androgens from the Leydig cells. However, Sertoli cell function is dynamically regulated by communication with the spermatogenic compartment involving physical interactions, various cytokines and other signaling molecules. Phagocytosis of senescent and apoptotic germ cells and residual cytoplasm transfers germ cell antigens to the Sertoli cell, which then allows these antigens access to the interstitial space. The intratesticular immune cell population of the testis, comprising the resident macrophages, dendritic cells (DC), T cells (T) and NK cells is confined to the interstitial tissue compartment. Resident macrophage number and function is maintained by the Leydig cells, under the influence of luteinizing hormone (LH), and by the Sertoli cells. In turn, the macrophages, Sertoli cells and Leydig cells, the latter through the production of androgens, regulate the trafficking and activity of the other immune cells within the interstitial tissue. In particular, presentation of germ cell antigens (or graft antigens in experimental models of immune privilege) by the antigen-presenting activity of the DC and resident macrophages to the T cells leads to a tolerogenic (type 2) response, under the immunoregulatory influence of the Sertoli cells and resident macrophages. This control involves several immunoregulatory cytokines produced by the somatic cells and immune cells of the testis, most notably transforming growth factor β (TGF β), activin A and interleukin 10 (IL10). As a back-up, various immunosuppressive mechanisms delete activated T cells that appear within the interstitial tissue environment [Fas ligand (FasL), indoleamine 2,3 dioxygenase (IDO),

lyso-glycerophosphatidylcholines (lyso-GPCs)]. Consequently, an environment is created where antigen-specific (adaptive) immunity is suppressed, while innate immune functions are retained or enhanced. Failure of this control, which might be triggered by chronic inflammation, physical trauma or certain infections, leads to antigen-specific reactions to intratesticular antigens (a type 1 response), resulting in sperm antibody formation and autoimmune orchitis, and eventually infertility⁶³.

Antibodies directed to sperm antigens can be detected in sperm cells and seminal plasma in men, as well as cervical mucus, oviductal fluid, or follicular fluid in women. They also occur in blood serum in men and women⁶⁴ but only the antibodies that are bound to sperm are considered to be of real significance for fertility^{6, 13}.

Evidence supporting the importance of studying sperm directly bound to sperm, in making the diagnosis of auto-immunity to spermatozoa, comes from a comparison of sperm antibodies detected in matched semen and serum specimens in men⁶⁵. In approximately 15% of cases, antibodies have been detected in serum but not on the sperm surface. The majority of these circulating anti-sperm antibodies that failed to enter seminal fluid were of low titer and directed against the sperm end piece. In addition, anti-sperm IgM does not enter the males genital tract secretions, even when present in high concentrations in blood⁶⁵. This immunoglobulin class of anti-sperm antibodies is only rarely encountered in sera of heterosexual men, through it is more common both in homosexual men and sera of women⁶⁵.

Sperm antibodies may be both transudates from the blood or secreted locally by plasma cells within the reproductive tract⁶⁶⁻⁶⁸. Sperm-reactive antibodies can also be present in serum yet undetectable in semen or within female reproductive tract secretions⁶⁹. Conversely, local immunity to sperm has also been demonstrated in the absence of detectable humoral antibodies in both men⁷⁰ and women⁷¹. Seminal plasma ASA not attached to the sperm surface are probably of little clinical significance. In humans, dogs, bulls and other species, seminal plasma components do not ascend beyond the vagina⁷², but this does not true for all species. Although the probable reason for the presence of unbound antibodies in seminal plasma traditionally is believed to be due to the excess of antibodies⁷³ relative to the patient's sperm specific antigens, the presence of unbound antibodies due to a reactivity different that found on the patient's spermatozoa cannot be excluded automatically when using indirect tests⁷⁴. Rasansen (1996)

concluded that the indirect measurement of sperm antibodies might be grossly unreliable as compared with the results obtained by direct methods⁷⁴.

In both infertile and fertile couples, 16% to 35% of men and women are found to have low levels of sperm antibodies directed primarily against the end piece. These antibodies do not interfere with sperm transport through cervical mucus or with fertilization of oocytes. Similarly, naturally occurring antibodies to intracellular components of sperm do not impair male fertility. Thus, the regional specificity of ASA must be determined for accurate diagnosis of the relevance of ASA to infertility⁷⁵.

In 5-12 % of infertile male partners, anti-sperm antibodies are found in the seminal plasma or attached to the surface of spermatozoa^{6, 76}. However, these antibodies are present also in approximately 1-2.5% of fertile men^{20, 77}.

The presence of “natural” anti-sperm antibodies in fertile humans⁷⁸, virgin girls⁷⁹ and boys before puberty⁸⁰, has questioned the authenticity of the serum anti-sperm antibodies. It has been suggested that molecular mimicry between bacteria and the sperm could be a major factor inducing anti-sperm immunological reactions⁷⁸. By the means of absorption experiments, it was established that “natural” sperm antibodies cross-react with bacteria, including *E. coli*⁸¹, *Salmonella sp* and *Shigella sp* in humans⁸², this has also been demonstrated in other species including rams⁸³, dogs⁸⁴, buffalos and bulls^{85, 86} with different pathogens.

Potential mechanisms of anti-sperm antibody-mediated infertility

Effects of ASA on sperm Transport

In humans, dogs, cats, rabbits, monkey, bulls, boars, and rams, semen is deposited into the vagina and must pass through the cervix before entering the uterus and oviducts¹⁹. In the periovulatory period, abundant, watery, mucus lines the cervical canal¹⁵. Sperm align themselves with the longitudinal axis of mucin fibers in the cervical mucus and their swimming is more vectoral than in culture medium⁸⁷. The cervical mucus functions as a guide for normal sperm to enter the uterus and may also act as a filter to prevent the passage of some very abnormal sperm into the upper reaches of the female reproductive tract. The ability of ASA to

disrupt normal interactions of sperm with the cervical mucus and inhibit sperm penetration into cervical mucus has been described in many studies in humans⁸⁸⁻⁹⁴. In several studies, the proportion of motile sperm or the percentages of ASA detected by Mixed Antiglobulin Reaction (MAR) or tray agglutination test (TAT) correlated with the inhibition of sperm penetration in the cervical mucus^{69,71,95,96}. Hence, the cervical mucus may aid in the selection of the most fertile sperm of an ejaculate by acting as an immunological filter preventing the passage of sperm coated with ASA. Anti-sperm antibodies may also be detected in the cervical mucus of up to 29.6% of women with unexplained infertility^{71,97} and at a lower incidence in a population of infertile women with known and unknown causes^{79,90,91}. Comparison of the isotype distribution of IgA in the sera or cervical mucus suggested that at least some of the cervical mucus antibodies were produced locally⁹⁸. These cervical mucus-derived ASA immobilized sperm and prevented passage through the cervical mucus^{71,97}. It has been suggested that sperm surface attached IgA are more important than IgG ASA in inhibiting penetration through the cervical mucus and further suggested that ASA reacting with the sperm head or tail principal piece but not those reacting with the tail end piece prevented mucus penetration⁹⁹⁻¹⁰¹.

Effects of ASA on capacitation and the acrosome reaction

After ejaculation, sperm undergo several changes in the female reproductive tract, which together are termed capacitation. Although the full spectrum of changes associated with capacitation is unclear, this process involves protein phosphorylation and lipid redistribution resulting in destabilization of the sperm membrane^{102,103}. Capacitation of sperm is essential to facilitate the acrosome reaction, an exocytotic event during which the sperm plasma membrane fuses with the outer acrosomal membrane allowing the exposure of the contents of the acrosome, such as acrosin and hyaluronidase that are important to allow penetration of the oocyte vestments by sperm. The acrosome reaction also results in the exposure of the inner acrosomal membrane proteins, such as the complement control protein CD46¹⁰⁴, to the exterior of the sperm. The ability of ASA to bind to sperm can be affected by capacitation while the binding of ASA to sperm can also conversely affect the processes of capacitation and acrosome reaction. Fusi and

Bronson (1990) suggested that the change in the sperm membrane induced by capacitation and acrosome reaction can affect ASA binding¹⁰⁵. These workers incubated ASA-containing sera with capacitated, acrosome-reacted sperm and found that 48% of ASA-positive and 20% of ASA-negative sera demonstrated a different pattern of binding using the indirect Immunobead test (IBT) with capacitated sperm¹⁰⁵. Evidence has also emerged that ASA might prevent membrane fluidity changes needed for capacitation before fertilization¹⁰⁶. In addition, sperm incubated with serum containing immobilizing ASA were found to have lower rates of spontaneous and induced acrosome reactions than sperm that were not incubated with the serum^{5, 107, 107}. Furthermore, ASA can inhibit the ability of sperm to undergo spontaneous capacitation as an antibody raised against a human sperm protein, BS-17, prevented capacitation of human sperm¹⁰⁸. The effects of ASA on acrosome reaction have been contradictory. For instance, Romano et al. found that the proportion of acrosome-reacted sperm was higher in ASA coated sperm¹⁰⁹, while other authors found no effect of ASA on acrosome reaction¹¹⁰. Lansford et al. found that IgG ASA from different individuals could inhibit the acrosome reaction, while other ASA initiated or had no effect on it¹¹¹. Likewise, Marin-Briggiler et al. found variable effects of ASA on the acrosome reaction¹¹². Francavilla et al. found 7 out of 12 ASA-containing sera inhibited the zona pellucida-induced acrosome reaction¹¹³. Taken together, these studies suggest that ASA have a variable effect on the acrosome reaction and capacitation; while some ASA can adversely alter the ability of sperm to undergo capacitation or acrosome reaction, other ASA do not¹⁵.

Complement as a mediator of ASA effects

Complement is a cascade of proteins of the innate immune system, which, among other functions, can bind to antibody/antigen complexes on a cell surface and cause lysis of the cell via deposition of a membrane attack complex. IgG isotype antibodies are efficient at stimulating complement while IgA is a relatively poor activator of complement. One study demonstrated that complement-fixing but not non-complement-fixing ASA reduced the ability of sperm to penetrate zona-free hamster oocytes¹¹⁴, while D'Cruz et al. have shown that incubating

complement activating ASA with normal sperm caused a significant reduction (from 87 to 43%) in mobility and also observed alterations in sperm morphology with subsequent sperm lysis in vitro¹¹⁵. D’Cruz et al.¹¹⁶ have also demonstrated that sperm-bound ASA obtained from the ejaculates of men with ASA are capable of activating complement. However, whether complement exists in the female reproductive tract at physiologically relevant concentrations is questionable. Price et al. (1979), measuring full-complement component lytic activity was measured using a hemolytic assay, have shown that complement is present in the cervical mucus, but at approximately one tenth of the concentrations found in the blood. This concentration of complement appeared to induce immobilization of 70% of ASA-coated sperm after 3 hours¹¹⁷. In contrast to the low levels of complement in the cervical mucus, follicular fluid has been shown to contain concentrations of complement approximately one half that found in plasma¹¹⁸. Other authors suggest that at least some of the major components of complement are present in follicular fluid at similar concentrations to plasma¹¹⁹⁻¹²¹.

Effects of ASA on fertilization

In order for sperm to fertilize an oocyte, several molecular interactions must take place. Firstly, the sperm must penetrate the cumulus cells that surround the oocyte. This process is facilitated by the sperm acrosomal enzyme hyaluronidase, also known as PH-20, which breaks down the cumulus cell matrix^{122, 123}. Interactions between PH-20 and the cumulus matrix also result in increased calcium concentrations in the sperm making the sperm more responsive to the subsequent zona pellucida-induced acrosome reaction¹²⁴. An acrosome intact sperm then attaches to the zona pellucida in a process referred to as primary binding. The acrosome reaction is triggered as part of this primary binding leading to secondary and more permanent binding of the sperm to the zona¹²⁵. Finally, having penetrated the zona pellucida, sperm bind to the outer membrane of the oocyte, the oolemma, so that the sperm nucleus enters the oocyte. Both primary and secondary binding to the zona pellucida and binding to the oolemma are thought to be mediated by specific protein or protein-carbohydrate interactions, which ASA could potentially disrupt¹⁵. Unfortunately, despite being many candidate proteins on sperm, the exact nature of the sperm ligands for the zona pellucida and oolemma remains unknown. There is, however,

scientific evidence of interference of ASA with recognition of sperm binding sites on the zona pellucida.

Bronson et al. reported binding of circulating head-directed IgG or IgA ASA to sperm reduced sperm binding to human zona pellucidae¹²⁶. Another study showed that donor sperm when incubated with ASA containing serum was unable to fertilize human oocytes or bind to the human zona pellucida. When the serum ASA were pre-absorbed by normal sperm, the serum no longer showed this negative effect on sperm–zona binding¹²⁷. Additional evidence that ASA can affect sperm binding to zona pellucida was provided by Mahony et al.¹²⁸ who, using the hemizona assay, demonstrated that seven ASA-containing sera tested reduced zona binding. Other authors reported that directly bound ASA could affect in vitro fertilization but not clinical pregnancy rates once fertilization had occurred, indicating that anti-sperm antibodies in the male interfere with sperm-egg fusion and subsequent fertilization but once fertilization has occurred, the pregnancy rate remains the same^{129, 130}. In another study, Clarke et al. showed that under controlled experimental conditions, sperm antibodies from female sera significantly inhibited in vitro fertilization of fresh human oocytes¹³¹. The fertilization rate was related to the IgG class sperm antibody titer. The inhibitory activity was removed by absorption of the highest titer serum with protein A sepharose to remove most of the IgG class sperm antibodies. The antibodies subsequently eluted from the protein A sepharose retained their ability to inhibit fertilization^{3, 129}.

Due to the scarce availability of human oocytes, many experiments have been undertaken using hamster oocytes. Numerous investigators have shown that circulating human ASA can inhibit the penetration of hamster oocytes by human sperm^{113, 132-135}. In addition, antibodies experimentally raised against specific sperm proteins are also capable of inhibiting sperm penetration of hamster oocytes¹³⁵⁻¹³⁷.

In contrast, some serum ASA have been found to promote the penetration and adhesion of human sperm to hamster oocytes^{138, 139}. Mixed results were also reported by Aitken et al.¹⁴⁰ who found that ASA could promote, inhibit or be neutral in their influence on sperm penetration of oocytes. Liu et al. observed in a preliminary study with a low number of patients that antibody-coated sperm from men with sperm autoimmunity had an impaired ability to bind to the human zona pellucida, but that oolemma binding was unaffected¹⁴¹.

Several proteins that are present in the sperm cell and are key in fertilization, such as GP20, SP-10 and a protein that binds D-mannose coupled to albumin, have been proposed as potential targets for ASA¹⁴². Several sperm proteins (or their isoforms) have been postulated to have more than one function in sperm–oocyte interaction^{124, 143, 144}. There is evidence that antibodies reactive with triosephosphate isomerase can inhibit both the secondary binding of sperm to the zona pellucida and also binding of sperm to the oolemmal membrane^{136, 143}. It is possible that not all ASA affect sperm–oocyte binding/fusion and it is likely that the antigenic specificity of ASA is important in their effects on fertilization.

Thus, the confusion regarding the functionality of ASA is exacerbated by the potential for an antibody reacting with a single protein to disrupt more molecular mechanisms involved in the sperm–oocyte interactions that are required for fertilization. It might also be possible that an ASA could react with a functional domain within a protein, which inhibits one function of that protein but not another function of the protein¹⁵. It is also important to note that most of the experiments have been performed by incubating semen with ASA positive serum, and the real presence of these antibodies at the time of in vivo fertilization is unknown.

Post fertilization effects of ASA on fertility

Definitive studies in various animal models have shown an association between circulating sperm antibodies and pre- or post-implantation embryonic degeneration¹⁴⁵. In one study on rabbits, reproductive tract secretions containing ASA were found to cross-react with rabbit morulae and blastocysts, resulting in embryotoxic effects during in vitro culture¹⁴⁶. The same authors demonstrated that only secretory IgA (sIgA) from the uterine fluid of semen-immunized does was embryotoxic during in vitro culture. In contrast, blood sera with high titers of anti-sperm antibodies were not embryotoxic nor were IgG fractions isolated from the immune uterine fluid (IUF)¹⁴⁶. Adsorption of IUF with either sperm or anti-sIgA removed the embryotoxicity, thereby providing evidence of specificity. Other experiments indicated that the sperm antigen stimulating the sIgA embryotoxic antibody in IUF was distinct from the antigen stimulating IgG and IgA class anti-sperm antibodies with the ability to inhibit fertilization. In unpublished observations, adsorption of the IUF with paternal lymphocytes did not abrogate the

embryotoxicity, therefore implying that transplantation antigens were unlikely to be involved. Additional investigations suggested that the antigen responsible for the sIgA-associated embryotoxicity was a subsurface component. Thus, immunization of does with isolated sperm membrane fractions resulted in reduced fertilization, whereas immunization with intracellular fractions caused only the post fertilization effects on embryos¹⁴⁶.

The sperm membrane is integrated as a mosaic into the zygote membrane during the process of fertilization so that sperm antigens are incorporated, although at relatively low densities, into the developing embryo¹⁴⁷. On the other hand, embryonic gene expression commencing from the four to eight cell stage results in the synthesis of various developmental antigens, which can cross-react with anti-sperm antibodies¹⁴⁸. Consequently, during embryo development and perhaps particularly around the time of blastocyst hatching, there is an opportunity for the ASA to bind to cross-reacting embryonic antigens and potentially cause embryo degeneration or possibly block implantation. With respect to deleterious effects, Jones first reported that around 50% of pregnancies conceived in women with ASA subsequently ended in first trimester spontaneous miscarriages¹⁴⁹.

In a study by Witkin and David, it was found that 7 of 16 (44%) women who miscarried had anti-sperm antibodies in their sera compared with only 2 of 17 (12%) women who had successful ongoing pregnancies¹⁵⁰. Examination of the immunoglobulin classes of the antibodies revealed that IgA was significantly more common in women who miscarried. It was suggested then that the IgA class antibodies in serum may be indicative of local secretory IgA in the female reproductive tract. However, despite the strong evidence in rabbits, it is not known whether sIgA class anti-sperm antibodies in humans are embryotoxic¹⁵¹. In a clinical study, it was found that of 173 women referred for a history of three or more consecutive spontaneous miscarriages, there was a significantly higher incidence of circulating sperm immobilizing antibodies when compared with the fertile group¹⁵¹. It is interesting to note that they also observed a higher incidence of serum anti-sperm antibodies in the group of women identified as having an immunological basis for their recurrent miscarriages (for example, couples sharing at least three human leukocyte antigen (HLA) determinants or couples with the female showing a relatively low response to her partner's lymphocytes in mixed lymphocyte culture). Other groups have reported a significant association between ASA and some autoantibodies such as antiphospholipid antibodies and it may be that it is these coincidental autoantibodies, which have

deleterious effects on the fetus rather than the ASA themselves^{152, 153}. In contrast to the studies cited above, other authors have not found a statistically significant association between ASA and miscarriage^{154, 155}. Further investigations in this area are warranted, particularly focusing on the possible involvement of intracellular sperm antigens, which react with IgA class anti-sperm antibodies. It is worth noticing that most of the research performed to date involved ASA present in serum samples, which may not affect in utero embryogenesis directly.

With respect to the positive effects of sperm immunity, there is evidence suggesting that directly bound and circulating anti-sperm antibodies are associated with increased implantation rates¹⁵⁶. The various isotypes of immunoglobulins (IgA, IgG, IgM) might have different effects on immunological infertility.

Detection of anti-sperm antibodies

Numerous tests have been developed for the detection of ASA in several species, being the majority related to human medicine because of the impact that ASA have on human fertility. An ideal assay would be an objective assay that detects the presence of ASA bound to sperm, their location, antibody load and isotype, with high sensitivity and specificity and that could be performed on live cells²⁵. Currently there is not a standardized and universally accepted assay for the detection of ASA, and this may be due to the lack of consensus on clinical consequences in human medicine¹⁵⁷.

Anti-sperm antibody detection methods are based on the identification of antibodies present in serum, seminal plasma, vaginal or cervical secretions, or follicular fluid, known as indirect methods, or the identification of antibodies bound to the sperm cell. One of the main problems with the indirect methods used to detect ASA in humans is the antigen (Ag) employed in the tests. In fact, because a purified molecular Ag does not exist, it is necessary to work with the whole cell. Moreover, in these tests, there is a risk of great inter-assay variability in the Ag used due to the fact that the tests utilize a cellular pool of spermatozoa from donors¹⁵⁸.

Some methods require previous fixation, and fixation effects on external antigens is controversial at this point. Fixation of spermatozoa may lead to nonspecific binding of IgG, detection of intracellular antigens, denaturation of sperm antigens or membrane damage resulting in false-positive or false-negative results¹⁵⁹. Use of live cells for ASA detection can also

compromise the interpretation of results obtained. It is known that cross-linking of surface antigens by multivalent antibodies or antigen-antibody complexes by second antibodies can cause aggregation of antigen-antibody complexes into patches and caps¹⁵⁹. The processes of capping that occur during interaction between ASA bound to sperm and secondary antibodies has been described by several authors¹⁶⁰⁻¹⁶². Capping may be accompanied by shedding of antigen-antibody complexes from the sperm surface and that may give a misleading picture of regions to which antibodies were directed or false negative results^{161, 163, 164}. The ability of the test to detect the specific region to which the antibody is bound is also important, since studies have suggested that head-bound ASA have greater impact on fertility¹⁶⁵.

The detection of the number of molecules of ASA per sperm may be important. Check in 2010,¹⁶⁶ stated that if ASA are directed to one specific antigen needed to accomplish fertilization, the antibody load may not be relevant, as the fact that it is affecting a key protein on the surface of the sperm cell is enough to cause infertility¹⁶⁶. On the other hand, there is a correlation in that the higher the percentage of sperm coated with ASA, the higher the concentration of ASA per sperm¹⁶⁶.

Some of the variability in conclusions as to the significance of ASA as a cause of infertility may be related to what percentage of sperm coated with ASA is considered a positive test. Some studies have considered a positive IBT test as > 20% antibody bound sperm, some > 50% since some normal fertile sperm donors have $\leq 50\%$ ASA bound sperm, and some consider $\geq 80\%$. Some studies have evaluated 100% of sperm showing ASA¹⁶⁶. Tests for ASA detection are classified as qualitative or quantitative, direct or indirect and some are limited due to the need for subjective interpretation.

Indirect methods

Agglutination techniques

In vitro tests, which have been developed to detect antibodies to human spermatozoa, have been frequently based on agglutination reactions, since agglutination is a traditional and sensitive demonstration of interaction between antibody and particulate antigen. However, there

are reports that spermatozoa can also be agglutinated by some mycoplasmas, viruses and non-antibody serum components. This means that control serum samples must always be included in each test series¹⁶⁷.

The agglutination test (gelatin agglutination test) is a quantitative method developed by Kibrick in 1952, and is an indirect test used in serum and other bodily fluids^{168, 169}. Agglutinates of spermatozoa are observed after patient's serum (and dilutions thereof) and donor spermatozoa are brought together in gelatin medium or on microslides. Disadvantages of this test include the possibility of agglutination caused by bacteria and amorphous material within semen, as well as non-immunoglobulin proteins in serum, leading to false positive reactions¹⁷⁰.

A gelatin agglutination test (Kibrick or K-B-M test) has been developed, originally using heterologous antisera to human spermatozoa. Experience with this technique in a number of laboratories has shown it to be useful with human iso and auto-antisera for macroscopic assessment of sperm agglutination. Results are reproducible within individual laboratories, and often in different laboratories. Fractionation of iso- and auto- antisera active in this test has shown the activity to be due to antibodies of either IgG or IgM classes¹⁶⁷. Semen specimens for use in this technique should be fresh and have actively motile sperm cells.

The gelatin agglutination test shows only a one or two tube difference where performed using semen from different, selected donors. Tests for sperm agglutinating antibodies have been performed satisfactorily using serum, seminal plasma, and cervical mucus extracts. The gelatin agglutination test has two disadvantages. Firstly, it uses moderately large volumes of reagents, of which the semen is the most important because of the difficulty of obtaining suitable specimens, and, secondly, the part of the spermatozoa involved in the agglutination (and, hence, the location of the relevant antigen) cannot be observed.

Capillary tube agglutination test¹⁷¹ is a procedure that utilizes unwashed spermatozoa, and, since these do not need to be motile, this is an advantage over the gelatin agglutination test. Gelatin can be omitted from the capillary tube test. In comparative studies, only some of the sera which were reactive in the gelatin agglutination test were reactive in the capillary tube test¹⁶⁷. Because of its lower sensitivity, the capillary tube agglutination test has not proved to be as useful as the gelatin agglutination test.

The tray agglutination test¹⁶⁹ has the advantages that small volumes of reagents are used, enabling many sera to be tested with one semen specimen. It is rapid to perform, so that there is

time to examine many serum specimens simultaneously, and the parts of the spermatozoa involved in agglutination can be observed. Consequently, the tray agglutination test appears to avoid the disadvantages of the gelatin agglutination test. In studies where the same sera were examined by both the tray and the gelatin agglutination tests, commonly the titers did not differ by more than one or two dilution steps¹⁶⁷. Further, many head-to-head agglutinins, detectable in the tray test, with titers of up to 128, were not detectable in the gelatin test. Thus, the tray test appeared to be more sensitive than the gelatin agglutination test¹⁶⁷.

Fractionation of a number of sera active in the tray test has shown that the active agents are antibodies, either IgG or IgM. However, a few sera containing non-immunoglobulin spermagglutinins showed activity when tested in the tray test; suggesting that this technique does not detect only anti-spermatozoal antibodies. All sera with a titer of 1: 32 or greater of sperm-agglutinating activity, were found to contain true antibodies to spermatozoa. In seminal plasma, IgA was commonly the agglutinin, but some activity was also found to be due to IgG. This test is limited because the need for skilled assistance²⁵.

A tube-slide agglutination test¹⁶⁸ has been widely used in a number of modified forms. It seems that, to obtain the best results with the technique, selection of semen specimens with the same characteristics as those required in the gelatin agglutination test is important. There is a one-way correlation between activity of sera in the tube-slide test and in the gelatin agglutination test or in the slide agglutination test. Sera positive in the gelatin agglutination test are also generally positive in the tube-slide test, whereas sera may be positive in the tube-slide test and yet negative in the gelatin agglutination test. One feature of this test is that incubation is performed in test tubes, and samples are transferred to microscope slides for immediate examination under the microscope. It seems that with many sera containing antibodies the agglutinates which are detectable in either the gelatin or the slide tests, are readily disrupted by the pipetting onto the microscope slide. Experience indicates that head-to-head agglutination is the form most readily detectable with this procedure¹⁶⁷.

Sperm immobilization tests

Complement-mediated sperm immobilization or cytotoxicity assays^{172, 173} count immobilized spermatozoa, when a complex of antibodies are linked with antigens on

spermatozoa in the presence of complement. These type of assays are limited in their usefulness for the detection of Ig isotype IgA containing ASA, since IgA does not fix complement. Moreover, many head-directed antibodies do not lead to a loss of motility, which might give false-negative results^{126, 174}. The early investigators of sperm immunogenicity utilized immobilization of spermatozoa as a measure of sperm antibodies. The reaction was found to be dependent upon complement for its completion. The interaction of antibody molecules with sperm antigens, presumably surface ones, activates the complement system resulting in a detrimental effect on the permeability and integrity of the cell membrane. The ultimate effect on the spermatozoa can be visualized by light microscopy as a loss of motility referred to as immobilization, and by staining with certain dyes referred to as cytotoxicity¹⁶⁷.

These characteristics have been utilized as clinical tests to study the occurrence of sperm antibodies in sera and seminal plasma of infertile men and sera and cervico-vaginal secretion of infertile women. The presence and titer of immobilizing and cytotoxic antibodies in serum appeared to have a definite relationship to infertility in men and women. Tests on sera of fertile men, and of pregnant women and women never exposed to seminal antigens, have given negative results. Sera with sperm immobilizing and cytotoxic activity usually also show agglutination, especially of the tail-to-tail and mixed agglutination types. However, some exceptions have been encountered. In general, complement-fixing antibodies belong to the IgM or most IgG classes of immunoglobulins, but not to the IgA class nor IgG₄ subclass¹⁶⁷.

There are two main types of immobilization tests that have been widely used. The first determines the time required for approximately 90% of the spermatozoa to become immotile in test sera and compares it with that for normal sera¹⁷⁵ and the method adapted from Isojima et al. (1968)¹⁷⁶.

The test sera should be titrated to gain quantitation of immobilizing antibody. This test has also been modified to enable standardized quantitative comparisons of different sera to be made. The procedure studies the relationship between dose of antiserum and response, and enables the calculation of 5000 sperm immobilizing units for different sera, which can then be compared. For the quantitative determination of sperm immobilizing antibody in women 50% sperm immobilization units (SI50) are used. This test was designed to detect quantitatively the complement-dependent sperm immobilizing antibody in women, using 50 per cent sperm immobilization¹⁷⁶.

In an attempt to achieve in vitro correlate of the post-coital test, a method was devised that measures the degree of sperm penetration into cervical mucus drawn into capillary tubes¹⁷⁷. It has been observed that spermatozoa from males who have serum anti-spermatozoal antibodies have reduced ability to penetrate cervical mucus in in vitro tests. The control cervical mucus is obtained at midcycle from healthy and fertile women and must be free from contamination with blood cells, bacteria, and other cellular debris. In tests with cervical mucus from infertile women with control sperm, the degree of sperm penetration has not been highly associated with the presence of circulating sperm antibodies in women. In vitro treatment of sperm with sera containing agglutinating and immobilizing activities inhibits the sperm penetration in the capillary tube method.

Techniques using labelled antibodies

The sensitive indirect immunofluorescent antibody technique has been applied for the study of sperm iso- and auto-immunization in several species. The source of antigen may be testicular tissue, or spermatozoa obtained from a spermatocele, from the vas deferens at vasectomy or from the fresh ejaculate. Despite extensive washing, these cells may still exhibit antigenicity which can be due to intrinsic spermatozoal components and possibly also to absorbed components from seminal plasma. The use of unfixed spermatozoa allows demonstration of staining of the midpiece, but is otherwise a less satisfactory substrate for immunofluorescent studies. The use of methanol-fixed spermatozoa, on the other hand, makes it possible to define fluorescent staining patterns involving anterior acrosome, equatorial segment, postacrosomal area (post-nuclear cap), tail principal piece and tail end piece¹⁶⁷.

Indirect immunofluorescent antibody technique for spermatozoa¹⁶⁰ require a good fluorescent microscope, preferably equipped with interference filters, adjusted for the fluorocromes used¹⁶⁷. Fixation with ethanol or acetone is unsatisfactory, since it destroys the antigen, but the absence of midpiece staining after methanol-fixation indicates that this treatment also leads to destruction of some antigens. Non-specific staining is common and dictates the careful use of control slides. Weak staining reactions of unknown character are often seen with undiluted sera. The use of monospecific fluorescent antisera demonstrates that the circulating anti-sperm antibodies detected in the indirect technique are most commonly in the IgG and IgM

classes, but IgA antibodies may also occur, particularly among antibodies against the equatorial segment. Acrosome staining occurs with both IgM and IgG, and there is evidence that separate antigens exist on the anterior acrosome and on the equatorial segment. Staining of the post-acrosomal area occurs with both IgG and IgM antibodies, which cross-react with bull spermatozoa. Specific staining of the principal piece stops short of the mid-piece. It is associated mainly with an IgG antibody, and the antigen involved is intrinsic to the spermatozoa. Staining of the tail end-piece may be observed occasionally and is due primarily to IgM antibodies¹⁶⁷.

The immunoperoxidase technique uses specific antibodies to localize antigens¹⁷⁸. Usually the IgG fraction of the immune serum contains most of the active antibody and the use of purified Fab fragments of IgG helps to ensure that antigen is localized specifically, rather than non-specific binding of the Fc piece or other serum proteins occurring. A second antiserum against the species of Fab used in the first step has to be raised, the Fab fragments are prepared, and these are covalently labeled with horseradish peroxidase¹⁶⁷.

The use of peroxidase as the label has advantages over fluorescent markers, notably: permanence of preparations, a fluorescence microscope is not required, and it can be taken to the Electron Microscopy level. In addition, there are no quenching or autofluorescence problems, although in some systems endogenous peroxidase activity can be troublesome. The method offers a marginal improvement in sensitivity compared with indirect immunofluorescence, and it may also be quantitated with moderate precision. It is laborious, however, and special care must be taken in the preparation and purification of reagents¹⁶⁷.

Immunoelectron microscopy provides a means of ultrastructural localization of antigen-antibody reactions involving spermatozoa. Sensitized erythrocytes can be used for scanning electron microscopy, and viruses, ferritin and peroxidase are useful labels for both transmission and scanning electron microscopy. In the study of antibodies against all surface antigens the reaction will take place on either live or fixed spermatozoa. If cytoplasmic antigens are involved the spermatozoa need to be fixed, and antibody labelling must be with peroxidase or other enzymes¹⁶⁷.

In flow cytometry (FCM)¹⁷⁹ fluorescein-tagged anti-human antibodies are used to detect serum antibodies that recognized antigens on the sperm surface. Living spermatozoa can be used for analysis. After incubation with the serum and the fluorescence labeled antibodies, samples are introduced into a cell sorter. FCM is an objective method to determine the proportion of

antibody-positive spermatozoa or the quantity of antibodies bound to the sperm surface. Dead cells can be easily excluded from the assay by means of staining and a greater number of cells can be objectively analyzed by the flow cytometer²⁵. Advantages and disadvantages of this technique are discussed later on this chapter.

Indirect immunobead test

The immunobead test is performed with spermatozoa washed three times by centrifugation and resuspension to remove the free immunoglobulins in the seminal plasma. These washed spermatozoa are mixed on a microscope slide with serum and latex beads coated with anti-immunoglobulin antibody molecules¹⁸⁰. The immunobead test is another technique currently used in the investigation of human infertility. Advantages include the ability to identify class- and region- specific sperm associated antibodies and to assess the proportion of motile sperm that are coated with antibody. Its disadvantages include the requirement for semen from a negative control donor tested to be negative for ASA, the subjective nature of the interpretation of results, and the technical differences in the protocols used by various authors¹⁸¹.

Enzyme-linked immunosorbent assays (ELISA)

Enzyme-linked immunosorbent assays (ELISA)¹⁸²⁻¹⁸⁵ use enzyme-linked anti-human antibodies, which bind to the antibodies on the sperm surface. A substrate for the enzyme is then added, and its product is measured colorimetrically. ELISA requires fixation of whole spermatozoa or use of membrane extracts. Fixation of spermatozoa may lead to denaturation of sperm antigens or membrane damage, resulting in both false-negative and false positive results; membrane extracts may not contain the relevant antigens associated with the process of fertilization⁶⁹. This test is also limited by the time involved, its cost, poor sensitivity, and inability to determine ASA location and isotype²⁵.

Direct methods for ASA detection

The mixed agglutination reaction (MAR)⁹⁶, is a direct test in which a washed suspension of Rhesus (Rh)-positive human red blood cells (RBCs) coated with Rh-directed human IgG or IgA are mixed with drops of semen. IgG- or IgA- bound sperm are then bridged with the IgG- or IgA coated RBCs after the addition of anti-human IgG or IgA antibody. When spermatozoa in the ejaculate are antibody-bound, they may form mixed agglutinates with the RBCs. Disadvantages of this test include the limitation on the ability of the test to determine the proportion of sperm bound or the regional specificity of antibody binding due to the relatively large size of agglutinates. Also in this test, non-specific immunoglobulins adhering to the sperm surface may be detected¹⁷⁰.

Other ways of detecting antibodies bound to spermatozoa is with antihuman antibody coated immunobeads. This test is called the immunobead test and is by forming agglutinates that it identifies the presence of ASA¹²⁶. The region of the sperm surface to which ASA are bound (by using antibodies specific for head, tail, and tail tip), the proportion of spermatozoa in the ejaculate that is antibody-bound and the isotypes of these antibodies can be determined (IgG, IgA, or IgM)¹⁷⁰. This test does not detect the concentration of ASA per sperm and is labor intensive, time consuming, and costly. A good correlation between the direct immunobead test and other direct and indirect tests was found by Dondero et al.¹⁸⁶. In order to compare these tests, the Gelatin Agglutination Test and Tray Agglutination Test were used to detect antibodies in blood serum and seminal plasma ("indirect methods"). A good concordance between the methods, measured by phi and K tests, was found and satisfactory mathematical models were established by regression analyses¹⁸⁶.

Immunofluorescence assays (IFA)^{160, 187} use fluorescein-tagged anti-human antibodies which bind to antibodies on the sperm surface (direct test). Internal sperm antigens exposed after plasma membrane damage or the obligatory use of methanol fixation may lead to false-positive results. Fixation is used to provide covalent binding of the proteins. Naturally occurring antibodies to these internal antigens occur commonly in men and women and probably play no role in subfertility¹⁷⁰. The determination of the regional specificity of sperm-directed antibodies is limited by the low resolution of this method¹⁸⁸.

The immunogold assay uses gold instead of fluorescein-tagged antibodies, and avoids the multiple washing/centrifugation/resuspensions (as with IFA) than can cause plasma membrane damage or loss¹⁸⁸.

Radiolabelled antiglobulin assays¹⁸⁹ differ from ELISA in that the antibody-linked enzyme has been replaced by a radioisotope. In contrast to ELISA, living spermatozoa can be used for analysis and fixation is not necessary. Radioisotopes and enzymes are quantitative probes; however, they provide no information about the proportion of antibody-bound spermatozoa or the regional specificity of ASA. Care must also be taken to ensure that a positive assay is secondary to sperm-associated antibodies and not antibodies on seminal leukocytes¹⁹⁰. This method is limited by an inability to determine specific ASA location, expense, and reliance on skilled labor²⁵. There is a close correlation between this assay and immunobead assays¹⁹¹.

Flow cytometry (FCM)¹⁷⁹, as previously described, uses fluorescein-tagged anti-human antibodies. This method can be used to evaluate ASA directly bound to sperm²⁵. The advantages, and disadvantages of using flow cytometry are further discussed in the next section.

Detection of anti-sperm antibodies by flow cytometry

Since the beginning of reproductive biology studies during the end of the 17th century, light microscopy has played a pivotal role in elucidating the complex mechanisms in male and female reproduction. In recent years, flow cytometry (FCM) has been applied to investigation of several aspects of reproductive physiology, pathology and toxicology¹⁹².

Several assays have been developed to identify, quantitate, and monitor immunoglobulins associated in vivo with the surface of sperm. With these methods, sperm are incubated with a labeled antiserum against human immunoglobulins. If human immunoglobulins are associated with the sperm's surface, the antiserum will attach to the immunoglobulin, and quantization of the label implies the amount of anti-sperm antibody that is present. Labels that have been utilized include red blood cells^{96, 193-195}, polyacrylamide beads¹⁹⁶, enzymes¹⁹⁷, and radioisotopes¹⁹⁸. Red blood cells and latex beads in many of these methods are identified using light microscopy; as a result, the site of antibody attachment can be determined, and a visual distinction can be made between labeled antiserum associated with sperm and labeled antiserum

associated with other seminal cells. When enzymes or radioisotopes are used as labels, the test result is objectively interpreted. However, it is not possible with these techniques to differentiate attachment of the labeled antiserum to sperm-associated immunoglobulins from attachment to immunoglobulins associated with other seminal cells unless samples of pure sperm are used as the antigen source. This can be a problem, because the abnormal semen parameters of men undergoing sperm antibody testing may make the isolation of a pure motile sperm population difficult, if not impossible.

The commonly used diagnostic tests for anti-sperm antibodies pose several diagnostic and technical problems. The sperm-immobilization test is specific but relatively insensitive and unable to detect anti-sperm antibodies of all immunoglobulin classes¹⁶⁷. The sperm agglutination test is non-specific, resulting in a high percentage of false positive results^{167, 199}. Mixed antiglobulin reaction and immunobead tests can only be performed on highly motile spermatozoa^{96, 200, 201}. All mentioned methods need microscopic estimation and do not provide objective measurements³². Anti-sperm antibodies detected by ELISA within seminal fluid may not be representative of the immunoglobulins detected on the surface of sperm from the same ejaculate^{202, 203}.

Flow cytometry, a method permitting quantitative estimation of ASA on the surface of live spermatozoa^{14, 32, 74, 179, 204-208}. The possibility of detecting ASA on the surface of living cells is considered a significant advantage of the MAR and FCM tests and both avoid the disadvantages of methods where fixed spermatozoa are used, e.g. ELISA^{182, 209}, immune fluorescence^{160, 187}, and radiolabelled antiglobulin assay¹⁸⁹. Fixation of spermatozoa may lead to nonspecific binding of IgG, detection of intracellular antigens, denaturation of sperm antigens or membrane damage resulting in false positive or false negative results¹⁵⁹. Flow cytometry allows for objective analysis of large numbers of cells with better quantization of antibody-positive sperm cells, especially when small percentages of cells are fluorescent^{182, 204, 209}. It is also a specific test because it is based on a highly specific interaction of monoclonal antibodies with different classes of immunoglobulins on the cell surface³². A unique feature of FCM is the possibility to detect cell heterogeneity³².

One of the essential factors for infertility seems to be the presence of the IgA class of antibody²¹⁰⁻²¹². Thus, information concerning the proportion of antibody-positive sperm in an ejaculate, the antibody class and its isotype, the sperm antibody load (i.e., the number of

antibody molecules per spermatozoa), as well as the ability to monitor reliably the sperm antibody load during and after therapy, should not only allow better planning of treatment but also objective comparisons between various treatment schedules¹⁴.

Use of live cells for ASA detection for indirect tests can also compromise the interpretation of results obtained. It is known that cross-linking of surface antigens by multivalent antibodies or Ag-Ab complexes by second antibodies can cause aggregation of Ag-Ab complexes into patches and caps; these phenomena have been observed in many cells types^{213, 214}. The processes of capping that occur during interaction between ASA bound to human spermatozoa and secondary antibodies has been described in several reports¹⁶⁰⁻¹⁶². It was shown that capping may be accompanied by shedding of Ag-Ab complexes from the sperm surface and that it gives a misleading picture of regions to which antibodies were directed^{161, 163, 164}.

Direct flow cytometry assays

Haas and Cunningham (1984) developed a direct sperm-associated and an indirect assays for measurement of plasma immunoglobulin G (IgG) utilizing fluorescein-labeled antihuman IgG and a flow cytometer¹⁷⁹. Good correlation was found between the direct sperm-associated flow cytometry assay and the direct radiolabeled antiglobulin assay in human semen for IgG, with good ability of the FCM test to discriminate between sperm cells and inflammatory or other cells. The technique was found to also be advantageous in quantifying presence of seminal granulocytes in individuals with chronic genital tract infections¹⁷⁹.

Detection of sperm-bound antibodies by direct FCM and assessment of antibody load (antibody molecules/spermatozoa) for IgG and IgA was performed by Rasansen et al. (1992). They excluded dead cells from the analysis because they can bind antibody nonspecifically, as shown by the difference in the percentages of antibody positive cells when these cells were considered (ranged from 10 to 58% increase in the number of positive cells when dead cells were included). Assessment of intra and inter assay variability by FCM method was less than 10% for the direct FCM readings, being this an advantage and these authors proposed the technique for use as an objective monitoring method for treatment of ASA with steroid therapy¹⁴. The use of F(ab)₂ fragments instead of complete immunoglobulin molecules was supported by the fact that

it avoided non-specific binding related to the presence of the Fc receptors on sperm. Use of polyclonal antibodies was also elected, since these would be expected to reach with all isotypes of antibodies, whereas monoclonal antibodies, by virtue of their specificity, react only with a particular epitope of a restricted isotype. If the epitope is masked due to the presence of seminal proteins, monoclonal antibodies may give negative results¹⁴.

Direct FCM has been shown to correlate well with the direct Mixed Antiglobulin reaction test (MAR) on fresh semen samples of infertile human patients⁷⁴. Percentages of positive sperm (Ab bound sperm) were lower in the FCM assay (86% MAR vs 55% FCM) and both tests had similar variability (stdev 25%). It was proposed that FCM could also be used on semen samples with poor motility⁷⁴. These authors also evaluated and compared direct FCM with indirect FCM, indirect Immunobead test, and indirect Mixed Antiglobulin Reaction test. There was no correlation between the direct FCM and the other methods. This finding was in agreement with that reported by Menkveld et al. (1991)²¹⁵, and could be explained by the different densities of antigens in the head vs the tail of the sperm cells and the different abilities of the methods to detect these. Also, the probable reason for the presence of unbound antibodies in seminal plasma traditionally is believed to be due to the excess of antibodies⁷³ relative to the patient's sperm specific antigens. The presence of unbound antibodies due to a reactivity different that found on the patient's spermatozoa cannot be excluded automatically when using indirect tests⁷⁴. This study concluded that the indirect measurement of sperm antibodies might be grossly unreliable as compared with the results obtained by direct FCM⁷⁴.

Nikolaeva et al. (1993) assessed the use of FCM analysis of living sperm and serum subjected to direct and indirect immunofluorescence, respectively, with monoclonal antibodies (IgG, IgA₂, and IgM). They also compared the correlation for IgG with direct and indirect IgG mixed antiglobulin reaction test. They found excellent agreement between the results of MAR and FCM when 10% of motile spermatozoa reacting with particles in the MAR test and 10% of antibody bound sperm were used as criterion levels of positivity. Using the cited criteria the FCM test showed high specificity (100%) and sensitivity (92% for semen, 100% for serum) with MAR positive serum and semen. The FCM test requires washing of the sperm cells and so it could reveal negative results in cases of insufficiently strong binding of antibody with the surface of the spermatozoa. This feature of the test could be important in decision making for therapy selection, since immovable antibodies may need to be approached differently. The authors

suggested that finding 25% or more living spermatozoa bound to IgG with FCM to be considered as evidence of a significant anti-sperm activity of semen and serum. When MAR results were around 100%, direct FCM percentages of positive cells varied from 40 to 93 %. The lower percentages of ASA positive cells found with the FCM test when compared with the MAR test, was attributed to the partial overlapping of green fluorescence of spermatozoa, the use of monoclonal antibodies, and unspecific binding by the negative controls³².

Rasansen et al. (1994)²⁰⁵ evaluated the direct FCM measurement of anti-sperm antibodies and compared it with the mixed antiglobulin reaction (MAR) and the serum tray agglutination test (TAT). The degree of MAR positivity but not that of the TAT correlated with the sperm antibody load. Removal of loosely bound antibodies to the sperm surface was also found to occur after the first wash, without further effect of washing on the % of ASA bound sperm and without affecting the % of dead sperm. This would explain why washing could improve the fertilization rates^{216, 217}, but is also in contradiction with Haas et al. (1988), that showed that the sperm bound immunoglobulins were not removed by several (3 to 18 times) washings²¹⁸. The fact that Haas et al. (1988) did 3 washings as a minimum may explain these results. Twenty six percent of the samples became negative (loss of antibodies attached to the sperm surface) after being washed. Good correlation was found between FCM measurements of paired semen samples obtained at an interval of 2 to 4 weeks, especially after washing of the native sperm sample. The authors evaluated the simultaneous presence of IgG and IgA on the surface of anti-sperm antibodies. 41% of the washed samples presented both immunoglobulins while 9% and 18% presented either IgG or IgA alone, respectively. This was in accordance with previous studies, in which a high level of IgG detected by MAR was also followed by a high IgA MAR test result¹³. The weakest correlation between MAR and FCM was obtained when native sperm were used, in agreement with the authors's previous experience¹⁴, where the high levels of free immunoglobulins in the ejaculate can saturate the added anti-IgG and there is very little to react with the sperm bound IgG. Direct FCM was useful to identify patients with a low sperm antibody load, and could help guide treatment, since other authors have found low levels of sperm antibodies to be clinically insignificant for fertility^{64, 219}.

During spermatogenesis and passage through the epididymal environment, the surface of the maturing sperm undergoes dramatic changes, resulting in antigenically distinct subpopulations of sperm in an ejaculate. It has been proposed that different Ig isotypes may have

affinity for different sperm subpopulations. Whether or not different Ig isotypes were directed to the same or different antigens on the sperm surface or ASA bound sperm was considered relevant, and removal of sperm associated with a particular isotype before insemination was proposed as a potential treatment for ASA²²⁰. Haas et al. (1990) used flow cytometry to determine if sperm bound IgG and sperm bound IgA occurred on the same sperm population in the ejaculates of infertile men with sperm-associated antibodies and found that both isotypes coincided on the surface of the same cells. This eliminated the feasibility of sperm separation procedures according to the sperm associated Ig isotype as a treatment modality for immune mediated infertility^{191, 220}.

To our knowledge, there have been no studies to date performed evaluating the effect of the use of a fixative solution on the percentages of ASA directly bound to sperm.

Indirect flow cytometry assays

Advantages of the use of indirect FCM would include the elimination of the need for same day flow cytometric analysis. It also would allow the seminal plasma samples to be collected and mailed from smaller hospitals to a specialized laboratory, where they could be frozen and objectively analyzed when convenient.

The technique developed by Haas and Cunningham (1984) for the measurement of IgG levels on serum from infertile patients showed a good correlation with the indirect radiolabeled antiglobulin assay. The indirect flow cytometric assay allowed for detection of ASA in serum with the use of low numbers of normal sperm, which is an advantage when compared with techniques that imply the use of light microscopy¹⁷⁹.

Comparison of indirect MAR test and the indirect FCM test showed a higher sensitivity of MAR test and a higher specificity of FCM test⁷⁴. Thus, Räsänen et al. (1996) obtained positive results with indirect MAR test in 9/11 (82%) IBT-positive semen samples with a high proportion of IgG positive spermatozoa (71%), whereas the FCM test result was positive only in five (45%) samples with a very low proportion of IgG positive spermatozoa (13%). A 95% false positive rate of ASA detection in serum of fertile donors by MAR test compared with FCM test has been demonstrated²²¹.

The study by Nikolaeva et al. in 2000 showed that false negative results of indirect FCM test might be due to disappearance of ASA from the sperm surface after shedding of serum ASA bound to sperm antigens¹⁵⁹. It is known that interaction of ASA with secondary antibodies can cause aggregation of cell surface molecules into patches and caps¹⁶⁰⁻¹⁶². The disappearance of the cap may be due to endocytosis and subsequent degradation and/or shedding of Ag-Ab complexes, as reported for many types of cells^{222, 223}. Endocytosis in mature spermatozoa is unlikely but there are data on shedding of Ag-Ab complexes from the sperm surface in other species^{163, 164, 224}. This study revealed the typical changes of fluorescence pattern from homogeneous distribution to the cap formation through patches followed by decrease of sperm antibody load, supporting the theory of Ag-Ab complex shedding. Small fluorescent particles, probably shed complexes in some samples, were detected both by fluorescent microscopy and FCM. These authors concluded that shedding was the most probable mechanisms that lead to removal (partial or complete) of Ag-Ab complex from the cell surface and to false negative results of FCM test¹⁵⁹.

The cross-linking of cells surface antigen (Ag) with bivalent antibodies and /or antigen-antibody (Ag-Ab) complexes with secondary antibodies may induce the reorganization of surface components (patching and capping) and result in their shedding from the sperm surface. Nikolaeva et al. (2000) used FCM and fluorescence microscopy to analyze swim-up spermatozoa of normozoospermic men incubated with ASA-positive sera from infertile patients and with secondary antibodies fluorescein isothiocyanate (FITC)-labelled goat anti human IgG polyclonal antiserum under different conditions. The objective of the study was to estimate the relationship between aggregation of Ag-Ab complexes on the sperm surface and the results of indirect FCM analysis¹⁵⁹. Low temperature, cytochalsin B, excess or lack of the primary and /or secondary antibodies and sperm fixation with paraformaldehyde may inhibit aggregation and shedding of Ag-Ab complexes and dramatically increase ASA quantity determined on the sperm surface. Inhibition of aggregation was observed only in a minority of ASA-positive samples and was poorly reproducibly using semen from different donors. The authors concluded that a high probability of Ag-Ab complex shedding from the sperm surface during experimental manipulation limits the use of indirect FCM test for quantitative ASA determination¹⁵⁹.

The studies performed to date using FCM have emphasized the value of this technique used as a direct test and have demonstrated that it is repeatable, relatively user friendly and

simple. These qualities make it a tempting option for application on the bovine reproductive market and the investigation of infertility. Obstacles to overcome consist on the evaluation of the effect of a fixative solution on directly bound anti-sperm antibodies, repeatability and effect of exclusion of dead cells, for it to become a reliable technique in the bovine species.

Anti-sperm antibodies in the bovine species

Infertility is a serious problem in animal production and accounts for great economic losses in the livestock industry. Causes of infertility may be infectious or non-infectious. Infertility investigation is usually requested by cattle farmers when the calf crop is low or when there is regular return to oestrus by cows that had been mated naturally or artificially inseminated. The significance of ASA for infertility and subfertility in mammalian species is poorly understood.

The reason for formation of ASA and their relationship to reproductive failures is unresolved. Generally, ASA formation can be induced primarily during infectious and non-infectious inflammations, or by obstruction of testicular efferent duct²²⁵. The formation of ASA has also been induced by breeding accidents in a stallion^{23, 226}; testicular biopsies, sperm granulomas, or epididymal aspirates in dogs^{227, 228}; very low temperature in swine²²⁹, cryptorchidism in boars²³⁰, vasectomy in ram²⁴, and excessive male exploitation in rabbits²³¹.

Despite numerous clinical studies and experimental efforts, over the past four decades, the precise role and significance of ASA in the pathogenesis of immunological infertility of both males and females still remains unclear. It is evident now that ASA may impair fertility to certain extent. The reason to the inability to explain immunological subfertility lies in the fact that the mechanisms involved in the gamete immune protection are very complex and the immune response to their antigens is never an “all or none” phenomenon²³².

It is well known that auto-immunization of the male and iso-immunization of the female with sperm cells can lead to significant impairment of fertility²³². Several studies have been undertaken to assess the effect of circulating anti-sperm antibodies on fertility. Nevertheless, no studies have been reported evaluating the effect ASA on the sperm cell to bovine fertility.

Studies on immunization of animals

Landsteiner (1899) reported that bovine spermatozoa were antigenic, based on the loss of motility once injected in the peritoneal cavity of guinea-pigs that had been previously immunized parenterally with bull spermatozoa²³³. This was confirmed by Metchnikoff (1899, 1900) who tested the serum of these animals for agglutinins and sperm immobilizing antibodies^{234, 235}. Henle et al. (1938), demonstrated head-specific and tail-specific antigens by means of complement fixation and slide agglutination²³⁶.

Menge and Christian (1971) auto- or iso-immunized bulls with bull semen or homogenized testis suspended in Freund's complete adjuvant³⁵. The effects ranged from little or none in three bulls to complete decrease of semen quality (evidenced by asthenozoospermia and pyospermia) and aspermatogenesis in three other bulls. Aspermatogenesis was induced only after prolonged periods of immunization. Intratesticular injection of complete Freund's adjuvant in three bulls resulted in immediate damage and aspermatogenesis of the injected testis and eventual aspermatogenesis of the contralateral testis in two of the bulls. Azoospermia after immunization appeared to be due to a heavy influx of leucocytes into the seminiferous tubules and rete testis that prevented passage of sperm into the epididymis. Sperm agglutinating and immobilizing antibodies were detected in the serum of the majority of bulls within 1 or 2 weeks after the first immunization³⁵.

Losos et al. (1968), Parsonson (1990) et al., and Wright (1980) immunized bulls with semen or testicular material and studied the resultant testicular and epididymal lesions. All three studies used adjuvants in the immunization process^{12, 237, 238}. Wright found circulating sperm agglutinins in the bulls seven days after the first injections. Only one bull showed significant changes in semen characteristics. Wright also examined 50 bulls in use at an artificial breeding center by macroscopic sperm agglutination for the presence of serum sperm-agglutinins and found none²³⁸.

In these studies using auto and iso immunization with sperm, testicular homogenates, or epididymal homogenates combined with Freund's adjuvant^{12, 35}, histologic testicular changes varied ranging from normal morphology to complete degeneration of the seminiferous tubules,

with occasional leukocytes seen in seminiferous tubules of a few bulls. Some animals in those studies had limited evidence of anti-sperm antibodies in seminal plasma³⁵.

Experimentally induced bull anti-sperm antibodies in 13-mo-old Holstein bulls significantly reduced *in vitro* fertilization rate⁸. Anti-sperm antibodies were generated by three subsequent auto-immunizations. All bulls produced serum anti-sperm subtypes IgG1 and IgG2 antibodies but no serum anti-sperm IgA nor seminal plasma anti-sperm antibodies of any isotype could be detected by ELISA. Serum samples from a reproductively normal bull population tested by ELISA had significantly higher levels of anti-sperm antibodies than did heifers. The bull population samples giving the highest ELISA values differed from those of the immunized bulls in that their anti-sperm antibodies were of the IgM isotype only. The authors proposed this to be secondary to mucosal exposure to the antigen due to homosexual behavior amongst bulls. Bull sperm were incubated with serum from the immunized and control bulls, and then added to bovine oocytes *in vitro*. Incubation of sperm with post-immunization serum reduced *in vitro* fertilization rate as evidenced by a lower sperm penetration rate to the oocyte. This study demonstrated that anti-sperm IgG1 and IgG2 generated by sperm auto-immunizations reduced fertility *in vitro*, and therefore naturally occurring anti-sperm antibodies might affect fertility in bulls⁸. The immunization regime was designed as a model of the ASA autoimmune responses that could result from testicular disease in bulls. Natural causes of testicular disease, including trauma and infection, can break down the blood-testis barrier and lead to exposure of sperm to leukocytes, with the subsequent formation of anti-sperm antibodies. The presence of anti-sperm antibodies in seminal plasma has been documented in cases of blood-testis barrier disruption resulting from testicular injury in a stallion²³. Kim et al. (1999) found no damage or pathology in the model and this might have accounted for the absence of ASA in the seminal plasma of the immunized bulls. A lack of detection by the ELISA technique could have also been a possibility or the seminal plasma could have interfered with the antibody recognition⁸.

Kim et al. (1999), Menge et al. (1971), and Losos et al. (1968) have reported the induction of circulating ASA in most of the bulls injected with autologous or heterologous semen^{8, 12, 35}. No seminal plasma ASA were found by Kim et al. (1999) using indirect ELISA. Bronson et al. (1987) expressed that the probable reason for the presence of unbound antibodies in seminal plasma traditionally is believed to be due to the excess of antibodies relative to the patient's sperm specific antigens⁷³. Rasansen et al. (1996) stated that the presence of unbound

antibodies due to a reactivity different that found on the patient's spermatozoa could not be excluded automatically when using indirect tests⁷⁴. This latter study concluded that the indirect measurement of sperm antibodies might be grossly unreliable as compared with the results obtained by direct FCM⁷⁴. This conclusion highlights the importance of the development of a reliable direct test for assessment of ASA in bovine male reproduction.

Summarizing, anti-sperm antibodies have been experimentally induced in bulls with varying degrees of responses, most likely due to the lack of a standardized method for induction as well as technique for anti-sperm antibody assessment, making the study of immune mediated infertility a difficult task. To date, only indirect techniques have been used for response assessment of induction of anti-sperm antibodies.

Studies on naturally occurring ASA

Studies in bulls

Several studies have been conducted to evaluate the presence of ASA in reproductively normal and infertile bulls. All of these have been performed on serum or seminal plasma, but none have evaluated the presence of antibodies directly attached to the sperm cell. Purswell et al. (1983) evaluated 25 satisfactory and 25 unsatisfactory potential breeder bulls' serum and seminal plasma with a modified Shulman sperm agglutination test for the presence of sperm agglutinating antibodies¹¹. This was done to determine whether antibodies to spermatozoa were a contributing factor to classification of the bull as an unsatisfactory potential breeder. A large number of bulls in both groups had detectable anti-sperm antibodies, although no correlation was found between classification as an unsatisfactory potential breeder and the presence of sperm agglutinating antibodies detected by the sperm agglutination test. In addition, indirect immunofluorescence was performed to detect isotypes of anti-sperm antibodies in the serum and seminal fluid of the bulls and no correlation was found between antibody class detected and breeding soundness classification. There was no correlation between the age of the bulls and titer of sperm agglutinating antibodies in serum or seminal fluid. The antibody class detected (IgM or IgG) by indirect fluorescent antibody test in serum and seminal plasma was not correlated with the titer of sperm-agglutinating antibodies¹¹. Wright (1980) was unable to find

ASA in serum from 50 bulls from an artificial insemination stud with varying semen quality, using a macroscopic sperm agglutination test (Kibrick method,²³⁹)²³⁸. Purswell and collaborators suggested that the reason for the lack of anti-sperm antibody detection in the stud bull population could have been the potential different sensitivity of the tests used to detect the antibodies. They also suggested that the different management practices and diversified backgrounds for the bulls in the two studies could have affected the results¹¹. Friberg has found antibodies of the IgA class most often to be responsible for sperm-agglutination in humans, antibody that was not tested in this experiment¹⁶⁹.

Perez and Carrasco (1964) reported two naturally occurring cases of agglutinating anti-sperm antibodies in bulls associated with reduced spermatozoal motility and infertility⁹. Clinical examination of bulls revealed the presence of uni- or bilateral seminal vesiculitis, and there was evidence of head to head agglutination on microscopic evaluation of the ejaculates. Agglutinating antibodies were evidenced by means of a ring precipitation test and complement fixation⁹. Zraly et al. (1998) analyzed sera from 2064 bulls randomly selected in artificial insemination stations and bull rearing stations using ELISA⁸⁶. Prevalence for positive bulls was 26.7%, at sera dilutions of 1:40, with a range of 9-39.7% for different investigation periods. There was no significant difference in the incidence of ASA positive bulls regarding the station type. Levels of antibodies to *Chlamydia sp.* were also measured and significantly increased ASA titers were found in bulls that were positive for *Chlamydia*. The authors suggested that environmental as well as infectious factors could be potential causes for ASA, since higher levels of ASA were present when high temperature periods and high serologic evidence for *Chlamydia* were present (45.1% ASA positive and 27.1% ASA positive in bulls that were positive and negative to chlamydial antibodies, respectively), suggesting a possible involvement of genital infection in ASA production, as proposed by Munoz and Witkin (1995) in humans³⁰. Poor nutrition could also represent a potential factor. This is supported by a study by Zraly et al. (1997), where cattle developed ASA two months after the onset of a high nitrate containing diet²⁴⁰. Palan and Naz (1996) found a significant linear correlation between the concentration of Beta-carotene in seminal plasma and IgG anti-sperm antibody titers in seminal plasma in men²⁴¹. It is important to note that the antigen used for the ELISA test consisted of bull sperm subjected to an ultrasound frequency of 20 kHz. This could have exposed internal antigens that are not relevant for fertility since they are not exposed in a physiologic situation, and this could have led

to misleading ASA titers in these bull populations. The authors highlighted the limitation of the study on the differentiation of clinically significant and non-significant antibodies⁸⁶.

Scarce literature is available on natural occurrence of anti-sperm antibodies in bulls. The existence of naturally occurring anti-sperm antibodies in bulls has been demonstrated by several authors, although only those present in serum or seminal plasma, but not those directly attached to sperm have been studied. Studies performed have failed to demonstrate a clear relationship between anti-sperm antibodies and bovine infertility, most likely due to the lack of a consistent and reliable test that allows for assessment of antibodies directly bound to sperm. Concomitant infections and environmental changes has been suggested as potential factors for the development of anti-sperm antibodies in bulls, but further research is needed on these topics.

ASA and age

The age-dependent differences between naturally occurring antibodies against autoantigens and antibodies against exogenous antigens, in terms of their incidence and titer, have been well established in humans. While the frequency of antibodies against self-antigens, which can be ignored in children and young adults, gradually increases after middle age²⁴², the antibodies against foreign antigens appear early, reach maximum titers before puberty, and then slowly decrease²⁴²⁻²⁴⁴. Studies in cattle have shown that the serum titer of sperm agglutinins increased during the first day after birth, reached a maximum during the third and fifth year, and then decreased. No sex differences were found²⁴⁵. In contrast, other investigators could not find sperm immobilizins in sera from bulls during the first 2-3 years of life, but the titer increased until the seventh to eleventh year, and then decreased again²⁴⁶. Purswell et al. (1983) when evaluating the presence (titer) of agglutinating antibodies in bulls that had passed and failed the BSE, found no correlation between the age of the bulls and titers of sperm agglutinating antibodies in serum or seminal fluid¹¹.

The presence of ASA (IgG and IgM) in sera of neonatal and young calves (up to 120 days) was evaluated by indirect immunofluorescence and sperm agglutination test by Lazarevic et al.²⁴⁷. The serum was evaluated against a pool of semen from three bulls processed in three different ways: washed sperm, washed sperm after freeze-thawing in TRIS egg yolk extender,

and washed sperm after freeze-thawing in Biociphos plus extender (IMV, France). Calves that had not ingested colostrum did not present ASA in serum detectable by immunofluorescence or sperm agglutination test. This was in agreement with studies by Jimenez et al. (1986), who also found no agglutinating factors in fetal calf sera. The first positive reaction was obtained in samples tested for the presence of anti-sperm IgG antibodies collected 48 hours after colostrum ingestion²⁴⁸. IgG and IgM titers increased with age thereon but remained at low titers. Sperm antigens seemed to be affected by the use of different semen extenders. The most likely reason proposed for the presence of anti-sperm antibodies at low titers up to the age of 120 days was cross reactivity with microbial agents²⁴⁷. The low titers were in agreement with previous results in a field study where sera and cervical mucus ASA titers were low in virgin heifers but increased with the number of artificial inseminations²⁴⁹.

In a study involving 612 bulls of two different breeds and several ages and uses, serum titers were obtained by the use of an ELISA assay²²⁵. The first occurrence of ASA was recorded in bulls aged 5 to 6 months, related to changes occurring in the developing reproductive system and in accordance to results in boars²⁵⁰. At the age of one year another increase of ASA titers was recognized, attributed to the intensive cells differentiation of the developed testis in puberty²⁵¹. Titters decreased between 13 and 24 months of age, when all developmental morphological and functional processes are terminated²²⁵. These changes in ASA titers could be connected to the presence of an immature blood testis barrier that is not yet fully functional. No significant changes were found in older bulls (greater than 25 months), unlike results obtained by Fayemi et al. (1992) and Flickinger et al. (1997), in boars and rats, respectively^{250, 252}.

The reason for the existence of contradicting results on the relationship between age and anti-sperm antibodies development is most likely the different sensitivities and specificities of the tests used and the different type of antibodies evaluated by the different tests. Colostral or maternal antibodies could be the source for these antibodies in young calves, but also cross reactivity with microbial agents has been proposed. Implications on infertility of these serologic titers is currently unknown.

Cross reactivity with bacterial and viral antigens

In humans, the presence of natural anti-sperm antibodies in fertile individuals, virgin girls⁷⁹ and boys before puberty⁸⁰ has raised questions about the origin of these antibodies. The age dependant pattern of these antibodies follow changes similar to those established for antibodies against exogenous antigens, rather than patterns typical for autoantigens²⁵³. This has raised the hypothesis that these antibodies may be cross-reacting antibodies produced against exogenous antigens (bacteria, fungi, viruses, allergens)⁸². Several reports suggested the presence of cross-reactive antigens between spermatozoa and the gastrointestinal bacteria *Escherichia coli*, *Salmonella typhi*^{78, 81}, and *Helicobacter pylori*²⁵⁴ in humans. Molecular biology approaches have demonstrated the existence of sequence homologies between genes encoding bacterial enzymes and mammalian sperm proteins^{255, 256}. Common antigenicity has been established for spermatozoa and *Staphylococcus aureus*, *Staphylococcus albus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*⁸¹, *Trichomonas vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, and *Candida albicans*^{257, 258}. A disease that disrupts the bowel epithelium, such as diarrheal infections or ulcerative colitis, could change its permeability²⁵⁹. As a result, bacterial antigens that have been limited to the bowel lumen may evade mucosal defenses and affect systemic inflammatory responses.

The presence of cross-reactions between infectious agents and sperm has been studied in the bovine species, but to a lesser extent than in humans. A different proposed mechanism is through natural infections that affect the reproductive tract⁸⁶. Natural infections with different pathogens are considered one of the most important environmental stressors for the bovine species²⁶⁰. Some virus and bacteria, such as Reft Valley virus²⁶¹, Infectious Bovine Rhinotracheitis Virus (IBRV)^{260, 262, 263}, Bluetongue virus^{227, 264, 265}, and *Brucella sp*²⁶⁶, affect the bovine reproductive tract and may play a role on immune mediated infertility⁸⁶. There is scarce information regarding the role of these pathogens on ASA development in the bovine species.

Hegazi and Ezzo (1995) evaluated the presence of ASA in buffalo and cattle naturally infected with IBRV and *Brucella abortus*⁸⁵. Results of agar gel precipitation test and serum neutralization tests were compared among buffaloes and cattle with confirmed natural infections of IBRV and *B. abortus*. A significantly higher response to sperm antigens was encountered in

animals infected by IBRV when compared with *Brucella abortus*, and anti-sperm antibody response in buffaloes was higher than in cattle to this pathogen. This might be attributed to the higher susceptibility of buffaloes to IBRV previously reported²⁶⁷.

Zraly et al. (1998) studied the level of antibodies to *Chlamydia sp* in sera from 590 bulls randomly selected in artificial insemination and bull rearing stations⁸⁶. Significantly increased ASA titers were found using ELISA in bulls that were also positive to antibodies to *Chlamydia sp*. The author suggested that infectious factors could act as potential causes for ASA, since higher levels of ASA were present when high serologic evidence for *Chlamydia sp* was also present⁸⁶.

Probably both the actual infection and the cross-reaction with bacterial and viral antigens take part as a cause of immune infertility in bovine, as suggested by several studies. Nevertheless, the real significance is still to be determined by means of the assessment of the presence of anti-sperm antibodies directly bound to sperm and their effect on fertility.

Antigenic proteins identified as potential candidates for ASA target

Bovine sperm antigens are capable of eliciting the production of anti-sperm antibodies. Identification and characterization of these antigens are important for understanding the mechanism involved in antibody mediated impairment of reproduction, to develop reliable diagnostic tests, and to prevent and treat the cases of unexplained immuno-infertility¹⁰.

Using bull-immune sera immuno-diffusion and immuno-electrophoresis in bulls auto- and iso-immunized with bull semen and testicular tissue, Menge and Christian (1971) indicated that bull sperm possess at least three auto-antigens³⁵. Similarly, by the use of rabbit antisera, three sperm-specific antigens were reported for bull semen, suggesting that bovine sperm-specific antigens are also auto-antigenic²⁶⁸⁷.

In a trial with experimentally induced bull anti-sperm antibodies that significantly reduced fertilization in vitro⁸, western blots were performed with immuno-purified IgG1 and IgG2 from pre- and post-immunization sera from one test bull after intramuscular injection of autologous semen. Both post-immunization IgG1 and IgG2 recognized a 45-kDa sperm antigen.

Similar size antigens, related to the intra-acrosomal SP-10 sperm protein and FA-1 protein in the post acrosomal region have been recognized in human medicine^{269, 270}. The significance of this is still unknown in bovine reproduction, but studies in humans have not demonstrated affected sperm-oocyte interaction in presence of monoclonal anti-sperm antibodies directed to surface and intra acrosomal proteins²⁷¹.

A monoclonal antibody (mAb) to the human sperm plasma membrane protein, fertilization antigen-1 (FA-1), was tested for its reactivity with bovine spermatozoa and its effects on bovine fertilization in vitro. Western blot analysis revealed that the FA-1 mAb reacted with proteins of similar molecular mass (53 +/- 2 kDa) in human and bovine sodium deoxycholate (DOC)-solubilized sperm extracts. Indirect immunofluorescence, using epifluorescence microscopy and laser scanning confocal microscopy, revealed that the FA-1 antigen is present in the post-acrosomal region of bovine spermatozoa, which is similar to human FA-1 localization. In bovine in vitro fertilization (IVF) trials, using oocytes obtained from slaughterhouse ovaries, addition of FA-1 monoclonal antibodies to the IVF medium resulted in a linear decrease in the fertilization rate. There was no inhibitory effect of the FA-1 monoclonal antibody on percent sperm motility or other motility characteristics tested, suggesting that human FA-1 monoclonal antibody inhibits bovine sperm cell function at some point after capacitation²⁷⁰.

The inhibiting effect on fertilization of antibodies to the intra-acrosomal protein SP-10 was studied in a bovine in vitro fertilization model. Indirect immunofluorescence showed that antibodies to human SP-10 localized to the acrosomal region of methanol-fixed, but not live, bovine spermatozoa, confirming the intra-acrosomal localization of bovine SP-10. The presence of the SP-10 protein in bovine spermatozoa was further established by immunoblotting of extracts of bovine spermatozoa with monoclonal antibodies and polyclonal antibodies to human SP-10. The antibodies identified a pattern of immune-reactive peptides similar to human, baboon, monkey and pig SP-10. Fertilization experiments in vitro demonstrated that monoclonal antibodies, as well as polyclonal antibodies, to human SP-10 significantly reduced fertilization rates of bovine oocytes by bovine spermatozoa²⁷⁰.

Tripathi et al. (1999) characterized the molecular weight of sperm polypeptides reactive with serum from hyperimmunized rabbits and calves, and their immune-reactivity with IgG in sera from immuno -infertile repeat breeder cows¹⁰. Repeat breeder cows presented significantly

higher seropositivity to sperm from different bulls compared to pregnant cows or heifers, but the occurrence showed marked variability (14.67-26.67%). This result was in accordance with Wang et al. (1990), who studied the relationship between ASA detected by ELISA and infertility in Chinese black and white dairy cows²⁷². The author found that 34.5% of infertile cows had anti-sperm antibodies in blood serum as compared to 6.7% in non-pregnant cows with a history of normal fertility²⁷². Most cows were reactive to one or two different bulls but fewer to most or all of them, possibly due to antigenic similarities between bulls used for breeding and testing, or because of potential cross-reactivity of antibodies. This may suggest that a potential solution for suspected immune infertility could be changing bulls used for breeding. Two of 16 total polypeptides identified reacted with sera from immune-infertile cows, and antigenicity was variable in different species and between sexes¹⁰.

As described previously, numerous authors have developed different tests to isolate and characterize sperm surface and internal antigens important for fertilization. The significance of isolated sperm-specific antigens on fertilization differs and some of the monoclonal antibodies raised against them may completely block fertilization while others may have no effect on it. Some antigenic and location similarities have been found with human proteins, but the real effect on fertility in vivo still remains unclear for the bovine species.

Effect on fertility in vitro

The ultimate objective of the study of anti-sperm antibodies is their effect on fertility in vivo. Assessment of the effects of circulating antibodies on in vitro fertilization has been used in human as well as bovine reproductive biology to gain more knowledge on the pathophysiology of immune mediated infertility in bovine and estimate their effects in vivo.

Bratanov et al. (1980) showed that sera containing anti-sperm antibodies from infertile cows and women with unexplained infertility inhibit acrosomal proteolytic activity in vitro and therefore suggested that anti-sperm antibodies could possibly affect fertilization²⁷³. In the study performed by Kim et al. (1999), where anti-sperm antibodies were experimentally induced in bull, the circulating antibodies obtained were found to significantly reduce the rate of sperm-oocyte penetration when semen from fertile bulls was incubated with anti-sperm antibody

positive serum. All bulls produced serum anti-sperm IgG1 and IgG2 antibodies. This study demonstrated that anti-sperm IgG1 and IgG2 generated by sperm auto-immunizations reduced fertility in vitro, and therefore the authors concluded that naturally occurring anti-sperm antibodies may affect fertility in bulls⁸.

The inhibiting effect on fertilization of antibodies to the intra-acrosomal protein SP-10 was studied in a bovine in vitro fertilization model. Indirect immunofluorescence showed that antibodies to human SP-10 localized to the acrosomal region of methanol-fixed, but not live, bovine spermatozoa, confirming the intra-acrosomal localization of bovine SP-10. SP-10 antibodies exerted their anti-fertilization effect by reducing sperm-zona secondary binding. Consistent with the accessibility of the SP-10 antigen following capacitation, SP-10 antibodies also reduced the ability of capacitated spermatozoa to complete the acrosome reaction. Furthermore, SP-10 antibodies affected the motility of capacitated spermatozoa, while not affecting the motility of non-capacitated spermatozoa²⁷⁰.

Leipute (2001) assessed the effect of ASA on the fertilization of bovine oocytes in vivo and in vitro. They found that the degree of agglutination of ASA containing sera from cows was unrelated to circulating levels of IgG and that multiple inseminations with the same antigens (same bull semen) caused infertility, preventing fertilization in vivo²⁷⁴. The high titers of IgG and ASA had no influence on the maturation of oocytes in vitro, but decreased their development after fertilization. These studies evidenced a counteractive effect of anti-sperm antibodies on in vitro fertility. Further research is warranted for assessment of the effects of sperm-bound antibodies on in vivo fertility and its real impact in bovine reproduction.

Effect of semen processing

In the technology of artificial insemination, the antigenic structure of sperm cells is changed due to the addition of different semen extenders, freezing and thawing procedures and reduction of seminal plasma volume. Studies that assess the effects of freezing on anti-sperm antibody load are scarce. The immunosuppressive activity of bull seminal plasma has been shown to be significantly reduced during semen preparation for artificial insemination and this could have a significant effect on posterior fertility in the face of anti-sperm antibody²⁷⁵. Lazarevic et al. (2002) also showed that the antigenicity of a single ejaculate can be altered by

the freezing process and the different semen extenders when assessing agglutinating antibodies and the subtypes IgG and IgM on serum from calves up to 120 days old²⁴⁷.

A great percentage of the cattle industry is based on genetic improvement through artificial insemination. Further research is needed to assess the effects of semen processing on sperm-bound antibodies and their effect on fertility.

Chapter 3 - Optimization of a flow cytometric assay for the detection of anti-sperm antibodies in bull semen

Introduction

Infertility is a serious problem in animal production systems and accounts for great economic losses in the livestock industry. Infertility can be caused by infectious or non-infectious diseases. Infertility investigations are most often done when the calf crop is low or when there is regular return to estrus by cows that had been mated either naturally or by artificial insemination. In the past, ASA's have not been assessed as part of these investigations. Thus, the significance of ASA as a contributor to infertility or subfertility in mammalian species is poorly understood and possibly underappreciated.

Despite numerous clinical studies and experimental efforts, over the past four decades, the precise role and significance of ASA in the pathogenesis of immunological infertility and subfertility of both males and females still remains unclear.

The existence of naturally occurring anti-sperm antibodies in bulls has been demonstrated by several authors, only those present in serum or seminal plasma, but there are no reports that demonstrate the presence of antibodies directly bound to sperm. Studies performed have not fully demonstrated a clear relationship between anti-sperm antibodies and bovine infertility, most likely due to the lack of a consistent and reliable test that allows for assessment of these antibodies. Concomitant infections and environmental changes have been suggested as potential factors for the development of anti-sperm antibodies in bulls. Probably both the actual infection and the cross-reaction with bacterial and viral antigens take part as a cause of immune infertility in bovine, as suggested by several studies. Nevertheless, the real significance is still to be determined by means of the assessment of the presence of anti-sperm antibodies directly bound to sperm and their effect on fertility.

As described previously, numerous authors have developed different tests to isolate and characterize sperm surface and internal antigens potentially relevant to the fertilization process. The significance of isolated sperm-specific antigens for fertilization differs and some of the monoclonal antibodies raised against them may completely block fertilization while others may have no effect on it. Some antigenic and location similarities have been found with human

proteins, but the real effect of their antigenicity on fertility in vivo still remains unclear for the bovine species.

Flow cytometry has been used extensively in hematology in veterinary medicine, and a method to detect and quantitate ASA has been developed in human medicine. Flow cytometry allows quantitative estimation of ASA on the surface of living spermatozoa and constitutes a sensitive, specific and repeatable test. However, use of flow cytometry to detect ASA bound to the surface of bovine spermatozoa was reported until recently²⁷⁶. This technique has many advantages over agglutination or ELISA tests previously described in bulls and its development will provide a valuable tool for diagnosis and treatment of immune-mediated infertility in bulls.

As stated before, antibodies of both the IgG and IgA class have been reported to have a deleterious effect on fertility in many species, including bulls. After the initial exposure to the immunological defense system IgM antibodies are produced, followed by IgG and IgA antibodies. IgM antibodies are not secreted into the genital tract because their size is too large to pass the epithelial barrier. IgG antibodies are able to enter the genital tract, come into contact with the spermatozoa, and attach to these³². In some cases -and more commonly indeed during infection – secretory IgA anti-sperm antibodies are produced locally in the genital tract (probably the epididymis)³³. Antibodies of the IgA class can then be detected on the ejaculated spermatozoa, but not in serum, and this has been associated with additional reduction of their fertilizing capacity³⁴.

A number of agglutination and immobilization tests have been developed in human and veterinary medicine for diagnosis of immune-mediated infertility. However, these tests are generally insensitive, nonspecific and too complex in some cases for routine laboratory analysis of ejaculates. In human medicine, direct immunofluorescence and ELISA techniques have been used, but these techniques require fixation of spermatozoa. Fixation has been shown to result in non-specific binding of antibodies, exposure of intracellular antigens, denaturation of sperm antigens or membrane damage, resulting in false-positive or –negative results when using an indirect flow cytometric technique¹⁵⁹. However, use of live cells for ASA detection can also compromise the interpretation of results obtained. It is known that cross-linking for surface antigens by multivalent antibodies or antigen-antibody complexes by second antibodies can cause aggregation of antigen-antibody complexes into patches and caps, phenomena observed in many cell types^{213,214}. The effect of fixation on a direct flow cytometric technique has not been evaluated to date.

Based on previous studies in humans, the inclusion of dead sperm cells in the analysis could falsely increase the percentage of positive cells detected due to the unspecific binding of antibodies

to the sperm surface secondary to changes in the configuration of the plasmatic membrane of dead sperm. This could severely affect the results of an infertility test and consequently the classification of a prospective breeder. Anti-sperm antibodies have been experimentally induced in bulls with varying degrees of responses, most likely due to the lack of a standardized method for induction as well as technique for anti-sperm antibody assessment, making the study of immune mediated infertility a difficult task. To date, only indirect techniques have been used for response assessment of anti-sperm antibody induction.

Based on the lack of a standardized, reliable and accurate method for detection of anti-sperm antibodies directly bound to sperm in the bovine species and the need for a technique that allows for a simple and relatively short time-consuming assessment on these antibodies, is that we have undertaken this research project, with the short term objective of developing a flow cytometric technique for detection of anti-sperm antibodies directly bound to sperm and the long term objective of providing a useful tool for the study of the relevance of anti-sperm antibodies in bovine fertility.

The specific objectives of these experiments were to determine the effect of immediate fixation of spermatozoa and inclusion of dead cells in the analysis on the proportion of IgG- and IgA-bound spermatozoa detected by flow cytometry; and to calculate intra- and inter-assay variability of flow cytometric detection and quantitation of IgG- and IgA-bound bovine spermatozoa.

We hypothesized that:

1. The percentage of antibody-bound spermatozoa in samples fixed with formalin buffer solution (FBS) or non-fixed was similar since fixation does not alter sperm membranes or surface antigens;
2. Including dead spermatozoa in the analysis would falsely increase the percentage of antibody-bound spermatozoa in the ejaculate due to non-specific binding of antibodies to membranes of dead cells;
3. Intra- and inter-assay variability of flow cytometric determination of ASA bound to bovine spermatozoa is <10%.

Materials and Methods

To carry out this project, four healthy yearling bulls were immunized with their own sperm. Once anti-sperm antibodies were detected on the sperm surface, the effects of fixation, exclusion of dead cells, and aliquot variability on the percentages of IgG and IgA bound sperm were assessed. Also, semen from nine reproductively normal bulls (Experiment 1, n= 4; Experiment 2, n=5) classified as satisfactory potential breeders during routine Breeding Soundness Examination were used as a low antibody group (healthy bulls).

Animals

Anti-sperm antibody generation: Four Aberdeen Angus healthy yearling bulls were used for induction of ASA through systemic (intramuscular) injection of autologous washed sperm with an adjuvant. The bulls were bought from a local producer and housed and fed in accordance with animal care guidelines of IACUC, Kansas State University. The bulls were allocated in individual or shared pens and fed brome hay and water ad libitum, and 2 lb of sweet feed twice daily for the duration of the study. All bulls were allowed for acclimation for one week before immunizations were performed.

Reproductively normal bulls: Ejaculates from Aberdeen Angus bulls (n= 9, aged 1 to 4 years) classified as satisfactory potential breeders during routine Breeding Soundness Examination (BSE) performed at the KSU-VMTH were used in the study.

Induction of anti-sperm antibodies in healthy bulls

Immunization of bulls

Immunizations were performed as described before³⁵ with some modifications. Semen was collected from each bull using electroejaculation and washed three times by centrifugation at 1000 x g for 15 min with phosphate buffer solution (PBS). Sperm cells were counted with a hemocytometer, and a total number of 1×10^9 spermatozoa, was suspended to 1 mL of PBS. This

suspension (1mL) was diluted in 1 mL of Freund's complete adjuvant (Freund's Complete Adjuvant, F5881, Sigma-Aldrich, St. Louis MO, USA). The antigen, 2 mL, containing autologous semen, was administered intramuscularly in the neck in four different aliquots (0.5 ml each).

Immunizations were repeated in 3 bulls once 17.5 ± 6.3 days (median \pm SD) after the first immunization using autologous sperm with Freund's incomplete adjuvant. Spermatozoa (1×10^9 sperm cells) were resuspended to 1 mL in PBS. The sperm suspension was further diluted in 1 mL of Freund's incomplete adjuvant (Freund's Incomplete Adjuvant, F5506, Sigma-Aldrich, St. Louis MO, USA). The antigen, 2 mL, was administered intramuscularly in the neck in four different aliquots (0.5 ml each). The experiments began at 6 ± 1.4 days after the second immunization, at which point the bulls were considered to be ASA positive. One bull did not receive a second immunization, due to the fact that based on the response from the previous bulls to initial immunization, it was not considered to be necessary. For this individual bull the experiments were started 22 days after the first immunization.

Breeding Soundness Examinations and semen collections

An examination for abnormalities of the bull's attitude, general appearance, body condition, and fecal characteristics was performed. The eyes were also examined. During the breeding soundness evaluation the bull's gait was observed, in search for any lameness or conformational abnormalities.

Palpation of external reproductive organs

The scrotum and its contents (testicle, epididymis, and spermatic cord) and prepuce were palpated for assessment of any pathologic abnormalities. When examining the contents of the scrotum, the temperature, size, texture, resilience and evenness of the testes and epididymis were determined. Presence of fat in the neck of the scrotum was determined. Free movement of the testes within the scrotum was also observed. The head and tail of the epididymis, as well as the body were palpated. The body of the epididymis was palpated on the medial aspect of the testis

by first sliding the opposite testis upward. The vasa deferentia were palpated throughout the scrotal neck. The spermatic cord was palpated up to the level of the inguinal ring for the presence of abdominal contents (scrotal hernia) or abnormalities of spermatic vasculature. The penis was exteriorized and evaluated, and palpation of the sigmoid flexures and assessment of the prepuce and preputial orifice was performed.

Scrotal circumference

After the testes and epididymis were palpated, the testes were positioned firmly into the lower part of the scrotum so that they were side by side. The testes were held down within the scrotum by placement of the fingers and thumb at the sides of the scrotal neck and a scrotal tape was placed around the greatest diameter of the scrotum and pulled snugly so that the tape was firmly in contact with the entire circumference. The measurements were recorded in centimeters.

Transrectal palpation of internal reproductive organs

The accessory sex glands (vesicular glands, prostate and bulbourethral glands), ampullae of the deferent ducts, urethra, and inguinal rings were palpated in search for pathologic abnormalities. Assessment of size, symmetry, consistency, and pain to palpation was performed on the accessory sex glands and ampullae. The urethra was assessed for size, consistency, and pain to palpation, while the inguinal rings were assessed for size and presence of bowel.

Semen collection via electroejaculation

An electroejaculator was used (SireMaster Original, ICE Corporation, Manhattan, KS) for electroejaculation. Rectal palpation of the internal organs was performed by prior manual evacuation of the feces in the rectum with a lubricated sleeve, and a rectal probe (6.5 cm in diameter) was lubricated and inserted transrectally with the electrodes over the ampullae (facing ventrally) after previous stimulation by massaging the pelvic urethra for 30 to 60 seconds and after the bull had shown penile protrusion. The area over the ampulla, prostate, and urethra was massaged by moving the clinician's fingers back and forth in a longitudinal direction, stimulation became evident by pulsation of the urethral muscle. The first stimulus was applied slowly until

the bull gave a minimal response (slight protrusion). Each successive stimulus were then increased slightly in intensity. Stimuli lasted 1 or 2 seconds, and then were discontinued for 0.5 seconds. The amount of electrical stimulation was gauged at all times by the bull's response. Extension of the penis pushing on the sigmoid flexure was performed when necessary if the bull failed to protrude the penis. Collection of the ejaculate was performed when the fluid became cloudy in a pre-warmed plastic collection tube. If urination occurred during the collection, a second sample was obtained after a period of 2-3 minutes of rest.

Semen quality evaluation

After semen collection, the sample was kept in an incubator at approximately 30°C until arrival to the laboratory. The samples were transported to the laboratory within 30 minutes of collection. Sperm density, mass and individual motility, progressive motility, sperm morphology, and concentration were assessed once in the laboratory. All manipulations that required live semen were performed with the use of a slide warmer.

Sperm density assessment

The semen samples were classified based on their macroscopic appearance and sperm density estimation as very good (creamy appearance, grainy semen, estimated 750 million to 1 billion or more sperm/ml), good (milk-like semen, estimated 400 to 750 million sperm/ml), fair (skim milk-like semen, estimated 250 to 400 million sperm/ml), or poor (translucent semen, estimated less than 250 million sperm/ml)²⁷⁷.

Sperm concentration

Semen was diluted 1:1000 using 3 successive dilutions of semen (100 ul of semen in 900 ul of diluent) in Formalin Buffer Solution and a Neubauer hemocytometer was used for assessment of the sperm concentration using phase contrast microscopy at 400X (Nikon Labophot 2, Nikon Instruments Inc., Melville, NY). To prepare the counting chamber the mirror-like polished surface was carefully cleaned with lens paper. The coverslip was also cleaned. The coverslip was placed over the counting surface prior to loading the cell suspension. The suspension was introduced into one of the V-shaped wells with a pasteur pipet. The area under the coverslip filled by capillary action. Enough liquid was introduced so that the mirrored

surface was just covered. The loaded counting chamber was then placed on the microscope stage and the counting grid was brought into focus at low power. Both counting grids were scanned and all cells were counted. The average of both counting grids multiplied by the dilution factor was obtained. Concentration of round cells was also assessed in the same manner.

Sperm motility assessment

Gross motility was determined using a 5 to 10 mm diameter, non covered, drop of semen examined at 40x to 125x magnification on a pre-warmed slide. The semen samples were classified based on their microscopic appearance and sperm motility estimated as very good (rapid dark swirls), good (slower swirls and eddies), fair (no swirls, but prominent individual cell motion), poor (little or no individual cell motion)²⁷⁷.

Individual motility assessment

Subjective analysis

Wet mounts were examined at 100 to 400x magnification, under phase contrast. The semen samples were classified based on their microscopic appearance and sperm individual motility estimated as follows very good (80 to 100 % motile sperm), good (60 to 79 % motile sperm), fair (40 to 59 % motile sperm), poor (less than 40 % motile sperm) (Barth, 2000).

Objective motility assessment via computer-assisted semen analyzer

Semen samples were diluted in Phosphate Buffer Solution to a concentration of 50×10^6 sperm/ml and evaluated for motility parameters described as % TM (total motility) and % PM (progressive motility), both performed by CASA instruments (Hamilton Thorne, Beverly, MA, USA). For each semen sample five random fields were observed, and a minimum of 100 cells per field was counted. With respect to the program parameters, spermatozoa with a VAP less than $10 \mu\text{m/s}$ were considered immotile, while spermatozoa with velocity greater than $15 \mu\text{m/s}$ were considered motile. Spermatozoa deviating less than 45 % from a straight line were designated as linearly motile.

Cytologic assessment

Sperm morphology

Nigrosin/eosin stain was used for morphologic assessment. Briefly, a drop of pre-warmed stain was mixed with a drop of semen on the slide warmer and a smear was performed. It was allowed to completely dry before examination of the slide at 1000X in oil immersion. A total of 100 cells were assessed, registering the morphologic features of these. Final percentages were calculated and recorded as percentage of normal sperm, abnormal head shape, abnormal mid pieces, coiled tails, kinked tails, acrosome abnormalities, proximal droplets, distal droplets, and detached heads.

Round Cell morphology

A modified Wright stain (Hemacol Protocol Hema solution, EMD Chemicals, Gibbstown, NJ, USA) was used for assessment of round cell morphology. Briefly, a smear with a drop of semen was performed in a slide and after allowing it to dry at room temperature, it was stained following the instructions of the manufacturer. The slides were assessed using 1000X immersion in oil. A total of 100 round cells were counted and classified as inflammatory cells (neutrophils, lymphocytes, macrophages) and germinal cells (spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids).

Breeding soundness exam classification

Bulls were classified based on the results of the breeding soundness examination as follows:

Satisfactory: Bulls that met the minimum requirements for physical soundness and semen quality. The minimum requirements consist on at least 30 % individual motility, 70 % morphologically normal sperm, and scrotal circumference that met minimum criteria for individual ages (greater than 32 cm for a yearling Angus bull).

Questionable: Bulls likely to perform adequately in mating but with below-normal fertility, or with undesirable trait with the potential for genetic transmission to offspring.

Decision Deferred: If there was evidence of disturbance of spermatogenesis from which recovery was expected before the onset of breeding season.

Unsatisfactory: Bulls whose use was expected to result in poor fertility in the next breeding season²⁷⁷.

Flow cytometric analysis of anti-sperm antibodies:

Effect of fixation and exclusion of dead cells on the percentage of antibody bound sperm

The effect of fixation with formalin buffer solution (FBS) and exclusion of dead cells in the analysis on the percentage of IgG- and IgA-bound spermatozoa detected by flow cytometry was evaluated. Formalin buffer solution was chosen as a fixative because most veterinary practitioners have access to FBS since the fixative is routinely used during semen analysis in other species. To improve accuracy of the analysis by including bulls with low and high ASA binding, both bulls classified as satisfactory potential breeders (n=4) and bulls with experimentally induced ASA (n=4) were used.

After semen collection, each ejaculate was divided into 12 aliquots (Table A.1). In non-fixed aliquots, 2 mL of raw semen was diluted to 50×10^6 sperm/mL in Phosphate Buffer Solution (PBS). In fixed aliquots, 2 mL of raw semen was diluted to 50×10^6 sperm/mL in a 1:10 dilution of Formalin Buffer Solution (FBS) in PBS. Fixed and non-fixed samples were incubated for 10 minutes at room temperature and washing and immune-fluorescent staining were performed immediately after as described below.

Immunofluorescent staining:

The aliquots diluted in PBS or FBS were washed three times by centrifugation at $900 \times g$ for 5 min in PBS. Washed spermatozoa, 2×10^6 , were resuspended in 320 μ L of PBS, and 0.1875 μ g/million sperm of FITC-labeled antibodies (FITC-labeled polyclonal goat anti-bovine IgG F(ab')₂, Jackson Immunoresearch Laboratories Inc, West Grove, PA, cat. No 101-096-003; FITC-labeled polyclonal rabbit anti-goat IgG F(ab')₂, Jackson Immunoresearch Laboratories Inc, West Grove, PA, cat. No 305-096-003; FITC-labeled polyclonal rabbit anti-bovine IgA, Bethyl

laboratories, Montgomery, TX, cat. No. A10-108F; or FITC-labeled polyclonal goat anti-mouse IgA, Bethyl laboratories, Montgomery, TX, cat. No. A90-103F) were added. The samples were incubated for 30 minutes at room temperature in the dark, followed by three washes by centrifugation at 900 x g for 5 minutes in PBS. After the last centrifugation, the pellet was resuspended in 0.5 mL of PBS and analyzed by FCM.

Flow Cytometry

Samples were analyzed by a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA). Sperm were analyzed at 488 nm (argon filter) to determine forward and right angle side scatter using a photo diode (forward scatter) and a photomultiplier tube (side scatter). These measures of light scatter correlate roughly with cell size (forward scatter) and density (side scatter), and a gate containing spermatozoa was set based on dot plot distribution of forward versus side scatter to eliminate debris and epithelial cells from the analysis. Fluorescence data was collected with logarithmic amplification for green (fluorescein isothiocyanate, FITC) and red (propidium iodide, PI) fluorescence. The FITC and PI signals were detected using standard argon filter (488 nm). Cells were simultaneously stained with 1 μ L/million cells of PI to facilitate exclusion of dead cells from the analysis. Color compensation was done by labeling sperm cells separately and together with PI and FITC-conjugated antibodies. The instrument was calibrated daily with standard beads so that the coefficients of variation of the forward scatter and fluorescence channels were $> 5\%$ on a daily basis. The control population (left quadrant) was marked on samples stained with the isotype control to include $<1\%$ of cells as positive (right quadrant). Quadrant settings were adjusted for each bull. The control quadrant (lower left, LL) was marked on isotype control stained samples to include $<1\%$ of cells as positive in the upper left (UL), upper right (UR) and lower right (LR) quadrants (Fig.B.1). Quadrant settings were adjusted for each bull. The LL, UL, UR and LR quadrants corresponded to antibody-negative live sperm, antibody-negative dead sperm, antibody-positive dead sperm and antibody-positive live sperm, respectively. A total number of 10,000 cells were analyzed from each sample at a rate of 1×10^3 to 2×10^3 cells/second using PBS as the sheath fluid. Data from these cells was collected using forward scatter as the trigger signal and a threshold of 48 of 1,024 channels.

Flow cytometric analysis of anti-sperm antibodies:

Inter and intra aliquot variability of direct flow cytometric detection of anti-sperm IgG- and IgA-bound spermatozoa

To determine inter- and intra-aliquot variability of the test was calculated. Semen was processed and analyzed in the most appropriate way as determined during the previous experiment: dilution in PBS and excluding dead cells. To improve accuracy of the analysis by including bulls with low and high ASA load, both bulls classified as satisfactory potential breeders (n=5) and bulls with experimentally induced ASA (n=4) were used. A complete semen evaluation was performed immediately after semen collection as described previously. Each ejaculate was divided into 14 aliquots (Table A.2). Aliquots 3 to 8 and 11 to 15 represent 5 replicates of the same sample, and were used to calculate inter-assay variability. Aliquots 3 and 11 were analyzed 5 times each, and were used to calculate intra-assay variability. Samples were processed as previously described. Immuno-fluorescent staining was performed as described previously.

Statistical analysis

All numerical data were expressed as mean \pm SE unless stated otherwise. Level of significance was set at 5%. All data analysis was performed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA).

Percentages of pre-induction antibody (IgG and IgA) bound live sperm was compared to percentages of post-induction ASA-bound live sperm by a paired t-test statistic in experimental bulls. A t-test statistic assuming unequal variances was used to compare percentages of antibody binding (IgG and IgA) in pre- or post- induction sperm samples of the experimental bulls with bulls classified as satisfactory potential breeders. Only the first post-induction sample was used for this statistical analysis in immunized bulls. Only live cells were included in the analysis.

Percentages of IgG- or IgA-bound fixed or non-fixed spermatozoa contained within the LR (live) or LR + UR (live + dead) quadrants were compared using ANOVA for repeated measures after a logarithmic transformation of the data.

Variability of the assay was estimated using the coefficient of variation (CV), calculated as %CV, defined as $\%CV = \frac{\text{Stdev}}{\text{Mean}} \times 100$. Results were assessed evaluating the coefficient of variation expressed as a percentage.

Results

Immunization of bulls, semen analysis, and Breeding Soundness Examination classification

Three bulls received one initial immunization with Freund's Complete Adjuvant, and a booster immunization with Freund's Incomplete Adjuvant, and achieved an adequate level of immunoglobulins. One bull did not receive a booster. This decision was made based on the response to initial immunization from the other bulls.

Among the experimental bulls, there was a 83.22 ± 10.84 % and a 56.88 ± 12.24 % increase for the percentage of IgG and IgA bound live sperm, respectively, when the first sample used for the experiments was compared with the pre-immunization samples ($p= 0.0046$ and $p= 0.0188$ for IgG and IgA, respectively) (Fig B.2 and B.3).

Baseline percentages of IgG-bound live sperm were 4.65 ± 2.15 % and 0.84 ± 0.13 % for pre-immunization semen samples from experimental bulls and satisfactory potential breeders, respectively ($p= 0.1748$). Percentages of IgA-bound live sperm were 6.74 ± 1.82 % and 2.85 ± 0.96 % for pre-immunization samples from experimental bulls and satisfactory potential breeders, respectively ($p= 0.1230$) (Fig B.4).

When post immunization samples from experimental bulls were compared with samples from reproductively normal bulls, there was a significant difference in the percentages of anti-sperm antibody bound live sperm ($p=0.0022$ and $p=0.0110$ for IgG- and IgA-bound sperm, respectively) (Fig B.5).

Evaluation of the effect of fixation and exclusion of dead cells on the percentage of ASA levels

The effect of fixation as well as the effect of exclusion of dead cells from the analysis on the percentage of antibody bound sperm was assessed in semen from satisfactory potential breeders and bulls auto-immunized with autologous semen (Table A.3). In semen samples from immunized bulls, there was no effect of fixation or exclusion of dead cells from the analysis on the percentages of IgG-bound sperm ($p=0.0922$). There was a significant effect of exclusion of dead cells from the analysis but not fixation for IgA-bound sperm ($p=0.0152$) in this group of bulls.

In semen samples from reproductively normal bulls, there was no effect of fixation or exclusion of dead cells from the analysis of the percentages of IgG-bound sperm ($p= 0.1525$). There was a significant effect of exclusion of dead cells from the analysis but not fixation for IgA-bound sperm ($p=0.0012$) in this group of bulls.

Variability of the flow cytometric assay

The inter and intra aliquot variability for the percentage of IgG- and IgA-bound live sperm was less than 5 % for the semen samples from the immunized bulls, while the variability was less than 10 % for semen samples from reproductively normal bulls (Table A.4).

Discussion

Immunization of bulls and flow cytometry

Immunization of bulls with autologous semen injected intramuscularly was successful in generating IgG and IgA anti-sperm antibodies directly bound to sperm, as proven by the magnitude of the increase in fluorescently labeled sperm using flow cytometry and this difference was statistically significant. When pre induction experimental bulls' immunoglobulin bound sperm percentages were compared with reproductively normal bulls that presented to the

VMTH-KSU and passed the BSE, the difference was not significant for either immunoglobulin (IgA and IgG).

The development of ASA directly bound to sperm secondary to autoimmunization with semen had, to our knowledge, not been reported before. Haas et al. (1990) used flow cytometry to determine if sperm bound IgG and sperm bound IgA occurred on the same sperm population in the ejaculates of infertile men with sperm-associated antibodies. Results from this study showed that both sperm-associated IgG and sperm-associated IgA were either singly stimulated or, if stimulated concomitantly, were found on the same population of sperm. This is in agreement with the finding of both IgG and IgA in semen from immunized bulls, although the concomitant presence in the same sperm cell cannot be ruled in or out, since the analysis for each immunoglobulin was performed separately. This eliminated the feasibility of sperm separation procedures according to the sperm associated Ig isotype as a treatment modality for immune mediated infertility²²⁰ and may present the same implications in bovine fertility.

Evaluation of the effect of fixation and exclusion of dead cells on the percentage of ASA levels

Fixation did not affect the percentage of ASA-bound sperm in our trial in bulls that passed the BSE as well as immunized bulls. Previous studies have assessed the effect of fixation on percentages of sperm bound antibodies and antibody load, but only when indirect flow cytometric techniques were used. The study by Nikolaeva et al. in 2000¹⁵⁹ showed that false negative results of indirect FCM test might be due to disappearance of ASA from the sperm surface after binding of ASA with antigens. It is known that interaction of ASA with secondary antibodies can cause aggregation of cell surface molecules into patches and caps¹⁶⁰⁻¹⁶². The disappearance of the cap may be due to endocytosis and subsequent degradation and/or shedding of Ag-Ab complexes, as reported for many types of cells^{222, 223}. Endocytosis in mature spermatozoa is unlikely but there are data on shedding of Ag-Ab complexes from the sperm surface in other species^{163, 164, 224}. The study by Nikolaeva et al. (2000) revealed the typical changes of fluorescence pattern from homogeneous distribution to the cap formation through patches followed by decrease of sperm antibody load, supporting the theory of Ag-Ab complex

shedding. Small fluorescent particles, probably the shed complexes in some samples, were detected both by fluorescent microscopy and FCM. These authors concluded that shedding was the most probable mechanisms that lead to removal (partial or complete) of Ag-Ab complex from the cell surface and to false negative results of FCM test¹⁵⁹. We did not assess changes in fluorescence patterns during our experiment, and so it is not possible to assess any changes in distribution of antibody-antigen complexes on the sperm surface. Our study showed that fixation does not affect the percentage of antibody-bound sperm, in either normal (low antibody load) or immunized (high antibody loads) animals. We used diluted formalin buffer solution of the fixative instead of 4 % paraformaldehyde and similar incubation times (10 vs 15 minutes) when compared to the study by Nikolaeva (2000). This could have influenced the different results but the two different times or the two different fixative solutions were not compared on this experiment. These results may be important for future management and transport of samples, considering that the regular bovine practitioner will be working in an adverse environment and laboratory availability would most likely be reduced, potentially allowing for transport of semen in diluted Formalin Buffer Solution. Further research assessing the effect of time on antibody bound sperm is needed.

Detection of sperm-bound antibodies by direct FCM and assessment of antibody load (antibody molecules/spermatozoa) for IgG and IgA was performed by Rasansen et al. (1992) in human samples¹⁴. They excluded dead cells from the analysis because they can bind antibody nonspecifically, as shown by the difference in the percentages of antibody positive cells when these cells were considered (ranged from 10 to 58% increase in positivity when dead cells were included). Our findings are contradictory. By means of exclusion of the dead cells, we obtained a significantly increased percentage of IgA-bound sperm in semen samples from immunized bulls, but a decreased percentage of IgA-bound sperm in samples from reproductively normal bulls. There was no difference when IgG-bound sperm was evaluated in either group.

The fact that we used different types of antibodies could explain our results, at least partially. Anti-bovine IgG antibodies consisted on the F(ab)₂ portion of the molecule, while IgA anti-bovine antibodies used consisted on whole molecules that included the Fc portion of the antibody. This may have increased the unspecific binding to the sperm surface, mediated by the presence of Fc-receptors (FcR), or FcR like proteins, on the plasmatic membrane of the spermatozoa^{278, 279}, although there are reports of greater affinity for the Fc portion of IgG, but not

other isotypes²⁷⁹. The fact that the percentages of IgA-bound sperm were higher when live and dead cells were considered in reproductively normal bulls may be related with the low ASA numbers present and the interaction of the Fc portion of the fluorescently labeled IgA antibody with these molecules on the sperm surface and the unspecific binding to the sperm membrane of dead cells. When semen from immunized bulls was obtained, the effects were opposite (greater percentage of IgA-bound sperm when considered only live cells for the analysis), and this could have been due to the fact that immunized bulls have antibodies bound to the sperm surface with high specificity that competes with unspecific binding to the cell surface or the presence of FcRs or FcR-like proteins.

Other possible reasons for the increase in the percentage of IgA-bound sperm when considering only live cells could rely on the fact that Rasansen et al. (1992) used samples that had been preserved in cold protected containers at room temperature for up to 8 hours. In our study, we processed the samples immediately after collection. It is unknown whether the time delay for the sample processing could have affected the membrane configuration of the dead cells, increasing antibody unspecific binding. On the other hand, it is likely that the sperm membrane physiology and changes after cell death are different for human and bovine sperm. Another important consideration was that the semen samples were only washed once before processing, compared to the three washings performed in our study. However, in a previous study by Rasansen (1994) using direct FCM assessment of ASA, it was found that removal of loosely bound antibodies to the sperm surface occurred after the first wash, without further effect of washing on the percentage of ASA-bound sperm and without affecting the percentage of dead sperm. This would explain why washing could improve the fertilization rates^{205, 216, 217}, but is also in contradiction with Haas et al. (1988), that showed that the sperm bound immunoglobulins were not removed by several washings (3 to 18 times)²¹⁸. The washing, the use of an F(ab)₂ portion of anti-bovine antibody, together with the use of an isotype control to exclude unspecific binding may have contributed to our results in the case of IgG.

Concluding, processing of fresh semen samples for direct flow cytometry is recommended using either PBS or diluted FBS and only analyzing the population of live cells to avoid the misleading results of the presence of dead cells, especially when analyzing IgA present on the surface of the sperm. Furthermore, it is important to highlight the need for the use

of an isotype control and F(ab)₂ portion of the antibody to allow for exclusion of any unspecific antibody binding.

Variability of the flow cytometric assay

The variability of the assay is in agreement with the variability reported in other human studies¹⁴. Assessment of intra and inter assay variability revealed these to be less than 10 % for the direct FCM readings, being this an advantage for objective diagnosis of ASA-bound sperm and potential monitoring of treatment under steroid therapy¹⁴. The use of F(ab)₂ fragments instead of complete immunoglobulin molecules was supported by the fact that it avoids any problems related to the presence of the Fc receptors on sperm. Unfortunately, there were no available fragments for fluorescently labeled anti-IgA antibodies and this may have accounted for some of the variability, although this is unknown unless a comparison between both types of antibodies is performed. Use of polyclonal antibodies was also elected, since these would be expected to reach with all isotypes of antibodies, whereas monoclonal antibodies, by virtue of their specificity, react only with a particular epitope of a restricted isotype. If the epitope is masked due to the presence of seminal proteins, monoclonal antibodies may give negative results¹⁴.

The FCM test requires washing of the sperm cells and so it could reveal negative results in cases of insufficiently strong binding of antibody with the surface of the spermatozoa³². On the other side this feature of the test could contribute to the low variability obtained and be important in decision making for therapy selection, since immovable antibodies may need to be approached differently than loosely bound ASA. As stated before, Rasasen (1994) assessed the effect of washing on sperm antibody load using direct flow cytometry in humans and this author found no further effect of washing on the percentage of ASA bound sperm, without changes on the percentage of dead sperm after the first wash²⁰⁵.

The fact that the variability for the positive bulls was less than the variability for the negative bulls could be explained by different affinity of the antibodies generated by immunization and the naturally occurring antibodies in normal bulls. Systemic exposure of the antigens to the immune system may have generated a different response than exposure of antigens by “leakage” during spermatogenesis and transport through the genital tract, exposure

of the spermatozoal antigens to mucosa, or the reported cross-reactivity with bacterial antigens. This may have led to a more specific antibody-antigen complex formation, leading to a stronger antigen-sperm binding.

Chapter 4 - Effect of freezing on Anti-sperm Antibody binding

Introduction

In the technology of artificial insemination, the antigenic structure of sperm cells is changed due to the addition of different extenders, freezing and thawing procedures and reduction of seminal plasma volume²⁸⁰. Studies that assess the effects of freezing on anti-sperm antibody load are scarce. The immunosuppressive activity of bull seminal plasma has been shown to be significantly reduced during semen preparation for AI and this may have a significant effect on posterior fertility in the case of ejaculates with high percentages of anti-sperm antibodies²⁷⁵. Lazarevic et al. (2002) also showed that the antigenicity of a single ejaculate can be altered by the freezing process and the different semen extenders when assessing agglutinating antibodies and the subtypes IgG and IgM on serum from calves up to 120 days old in a study that evaluated the same serum samples with ejaculates diluted with different extenders²⁴⁷.

Artificial insemination is widely used in beef and dairy production. Use of frozen semen is the most common way of performing artificial insemination in cattle, increasing the offspring for a single bull and favoring selection towards that genetic group. The presence of anti-sperm antibodies in bovine frozen semen has not been studied to our knowledge. The objective of this experiment was to assess the effect of freezing on Ab binding, using a commercial freezing protocol for bull sperm and the previously standardized protocol. We hypothesized that freezing would not alter the percentages of ASA-bound sperm for IgG and IgA.

Materials and Methods

Semen collection and initial evaluation

A Breeding Soundness examination was performed on each bull at the time of the experiment. Semen was collected by electroejaculation as previously described. A complete semen evaluation was performed immediately after semen collection.

A breeding soundness exam and semen collection for freezing was performed in 4 bulls previously immunized with autologous semen and adjuvant, as described before, once ASA were detected on the sperm surface. A single ejaculate from each bull was collected and an aliquot (2 mL) of semen was separated to analyze pre-freezing percentages of IgG- and IgA-bound sperm. The remaining semen was immediately transferred to the laboratory for freezing. The samples were held at 37 °C in water bath for 15 min before dilution for further freezing.

Semen processing for freezing

Upon collection and incubation at 37 °C, semen concentration was determined using a spectrophotometer. Each sample was diluted (based on initial concentration and motility and to a final concentration of 80 million sperm per mL, with a ratio of at least 1:1) with extender #1 (Bioxcell QSF I, IMV Technologies, Maple Grove, MN, USA), submerged in 37°C water bath jars and placed in cold room for 2 hours (5°C). Extender #2 (Bioxcell QSF, IMV Technologies, Maple Grove, MN, USA) was added slowly to final concentrations of 40 million sperm per mL. The extended semen was loaded in 0.5 mL plastic straws and placed on freezing trays. Semen straws were then held cold (5° C) in room for a minimum of 4 hours from time of collection until freezing. Trays were set above liquid nitrogen vapors (1 to 2 inches above the liquid nitrogen), for 10 to 15 min and were subsequently plunged into the liquid nitrogen (-196 °C). The straws were kept in liquid nitrogen until thawing. Thawing was performed when necessary in a water bath at 37 °C for 30 seconds.

Flow cytometric analysis

After collection through electroejaculation, 2 mL of raw semen was diluted to 50×10^6 sperm/mL in phosphate buffer solution (PBS). The diluted ejaculate was washed three times by centrifugation at $900 \times g$ for 5 min in PBS. Washed spermatozoa, 2×10^6 , was resuspended in 320 μ L of PBS, and 0.1875 μ g/million sperm of FITC-labeled antibodies (FITC-labeled polyclonal goat anti-bovine IgG F(ab')₂, Jackson ImmunoResearch Laboratories Inc, West Grove, PA, cat. No 101-096-003; FITC-labeled polyclonal rabbit anti-goat IgG F(ab')₂, Jackson

Immunoresearch Laboratories Inc, West Grove, PA, cat. No 305-096-003; FITC-labeled polyclonal rabbit anti-bovine IgA, Bethyl laboratories, Montgomery, TX, cat. No. A10-108F; or FITC-labeled polyclonal goat anti-mouse IgA, Bethyl laboratories, Montgomery, TX, cat. No. A90-103F) were added. The samples were incubated for 30 minutes at room temperature in the dark, followed by three washes by centrifugation at 900 x g for 5 minutes in PBS. After the last centrifugation, the pellet was resuspended in 0.5 mL of PBS and analyzed by FCM.

Samples were analyzed by a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA). Sperm were analyzed at 488 nm (argon filter) to determine forward and right angle side scatter using a photo diode (forward scatter) and a photomultiplier tube (side scatter). These measures of light scatter correlate roughly with cell size (forward scatter) and density (side scatter), and a gate containing spermatozoa was set based on dot plot distribution of forward versus side scatter to eliminate debris and epithelial cells from the analysis. Fluorescence data was collected with logarithmic amplification for green (fluorescein isothiocyanate, FITC) and red (propidium iodide, PI) fluorescence. The FITC and PI signals were detected using standard argon filter (488 nm). Cells were simultaneously stained with 1 μ L/million cells of PI to facilitate exclusion of dead cells from the analysis. Color compensation was done by labeling sperm cells separately and together with PI, and FITC-conjugated antibodies. The instrument was calibrated daily with standard beads so that the coefficients of variation of the forward scatter and fluorescence channels were > 5 % on a daily basis. The control population (left quadrant) was marked on samples stained with the isotype control to include < 1 % of cells as positive (right quadrant). Quadrant settings were adjusted for each bull. The control quadrant (lower left, LL) was marked on isotype control stained samples to include < 1 % of cells as positive in the upper left (UL), upper right (UR) and lower right (LR) quadrants (Fig.1). Quadrant settings were adjusted for each bull. The LL, UL, UR and LR quadrants corresponded to antibody-negative live sperm, antibody-negative dead sperm, antibody-positive dead sperm and antibody-positive live sperm, respectively. For a more simplified analysis, the live sperm were gated and analyzed separately, using the lower left (live, non ASA-bound sperm) and lower right (live, ASA-bound sperm). A total number of 10,000 cells were analyzed from each sample at a rate of 1×10^3 to 2×10^3 cells/second using PBS as the sheath fluid. Data from these cells was collected using forward scatter as the trigger signal and a threshold of 48 of 1,024 channels.

One straw from each bull was thawed at 37 °C for 30 seconds, processed for flow cytometry and analyzed as described before to determine post-thawing percentage of ASA-bound sperm for IgG and IgA.

Statistical analysis

All numerical data were expressed as mean \pm SE unless stated otherwise. Level of significance was set at 5 %. All data analysis was performed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA). Pre-freezing antibody bound live sperm (IgG and IgA) was compared to post-thawing samples by means of a paired t-test statistic.

Results

Effect of freezing on percentage of ASA bound sperm

There was no significant difference on the percentage of antibody bound live sperm between pre- and post-freezing samples for IgG ($p= 0.1287$, Fig B.10) as well as IgA ($p= 0.4175$, Fig.B.11) on ejaculates from immunized bulls, using a commercial freezing protocol.

Discussion

Freezing-thawing seems not to affect ASA binding in sperm from bulls that have been immunized against sperm. This is in agreement with a previous trial in human sperm²⁸¹. Haas and Cunninham (1984) performed an experiment in which previously frozen and fresh sperm from infertile and fertile men were assayed for sperm-associated immunoglobulin G (IgG) to determine the effect of a simple freezing technique on the assay results. A direct radiolabelled antiglobulin assay (DRAA) and a direct fluorescein-labeled antiglobulin assay (DFAA) utilizing a fluorescein-activated flow cytometer were employed. The amount of IgG detected by the DRAA and the percentage of sperm that fluoresced in the DFAA generally declined when frozen sperm were used, but only the DFAA showed a statistically significant decrease. However, the

degree of decline was predictable ($r = 0.91$ in both assays), and whether the test remained positive or negative when frozen sperm were used was not significantly affected. Our trial, on the other hand, showed no significant difference for pre- and post-thawing samples for IgG and IgA. It is important to recognize the limitation of the number of bulls evaluated (4 bulls) and the fact that only one ejaculate from each bull was used for this trial.

Not only the low number of bulls used in the present study but also the high variability between bulls has to be taken into consideration. The variability on the ASA binding percentages could be also affected by freezing, as Ollero et al. (1998) showed that surface plasma membrane protein composition changed in bull sperm after freezing-thawing²⁸². Köss et al. (2008) also examined intact plasma membranes, high mitochondrial membrane potential, and positive acrosomal status before and after cryopreservation between bulls and between ejaculates within bulls²⁸⁰. These did not only vary between bulls, but also between ejaculates within bulls. The authors concluded that as there are no high relationships in these sperm parameters between times before and after cryopreservation, each ejaculate should be examined after cryopreservation in order to receive reliable information about the quality of cryopreserved sperm. It is expected that the levels of ASA bound sperm would follow the same rules, since they bind to proteins present on the sperm plasma and acrosomal membranes.

Chapter 5 - Conclusion

We were able to reproduce previous reports of induction of ASA in bulls, but in our report we proved the presence of antibodies (IgG and IgA) directly bound to sperm. We observed similar changes to what other authors have found regarding response to intramuscular injection of semen.

Flow cytometry, a technique of widespread use in veterinary medicine was adapted to the detection of anti-sperm antibodies directly bound to sperm in the bovine species. Our results showed good repeatability. There was no evidence of unspecific binding, even when the diluted formalin buffer solution was added into the equation. The use of an isotype control is warranted in any technique that involves flow cytometry.

Fixation does not seem to alter the results, being this very important in the case of the bovine veterinarian. Formalin buffer solution is the most common fixative used in the field for semen evaluation of other species and this is the reason why we selected it. Previous trials had shown that fixatives may affect outcome, and that is the reason why we elected to dilute the fixative. Fixation of cells for transport may be required to allow samples to be processed elsewhere. Further studies are required for evaluation of the effect of time on fixed samples.

Based on the results observed in satisfactory potential breeders during the project, and also found in our preliminary data, it seems that bulls have normally low percentages of ASA-bound sperm. The normal frequency of these antibodies is unknown since this is the first study evaluating ASA directly bound to sperm.

Samples for evaluation of ASA-bound sperm should be processed using either phosphate buffer solution or diluted formalin buffer solution, and only live cells should be evaluated for the analysis of percentages of IgG- and IgA-bound sperm, using labeled antibodies that only contain the F(ab)₂ portion of the molecule. The use of formalin buffer solution may add an additional step to the sample processing but its effect on overall time consumption is minimal. The technique is easy to perform, and requires moderate expertise and time. Flow cytometry has the advantage of being objective and evaluating a higher number of cells when compared with techniques that involve microscopy.

The importance of the use of frozen semen is related to the breeding techniques currently used in the bovine market. The bovine sperm is transported in liquid nitrogen and the ability to use this sample for assessment of immune mediated infertility would make the technique available for a greater spectrum of clients, allowing the samples to travel from one state to another, if a flow cytometer is not available nearby. Results suggest that freezing may not affect ASA load, although further research is needed in this field. A gold standard was not used for ASA detection. This is a great weakness of our study, since there was no corroboration of the presence of anti-sperm antibodies on the surface of the cells by other methods. Potential techniques to be used as for comparison could have been direct observation of the sperm under fluorescent microscopy, or confocal microscopy. Direct immunobead test would have been also appropriate, being this the most commonly used technique in human andrology for detection of anti-sperm antibodies. The antibody load and distribution on the sperm surface could have been assessed as well by means of calculation of fluorescence emitted by the sperm relative to molecules of fluorescent probes present per antibody.

Concluding, processing of sperm should include dilution in phosphate buffer solution or diluted formalin buffer solution, dead cells should be excluded from the analysis and the presence of antibody bound sperm can be assessed in frozen/thawed semen samples. Comparisons between aliquots have shown low variability, suggesting low overall assay variability. Good accuracy is expected based on our results. This protocol was able to detect the presence of IgG- and IgA-bound sperm in satisfactory potential breeders and immunized bulls, and to discriminate between antibody-positive and –negative bulls. This research project provided the building blocks necessary for further research in this field.

Chapter 6 - Bibliography

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Appendix A - Supplementary Tables

Table A.1 Description of processing of each aliquot from each ejaculate for evaluation of the effect of fixation and exclusion of dead cells from the analysis.

Aliquot	Fixation with FBS	FITC-labeled antibody	Treatment group
1	yes	No	Negative control IgGF
2	yes	Rabbit anti-goat IgG	Isotype control IgGF
3	yes	Goat anti-bovine IgG	Treatment IgGF
4	no	No	Negative control IgGN
5	no	Rabbit anti-goat IgG	Isotype control IgGN
6	no	Goat anti-bovine IgG	Treatment IgGN
7	yes	No	Negative control IgAF
8	yes	Rabbit anti-mouse IgA	Isotype control IgAF
9	yes	Rabbit anti-bovine IgA	Treatment IgAF
10	no	No	Negative control IgAN
11	no	Rabbit anti-mouse Ig	Isotype control IgAN
12	no	Rabbit anti-bovine IgA	Treatment IgAN

Table A.2 Description of processing of each aliquot for inter- and intra-assay variability analysis.

Aliquot	FITC-labeled antibody	Treatment group
1	no	Negative control IgG
2	Rabbit anti-goat IgG	Isotype control IgG
3 to 8	Goat anti-bovine IgG	Treatment IgG
9	no	Negative control IgA
10	Goat anti-mouse IgA	Isotype control IgA
11 to 15	Rabbit anti-bovine IgA	Treatment IgA

Table A.3 Effects fixation and exclusion of dead cells from the analysis of %IgG and %IgA.

Sperm-bound Immunoglobulin	Bull group	Treatment (Logarithm \pm SE (% \pm SE))			
		LF	LNF	LDF	LDNF
IgG	Immunized (n=4)	1.97 \pm 0.016 ^a (94.66 \pm 3.48)	1.96 \pm 0.01 ^a (92.78 \pm 4.02)	1.66 \pm 0.29 ^a (70.12 \pm 1.74)	1.56 \pm 0.32 ^a (60.1 \pm 19.87)
	Reproductively normal (n=4)	-0.04 \pm 0.12 ^a (1.01 \pm 0.29)	-0.09 \pm 0.10 ^a (0.86 \pm 0.17)	0.30 \pm 0.08 ^a (2.14 \pm 0.43)	-0.09 \pm 0.10 ^a (0.88 \pm 0.24)
IgA	Immunized (n=4)	1.89 \pm 0.04 ^a (79.99 \pm 7.51)	1.83 \pm 0.06 ^a (70.66 \pm 1.00)	1.30 \pm 0.22 ^b (29.64 \pm 4.33)	1.26 \pm 0.12 ^b (20.95 \pm 5.58)
	Reproductively normal (n=4)	0.19 \pm 0.11 ^b (1.75 \pm 0.45)	0.30 \pm 0.22 ^b (2.83 \pm 1.24)	1.08 \pm 0.18 ^a (15.43 \pm 5.80)	0.96 \pm 0.03 ^a (9.25 \pm 0.73)

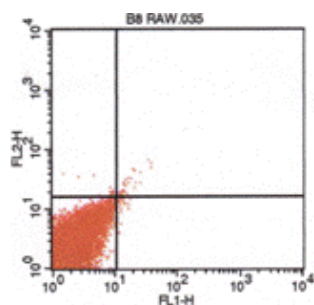
Percentage of IgG- or IgA-bound sperm from semen samples of immunized and reproductively normal bulls. Different superscripts mean statistical significance. (LF= Live cells and fixed; LNF= Live cells and not fixed; LDF= Live and dead cells, and fixed; LDNF= Live and dead cells, not fixed).

Table A.4 Inter- and intra-aliquot variability for IgG- and IgA-bound live sperm.

% CV	IgG		IgA	
	Inter-aliquot	Intra-aliquot	Inter-aliquot	Intra-aliquot
Immunized bulls	2.27	0.60	3.99	1.37
Reproductively normal bulls	9.74	6.57	8.74	4.02

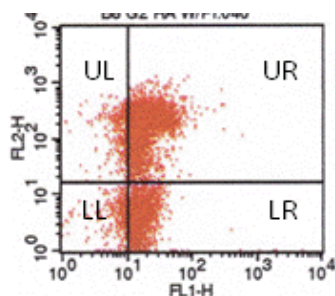
Percentage of coefficient of variation of the percentages of IgG- and IgA-bound live sperm in immunized bulls and reproductively normal bulls between (inter) aliquots and within (intra) the same aliquot.

Appendix B - Supplementary Figures



File: B8 RAW.035
 Sample ID: B8 RAW
 Patient ID:
 Acquisition Date: 08-Dec-08
 Gate: G3
 Gated Events: 8435
 Total Events: 10000
 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total
UL	20	0.24	0.20
UR	32	0.38	0.32
LL	8348	98.97	83.48
LR	35	0.41	0.35



File: B8 G2 RA W/PI.046
 Sample ID: B8 G2 RA W/PI
 Patient ID:
 Acquisition Date: 08-Dec-08
 Gate: G3
 Gated Events: 8633
 Total Events: 10000
 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total
UL	433	5.02	4.33
UR	5517	63.91	55.17
LL	433	5.02	4.33
LR	2250	26.06	22.50

Figure B.1 Example of the flow cytometric analysis for each sample.

Control quadrant marked on the unstained sample (Left). FITC- and PI-labeled sample from the same bull containing IgG-negative live sperm (LL), IgG-negative dead sperm (UL), IgG-positive dead sperm (UR) and IgG-positive live sperm (LR) (Right).

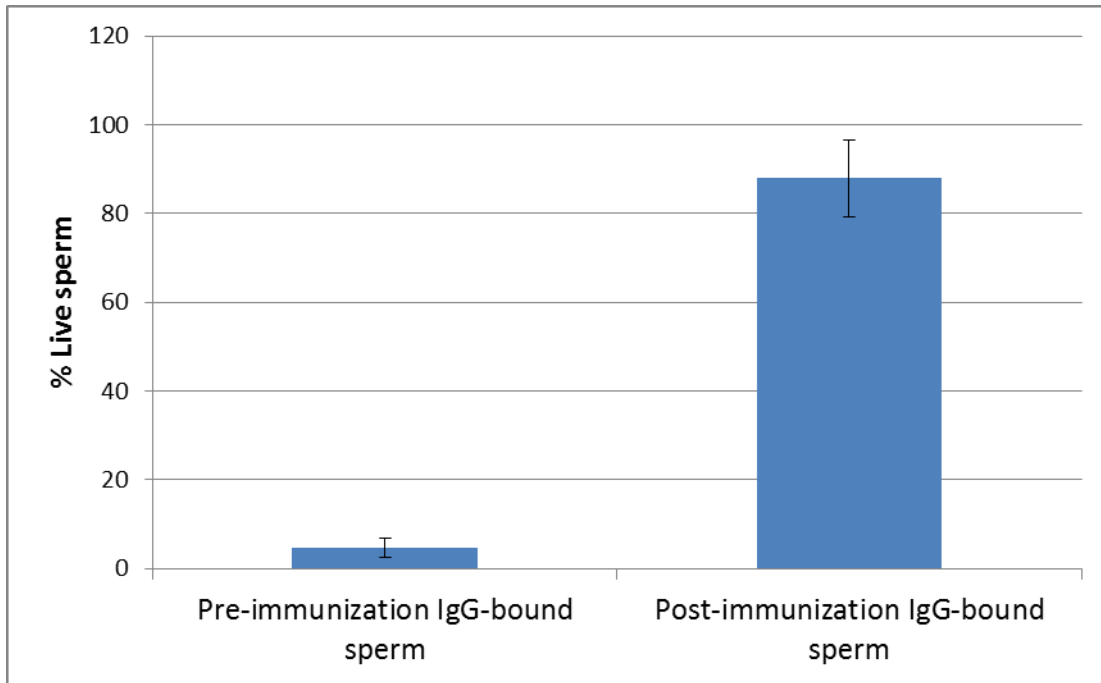


Figure B.2 Percentages of live IgG-bound sperm in experimental bulls before and after immunization.

The percentages of pre- and post- immunization IgG-bound live sperm in samples from experimental bulls are shown above. There was a significant increase in the percentage of IgG-bound live sperm after intramuscular injection of autologous semen ($p=0.0046$) (Mean \pm SE).

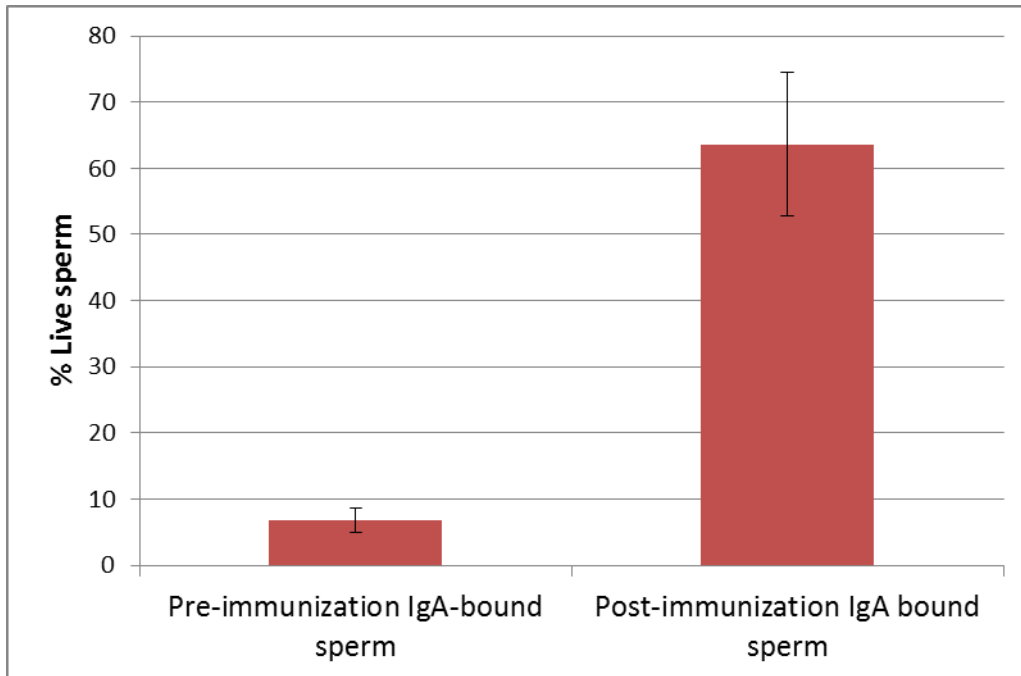


Figure B.3 Percentages of live IgA-bound sperm in experimental bulls before and after immunization.

The percentages of pre- and post- immunization IgA-bound live sperm in samples from experimental bulls are shown above. There was a significant increase in the percentage of IgA-bound live sperm after intramuscular injection of autologous semen ($p=0.0188$) (Mean \pm SE).

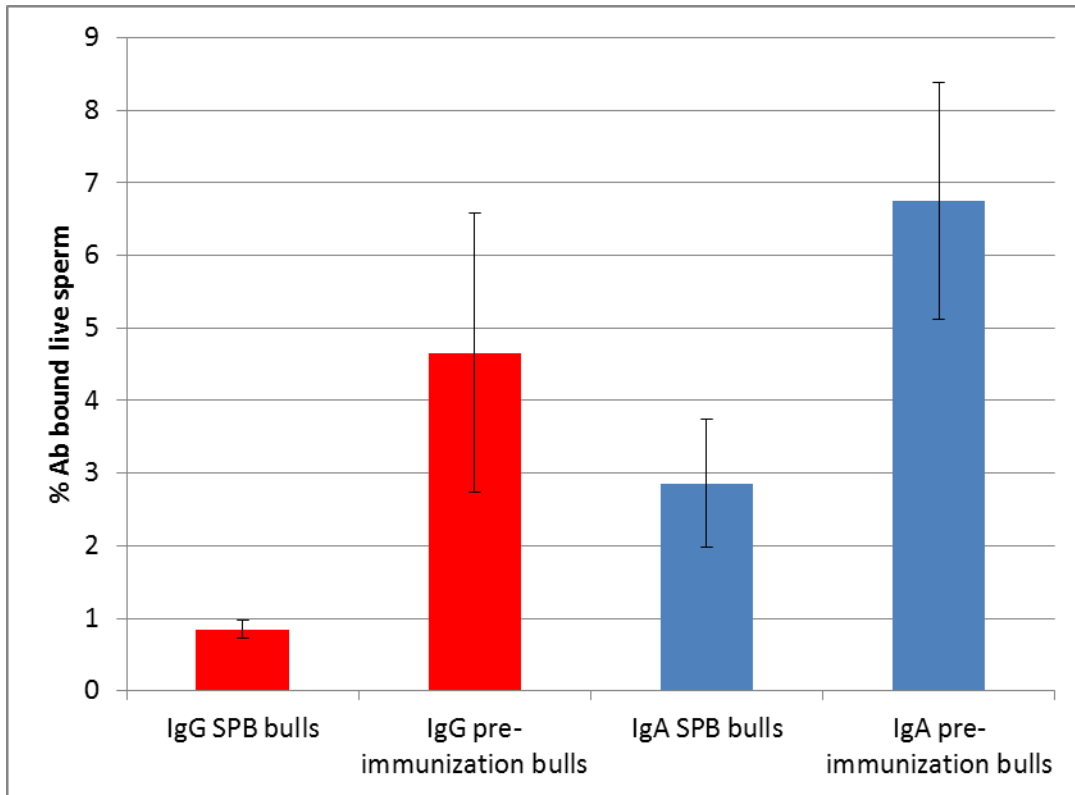


Figure B.4 Percentages of IgG- and IgA-bound live sperm in semen samples from satisfactory potential breeders (SPB) and pre-immunization experimental bulls.

There was no difference on the baseline percentages of IgG-bound live sperm ($p= 0.1748$) and IgA-bound live sperm ($p= 0.1230$) between semen samples from the two bull groups (Mean \pm SE).

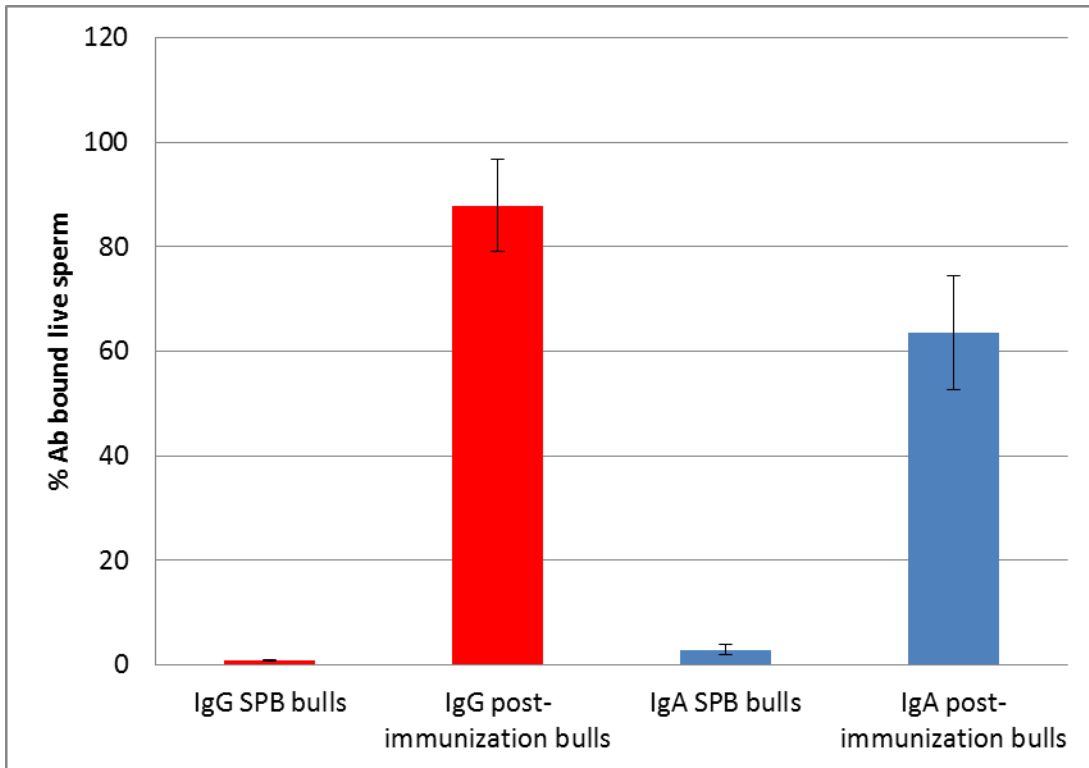


Figure B.5 Percentages of IgG- and IgA-bound live sperm in semen samples from satisfactory potential breeders (SPB) and post-immunization experimental bulls.

There was a significant difference when the percentages of IgG- ($p=0.0022$) and IgA- ($p=0.0110$) antibody bound live sperm in semen samples from reproductively normal bulls that passed the breeding soundness examination (classified as satisfactory potential breeders, SPB) and post immunization experimental bulls was compared (Mean \pm SE).

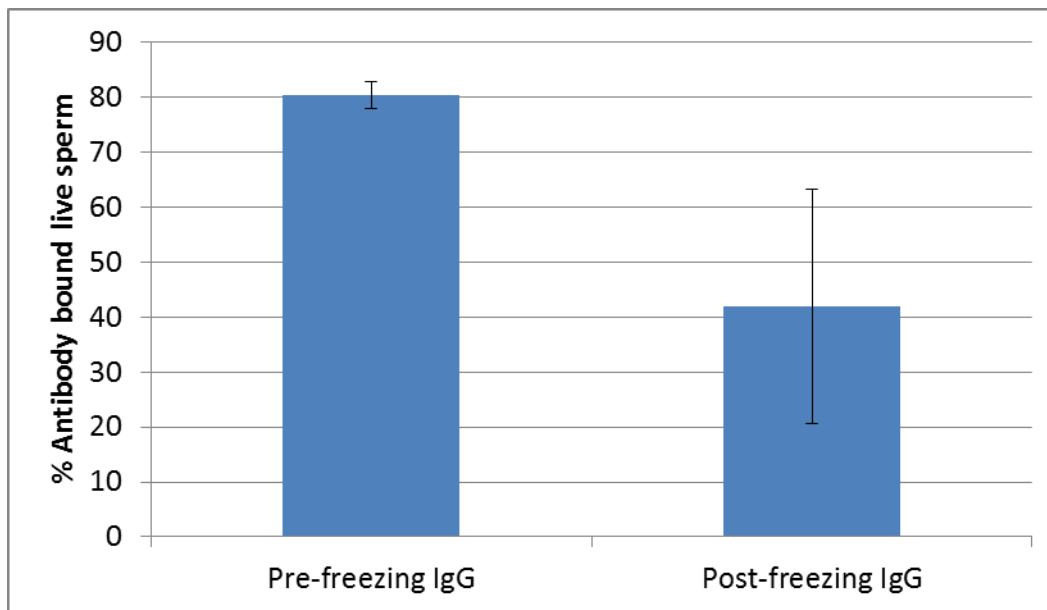


Figure B.6 Effect of freezing on the percentage of IgG-bound live sperm in semen samples from immunized bulls.

There was no significant difference on the percentage of antibody bound live sperm before and after freezing an ejaculate with a commercial freezing protocol for IgG ($p= 0.1287$) (Mean \pm SE).

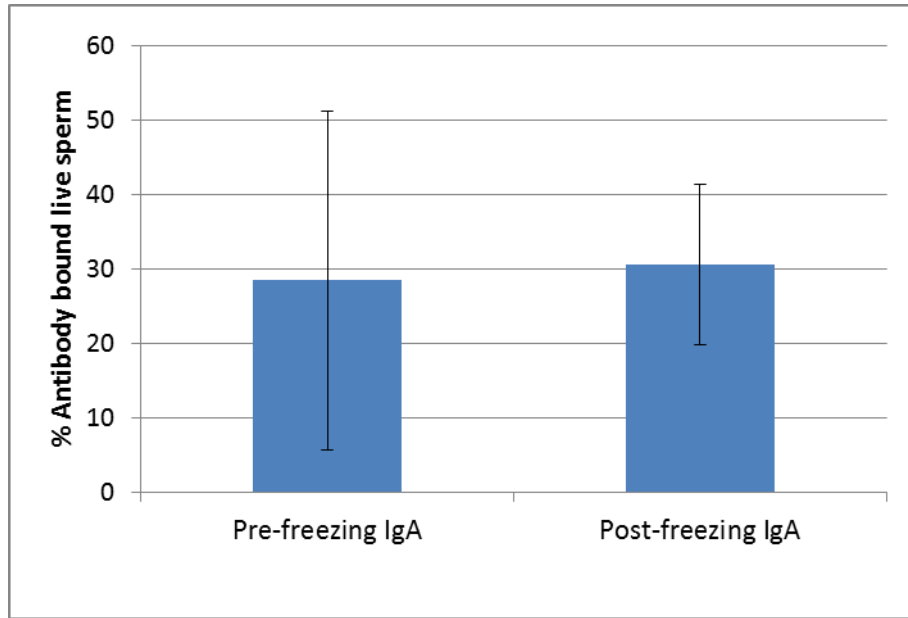


Figure B.7 Effect of freezing on the percentage of IgA-bound live sperm in semen samples from immunized bulls.

There was no significant difference on the percentage of antibody bound live sperm before and after freezing an ejaculate with a commercial freezing protocol for IgA ($p= 0.4175$) (Mean \pm SE).