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Standardization of a method to detect bovine sperm-bound anti-sperm antibodies by flow cytometry

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Abstract

The objectives of this study were to standardize some methodological and analytical aspects of a direct technique to detect sperm-bound anti-sperm antibodies (ASAs) in bovine semen using flow cytometry. Four ASA-positive bulls with experimentally induced ASAs and 10 reproductively normal ASA-negative bulls were included in the study. The effect of pre-fixation of sperm membranes with formalin buffer solution and inclusion of dead cells in the analysis was evaluated. Fixation of sperm membranes had no significant effect on the percentage of IgG- or

IgA-bound spermatozoa detected by flow cytometry. Including dead cells in the analysis increased the percentage of IgG-bound spermatozoa in fixed (live and dead 18.6 ± 9.7 % and live 1.3 ± 0.5 %) and non-fixed samples (live and dead 18.8 ± 9.2 %, live 1.5 ± 0.6 %) (P = 0.0029), as well as IgA-bound spermatozoa in fixed (live and dead 16.3 ± 6.4 %, live 0.3 ± 0.5 %) and non-fixed samples (live and dead 21.4 ± 4.6 %, live 1.0 ± 0.5 %) (P = 0.0041) (median ± SE) in semen from ASA-negative bulls. Intra-sample, intra-assay and inter-assay coefficients of variation (CV) for determination of sperm-bound IgG were 0.8 %, 4.6 % and 5.3 %, respectively. For determination of sperm-bound IgA, intra-sample, intra-assay and inter-assay CV were 2.8 %, 8.4 % and 40.3 %, respectively. In spite of the high inter-assay CV for IgA determination, all ASA-positive bulls had high percentages of IgA-bound spermatozoa at all times. Flow cytometry correctly identified ASA-positive bulls. Confocal laser microscopy confirmed the binding of ASAs to the sperm head and cytoplasmic droplets, and less frequently to the mid and principal piece. It was concluded that fixation was not necessary. Dead cells should be excluded from the analysis since ejaculates with large numbers of dead cells can yield false-positive results. Flow cytometry was accurate and reliable for detection of sperm-bound IgG and IgA and discrimination between ASA-positive and ASA-negative bulls.

Keywords: Flow cytometry; anti-sperm antibodies; sperm-bound antibodies; immunoinfertility; bovine

1. Introduction

During spermatogenesis, developing germ cells express new surface antigens that are not recognized as self. Sperm-specific surface antigens first appear on pachytene primary spermatocytes [1]. The blood-testis barrier (BTB), removal of antigenic apoptotic cells by phagocytosis and immunosuppressive factors released by Sertoli cells confer the testes an immune privileged status. Disruption of the BTB induced by infectious, inflammatory or degenerative conditions exposes sperm antigens to the immune system and results in formation of anti-sperm antibodies (ASAs) [2]. In bulls, genital infection with *Chlamydia sp*., *Brucella abortus* and Infectious Bovine Rhinotracheitis Virus was associated with concomitant presence of ASAs [3,4]. Antisperm antibodies were also detected in bulls with seminal vesiculitis [5] and orchitis [6]. The ASAs persisted in a bull with orchitis for 18 m after initial presentation [6]. Persistence of ASAs can account, at least in part, for the long-term effects of genital infection on fertility. Exposure to electromagnetic pulses was also shown to alter the BTB and result in formation of ASAs in mice [7]. Exposure to electromagnetic pulses from electric transmission lines, generators and fences could represent an unidentified risk factor for immune-mediated infertility in bulls.

Bovine ASAs can reduce penetration and fertilization of oocytes *in vivo* and *in vitro*, sperm-zona pellucida secondary binding, the ability of capacitated spermatozoa to complete the acrosome reaction and the motility of capacitated and non-capacitated spermatozoa [8-10]. Antisperm antibodies can impair fertility by contributing one more factor to an already compromised semen sample, or by being the primary cause of idiopathic infertility. Their effect on fertility depends on the location of the ASAs, their regional specificity, the antibody class, isotype and load, and the antigen specificity [11-13]. Antibodies directed against sperm antigens can be detected free in seminal plasma or serum. However, only those bound to the surface of spermatozoa are of significance for fertility [13]. Both IgA and IgG, but not IgM, have a proven negative effect on fertility [11,14]. Therefore, an ideal diagnostic test should be able to identify sperm-bound ASAs and provide information about the proportion of ASA-bound spermatozoa in an ejaculate, the antibody class and load, and the regional specificity [15].

To date, most reports in veterinary medicine have involved the use of indirect techniques to detect ASAs in serum or seminal plasma. Sperm agglutination [16,17] and immobilization tests [18] have been used to detect ASAs in bulls. However, these tests are insensitive and nonspecific [15]. Immunofluorescence [17], immunocytochemistry [3,19] and enzyme-linked immunosorbent assay [8,20] have also been used in bulls. These techniques require fixation of the cell membranes. 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 [15,21]. The mixed antiglobulin reaction and immunobead-binding tests are most commonly used in human medicine [22]. These tests provide a semi-quantitative estimation of the proportion of ASA-positive spermatozoa, and information on the antibody class and its location on the spermatozoa. However, both tests are based on counting motile spermatozoa bound to beads or latex particles. Therefore, the estimation is subjective. The tests require good sperm motility in the samples from the infertile patients if a direct test is used, or availability of a semen donor with good sperm motility if the indirect test is used [15]. Instead, flow cytometry allows objective and quantitative estimation of ASAs on the surface of living spermatozoa and is a sensitive, specific and repeatable test [15]. Flow cytometry also allows identification of the antibody class, isotype and load [15].

The use of flow cytometry to detect ASAs in bulls was only recently reported [6]. Moreover, a standardized direct technique to detect sperm-bound ASAs has not been developed in veterinary medicine. How the samples are processed and analyzed can have a significant impact on the reliability of the results. When analyzing live cells, cross-linking of surface antigens by multivalent antibodies, or of antigen-antibody (ag-ab) complexes by secondary antibodies can cause aggregation of ag-ab complexes into patches and caps [21,23]. Patching and capping is followed by shedding of the ag-ab complexes. Patching and capping can be prevented by fixing the cell membranes prior to incubation with antibodies [21,23]. However, as mentioned before, fixation can alter the membranes or antigens also giving misleading results [15,21]. Another source of error is nonspecific uptake of antibody by dead spermatozoa. Nonspecific binding can yield false-positive results if the proportion of dead cells in the ejaculate is high [15].

The objectives of this study were to standardize some methodological and analytical aspects of a direct technique to detect sperm-bound ASAs in bovine semen using flow cytometry. The effect of fixation and inclusion of dead cells in the analysis were evaluated, coefficients of variation for the standardized protocol were calculated and binding of ASAs to bovine spermatozoa was confirmed with confocal laser scanning microscopy.

2. Materials and Methods

2.1. Animals

Four 1-year old *Bos Taurus* bulls of Angus breed were purchased from local producers. The bulls were housed individually or in pairs in pens, and fed brome hay and water *ad libitum*, and 2 lb of sweet feed twice daily. Bulls were allowed to acclimate for one week prior to starting the experiments. To provide a known ASA-positive control, the bulls were immunized with autologous spermatozoa as described below. When the percentage of ASA-bound spermatozoa was ≥ 20 %, bulls were considered to have a positive response [24] and experiments were initiated. Additionally, ten privately owned Angus bulls (standardization of the technique, n = 5; calculation of coefficients of variation, n = 5) classified as satisfactory breeders during routine breeding soundness examination [25] were included as ASA-negative control bulls. Bulls were considered satisfactory breeders if they had no gross abnormalities of their internal and external genitalia, a scrotal circumference above the minimum recommended value for the age, ≥ 30 % individual sperm motility and ≥ 70 % morphologically normal spermatozoa [25]. The study was performed following Kansas State University's Institutional Animal Care and Use Committee's guidelines. The bulls with experimentally-induced antibodies were euthanized at the end of the study.

2.2. Semen collection and evaluation

Semen was collected using electroejaculation (SireMaster Original, ICE Corporation, Manhattan, KS, USA). The accessory sex glands were massaged transrectally with a gloved hand for 30 to 60 sec. A 6.5-cm in diameter lubricated rectal probe was inserted into the rectum with the electrodes facing ventrally. Electrical stimulation was applied with increasing intensity until ejaculation [25]. A complete semen evaluation [25] was performed immediately after collection. Semen was then used for immunization or for the experiments.

2.3. Immunization of bulls

Immunizations were performed as described before with some modifications [18]. Ejaculated spermatozoa were washed three times by centrifugation at 900 x g for 10 min diluted in warm Dulbecco's phosphate buffered saline (DPBS, Invitrogen, Grand Island, NY, USA). Washed spermatozoa, 1 x 10^9, were re-suspended to 1 mL in DPBS. One milliliter of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) was then added. Each bull was immunized with 2 mL of inoculum containing 1 x 10^9 autologous spermatozoa. The inoculum was administered intramuscularly in the neck in four different aliquots of 0.5 mL each. Booster immunizations were administered to three bulls 22 d after the primary immunization. Semen was processed in the same way as for primary immunizations but Freund's Incomplete Adjuvant (Sigma-Aldrich) was used instead of Freund's Complete Adjuvant. One bull did not receive a booster immunization since the response to the primary immunization was satisfactory.

2.4. Standardization of flow cytometry for detection of ASAs

The effect of fixing spermatozoa with formalin buffer solution prior to labeling on the ability to detect sperm-bound ASAs was evaluated. One ejaculate was collected from each bull with experimentally-induced ASAs (n = 4) and each ASA-negative bull (n = 5). Each ejaculate was initially divided into two aliquots. Semen was diluted to 50 x 10^6 spermatozoa /mL in DPBS (non-fixed samples) or formalin buffer solution (FBS, Animal Reproduction Systems, Chino, CA, USA) (fixed samples). Formalin buffer solution had been previously diluted 1:10 in DPBS. After 10 min at room temperature, samples were washed three times by centrifugation and labeled with fluorescein isothiocyanate (FITC)-labeled anti-bovine IgG or IgA, or their respective isotype control antibodies as described below. Samples were analyzed by flow cytometry. The percentage of IgG- and IgA-bound spermatozoa was calculated including the entire cell population (live and dead cells) or live cells only. Comparisons were made among treatment groups: non-fixed samples including live cells only in the analysis, non-fixed samples including both live and dead cells in the analysis, fixed samples including live cells only in the analysis, and fixed samples including live and dead cells in the analysis.

2.5. Calculation of coefficients of variation

It was determined in the previous experiment that fixation was not necessary and that including dead cells in the analysis yielded false-positive results. Therefore, non-fixed samples were used and only live cells were included in the analysis for calculation of coefficients of variation (CV). One ejaculate from each ASA-negative (n = 5) and ASA-positive bull (n = 4) was divided into five aliquots and processed in five replicates to calculate intra-assay CV. One of the aliquots was evaluated five times to assess intra-sample CV. Only semen from ASA-positive bulls was available for assessment of the inter-assay CV. Inter-assay CV was calculated retrospectively from two ejaculates collected from each bull 6 to 20 d apart. The CVs were calculated with the following formula: CV (%) = mean of standard deviations / mean x 100.

2.6. Antibody labeling

Semen was diluted to a concentration of 50 x 10^6 spermatozoa /mL in warm DPBS, and was washed three times by centrifugation at 900 x g for 10 min in DPBS. Then, 2.5 x 10^6 of washed spermatozoa were added to each of four tubes containing 320 µL of DPBS. The corresponding antibodies were added to each tube: IgG = 30 µL of FITC-labeled polyclonal goat anti-bovine IgG F(ab')$_2$ (12.5 µg/mL; Cat. No. 101-096-003, Jackson Immunoreseach Laboratories Inc., West Grove, PA, USA); IgG isotype control = 30 µL of FITC-labeled polyclonal rabbit anti-goat IgG F(ab')$_2$ (12.5 µg/mL; Cat. No. 305-096-003; Jackson Immunoreseach Laboratories Inc.); IgA = 20 µL of FITC-labeled polyclonal rabbit anti-bovine IgA (12.5 µg/mL; Cat. No. A10-108F; Bethyl Laboratories, Montgomery,TX, USA); or IgA isotype control = 20 µL of FITC-labeled polyclonal goat anti-mouse IgA (12.5 µg/mL; Cat. No. A90-103F; Bethyl laboratories). A preliminary study was performed to evaluate saturating concentrations and select the appropriate concentration of each antibody (data not shown). The samples were incubated for 30 min at room temperature in the dark, followed by three washes by centrifugation at 900 x g for 10 min in DPBS. Propidium iodide (PI, viability stain), 5 µL, was then added for simultaneous staining of dead cells.

2.7. Flow Cytometry

The percentage of IgG- and IgA-bound spermatozoa was assessed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose CA, USA). From each sample, 10 000 cells were analyzed at a rate of 1 to 2 x 10^3 cells /sec using DPBS as the sheath fluid. Data from these cells were collected using forward scatter as the size parameter. A gate containing spermatozoa was selected based on dot plot distribution of forward (size) versus side scatter (complexity parameter) to eliminate debris and epithelial cells from the analysis (Fig. 1). The FITC and PI signals were detected using a standard argon laser (488 nm) and emission filters (535 ± 30 nm for FITC and 585 ± 30 nm for PI). The instrument was calibrated daily with standard beads so that the CV of the forward scatter and fluorescence channels were < 5 % on a daily basis. Compensation for FITC emission into the PI detector or vice versa was done by establishing quadrants on spermatozoa labeled only with PI or FITC-conjugated antibodies, followed by electronic substraction of the FITC emission into the PI detector and PI emission into the FITC detector. After color compensation, fluorescence emission data were collected with logarithmic amplification for green fluorescence (FITC using FL1 detector) and orange-red fluorescence (PI using FL2 detector). Quadrant settings were adjusted for each sample. The control quadrant (lower left, LL) was marked on samples labeled with the isotype control to include < 1 % of cells as positive in the upper left (UL), upper right (UR) and lower right (LR) quadrants (Fig. 1). The ASA-negative dead cells (PI stained) appeared in the UL quadrant, ASA-negative live cells (no stain) in the LL quadrant, ASA-positive dead cells (dual stained) in the UR quadrant, ASA-positive live cells (FITC stained) in the LR quadrant (Fig. 1). The percentage of ASA-positive live spermatozoa (LR quadrant) was calculated considering only live cells (PI negative cells in LL and LR quadrants) in the analysis. When including dead cells (PI positive cells), the percentage of ASA-positive spermatozoa (LR and UR quadrants) was calculated considering all quadrants.

2.8. Confocal laser scanning microscopy

Labeled spermatozoa from bulls with experimentally-induced antibodies were evaluated under confocal laser scanning microscopy to confirm binding of the antibodies to the sperm surface. No attempts were made to quantitatively evaluate the percentage of ASA-bound spermatozoa or the relative distribution of the binding sites. Spermatozoa were labeled with FITC-labeled anti-bovine IgG or IgA as described above. After labeling, 10 µL of FBS was added to inhibit sperm motility and facilitate visual evaluation. A drop of sperm suspension was evaluated on a microscope slide under a cover slide. The FITC signal was excited at 488 nm and was collected with a band pass filter at a wavelength of 505-550 nm. Samples were assessed at X20 and X40 and optical sections were collected (LSM 710 META, Carl Zeiss MicroImaging, Thornwood, NY).

2.9. Statistical analysis

Statistical analysis was performed using SAS package (SAS Institute, Cary, NC, USA). Distribution of the data was tested for normality using a Shapiro Wilk test. Data were not normally distributed. To determine response to immunization, percentages of IgG- and IgA-bound spermatozoa before and after the last immunization were compared using a Wilxocon signed test. Only non-fixed live spermatozoa were included in this analysis. To assess the effect of fixation and inclusion of dead cells in the analysis, differences in median percentages of IgG- and IgA-bound spermatozoa among treatment groups were compared using a Friedman test. The Friedman test is a non-parametric test that compares median values across treatments controlling for bull. Since non-parametric tests were used, data were reported as median ± SE. Differences were considered significant at $P < 0.05$.

3. Results

Immunization with autologous spermatozoa induced a significant increase in the percentage of both IgG-bound spermatozoa and IgA-bound spermatozoa. The percentage of IgG-bound spermatozoa was 2.9 ± 2.1 % and 89.8 ± 4.6 % before and after immunization, respectively (P = 0.0209). The percentage of IgA-bound spermatozoa was 7.7 ± 2.2 % and 75.7 ± 18.9 % before and after immunization, respectively (P = 0.0433) (median ± SE).

There was no significant difference in the percentage of IgG- or IgA-bound spermatozoa between samples fixed with FBS and non-fixed samples (Fig. 2). Including dead cells in the analysis increased the percentage of IgG- (P = 0.0029) and IgA-bound spermatozoa (P = 0.0041) detected in semen samples from ASA-negative bulls (Fig. 2). However, median percentages of ASA-bound spermatozoa did not differ among semen samples from ASA-positive bulls when dead cells were included in the analysis (Fig. 2).

Intra-sample CV for determination of sperm-bound IgG was 0.8 %, intra-assay CV was 4.6 % and inter-assay CV was 5.3 %. For determination of sperm-bound IgA, intra-sample CV was 2.8 %, intra-assay CV was 8.4 % and inter-assay CV was 40.3 %. Both antibody classes bound to the acrosomal, equatorial and post-acrosomal areas of the sperm head, and to cytoplasmic droplets (Fig.3). Least frequently, ASAs bound to the sperm midpiece and principal piece.

4. Discussion

Systemic immunization with autologous spermatozoa induced an immune response in all bulls characterized by an increase in sperm-bound IgG and IgA. Immunoglobulin G in genital secretions is mostly derived from systemic circulation [26]. In the presence of an intact blood-testis or blood-epididymis barrier, IgG reaches the genital tract and binds to spermatozoa at the rete testis or at ejaculation when spermatozoa contact the secretions of the accessory sex glands [27-29]. On the other hand, IgA is produced locally [26]. Systemic immunization can result in increased production of IgA within the genital tract, and increases in antigen-specific B cells in the testis [29]. It is possible that systemic immunization of bulls with spermatozoa induced both a systemic and mucosal immune response here. It is also possible that migration of activated IgA-committed B cells from lymph nodes draining the injection site to the genital mucosa contributed to the increase in sperm-bound IgA after immunization, as described in humans [30].

Recommendations for processing and evaluating bovine semen samples for detection of sperm-bound ASAs by flow cytometry can be made based on the results of this study. Polyclonal antibodies and $F(ab')_2$ fragments were used here. Since they are expected to react with all subclasses, use of polyclonal antibodies may decrease the likelihood of obtaining false-negative results [15]. Use of $F(ab')_2$ fragments is also preferred to prevent non-immune binding of the Fc portion of the IgG molecule to the sperm membrane [15], which occurs via disulfide rearrangement at the cell surface in bulls [31]. Fixation of sperm membranes with formalin buffer solution prior to labeling did not affect the ability to detect sperm-bound ASAs. Fixation was performed to potentially prevent patching or capping of ag-ab complexes, which would have yielded false-negative results. Mature spermatozoa have both mobile and non-mobile surface antigens [32,33]. Patching and capping involve redistribution of mobile antigens in response to multivalent ligands. Patching is a local clustering of molecules, while capping is the aggregation of the clusters to a single area of the membrane. Following capping, molecules are shed from the cell membrane [23]. While these phenomena were demonstrated in early spermatogenic cells [1], patching and capping were not observed in late spermatids [1] or mature spermatozoa [34]. It was speculated that non-mobile antigens are inserted later in germ cell development, and that cross-linking between mobile and non-mobile antigens results in loss of capping in late spermatids [1] and spermatozoa. While fixation may be necessary to prevent lateral mobility of antigens, it can also alter the results by causing non-specific binding of antibodies, exposure of intracellular antigens, denaturation of sperm antigens and membrane damage [15,21]. It was concluded that since fixation of sperm membranes prior to labeling did not affect the results but increased processing times, this procedure could be avoided.

When dead cells were included in the analysis, false-positive results were obtained in samples from ASA-negative bulls. It is likely that non-specific binding of antibodies to dead cells or increased autofluorescence displayed by dead cells accounted for the increase in the percentage of fluorescently-labeled spermatozoa among ASA-negative bulls [15,35]. In ASA-positive bulls, the percentage of ASA-bound spermatozoa was already high. Even when non-specific binding to dead cells may have occurred, the difference may not have been large enough to be significant. It was concluded that dead cells should be excluded from the analysis to prevent false-positive results in ASA-negative bulls. This limits the use of flow cytometry to detect ASAs in bulls with necrozoospermia.

Coefficients of variation were all < 10 %, except for inter-assay CV for IgA-bound spermatozoa. It is not known if this high CV resulted from the low number of samples available, or from different frequencies of ejaculation that resulted in varying storage times and contact with ASA-loaded genital secretions. It is also possible that the variation reflected changes in antibody titers at different times post-immunization and was inherent to the model used rather than the test itself. The reason for the high inter-assay CV of the IgA test requires further investigation with more standardized sampling times. Nonetheless, the percentage of IgA-bound spermatozoa was ≥ 20 % in all samples from all ASA-positive bulls. In spite of the high inter-assay CV, the test was able to correctly identify IgA-positive bulls. With this exception, CVs in this study were similar to those reported in the human literature [15]. It was concluded that flow cytometry was accurate and reliable for detection of sperm-bound ASAs and discrimination between ASA-positive and ASA-negative bulls.

Confocal laser microscopy confirmed binding of ASAs to the sperm surface. The combination of flow cytometry and fluorescence microscopy provided an ideal diagnostic approach. Flow cytometry allowed identification of sperm-bound ASAs and provided objective and quantitative information about the antibody class and load. Additional use of fluorescence microscopy provided information about the regional specificity of the ASAs. Due the lack of reports on presence and behavior of naturally-occurring sperm-bound antibodies in bulls, it is difficult to determine how detection of experimentally-induced antibodies compares with detection of sperm-bound ASAs produced during bacterial infection or trauma. Studies are under way to determine the reference ranges and prevalence of naturally-occurring sperm-bound ASAs in satisfactory breeder beef bulls and bulls with reproductive pathology.

In conclusion, a direct technique to detect sperm-bound ASAs in bull semen was developed. Flow cytometry was accurate and reliable for detection of sperm-bound ASAs and discrimination between ASA-positive and ASA-negative bulls. When combined with fluorescence microscopy, this method provided an ideal diagnostic approach for objective and quantitative evaluation of sperm-bound ASAs in bulls.

Disclosure statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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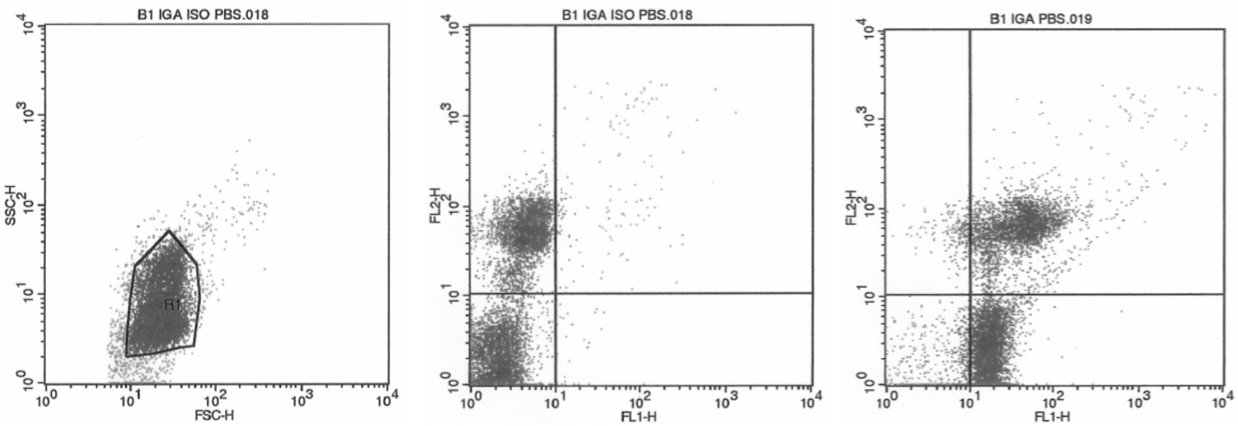

Fig. 1. Example of dot plot distribution of forward (FSC-H) and side scatter (SSC-H) of a washed sperm sample (left panel). The cells within gate 1 (R1) represent the population of spermatozoa. Example of dot plot distribution of two-color analysis of a sperm sample from a bull with experimentally-induced anti-sperm antibodies stained with FITC-labeled anti-mouse IgA (isotype control) (central panel) or FITC-labeled anti-bovine IgA (right panel). Fluorescence data was collected with logarithmic amplification for green (FITC; FL1-H) and red (PI; FL2-H) fluorescence. The anti-sperm antibody (ASA)-negative dead sperm appeared in the upper left (UL) quadrant, ASA-negative live sperm in the lower left (LL) quadrant, ASA-positive dead sperm in the upper right (UR) quadrant, and ASA-positive live sperm in the lower left (LR) quadrant.

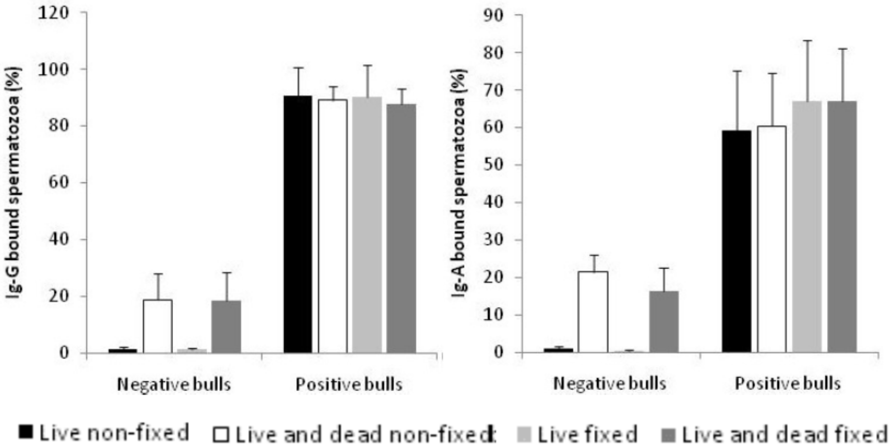

Fig. 2. Percentage of anti-sperm antibody (ASA)-bound spermatozoa in samples fixed with formalin buffer solution and non-fixed samples, and including live only or live and dead cells in the analysis. [a,b]Values with different superscript differ significantly among treatments within ASA-negative bulls (Median ± SE).

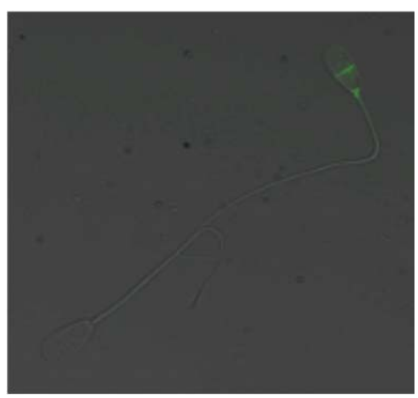

Fig. 3. Antibody-negative spermatozoa (left) and spermatozoa with IgG binding to the equatorial area and the junction between the sperm head and midpiece (right).