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Increased Conception Rates in Beef Cattle Inseminated with Nanopurified Bull Semen[1]

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ABSTRACT

Aberrant sperm phenotypes coincide with the expression of unique sperm surface determinants that can be probed by objective, biomarker-based semen analysis and targeted as ligands for semen purification. This study evaluated a nanoparticle-based magnetic purification method that removes defective spermatozoa (~30% of sample) from bull semen and improves sperm sample viability and fertilizing ability in vitro and in vivo. Two types of nanoparticles were developed: a particle coated with antibody against ubiquitin, which is present on the surface of defective spermatozoa, and a particle coated with the lectin peanut agglutinin, which binds to glycans exposed by acrosomal damage. In a 2 yr artificial insemination field trial with 798 cows, a conception rate of 64.5% ± 3.7% was achieved with a 10×10^6 sperm dose of peanut agglutinin-nanopurified spermatozoa, comparable to a control nonpurified full dose of 20×10^6 spermatozoa per dose (63.3% ± 3.2%) and significantly higher than a 10×10^6 sperm dose of nonpurified control semen (53.7% ± 3.2%; $P < 0.05$). A total of 466 healthy calves were delivered, and no negative side effects were observed in the inseminated animals or offspring. Because the method is inexpensive and can be fully integrated in current protocols for semen cryopreservation, it is feasible for use in the artificial insemination industry to improve fertility with reduced sperm dosage inseminations. Spermatology will benefit from nanopurification methodology by gaining new tools for the identification of candidate biomarkers of sperm quality such as binder of sperm protein 5 (BSP5), described in the present study.

artificial insemination, conception rate, fertility, livestock, nanoparticle

INTRODUCTION

Samples of semen of eutherian mammals, including cattle, contain spermatozoa of varying levels of fertility. It has been increasingly noted that certain types of abnormal spermatozoa express unique cell surface constituents that are not found in morphologically normal spermatozoa capable of fertilizing oocytes [1–5]. Such sperm surface constituents, dubbed negative biomarkers of sperm quality and fertility [1], can be detected using specific probes and ultimately targeted as ligands for semen purification [6]. For example, some surface proteins of aberrant spermatozoa become ubiquitinated in the epididymis as part of an internal quality control system, [2, 4, 7]. Ubiquitination occurs via an apocrine secretory mechanism in which apical blebs detach from the surface of epididymal epithelial cells and deliver to spermatozoa unconjugated ubiquitin and the enzymes necessary for its covalent ligation to sperm surface proteins [2]. Protein ubiquitination is a stable covalent posttranslational protein modification that most often occurs by tandem ligation of one or more multiubiquitin chains to the substrate protein [8]. Defects detected in the surface-ubiquitinated bull spermatozoa include abnormal morphology, DNA fragmentation [9], and acrosomal damage [10] as well as the dysregulation and ectopic accumulation of the fertility associated proteins PAFR [11] and PAWP [12].

In addition to ubiquitin, ligands of lectins such as PNA (peanut agglutinin from *Arachis hypogaea*), PSA (*Pisum sativum* agglutinin), and *Lens culinaris* agglutinin, are either more abundant or present exclusively on the surface of defective spermatozoa compared to normal spermatozoa capable of fertilizing oocytes [10, 13]. Ubiquitin and other molecules present on a defective sperm surface can therefore be exploited to measure and grade bull fertility/semen quality. This novel approach to sperm phenotyping is now referred to as the negative marker approach to sperm quality/male fertility evaluation [1]. Furthermore, probes used as negative markers can be adapted for use in removing defective spermatozoa from semen used for artificial insemination (AI) in cattle.

The use of synchronized ovulation and fixed-timed AI in breeding programs has necessitated improved pregnancy rates in the U.S. cattle industry [1]. However, accurate assessment and prediction of bull fertility in natural service or AI is lacking. An additional increase in conception rates (CRs) could be achieved by improving measures of bull fertility/semen quality, particularly in AI sires with acceptable but suboptimal

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sperm quality. Also, the introduction of genomic selection to cattle breeding has resulted in a shift in market demand toward sires of younger ages. To satisfy this market demand, AI centers might require technologies that allow timely estimates of sire fertility before males reach puberty as well as more efficient sperm utilization and extension rates [14]. These technologies should increase CRs after AI and allow for the reduction of sperm number per AI dose, thus increasing the numbers of AI doses from bulls with valuable genomes [15]. Such purification methods could also allow bull studs to salvage spermatozoa from suboptimal semen collections from bulls with high genetic value.

Current standard sperm preparation techniques depend on sedimentation or a migration approach to isolate fertile spermatozoa based on their density or motility, thus improving the fertility of the sample. Nevertheless, biochemical properties of abnormal spermatozoa, such as lack of plasma membrane integrity, sperm surface ubiquitination, and abnormal surface glycosylation patterns [10], have been overlooked in developing these procedures. Removing spermatozoa based on these abnormalities could improve the fertility of the sample. One way to remove biochemically aberrant spermatozoa is to use magnetic beads coated with probes (antibodies or lectins) for specific surface markers; aberrant spermatozoa can then be pulled out of a sample using magnetic force. Magnetic sperm separation offers advantages of simplicity of operation, low cost, and specificity and sensitivity afforded by use of specific probes [16]. However, a disadvantage that limits magnetic bead efficiency is the limited surface area to which cells can bind on a round particle. Consequently, small nanoparticles with flat, rather than spherical shapes have been enriched with ferritin to increase surface area and binding and thus improve sperm purification efficiency. While such particles are currently widely used for blood and stem cell separation, they have not been tested for sperm purification. Nanotechnology based on magnetic separation of abnormal spermatozoa and their fragments by antibody and lectin-conjugated nanoparticles might provide a simple and useful alternative/addition to sperm purification methods that are currently in use.

Nanotechnology is defined as science, engineering, and technology conducted at a scale of 1–100 nm [17], and is used widely in electronics, energy production, medicine, and increasingly in agriculture. Nanoparticles are ultrafine particles of 1–100 nm created by a chemical or physical process that have found a variety of uses in medicine and biology that are related to the fields of reproductive biology [6]. Thus far, the potential use of magnetic nanoparticles for semen purification has been unexplored as an alternative to sedimentation-, filtration-, and centrifugation-based semen purification techniques.

The objective of experiments described here was to enhance bull semen quality by removing defective and/or prematurely capacitated spermatozoa prior to cryopreserving semen for AI. To achieve this depletion with anti-ubiquitin antibody and/or lectin-conjugated magnetic particles, we have developed and validated an industrial scale protocol for magnetic depletion of defective spermatozoa from bull semen, prior to dilution of semen in cryoprotectants. We have also utilized proteomic analysis of the supernatant and pellet fractions obtained by nanopurification to further identify potential differences in protein makeup of normal and defective spermatozoa in these fractions.

MATERIALS AND METHODS

Nanoparticles and Magnetic Separator

Nanoparticles coated with lectins and antibodies showing high affinity to cell surface determinants of defective spermatozoa were prepared for experiments aimed at the depletion of defective spermatozoa from freshly collected semen for field AI trials or at the depletion of defective spermatozoa from frozen-thawed semen for laboratory analyzes and in vitro fertilization (IVF). Two types of nanoparticles were developed: particles coated with a monoclonal anti-ubiquitin antibody (clone MK-12-3; MBL Co.) [2] and particles coated with the lectin PNA/PSA (Sigma) that binds to glycans exposed by the remodeling of the sperm acrosome caused by premature capacitation or acrosome reaction or by mechanical, osmotic, and/or cryogenic damage to the acrosome [10]. Irregular-shaped magnetite F_2O_3 core nanoparticles were conjugated to the above lectins or antibodies and washed stringently following an undisclosed proprietary protocol of the manufacturer (Clemente Associates, www.clemente-associates.com). Particles were purchased and delivered at a concentration of 0.1 mg/ml of 10 mM phosphate buffer without sodium azide, with $\sim 10^{15}$ particles/g and surface area > 24 m^2/g. Nanopurification was performed on an msp Magnetic Separator with three permanent magnets fixed in polycarbonate (Clemente Associates) (Fig. 1, a–c). Enrichment of ubiquitin- and PNA-positive spermatozoa in the pellet fraction after nanopurification was confirmed by immunofluorescence (Fig. 1d).

Nanopurification of Cryopreserved Semen for In Vitro Analyses and IVF

The IVF trial was conducted with cryopreserved semen that was nanopurified after thawing. The goal was to establish whether the nanopurification of extended, frozen-thawed semen had a positive effect on sperm fertilizing potential reflected by fertilization rates in vitro. These experiments were performed before the field trials and provided baseline information on nanoparticle concentration and incubation times that was useful for the development of nanopurification procedure for field AI use. Semen samples from different collection dates (n = 10/bull from 6 bulls) were randomly chosen from existing stocks of cryopreserved semen available at Select Sires Inc. Samples were thawed at 37°C for 1 min, pooled per bull, and diluted with equal volumes of PBS (1.5 ml sample + 1.5 ml PBS composed of 8 g NaCl, 0.2 g KCl, 0.26 g K_2HPO_4, and 0.5 g NaN_3 per liter of ultrapure water, pH 7.2). Each pooled sample was assigned to the following nanopurification treatments in duplicate: ubiquitin-binding nanoparticles, lectin PNA nanoparticles, ubiquitin-binding nanoparticles + lectin PNA nanoparticles, control with untreated nanoparticles, or control with no particles (not nanopurified). Each 750 μl of the pooled samples was incubated at room temperature with 2 ml warm PBS (37°C) and 50 μl magnetic particles for 30 min on a Dynal rotator set at the lowest possible speed. Thereafter, the samples were placed on magnets and allowed to sediment for 15 min at room temperature. Control samples without nanoparticles were kept under the same conditions as the nanopurified samples for the entire duration of the experiment. Two sperm fractions were obtained from the supernatant solutions of the treated samples after nanopurification: Supernatant fractions (SFs) were obtained from the top half of the depleted samples while the particle fractions (PFs) obtained from the bottom of the supernatant without disturbing the magnetic particles. Spermatozoa from these fractions and control samples were concentrated by centrifugation at $2000 \times g$ for 5 min at room temperature in a microcentrifuge and resuspended in 100 μl PBS.

Immunofluorescence

Immunofluorescence was used to confirm the enrichment of surface ubiquitinated-defective spermatozoa by the anti-ubiquitin-conjugated nanoparticles and enrichment of spermatozoa with damaged acrosomes by the PNA-conjugated nanoparticles in the pellet fractions obtained by nanopurification of both frozen-thawed and freshly collected semen. As will be detailed later, immunofluorescence was also used to characterize the phenoptypes of BSP5 protein in normal and defective spermatozoa. Control, pellet, and SFs obtained from the previous step were incubated with MK-12-3 at room temperature for 1 h using the following dilutions: 9.2 μl sample (1:200) + 390 μl PBS + 0.8 μl MK-12-3 (1:250). This antibody recognizes multiubiquitin chains present on the surface of bull spermatozoa with gross morphological defects [4], spermatozoa with fragmented DNA [9], as well as spermatozoa with damaged acrosomes [10] or abnormal content of fertility-associated sperm proteins PAFR [11] and PAWP [12]. Thereafter, samples were incubated with a fluorescein isothiocyanate (FITC)-labeled secondary goat antibody against mouse immunoglobulin G (GAM-IgG-FITC; Zymed/Invitrogen) at room temperature for 30 min. Nonimmune mouse serum was used in place of MK-12-3 for negative controls. Fluorescence properties were evaluated using EasyCyte Plus flow cytometer (Guava Technologies) operating on a Cytosoft platform (IMV Technologies). The photomultiplier tube voltages during acquisition were set at 500 V for forward scatter (FSC) and 485 V for fluorescein fluorescence, and the threshold value was set at 10 units of FSC.

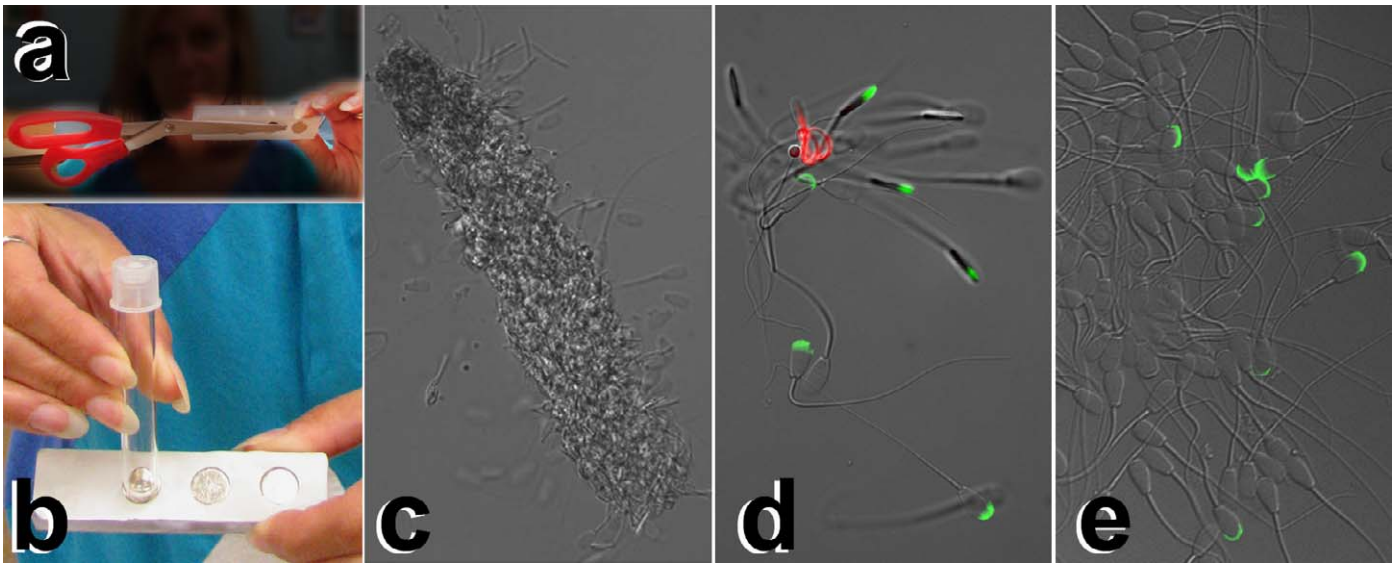

FIG. 1. Magnetic nanopurification of defective spermatozoa. **a**) An msp Magnetic Separator with three permanent magnets fixed in polycarbonate was used to attract the nanoparticle-coated defective sperm to the bottom of collection tube. **b**) Placement of collection tube on top of magnet. Floating polystyrene racks fashioned out of Falcon tube packaging racks were used to hold magnets with tubes inside a water incubator during fresh semen nanopurification. **c**) Discarded pellet of defective spermatozoa, sperm fragments, and nanoparticles. **d**) Spermatozoa from the discarded pellet fraction labeled with fluorescein-conjugated lectin PNA (green; labels damaged acrosomes) and anti-ubiquitin antibody MK-12-3 (red; labels defective sperm surface). The same anti-ubiquitin antibody was used to prepare the ubiquitin binding nanoparticles. **e**) Spermatozoa from the recovered SF are mostly morphologically normal. Note that only eight spermatozoa are positive for lectin PNA (green; acrosomal damage). Original magnification ×400 (**c**) and ×600 (**d** and **e**).

The sample flow rate was set at medium (0.59 μl/sec). Control, pellet, and SFs were screened and photographed by using a Nikon Eclipse E800 epifluorescence microscope equipped with differential interference contrast optics, charge-coupled device camera, and MetaMorph image-acquisition system. Images were edited by Adobe Photoshop CS5.

Flow Cytometric Testing of Nanopurified Sperm Fractions for Sperm Viability, Ubiquitination, Count, and Cell Population Homogeneity

Flow cytometry was used for the analysis of sperm viability and mitochondrial potential and for the quantification of defective sperm biomarkers (ubiquitin and lectin ligands) in normal and defective sperm fractions obtained by nanopurification of frozen-thawed semen. Sperm viability was evaluated from the control and SFs using the live/dead cell viability kit (Invitrogen). Briefly, sperm samples were diluted in HEPES-buffered saline-bovine serum albumin (10 mM HEPES-buffered saline and 20 mg/ml BSA; 1:20) and incubated with 5 μl of dilute $SYBR_{14}$ (1:50) at 37°C for 10 min. The samples were then counterstained with 5 μl of propidium iodide (PI) at 37°C for 10 min. Sample aliquots (1:20) were loaded on to 96-well plates and evaluated for DNA fluorescence by using the EasyCyte Plus flow cytometer. To determine viability, the plots were first gated and spermatozoa selected on the basis of their size (FSC) and green fluorescence ($SYBR_{14}$ staining of live nucleated cells). The resultant plots were further gated by green fluorescence and red fluorescence (PI staining incorporated exclusively in the nuclei of dead cells). Viable cells were characterized by positive staining for $SYBR_{14}$ and negative staining for PI. Viability was thereafter determined by the following formula: Percent viability = (Viable cells/[Viable cells+Dead cells]) × 100 where Viable = $SYBR_{14}$-positive cells and Dead = PI-positive cells.

To analyze the efficiency of defective sperm removal by nanopurification with lectin- and antibody-conjugated nanoparticles, sample fractions from the supernatant (SF) and fractions closer to the particles (PF) were incubated with anti-ubiquitin antibody, MK-12-3, for 1 h and probed with GAM-IgG-FITC for 30 min. Immunofluorescence was evaluated using EasyCyte Plus flow cytometer as described above. Means (± SEM) of the number of spermatozoa ($\times 10^3$) per unit volume of supernatant following nanopurification with particles conjugated to anti-ubiquitin antibody or lectins PNA/PSA were determined by sample flow rate throughout the capillary tube of the flow cytometer set at 3000 events/min.

The percent homogeneity in sperm cell populations following nanopurification with magnetic nanoparticles conjugated to MK-12-3, lectin PNA, or PSA was evaluated by flow cytometry. Sample fractions were incubated with MK-12-3 antibody and lectin PNA-FITC (Molecular Probes) for 30 min and probed with GAM-IgG-TRITC (Zymed/Invitrogen) for 30 min. Immunofluorescence was evaluated using a Guava EasyCyte Plus flow cytometer. Population characteristics were determined by percent side-scatter fluorescence.

Flow Cytometric Data Analysis

Data on sperm fluorescent properties were analyzed by the general linear model procedures of SAS (SAS Institute Inc.) for nested designs. Parameters of interest included total positive immunofluorescence (%M3-total), total negative immunofluorescence (%M2-total), intensity of positive immunofluorescence (M3-median), and intensity of negative immunofluorescence (M2-median). The statistical model included the fixed effects of treatments nested within replicates and random effects of the bull. Interactions between the main effects were evaluated and discarded when they appeared nonsignificant in the final analyses. Duncan multiple comparison tests were used to determine differences at $P < 0.05$.

In Vitro Fertilization

The IVF studies were performed by using pooled semen from three fertile AI bulls in four replicates with a total of 228 oocytes scored for fertilization. Bovine IVF was performed as described previously [18]. Spermatozoa were not washed free of semen and extender before IVF in order to mimic the conditions of field semen collection during which the semen doses subjected to nanopurification also contained seminal plasma and first extender fraction. Oocytes were fixed in 2% formaldehyde at 16 h after fertilization, permeabilized, and processed with the blue-fluorescent DNA stain 4′,6-diamidino-2-phenylindole (DAPI) to reveal the presence of a metaphase II chromosome plate in the unfertilized oocytes and the presence of male and female pronuclei in zygotes [19]. Slides with oocytes were prepared using a standard technique and examined under Nikon Eclipse E800 microscope with epifluorescence illumination and differential interference contrast optics. Percentages of fertilized oocytes for individual sperm treatments were calculated from three replicates with a total of 179 scored oocytes and were analyzed by the Student *t*-test assuming equal variance.

Identification and Characterization of BSP5 in Nanopurified Sperm Fractions

Proteomic trials aimed at comparing the proteomes of normal versus defective spermatozoa separated by nanopurification were performed to

identify major proteins associated predominantly or exclusively with defective bull spermatozoa. Such fractions were initially subjected to Western blot (WB) analysis with anti-ubiquitin antibodies, to identify the approximate masses of ubiquitinated proteins enriched in the defective sperm fractions. Based on this information, corresponding protein bands were excised from PAGE gels of source sperm extracts and subjected to tandem mass spectrometry (MS/MS). Such studies employed routine methods described previously [20]. Initially, spermatozoa were pelleted from the supernatant and pellet fractions obtained by nanopurification with ubiquitin-binding nanoparticles by centrifugation at 800 $\times$ *g* for 10 min and boiled in loading buffer (50 mm Tris, pH 6.8, 150 mm NaCl, 2% SDS, 20% glycerol, 5% β-mercaptoethanol, and 0.02% bromophenol blue) at 95°C for 5 min. Sperm extracts were centrifuged at 3000 $\times$ *g* for 10 min and resolved on 4%–20% gradient gels (PAGEr Precast gels; Lonza Rockland Inc.), followed by transfer to polyvinylidene fluoride membranes (Millipore) using an Owl wet transfer system (Fisher Scientific) at a constant 50 V for 4 h. The membranes were sequentially blocked with 5% nonfat milk for 1 h at room temperature and incubated with MK-12-3 (1:1000 dilution) at 4°C, overnight. The membranes were then incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (GAM-IgG-HRP, dilution 1:10 000). The membranes were then reacted with chemiluminiscent substrate (SuperSignal; Pierce) and visualized by exposing to Kodak BioMax Light film (Kodak). A monoclonal antibody, raised against tubulin (TUBB) (E7; DSHB), was used to normalize the protein load for densitometry.

Following PAGE and WB analysis, several unique bands were observed in the defective sperm fraction prompting further analysis by MS/MS and verification by WB analysis. The most prominent 27–30 kDa protein band (Supplemental Fig. S1; Supplemental Data are available online at www.biolreprod.org), identified later as bovine BSP5, was excised from the PAGE gels, washed by 50 mM ammonium bicarbonate/50% (vol/vol) methanol in water, dried by vacuum centrifugation, and incubated overnight at 37°C in 140 ng of sequencing-grade trypsin. Tryptic digests were analyzed by capillary liquid chromatography-nanoelectrospray ionization-tandem mass spectrometry (Thermo Finnigan). Proteins were identified by MS/MS ion searches performed on the processed spectra against the SwissProt and National Center for Biotechnology Information protein databases using a Bioworks Browser 3.1 (Thermo Finnigan) search engine. The identification of BSP5 protein was confirmed when the Bioworks confidence interval was greater than 95%. Identity was further verified by WB analysis with a custom-made anti-BSP5 antibody raised against the N-terminal amino acid residues 1–25 of bovine BSP5 (National Center for Biotechnology Information sequence NP_777267.1) as described previously [21].

Nanopurification and Processing of Freshly Collected Semen for Field AI

Four fertile Angus bulls were selected for the nanopurification experiments from the pool of bulls available for commercial AI service by Select Sires Inc. Two ejaculates were collected from each bull, combined for each bull, and reevaluated for concentration and total volume (number of cells). A flow chart of the nanopurification protocol is shown in Supplemental Fig. S1. From each fresh semen sample, two aliquots to serve as controls were extended in 20% egg-yolk + 2.9% sodium citrate extender and cryopreserved in AI straws using standard industry protocols; one was packaged at 20×10^6 sperm per straw (20M CON) and the other at 10×10^6 sperm per straw (10M CON). For nanopurification, additional aliquots of the same semen sample were treated with nanoparticles conjugated with PNA-lectin (10M PNA) to remove acrosome-reacted spermatozoa or with nanoparticles conjugated with anti-ubiquitin antibody (10M UBI) to remove spermatozoa containing ubiquitin protein on the cell membrane (indicative of cell damage) before they were extended and cryopreserved in doses of 10×10^6 sperm per AI straw using standard industry protocols.

Specifically, two aliquots were prepared for each sire, containing 0.67×10^9 sperm in 5 ml tubes. These aliquots were diluted to a final volume of 4.5 ml (150×10^6 spermatozoa/ml) in 20% egg-yolk + 2.9% sodium citrate extender at 35°C. To each of these aliquots, 670 µl of anti-ubiquitin antibody- or PNA-conjugated nanoparticles (0.01 mg particles in 100 µl buffer added per 100×10^6 spermatozoa) were added at room temperature. Aliquots were incubated for 15 min at room temperature while slowly rotating on Dynal tube rotator. Subsequently, tubes were placed on top of a magnet (Clemente Associates) for 15 min. After the completion of sedimentation, indicated by the clustering of opaque nanoparticles on the tube bottoms, viable spermatozoa were carefully recovered from supernatant by pipetting into appropriately labeled 50 ml tubes. Approximately 200 µl of supernatant was left with PF on the bottom of the tubes to avoid the uptake of nanoparticle clusters in the pipette. Tubes with spermatozoa were placed in a 200 ml water bath and cooled to 5°C over 1.5 h. Postsort sperm concentration was reassessed by hemocytometer and adjusted to 40×10^6 spermatozoa/ml. Following nanopurification, an equal volume of egg-yolk citrate extender containing 14% glycerol were added in three aliquots of 20%, 30%, and 50% of the 0.5-volume amount. Color-coded straws were filled with 10M CON, 10M UBI, 10M PNA, or 20M CON and cryopreserved using a standard industry protocol and equipment. Straws were stored and shipped to the field trial site in liquid nitrogen. All the straws were used for AI.

Artificial Insemination Field Trials with Nanopurified Semen

The goal of this trial was to compare the in vivo fertility of a half-dose of nanopurified semen (10M UBI and 10M PNA) with that of full dose of control semen (20M CON) from the same group of bulls. Artificial insemination was performed and monitored in accordance with applicable industry guidelines and standards. Predominantly Hereford-Angus crossbred cows (n = 798) of mixed ages at the USDA-ARS Fort Keogh Livestock and Range Research Laboratory were inseminated with 20 or 10×10^6 spermatozoa per straw, prepared as described above. Field trials were conducted during the 2011 and 2012 breeding season. Both cows and heifers were inseminated during the 2011 season and no heifers were inseminated during the 2012 breeding season. Three bulls were used during the first breeding season, but only one sire was used during the second breeding season because semen from two other sires selected for the second trial did not meet minimum postthaw motility and viability criteria for distribution and use in field trials. Estrous cycles of cows and heifers were synchronized using the 5 day cosynchronization + CIDR (controlled intravaginal progesterone-releasing) protocol [22]. Briefly, CIDR devices (Zoetis Animal Health) were inserted in cows and followed by injections of GnRH (100 µg intramuscularly [i.m.]) on Day 8 before breeding. Thereafter, the CIDRs were removed and two injections of PGF (50 mg dinoprost tromethamine, i.m.) were given on Day −3 before breeding. Females observed in estrus within the first 60 h received AI approximately 12 h later and all the remaining females (not observed in estrus) received timed AI with an injection of GnRH (100 µg i.m.) at 72 h after PGF by one of two AI technicians using semen packaged as described above from one of four Angus bulls. Bull and semen treatment order were randomized and assigned before breeding based on the order in which females exhibited estrus or chute order at timed AI. Inseminations were preassigned in this order to account for different numbers of straws available from each sperm sorting treatment so that an approximately equal distribution of estrus AIs and timed AIs occurred with each bull and sperm treatment (n = 240, 228, 170, and 160 for 20M CON, 10M CON, 10M PNA, and 10M UBI, respectively). Pregnancies resulting from AI were diagnosed using ultrasound on Days 38 and 34 and confirmed on Days 80 and 103 after timed AI in heifers and cows, respectively. Pregnancy maintenance was similar between the first and second pregnancy diagnoses across all the treatments. Likewise, none of the treatments resulted in pregnancy loss between the final pregnancy diagnosis and calving. Uterine infection was not measured, per se, but breeding season (50 days including AI plus cleanup breeding with natural service) pregnancy rates were similar across treatments, so no uterine infections were suspected. Data from all the bulls within treatments were pooled for analysis. Technicians did not differ across years or between heifers and cows. Approximately equal numbers of heifers bred for the first time and cows that delivered calves previously were bred to each bull and treatment within bull. The general linear model in SAS was used to detect differences between treatments. The original model included year, semen treatment, technician $\times$ semen treatment, and semen treatment within sire as independent variables. Stepwise elimination of nuisance variables ($P > 0.10$) was used to arrive at the final model, which contained only semen treatment as the independent variable.

RESULTS

In Vitro Testing of Cryopreserved Nanopurified Semen

To determine beneficial effects of nanopurification on sperm quality and viability, laboratory analyses of nanopurified semen were conducted using cryopreserved semen as a convenient source of same-batch spermatozoa from fertile bulls. Immunofluorescence of frozen-thawed spermatozoa nanopurified by using msp Magnetic Separator (Fig. 1, a–c) revealed the enrichment of ubiquitin- and PNA-positive spermatozoa in the pellet fraction after nanopurification with anti-ubiquitin antibody- or PNA-conjugated nanoparticles (Fig. 1d). Further analyses were conducted by dual flow cytometric ubiquitin-PNA assay of fluorescently labeled spermatozoa (Fig. 2). In some instances, the lectin PSA was used instead of PNA and analyzed separately from PNA-labeled samples with

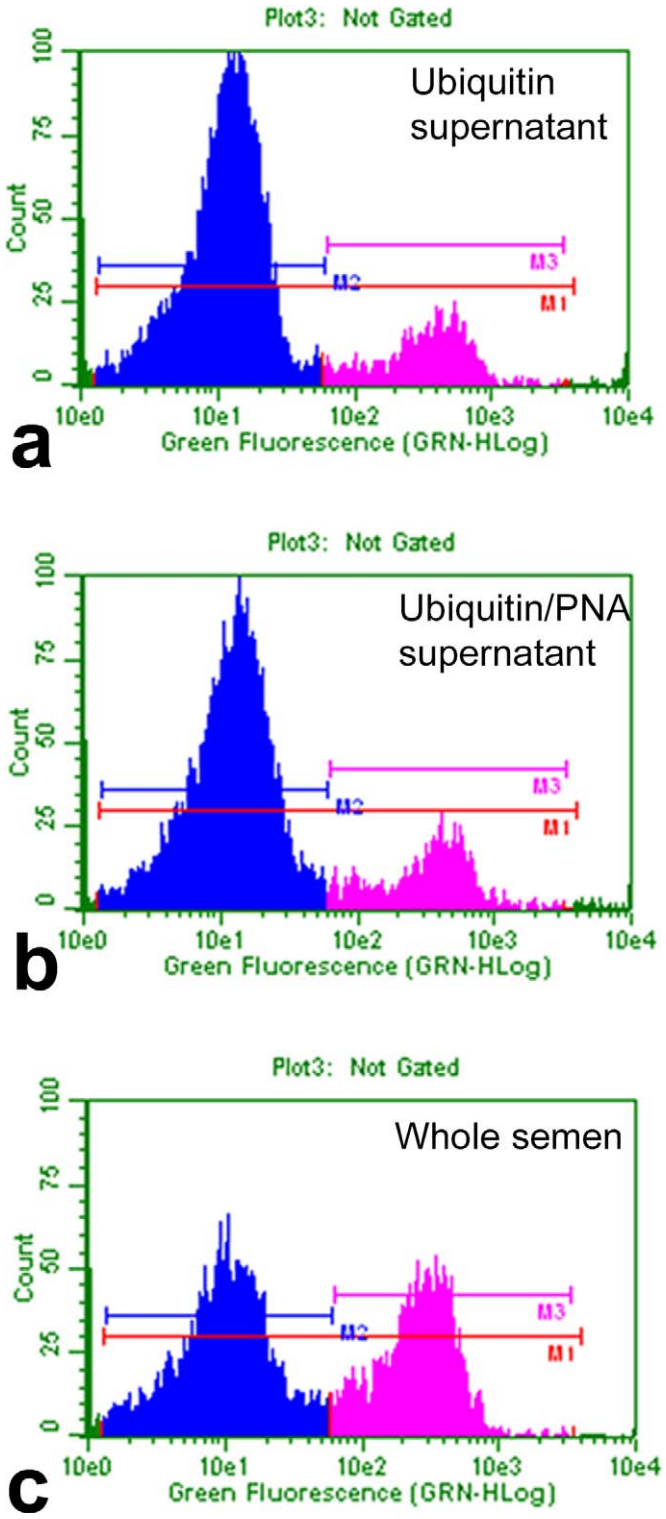

FIG. 2. Ubiquitin-induced fluorescence of spermatozoa after purification with nanoparticles conjugated with anti-ubiquitin antibody MK-12-3 (**a**, SF), MK-12-3/PNA (**b**, SF), and nonpurified raw control (**c**). Samples were incubated with MK-12-3 antibody for 1 h and probed with GAM-IgG-FITC for 30 min. Fluorescence was measured using a Guava EasyCyte Plus flow cytometer. M3 (pink) indicates spermatozoa with positive ubiquitin-induced fluorescence (damaged spermatozoa); M2 (blue) indicates spermatozoa displaying negative, background-level fluorescence.

similar or identical results. A main effect of bull and its interaction with treatments did not have a significant effect on sperm properties assessed by fluorescently tagged lectin PNA ($P = 0.73$). Therefore, subsequent analyses were carried out on the main effects of treatments. The percentage of ubiquitinated spermatozoa in samples %M3) was significantly reduced ($P < 0.0001$) (Fig. 3a) by sperm nanopurification using nanoparticles conjugated with anti-ubiquitin MK-12-3, PNA, and a mixture of MK-12-3 and PNA particles. These differences accounted for 49% of total variation in ubiquitin-induced fluorescence in this trial. However, fluorescence intensity of the ubiquitinated sperm population (M3-median) did not differ among treatments ($P = 0.92$). As expected, the percentage of presumed normal spermatozoa lacking ubiquitin-induced fluorescence (%M2) was significantly higher ($P < 0.001$) in nanopurified samples than in controls and accounted for 44% of total variation in ubiquitin negative immunofluorescence in the study (Fig. 3b).

The number of spermatozoa per unit volume were higher ($P < 0.001$) in control frozen-thawed samples compared to nanopurified frozen-thawed samples but did not differ significantly among the various treatment groups (Fig. 3c). However, based on flow cytometric side scatter characteristics, cell populations were more homogeneous ($P < 0.001$) in the nanopurified samples compared to the control (Fig. 3d). Sperm viability was significantly increased ($P < 0.01$) in samples nanopurified with PNA particles but remained fairly even between MK 12-3, PSA, and control samples (Fig. 3e). A significant increase in sperm viability was also observed with a mixture of anti-ubiquitin antibody- and lectin PNA-coated particles, compared to anti-ubiquitin antibody-coated particles alone (Fig. 3f).

In an IVF trial (Fig. 4), fertilization rate was greater ($P < 0.01$) for sperm that were nanopurified using ubiquitin-binding nanoparticles than for sperm that were nanopurified with PNA, PSA, and mixed ubiquitin/PNA nanoparticles. Overall fertilization rate was lower in this study than in typical IVF trials that use frozen-thawed semen stripped of extender and precleaned by gradient separation or sperm swim up.

Proteomic Analysis of Nanopurified Semen Fractions

To identify potential differences in protein makeup of normal and defective spermatozoa, the supernatant and pellet fractions obtained by nanopurification with ubiquitin-binding nanoparticles were resolved on one-dimensional PAGE gels and subjected to analysis by WBs and MS/MS. As expected, WB analysis with anti-ubiquitin antibodies revealed differences in in the pattern of protein ubiquitination between the fractions. The most obvious protein band, differentially accumulated in the pellet/defective sperm fractions, was isolated and identified by MS/MS as binder of sperm protein 5 (BSP5; alternative name BSP30kDa) (Fig. 5, a–c; gel and amino acid sequence coverage are shown in Supplemental Figs. S2 and S3, respectively). The BSP5 protein has been implicated in sperm binding to oviductal epithelial cells [23] and the sperm capacitation process that prepares spermatozoa for fertilization [24]. Immunofluorescence analysis revealed an altered pattern of BSP5 localization between morphologically normal and defective spermatozoa (Fig. 5, d and e, and Supplemental Fig. S4). Immunolabeling of BSP5 was also detected in the presumed amyloid aggregates of epididymal origin (Supplemental Fig. S4m; see [25]) and in the decayed somatic cells (Supplemental Fig. S4n), which are present occasionally in bull semen. Some, but not all spermatozoa with altered BSP5 SD 3

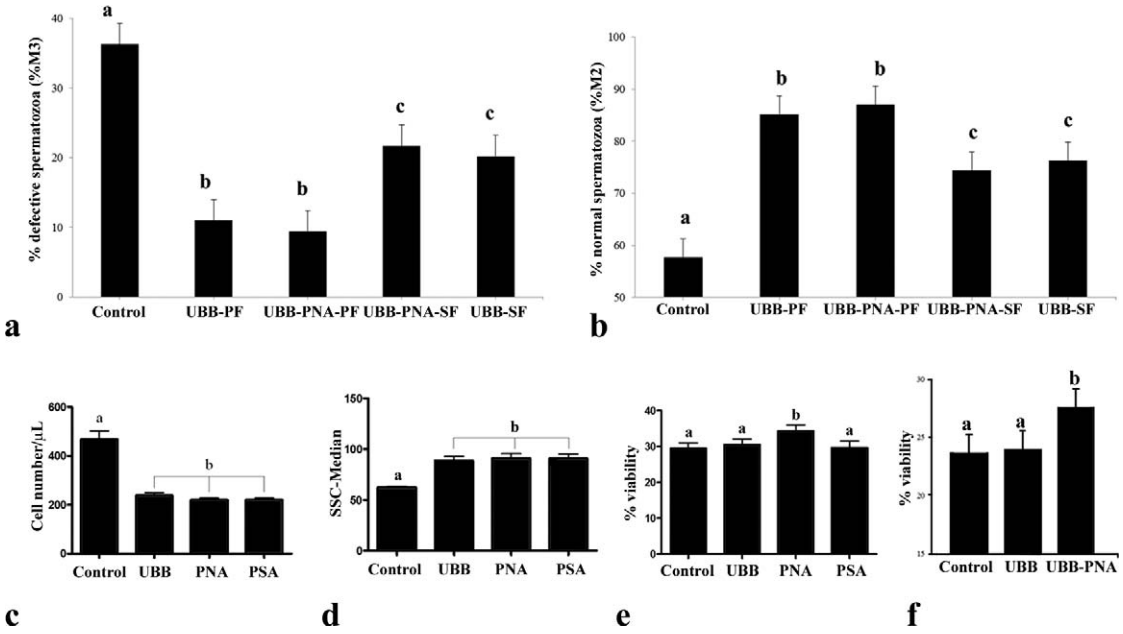

FIG. 3. Effect of nanopurification on semen content of ubiquitinated spermatozoa and sperm count, sperm size/homogeneity, and sperm viability. **a**, **b**) Means (± SEM) percent of ubiquitinated spermatozoa (%M3) and normal spermatozoa without surface ubiquitination (%M2) following nanopurification with magnetic nanoparticles conjugated to anti-ubiquitin antibody (UBB) or lectin PNA (PNA) or a mixture of UBB and PNA particles (n = 6 bulls, 10 samples/bull). It is important to note that the defective spermatozoa and sperm fragments brought to the bottom of the tube with the nanoparticles cannot be recovered for flow cytometry (clumps of spermatozoa and nanoparticles congest flow cytometer). Consequently, the pellet fraction in this figure refers to the pellet of mostly normal, viable spermatozoa collected from medium immediately above the particle fraction (PF). Thus, both PF and supernatant fraction (SF) spermatozoa show significant reduction in defective sperm content over control. Means differed *a* versus *b*, versus *c* among the treatments in **a** ($P < 0.0001$; $r^2 = 0.49$). Means differed *a* versus *b*, versus *c* among the treatments in **b** ($P < 0.001$; $r^2 = 0.44$). **c**) Means ± SEM number of sperm cells (× 103) per unit volume of SF following nanopurification with magnetic nanoparticles conjugated to UBB, PNA, or PSA. Means differed *a* versus *b* among the treatments ($P < 0.001$). **d**) Percent homogeneity in sperm cell population following nanopurification with magnetic nanoparticles conjugated to UBB, lectin PNA, or lectin PSA, as determined by percent side scatter fluorescence (SSC) in flow cytometer. Means differed *a* versus *b* among the treatments ($P < 0.001$). **e**, **f**) Sperm viability in sperm cell population following nanopurification with magnetic nanoparticles conjugated to UBB, lectin PNA, or lectin PSA (**e**) or with a mixture of UBB and PNA nanoparticles (**f**). Viable cells were characterized by positive staining for $SYBR_{14}$ and negative staining for PI using the live/dead cell viability test and the ViaCount program of the Guava EasyCyte Plus flow cytometer. Means differed among the treatments ($P < 0.01$ in **e** and **f**).

localization also displayed labeling with anti-ubiquitin antibodies, associated with sperm-morphological defects and sperm-surface alterations (Supplemental Fig. S5), and high affinity for lectin PNA indicative of acrosomal damage (Supplemental Fig. S6).

Artificial Insemination Field Trial with Nanopurified Semen

To prepare a semen dose for AI field trials, the nanoparticles were mixed with freshly collected semen for 15 min during the initial semen cooling step prior to cryopreservation (Supplemental Fig. S7). The nanoparticle-decorated defective spermatozoa were removed by an msp Magnetic Separator prior to the final dilution in cryoprotectant (semen extension), packaging in standard 0.5 ml straws, and cryopreservation of the semen. The efficiency of particle and defective sperm removal was checked by examining the pellet fraction under a light microscope.

Two AI field trials were conducted in 2011 and 2012, inseminating a total of 798 mixed age Angus-Hereford cows (Table 1, a, b). Four AI treatments were selected: 1) a 20×10^6 sperm dose of nonpurified spermatozoa; 2) a 10×10^6 sperm dose of nonpurified spermatozoa; 3) a 10×10^6 sperm dose of spermatozoa purified with PNA particles; and 4) a 10×10^6 sperm dose of spermatozoa purified with ubiquitin-binding particles. It was hypothesized that the 10×10^6 sperm dose of semen from beef bulls would approach thresholds for compensable sperm numbers and thereby enhance the experimental power to detect differences in fertility among treatments [14]. Data on pre- and postnanopurification sperm concentration, dose number, and postthaw motility and viability of control and nanopurified semen are shown in Table 1, c–h.

Semen treatment affected AI pregnancy rates ($P = 0.01$). Cumulative data from 2 yr show that semen nanopurification with PNA particles resulted in an average CR of 64.5% ± 3.8% with a 10×10^6 dose of nanopurified spermatozoa, equivalent to a control nonpurified dose of 20×10^6 spermatozoa (CR = 63.3% ± 3.2%) and greater ($P < 0.05$) than a 10×10^6 dose of nonpurified control spermatozoa (CR = 53.7% ± 3.2%; Table 1b). The CR achieved with the 10×10^6 dose of spermatozoa purified by ubiquitin-binding particles (CR = 51.3% ± 3.9%) was not different from the 10×10^6 dose of nonpurified semen, but was significantly lower ($P < 0.05$) than 20M CON and 10M PNA treatments (Table 1b). The average postthaw motility and viability was comparable in all four groups (Table 1, g and h). A positive effect of PNA-binding particles was not observed in some bulls, suggesting an effect of bull on the benefit of nanopurification (Supplemental Table S1). The average sperm recovery rate after nanopurification was near 70% for both ubiquitin- and PNA-binding nanoparticles (Table 1c). Importantly, no adverse abnormalities were noted in inseminated animals in either trial. Offspring from ubiquitin- and PNA-nanopurified semen treatments appeared normal. Based on AI pregnancy outcomes, heifer calves were similarly fertile across all the semen treatments (data not shown).

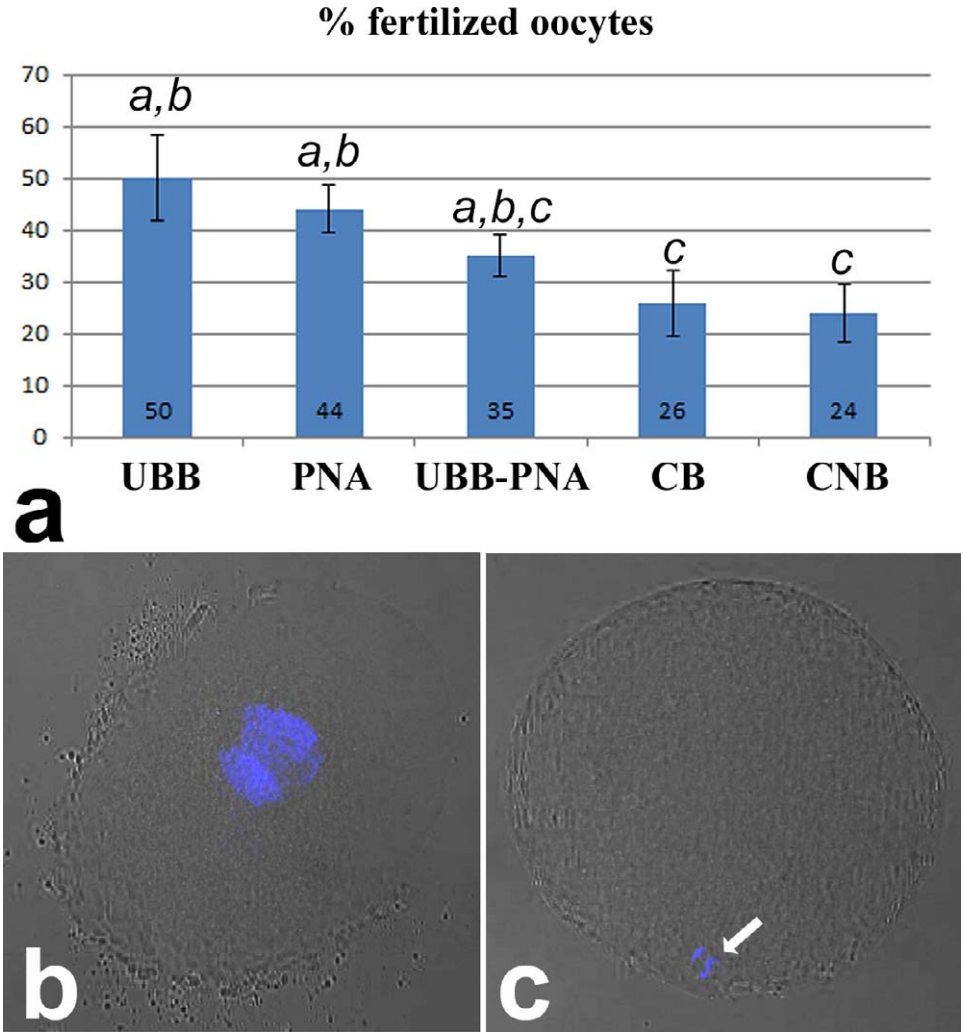

FIG. 4. Results of IVF with extended, frozen-thawed spermatozoa treated with anti-ubiquitin antibody (UBB) and lectin PNA-coated (PNA) nanoparticles. Control treatments include a 50:50 mixture of UBB and PNA particles, control particles not conjugated to lectins/antibodies (CB), and control IVF without any particles (CNB). **a**) Three replicate experiments, representing a total of 179 oocytes are summarized; columns *a, b,* and *c* differ significantly. *P*-values of differences between treatments are as follows: $P^{\text{UBB-CB}} = 0.02$; $P^{\text{UBB-CNB}} = 0.01$; $P^{\text{PNA-CB}} = 0.02$; $P^{\text{PNA-CNB}} = 0.01$. **b**, **c**) Typical results of fertilization assessment showing a successfully fertilized oocyte/zygote with two apposed pronuclei (**b**) and a metaphase-II oocyte that had failed to fertilize (**c**; arrow = metaphase II plate); chromatin is stained with DAPI. Original magnification ×400 (**b** and **c**).

DISCUSSION

Based on their specific affinity to glycans concealed in the interior of sperm acrosome, lectins PNA and PSA are reliable markers of acrosomal damage in mammalian spermatozoa [13]. Similarly, sperm surface ubiquitination has been associated with a variety of sperm defects and negatively correlated with male fertility in several mammalian species, including cattle and humans (reviewed in [1, 6]). In a previous study, we demonstrated that ubiquitin and lectin PNA immunofluorescence can be incorporated in flow cytometric protocols that enhance the semen evaluation processes [10]. The present study was designed in part to complement the previous work by evaluating the level of reduction in ubiquitin immunofluorescence achieved by depleting ubiquitinated spermatozoa and acrosome-damaged spermatozoa by using magnetic nanoparticles conjugated to anti-ubiquitin antibodies and lectin PNA. Attempts were also made to correlate the observed fluorescent characteristics to sperm viability with these treatments. As indicated in the results, ubiquitin immunofluorescence of the samples was greatly reduced and sperm viability increased following the nanopurification of frozen-thawed extended semen.

Mirroring their differential fertilizing ability in vitro, differences were observed between sperm proteomes of normal and defective sperm fractions obtained by nanopurification with ubiquitin-binding nanoparticles. Such proteomic differences were highlighted by altered pattern of sperm protein ubiquitination observed in the defective sperm fraction. Protein ubiquitination is a stable, covalent posttranslational modification that is common in male and female reproductive systems where it regulates gametogenesis, fertilization, and preimplantation embryo development [26]. Polyubiquitination, the most commonly studied form of protein ubiquitination occurs by tandem ligation of one or more multiubiquitin chains to the internal Lys residues of the substrate protein. Each ubiquitin molecule within the substrate bound multiubiquitin chain increases the mass of substrate protein by 8.5 kDa, which is the mass of unconjugated monoubiquitin [8]. Consequently, a small protein of 20 kDa can migrate well above 100 kDa if it is polyubiquitinated. Ubiquitin and its enzymatic conjugation machinery within the mammalian epididymal fluid may fulfill a

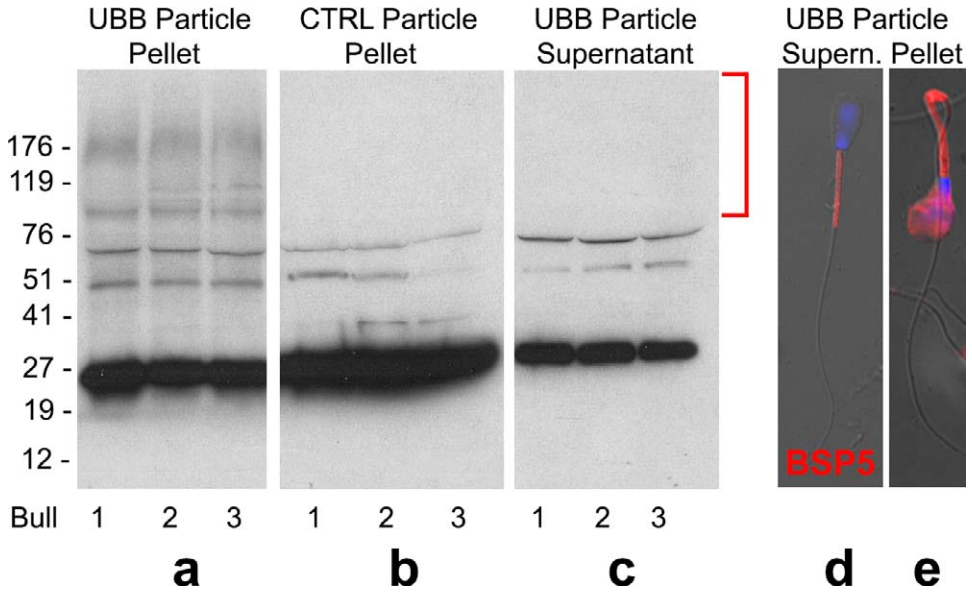

FIG. 5. Differential expression of BSP5 in normal sperm fraction (supernatant) and defective sperm fraction (pellet) obtained by depletion with ubiquitin-binding nanoparticles. **a**–**c**) WB analysis shows unique, presumably polyubiquitinated BSP5 bands above the 76 kDa marker (red bracket) in the ubiquitin (UBB) particle pellet fraction (**a**) that are missing from the control particle pellet (**b**) and supernatant (**c**) fractions that contain few defective spermatozoa. Polyubiquitination increases the mass of substrate protein by multiples of 8.5 kDa. **d**, **e**) Representative patterns of BSP5 labeling (red fluorescence) in morphologically normal and defective spermatozoa (sperm heads are stained with DAPI). Labeling in the normal spermatozoa (**d**) is restricted to sperm tail midpiece; defective spermatozoa (**e**) display intense labeling both on the sperm head and on the sperm tail midpiece. Original magnification ×400 (**d** and **e**).

dual role in epididymal sperm maturation by embalming dead, defective spermatozoa [2] and by contributing to the aggregation and turnover of superfluous epididymal secretory proteins [27]. It is not known at present whether seminal plasma also contains an ubiquitin-conjugating enzymatic system.

In the present study, the sperm-adsorbed seminal plasma protein BSP5 accumulated in the defective sperm fractions isolated with the ubiquitin-binding nanoparticles. In the absence of posttranslational modifications other than glycosylation, the 183 amino-acid residue BSP5 migrates as a major band of ~30 kDa [21, 28], as also observed in the nanopurified sperm SFs in the present study. The prevalent (by WB band density) 30 kDa BSP5 species and minor bands in the 50–75 kDa range were present in both pellet and SFs after nanopurification. A ladder of BSP5-immunoreactive bands migrating at and above 75 kDa, similar to the typical laddering seen in polyubiquitinated proteins, was observed in the pellet fractions rich in defective, ubiquitinated spermatozoa. Based on the unexpectedly high mass of such BSP5 bands copurified with ubiquitin-binding nanoparticles, it is plausible to hypothesize that the sperm surface BSP5 protein may become ubiquitinated in defective spermatozoa. The BSP5 protein of seminal plasma origin could become ubiquitinated after ejaculation or the ubiquitinated BSP5 could be secreted by sex accessory glands and adsorbed ectopically onto defective sperm surface.

Removal of defective spermatozoa from collected semen may have dual benefit of mitigating the dilution of total sperm count per AI dose by defective spermatozoa and of reducing the reactive oxygen species released from decaying spermatozoa that might affect the fertilizing ability of the normal spermatozoa [29]. Our laboratory tests demonstrated a statistically significant improvement in sperm viability after semen nanopurification with ubiquitin-binding nanoparticles. Enrichment of defective spermatozoa was documented in the nanoparticle fraction discarded after depletion. Tests of frozen-thawed spermatozoa by IVF demonstrated an improved fertilization rate using sperm fractions purified with ubiquitin-binding nanoparticles compared to control, inactive nanoparticles, or particles coated with lectin PNA. In contrast, the best results in the AI field trials were obtained with PNA-coated nanoparticles. Possible reasons for this discrepancy could include immunosaturation of particle-bound anti-ubiqui-

TABLE 1. Summary of semen recovery data and CRs of four sires used for field AI trials in two consecutive years.

Item	PNA 10×10^6	Ubiquitin 10×10^6	Control 20×10^6*	Control 10×10^6*
a: Number of inseminated cows	169	160	240	229
b: Percent CRs	64.5 ± 3.8^a	51.3 ± 3.9^b	63.3 ± 3.2^a	53.7 ± 3.2^b
c: Average initial sperm concentration (10^9/ml)	0.94 ± 0.11	0.94 ± 0.11	0.94 ± 0.11	0.94 ± 0.11
d: Average postrecovery sperm concentration (10^9/ml)	0.27 ± 0.14	0.24 ± 0.09	N/A	N/A
e: Percentage of sperm recovered	68.5 ± 6.11	72.2 ± 8.1	N/A	N/A
f: Number of semen doses produced	393	442	667	667
g: Postthaw motility	57.5 ± 5.4	64.2 ± 3.5	60.8 ± 3.7	66.7 ± 1.7
h: Postthaw viability	41.8 ± 4.4	34.2 ± 7.6	41.8 ± 4.9	44.8 ± 4.4

* N/A, not assessed.
[a,b] Superscripts denote statistically significant difference between columns at $P < 0.05$.

tin antibodies by proteins present in seminal plasma and/or semen extender. For example, egg yolk or milk components of the semen extender may contain ubiquitinated proteins. Alternatively, antibodies but not lectins absorbed onto nanoparticle surface could have an effect on sperm viability; the only treatment that significantly improved sperm viability in vitro was nanopurification with PNA particles. Another possible reason for the discordant results between AI and IVF is that different populations of spermatozoa that exist within bull semen [30] might fertilize oocytes at different rates in vivo versus in vitro. For IVF, the partially damaged and/or precapacitated spermatozoa might still be able to fertilize. In vivo, those spermatozoa likely die and are degraded by phagocytosis before reaching the fertilization site in the oviduct [31], resulting in lower pregnancy rates following AI as observed with this treatment.

The nanopurification of freshly collected bull semen prior to cryopreservation does not require extensive semen manipulation, is neither equipment nor labor intensive and uses commercially available components adding only cents to the cost per AI dose. Importantly, this procedure is fully compatible with current industry protocols for semen collection and processing and was in fact tested in the setting of a large commercial bull stud. Moreover, the nanopurification procedure does not interfere with fertilization or pregnancy establishment after AI and does not increase the risk of reproductive tract infection. Importantly, no adverse effects on animal reproductive health and overall health have been observed after AI with nanopurified semen. Data collected from these trials provide proof of concept for semen nanopurification and a rationale for additional field trials aimed at the optimization of the procedure.

Dissemination of new approaches to sire fertility management could increase profitability in the cattle industry by eliminating subfertile young bulls during breeding soundness evaluations and facilitating continuous fertility/semen quality monitoring in AI service by biomarker-based sperm flow cytometry [32–34]. To complement improved semen analysis and bull fertility evaluation, purification of spermatozoa in semen, such as by nanoparticles, could increase the quality of individual AI dose by the removal of defective spermatozoa that dilute the concentration of viable, fertile spermatozoa in semen and may actually impair fertile spermatozoa by producing harmful decay products [35, 36]. It is also possible that removal of defective spermatozoa from the AI sample leads to fertilization by a more optimal spermatozoon that leads to increased embryonic survival. Our results indicate that removal of approximately 30% of spermatozoa from a semen collection is compatible with high CR when such nanopurified semen is used for AI at a low dose. Consequently, nanopurification may allow for the production of a larger number of fully fertile AI doses per bull per collection from bulls with superior genetics and reproductive performance. The benefits of such technology may extend to human reproductive medicine wherein semen nanopurification could improve sperm selection for assisted reproductive therapy by AI, IVF, or intracytoplasmic sperm injection.

On the opposite end of reducing the minimum number of spermatozoa per AI dose necessary to achieve pregnancy, it is possible that by removing defective spermatozoa from freshly collected semen, CRs could actually be improved compared to semen samples where the defective spermatozoa have not been removed. This procedure could be very advantageous depending on the efficiency of sperm harvest from the sorting procedure, minimum sperm dosages required to obtain normal conception for both sorted and for unsorted samples, and whether or not the inseminated cow can actually translate improved semen quality in the insemination dose into improved CRs on the farm. Further research is warranted with nanopurified semen at higher numbers per straw than the 10×10^6 used in these studies to determine if even greater pregnancy rates can be achieved.

Nanopurification could also be useful for recovery of fertile spermatozoa from inferior quality semen samples caused by inclement environmental conditions, such as summer heat stress. In some breeds that are particularly sensitive to adverse weather/climate, this protocol could extend the semen collection season by several months. Nanopurification applied prior to the separation of X and Y chromosome-bearing spermatozoa by semen sexing could increase the speed of sorting X and Y spermatozoa, while also improving the input sperm quality and the viability of cell-sorted sexed sperm output. The need for increased semen dosage remains a limiting factor in the advancement of semen-sexing technology [37]. Even if improved conception cannot be confirmed, improved efficiency of semen utilization could still be a valuable application for this technology. However, very intensive dose titration studies will be required for sorted and unsorted samples, and effects of individual sire may affect the results, as also noted in the present study (see Supplemental Table SD2). Some bulls appeared to have maintained normal fertility at 10×10^6 sperm/dose even without nanopurification, and other intensive sperm dose titration studies have shown that many bulls are already capable of maintaining normal levels of conception at dosages as low as $1–5 \times 10^6$ total spermatozoa [38]. Thus, as opposed to the simple question of improved fertility, improved efficiency of semen utilization in the absence of improved fertility will require much more extensive research to validate.

In summary, our study describes successful field testing of a new nanotechnology for the improvement of AI in cattle. Offspring born from PNA- and ubiquitin-binding nanopurified semen appeared completely normal. Heifers born from the first year of this AI field trial displayed normal fertility compared to heifers sired by control, nonpurified semen. Further efforts will be focused on optimization and safeguarding of this technology with the goals of increasing the reproductive efficiency of cattle herds in the United States and internationally.

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REFERENCES

1. Sutovsky P, Lovercamp K. Molecular markers of sperm quality. Soc Reprod Fertil Suppl 2010; 67:247–256.
2. Baska KM, Manandhar G, Feng D, Agca Y, Tengowski MW, Sutovsky M, Yi YJ, Sutovsky P. Mechanism of extracellular ubiquitination in the mammalian epididymis. J Cell Physiol 2008; 215:684–696.
3. Olson GE, Winfrey VP, NagDas SK, Melner MH. Region-specific expression and secretion of the fibrinogen-related protein, fgl2, by epithelial cells of the hamster epididymis and its role in disposal of defective spermatozoa. J Biol Chem 2004; 279:51266–51274.
4. Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Thompson WE, Schatten G. A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. J Cell Sci 2001; 114:1665–1675.
5. Ibrahim NM, Gilbert GR, Loseth KJ, Crabo BG. Correlation between clusterin-positive spermatozoa determined by flow cytometry in bull semen and fertility. J Androl 2000; 21:887–894.
6. Sutovsky P, Kennedy CE. Biomarker-based nanotechnology for the

improvement of reproductive performance in beef and dairy cattle. Ind Biotechnol 2013; 9:24–30.
7. Lovercamp KW, Safranski TJ, Fischer KA, Manandhar G, Sutovsky M, Herring W, Sutovsky P. Arachidonate 15-lipoxygenase and ubiquitin as fertility markers in boars. Theriogenology 2007; 67:704–718.
8. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002; 82: 373–428.
9. Sutovsky P, Neuber E, Schatten G. Ubiquitin-dependent sperm quality control mechanism recognizes spermatozoa with DNA defects as revealed by dual ubiquitin-TUNEL assay. Mol Reprod Dev 2002; 61:406–413.
10. Odhiambo JF, Sutovsky M, DeJarnette JM, Marshall C, Sutovsky P. Adaptation of ubiquitin-PNA based sperm quality assay for semen evaluation by a conventional flow cytometer and a dedicated platform for flow cytometric semen analysis. Theriogenology 2011; 76:1168–1176.
11. Sutovsky P, Plummer W, Baska K, Peterman K, Diehl JR, Sutovsky M. Relative levels of semen platelet activating factor-receptor (PAFr) and ubiquitin in yearling bulls with high content of semen white blood cells: implications for breeding soundness evaluation. J Androl 2007; 28: 92–108.
12. Kennedy CE, Krieger KB, Sutovsky M, Xu W, Vargovic P, Didion BA, Ellersieck MR, Hennessy ME, Verstegen J, Oko R, Sutovsky P. Protein expression pattern of PAWP in bull spermatozoa is associated with sperm quality and fertility following artificial insemination. Mol Reprod Dev 2014; 81:436–449.
13. Cross NL, Watson SK. Assessing acrosomal status of bovine sperm using fluoresceinated lectins. Theriogenology 1994; 42:89–98.
14. Amann RP, DeJarnette JM. Impact of genomic selection of AI dairy sires on their likely utilization and methods to estimate fertility: a paradigm shift. Theriogenology 2011; 77:795–817.
15. Mitchell JR. Developing an NAAB semen quality control program. In: Association of Applied Animal Andrology Conference, Vancouver, 2012. Assoc Appl Anim Androl 2012; 1–6.
16. Said TM, Agarwal A, Zborowski M, Grunewald S, Glander HJ, Paasch U. Utility of magnetic cell separation as a molecular sperm preparation technique. J Androl 2008; 29:134–142.
17. United States National Nanotechnology Initiative [Internet]. Washington DC. http://www.nano.gov/nanotech-101/what/definition. Accessed 10 September 2014.
18. Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos. Biol Reprod 2000; 63:582–590.
19. Sutovsky P. Visualization of sperm accessory structures in the mammalian spermatids, spermatozoa, and zygotes by immunofluorescence, confocal, and immunoelectron microscopy. Methods Mol Biol 2004; 253:59–77.
20. Odhiambo JF, Dailey RA. Characterization of proteins in cryopreserved and non-cryopreserved seminal plasma of dairy bulls of differing fertility. Open J Anim Sci 2011; 1:33–40.
21. Hung PH, Suarez SS. Alterations to the bull sperm surface proteins that bind sperm to oviductal epithelium. Biol Reprod 2012; 87:88.
22. Bridges GA, Helser LA, Grum DE, Mussard ML, Gasser CL, Day ML. Decreasing the interval between GnRH and PGF2alpha from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows. Theriogenology 2008; 69:843–851.
23. Gwathmey TM, Ignotz GG, Mueller JL, Manjunath P, Suarez SS. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. Biol Reprod 2006; 75: 501–507.
24. Jois PS, Manjunath P. The N-terminal part of Binder of SPerm 5 (BSP5), which promotes sperm capacitation in bovine species is intrinsically disordered. Biochem Biophys Res Commun 2010; 394:1036–1041.
25. Cornwall GA, von Horsten HH, Swartz D, Johnson S, Chau K, Whelly S. Extracellular quality control in the epididymis. Asian J Androl 2007; 9: 500–507.
26. Sutovsky P. Sperm proteasome and fertilization. Reproduction 2011; 142: 1–14.
27. Cornwall GA, Von Horsten HH, Whelly S. Cystatin-related epididymal spermatogenic aggregates in the epididymis. J Androl 2011; 32:679–685.
28. Calvete JJ, Mann K, Sanz L, Raida M, Topfer-Petersen E. The primary structure of BSP-30K, a major lipid-, gelatin-, and heparin-binding glycoprotein of bovine seminal plasma. FEBS Lett 1996; 399:147–152.
29. Aitken RJ, Baker MA. Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. Int J Dev Biol 2013; 57:265–272.
30. D'Amours O, Bordeleau LJ, Frenette G, Blondin P, Leclerc P, Sullivan R. Binder of sperm 1 and epididymal sperm binding protein 1 are associated with different bull sperm subpopulations. Reproduction 2012; 143: 759–771.
31. Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. Hum Reprod Update 2006; 12:23–37.
32. Hossain MS, Johannisson A, Wallgren M, Nagy S, Siqueira AP, Rodriguez-Martinez H. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. Asian J Androl 2011; 13: 406–419.
33. Petrunkina AM, Harrison RA. Cytometric solutions in veterinary andrology: developments, advantages, and limitations. Cytometry A 2011; 79:338–348.
34. Thomas CA, Garner DL, DeJarnette JM, Marshall CE. Effect of cryopreservation of bovine sperm organelle function and viability as determined by flow cytometry. Biol Reprod 1998; 58:786–793.
35. Blondin P, Coenen K, Sirard MA. The impact of reactive oxygen species on bovine sperm fertilizing ability and oocyte maturation. J Androl 1997; 18:454–460.
36. Aitken RJ, Jones KT, Robertson SA. Reactive oxygen species and sperm function—in sickness and in health. J Androl 2012; 33:1096–1106.
37. Dejarnette JM, Leach MA, Nebel RL, Marshall CE, McCleary CR, Moreno JF. Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: is comparable fertility of sex-sorted and conventional semen plausible? J Dairy Sci 2011; 94:3477–3483.
38. Den Daas JH, De Jong G, Lansbergen LM, Van Wagtendonk-De Leeuw AM. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. J Dairy Sci 1998; 81:1714–1723.