Monoclonal Antibody Detection of Heparin-Binding Proteins on Sperm Corresponds to Increased Fertility of Bulls[1,2]

Mary E. Bellin*, Hal E. Hawkins†, Janice N. Oyarzo*, Russell J. Vanderboom*, and Roy L. Ax*,[3]

*Department of Animal Sciences, University of Arizona, Tucson 85721 and †King Ranch, Kingsville, TX

ABSTRACT: A monoclonal antibody (M1) was produced against seminal fluid heparin-binding proteins (HBP) from a vasectomized bull. In the first part of this study, the presence of HBP in sperm or seminal fluid was determined for 53 bulls with an ELISA using M1. Bulls (8 to 18 per pasture) were bred to 1,114 cows at ratios of 1 bull:25 cows. Bulls with detectable HBP on sperm membranes were 11 percentage points more fertile than bulls with undetectable HBP in sperm membranes. In the second part of this study, three sperm membrane HBP approximately 30, 24, and 21.5 kDa were identified with Western blots using M1. Santa Gertrudis bulls (n = 64) were bred to 1,354 Santa Gertrudis cows in groups with 2 to 11 bulls. Bulls with those three HBP (Group A) or a single 30-kDa HBP (Group B) in sperm membranes had the greatest fertility, ranging from 74.4 to 89.9% (mean = 81.5%) of the palpated cows that were pregnant. Bulls with the 21.5- and 30-kDa HBP (i.e., the 24-kDa HBP was absent; Group C) had a reduced fertility of 61.3%. Bulls without detectable HBP (Group D) resulted in 41.9% of 186 cows palpated pregnant. Bulls in Groups A and B were more ($P < .01$) fertile than all other groups. In conclusion, the presence of HBP in sperm membranes was indicative of the fertility potential of bulls.

Key Words: Bulls, Fertility, Heparin-Binding Proteins, Spermatozoa, Monoclonal Antibodies

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Introduction

Heparin is chemically similiar to other sugars, known as glycosaminoglycans, secreted in the female reproductive tract (Lee et al., 1985). Heparin and glycosaminoglycans from the reproductive tract bind to proteins on bovine sperm and induce capacitation (Handrow et al., 1982; Lenz et al., 1983; Lee et al., 1985). Bulls with greater fertility produce sperm with greater affinity for heparin than less-fertile bulls (Marks and Ax, 1985). The increase in heparin affinity corresponds to an increased rate of acrosome reaction (Ax et al., 1985; Ax and Lenz, 1987; Lenz et al., 1988). Heparin-binding proteins (**HBP**) are produced by male accessory glands and are secreted into seminal fluid. The HBP combine to form complexes with five affinities for heparin (Miller et al., 1990; Nass et al., 1990). Bulls with the greatest heparin affinity proteins (HBP-B5) in sperm membranes, but not in seminal fluid, were 17 percentage points more fertile than groups of bulls with other HBP-B5 profiles (Bellin et al., 1994). The presence of specific HBP on sperm indicates affinity of sperm to heparin, the subsequent ability of sperm to acrosome react, and thus the fertility potential of a bull.

The objectives of these studies were to 1) produce a monoclonal antibody to HBP with greater affinity for heparin and 2) determine whether the presence of HBP recognized by the monoclonal antibody in sperm membranes was related to fertility of bulls.

Materials and Methods

Production of Monoclonal Antibody

Coupling Heparin to Sepharose Beads. Heparin (sodium salt from porcine intestinal mucosa; Scientific Proteins Lab, Waunakee, WI) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia,

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[3]To whom correspondence should be addressed.

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Piscataway, NJ). Sepharose (3 g) and heparin (300 mg) were incubated at room temperature for 2 h and then centrifuged (2,000 × *g* for 10 min 4°C) to remove unbound heparin. The beads were washed with 40 m*M* T (TRIZA® base), pH 7.4 (Sigma Chemical, St. Louis, MO). Then, the Sepharose was equilibrated in 40 m*M* T with 2 m*M* $CaCl_2$, .05% sodium azide, 1 m*M* phenylmethylsulfonylfluoride, and 1 μM pepstatin A (TC, Sigma Chemical). The TC buffer contained protease inhibitors to prevent protein degradation during isolation of HBP.

Isolation of Heparin-Binding Proteins. Heparin-binding proteins were initially purified using heparin-coupled Sepharose beads. Commercially prepared heparin-coupled agarose beads (Sigma Chemical) were also used to prepare heparin-binding proteins. Briefly, seminal plasma (10 mL) from a vasectomized Holstein bull (provided by 21st Century Genetics, Shawano, WI) was added to approximately 1 g of heparin-coupled beads and gently mixed for 10 min. Unbound protein was removed by centrifuging (2,000 × *g*) the mixture for 10 min with 50 mL of TC buffer; three additional centrifugations with 10 mL of TC buffer were performed. Proteins bound to heparin beads (heparin-binding proteins, HBP) were eluted from the beads with 20 mL of TC buffer containing 2 *M* NaCl. Salt was removed from HBP by dialysis in Spectra/Por molecular porous membrane tubing with a 10,000 molecular weight cut-off (Spectrum Medical Industries, Houston, TX) in one L of 50 m*M* ammonium carbonate buffer (Sigma Chemical) for 18 h at 4°C; the buffer was changed at least four times. Dialyzed HBP was lyophilized and stored as a powder. The yield was approximately 7 mg of HBP/mL of seminal fluid.

Immunization of Mice with Heparin-Binding Proteins. Monoclonal antibodies to HBP were generated at the Hybridoma Technology Core Support Facility at the University of Arizona according to the procedures of Enriquez et al. (1991). Three female (8 to 12 wk old) Balb/cByJ mice (Jackson Laboratories, Bar Harbor, ME) were immunized intraperitoneally six times at 14-d intervals with approximately 60 μg of purified HBP antigen mixed in Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for the following injections in 200 μL of volume. Mice were sedated with carbon dioxide and bled from the retroorbital plexus. Serum was tested for the presence of antibodies to HBP using an ELISA and specifically to individual HBP using Western blot analysis (described later). The mouse with the greatest antibody titer to the HBP was selected for the fusion. Serum was stored at –80°C and used as the positive control during screening of hybrids. Three days after the final immunization, the selected mouse was killed by cervical dislocation, and the spleen was aseptically removed. Spleenocytes were dispersed into a single cell suspension.

Hybridization of Cells. Spleen cells (1×10^8) and SP2/0 myeloma cells (1×10^7) in RPMI-1640 complete media were fused with polyethylene glycol (4,000 M_r; EM Science, Gibbstown, NJ; Kohler and Milstein, 1975). Hybridized cells were diluted to 100 mL, pipetted into 24-well culture plates at approximately 1×10^5 cells/mL, and cultured for 10 to 14 d before supernatants were assayed for antibody production with ELISA and Western blots. Cells identified as producing antibody to the 30-kDa HBP were further cloned or frozen in 10% DMSO and stored in liquid nitrogen.

Cloning of Cells. Positive hybrids were cloned three times by limiting dilution. Briefly, a selected positive hybrid (50 μL of 1×10^4 cells/mL) was diluted to 5 mL, then 100 μL was pipetted into each well of the first two rows (Rows A and B with 12 wells/row) of a 96-well plate (10 cells/well). An additional 2.5 mL of fresh media was added to the cells, then 100 μL was pipetted into the third and fourth rows (Rows C and D, 5 cells/well). This procedure was repeated until the 96-well plate was completed with 1 cell/well in rows G and H. Cells received RPMI media with 10% BSA on d 5 and 10 and were monitored for antibody production on d 10 with ELISA and Western blots.

Development of Monoclonal Antibody. One monoclonal antibody (S1A5C8G10H12, refered to as M1) recognized the 30-kDa HBP and was subsequently developed by transferring cells to a well in a 24-well plate and cultured in RPMI complete media. Media were changed three times per week. Cells were monitored daily, and, when cells seemed to have healthy growth, they were transferred to flasks in 10 mL of media and allowed to grow for approximately 2 wk before monoclonal antibody was harvested. Hybridomas were monitored periodically for secretion and cloned approximately once every 6 to 8 wk to remove nonsecreting cells.

Enzyme-Linked Immunosorbent Assay. Enzyme-linked immunosorbent assay plates (Falcon, Fisher Scientific, Pittsburgh, PA) were coated with 50 μL of HBP (100 μg/mL) by incubating overnight at 4°C. Wells were washed twice with PBS with .05% Tween (polyoxyethlyenesorbitan; Sigma Chemical), then open sites were blocked with 1% nonfat dry milk in PBS for 30 min. Mouse serum (1:1,000) or cloned cell supernatant (100 μL) was added and incubated at 37°C for 1 h, then washed twice with PBS-Tween. Goat anti-mouse second antibody (1:500; TAGO, Burlingame, CA) conjugated to horseradish peroxidase was added and incubated for 1 h at 37°C. Wells were washed twice with PBS-Tween, then 50 μL of substrate (ABTS Substrate system; Kirkgaard and Perry, Gaithersburg, MD) was added; the color developed in positive samples in minutes. The absorbance for each well was determined with an ELISA reader at 490 nm (Dynatech 700; Chantilly, VA).

Gel Electrophoresis and Western Blots. The HBP were identified using SDS-PAGE according to the

method of Laemmli (1970) and Western blots as described by Deutscher (1990). Isolated HBP in PBS were vortexed vigorously and added to sample buffer (1:1; 10% glycerol, 2% SDS, .001% bromophenol blue in 62.5 m*M* TRIS), then the tubes were placed in boiling water for 5 min. Samples (50 μg of HBP per lane) were pipetted onto a SDS 5% polyacrylamide stacking gel. Fifteen microliters of prestained molecular weight markers were applied to one lane per gel (Bio-Rad Laboratories, Hercules, CA). Samples were size-fractionated with denaturing SDS 12% polyacrylamide gel electrophoresis. Gels were run in duplicate, and one gel was stained with Coomassie brilliant blue G stain (Sigma Chemical) to visualize proteins.

Proteins on the second gel were transferred electrophoretically to Immobilon-PVDF membranes (Millipore, Bedford, MA). Membranes were blocked overnight at 4°C or for 2 h at room temperature in PBS containing 3% (vol/vol) Tween-20 and 5% (wt/vol) BSA (PBST-5% BSA). Membranes were rinsed five times in PBST-1% BSA and then incubated with mouse serum (1:1,000) or cloned cell supernatant (1 mL/lane). After an overnight incubation at 4°C, blots were washed five times in PBST-1% BSA. Blots were then incubated for 2 h at room temperature with a 1:3,000 dilution of second antibody, goat anti-mouse IgG conjugated to horseradish peroxidase (TAGO). Immobilized specific antigens were then detected using ImmunoSelect 4-chloro-1-naphthol (4CN; Gibco BRL, Life Technologies, Gaithersburg, MD). Other detection systems for Western Blots using alkaline phosphatase-conjugated secondary antibody with Sigma Fast Red TR/Naphthol AS-MX substrate (Sigma Chemical) or goat anti-mouse IgG conjugated to horseradish peroxidase secondary antibody (TAGO) with enhanced chemical luminescence (ECL, Amersham, Northbrook, IL) were also used according to the manufacturers' directions in these studies.

Determining Proteins That Bind Heparin and Monoclonal Antibody. Sperm membrane and seminal fluid proteins that bind heparin and M1-coated affinity beads were compared. Heparin beads were prepared as described previously, and M1 affinity beads were prepared by binding M1 to Affi Prep Hz Hydrazide support (Bio-Rad) according to the manufacturer's directions. Seminal fluid (2 mL of semen) from a Santa Gertrudis bull was removed from sperm by centrifugation at 1,400 × *g* for 6 min, sperm were then washed three times and resuspended in 2 mL of isolation buffer (40 m*M* T, 250 m*M* sucrose, 2 m*M* EDTA, 5 m*M* benzamidine, 1 μ*M* phenylmethylsulfonyl fluoride, 1μM pepstatin A, and .01% sodium azide; pH 7.35). Cells were disrupted by nitrogen cavitation in a Parr Bomb (Parr Instrument, Moline, IL) for 10 min at 4,135 kPa (Noland et al., 1983). Suspensions of cavitated cells were microcentrifuged at 16,000 × *g* for 20 min. Supernatants containing plasma membrane proteins (200 μL) or seminal fluid (200 μL) were added to 200μL of heparin or M1 affinity beads, incubated for 10 min at 4°C, then washed three times with PBS with .05% Tween. Beads were suspended in 100 μL of buffer, and 100 μL of sample buffer was added. Samples were boiled for 5 min, and 40 μL per lane was applied to SDS 12%-polyacrylamide gels. Western blots using M1 were then performed as described previously.

Study 1: Evaluating Fertility of Bulls Grouped According to Heparin-Binding Proteins in Sperm Membranes and Seminal Fluid Determined with an Enzyme-Linked Immunosorbent Assay Using a Monoclonal Antibody

Semen Collection. A programmable electroejaculator (Electrojac, Chicago, IL) was used to collect semen samples from 53 Santa Gertrudis bulls at a commercial ranch (King Ranch, Kingsville, TX). Semen collection was part of the routine breeding soundness examinations (**BSE**; Chenoweth et al., 1992) performed before spring breeding. Bulls were 3 to 7 yr of age. Sperm samples were examined visually for motility using a phase contrast microscope. One hundred sperm from an ejaculate were examined and visually identified as having normal or abnormal structure using a morphology stain (Society for Theriogenology, Hastings, NE) and a light microscope (1,000× magnification). Sperm were further characterized as having primary (head) or secondary (tail) abnormalities. Bulls with less than 70% normal sperm were removed from the study and not used for breeding. Semen samples were covered in ice, then at 4-h intervals samples were frozen (–20°C). Samples were shipped with Dry Ice to the University of Arizona (Tucson, AZ) and stored at –20°C until samples were analyzed for HBP. In addition to BSE, scrotal circumference for each bull was measured.

Seminal Fluid Proteins. Semen (2 mL) samples were thawed and centrifuged at 1,200 × *g* for 15 min at 4°C. Supernatants were applied to a PD-10 Sephadex G-25M column (Bio-Rad) and washed with .05 *M* ammonium bicarbonate (pH 8.5) to remove excess salt as described by Nass et al. (1990). Samples were lyophilized and stored at –20°C until they were analyzed for HBP with ELISA. The sperm pellets were frozen (–70°C) until proteins were isolated.

Sperm Membrane Proteins. Sperm pellets were thawed and washed three times with 1 mL of TC buffer at 4°C. To determine the number of sperm/mL of semen, 50 μL from the last wash was pipetted and diluted to 250 μL with TC buffer, and sperm in 10 μL were counted using a hemacytometer. Centrifuged pellets were suspended in 1 mL of TC buffer containing .1% Triton X-100 (vol/vol) and incubated for 1 h at 4°C. Then, 3 mL of .1% TC buffer was added, and the cells were centrifuged at 50,000 × *g* for 30 min to remove the cellular debris (Nass et al., 1990).

Supernatants containing sperm membrane proteins were lyophilized and stored at –20°C until they were analyzed for HBP with ELISA.

Testing for Presence of Heparin-Binding Proteins. Seminal fluid and sperm membrane proteins were solubilized in 500 and 200 μL of deionized distilled water, respectively. Seminal fluid or sperm membrane proteins were added (100 μL) to an ELISA plate in duplicate. Monoclonal antibody (100 μL of M1) was added to each well and the ELISA were performed as previously described.

Grouping of Bulls According to Heparin-Binding Protein Profiles. Trials were conducted at a large commercial ranch (King Ranch, Kingsville, TX), and it was not possible to test fertility of individual bulls. Instead, bulls were identified and grouped according to the presence or absence of HBP in sperm membranes and seminal fluid as determined with ELISA. Group 1 was composed of bulls with HBP on sperm but with undetectable HBP in fluid. Group 2 had bulls with HBP detectable in sperm and in fluid. Bulls with undetectable HBP in fluid and sperm were in Group 3, and bulls with HBP in fluid but not in sperm were in Group 4.

Pasture Assignments. Groups of Santa Gertrudis bulls (8 to 18) were assigned to a pasture based on the number of cows in the pasture to achieve a ratio of 1 bull:25 cows. To eliminate bias of selection of pastures, actual HBP characteristics for each group were coded and not identified to King Ranch personnel until completion of the trials. Bulls were identified by ear tags, matched to their coded HBP group, and the appropriate pasture assignment was spray-painted on their backs. Bulls were transported to each pasture. All cows used in these studies were Santa Gertrudis and had calved in the spring before the trial. Pastures contained native grasses, and cattle were supplemented with Mintrate mineral blocks (MoorMan's Mfg., Quincy, IL).

Determination of Fertility. On approximately May 1, bulls were pastured with cows, left for 60 d, and then removed. After an additional 60 d, cows were checked for pregnancy by palpation per rectum. Because the pastures were large, not all cows were recovered from each pasture at the time of palpation. Fertility for each group of bulls was computed as the number of cows determined pregnant divided by the total number of cows palpated.

Study 2: Evaluating Fertility of Bulls Grouped According to Heparin-Binding Proteins in Sperm Membranes as Determined with Western Blots Using a Monoclonal Antibody

Semen Collection. Semen samples were collected from 62 (3 to 7 yr old) and 30 (2 yr old) Santa Gertrudis bulls by electroejaculation and processed as described previously. In addition, five Santa Cruz bulls (1/2 Santa Gertrudis, 1/4 Red Angus, and 1/4 Gelbvieh) were also evaluated and assigned to three pastures.

Sperm Membrane Proteins. Semen samples were thawed, and 200 μL of each sample was washed once with 1 mL of PBS at 4°C in a 5415 C Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY) at 6,000 × *g* for 5 min. Samples (30 μL of sperm pellets suspended in .1 mL of PBS buffer and .1 mL of sample buffer) were applied to each lane of SDS 12% polyacrylamide gels, and Western blots with indirect second antibody and Sigma Fast Red detection of HBP were used as described previously.

Grouping of Bulls According to Heparin-Binding Protein Profiles. The presence of three HBP in sperm samples of approximately 30, 24, and 21.5 kDa were analyzed. Bulls were identified and grouped according to the number of HBP bands recognized in sperm by M1. Group A bulls had the three HBP bands (30, 24, and 21.5 kDa); Group B bulls had a single 30-kDa HBP band; Group C bulls had 21.5- and 30-kDa HBP bands (i.e., the 24-kDa HBP was absent); and Group D bulls had no HBP bands in sperm.

Pasture Assignments and Determination of Fertility. Groups of Santa Gertrudis bulls (8 to 18) were pastured and bred to Santa Gertrudis cows as described previously. Additionally, one or two Santa Gertrudis and Santa Cruz crossbred bulls with known HBP profiles were assigned to smaller pastures.

Statistical Analyses

Chi-square 2 × 2 contingency tests were used to determine differences in pregnancy outcomes for cows bred to bulls with different patterns of HBP assayed with ELISA or Western blots.

Results

Sperm membranes and seminal fluid yielded similiar protein bands on SDS-PAGE (Figure 1, top panel), even though relative darkness of staining indicated that less protein was applied from sperm membranes (lanes 1, 2, and 6) compared with seminal fluid (lanes 10, 12, and 13). Western blots indicated that M1 recognized multiple bands at approximately 112, 106, 99, 75 to 84, 55 to 66, 35, 30, 24, 21.5, and 12 kDa in seminal fluid (Figure 1, bottom panel; lanes 11, 13, and 14) and at 75 to 84, 55 to 66, 49 to 51, 30, 24, 21.5, and 12 kDa in sperm membranes (Figure 1, bottom panel; lanes 1, 2, and 6).

Heparin and M1 affinity purifications of proteins were performed to determine which HBP were recognized by M1. Heparin and M1 purifications of seminal fluid proteins before application to PAGE/Western blots resulted in detection of similar proteins at 55 to 66, 30, 24, and 21.5 kDa (Figure 1, bottom panel;

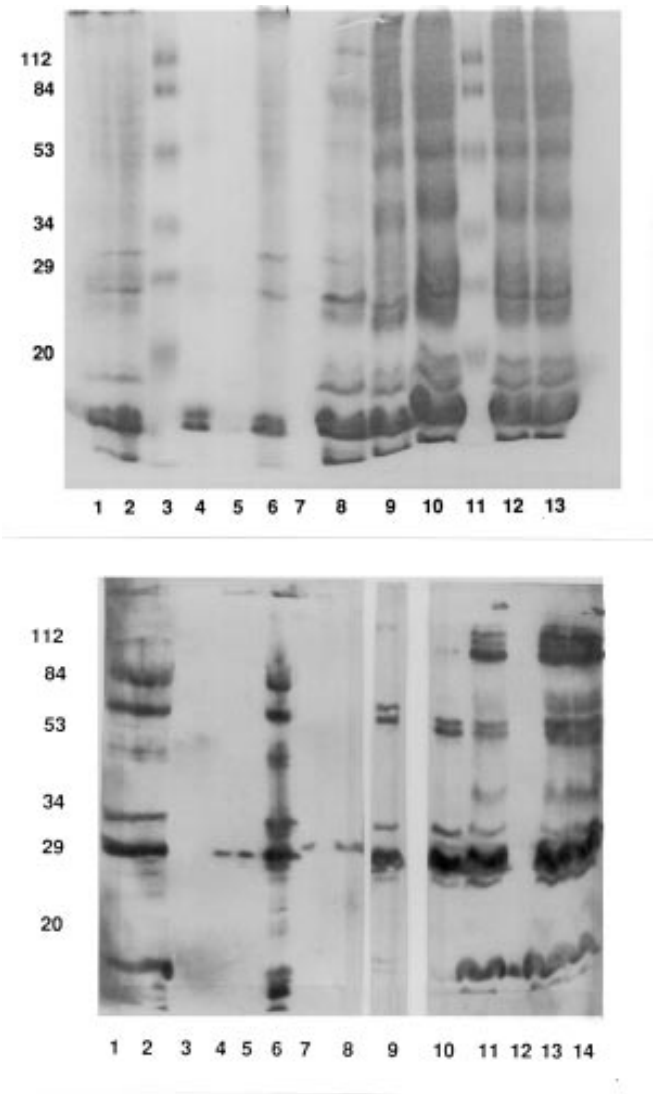

Figure 1. (Top panel) Coomassie blue-stained SDS-PAGE gel of sperm membranes (lanes 1, 2, and 6) and seminal fluid (lanes 10, 12, and 13) from a Santa Gertrudis bull. Proteins from sperm membranes and seminal fluid were affinity-purified with heparin (lanes 5 and 9, respectively) and monoclonal antibody (M1, lanes 4 and 8, respectively). Heparin and M1 affinity-purified proteins were similar. Molecular masses of standards (lanes 3 and 11) and a 29-kDa marker (lane 7) are indicated on the left. (Bottom panel) Autoradiograph of Western blot hybridization using M1 to HBP was developed with enhanced chemical luminescence. Proteins bands from sperm membranes (lanes 1, 2, and 6) and seminal fluid (lanes 11, 13, and 14) were from a Santa Gertrudis bull. Sperm membranes and seminal fluid proteins purified by heparin (lanes 5 and 10, respectively) and M1 (lanes 4 and 9, respectively) affinity before being applied to PAGE/Western blot indicated that similar-sized proteins bound to heparin and M1. Molecular masses of standards (lanes 3 and 12) are indicated on the left, and a 29-kDa marker recognized by enhanced chemical luminescence was in lanes 7 and 8.

Table 1. Pasture assignments including the number of Santa Gertrudis bulls grouped according to presence or absence of heparin-binding proteins (HBP) in seminal fluid and sperm membranes as determined with an ELISA using monoclonal antibody (M1) to HBP, number of Santa Gertrudis cows, and pasture size for each group

	Sperm membrane HBP	
Seminal fluid HBP	Present	Absent
	Group 1	Group 3
Absent	8 bulls	18 bulls
	207 cows	441 cows
	2,274 ha	2,743 ha
Present	Group 2	Group 4
	10 bulls	8 bulls
	257 cows	209 cows
	2,274 ha	1,489 ha

lanes 10 and 9, respectively). Seminal fluid protein bands at 99 to 112, 75 to 84, 35, and 12 kDa seemed not to recognize M1 (lane 9) or heparin (lane 10) attached to an affinity matrix. Heparin and M1 affinity purification before application to PAGE/Western blots required Parr Bomb isolation of sperm; therefore, concentrations of proteins were greatly reduced (Figure 1, top panel; lanes 5 and 4, respectively), and thus only the 24-kDa HBP was detected with Western blots using M1 (Figure 1, bottom panel; lanes 5 and 4, respectively).

In conclusion, M1 recognized multiple HBP (49 to 66, 30, 24 and 21.5 kDa). Detection of proteins with Fast Red and ECL detection systems for Western blots did not differ. Western blots stained with Fast Red were used in Study 2 and are shown in Figure 2.

In the first fertility study, the presence of multiple HBP in sperm or seminal fluid was rapidly determined with an ELISA using M1. Semen from 63 Santa Gertrudis bulls was tested and resulted in four groupings. Twelve bulls (19%) had HBP on sperm but with undetectable HBP in fluid (Group 1). Group 2 had bulls (25%, n = 16) with HBP detectable in sperm and in fluid. Bulls (40%, n = 25) with undetectable HBP in fluid and sperm were assigned to Group 3, and bulls (16%, n = 10) with HBP in fluid but not in sperm were assigned to Group 4. Pasture assignments resulted in 8, 10, 18, and 8 bulls from Groups 1, 2, 3, and 4 with 207, 257, 441, and 209 Santa Gertrudis cows in each pasture, respectively (Table 1). Concentrations of sperm and BSE scores did not differ among groups ($P > .05$; data not shown).

Bulls that had positive identification of HBP by M1 in sperm membranes were more ($P < .01$) fertile. Pregnancy rates were 89.1 and 89.2% for cows bred to bulls in Groups 1 and 2, respectively, and 78.1 and 79.1% for cows bred to bulls in Groups 3 and 4, respectively (Table 2). The presence of HBP in seminal fluid was not indicative of fertility. Therefore,

Table 2. Fertility (percentage of Santa Gertrudis cows pregnant of total number of palpated cows) and number of pregnant/total palpated cows (in parentheses) among Santa Gertrudis bulls grouped according to presence or absence of heparin-binding proteins (HBP) in seminal fluid and sperm membranes as determined with an ELISA using monoclonal antibody (M1) to HBP

	Sperm membrane HBP	
Seminal fluid HBP	Present	Absent
Absent	Group 1 89.1%[a] (147/165)	Group 3 78.1% (200/256)
Present	Group 2 89.2%[a] (174/195)	Group 4 79.1% (140/177)

[a]$P < .01$ compared with other values in the same row.

bulls with detectable HBP on sperm membranes were 11 percentage points more ($P < .01$) fertile than bulls with undetectable HBP on sperm membranes as determined with ELISA.

In the second fertility study, the presence of three HBP (30, 24, and 21.5 kDa) in sperm membranes was determined with Western blots using M1. Four band patterns were detected, and bulls were classified as Groups A to D (Figure 2). Santa Gertrudis bulls were grouped according to the pattern of three HBP in sperm (Table 3). Of the 49 older bulls (3 yr or older) in which HBP was determined, 67.3% had three HBP bands (30, 24, and 21.5 kDa; Group A) and 4.1% had a single 30-kDa HBP band (Group B). Some bulls (12.2%) had only the 21.5- and 30-kDa HBP (i.e., the 24-kDa HBP was absent; Group C). One group of bulls (16.3%) had no HBP bands present in sperm (Group D). There were 30 young bulls (2-yr) tested for HBP. Twenty of the younger bulls were classified as Group A (66.7%) and 10 bulls (33.3%) had no visible HBP bands (Group D). Concentrations of sperm and breeding soundness scores did not differ among Groups ($P > .05$; data not shown).

There were 41 older and 14 younger bulls grouped, pastured, and bred to 1,354 Santa Gertrudis cows (Table 4). Fewer bulls were actually used in the study (Table 4) than were tested for HBP (Table 3). One group of bulls (n = 11; Group E) was randomly selected from bulls that had passed the BSE but for which HBP patterns were not determined. Group E bulls were included for reference, because they were representative of how the rest of the bulls are selected and pastured at the ranch.

Groups of bulls with different HBP bands present in sperm membranes varied in fertility (Table 4). Group A and B bulls had the greatest fertility, ranging from 74.4 to 89.9% (mean = 81.3%) of the palpated cows pregnant. Group C bulls had a reduced fertility of 61.3%. Bulls without any detectable HBP bands present in sperm (Group D) had a fertility value of 41.9%. The bulls that had not been tested for HBP (Group E) had fertility of 75.1%. Group A and B bulls were more ($P < .01$) fertile than bulls in all other groups.

Four Santa Gertrudis and five Santa Cruz bulls with Group A patterns and one Santa Gertrudis bull with a Group D pattern were selected and used in six small research pastures containing one or two bulls (Table 5). Fertility ranged from 80 to 100%; the average fertility was 92.7% of the palpated cows pregnant.

Discussion

Fertility potential of bulls can be predicted by identifying HBP on sperm membranes. Experiments in these studies were performed as a continuation of previously published fertility studies (Bellin et al., 1994). Collectively, a total of four breeds of 343 range beef bulls bred to 8,352 cows in 31 pastures on a total of 65,046 ha have been evaluated. Bulls used in these studies had acceptable and comparable BSE values and sperm concentrations. Identifying and separating bulls of similar physical characteristics by HBP content of sperm membranes resulted in differences in fertility of as much as 40 percentage points. Identifying HBP on sperm membranes to determine fertility potential was performed using frozen ejaculates and was independent of incubating sperm for acrosomal

Table 3. Number and percentage of total older (3 to 7 yr) and younger (2 yr) bulls in each group as determined with Western blots using monoclonal antibody (M1) to heparin-binding proteins (HBP)

HBP		Mature bulls		Young bulls	
Group	Bands, kDa	n	%	n	%
A	30, 24, and 21.5	33	67.3	20	66.7
B	Only 30	2	4.1	0	0
C	30 and 21.5	6	12.2	0	0
D	No bands	8	16.3	10	33.3
Total		49		30	

Table 4. Fertility (percentage of Santa Gertrudis cows pregnant of total number of palpated cows) for each pasture containing Santa Gertrudis bulls grouped according to presence of heparin-binding protein (HBP) in sperm as determined with Western blots using monoclonal antibody (M1) to HBP

Pasture	Group[a]	No. of bulls pastured	No. of cows Pastured	No. of cows Palpated	Fertility, %
1	A	8	191	166	84.3
2	A	9	226	199	73.4
3	A	6	138[e]	127	89.9
4	A	11[b]	270	218	84.9
5	A	5[c]	132[e]	119	74.8
Average	A				81.3
6	B	2	53	46	86.9
7	C	6	155	124	61.3[f]
8	D	7[d]	189	186	41.9[f]
9	E	11	265	213	75.1[g]

[a]Group A bulls had the 30-, 24-, and 21.5-kDa HBP bands; Group B bulls had the 30-kDa HBP band; Group C bulls had the 30- and 21.5-kDa HBP bands; Group D bulls had no HBP bands; Group E bulls were not tested for HBP.
[b]Bulls were 2 yr old.
[c]Three bulls were 2 yr old, and two bulls were 2 to 7 yr old.
[d]Pasture contained six bulls with no HBP bands and one Group A (30, 24, and 21.5 kDa) bull.
[e]Cows were 2 yr old and had calved once.
[f]Values differ from Group A, $P < .001$.
[g]Values differ from Group A, $P < .05$.

Table 5. Fertility (percentage of Santa Gertrudis cows pregnant of total number of palpated cows) for each pasture containing one or two Santa Gertrudis and Santa Cruz bulls grouped according to the presence of heparin-binding proteins (HBP) in sperm as determined with Western blots using monoclonal antibody (M1) to HBP

Pasture no.	HBP group[a]	No. of Bulls pastured	No. of Cows palpated	Pregnant cows, %
10	A/D	2[b]	43	95.0
11	A	1[b]	23	100.0
12	A	2[b]	51[d]	80.3
13	A	2[c]	44[e]	88.6
14	A	1[c]	33	96.9
15	A	2[c]	57	89.9

[a]Group A bulls had the 30-, 24-, and 21.5-kDa HBP bands; Group D bull had no HBP bands.
[b]Santa Gerturdis bulls bred to Santa Gertrudis cows.
[c]Santa Cruz bulls bred to Santa Cruz cows.
[d]Cows were between 8 and 14 yr old.
[e]Cows were visually very thin at the start of the breeding season.

reaction or other tests to determine sperm functions.

Heparin-binding proteins are secreted by the male accessory glands and bind sperm at ejaculation (Miller et al., 1990; Nass et al., 1990). Multiple HBP form complexes with different affinities for heparin (Miller et al., 1990). Heparin-binding proteins bind to epididymal sperm and increase the ability of epididymal sperm to acrosome react in response to heparin and zona pellucida proteins (Miller et al., 1990). Total concentrations of HBP in seminal fluid or on sperm membranes do not differ among bulls (Bellin et al., 1994), and the number of heparin-binding sites per sperm does not vary among bulls (Marks and Ax, 1985). But, bulls with sperm with greater affinity for heparin have increased fertility (Marks and Ax, 1985).

High performance liquid heparin affinity chromatography (HPLC) was initially used to identify bulls with sperm that had measurable amounts of a complex of proteins with the greatest affinity for heparin, known as HBP-B5 (Bellin et al., 1994). Groups of bulls with HBP-B5 in sperm membranes, but not in seminal fluid, were 17 percentage points more fertile than groups with other HBP-B5 profiles. The HBP-B5 peak is composed of multiple proteins (31, 24, and 14 to 18 kDa; Miller et al., 1990). The HBP with larger molecular weight (24 and 31 kDa) are detected in peaks with greater affinity for heparin (HBP-B4 and HBP-B5; Miller et al., 1990). The larger (31 kDa) protein has been reported as 30 kDa (Chandonnet et al., 1990) and is often detected between 29 and 31 kDa. The approximate value of 30 kDa was used in this report.

In the current study, we used a monoclonal antibody (M1) that recognized 21.5-, 24-, and 30-kDa HBP, but not the smaller 14- to 18-kDa HBP, to select Santa Gertrudis bulls. Groups of bulls with HBP in sperm membranes, as identified with an ELISA using M1, were 11 percentage points more fertile than groups of bulls without detectable HBP in sperm. Fertility was not correlated with detection of HBP by M1 in seminal fluid. In comparison, for Santa Gertrudis bulls separated based on HBP-B5 profiles in the first study, there was a 9 percentage point difference in fertility between the most and least fertile groups (Bellin et al., 1994). The ELISA was used to determine whether proteins were present and not to identify individual proteins. When Western blots using M1 were used to detect the presence of individual proteins in sperm membranes, bulls with 30-, 24-, and 21.5-kDa HBP or just the 30-kDa HBP were 40 percentage points more fertile than bulls without those three HBP. Bulls with the 30- and 21.5-kDa HBP in sperm (i.e., the 24-kDa protein was absent; Group C), had intermediate fertility of 61.3%; thus, the interaction of 30- and 24-kDa HBP may be important for fertility. In conclusion, we used three methods to measure HBP in sperm membrane, and, as the sensitivity of the assays to determine specific HBP related to heparin affinity increased, our ability to determine differences in fertility increased.

Experiments to identify and characterize the HBP related to fertility (21.5, 24, and 30 kDa) have not

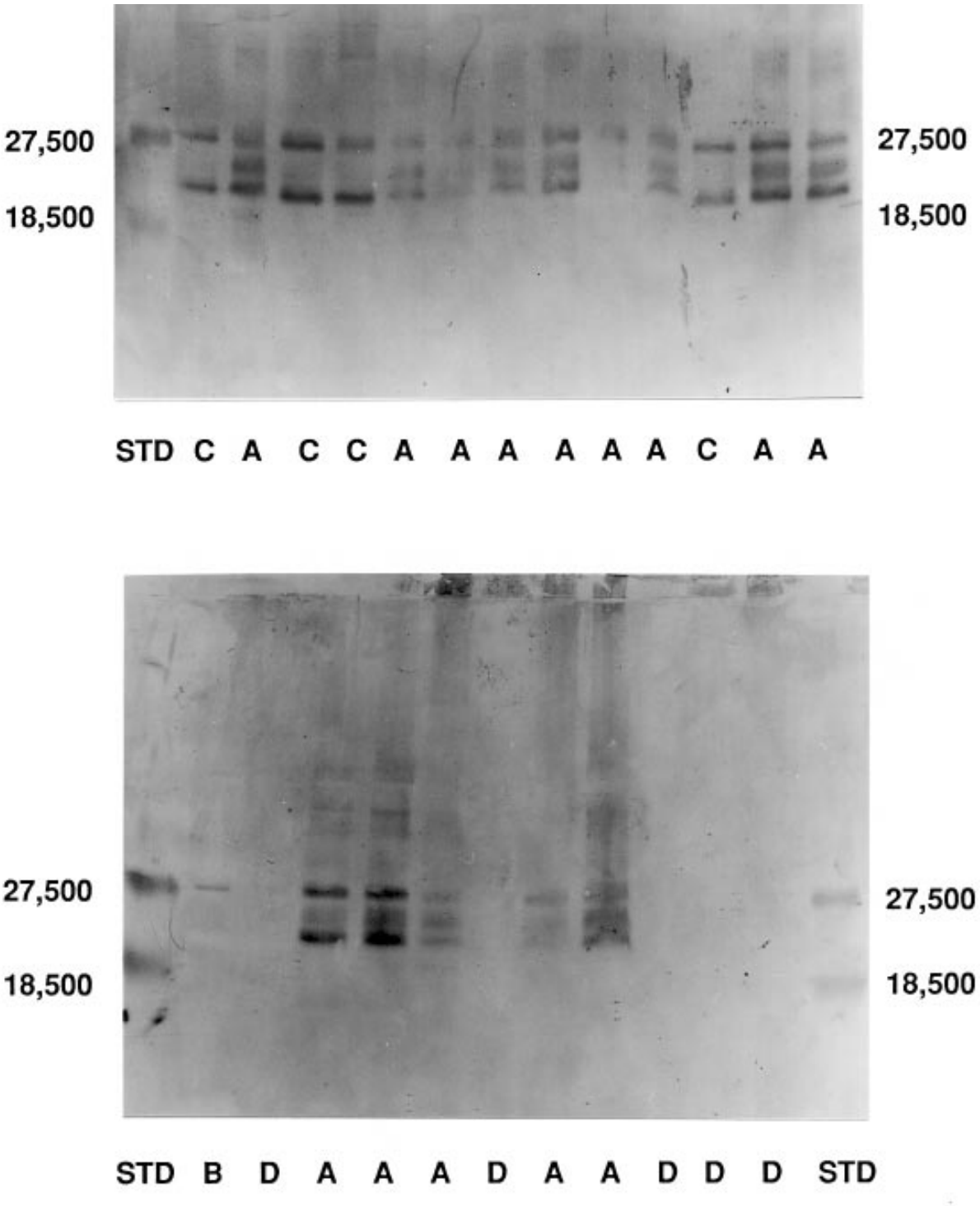

Figure 2. Western blot hybridization using monclonal antibody (M1) to heparin-binding proteins (HBP) was developed with the Sigma Fast Red detection system. Each lane represents sperm from one bull. Samples were applied to gel lanes in corresponding order in which bulls were collected for semen. Therefore, two blots containing profiles of 24 bulls were necessary to observe all four patterns. Group A bulls had the three HBP bands (30, 24, and 21.5 kDa); Group B bulls had a single 30-kDa HBP band; Group C bulls had 21.5- and 30-kDa HBP bands (i.e., the 24-kDa HBP was absent); Group D bulls had no HBP bands present in sperm. Molecular masses of standards (STD) are indicated on each side.

been performed. Many proteins have heparin-binding sites (e.g., growth factors, coagulation factors, DNA- and RNA-specific enzymes, lipase, lipoproteins, and proteases). Preliminary data indicated that M1 and heparin do not bind to the same site, because heparin binds to reduced proteins (Miller et al., 1990), but reduced HBP were not recognized by M1 (data not shown). This study demonstrated that M1 also recognized multiple HBP greater than 31 kDa, whereas HBP greater than 31 kDa have not been previously reported (Manjunath and Sairam, 1987; Chandonnet et al., 1990; Miller et al., 1990). Because M1 was a single antibody produced by a clone derived from one hybridized mouse spleen cell, the HBP recognized by M1 may be closely related. Protein patterns may possibly be due to variations in hydrolysis of larger HBP in which the heparin- and M1-binding sites were not destroyed. If hydrolysis of smaller HBP less than 31 kDa plays a role in fertility, it probably would occur before binding to sperm membranes; iodinated seminal HBP bound to sperm and retained their molecular size and ability to bind heparin after being extracted from sperm membranes (Miller et al., 1990).

Different HBP patterns may not be due to hydrolysis of proteins but to protein production by different accessory glands. Rat accessory glands (seminal vesicles, prostate, and Cowper's) produced HBP under androgen control (Nass et al., 1990). Patterns of HBP sorted on the basis of affinity to heparin, followed by electrophoresis, varied with source of accessory organ. Therefore, the different HBP recognized by M1 may reflect relative contributions of peptides of similar sizes from a particular secretion from accessory sex glands.

In another study, the ability of accessory sex gland fluid to affect cauda epididymal sperm penetration of zona-free bovine oocytes was evaluated for 10 bulls that ranged in fertility from 6% less than to 6% greater than the average (Henault et al., 1995). Protein content of seminal fluid was not evaluated, but samples of cauda epidiymal sperm mixed with accessory sex gland fluid from the more-fertile bulls had greater oocyte-penetrating ability than samples mixed with fluid from less-fertile bulls. Smaller populations of samples (11 of 42 combinations) indicated no advantage in penetration ability of epididymal sperm mixed with fluid obtained from bulls of varying fertility, and another 10 comparisons indicated that oocyte-penetrating ability was increased when seminal fluid from bulls of decreased fertility was added to epididymal sperm. In conclusion, seminal fluid contained components that translated into increased sperm fertility, but sperm may have a predetermined ability to bind these components. Our current and previous studies (Bellin et al., 1994) indicated that HBP with increased heparin affinity may be present in fluid, but if those proteins were not bound to the sperm, fertility would be subsequently reduced. How HBP interacted with other components in sperm membranes and translated into greater fertility is not known.

Methods used in our studies measured HBP in detergent extracts of sperm membranes. Attempts to measure sperm directly with ELISA were unsuccessful. Fluorescent microscopic localization of M1-binding to the anterior head of sperm was negatively correlated with fertility (unpublished observations, McCauley et al., 1995). Western blots of those sperm samples indicated that only the 24-kDa HBP was present in sperm membranes. Thus, the region on HBP recognized by M1 on sperm surfaces may be hydrophobic and oriented away from the surface of the sperm. Additional studies are needed to identify the peptides with the epitope for M1 binding.

In these studies, bull fertility was defined by the percentage of pregnant cows after a 60-d mating season. The actual fertilization rate or the ability of pregnancy to occur at a single mating was not determined. Mathematically, Group A bulls could have a fertilization rate of approximately 50% and still have 84% of the 25 cows pregnant after 60 d (approximately three reproductive cycles; 12 cows pregnant the first cycle; six the second; three the third; or 21 pregnant cows per 25 total cows bred). Bulls without the 24-kDa HBP (Group C) and bulls without HBP (Group D) on sperm membranes may have had decreased fertilization rates (approximately 30 and 20%, respectively). Fertility of multiple bulls within a pasture was examined. Fertility of one or two Group A bulls was also determined, although no comparisons among other group types were made. Pregnancy rates in pastures with individual or two bulls may be slightly greater than rates in pastures with multiple Group A bulls. One pasture had a Group A and a Group D bull; if a bull only bred a particular 25 cows and no others, then we would expect fertility to be decreased compared with that in pastures with two Group A bulls. But, when only two bulls are competing for 50 cows, the actual fertility of the pasture group should not change significantly from that of the bull of greater fertility. In comparison, Group E contained a mixture of bulls that had passed the BSE but were not tested for HBP and should have been representive of the average for the ranch. Approximately 70% of the bulls would be Group A or B (Table 1); we would expect that fertility of Group E would not be greatly reduced from the fertility of Group A or B bulls. In these studies, fertility of Group E bulls was six percentage points less than the average fertility for Group A bulls, and our previous study indicated that the more-fertile Santa Gertrudis bulls (Group 1) differed from the calculated average fertility by five percentage points (Bellin et al., 1994).

In conclusion, the presence of 21.5-, 24-, and 30-kDa HBP in sperm membranes is an important

determinant of fertility potential of bulls. The sources of those proteins, control of their synthesis, secretion, and mechanisms for binding to sperm to affect differences in fertility of males will be important topics for future research.

Implications

Heparin-binding proteins (HBP) are produced by male accessory glands and bind to sperm at ejaculation. Ability of accessory glands to produce HBP and sperm to bind HBP differs among males. Identifying the presence of a 21.5-, 24-, and 30-kDa HBP in sperm membranes with a monoclonal antibody allowed us to separate bulls into groups that differed in fertility by 40 percentage points. Thus, fertility potential of bulls can be predicted by HBP content of sperm membranes.

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