BIOLOGY OF REPRODUCTION (2016) **95**(6):122, 1–12
Published online before print 5 October 2016.
DOI 10.1095/biolreprod.116.138792

Cauda Epididymis-Specific Beta-Defensin 126 Promotes Sperm Motility but Not Fertilizing Ability in Cattle[1]

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ABSTRACT

Bovine beta-defensin 126 (BBD126) exhibits preferential expression for the cauda epididymis of males, where it is absorbed onto the tail and postacrosomal region of the sperm. The aim of this study was to examine the role of BBD126 in bull sperm function. Fresh and frozen-thawed semen were incubated in the presence of different capacitating agents as well as with phosphatidylinositol-specific phospholipase C. These treatments, which have been successful in releasing beta-defensin 126 from macaque sperm, proved to be ineffective in bull sperm. This finding suggests that the protein behaves in a different manner in the bovine. The lack of success in removing BBD126 led us to use corpus epididymis sperm, a model in which the protein is not present, to study its functional role. Corpus sperm were incubated with cauda epididymal fluid (CEF) in the absence or presence of BBD126 antibody or with recombinant BBD126 (rBBD126). Confocal microscopy revealed that rBBD126 binds to corpus sperm with the same pattern observed for BBD126 in cauda sperm, whereas an aberrant binding pattern is observed when sperm are subject to CEF incubation. Addition of CEF increased motility as well as the number of corpus sperm migrating through cervical mucus from estrus cows. However, it decreased the ability of sperm to fertilize in vitro matured oocytes. The presence of the antibody failed to abrogate these effects. Furthermore, when rBBD126 was added in the absence of other factors and proteins from the CEF, an increase in motility was also observed and no negative effects in fertility were seen. These results suggest that BBD126 plays a key role in the acquisition of sperm motility in the epididymis.

epididymis, in vitro fertilization (IVF), sperm

INTRODUCTION

The process of spermatogenesis, which takes place in the testes postpuberty, produces a nonfunctional male gamete that has no transcriptional or translational abilities due to a highly condensed chromatin [1]. To acquire motility and fertilizing ability, sperm must transit through the epididymis, a convoluted tube that connects the efferent ducts in the testis to the vas deferens. Along this journey, sperm undergo maturation, which consists of a series of sequential biochemical modifications as a result of subtle interactions with the extracellular milieu of the epididymis [2].

Based on anatomical differences, the epididymis can be grossly divided into three regions, including the caput, corpus, and cauda. The interplay of steroid hormones, testicular factors, and other elements such as temperature, pH, and osmotic pressure creates a unique physiological environment in each of these areas [3, 4]. Each region exhibits distinctive gene expression profiles within the epithelium that dictate segment-specific secretion of proteins into the luminal fluid that affect sperm maturation directly or indirectly [5]. Although there are differences between species regarding the epididymal region in which sperm first exhibit motility, in most mammals this ability increases progressively from the corpus to the cauda, with testicular and caput epididymis showing either weak or no tail beating [6, 7]. Several studies in different species have shown that forward motility can be induced in immature sperm in vitro by inhibiting cyclic adenosine monophosphate (cAMP) phosphodiesterases or by exposing demembranated sperm to media containing cAMP or ATP [8–10]. Thus, it would seem that immature sperm possess the machinery required for motility and that the activation of this machinery is dependent on intracellular cAMP levels. However, maturation of this machinery along the epididymis seems to occur to some degree, as the percentage of demembranated sperm activated increases and the pattern and intensity of such activation varies depending on the region from which sperm are recovered [9].

Amongst the proteins secreted by the epithelium of the epididymis that bind to the sperm surface, several members of the β-defensin family have been identified [11–14]. β-Defensins are a family of host-defense peptides produced by multiple epithelial tissues and immune cells, which are involved in the innate immune response [15]. In the epididymis, the expression of β-defensin genes exhibits a clear region-specific pattern [16–19]. For example, in the rat and mouse, β-defensin 12 (*Defb12*), and *-15* are more abundantly expressed in the caput epididymis, whereas expression of *Defb28* and *-40* is restricted to the caudal region [18, 19]. Furthermore, species-specific variation in gene expression has been documented, with preferential expression of *Defb13* in the cauda epididymis of the rat [19] but in the corpus in the mouse [18]. In contrast to rodents, predominant expression of the human ortholog (*HBD13*, also known as *DEFB13*) is found in the testis [20].

[1]This work was supported by a grant from the Irish Department of Agriculture, Food and The Marine under the Research Stimulus Programme (Grant No. 11/S/104).
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Received: 19 January 2016.
First decision: 15 February 2016.
Accepted: 4 October 2016.
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eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

Although most studies on these proteins have focused on their antimicrobial characteristics, there is growing evidence for a role for β-defensins in reproductive function. In recent years, specific members of this protein family have been associated with sperm maturation and motility in rodents [12, 14]. The β-defensin-like protein, Bin1b, which is exclusively expressed in the epididymis of the rat, binds to the sperm surface and induces Ca^{2+} uptake, resulting in sperm acquiring progressive motility [14]. Knock down of another rat β-defensin found in the same cluster, *Defb15,* led to a decline in both total and progressive motility [12]. Moreover, lower levels of DEFB1 on human sperm has been associated with reduced motility as well as lower bactericidal activity [21]. In the macaque, DEFB126 is secreted in the corpus and cauda epididymis where it has been reported to bind to the sperm surface [13]. This coating protein contains multiple sialylated oligosaccharides that play a role in the migration of sperm through the cervical mucus (CM). By increasing the negative charge on the sperm, DEFB126 enables them to move through the electronegative mucus more efficiently [22]. Release of DEFB126 during the capacitation process, which in the macaque has been shown to be induced by treatment with caffeine and dibutyryl-cAMP (dbcAMP), is required for sperm to be able to interact with the zona pellucida [23]. Furthermore, the lack of DEFB126 on the sperm surface elicits a dramatic increase in immune recognition of a variety of sperm proteins [24]. In humans, a sequence variation in *DEFB126* was correlated to a reduction in glycosylation levels and in the rate of mucus penetration of sperm [25]. These alterations ultimately lead to impaired reproductive function in individuals containing this variation in their genome [25].

Our group has recently discovered and profiled the expression of a cluster of 19 (cattle) and 13 (horses) novel β-defensin genes along the male and female reproductive tracts [26, 27]. Studies in cattle have shown that these genes are preferentially expressed in the reproductive tract [28]. A subgroup of these genes, made up of bovine β-defensin 132 (*BBD132*), *-129, -128, -127, -126, -125,* and *-125a*, was found to be uniquely expressed in males, preferentially in the epididymis [28]. This pattern of expression suggests a role in male reproductive function.

Despite the growing body of evidence linking defensins to sperm maturation in rodents and primates, there is currently, to our knowledge, no information on their role in bovine sperm function. Cattle represent an excellent model in which to study issues of male fertility due to the large national databases available, the high number of progeny per sire, and the consequent high confidence associated with fertility estimates. BBD126 has been identified as an epididymal-specific protein that is secreted in the cauda epididymis of the bull [29]. However, neither its distribution on sperm nor its potential reproductive role has been studied. The objective of the present study was to characterize the role of BBD126 in bovine sperm function. We show that BBD126 binds to the tail and postacrosomal regions of sperm, where it remains after capacitation, suggesting a potential role of BBD126 in sperm function. We explore this hypothesis through a model based on the incubation of immature corpus sperm with epididymal fluid recovered from the cauda (CEF) or recombinant BBD126 (rBBD126).

MATERIALS AND METHODS

Reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. unless stated otherwise.

Experiment 1: Characterization of BBD126 on Fresh and Frozen-Thawed Bovine Sperm

The aim of experiment 1 was to characterize the presence and binding pattern of BBD126 on both fresh and frozen-thawed sperm. For each of the three replicates carried out, freshly ejaculated semen (1 ml) was obtained from three bulls at an artificial insemination center (National Cattle Breeding Centre, Enfield, Ireland), pooled, and transported undiluted back to the laboratory at 37°C. Frozen semen straws obtained from the same bull, and diluted in BullXCell (IMV Technologies), were placed in a warm water bath at 37°C for 30 sec to thaw. Motile sperm from both fresh and frozen-thawed samples were selected by centrifugation through a 95%–45% discontinuous Percoll gradient (Pharmacia) for 10 min at 700 × *g*, followed by a second centrifugation in HEPES-buffered Tyrode medium at 200 × *g* for 5 min. Concentration was assessed using a hemocytometer and diluted according to the analysis that would be performed. The presence of BBD126 on the sperm samples was determined by Western blot. The presence and localization of the protein were confirmed by confocal microscopy. Furthermore, the possibility that the protein was glycosylphosphatidylinositol (GPI)-anchored or associated to a GPI surface protein was explored through incubation of the frozen-thawed sperm with 0.1 or 1 IU/ml phosphatidylinositol-specific phospholipase C (PIPLC). After 1-h incubation with the enzyme at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity, the sperm and supernatants were analyzed by Western blot.

Antibody

A custom monoclonal antibody specific for BBD126 (α-BBD126 antibody [Ab]) was ordered from GeneScript, and generated as described by Narciandi et al. [29]. Briefly, five BALB/c mice were inoculated with a 14-amino acid chemically produced peptide (RNGERVINPPTGMC). Immune response was confirmed by binding of serum to the antigen in an enzyme-linked immunosorbent-type assay, and the cells were isolated for cell fusion and hybridoma production. Unpurified antibodies produced by each of the four hybridoma clones, selected and tested in an enzyme-linked immunosorbent assay against the peptide, were tested against BBD126 on Western blot. Clone 6A11E2 was selected for large-scale production and purification. The specificity of the antibody was validated using a peptide competition assay where a sperm lysate sample was blotted with α-BBD126 Ab in the presence of recombinant BBD126 (rBBD126) or rBBD117 (another bovine beta-defensin found in the same gene cluster). The specificity of the antibody was tested further by transfecting human embryonic kidney-derived cells (HEK293) with a transient expression vector containing the coding sequence for BBD126 [29]. When analyzed by Western blot, only cells transfected with BBD126 showed a band of the predicted protein size.

Western Blot Analysis

After washing the sperm in HEPES-buffered Tyrode medium, sperm concentration was assessed and adjusted to 15×10^6 sperm/ml. Samples were then centrifuged at 16 000 × *g* for 10 min and the supernatants were discarded, except those obtained from the PIPLC-treated samples that were stored at −20°C, and the cell pellets were stored at −20°C until analysis. Following thawing of the samples on ice, sperm were lysed with lysis buffer (50 mM Hepes, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 10% glycerol, 0.5% NP40, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM phenylmethane sulfonyl fluoride, and 1mM Na_3VO_4) for 30 min and centrifuged at 16 000 × *g* for 30 min. Supernatants were recovered and boiled at 95°C for 5 min. Supernatants obtained from the PIPLC-treated samples were assessed for total protein concentration with a Pierce bicinchoninic acid protein assay kit (Life Technologies) and adjusted to 15 μg of total protein loaded per sample. Sperm and supernatant proteins were separated by a 4%–12% SDS-PAGE gel under reducing conditions and transferred to a polyvinyl difluoride membrane (Life Technologies). The membrane was blocked with 2% BSA/PBS-Tween for 1 h before adding the α-BBD126 Ab (1.08 μg/ml) and leaving it to incubate overnight at 4°C. Goat antimouse Alexa Fluor 680 (1:10 000 dilution; Life Technologies) was used as secondary antibody, and its infrared fluorescence was read on an Odyssey Imaging System (LI-COR Biosciences).

Fluorescent Immunolocalization of BBD126

Diluted sperm (300 μl, 1×10^6 sperm/ml) was incubated overnight in 8-well glass slides (Merck Millipore) at 39°C under an atmosphere of 5% CO_2 in air and maximum humidity to ensure sperm binding to the slides. After incubation, sperm cells were fixed with 4% paraformaldehyde/PBS for 30 min on ice and permeabilized for 15 min in 0.2% Triton X-100/PBS. Sperm were

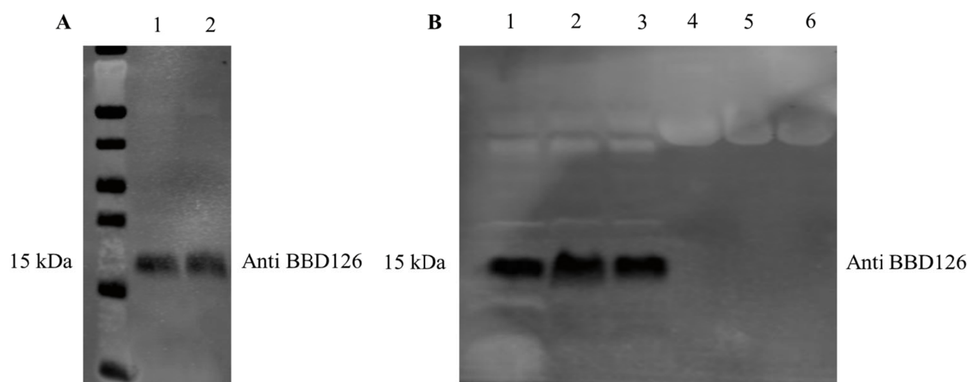

FIG. 1. **A**) Western blot analysis showing an intense band corresponding to BBD126 on both fresh (lane 1) and frozen-thawed (lane 2) sperm. **B**) Detection of BBD126 in sperm incubated with 0.1 IU/ml (lane 2) or 1 IU/ml (lane 3) PIPLC by Western blot. The supernatant (lanes 5 and 6, respectively) show no reactivity to the α-BBD126 Ab. Control sperm and media samples are in lanes 1 and 4, respectively). Experiment 1.

subsequently blocked with blocking buffer (4% BSA and 0.02% Triton X-100/PBS) for 1 h at room temperature. Samples were then incubated with α-BBD126 Ab (1:250 dilution) overnight at 4°C. After three 5 min washes in PBS, sperm were incubated for 1 h with goat antimouse IgG-biotin antibody (1:500 dilution; Life Technologies) as a secondary antibody, washed three times in PBS, and incubated for an additional 1 h with Alexa Fluor 488-streptavidin conjugate (1:500 dilution; Life Technologies). Finally, samples were washed three additional times in PBS and incubated with Hoechst 33342 for 10 min. The slides were mounted with Mowiol, and sperm were then observed under an Olympus FluoView FV1000 confocal microscope equipped with a 60×/1.35NA oil immersion objective. Images were acquired at a resolution of 1024 × 1024 pixels, and a pixel dwell time of 12.5 μsec. Sequential acquisition mode was used in all cases.

In order to confirm that permeabilization of sperm did not alter the localization pattern of BBD126, a representative number of sperm samples were stained skipping this step in the protocol. No differences were observed in the localization pattern of BBD126 when either protocol was used.

Experiment 2: Removal of BBD126 from Bovine Sperm Using Various Capacitating Agents

The aim of Experiment 2 was to assess the effect of the removal BBD126 from the surface of sperm on sperm function. Pooled fresh sperm from three bulls, or frozen-thawed sperm diluted in BullXCell, were incubated with various capacitation-inducing factors, previously reported to remove BDEF126 from macaque sperm [13]. Briefly, sperm were diluted to 20×10^6 sperm/ml and incubated with either 2 mM caffeine, 2 mM dbcAMP, or a combination of 1 mM caffeine and 1 mM dbcAMP. An additional treatment group consisting of 10 μg/ml heparin (Calbiochem) was used because this is a routine method for stimulating capacitation during bovine in vitro fertilization (IVF). After 1 h incubation at 39°C and 5% CO_2, samples were washed by centrifugation in HEPES-buffered Tyrode medium at 200 × *g* for 5 min. Sperm concentration was adjusted to either 2×10^6 sperm/ml to perform IVF or to 15×10^6 sperm/ml to confirm presence or absence of BBD126 by Western blot. Samples for Western blot analysis were analyzed as described above. The experiment was replicated three times.

Calcium Ionophore A23187-Induced Acrosome Reaction

In order to determine if the localization of BBD126 changes after the acrosome reaction, sperm were incubated in the presence of calcium ionophore. Frozen-thawed sperm were centrifuged through a 95%–45% discontinuous Percoll gradient for 10 min at 700 × *g* followed by a second centrifugation in HEPES-buffered Tyrode medium at 200 × *g* for 5 min. Sperm concentration was adjusted to 30×10^6 sperm/ml in HEPES-buffered Tyrode medium containing 10 μM calcium ionophore A23187. After 3 h incubation with the ionophore, sperm were centrifuged at 200 × *g* for 5 min. Concentration was reassessed and adjusted to 1×10^6 sperm/ml. Sperm were stained as described earlier. Peanut agglutinin conjugated with fluorescein isothiocyanate was used to label the acrosome of sperm. Goat antimouse Alexa Fluor 647 (1:500 dilution) was used as secondary antibody against α-BBD126.

IVF

Ovaries from cows and heifers were collected at a commercial abattoir, and surface visible follicles (>2 mm) were aspirated to recover cumulus-oocyte complexes (COCs). Good quality COCs (n = 50 per treatment, four replicates) were matured in TCM-199 supplemented with 10% (v/v) fetal calf serum and 10 ng/ml epidermal growth factor (n = 50 COCs per well) for 24 h at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. Matured COCs were fertilized with 1×10^6 epididymal sperm treated as described earlier. COCs and sperm were co-incubated at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. Approximately 20 h postfertilization, cumulus cells were removed, and presumptive zygotes were cultured in 25 μl droplets of synthetic oviduct fluid supplemented with 5% fetal calf serum (n = 25 per drop) under mineral oil until Day 7 (Day 0 = day of fertilization). Culture dishes were kept at 39°C under an atmosphere of 5% CO_2 and 5% O_2 in air with maximum humidity. Cleavage was assessed 48 h postfertilization and blastocyst development was recorded on Day 7 and expressed over the total number of oocytes fertilized.

Experiment 3: Characterization of Epididymal Sperm Function

Due to the lack of success in removing BBD126 by inducing sperm capacitation (experiment 2), we used corpus epididymal sperm, a model in which the protein is not present [29], to study its functional role in cattle. The aim of experiment 3 was to characterize BBD126 in sperm from the caput, corpus, and cauda regions of the epididymis. Testes were recovered from three mature bulls postslaughter and transported within 2 h to the laboratory at ambient temperature. To recover sperm from the cauda epididymis, a small incision was made in the cauda, and the lumen of the deferent duct was cannulated with a blunted 22 gauge needle. Sperm cells were then gently flushed through the cauda with a 5 ml syringe loaded with PBS at 38°C, as described by Druart et al. [30]. Due to the small diameter of the epididymal tubule, flushing of caput and corpus sperm was not possible. The caput and the corpus epididymis were each isolated and minced with a scalpel blade in a dish of PBS at 38°C. Sperm from caput and corpus epididymis from each bull were pooled and separately washed in HEPES-buffered Tyrode medium by centrifugation at 200 × *g* for 5 min. Sperm concentration was assessed using a hemocytometer and adjusted to 2×10^6 sperm/ml for IVF and immunofluorescence analysis, to 15×10^6 sperm/ml for Western blot analysis, or to 20×10^6 sperm/ml for computer-assisted sperm analysis (CASA). Western blots (three replicates), immunofluorescent labeling (two replicates), and IVF (n = 50 COCs per treatment per replicate, three replicates) were carried out as described previously.

CASA

Sperm from different epididymal regions were assessed for motility using a CASA system (Sperm Class Analyser; Microptic). A sample from each treatment was placed on a 10 μl four-chamber Leja slide (Micro Optic) and evaluated. Percentage of motile sperm and percentage of sperm exhibiting progressive motility were evaluated. Two slides were assessed per sample with a minimum of 100 sperm cells counted in a minimum of four fields of view per slide. The experiment was replicated four times.

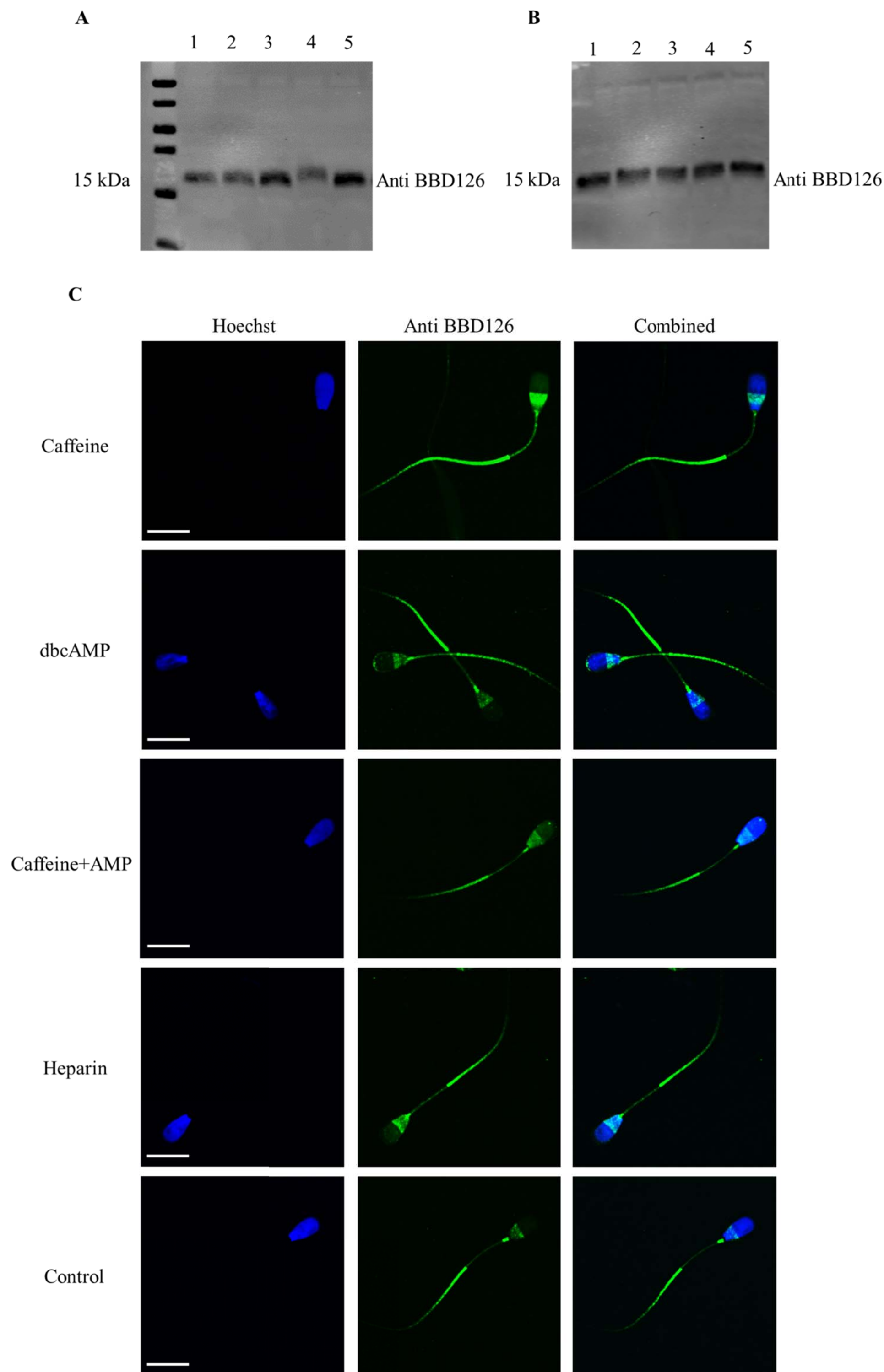

FIG. 2. Western blot of fresh (**A**) and frozen-thawed (**B**) sperm after treatment with caffeine (lane 1), dbcAMP (lane 2), a combination of caffeine and dbcAMP (Caffeine+AMP; lane 3), or heparin (lane 4) (control: lane 5). **C**) Representative confocal images of frozen-thawed sperm incubated with the same treatments. Bars = 10 μm. Experiment 2.

Experiment 4: Effect of CEF on Motility of Corpus Epididymal Sperm

The aim of experiment 4 was to determine the effect of incubating immature corpus sperm in the presence of CEF, with or without α-BBD126 Ab, on sperm motility and fertilizing ability. Because BBD126 is exclusively secreted in the final (cauda) region of the epididymis [29], the differences in functional ability observed in bull sperm from different epididymal regions may be partly explained by the activity of this protein.

Sperm from the corpus and cauda epididymis were recovered as described earlier, and their concentration was adjusted to 20×10^6 sperm/ml in HEPES-buffered Tyrode medium. CEF was separated from cauda sperm by centrifuging the fluid obtained in the flushing process at $15\,000 \times g$ for 10 min. The supernatant was recovered and passed through a 0.22 μm filter to ensure no cauda sperm remained. Four treatment groups were analyzed: 1)

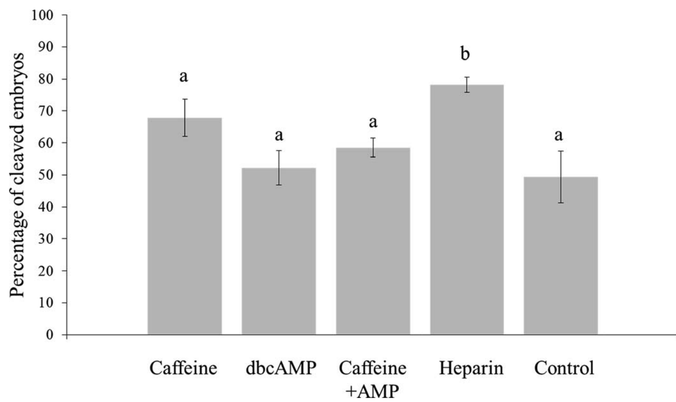

FIG. 3. Percentage of cleaved zygotes observed on Day 2 (Day 0 = day of fertilization) after fertilizing in vitro matured oocytes (n = 50 per treatment, four replicates) with frozen-thawed sperm incubated with caffeine, dbcAMP, a combination of caffeine and dbcAMP (Caffeine+AMP), or heparin. Sperm incubated in HEPES-buffered Tyrode medium were used as control. Data reported as least-squares means ± SEM. Different superscripts indicate a significant difference ($P < 0.001$). Experiment 2.

corpus sperm incubated with CEF (1:20) (CEF), 2) corpus sperm incubated with 1:20 CEF previously blocked with α-BBD126 Ab (1:20 dilution; final antibody concentration in CEF = 5.75 μg/ml) (CEF + Ab), 3) untreated corpus sperm (corpus), and 4) untreated cauda sperm (cauda). All treatments were incubated for 1 h in an incubator at 38.5°C and 5% CO_2, before performing Western blot (three replicates), immunofluorescent labeling (two replicates), CASA (four replicates), mucus penetration assays (four replicates), or IVF (five replicates).

Cervical Mucus Penetration

Cervical mucus was collected from cows observed in standing estrus. A polyethylene catheter attached to a 20 ml syringe was inserted into the vagina and guided toward the cervical os. Once the catheter was in position, gentle suction was applied to recover the CM. Mucus from three different cows was pooled, aliquoted, and stored at −20°C. Cervical mucus from the same pool was used throughout, and the mucus penetration test was carried out as described by Kiernan et al. [31]. Flattened capillary tubes (0.3 mm × 3.0 mm × 100 mm; Composite Metal Services Ltd.) were marked at 10 mm intervals between 10 and 90 mm, filled with thawed mucus, and put in an incubator at 38.5°C while sperm were processed. Treated epididymal sperm were diluted to a final concentration of 20×10^6 sperm/ml in HEPES-buffered Tyrode medium containing Hoechst 33342 fluorescent stain (10 μg/ml), and incubated for 10 min at 38.5°C to ensure uptake of the stain. Following incubation, two mucus-filled capillaries were placed vertically into an Eppendorf tube containing 250 μl of the stained sperm dilution and left for 30 min in an incubator at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. Capillary tubes were then removed and placed on a hot plate at 70°C for 3 min to immobilize the sperm. Sperm were counted across the width of the tube, one field of view wide, at each 10 mm interval between 10 and 90 mm using a fluorescent microscope (Nikon Eclipse TE2000s). Four replicates were completed with two capillary tubes representing each treatment in each replicate.

Experiment 5: Effect of rBBD126 on Corpus Sperm Function

Because the α-BBD126 Ab was not able to block the protein from binding to the sperm surface, a different approach was adopted to study the function of β-defensin 126 on bull sperm. The aim of experiment 5 was to study the motility and fertilizing ability of corpus sperm incubated in the presence of recombinant BBD126 (rBBD126).

Recombinant BBD126 Expression

Because BBD126 is an antimicrobial peptide, protein expression in an *Escherichia coli* host was only possible with the aid of a carrier protein, as described by Narciandi et al. [29]. Briefly, the *BBD126* coding sequence was amplified by PCR (forward primer: 5′-GGTAATTGGTATGTGAGAAA-3′; reverse primer: 5′-AGCAATGCCTGTTGTAGATC-3′) using a Platinum Taq DNA polymerase (Life Technologies). The resulting PCR product was cloned with the pBAD/TOPO Thiofusion kit (Invitrogen Ltd.) following the manufacturer's instructions. The resulting rBBD126 protein has a thioredoxin fusion at the N-terminus and a hexa histidine tag at the C-terminus. The sequence was confirmed by Sanger sequencing of the resulting plasmid (GATC Biotech). Luria-Bertani broth was used as the culture medium, and ampicillin was added at a concentration of 100 μg/ml. Bacteria were incubated overnight at 37°C before adding 50% glycerol Luria-Bertani broth (1:1) and storing them at −20°C as a starting culture. For the production of the rBBD126, the *E. coli* starter culture was grown in 1 L of medium at 37°C to an optical density of 0.5 at 600 nm. Protein expression was activated by adding 1 ml 20% L-arabinose, and the culture was grown at 28°C for 4 h. After production, the bacteria were sonicated using a sonicator equipped with a microtip. The sample was then centrifuged to recover the rBBD126-rich supernatant. The extracted solution was mixed with 1.5 ml nickel-nitrilotriacetic acid magnetic beads (Qiagen), beads were washed several times with PBS, and protein was eluted with a 300 mM imidazole solution. Pooled sample was injected into an AKTA gel filtration system set up with a Hiprep 16/60 Sephacryl S100HR column (GE Healthcare). Fractions were tested by Western blot using α-BBD126. The resulting fusion protein was treated with enterokinase protease (New England Biolabs) to cleave the N-terminus thioredoxin tag following the manufacturer's protocol. Pooled fractions of the nickel-nitrilotriacetic acid purification were injected into a Hiprep 16/60 Sephacryl S100HR column (GE Healthcare) to purify the rBBD126. Coomassie gel staining and Western blot using α-BBD126 were analyzed to determine the purity of the resulting rBBD126.

Incubation of Corpus Sperm with rBBD126

Corpus and cauda sperm were adjusted to a concentration of 20×10^6 sperm/ml, and the following treatments were assessed: 1) corpus sperm incubated with 30 μg/ml rBBD126 (rBBD126), 2) untreated corpus sperm (corpus), and 3) untreated cauda sperm (cauda). The concentration of rBBD126 added to the corpus sperm was based on previous studies of DEFB126 in the macaque by Tollner et al [22, 23, 32]. After 1 h at 38.5°C and 5% CO_2, the samples were assessed by Western blot (three replicates), immunofluorescent labeling (two replicates), CASA (four replicates), mucus penetration (four replicates), and IVF (four replicates).

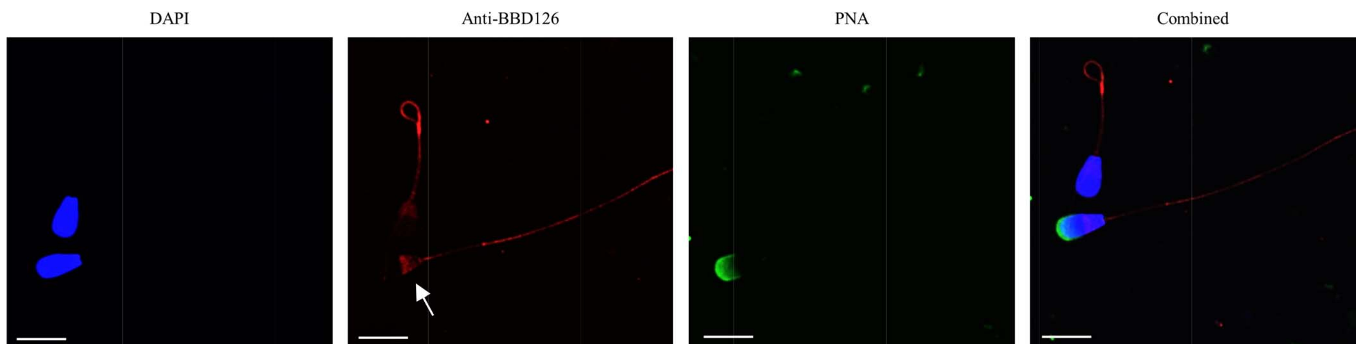

FIG. 4. Representative image of an acrosome-intact (arrow) and acrosome-reacted sperm after incubation with calcium ionophore. Bars = 10 μm. Experiment 2.

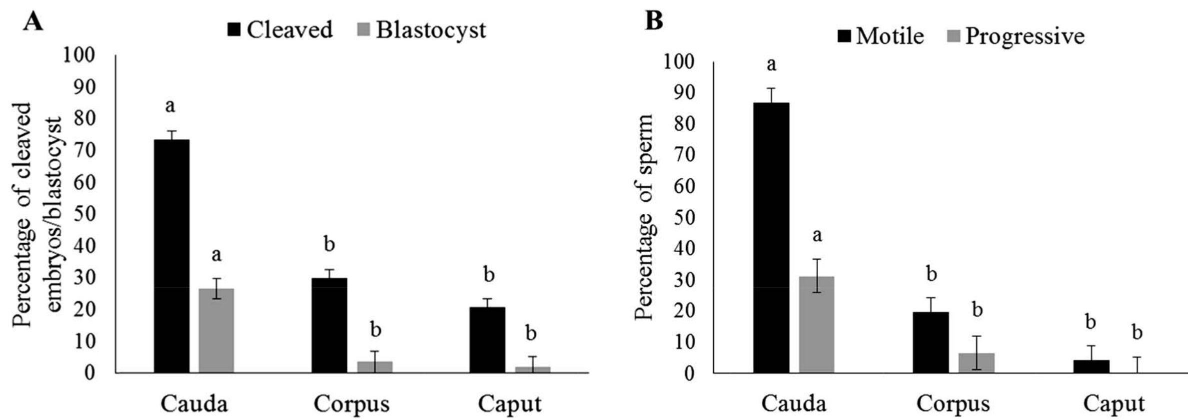

FIG. 5. Functional analysis of sperm from different epididymal regions (cauda, corpus, and caput). **A**) Percentage of cleaved embryos at Day 2 (Day 0 = day of fertilization) and blastocysts on Day 7 (black and gray bars, respectively) obtained after fertilization with sperm from the caput, corpus, and cauda epididymis (n = 100 oocytes per group). Data reported as least-squares means ± SEM. Different superscripts indicate a difference within cleavage or blastocyst rate ($P < 0.05$). **B**) Overall (black bars) and progressive (grey bars) motility analyzed by CASA. Data reported as least-squares means ± SEM. Different superscripts indicate a significant difference within motile or progressive sperm ($P < 0.05$). Experiment 3.

Statistical Analysis

Data were checked for normality and homogeneity of variance using histograms, qplots, and formal statistical tests in the Univariate procedure (version 9.1.3; SAS Institute). Data that were not normally distributed were subsequently transformed prior to analysis.

The sperm mucus penetration data and motility required a square root transformation to normalize the residuals because preliminary analyses revealed that the distribution of values was positively skewed. Additionally, the data from IVF (cleavage rate and blastocyst rate) were subjected to arcsin transformation prior to analysis. The transformed data were used to calculate P values. However, the corresponding least-squares means and SEM of the nontransformed data are presented in the results for clarity.

For all individual experiments, IVF data for the proportion of oocytes that cleaved and reached the blastocyst stage were analyzed using the Mixed procedure of SAS (SAS Institute) with a model that included treatment imposed as a fixed effect and bull as a random term. Sperm mucus penetration was analyzed using the Mixed procedure of SAS (SAS Institute). The model had fixed effects for treatment, position along the capillary tube, and a two-way interaction, and a random term for bull was fitted. Interaction terms if not statistically significant ($P < 0.05$) was subsequently excluded from the final model. Additionally, sperm mucus penetration counts along the capillary tube (10 mm to 90 mm) were cumulatively assessed and analyzed thereafter using the Mixed procedure of SAS with a model that included treatment imposed as a fixed effect and bull as a random term. Differences among means were determined by F-tests using Type III sums of squares. The PDIFF option (SAS Institute) and the Tukey test were applied to evaluate pairwise comparisons between means.

RESULTS

Experiment 1: Characterization of BBD126 in Fresh and Frozen-Thawed Bovine Sperm

BBD126 was confirmed to be present on both fresh and frozen-thawed sperm, as evidenced in Western blot by an intense band at the appropriate molecular weight (Fig. 1A). Furthermore, treatment of sperm with the PIPLC enzyme was not successful in removing BBD126 from the sperm surface (Fig. 1B) because it was detected in all sperm treatments and was absent from all the supernatants recovered.

Experiment 2: Removal of BBD126 from Bovine Sperm Using Various Capacitating Agents

Treatment of fresh and frozen-thawed sperm samples for 1 h with either 2 mM caffeine, 2 mM dbcAMP, a combination of 1 mM caffeine and 1 mM dbcAMP, or with 10 μg/ml heparin failed to remove BBD126 from the sperm surface, as evidenced in Western blot by the presence of an intense band corresponding to this protein in all treatment groups (Fig. 2, A and B). This finding was confirmed by confocal microscopy, where fluorescent staining of the postacrosomal and tail region was evident in all treatment groups (Fig. 2C). Increasing the time of incubation with the capacitating factors from 1 to 5 h, incubating the sperm overnight prior to exposing them to the treatments (as described by Yudin et al. [13]), or increasing the compound's concentration 10-fold did not alter the presence of the protein on sperm (data not shown). However, sperm treated with heparin for 1 h were more successful in fertilizing oocytes than sperm incubated in the absence of capacitating factors, as evidenced by an increase in cleavage rate in this treatment group in comparison to the control (80% vs. 46%, respectively; $P < 0.001$; Fig. 3), suggesting that sperm from that group were fully capacitated.

To determine if the localization of BBD126 changes after sperm undergo the acrosome reaction, frozen-thawed sperm were incubated for 3 h in the presence of calcium ionophore. The binding pattern of BBD126 did not change after the acrosome reaction (Fig. 4).

Experiment 3: Characterization of BBD126 in Epididymal Sperm and Sperm Motility and IVF

Sperm recovered from caput, corpus, and cauda epididymis were subjected to IVF and motility analysis. Co-incubation of in vitro matured oocytes with cauda sperm resulted in a higher fertilization rate than with caput or corpus sperm, as evidenced by a higher percentage of cleaved embryos observed in this group (73.5% vs. 20.7% and 30.0%, respectively; $P < 0.0001$). However, no differences were observed in cleavage rate between caput and corpus sperm ($P = 0.10$) (Fig. 5A). The percentage of blastocysts on Day 7 of development was highest in the cauda group (26.6%; $P < 0.0001$) compared to the other treatment groups. No differences were observed in Day 7 blastocyst rate between the corpus and caput groups (3.5% vs. 1.8%; $P = 0.40$).

Following CASA, cauda sperm exhibited the highest percentage of overall and progressive motility (86.7% and 31.3%, respectively; $P < 0.005$). Corpus sperm, in turn, exhibited a tendency toward higher overall motility than caput sperm (19.6% vs. 4.3%, respectively; $P = 0.05$) (Fig. 5B). Furthermore, whereas 6.5% of corpus sperm presented

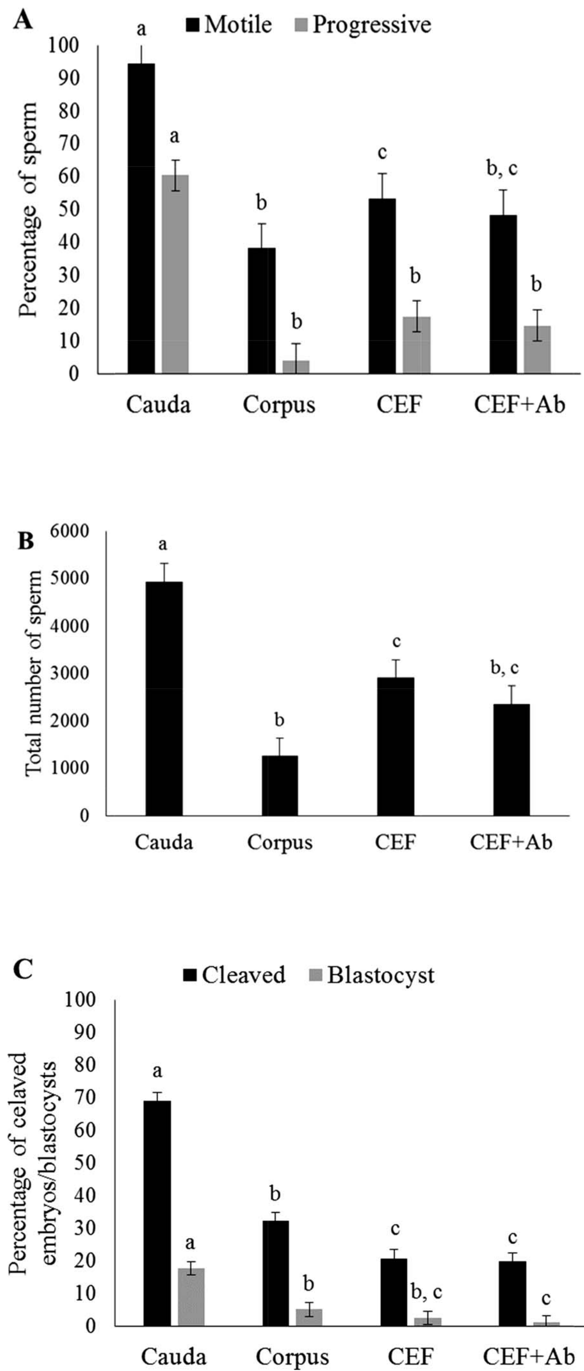

FIG. 6. Results of functional analysis performed on corpus sperm treated with CEF in the absence (CEF) or presence (CEF+ Atb) of α-BBD126 Ab (untreated corpus and cauda sperm used as controls). **A**) Black bars represent the percentage of overall motile sperm, and gray bars show the percentage of progressively motile sperm. Data reported as means ± SEM. Different superscripts between treatment groups indicate a difference within motile or progressive sperm ($P < 0.0001$). **B**) The number of sperm found along each 10 mm marking was recorded; the average number for two capillaries in each of the four replicates is reported. **C**) Percentage of cleaved embryos on Day 2 (Day 0 = day of fertilization) and blastocysts at Day 7 (black and gray bars, respectively) obtained after fertilization with treated sperm (n = 200 oocytes per treatment). Data reported as least-squares mean ± SEM. Different superscripts between treatment groups within cleavage or blastocyst rate indicate a difference ($P < 0.001$). Experiment 4.

progressive motility, no caput sperm were found to be progressively motile in any of the replicates performed. However, due to the high variation in the corpus group, the difference in progressive motility between corpus and caput sperm was not statistically significant ($P = 0.50$).

Experiment 4: Effect of CEF on Motility of Corpus Sperm

Consistent with experiment 3, when motility was analyzed by CASA, cauda sperm had higher overall and progressive motility than corpus sperm (94.6% and 60.4% vs. 38.1% and 3.8%, respectively; $P < 0.0001$). Addition of CEF to corpus sperm increased the percentage of sperm exhibiting overall motility from 38.1% to 53.4% ($P < 0.05$) (Fig. 6A). The α-BBD126 Ab failed to inhibit this increase in sperm motility. This translated into an increased ability to penetrate CM, as evidenced by an increased number of total sperm counted in the capillary tube (2911 vs. 1255; $P < 0.005$) However, this number was still much lower than that found in the cauda group (4935; $P < 0.001$). Addition of the α-BBD126 Ab to corpus sperm in combination with CEF did not alter the number of sperm migrating through the mucus compared to the corpus sperm with CEF alone (2350 vs. 2,911; $P = 0.20$) (Fig. 6B). Interestingly, a high level of agglutination was observed in the control corpus sample, which was reduced with the addition of CEF (Fig. 7). Furthermore, when corpus sperm were incubated in the presence of CEF (with or without α-BBD126 Ab) their ability to fertilize oocytes in vitro decreased, as evidence by a lower cleavage rate than the corpus group (19.8% and 20.8% vs. 32.4%, respectively; $P < 0.01$) (Fig. 6C).

Confocal microscopy analysis revealed that the binding pattern of BBD126 in the corpus sperm treated with CEF differed from the cauda sperm, as evidenced by a faint fluorescence in the head and midpiece of the CEF-treated sperm (Fig. 8). Moreover, BBD126 was detected in sperm incubated in the presence of α-BBD126 Ab, which indicated a failure of the antibody to block the protein binding to the sperm surface.

Experiment 5: Effect of rBBD126 on Corpus Sperm Function

A concentration of 30 μg/ml rBBD126 was added to corpus sperm. After 1 h incubation, a high level of agglutination was observed in the corpus sperm (control treatment), which was reduced in the presence of the rBBD126, although not as dramatically as when CEF was used in the previous experiment (Fig. 7E). Confocal microscopy of the treated samples showed that rBBD126 bound to corpus sperm in a similar pattern to that observed for BBD126 in cauda sperm (Fig. 8). Incubation with rBBD126 increased overall motility of corpus sperm from 11.8% to 21.8% ($P < 0.001$; Fig. 9A). No differences were observed in progressive motility between both groups. Furthermore, the increase in overall motility detected in the rBBD126 group did not translate to an enhance ability of the sperm to migrate through CM because no significant differences were observed (Fig. 9B). IVF of oocytes with rBBD126 treated or untreated corpus sperm showed no differences in their cleavage or blastocyst rate ($P = 0.80$) (Fig. 9C).

DISCUSSION

As immature sperm migrate through the epididymis, they are bathed in region-specific epididymal fluid that leads to a sequential addition, deletion, and modification of their surface

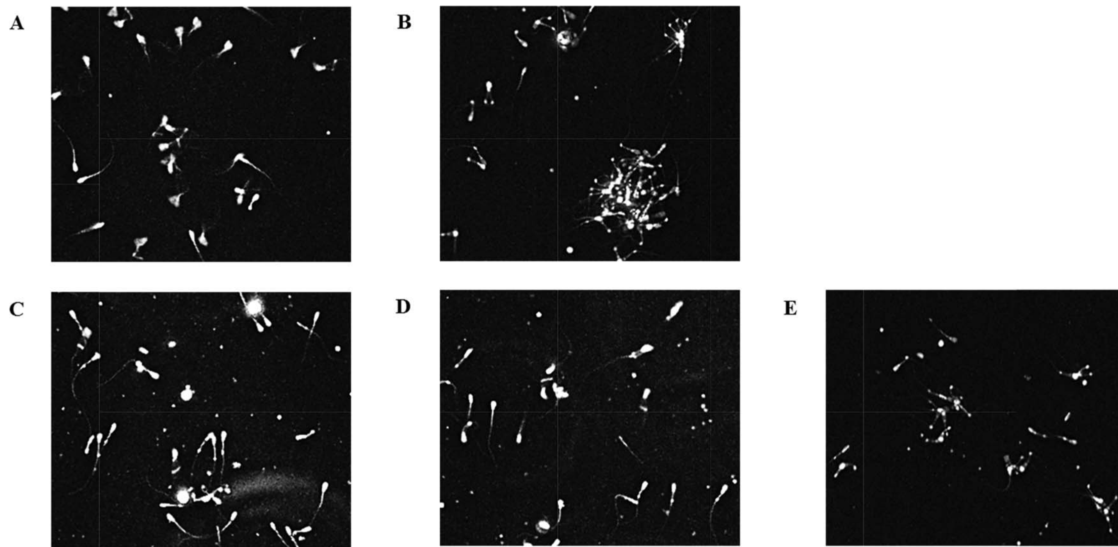

FIG. 7. Representative images showing the different levels of agglutination in the different sperm samples. Cauda sperm do not agglutinate (**A**) but corpus sperm do (**B**). Addition of CEF in the absence or presence of α-BBD126 Ab appeared to reduce this effect (**C** and **D**, respectively). Incubation of corpus sperm with rBBD126 also decreases agglutination (**E**). Images taken during CASA (100× magnification). Experiments 4 and 5.

proteins, which ultimately results in the acquisition of motility and fertilizing abilities [33–35]. In primates, as well as in rodents, evidence is now accumulating that pleiotropic β-defensin molecules are preferentially expressed in the male reproductive tract and also play an important role in sperm motility and fertility [12, 21, 36, 37]. Our group previously discovered a cluster of β-defensin genes that showed high epididymal mRNA expression in bulls [28], and we recently characterized β-defensin 126 protein expression on sperm and in epididymal epithelium [29]. However, the role of these host defense peptides in mediating sperm function has not previously been investigated in cattle.

This study has shown that 1) BBD126 is present on bovine fresh and frozen-thawed sperm, preferentially located on the postacrosomal region and tail; 2) capacitating agents previously reported to remove BBD126 from macaque sperm failed to remove the bovine ortholog; 3) CEF, which contains BBD126, increases progressive motility of corpus sperm, which translates into an increase in mucus penetration ability, an effect that is not inhibited by addition of α-BBD126 Ab; and 4) rBBD126 increases overall motility of corpus sperm but not progressive motility or mucus penetration abilities.

BBD126, a protein that is exclusively secreted in the cauda epididymis [29], exhibits a clear preference for the tail and postacrosomal region of the head of both fresh and frozen-thawed sperm. This binding pattern differs from that described for the macaque ortholog, which coats the entire sperm surface [13], and the mouse ortholog, which is present over the entire sperm surface except for the equatorial segment [11]. Another difference between the bovine and macaque orthologs is observed when sperm from both species are subject to treatment with PIPLC, an enzyme that cleaves off GPI-anchored proteins. GPI-anchored proteins are a major component of the lipid rafts in sperm and are involved in the membrane reorganization that takes place during the capacitation of the male gamete in the female reproductive tract [38]. In the macaque, β-defensin 126 appears to be attached to GPI-anchored proteins, as PIPLC treatment releases β-defensin 126 along with these proteins [13]. However, data in the current study would suggest that this is not the case in bovine because sperm treated with PIPLC retained BBD126 in their structure.

In the present study, freshly ejaculated and frozen-thawed sperm were incubated with either caffeine, dbcAMP, a combination of caffeine and dbcAMP, or heparin, which is routinely used to stimulate capacitation during bovine IVF. A previous study in the macaque demonstrated that a combination of caffeine and dbcAMP synchronizes sperm capacitation in this species and, as a result, removes DEFB126 from their surface [23]. Interestingly, incubation of bovine sperm with the different treatments failed to remove BBD126 as evidenced by its detection by Western blot and confocal microscopy in all samples. Increasing the duration of incubation, the concentration of the different reagents, or incubating sperm overnight prior to exposing them to the treatments did not result in a loss of the protein. Because the ultimate sign of sperm being capacitated is their ability to interact with the oocyte and fertilize it, we used the treated sperm in IVF. This allowed us to determine whether the results observed in the Western blot were due to an unsuccessful sperm capacitation or to BBD126 not being released during this process. As expected, heparin-treated sperm were more successful in fertilizing matured oocytes, as evidenced by an increase in cleavage rate compared to the other treatments. This indicates that at least this treatment is capable of inducing bull sperm capacitation, which in turn suggests that bovine BBD126 is retained after sperm undergo this process, in contrast to the macaque [23]. In addition, induction of the acrosome reaction by incubation with calcium ionophore did not have an effect on the binding pattern of BBD126. This observation differs from the results in both macaque and mouse orthologs. In the mouse, sperm recovered from both the oviduct and the surface of COCs showed immunorecognition of Defb22 over their entire surface [11]. However, sperm found within the cumulus cell matrix had lost most of the immunofluorescence over the sperm head, while retaining it along the tail and midpiece [11]. As mentioned before, macaque sperm lose β-defensin 126 after capacitation [23]. This suggests that in the mouse and macaque there is a region-specific loss of Defb22, probably induced by capacitation, that does not occur in bovine sperm.

The sequential nature of sperm maturation is highlighted by the differences observed in motility and fertilizing ability of sperm recovered from different epididymal regions. Epididy-

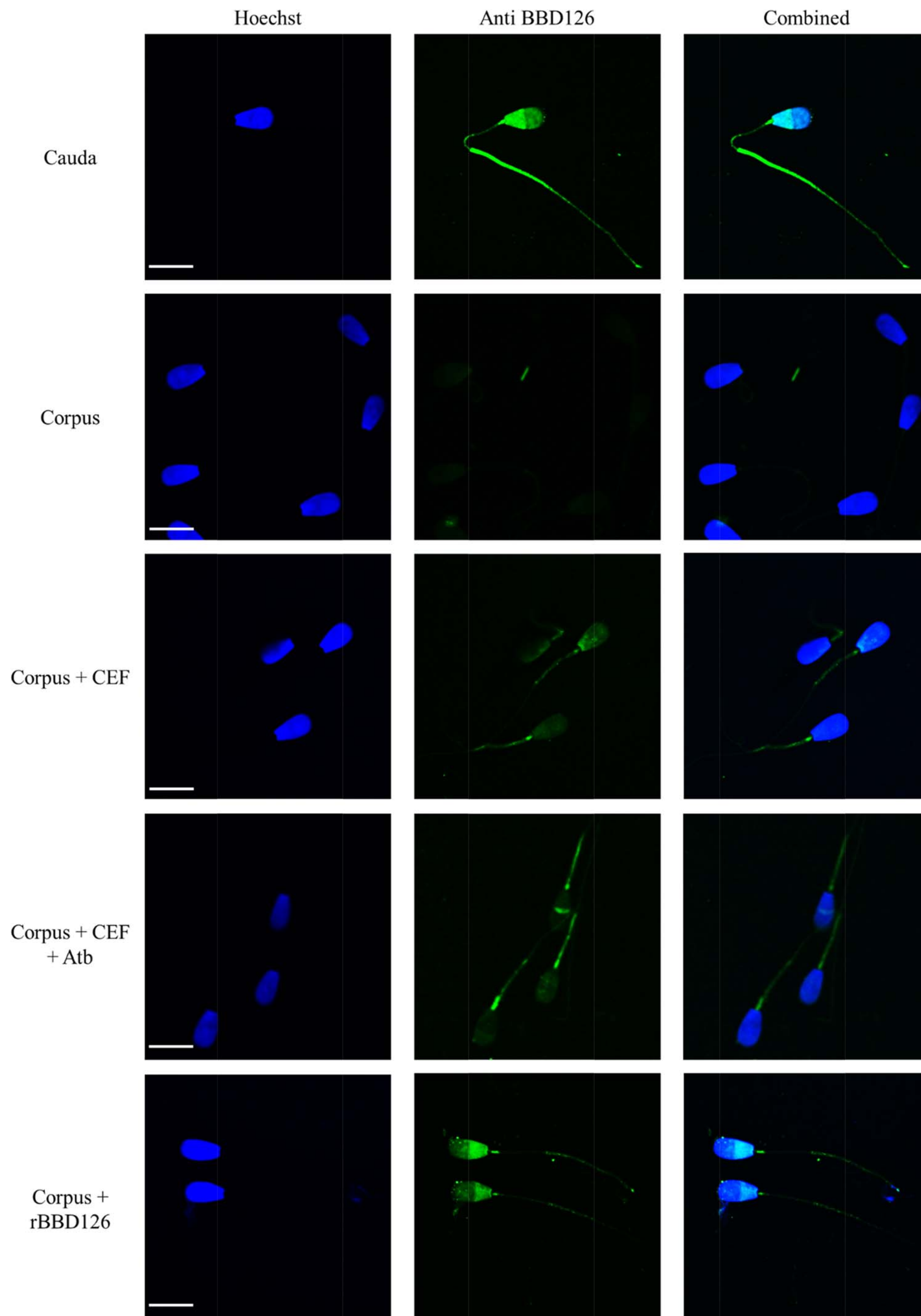

FIG. 8. Representative confocal images of corpus sperm treated with CEF in the presence (CEF+Ab) or absence (CEF) of α-BBD126 Ab, and with rBBD126. Untreated corpus and cauda sperm were used as controls. Bars = 10 μm. Experiments 4 and 5.

mal sperm exhibit an increase in motility and fertilizing abilities from proximal to more distal regions. Sperm obtained from the cauda had the highest fertilizing ability as well as total and progressive motility. Both motility and fertilizing ability declined in corpus sperm and caput sperm. These results are consistent with results of previous studies in which ewes [39] and rabbits [40] were inseminated with sperm from different epididymal region as well as with the observations of motility patterns in epididymal sperm from different species [6, 39, 41]. This suggests that in order to be fully competent, sperm must interact with the cauda environment. Because BBD126 in the bull is exclusively secreted in the cauda, where it binds to the sperm surface, we hypothesized that it might be involved in the maturation of sperm. The localization of the protein to the tail of the sperm suggests a role in sperm motility in the bull.

Due to the inability to remove BBD126 from the surface of bull sperm by inducing capacitation, corpus epididymal sperm, which do not express the protein [29], were used as an alternative model to study the protein's role in sperm function. When immature corpus sperm were incubated in the presence of fluid recovered from the cauda, an increase in motility was observed. Furthermore, when these sperm were challenged to swim through a capillary filled with CM collected from estrus cows, they were more competent than the untreated corpus sperm. Addition of BBD126 antibody to the CEF did not abrogate this effect. The fact that CEF positively affects the

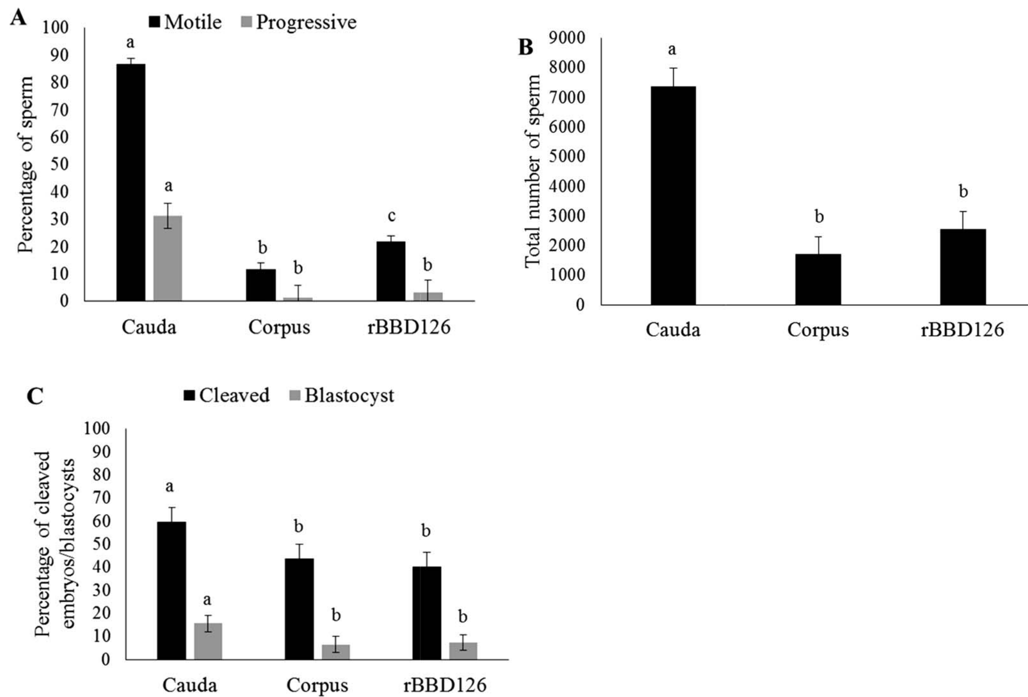

FIG. 9. Functional analysis of sperm treated with rBBD126. **A**) CASA of overall (black bars) and progressive (grey bars) motility of corpus sperm incubated with rBBD126 (cauda and corpus sperm used as controls). Data reported as least-squares mean ± SEM. Different superscripts indicate a significant difference ($P < 0.01$). **B**) Total number of sperm in the mucus-filled capillary tube after incubation with rBBD126 (cauda and corpus sperm used as controls). **C**) Percentage of cleaved embryos on Day 2 (Day 0 = day of fertilization) and blastocysts at Day 7 (black and gray bars respectively) obtained after fertilization with treated sperm (n = 200 oocytes per treatment). Data reported as least-squares mean ± SEM. Different superscripts between treatment groups within cleavage or blastocyst rate indicate a difference ($P < 0.05$).

motility of corpus sperm is in agreement with previously published papers that found epididymis-secreted proteins that aid in maintaining motility in sperm [42] and a cauda-secreted protein that stimulates forward motility in caput sperm [41, 43]. Confocal microscopy revealed faint fluorescence in sperm incubated with CEF, with or without α-BBD126 Ab, which indicates that the Ab does not block BBD126 from attaching to the sperm surface, possibly because it binds an epitope that does not interfere with this process. This would explain the lack of differences observed between the treatment groups containing or lacking α-BBD126 Ab. Another important finding was the fact that BBD126 shows an aberrant binding pattern in corpus sperm treated with CEF. This is perhaps not surprising when one considers that, in vivo, sperm maturation is a highly regulated sequential process involving addition, modification, and deletion of surface proteins [5], while, in vitro, CEF was added in one step, possibly leading to modification of the localization and structure of different proteins, including BBD126. This alteration of the sperm surface could help explain the lower cleavage rate observed for corpus sperm incubated in the presence of CEF because proteins involved in sperm-egg recognition and fertilization could have also been modified.

When corpus sperm were incubated in the presence of rBBD126, as opposed to CEF, the recombinant protein's binding pattern mimicked that of BBD126 in cauda sperm. An increase in the total motility of corpus sperm was observed after they were incubated in the presence of rBBD126, but no differences were observed in progressive motility or in the ability of sperm to swim through CM. These results suggest that the increase in motility observed after treatment of corpus sperm with CEF could be explained in part by the addition of BBD126. Increases in motility due to defensins have been described in rodents and humans [12, 21]. However, the analysis of motility in all corpus treatments proved to be difficult due to the high level of sperm agglutination. The phenomenon of corpus sperm agglutinating has been well documented in different species [44, 45]. It is not observed in other epididymal regions, and it is thought to be due to changes in membrane composition required for normal sperm maturation [45, 46]. In boar and ram sperm, agglutination appeared 15 min after isolation of the cells and was maximal after 1 h of incubation [45], which is consistent with our observations. Furthermore, proteins that are present in the cauda epididymis have been reported to reduce agglutination [45, 47]. Thus, it is possible that the increase in motility observed after incubation with rBBD126 is due to the sperm being disengaged from one another and allowed to swim freely rather than a direct effect on tail movement.

Furthermore, unlike in the macaque, bull and mouse BBD126/Defb22 is retained after capacitation, which might suggest an additional role of this protein in sperm-oocyte interaction in these species. However, based on the lack of effect of rBBD126 on cleavage rate, it would seem that in the bull this protein is not involved in the oocyte-recognition and fertilization process. An additional experiment was planned to incubate corpus sperm in the presence of CEF depleted of BBD126. CEF was run through a chromatography column coated in α-BBD126 or α-IgG (control) Ab. To our surprise, incubation of sperm with fluid that had passed through either column rendered them immotile. The reason for this is unclear at present, but it is likely to be due to an artifact of the method used for depletion.

The results presented in this study suggest that the role of BBD126 in cattle is different to that observed in primates. However, it is important to remember that BBD126 is part of a

cluster of 19 genes, all of which are expressed in the male reproductive tract [28]. Furthermore, the bovine genome exhibits a defensin gene expansion in this cluster, with a number of recent gene duplicates that have not been found in the human genome [48]. This means that functional redundancy between different defensins present on the sperm surface is highly likely. This redundancy has been used to explain the lack of a dramatic immunological phenotype in *Defb1* knockout mice [49, 50]. Furthermore, the main effect of deletion of nine defensin genes located together at the end of the gene cluster was male infertility [14]. Sperm produced by mutant males had reduced motility, underwent precocious capacitation and acrosome reaction, and exhibited defects in the microtubule structure of the axoneme that could only be observed in sperm recovered from the cauda [14].

In conclusion, we have demonstrated that rBBD126 binds to the sperm with the same pattern as the one observed for BBD126 in cauda sperm and that addition of the recombinant protein induces an increase in motility of immature corpus sperm. The role of the epididymis in sperm maturation is well established; however, the underlying molecular events regulating this process remain largely unknown. Understanding these mechanisms is the first step toward a more complete understanding of the molecular nature of sperm fertility defects or the identification of biomarkers for accurately predicting fertility. The growing body of evidence linking defensins and male fertility suggests that these proteins are essential in the creation of a male gamete capable of reaching and fertilizing an oocyte. Further research on BBD126 and related β-defensins will help expand our understanding of acquisition of fertilizing abilities and infertility in the bovine sperm.

ACKNOWLEDGMENT

The authors acknowledge the technical assistance of Mary Wade and the Conway Institute Core Imaging Facility (Professor Jeremy Simpson).

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