

Assessment of binder of sperm protein 1 (BSP1) and heparin effects on *in vitro* capacitation and fertilization of bovine ejaculated and epididymal sperm

Research Article

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Summary

The present study evaluated the effect of binder of sperm protein 1 (BSP1) and/or heparin on *in vitro* bovine capacitation and fertilization rates using epididymal and ejaculated bovine sperm. Frozen–thawed sperm were selected and used in the following treatments. Control group: Fert-TALP medium without heparin; heparin (HEP) group: Fert-TALP with heparin (10 UI/ml); BSP1 group: Fert-TALP medium with BSP1 (10 µg/ml for ejaculated sperm; 40 µg/ml for epididymal sperm); HEP + BSP1 group: Fert-TALP medium with heparin (5 UI/ml) and BSP1 (5 µg/ml for ejaculated sperm; 20 µg/ml for epididymal sperm) and determined *in vitro* capacitation rates in different interval times (0, 15, 30 and 60 min) using the chlortetracycline fluorescence (CTC) method. Also, we evaluated the development rates of oocytes fertilized with ejaculated or epididymal sperm into the same treatments. Capacitation was greater and faster when ejaculated sperm were treated for 60 min with heparin compared with other treatments. However, developmental rates were similar in all treatments. For epididymal sperm, the treatments with BSP1 presented higher capacitation and fertilization rates compared with heparin ($P < 0.05$). The effects of heparin + BSP1 on capacitation and developmental rates did not cause any increase in capacitation or blastocyst rates compared with other groups for ejaculated or epididymal sperm. In conclusion, this study confirmed that either BSP1 and heparin can be used as capacitator agents for bovine ejaculated sperm during IVF. However, BSP1 seems to be more efficient compared with heparin for epididymal sperm. Furthermore, BSP1 and heparin have no synergic effects on sperm capacitation.

Introduction

In mammals, sperm are formed in the seminiferous tubules and undergo complex modifications while transiting in the epididymis and after ejaculation to become able to fertilize the oocyte (Gadella, 2008). Capacitation is one of the major physiological events that affects sperm after ejaculation and involves increases in the fluidity of the sperm membrane, cholesterol efflux, alteration of membrane potential and induction of hyperactivation. All these events are necessary for sperm to acquire the competence to fertilize an egg (Córdoba *et al.*, 1997; Rodríguez-Martínez *et al.*, 2011; Visconti *et al.*, 2011). In many mammalian species such as bovine, sperm are unable to fertilize oocytes immediately after ejaculation and they must undergo capacitation in the female reproductive tract (*in vivo*) or by capacitating agents (*in vitro*) to acquire fertilizing capability (Yanagimachi, 1994; Visconti *et al.*, 2011). At this time, several substances are being used to induce bovine sperm capacitation *in vitro*, such as bicarbonate (Harrison and Gadella, 2005), bovine serum albumin (Xia and Ren, 2009), calcium (Visconti *et al.*, 2011), binder of sperm protein 1 (BSP1; Plante *et al.*, 2014; Rodríguez-Villamil *et al.*, 2016) and glycosaminoglycans (Parrish *et al.*, 1989). Nevertheless, heparin is the most commonly used compound for sperm capacitation in IVF systems for both research and commercial production of bovine embryos (Parrish, 2014). Despite this spread of use of heparin, the mechanism by which it induces capacitation is largely unknown. Heparin is present in the female genital tract, follicular and oviductal fluid and binds to bull spermatozoa as a typical receptor–ligand interaction, thereby promoting capacitation (Ax and Lenz, 1987). BSP1 is one of the major proteins of bovine seminal plasma (Manjunath *et al.*, 2009; Rego *et al.*, 2014) and binds to sperm membrane at ejaculation. BSPs promote efflux of membrane cholesterol and phospholipids, an essential step for sperm capacitation (Plante *et al.*, 2016).

Some authors have suggested that heparin and BSP1 act in conjunction to induce sperm capacitation. This hypothesis is supported by the fact that heparin and BSPs have biochemical affinity and interact during chromatography assays (Parrish *et al.*, 1989; Manjunath and Thérien, 2002; Parrish, 2014; Rodríguez-Villamil *et al.*, 2016; our unpublished results). Our previous study showed that addition of BSP1 to the fertilization medium without the presence of heparin induced capacitation and the acrosome reaction of bovine sperm. Also, when compared with heparin, BSP1 alone added to the IVF medium was associated with better *in vitro* embryo development after fertilization with epididymal sperm (Rodríguez-Villamil *et al.*, 2016). When we used ejaculated sperm in the same experiment, BSP1 induced sperm capacitation similarly to the effects of heparin, with similar rates of embryo development (Rodríguez-Villamil *et al.*, 2016). Therefore, the study on the interaction among capacitator agents is of interest because of the important role played by heparin for bovine IVF and all the known effects of BSPs on sperm function (Parrish *et al.*, 1989; Thérien *et al.*, 2005; Plante *et al.*, 2016; Rodríguez-Villamil *et al.*, 2016). Given this scenario, the aim of the present study was to evaluate the effect of heparin and BSP1, as well as their potential interaction, on sperm capacitation and fertilization rates using epididymal and ejaculated bovine sperm.

Materials and methods

Effects of BSP1 and/or heparin on sperm capacitation

Pools from frozen–thawed ejaculated ($n = 4$) and epididymal ($n = 6$) bull sperm were thawed at 37°C for 30 s and separated through Percoll gradients (45–90%), as described before (Parrish *et al.*, 1995; Rodríguez-Villamil *et al.*, 2016). Briefly, Percoll solution (90%; 400 μ l) was placed in two 1-ml Eppendorf tubes, and 400 μ l of 45% Percoll was layered smoothly over that. An aliquot of 100 μ l of pooled frozen–thawed semen was layered on top of the gradients and then tubes were centrifuged for 10 min at 900 *g*. The pellets were re-suspended in the same amount of sperm-TALP medium and centrifuged for 5 min at 600 *g*. The supernatant was removed and incubated in accordance with the following treatments. Control group: Fert-TALP medium without heparin; heparin group: Fert-TALP with heparin (10 UI/ml); BSP1 group: Fert-TALP medium with BSP1 (10 μ g/ml for ejaculated sperm; 40 μ g/ml for epididymal sperm); HEP + BSP1 group: Fert-TALP medium with heparin (5 UI/ml) and BSP1 (5 μ g/ml for ejaculated sperm; 20 μ g/ml for epididymal sperm).

The percentage of capacitated spermatozoa was determined by the method of chlortetracycline fluorescence (CTC), as described by Fraser (1995) and validated in our previous work (Rodríguez-Villamil *et al.*, 2016). Briefly, CTC was added to the medium containing the capacitated sperm and the control sample. Glutaraldehyde was then added to the mixture to a final concentration of 0.1% (w/v). Sperm capacitation rates were estimated by evaluating the CTC pattern, identifying intact capacitated sperm, the ones with a fluorescence-free band in the post-acrosomal region; and acrosome-reacted sperm, and the ones with a soft fluorescence over the whole spermatozoon except for the thin bright punctate fluorescent band in the equatorial segment. Each sample was evaluated at 0, 15, 30 and 60 min after treatment. Slides were examined at $\times 400$ magnification using excitation at 410 nm on an epifluorescence microscope. The percentages of

live and dead sperm for each treatment were determined by counting 100–200 sperm per slide.

In vitro embryo production

Bovine ovaries were obtained at a local abattoir and kept at 32°C during transport to the laboratory. Cumulus–oocyte complexes (COCs) were aspirated with a disposable syringe from follicles with 3–8 mm diameter and washed in Tissue Culture Medium 199 (TCM-199; Sigma-Aldrich, USA), as reported before (Rodríguez-Villamil *et al.*, 2012). In summary, oocytes were transferred to micro-drops containing 100 μ l maturation medium (10–20 COCs per drop) and matured for approximately 24 h at 38.8°C in an atmosphere of saturated humidity and 5% CO₂ in air. The maturation medium consisted of TCM-199 supplemented with 10% (v/v) fetal bovine serum, 0.2 mM sodium pyruvate, 25 mg/ml porcine follicle stimulating hormone (FSH) (Follitropin-V; Bioniche, Canada) and penicillin/streptomycin (10 μ l/ml; Schering, USA). After *in vitro* maturation, COCs were assigned randomly to the different experimental groups.

After each straw of frozen semen (ejaculated or epididymal) was thawed for 1 min at 37°C, sperm were prepared for fertilization using Percoll discontinuous gradient (45% and 90%), as described before (Parrish *et al.*, 1995). Fertilization medium consisted of Fert-TALP medium supplement with the different capacitator factors and COCs were fertilized in 50 μ l droplets (8–20 COC/droplet) covered with mineral oil. A 3 μ l aliquot of 1×10^6 sperm/ml was used to fertilize the COCs. For culture synthetic oviductal fluid (SOF) medium supplemented with amino acids, 1.5 mM D-glucose and 0.4% of BSA was used. After 18–20 h, COCs were pipetted to remove the cumulus cells and excess sperm cells, washed once in SOF and transferred into 50 μ l droplets of SOF medium under mineral oil in a controlled atmosphere (5% CO₂, 5% O₂ and 90% N₂) at 38.8°C. Zygotes were cultured *in vitro* in SOF medium for 7 days at 38.8°C, using a humidified incubator with 5% CO₂ and 5% O₂ in air. Cleavage and blastocyst rates were evaluated on days 2 and 7 after fertilization, respectively (Robertson and Nelson, 2007). Oocyte maturation and embryo production were performed under the same conditions for epididymal and ejaculated sperm.

Fertilization rates of frozen–thawed ejaculated sperm after treatment with BSP1 and/or heparin

Matured COCs ($n = 1079$) were allocated randomly into treatments and fertilized with frozen–thawed ejaculated sperm from four different bulls from a commercial artificial insemination centre. For fertilization, COCs were incubated with ejaculated sperm into the different treatments groups. Treatments were defined as: Control group: Fert-TALP medium without heparin; Heparin group: Fert-TALP with heparin (10 UI/ml); BSP1 group: Fert-TALP medium with BSP1 (10 μ g/ml); HEP + BSP1 group: Fert-TALP medium with heparin (5 UI/ml) and BSP1 (5 μ g/ml) for 18 h. For each *in vitro* fertilization routine, conducted weekly, we used the pool of four different bulls for all treatments.

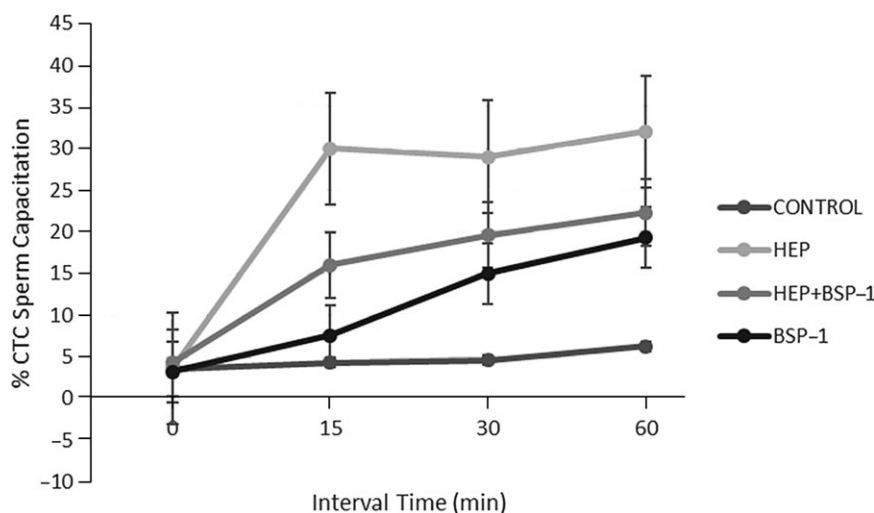
Fertilization rates of epididymal frozen–thawed sperm after treatment with BSP1 and/or heparin

Matured COCs ($n = 1014$) were allocated randomly into treatment groups and fertilized with a pool of frozen–thawed epididymal sperm from six different bulls. Cauda epididymal sperm used in

Table 1. Cleavage and blastocyst rates (means \pm standard error of the mean (SEM)) after *in vitro* fertilization with bovine ejaculated sperm treated with heparin and/or BSP1

Treatment	Oocytes	% Cleavage	% Blastocyst
Control	267	64.7 \pm 2.0 ^a	21.8 \pm 3.9 ^a
Heparin	271	77.0 \pm 2.2 ^b	40.0 \pm 1.5 ^b
BSP1	256	79.1 \pm 5.3 ^b	41.7 \pm 3.1 ^b
Hep + BSP1	285	76.4 \pm 2.8 ^b	37.7 \pm 5.7 ^b

Control: Fert-TALP medium without heparin; Heparin: Fert-TALP with heparin (10 UI/ml); BSP1: Fert-TALP medium with BSP1 (10 μ g/ml); Hep + BSP1: Fert-TALP medium with heparin (5 UI/ml) and BSP1 (5 μ g/ml).

**Figure 1.** Sperm capacitation rates (means \pm standard error of the mean (SEM)) of frozen-thawed bovine ejaculated sperm *in vitro* capacitated into different treatments, evaluated during a period from 0 to 60 min. Control: Fert-TALP medium without heparin; HEP: Fert-TALP with heparin (10 UI/ml); BSP1: Fert-TALP medium with BSP1 (10 μ g/ml); HEP + BSP1: Fert-TALP medium with heparin (5 UI/ml) and BSP1 (5 μ g/ml).

this experiment were collected and frozen as described before (Rodríguez-Villamil *et al.*, 2016). For fertilization, COCs were incubated with epididymal sperm into the different four treatments groups. Treatments were defined as: Control group: Fert-TALP medium without heparin; Heparin group: Fert-TALP with heparin (10 UI/ml); BSP1 group: Fert-TALP medium with BSP1 (10 μ g/ml) and HEP + BSP1 group: Fert-TALP medium with heparin (5 UI/ml) and BSP1 (5 μ g/ml) for 18 h.

Statistical analysis

Sperm capacitation rates were compared among all treatments by Fisher's exact test. Data related to cleavage and blastocyst rates were transformed by square root and then analyzed by analysis of variance (ANOVA), considering bulls and BSP1 and/or heparin concentrations as the main effects as well their interactions. The protected least significant difference (LSD) test was used for subsequent multiple comparisons when ANOVA revealed statistically significant differences ($P < 0.05$). All data were analyzed using the Info-Stat software (UNC, Argentina).

Results

Capacitation was greater when ejaculated sperm were treated for 60 min with heparin (32 \pm 1.5%) in comparison with the other treatments (Control: 6.3 \pm 0.6%; HEP + BSP1: 22.3 \pm 1.4%;

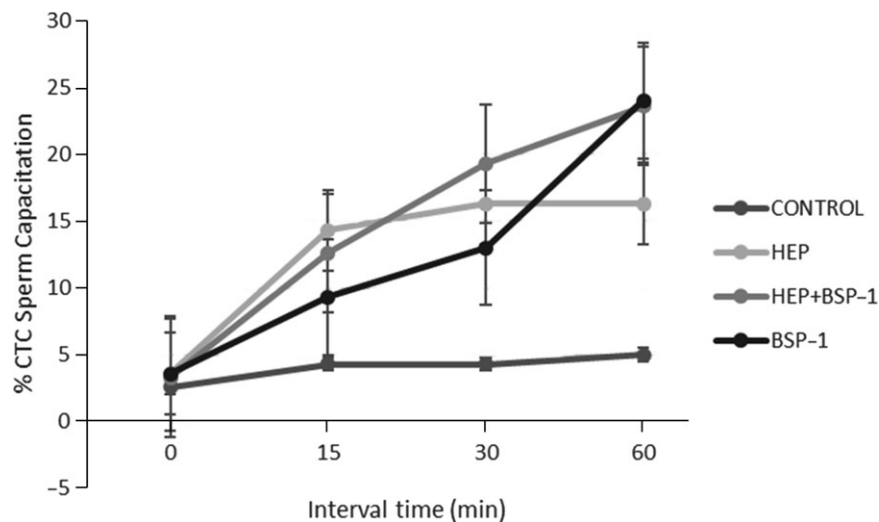
BSP1: 19.3 \pm 2.9%). Furthermore, capacitation of ejaculated sperm happened faster during the first 15 min of incubation with heparin compared with HEP + BSP1 or with BSP1 only ($P < 0.03$; Fig. 1). Nevertheless, cleavage rates and blastocyst formation were similar after ejaculated sperm were incubated with heparin, Hep + BSP1 or BSP1 ($P > 0.05$; Table 1).

Capacitation rates of epididymal sperm gradually increased over time (15–60 min) after incubations with BSP1, reaching the highest rates at 60 min. Capacitation rates after treatment with heparin had sharp increases in the first 15 min but, after 60 min, those rates were significantly lower ($P < 0.05$) compared with the ones detected with BSP1 treatment (Fig. 2). Also, the combination of BSP1 with heparin did not increase ($P > 0.05$) capacitation rates of epididymal sperm beyond that detected with BSP1 alone at 60 min of incubation (BSP1: 24 \pm 1.1%; Hep + BSP1: 23.6 \pm 1.7%). When using epididymal sperm, only BSP1 significantly enhanced cleavage rates above the other groups (Table 2). However, effects of heparin + BSP1 on cleavage rates were equivalent to those achieved with either heparin or BSP1 alone ($P > 0.05$). The percentage of blastocyst formation was greater after BSP1 (29.7 \pm 2.4) or BSP1 + Hep (30.4 \pm 2.1) treatment in comparison with those obtained with heparin alone (19.4 \pm 2.2%) and with the control treatment (13.8 \pm 1.3%; Table 2). The combination of BSP1 with heparin had similar results compared with BSP1 by itself in blastocyst formation when IVF was carried out with frozen-thawed epididymal sperm.

Table 2. Cleavage and blastocyst rates (means \pm standard error of the mean (SEM)) after *in vitro* fertilization with bovine epididymal sperm treated with BSP1 and/or heparin

Treatment	Oocytes	% Cleavage	% Blastocyst
Control	250	58.2 \pm 4.7 ^a	13.8 \pm 1.3 ^a
Heparin	253	60.5 \pm 3.0 ^{a,b}	19.4 \pm 2.2 ^b
BSP1	253	71.7 \pm 5.3 ^b	29.7 \pm 2.4 ^c
Hep + BSP1	258	70.5 \pm 1.9 ^{a,b}	30.4 \pm 2.1 ^c

Control: Fert-TALP medium without heparin; Heparin: Fert-TALP with heparin (10 UI/ml); BSP1: Fert-TALP medium with BSP1 (10 μ g/ml); Hep + BSP1: Fert-TALP medium with heparin (5 UI/ml) and BSP1 (5 μ g/ml).

**Figure 2.** Sperm capacitation rates (means \pm standard error of the mean (SEM)) of frozen-thawed bovine epididymal sperm *in vitro* capacitated into different treatments, evaluated during a period from 0 to 60 min. Control: Fert-TALP medium without heparin; HEP: Fert-TALP with heparin (10 UI/ml); BSP1: Fert-TALP medium with BSP1 (40 μ g/ml); HEP + BSP1: Fert-TALP medium with heparin (5 UI/ml) and BSP1 (20 μ g/ml).

Discussion

Binder of sperm proteins are the predominant protein of the bovine seminal plasma, are mainly secreted by the accessory sex glands and bind to sperm at the moment ejaculation occurs. In the present study, we have shown that responses of epididymal and ejaculated sperm to heparin and BSP1 are quite different, as evaluated by capacitation rates as well as by cleavage rates and early embryo development. To our knowledge, this is the first study focused on the evaluation of combined effects of heparin and BSP1 on both epididymal and ejaculated sperm capacitation and fertilization capacity *in vitro*.

Treatments with either heparin or BSP1 induced *in vitro* capacitation of bovine sperm (ejaculated or epididymal). However, capacitation rates were significantly affected by the duration of treatment and type of sperm (ejaculated or cauda epididymal sperm) used. As well known, both BSP1 and heparin promote capacitation and acrosome reaction of bovine sperm (Thérien *et al.*, 1995; Sankhala *et al.*, 2011; Parrish, 2014; Plante *et al.*, 2016; Rodríguez-Villamil *et al.*, 2016). In the present experiment, we demonstrated that heparin induced the highest rates of capacitation in frozen-thawed ejaculated sperm, as described by other authors (Parrish *et al.*, 1998, 2014; Visconti and Kopf, 1998). In fact, heparin is, at this time, the main agent used to capacitate bovine sperm *in vitro*. Furthermore, our study demonstrated that BSP1 presented a different performance for capacitation rates

throughout the time assayed. During the first minutes of incubation, treatments with BSP1 induced lower capacitation rates in comparison with heparin. In the case of ejaculated sperm, treatments with BSP1 increased the rates over the time, but did not achieve the same rates induced by heparin. Therefore, BSP1 could be used for sperm capacitation but it requires more time to exert its effect when compared with heparin, as also shown by previous studies (Manjunath *et al.*, 2002, 2007; Plante *et al.*, 2012, 2014; Therrien *et al.*, 2013; Rodríguez-Villamil *et al.*, 2016). The reason for this delayed effect of BSP1 is probably related to the time necessary for BSP1 to interact with and remove both cholesterol and phospholipids from sperm membrane (Thérien *et al.*, 1999; Manjunath and Thérien, 2002; Manjunath *et al.*, 2007; Therrien *et al.*, 2013). Furthermore, the use of heparin and BSP1 together did not present any synergic effect on the capacitation rates of ejaculated sperm. The precise pathways for BSP-induced sperm capacitation are still unknown, but it is known that BSP promotes removal of cholesterol from the sperm plasma membrane to promote capacitation (Xia and Ren, 2009; Parrish, 2014).

BSP1 had greater effects on capacitation rates of cauda epididymal, allowing further acrosome reaction and normal fertilization. This finding has been confirmed by other authors, who used exogenous BSP1 to trigger capacitating-related events on epididymal sperm (Gadella and Luna, 2014; Parrish, 2014; Rodríguez-Villamil *et al.*, 2016). In the present experiment, epididymal sperm treated with BSP1 had an initial delayed capacitation, but

differences in comparison with heparin gradually decreased over time, as BSP1 achieved greater effect on sperm capacitation after 60 min of incubation. These observations confirmed that BSP1 needs more time to induce capacitation when compared with heparin, regardless the type of sperm cells (epididymal or ejaculated). Also, we showed that addition of heparin to BSP1 treatment did not cause a significant increase in capacitation rates of epididymal sperm. Therefore, it became certain that BSP1, and not heparin, was the component causing the main effect on capacitation of epididymal sperm. Heparin was associated with lower rates of epididymal sperm capacitation in comparison with treatments with BSP1 because of the type of heparin interaction with the sperm membrane (Parrish, 2014). Therefore, heparin induced higher capacitation rates in ejaculated compared with epididymal sperm probably because heparin induces capacitation by removing BSP-bound molecules present in the ejaculated sperm (Parrish *et al.*, 1989; Plante *et al.*, 2016). Given that bovine epididymal sperm do not have BSP molecules bound to them (Souza *et al.*, 2008), capacitation rates were greater only with treatments including BSP1.

Effects of heparin and BSP1 on cleavage rates after IVF using ejaculated and epididymal sperm were similar to those described previously (Thérien *et al.*, 1999; Plante *et al.*, 2016; Rodríguez-Villamil *et al.*, 2016). Furthermore, embryo developmental rates produced by epididymal and ejaculated sperm treated with BSP1 were similar or higher compared with heparin, probably due to the capacity of BSP1 to help sperm–oocyte interaction. It is known that BSPs bind to bovine zona pellucida (Liberda *et al.*, 2002) and BSP absence may even inhibit the sperm–oocyte adhesion (Turmo *et al.*, 2009). Therefore, it is possible that some of the remaining BSP1 bound to the sperm surface after capacitation also participates in sperm–oocyte adhesion (Manjunath *et al.*, 1987; Plante *et al.*, 2016). We have reported before that BSP molecules remain bound to bovine sperm even after the capacitation/acrosome reaction and after contact of sperm with secretions of the oviductal fluid (Souza *et al.*, 2008).

BSP1 and heparin presented similar effects on embryo development when we used ejaculated sperm for IVF, as reported before (Rodríguez-Villamil *et al.*, 2016). Nevertheless, BSP1 + heparin did not have any synergic effect on embryo development when we used ejaculated sperm for IVF. This result confirmed that these two agents probably had the capacity to induce capacitation through the same pathway, producing the efflux of cholesterol efflux and membrane modifications (Thérien *et al.*, 1999; Plante *et al.*, 2016; Rodríguez-Villamil *et al.*, 2016). Therefore, it seems that BSP1 and heparin compete for the same adhesion sites on the sperm membrane, suggesting that there is a competitive relationship among them instead of a synergic effect. When we used IVF systems with epididymal sperm, formation of blastocysts was greater after treatment with BSP1 in comparison with heparin. It appears therefore that heparin needs an interaction with sperm membrane-bound BSP1 to induce capacitation (Gadella and Luna, 2014; Parrish, 2014). Given that bovine epididymal sperm did not have any BSP1 attached to them, these cells were unable to respond to heparin alone.

In conclusion, the present study confirms that either BSP1 or heparin can be used as a capacitator agent for bovine ejaculated sperm during *in vitro* fertilization, also associated with proper embryo development. However, BSP1 seems to be more efficient compared with heparin to induce capacitation in epididymal sperm and formation of blastocysts. Such results clearly indicate

that ejaculated and epididymal sperm have distinct physiological status, related to the presence, or not, of BSP1. We also report that BSP1 and heparin have no synergic effects on sperm capacitation.

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Conflict of Interest. No conflict of Interest.

Ethical Standards. The study was conducted in accordance with the ethical and animal welfare statements approved by the Department of Animal Science of the Federal University of Ceará, Fortaleza, Brazil, on August 30, 2010.

Data Availability. The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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