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Evaluation of porcine beta defensins-1 and -2 as antimicrobial peptides for liquid-stored boar semen: Effects on bacterial growth and sperm quality

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ABSTRACT

The present study evaluated whether two different antimicrobial peptides (AMP): porcine beta defensins-1 (PBD1) and −2 (PBD2) at three concentrations (1.5 μM, 3 μM and 5 μM) could be a suitable alternative to antibiotics in liquid-stored boar semen. Two separate experiments were conducted with liquid-stored boar semen preserved at 17 °C for 9–10 days. In the first one, we evaluated the impact of adding three concentrations of each AMP on the bacterial growth and sperm quality of boar semen stored for 10 days. In the second experiment, the ability of these AMPs to control bacterial growth was determined over a 9-day period, following artificial inoculation with *Escherichia coli* at 10^7 and 10^8 CFU mL^{-1}. In both experiments, sperm viability was assessed through flow cytometry, sperm motility was determined with Computer Assisted Sperm Analysis (CASA) and the inhibitory effect on microbial growth was evaluated by bacteria culture on Luria Bertani agar. PBD1 and PBD2 were found to significantly ($P < 0.05$) decrease sperm motility at 5 μM (% total motile spermatozoa at day 10, Control: 31.6% ± 1.2% *vs.* PBD1: 6.5% ± 0.3% and PBD2: 5.6% ± 0.4%). Although the highest inhibitory effect on bacterial growth was observed at 3 μM (day 10, PBD1: $1.4 \times 10^6 \pm 6.2 \times 10^5$ CFU mL^{-1} and PBD2: $9.1 \times 10^5 \pm 2.4 \times 10^5$ CFU mL^{-1}) and 5 μM (day 10, PBD1: $1.2 \times 10^5 \pm 5.1 \times 10^4$ CFU mL^{-1}; PBD2: $8.7 \times 10^4 \pm 2.9 \times 10^4$ CFU mL^{-1}), the control with antibiotic was found to be more effective (day 10, $8.3 \times 10^3 \pm 1.6 \times 10^3$ CFU mL^{-1}). In conclusion, PBD1 and PBD2 may be added to antibiotic-free extenders for boar semen at a concentration of 3 μM, but do not completely control all bacterial growth.

1. Introduction

Pig breeding is mainly conducted through artificial insemination with liquid-stored boar semen in Western Countries [1,2]. Due to the composition of their plasma membrane, boar spermatozoa are preserved at 15-20 °C in liquid state, which differs from other species, such as equine, whose semen can be stored at 4 °C [3]. There are different extenders for liquid boar semen that preserve sperm cells for the short- (3–4 days), long- (5–9 days) and extra-long term (>10 days). In all cases, these extenders contain antibiotics as potential bacterial growth is more likely to occur at 15-17 °C than at 4 °C [4].

Despite mounting restrictions within the European Union (EU), a cocktail of broad-spectrum, highly potent antibiotics is still included in boar semen extenders, especially in the long-term ones [5,6]. In 2016, the European Medicines Agency (EMA) devised a plan to reduce the proliferation of antimicrobial resistances and develop alternatives to antibiotics. The restriction in the use of antibiotics is related to the fact that most of the bacteria isolated from animal samples exhibit resistance to common antibiotics, as is the case of seminal doses [7]. Therefore, finding alternatives to common antibiotics is urgent and antimicrobial peptides (AMPs) appear to be a promising strategy. While one of the main limitations in the use of antibiotics is the high number of bacterial resistances, very few and with moderate effects have been described for antimicrobial peptides [8].

Defensins are AMPs and a rabbit defensin was the first antimicrobial peptide isolated from an animal in 1956 [8]. Defensins are

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cationic peptides from the innate immunity system of mammals, forming a β-sheet on a frame of six cysteines residues linked by three disulphide bonds: 1–5, 2–4 and 3–6 [9]. These cationic peptides interact with the anionic lipids of the bacterial outer membrane, which results in the formation of pores in the membrane and ultimately leads bacterial cell to death [10]. Defensins are divided into three different classes: α, β and θ [11]. β-defensins, such as Porcine β-defensin 1 (PBD1) and Porcine β-defensin 2 (PBD2), present high diversity in terms of biological functions, which makes them as ideal candidates for new therapeutic treatments against infections and the resulting injuries and diseases [12].

Thus far, only few studies have investigated the effects of replacing antibiotics by AMPs in boar semen extenders. Schulze et al. [13] and Speck et al. [14] evaluated cationic AMPs and found a peptide-concentration dependent effect upon sperm integrity and motility. While these AMPs were also found to inhibit bacterial growth, results were especially good when AMPs were combined with gentamycin [14]. On the other hand, another work compared three AMPs (PMAP36, PMAP37 and PR39) and found that, despite PMAP37 being a promising agent to replace antibiotics in boar semen extenders, results obtained with an antibiotic-containing medium were better [15].

The present work sought to determine whether two AMPs, belonging to the defensin family (PBD1 and PBD2), are a safe and suitable alternative to antibiotics for liquid-stored boar semen. As safety and suitability mainly relies upon spermicidal and bactericidal effects, both sperm motility and viability and bacterial growth were evaluated. Two separate experiments were conducted. In the first one, the effects of three concentrations (1.5 μM, 3 μM and 5 μM) of each AMP were tested in extended boar semen. In the second one, the ability of these two AMPs to control bacterial growth was evaluated following artificial inoculation with *Escherichia coli* at 10^7 or 10^8 Colony Forming Units per mL (CFU·mL^{-1}).

2. Materials and methods

2.1. Semen samples

Semen samples were purchased from a local boar stud. Since samples were bought from a commercial company rather than obtained from animals kept for experimental purposes, no specific authorization by an Ethics Committee was required. We can confirm that the farm handled the animals by strictly following the Animal Welfare Rule issued by the Regional Government of Catalonia, Spain (D 214/1997, DOGC 1997; 2450: 9169–9174) and the Spanish welfare and protection standards in swine (RD 1392/2012, BOE 2012; 241: 71,380–71382).

A total of eighteen ejaculates (nine per experiment) was used, each coming from a separate Piétrain boar. Boars, which were all mature and healthy, were kept under adjusted conditions of temperature and humidity and fed a standard diet with water provided *ad libitum*. The rhythm of collection for all animals was twice a week and no fertility problems were recorded by the boar stud. Semen was collected using the gloved-hand technique [16] and the sperm-rich fraction was diluted with Beltsville Thawing Solution (BTS) without antibiotic (205 mM glucose, 10 mM KCl, 20.4 mM $Na_3C_6H_5O_7$, 15.0 mM $NaHCO_3$ and 3.36 mM EDTA; pH = 7.2 [17]). Sperm concentration was adjusted with a Neubauer counting chamber to 3×10^7 spermatozoa·mL^{-1}. Samples were sent to our laboratory at 17 °C and arrived within 12 h post-collection.

When received at our laboratory, seminal samples were evaluated by assessing sperm motility, morphology and membrane integrity. All samples were confirmed to be over the following sperm quality thresholds: 80% viable spermatozoa, 80% total motile spermatozoa and 85% morphologically normal spermatozoa. Each semen sample was split into separate aliquots of 3 mL that were stored at 17 °C and used to test the AMPs as described below.

2.2. Antimicrobial peptides (PBD1 and PBD2)

As previously stated, two AMPs were tested: porcine β-defensin 1 (PBD1) and Porcine β-defensin 2 (PBD2). Both AMPs are from porcine origin and target gram-negative and gram-positive bacteria [18–21]. The two AMPs were purchased from BioWORLD® (Dublin, Ohio, USA) and were dissolved with DMSO following the manufacturer's instructions. Purities of PBD1 and PBD2 were 92.23% and 92.27%, respectively. Preliminary experiments were performed prior to setting the concentrations that were finally assayed (1.5, 3 and 5 μM). Those experiments, which tested 10 nM, 100 nM, 1 μM and 10 μM, let us to conclude that concentrations of both peptides lower than 1 μM had no effect on bacterial growth and those higher than 10 μM were cytotoxic for sperm.

2.3. Experimental design

The first experiment compared the effects of adding the semen extender with PBD1 or PBD2 at 1.5, 3 and 5 μM or with antibiotic (kanamycin; 50 μg mL^{-1}). Negative control was the extender without antibiotic or AMP. Conventional sperm parameters and bacterial growth of samples were evaluated at 1, 2, 3, 5, 7, 8, 9 and 10 days of liquid storage at 17 °C.

The second experiment tested the capability of PBD1 and PBD2 to keep bacterial growth at minimum following artificial inoculation with *Escherichia coli* (O157:H7 strain) purchased from the Spanish collection of microbiological type-cultures (CECT, Valencia, Spain [22]). Two bacterial loads were inoculated: 10^7 CFU mL^{-1} and 10^8 CFU mL^{-1}. Prior to inoculation of semen doses, *E. coli* was cultured in Luria-Bertani (LB) liquid medium (Tryptone 10 g L^{-1}, NaCl 10 g L^{-1}, Yeast extract 5 g L^{-1}; pH = 7.2) using a shaking water bath (Memmert; Schwabach, Germany), under aerobic conditions at 37 °C for 24 h. A tube containing LB medium without *E. coli* was used as a control. Bacteria were subsequently pelleted by centrifugation at 4800 × g for 10 min, resuspended with BTS without antibiotic and again centrifuged at 4800 × g for 10 min. The pellet was resuspended with BTS without antibiotic and bacteria concentration was evaluated with a spectrophotometer (SmartSpec™ Plus, Bio-Rad, California, USA) at a wave-length of 600 nm (optical density, OD600). Following this, bacterial inocula at final concentrations of 10^7 and 10^8 CFU mL^{-1} were added to semen doses. As in Experiment 1, bacterial growth, sperm motility and viability were evaluated in control samples (without antibiotic and AMPs), samples containing antibiotic (50 μg mL^{-1} kanamycin) and treatments (PBD1 and PBD2 at 1.5 μM, 3 μM and 5 μM). Semen was stored at 17 °C and evaluated at 1, 4, 7 and 9 days post-inoculation.

2.4. Evaluation of sperm motility

Sperm motility was determined through a CASA system (Integrated Sperm Analysis System V1.0; Proiser S.L.; Valencia, Spain) and a phase-contrast microscope at 100 × magnification (Olympus BX41 microscope, Olympus Europe GmbH, Hamburg, Germany) equipped with a warm stage. Prior to evaluation, 100 μL of each sample was warmed at 37 °C for 15 min in a water bath. Following this, one 5-μL drop per sample was placed onto a warmed (37 °C) Makler® counting chamber (Sefi-Medical instruments; Haifa, Israel) and analysed using the CASA system. A minimum of two replicates per sample were analysed evaluating, at least, 1000 spermatozoa. The system records the following parameters: total and progressive sperm motilities, curvilinear velocity (VCL,