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An Investigative Immunoassay Targeting Two Osteopontin Epitopes in Boar Semen

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Abstract

The identification of superior sire is largely dependent on the semen quality evaluations, currently through computer-aided sperm class analyser (CASA). Immunoassays present a viable method for analysis of proteins associated with fertility, such as osteopontin. We have targeted osteopontin at an upstream epitope at position 249 - 267 amino acids and a downstream epitope at position 59 - 74 amino acids of full osteopontin protein in raw, BTS, Kobidil, Tris and Citrate liquid preserved Large white and Kolbroek boar semen. The Genetool analysis software was used to quantify the amount of osteopontin detected. Three boars per breed were used and experiments were repeated five times. Our results revealed that the downstream 15 amino acid epitope was more sensitive to osteopontin antibody. Between total motility and osteopontin, Kolbroek boar semen preserved in Kobidil, Citrate, BTS and Tris extenders revealed positive correlations of 0.564, 0.471, −0.960 and 0.330, respectively. Large white boars semen showed correlations of −0.043, 0.655, 0.778, and 0.202 Kobidil, Citrate, BTS and Tris, respectively. For rapid motility and osteopontin, Kolbroek boar semen revealed positive correlations of 0.842, 0.601, 0.164 and 0.202, respectively while large white boars semen showed correlations of −0.909, −0.144, −0.210, and 0.089, respectively. Only BTS and Kobidil showed high negative correlations between osteopontin and viability in Kolbroek (−0.975) and Large white (−0.746) boar semen, respectively. No significant correlations with osteopontin were observed when Tris extender was used in both breeds. In conclusion, Kolbroek boar semen requires osteopontin in Kobodil extender while large white boar semen requires osteopontin in BTS extender.

Keywords

Extenders, Osteopontin, Boar Semen, Kolbroek, Large White

1. Introduction

The ultimate aim of any pig breeding programme is to increase the profitability of commercial pork production. Artificial insemination practices dominate reproduction management on pig farms in South Africa because it brings superior sire lines into the herd and across many sows [1]. The identification of superior sire is largely dependent on the semen quality evaluations and the reproductive performance of sires [2]. Consequently, boar semen fertility remains an area of interest in the pig farming and industries alike [2] [3]. Large White and Kolbroek boars are popular with our local pig farmers due to their desirable traits including their superior semen motility parameters accompanied by high sperm cell viabilities [4] [5]. The current semen evaluation techniques, such as CASA, provide a very conservative estimate of their relative fertility [4] [5]. These observations have led to interests in the investigations of fertility markers in boars [3] [6] [7] [8]. The seminal plasma proteins, mainly osteopontin, have been recognized as a potential fertility marker in boars [6]. It has been found in the reproductive tract and ejaculated semen, where it modifies semen on route to ejaculation [6] [9]. These sperm modifications by osteopontin influence capacitation, motility and viability of sperm cells [7] [9]. Antibodies against osteopontin revealed that osteopontin was localized in the post-acrosomal region of ejaculates spermatozoa [10] [11] [12]. Also, in adult boars, osteopontin has been found in the testis, specifically, in the early and late germ cells, including spermatids [6] [11]. The most profound illustrated function of osteopontin in boars during fertilization was shown when osteopontin decreased polyspermy, apoptosis and improved fertilization [3] [7]. The structure of osteopontin reveals that it is a long protein of 322 amino acids [13]. It consists of RGD sequence important for adhesion functions and SVVYGG for thrombin cleavage (**Figure 1**). The down-stream osteopontin epitope at region 59 - 74 with 15 amino acids sequence, and the up-stream epitope at position 249 - 267 with 18 amino acids sequence of full osteopontin protein, provide great targets for osteopontin immunoassays.

Liquid preservation of boar semen in extenders like Tris, Kobidil, BTS and Citrate, remains a preferred method of storage over short or extended periods [5] [14] [15]. While these extenders have been proven to be ideal for liquid preservation of semen from these breeds, motility parameters are severely compromised over time in an extender and period of incubation dependent manner [4] [5] [16]. Techniques such as ELISA and Western blot analysis can be used to assess

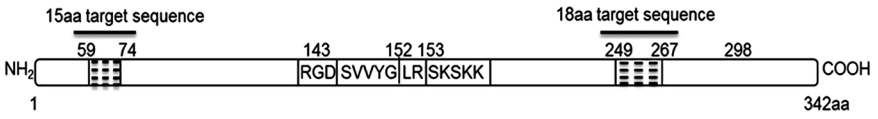

Figure 1. The structure of an osteopontin protein showing the full protein with the downstream 15 amino acid (aa) target site at position 59 - 74 and the 18 amino acid upstream target site at position 249 - 267. (Adopted from Wei *et al.*, 2017, with modification).

the presence or absence of osteopontin in liquid preserved semen [17]. However, in the absence of these techniques, the dot-blot analysis becomes a promising alternative technique. The advantage of using this technique includes the fact that it is simple, quick, and cheap and multiple samples can be analysed at once [17]. The absolute requirement for applying this technique is the use of highly specific antibodies due to the presence of "junk" proteins which might compromise the specificity and sensitivity of the assays hence a densitometric analyser is often required [17]. Genetool software provides a spot blot analysis using a spot grid, and can perform quantitative and incidence analysis plus multiple background noise subtraction.

Tris, Kobidil, BTS and Citrate extenders show low motilities and viabilities over time, is it imperative to investigate whether the osteopontin can be associated with these decreases. Hence, the objective of this study was to determine the correlation between osteopontin detected with total motility, rapid motility and viability of liquid preserved Kolbroek and large white boar semen.

2. Materials and Methods

2.1. Animal Care

The experiments were carried out at the Pig Research Unit of Germplasm Conservation and Reproductive Biotechnologies Programme at the Agricultural Research Council (ARC), Irene, South Africa. The ARC-AP campus is located at 25˚55' South; 28˚12' East. The campus is located in the Highveld region of South Africa and situated at an altitude of 1525 m above sea level. The experimental protocols were evaluated and approved by the Animal Ethics Committee of the Agricultural Research Council-Animal Production (APIEC13/002).

2.2. Semen Collection

Three South African indigenous Kolbroek boars and three exotic Large White boars with ages ranging from nine to ten months were utilized in this study. Boars were in good health and were fed grower diet and water was given ad libitum throughout the duration of the study. Semen was collected once a week for five weeks from each boar by the gloved hand technique, as outlined by [5]. The sperm-rich fraction was collected using a thermos-flask containing warm water (39˚C) and a glass beaker covered with a gauze filter to separate the gel fraction from sperm-rich fraction [4]. Within two hours of collection semen was transported to the laboratory for evaluation. A drop of fresh semen was placed on a microscopic slide and evaluated with the aid CASA system. The semen was diluted using four different short term extenders, namely: BTS, Kobidil, Citrate and Tris-based extenders at a ratio of 1:1 (v/v). The composition of these extenders is shown (**Table 1**) [4].

Sperm motility and viability were evaluated at 0, 3, 24 and 48 hours (hrs) interval. For viability, cells were stained with SYBR-14/PI as previously demonstrated [18]. Briefly, 50 µl of semen was diluted with pre-warmed BO-Wash to

Table 1. Chemical composition of BTS, Citrate, Tris and Kobidil extenders.

Composition (g/L)	BTS	Citrate	Tris	Kobidil
Glucose	37.0	10	10	37.0
EDTA	1.25	-	-	1.25
Sodium Citrate	6.0	18.56	-	6.0
Tris	-	-	24.22	-
Citric Acid	-	-	13.60	-
Sodium Bicarbonate	1.25	-	-	1.25
Potassium Chloride	0.75	-	-	0.75
Gentamycin	-	1.0	1.0	2.0

BTS: Beltsville Thawing Solution; EDTA: Ethylenediaminetetraacetic Acid; Tris: Tris(hydroxymethyl) aminomethane.

1 mL and 5 µl of a 50 times diluted SYBR-14 was added to the cells followed by incubation at 37˚C for 10 minutes. After 10 minutes, 5 µl of propidium iodide was added to the cells followed by incubation for an additional 10 minutes. After 10 minutes, 5 µl of cells were immediately placed on pre-warmed glass slide and observed under a fluorescent microscope (Olympus, model BX51).

2.3. Dot Blot Analysis

A dot blot assay is a technique in molecular biology used to detect biomolecules such as proteins using specific antibodies. Freshly collected semen and extended semen was centrifuged at 500 × g for 10 minutes at 5˚C. The precipitated semen was then resuspended in in the semen lysis buffer containing 0.1 M Tris-HCl pH 8.0, 0.14 M NaCl, 10% Glycerol and 1% NP-40. After vortexing, the protein concentration was determined using the Bradford assay and a standard curve was established to determine the semen total protein concentrations [19]. Bovine Serum Albumin (sigma cat#A3311) the standard osteopontin protein (sigma cat#O3514) and the lysates from freshly collected semen was loaded onto the nitrocellulose membrane at protein concentrations of 3 µg/ml, 12 µg/ml and 60 µg/ml at 5 µl per sample. For the liquid preserved semen in BTS, Kobidil, Citrate and Tris-based extenders for 0, 3, 24, and 48 hrs, 5 µl was applied to the high protein affinity nitrocellulose membrane and allow to air dry at room temperature. The high protein affinity nitrocellulose membrane was then blocked for 30 minutes with a 0.1% BSA in 0.05% Tween-20 in 20 mM Tris-HCl, 150 mM NaCl at pH 7.5 (BSA-TBS-T-buffer) solution to prevent non-specific binding of the antibodies to be used in the assay.

The membrane was then washed three times for five minutes each with a washing buffer containing 0.05% Tween-20 in 20 mM Tris-HCl, 150 mM NaCl (TBS-T-buffer). The washed membrane was then incubated with a primary antibody, namely, a polyclonal anti-osteopontin antibody raised in rabbits at a dilution of 1:1000 in TBS-T-BSA buffer for 1 hour at room temperature. Two primary antibodies were used, the 15 amino acid antibody corresponding to the 59

- 74 amino acid sequence of human osteopontin (Abcam, ab14175) and the 18 amino acid antibody corresponding to the 249 - 267 amino acid sequence of human osteopontin (Sigma, O7264). After an hour, the membrane was washed three times for five minutes each with TBS-T. The washed membrane was then incubated with the secondary antibody; the horse reddish peroxidase conjugated anti-rabbit IgG (sigma), at 1:1000 dilutions for 30 minutes at room temperature. The membrane was then washed twice for five minutes each with TBS-T and then incubated with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) diluted 3,3'-diamino-benzamide-urea-hydrogen peroxide tablets until brown colour development, according to manufacturer's protocol (Sigma, D4168). After colour development, the membrane was rinsed once in TBS and allowed to air dry. The brown colour developed indicated the presence of osteopontin in the semen protein lysates.

2.4. Genetool Software Analysis of Osteopontin

The syngene genetool software version 4.03.00 was used to assess the differences in the osteoponin levels among the samples. It features novel algorithms for background subtraction, noise filtering, precise alignment, spot detection, rapid matching and reduced image editing time. Using its powerful spot detection algorithm, it instantly locates and analyses protein spots. The entire analysis process from background correction to spot matching results and reporting takes minutes, making this the fastest analysis package currently available. The colour developed nitrocellulose membranes were placed on a stage of the syngene genetool platform and scanned to capture the image. Tracks, with a track identity number, were developed to indicate the areas to be analyzed and their positions. The scanned and analyzed image tracks were allocated numbers corresponding to the intensity due to the developed colour due to osteopontin. The intensity of the colour developed can be expressed as peaks or raw volume indicating the intensity and share volume due to osteopontin levels. Both the raw semen arbitrary osteopontin values and the liquid preserved semen osteopontin arbitrary values were captured and analyzed.

2.5. Statistical Analysis

All experiments were repeated 3 times. Pearson's correlation coefficient was used to establish relationships between different parameters. Data were presented as the mean ± SEM. Student's t-test was done wherever applicable, and significance was expressed as a $p \leq 0.05$. The syngene genetool software version 4.03.00 was used to assess the differences in the osteoponin levels among the samples. Statistical analyses were done using the software GraphPad InStat Version 3.06 (GraphPad Software).

3. Results

CASA analysis of boar semen revealed different colours in accordance to semen

motility properties (Figure 2a). Also, The SYBR-14/PI stain proved definitive in distinguishing between live and dead boar sperm cells (Figure 2b).

Raw semen from Kolbroek and Large White boars show high and comparable percent total motility and percent viability, however, the rapid motility of Kolbroekboar raw semen was superior to that of large white boars (Table 2).

The genetool software analysis provides a good estimation of the amount of antigen detected in an immunoassay. The output show peaks corresponding to detected spots were the peak volume show the amount of antigen. In this study we have targeted two osteopontin epitopes, namely, the downstream 15 amino acid sequence at position 59 - 74 and the upstream 18 amino acid sequence at position 249 - 267 of the full osteopontin protein and quantitated the amount of osteopontin with the use of the genetool software. To prove the reliability of the software, a standard curve using BSA (negative control), osteopontin protein (positive control), and semen extracted proteins were analysed targeting both epitopes revealed acceptable correlation coefficients between antigen and amount of protein used (Table 3).

The anti-osteopontin antibody targeting the 15 amino acids sequence at position 59 - 74, revealed that on average, 3 µg/ml, 12 µg/ml and 60 µg/ml of raw Kolbroek boar semen total protein show average osteopontin values of 4839.7, 15,964 and 24,286.33, respectively (Table 4). At similar semen total protein concentrations, the same antibody revealed values of 1038.8, 11,992, and 14,717, respectively, for raw Large White boar semen. In contrast, at similar concentrations, the anti-osteopontin antibody targeting the 18 amino acids sequence at position 249 - 267, revealed average values of 1961, 3996.7 and 6682.3 respectively,

Table 2. The total motility, rapid motility and viability of raw Kolbroek and Large White boar semen (mean ± SEM).

	Kolbroek boars	Large White boars
Total Motility	97 ± 0.82[a]	95 ± 2.16[a]
Rapid Motility	60.67 ± 10.21[a]	31.67 ± 6.94[b]
Viability	92.33 ± 2.055[a]	89 ± 2.95[a]

Superscripts (a, b) indicates significant difference across the raw between the two breed.

Table 3. The Pearson's correlation coefficients following dot-blot and genetool analysis of osteopontin targeted at the 15 amino acid epitope or 18 amino acid epitope in BSA, human osteopontin protein, Kolbroek semen and Large White semen.

	15 amino acids target epitope	18 amino acids target epitope
BSA	−0.003	0.002
Osteopontin	0.993	0.981
Kolbroek boar semen	0.972	0.574
Large White boar semen	0.446	−0.011

Table 4. Genetool osteopontin raw values obtained from Kolbroek and Large White boar semen targeted at the 15 amino acid or the 18 amino acid osteopontin epitopes.

	15 amino acids target epitope	18 amino acids target epitope
Kolbroek boar semen	24,286.3 ± 19,924.93[a]	6682.3 ± 521.81[b]
Large White boar semen	23,568.7 ± 13794.7[a]	7101.67 ± 4691.5[b]

Superscripts (a, b) indicates significant difference across the raw between the two osteopontin epitopes.

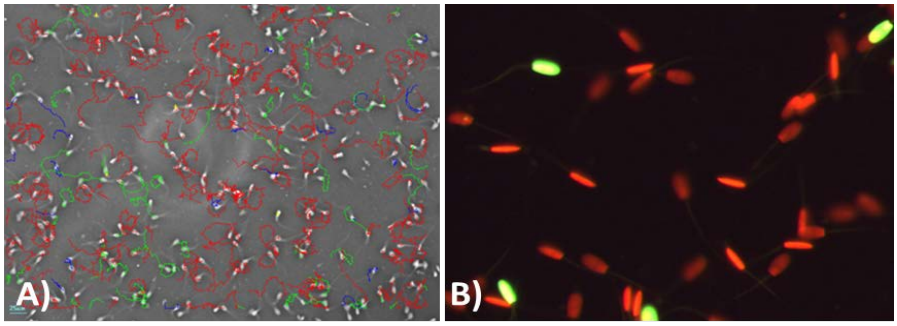

Figure 2. The images showing (A) the semen motility analysis of boar using the CASA system showing motility (μm/s) in different colours or (B) the boar semen viability following SYBR-14/PI staining showing live (green) and dead (red) sperm cells.

for raw Kolbroek boar semen and average values of 985.3, 1869 and 7101.67 respectively, for raw Large White boar semen. At the highest protein concentrstion, these specificities were demonstrated (Table 4).

For raw semen, the downstream 15 amino acid targeted osteopontin epitope was more sensitive to the antibody in Kolbroek boar semen than Large White boar semen revealing a greater correlation coefficient, 0.972 and 0.446, respectively (Table 4). Consequently, only the 15 amino acid epitope was targeted throughout the liquid preservation experiments.

Our data revealed that depending on the extender used, the osteopontin detected varied. The relationship between effect of extenders used for boar semen preservation and osteopontin has never been shown. Our data also show that BTS is the only extender that maintained osteopontin levels up to 24 hrs whereas other extender maintained osteopontin levels only up to 3 hrs in Kolbroek boar semen. Interestingly, in Large White boars, Tris -based extender maintained osteopontin levels up to 24 hrs, although the same was observed for Kobidil liquid preserved semen, but to a much lesser extent. After 3 hrs, Kobidil liquid preserved Kolbroek semen showed high osteopontin levels and highest total motility. This observation is not true for Large White boar for all extenders after 3 hrs, although an incline in osteopontin is observed with Tris liquid preserved Large White boar semen.

After 48 hrs, semen liquid preserved with all extenders showed low osteopontin levels in Kolbroek boar semen with Kobidil showing the lowest osteopontin levels despite Tris liquid preserved semen having high total motility. For large white, all liquid preserved semen showed low and comparable osteopontin levels

despite BTS and Kobidil showing high motility. Kobidil and BTS have similar chemical composition except for the presence of gentamycin in Kobidil extender (Table 1). Essentially, Kobidil maintains osteopontin to 24 hrs in Large White boar semen like BTS in Kolbroek boar semen, although BTS maintain osteopontin at 15 times as much in Large White boars for the same period. Even more fascinating is that the total motility for BTS liquid preserved Kolbroek semen at 24 hrs is moderately high while Kobidil liquid preserved Large White semen is high as is the Tris liquid preserved Kolbroek semen after 24 hrs which is high. This indicates that in Large White boar semen, osteopontin is required to maintain motility after 24 hrs in Kobidil liquid preserved semen. On the contrary, in Kolbroek boar semen, osteopontin is not required to maintain motility in BTS liquid preserved semen for the same period. The same is true for Tris liquid preserved Kolbroek semen after 24 hrs.

We have established the correlation coefficient in liquid preserved Kolbroek and large white boar semen relating to total motility of osteopontin detected. Our data show that when Kolbroek semen is preserved in BTS, there in high negative correlation between total motility and osteopontin detected (−0.960) while large white boar semen show moderately high positive correlation. In Kolbroek boar semen preserved in Kobidil, Citrate and Tris extenders, positive correlations of 0.564, 0.471 and 0.330, respectively, were obtained. Kobidil preserved semen showed that there was no relationship between total motility and osteopontin detected in large white boars (−0.043) (Table 5).

Rapid motility of Kolbroek boar raw semen is twice as much compared to large white boar raw semen. Under Kobidil preservation conditions, Kolbroek boars showed a high positive correlation of 0.842 between rapid motility and osteopontin while large white boars showed highly negative correlation of −0.909. When citrate extender is used, Kolbroek boar semen showed positive correlation between rapid motility and osteopontin (0.601) while large white boar semen showed low negative correlation (−0.144) (Table 6). BTS and Tris revealed low correlation between rapid motility and osteopontin detected in both breeds.

For viability, Kolbroek boars show highly negative correlation (−0.975) between viability and osteopontin when BTS was used while a moderately high negative correlation (−0.746) was observed with Large white boar semen albeit with Kobidil extender (Table 7).

Table 5. The pearson's correlation coefficient between total motility and osteopontin in Kolbroek and Large White boar semen extended with Kobidil, Citrate, BTS and Tris extenders.

	Kolbroek boars	Large White boars
Kobidil	0.564	−0.043
Citrate	0.471	0.655
BTS	−0.960	0.778
Tris	0.330	0.202

Table 6. The pearson's correlation coefficient between rapid motility and osteopontin in Kolbroek and Large White boar semen extended with Kobidil, Citrate, BTS and Tris extenders.

	Kolbroek boars	Large White boars
Kobidil	0.842	−0.909
Citrate	0.601	−0.144
BTS	0.164	−0.210
Tris	0.202	0.089

Table 7. The pearson's correlation coefficient between viability and osteopontin in Kolbroek and Large White boar semen extended with Kobidil, Citrate, BTS and Tris extenders.

	Kolbroek boars	Large White boars
Kobidil	0.291	−0.746
Citrate	0.359	0.428
BTS	−0.975	0.604
Tris	−0.189	0.094

4. Discussion

The sensitivity of the antibody developed from 15 amino acid peptide versus the 18 amino acid peptide has shown that only the 15 amino acid downstream epitope appears to have a dominant sensitivity as shown by the highly positive correlation coefficient of osteopontin detected raw semen extracted proteins from both breeds. Hence, only the downstream epitope was targeted for further analysis. It is also possible that this downstream epitope sequence was different between Kolbroek and Large White boar semen full osteopontin protein since this was not investigated.

The distinction in the extender composition is important for the interpretation of data as a pattern of osteopontin detected and its correlations appear to favors use of certain extenders. BTS extenders show high negative correlation between osteopontin, total motility and viability in Kolbroek boar semen. In contrast, large white boars show a relatively high correlation between osteopontin and total motility plus viability with same extender. This contrasting effect appears to be breed dependent which then raises questions about the differences between Kolbroek and large white boar semen response mechanism to these extenders. Another interesting observation is that Kobidil preserved Kolbroek semen show high correlation between rapid motility and osteopontin while Large White boar semen showed highly negative correlation with this extender. This observation seems to suggest that osteopontin is required for Kolbroek rapid motility but not in large white boar semen rapid motility.

The citrate extenders show positive correlations between osteopontin and total motility plus viability in both breeds. However, only Kolbroek boar semen

shows a positive correlation between Rapid motility and osteopontin. In both breeds, Tris extenders did not reveal any significant correlation coefficient between osteopontin with total motility, rapid motility and viability. Speculation is that for both citrate and Tris, the buffering capacity and calcium chelation when Tris extender is used is compromised hence cell function is impeded.

5. Conclusion

The role of osteopontin in boars is not understood, however, the fact that osteopontin is present in Kolbroek and Large White boar semen suggests that it might have a role in the boar semen fertilizing ability. Osteopontin is not required for rapid motility in large white boar semen but appears to be required for Kolbroek boar semen rapid motility. This then raises questions about whether the extenders used for semen cryopreservation for these breeds require further scrutiny. The importance of the buffering capacity, chelation capacity and salt content appear to influence motility and viability in a breed dependent manner. This could imply that certain extenders may not be preferable for certain breeds. The limitation of this study is whether these observations can translate to semen fertility when osteopontin is at high levels. To prove this, an *in vitro* fertilization (IVF) assay must be performed to assess fertilizing ability of boar semen. More research is required to establish the mechanisms of function of osteopontin during boar semen fertilization.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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