Antioxidative Potential of Aqueous Neem Bark Extract (Azadirachtaindica A. Juss) on Spermatozoa Quality in Extended Porcine Semen

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Abstract

The antioxidative potential of Neem bark in relation to spermatozoa quality is not fully understood. Thus, this present study was conducted to investigate the antioxidative potential of aqueous neem bark extract (ANBE) on spermatozoa quality in extended Porcine Semen.Fresh semen was collected from a mature and intact boar (age, breed, body condition score, health status) using the glove-hand technique. The collected semen samples were diluted and allotted to five treatments with three replicates per treatment in a completely randomized design and evaluated at 0, 24 and 48 h of refrigeration at 17°C. Semen quality parameters such as progressive motility (

Index terms—
Fresh semen was collected from a mature and intact boar (age, breed, body condition score, health status) using the glove-hand technique. The collected semen samples were diluted and allotted to five treatments with three replicates per treatment in a completely randomized design and evaluated at 0, 24 and 48 h of refrigeration at 17°C. Semen quality parameters such as progressive motility (%), liveability (%), morphology (%), acrosome integrity (%), pH, and lipid per oxidation were evaluated.

The results of effect of ANBE on spermatozoa quality in extended porcine semen indicate that progressive motility, liveability, morphology, were lowest ($p<0.05$) in the treatment groups than the control group throughout the period of mean values were observed in spermatozoa progressive motility and liveability across the treatments with T5 given the lowest mean values in progressive motility (48.00±2.00) and liveability (43.33±2.89) respectively. There was no significant difference ($P>0.05$) in morphology across the treatments. However, all the treatments gave mean values within the acceptable normal range.

The results of effect of ANBE on spermatozoa fertilizing potential of extended porcine semen reveal that acrosome integrity and lipid peroxidation were lowest ($p<0.05$) in the treatment groups than the control group throughout the period of preservation. At 48 hours, there was no significant difference ($P>0.05$) in pH across the treatments. Significant difference ($P>0.05$) was observed in acrosome integrity and lipid peroxidation across the treatments. The lower level of lipid peroxidation recorded in this study for all the treatments throughout the period of preservation is an indication of antioxidative potential of ANBE on spermatozoa quality.

The results of this study suggest that 0.75mL of ANBE can be used in boar semen extension up to 48 h as indicated by observed mean values of all parameters, which fall within the acceptable range of normal values indicative of good semen quality.

1 I. Introduction

xidative stress (OS) due to imbalance between oxidants and antioxidants in the semen can results to sperm damage, impairs the structure and function of spermatozoa and eventually male infertility (Agarwal et al., 2009). Oxidative stress is an important factor which influences fertility potential of spermatozoa by lipid peroxidation which may result in sperm dysfunction (Abasalt, et al., 2013).

The supplementation of a cryopreservation extender with antioxidant has been shown to provide a cryo protective effect on mammalian sperm quality (Amrit et al., 2011).

However, high cost of synthetic antioxidants necessitate a search for novel and more sustainable natural antioxidants to maintain a balance between the reactive oxygen species (ROS) and antioxidants in the body so as to prevent to sperm damage, deformity, and male infertility.

There are abundance of literature on antioxidative potential of Neem bark. Neem bark plays the role of free radical scavenger due to rich source of antioxidants. Hassain et al. (2013)

2 b) Preparation of Aqueous Extracts from Fresh Neem Leaves

The extracts from fresh neem leaves were prepared immediately after sample collection with the following procedure; 1 kg of fresh leaves was collected, washed with distilled water and then chopped into small pieces. These were soaked into 1000 mL of distilled water in overnight and were then filtered with a cheese cloth. The filtrate was then centrifuged to remove remaining fibre in the extract, thus enhancing the visibility of spermatozoa during the microscopic evaluation and then stored at 5°C (Ilori et al., 2018).

3 c) Preparation of the Boar, Semen Collection and Extension

Prior to collection of semen, the boar was thoroughly washed and the preputial pouch was cleaned with water by a milking action, to remove urine and other materials that could contaminate semen during collection. Semen was collected using the gloved hand method into a US bag inserted in a collection cup such that the pre and post sperm fractions were separated from the sperm-rich fraction. Semen and extender was mixed in a ratio 1:4, 1:0.25, 1:0.75, 1:0.5, 1:1 as described by (Althouse, 2008). The mixture was refrigerated at 17°C. (Althouse et al., 2000, Althouse, 2008).

4 d) Semen Evaluation

Semen evaluation was carried out using the following parameters; pH, progressive motility, liveability, morphology, acrosome integrity and lipid peroxidation at 0, 24 and 48 h of preservation (17°C).

5 e) Progressive Motility

This was assessed by putting a drop of semen on a clean glass slide, covered with a cover slip and examined with a microscope under at 400X (B100,AmScope, USA), The progressive motility of the spermatozoa was subjectively estimated and rated between 0 and 100 (Yi et al., 2008). 0 means low percentage of motile spermatozoa and 100 means a high percentage of motile spermatozoa which indicate that the spermatozoa have not been damaged by the process of dilution and storage (Althouse, 2008).

6 f) Liveability

This was determined by mixing a drop of semen with a drop of a staining solution (eosin-nigrosin) on a clean glass slide gently and a smear developed using the edge of another clean slide, air-dried and examined with a microscope at 400X (Althouse, 2008).

7 g) Morphology

This was determined following the same method for liveability. Spermatozoa with coiled or double tail, damaged mid-piece and damaged head were considered abnormal (Levis, 2000).

8 h) Acrosome Integrity

Sperm was fixed with 1% glutaraldehyde in Beltsville thawing solution (BTS; 3.71 g glucose, 0.60 g trisodium citrate, 1.25 g ethylenediaminetetraacetic acid, 1.25 g sodium bicarbonate, 0.75 g potassium chloride and 100.0 ml distilled water) so as to examine acrosome integrity according to (Yi et al., 2008).

9 i) pH

A pH meter (Mettler Toledo Switzerland) was used to measure the hydrogen ion concentrations produced by spermatozoa metabolic activities during the storage period.

10 j) Lipid Peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) levels as described by Hunter et al. (1963) modified by **??**utteridge and Wilkins (1980).

11 k) Experimental Treatments and Design

A completely randomized design was utilized for the study, such that diluted semen was allotted to six treatments with three replicates per treatment and evaluated at 0, 24 and 48 h: Treatment 1 (Control): Semen + Beltsville

Thawing Solution (BTS) Extender. Treatment 2: Semen + BTS + 0.25 mL ANBE. Treatment 3: Semen + BTS + 0.50 mL ANBE. Treatment 4: Semen + BTS+ 0.75 mL ANBE. Treatment 5: Semen + BTS + 1.00 mL ANBE.

12 III. Results

13 a) Effect of ANBE on Spermatozoa Quality in Extended

Porcine Semen at 0, 24 and 48 Hours The data on effect of ANBE on spermatozoa characteristics of extended porcine semen at 0, 24 and 48 hours of refrigeration at 17°C is as shown in Tables 1, 2 and 3 respectively.

At 0 hour, there was no significant difference (P<0.05) inspermatozoa progressive motility, morphology and liveability across the treatments with the exception of T5 with slight reduction in mean values. Mean vales of T5 were found to be (61.67±2.87), (93.67±3.21) and (90.00±0.00) for spermatozoa progressive motility, morphology and liveability respectively.

At 24 hours, significant difference (P<0.05) was observed in progressive motility across the treatments with T5 (76.33±5.00) being significantly lower than other treatments. There was no significant difference (P>0.05) in morphology across the treatments with the exception of T5 (83.00±2.00) with slight reduction in mean value. There was no significant difference (P>0.05) in liveability across the treatments with the exception of T4 (84.33±1.15) and T5 (80.33±0.58) with slight reduction in mean values.

At 48 hours, significant difference (P<0.05) in mean values were observed in spermatozoa progressive motility and liveability across the treatments with T5 given the lowest mean values in progressive motility (48.00±2.00) and liveability (43.33±2.89) respectively. There was no significant difference (P>0.05) in morphology across the treatments. However, all the treatments gave mean values within the acceptable normal range. At 0 h, there was no significant difference (P>0.05) in the pH across the treatments. A significant difference (P<0.05) was observed in acrosome integrity and lipid peroxidation across the treatments. However, mean vales of T5 were found to be lower (93.67±3.21) and (0.55±0.18) in acrosome integrity and lipid peroxidation respectively than in the other treatments. At 24 hours, there was no significant difference (P>0.05) in pH and lipid peroxidation across the treatments. Significant difference (P<0.05) was observed in acrosome integrity with T5 (88.33±2.08) and T4 (91.00±1.00) being significantly lower than the other treatments.

At 48 hours, there was no significant difference (P>0.05) in pH across the treatments. Significant difference (P>0.05) was observed in acrosome integrity and lipid peroxidation across the treatments. IV. Discussion a) Effect of ANBE on Spermatozoa Quality in Extended Porcine Semen at 0, 24 and 48 Hours Spermatozoa progressive motility is one of the major determinants of fertility of male animals such as boar **??**Haugan et al., 2004). Progressive motility of spermatozoa has always been considered a primary requirement for egg fertilization. It is known to be an important characteristic in predicting the fertilizing potential of an ejaculate (Gadea, 2005). The results of this study showed that ANBE has potential to maintain spermatozoa progressive motility throughout the periods of preservation and this may probably be due to antioxidants activities of neem bark (Gayatri et al., 2010).

A. indica has been reported to contain polyphenolic compounds which possess remarkable antioxidant activities (Siddiqui, et al., 1992;Sultana, et al., 2007;Gayatri et al., 2010). All the treatments gave mean values within acceptable normal range throughout the period of preservation with the exception of 1.00 m Linclusion level of ANBE which gave the mean values below acceptable normal range at 48 hours of storage. The high percentages of motile spermatozoa recorded with the inclusion of ANBE in boar semen is in accordance with findings of Levis, (2000), Roca et, al, (2006); Vytet, al, (2008) who reported that motility above 60% is enough for fertilization to take place provided that all other semen parameters are good.

The antioxidants activities of ANBE was found to enhanced spermatozoa morphology throughout the periods of preservation. Morphological abnormalities of spermatozoa that can severely influenced fertilization and embryonic development was found to be corrected due to presence of polyphenolic compounds in neem barks which possess significant antioxidant activities (Siddiqui, et al., 1992;Sultana, et al., 2007;Gayatri et al., 2010).All the treatments gave mean values within acceptable normal range throughout the period of preservation and this support the findings of Maes et al., (2010) who reported that ejaculates should have greater than 70% normal sperm with no more than 20% sperm with primary abnormalities. This findings agrees with Cerolini et al. (2000) who reported that inclusion of antioxidant into storage diluents could prevent deterioration of boar spermatozoa quality and provided protection to the cells up to 5 days of storage through its prevention of oxidative reduction in the levels of major polyunsaturated fatty acid.

The percent live spermatozoa was found to be enhanced G of good semen quality throughout the period of preservation with the exception of 1.00 Ml inclusion level which gave the mean values below acceptable normal range at 48 hours of storage. This decline in percent spermatozoa liveability at this hour of preservation may indicate a gradual reduction of antioxidant activities of neem barks.However, the high percent live spermatozoa recorded with the inclusion of ANBE in boar semen corroborates the findings of Maes et al., (2010) who reported that semen samples should have more than 70% viable sperm by a vital stain assay prior to processing. This is in line with findings of Cerollini et al., **??**2000) reported that the inclusion of an antioxidant into the diluent could prevent the significance reduction in viability of cells, and this could lead to high percent live spermatozoa

recorded for this study. This indicates that ANBE can be used as exogenous antioxidant in extender to inhibit lipid peroxidation.

14 b) Effect of ANBE on Spermatozoa Fertilizing Potential ofExtended Porcine Semen at 0, 24 and 48 Hours

The antioxidants activities of ANBE was found to enhanced acrosome integrity throughout the period of preservation as indicated by high percentages recorded for acrosome integrity which falls within acceptable range of normal values indicative of good semen quality and this is in line with the findings of Maes et al. (2010) who reported that semen samples with less than 70% sperm with intact acrosomes should be discarded before processing. This finding is agreement with the findings of Ilori et al., (2018) who reported that neem has the potential of maintaining acrosome integrity of boar semen by protecting acrosome from undergoing capacitation during preservation.The antioxidants activities of ANBE was found to maintain the pH throughout the period of preservation. It is important for pH to be maintained because when the pH of the semen is declined; the internal pH of the spermatozoa is also reduced leading to a decrease in sperm metabolism and mobility (Gadea, 2005). This result is in compliance with of Frunza et al., (2008) who recorded a higher proportion of live normal sperm in a neutral and alkaline pHlevel (7.0 and 8.2).

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) levels as described by Hunter et al. (1963) modified by **??**utteridge and Wilkins (1980).Malondialdehyde (MDA) is one of the reactive and mutagenic aldehyde products of lipid peroxidation in seminal plasma (Shang et al., 2004). Toxic lipid peroxides are known to cause different impairments of sperm cells and may play a main role in the etiology of male infertility (Abasalt et al., 2013). Malondialdehyde (MDA) is an indicator of lipid peroxidation which may be a diagnostic tool for the analysis of infertility (Tavilani et al., 2008) High lipid peroxidation levels have been reported to reduced sperm functionality such as motility, acrosomal reaction, fertilization, membrane degradation and sperm oocyte fusion (Abasalt et al., 2013; **??**oolsbyet al., 2014). However, in this study, the MDA level recorded as an indicator of lipid peroxidation for all the treatments were found to be lower throughout the period of preservation. This could be attributed to antioxidative properties of ANBE such as presence of phenols and other inhibiting compounds which help to inhibit lipid peroxidation throughout the period of preservation. This assertion is in compliance with Cal et al., **??**2004) who reported that phenols are responsible for the variation in the antioxidant activity of Neem bark. This is also justified by Pitchaon et al., (2007) and Pokorney et al., (2001) who opined that neem plant exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals. Phenolic compounds are considered to be the most important antioxidant components of herbs and other plant materials and a good correlation between the concentration of plant phenolic and total antioxidant capacities has been reported (Madsen et al., 1996;Pellegrini et al., 2000).

The results of this study suggest that 0.75mL of ANBE can be used in boar semen extension up to 48 h as indicated by observed mean values of all parameters, which fall within the acceptable range of normal values indicative of good semen quality. [1]

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Figure 1:

1

Inclusion Level of ANBE

[Note: b Mean values on the same row with different superscript (a, and b) are significantly different (p<0.05), SD = Standard Deviation]

Figure 2: Table 1 :

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2

Inclusion Level of ANBE

Figure 3: Table 2 :

3

Inclusion Level of ANBE

Figure 4: Table 3 :

4

	Inclusion Level of ANBE				
Parameters	0 mL	0.25 mL	0.50 mL	0.75 mL	1.00 mL
pH	6.96±0.58	6.93±0.58	7.00±0.00	6.96±0.58	6.93±0.58
AI	98.00±0.00 a	98.33±0.58 a	97.67±0.58 a	96.33±0.58 a	93.67±3.21 b
LP	0.84±0.24 a	0.66±0.23 b	0.63±0.27 b	0.62±0.27 b	0.55±0.18 c

Mean values on the same row with different superscript (a, b, and c) are significantly different ($p<0.05$), SD = Standard Deviation, AI = Acrosome Integrity, LP = Lipid Peroxidation

Figure 5: Table 4 :

5

	Inclusion Level of ANBE				
Parameters	0 mL	0.25 mL	0.50 mL	0.75 mL	1.00 mL
pH	7.00±0.00	7.03±0.58	7.00±0.10	7.00±0.10	7.03±0.06
AI	96.67±2.31 a	92.67±0.58 b	92.00±0.00 b	91.00±1.00 b	88.33±2.08 c
LP	0.79±0.30	0.77±0.20	0.75±0.30	0.71±0.25	0.70±0.09

Mean values on the same row with different superscript (a, b, and c) are significantly different ($p<0.05$), SD = Standard Deviation, AI = Acrosome Integrity, LP = Lipid Peroxidation

Figure 6: Table 5 :

14 B) EFFECT OF ANBE ON SPERMATOZOA FERTILIZING POTENTIAL OFEXTENDED PORCINE SEMEN AT 0, 24 AND 48 HOURS

6

	Inclusion Level of ANBE				
Parameters	0 mL	0.25 mL	0.50 mL	0.75 mL	1.00 mL
pH	7.03±0.06	7.03±0.06	7.06±0.06	7.06±0.06	7.03±0.06
AI	89.33±1.15 a	82.33±0.58 b	81.67±1.15 b	80.00±0.00 b	79.33±0.58 b
LP	0.57±0.03 a	0.47±0.05 b	0.43±0.05 b	0.43±0.08 b	0.21±0.03

[Note: c Mean values on the same row with different superscript (a, b, and c) are significantly different ($p<0.05$), SD = Standard Deviation, AI = Acrosome Integrity, LP = Lipid Peroxidation]

Figure 7: Table 6 :

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