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EFFECTS OF MUCUNA SEED EXTRACT ON SPERM FUNCTIONAL INTEGRITIES AND SEMINAL OXIDATIVE STRESS INDICES OF VITRIFIED GOAT SEMEN

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Abstract: Effects of mucuna seed extract (MSE) in tris-extenders on sperm functional integrities and seminal oxidative stress indices of vitrified sperm of West African Dwarf (WAD) goat bucks were investigated. Varying levels (0, 0.25, 0.5, 0.75, 1 g/100 mL of diluent) of MSE in tris-extenders were diluted with semen specimens. Diluted semen specimens were vitrified and preserved in liquid nitrogen. The results showed that sperm functional integrities (acrosome and membrane integrities) in MSE extenders were comparable. However, seminal oxidative stress indices (malondialdehyde [MDA] and acrosin activity) differed ($P<0.05$) among the various treatments and the control. The results revealed that semen vitrified with MSE had reduced ($P<0.05$) MDA concentrations compared to the control. Higher ($P<0.05$) acrosin activities were recorded in semen vitrified with MSE compared to the control except at 0.25g MSE and optimal values were recorded at 1g MSE. The findings indicated that MSE in the tris-extenders reduced MDA concentration with congruent elevated acrosin activity and consequently maintained sperm functional integrities of vitrified spermatozoa of the WAD goat.

Key words: antioxidant, freezing, mucuna, oxidative stress, sperm integrity.

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Introduction

Cryopreservation is a major development made in artificial insemination to preserve sperm cells in order to multiply important genetic material in species of economic interest (Blash et al., 2005). The utilization of this proven technology to preserve sperm cells as a method of improving goat production (*Capra hircus*) is necessary to alleviate the problem of protein malnutrition. The protocols of cryopreservation, however, can cause harmful effects on sperm viability (Pegg, 2007; Bagchi et al., 2008). The conventional constituents of semen extenders such as milk and egg yolk are animal products (Correa et al., 2006; Purdy, 2006) and they are sometimes susceptible to microbial damage. In addition, an enzyme that coagulates egg yolk in the bulbourethral gland (Iritani and Nishikawa, 1961) or SBUIII (a protein fraction) that interacts with milk components in extenders causes reduced viability of buck sperm cells (Leboeuf et al., 2000). Therefore, semen diluents void of these constraints could be a possible way to preserve goat semen. Inclusion of plant products like fruit juices, coconut water and coconut milk as constituents of semen extenders has recently been proved to improve the quality of cryopreserved spermatozoa (Daramola et al., 2016 a, b, c). The seeds of *Mucuna pruriens* are sources of important compounds such as antioxidants (Sharma et al., 1978). The seeds contain flavonoids, magnesium, copper, zinc, manganese and iron (Misra and Wagner, 2007). Daramola et al. (2015) recently investigated the potential effect of *Mucuna pruriens* seed powder on spermiograms of West African Dwarf goats and observed improved testicular measurements and sperm quality. However, the protective effects of MSE on stored semen have not been reported. The objective of this experiment was therefore to assess the effects of MSE in extenders on sperm functional integrities and oxidative stress indices of WAD goats (*Capra hircus*) during vitrification.

Materials and Methods

Experimental location and animal management

The Directorate of University Teaching and Research Farm, Federal University of Agriculture, Abeokuta in south-western part of Nigeria was used for the research. The animals used for this study consisted of four WAD goat bucks aged 3–5 years. The bucks had access to the concentrate feed and guinea grass (*Panicum maximum*) under intensive system.

Preparation of mucuna seed extract

Dried seeds of *Mucuna pruriens* were ground into granules. The granules (518g) were then soaked in 1750 mL of methanol for 17 hours with intermittent

stirring. The soaked mucuna granules were filtered and the resultant extract solution was concentrated on a water bath by evaporation at 54^{o}C. The extract was then transferred into a beaker for use with a tris-based extender.

Semen collection, dilution and vitrification

An artificial vagina was used to collect semen specimens from the bucks. A total of four semen specimens from the bucks were pooled (Bucak and Tekin, 2007). The pooled semen specimens were extended at room temperature using a tris-based extender. The specimen was divided into 5 equal portions, diluted with varying levels of MSE (0, 0.25, 0.50, 0.75, 1.00g/100 mL of the diluent) in tris-extenders. The tris-extenders consisting of 2 M tris-hydroxymethyl-aminomethane (2.42 g), 0.5 M glucose (1 g), 0.0005 M penicillin (0.028 g), egg yolk (20 ml) and distilled water made up 100 ml and were supplemented with the varying levels of MSE. The diluted semen specimens were vitrified at room temperature using separate cryoprotectants as described by Srirattana et al. (2013) with some changes. Phosphate-buffered saline (PBS) was supplemented with bovine serum albumin (20%) (v/v) as a holding medium (HM). Ethylene glycol (EG), dimethyl sulfoxide (DMSO) and HM were used to prepare vitrification solution I (VS-I) and vitrification solution II (VS-II), respectively. VS-I was made by mixing 0.2 M EG (12.5%), 0.15 M DMSO (12.5%) and the HM in the ratio of 1:1:6 while VS-II was made by mixing 0.4 M EG (25%), 0.3 M DMSO (25%) and the HM in the ratio of 1:1:2. The semen specimen previously diluted was added to the separate cryoprotectants sequentially for each treatment. The specimen (1 mL) was first added to 1 mL of HM for 10 min. The mixture (diluted semen specimen + HM) was then placed in 1 mL of VS-I for 4 min, and the mixture (diluted semen specimen + HM + VS-I) was finally put into 1 mL of VS-II for 1 min. Following equilibration in VS-II, the specimen was loaded into 0.2 mL straws and treatment was replicated four times (4 straws per treatment) and preserved in liquid nitrogen for 7 days.

Evaluation of sperm functional indices

Following storage for 7 days, thawing of the straws was carried out in a water bath at 38°C and assessed for acrosome integrity and membrane integrity. Assessment of acrosome integrity was carried out in a formalin citrate solution consisting of 2.9% sodium citrate (96 mL) and 37% formaldehyde (4 mL) as described by Ahmad et al. (2003) to identify the acrosome that showed a normal apical ridge of sperm cells. Membrane integrity of the sperm cells was evaluated in a hypo-osmotic solution consisting of 9 g fructose plus 4.9 g sodium citrate mixed with 1000 mL of distilled water as described by Zubair et al. (2013) to

identify swelled spermatozoa with a characteristic coiled tail as the intact plasma membrane.

Evaluation of seminal oxidative stress indices

The malondialdehyde concentrations in the preserved semen were evaluated using a thiobarbituric acid reactive substance as described by Pipan et al. (2014) while evaluation of the acrosin activity using the specific substrate for acrosin consisting of N-a-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) was carried out as described by Rosatti et al. (2004).

Statistical analysis

Estimations were performed for the stored semen specimens for each treatment consisting of four straws and four different measurements made for each straw (n=16). One-way analysis of variance in the SAS 2002 package was used to analyze the data obtained and all significant means were separated by Duncan's multiple range test (Duncan, 1955).

The model used for the experiment included:

$Y_{ij} = \mu + E_i + \sum_{ij}$,

where Y_{ij} = Dependent variables,

μ = Population mean,

E_i = Effect due to the j^{th} level of MSE inclusion (0, 0.25, 0.50, 0.75, 1.00),

$\sum_{ij}$ = Experimental error.

Results and Discussion

The sperm functional integrities (acrosome and membrane integrities) of WAD goat semen vitrified with MSE are presented in Figures 1 and 2, respectively. The results showed that the acrosome integrity and membrane integrity in MSE extenders were comparable. However, seminal oxidative stress indices such as malondialdehyde and acrosin activity differed ($P<0.05$) among the various treatments and the control (Figures 3 and 4). The results revealed that semen vitrified with MSE had reduced ($P<0.05$) malondialdehyde concentrations compared to the control (Figure 3) while higher ($P<0.05$) acrosin activities were observed in semen vitrified with MSE compared to the control except at 0.25g MSE and optimal values were recorded at 1g MSE (Figure 4).

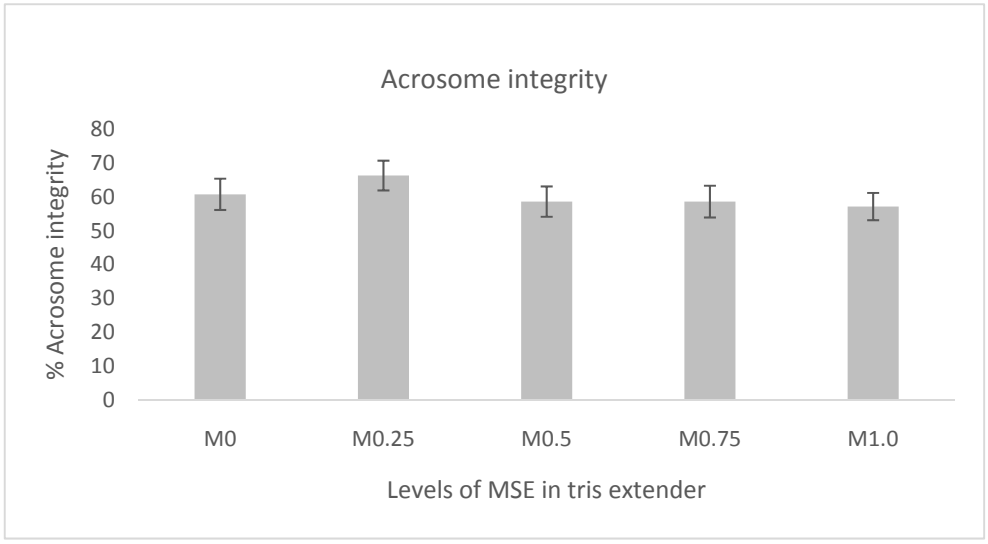

Figure 1. Acrosome integrities of semen vitrified with MSE extenders (n=16). MSE = Mucuna seed extract.

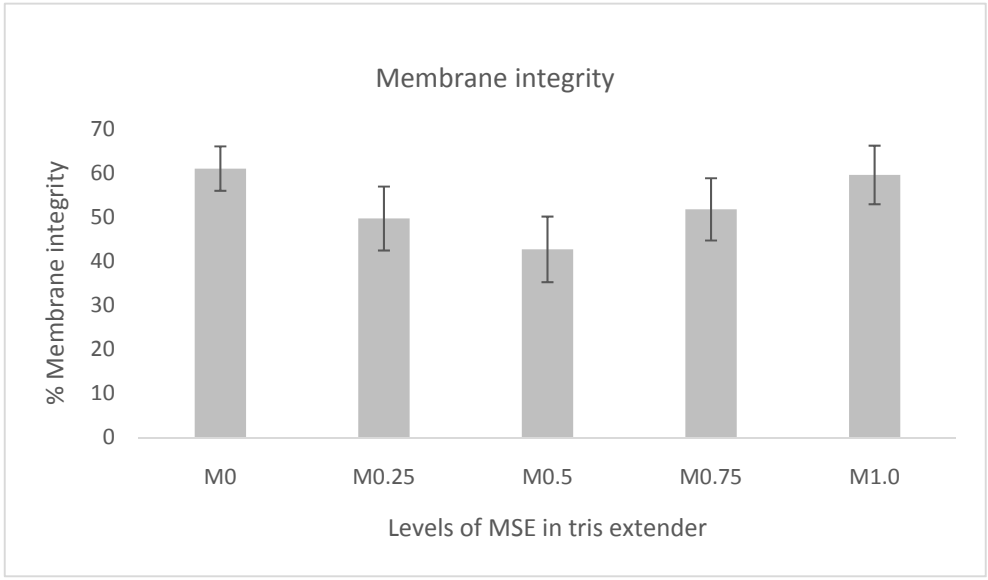

Figure 2. Membrane integrities of semen vitrified with MSE extenders (n=16). MSE = Mucuna seed extract.

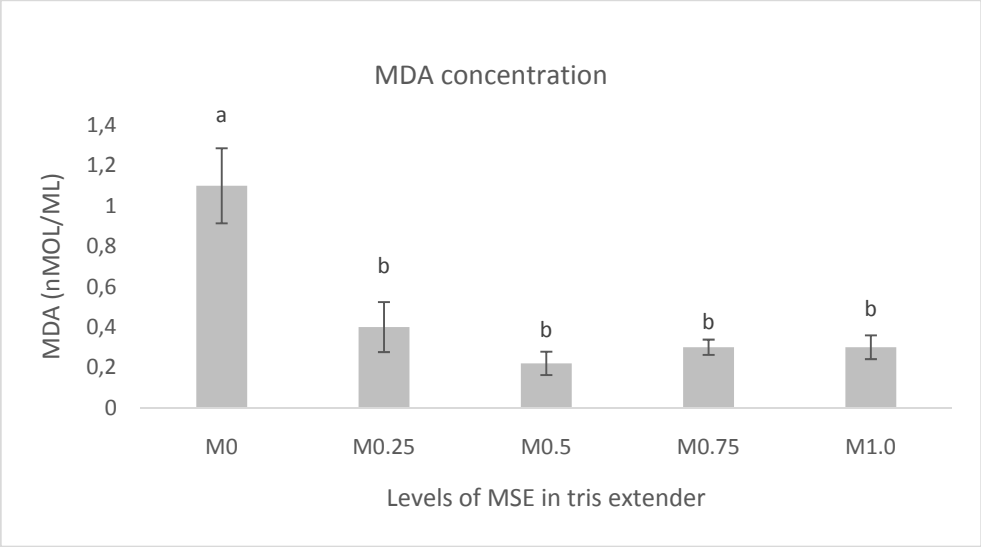

Figure 3. Malondialdehyde concentrations of semen vitrified with MSE extenders (n=16).
[a,b] Means with different superscripts differ significantly (P<0.05).
MSE = Mucuna seed extract; MDA = Malondialdehyde.

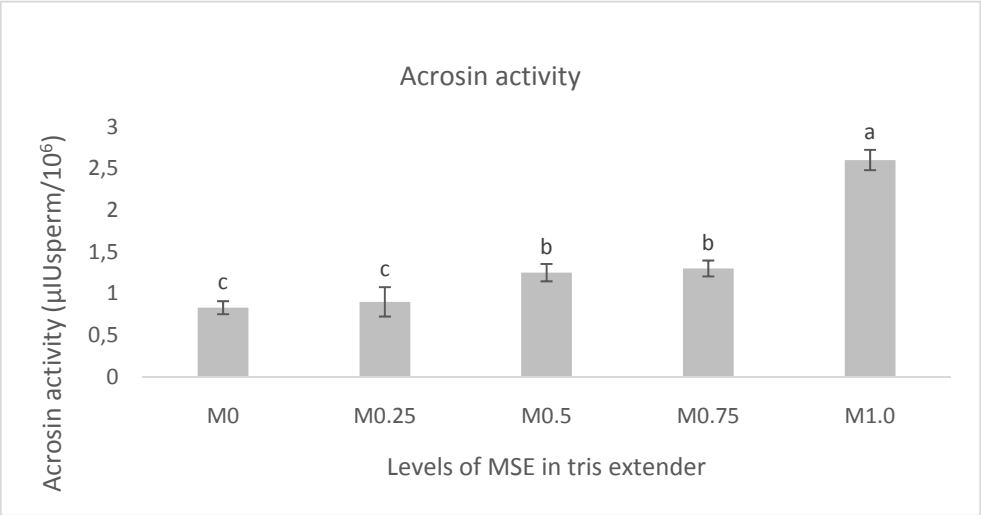

Figure 4. Acrosin activity of semen vitrified with MSE extenders (n=16).
[a,b,c] Means with different superscripts differ significantly (P<0.05).
MSE = Mucuna seed extract.

The reduced MDA and congruent elevated acrosin activity in extenders supplemented with MSE might probably be due to protective action of the antioxidant in *Mucuna pruriens* seed against oxidative stress during the freezing process. *Mucuna pruriens* seeds contain many bioactive components such as alkaloids, coumarins, flavonoids and alkylamines which perform an important function in elevating the antioxidant ability (Misra and Wagner, 2007). In addition, seeds of *Mucuna pruriens* have antioxidant, lipid reducing and neuroprotective functions (Sharma et al., 1978; Longhi et al., 2011). Moreover, phenolic substances have high antioxidant potential that is attributed to their ability to reduce oxides, which perform a vital function in the adsorption or neutralization of free radicals (Basile et al., 2005). The total phenolic content (24 ± 0.2 g/100 g) that translates to 6.48 g of total phenolic in 100 g of mucuna seed has been observed in *M. pruriens* extract (Longhi et al., 2011). *M. pruriens* with its characteristic hydrogen-donating ability possesses an excellent reducing power and its extract has been observed to possess the potential scavenging activity on the free radical-scavenging activity (Longhi et al., 2011).

Production of free radicals and reactive oxygen species during the oxygen metabolism leads to body cell damage (Grace, 1994). Although the mechanisms by which free radicals obstruct activities of cells have not been well elucidated yet, cell membrane damage by lipid peroxidation seems to be an important event to reckon with (Nijveldt et al., 2001; Lobo et al., 2010). Flavonoids like other antioxidants exert their effects on cell functions by different mechanisms including electron donation, metal ion chelating, co-antioxidants, or by gene expression regulation (Krinsky, 1992; Misra and Wagner, 2007). Oxidation of flavonoids by radicals leads to a more stable, less reactive radical that stabilizes the reactive oxygen species (Nijveldt et al., 2001). The high reactivity of the hydroxyl group of the flavonoids makes radicals inactive (Korkina and Afanas'ev, 1997). The protective action on spermatozoa in this study could therefore be linked to the antioxidant property of flavonoids in *M. pruriens* (Misra and Wagner, 2007).

The protective action of extenders supplemented with MSE on spermatozoa in this study could further be linked to the antioxidant property of metals such as magnesium, copper, zinc, manganese and iron that are abundant in MSE (Misra and Wagner, 2007). Metal ions perform important functions in different cellular activities including homeostasis (Bertini and Cavallaro, 2008). *M. pruriens* is rich in copper (Misra and Wagner, 2007), and as an antioxidant, copper scavenges or neutralizes free radicals and helps to reduce some of the damage they cause (Araya et al., 2006; Bonham et al., 2002). The role of copper as an antioxidant supported by higher content of MDA compared to copper deficiency and reduced MDA with the increasing copper intake has been reported (Duan et al., 2010). Furthermore, copper as an antioxidant acts as a cofactor of some enzymes, such as Cu/Zn SOD with a protective effect on spermatozoa against peroxidative damage of its cellular

enzymes and structures from reactive oxygen species (Bray and Bettger, 1990; Menella and Jones, 1991; Sikka, 2001; Tabassomi and Alayi-Shoushtari, 2013). Reports have revealed that copper ion is important for optimal superoxide dismutase activity that leads to less free radical production during spermatogenesis (Abdul-Rasheed, 2010).

Moreover, in addition to copper as an antioxidant, findings suggest that zinc has antioxidant properties and protects cells from oxidative damage (Ho and Ames, 2002; Sakaguchi et al., 2002). Zinc performs a vital role in cell functions and as an important constituent of several proteins involved in the defense against oxidative stress (Song et al., 2009). The mechanisms by which zinc performs antioxidative activity involve the protection of sulfhydryl groups of proteins and reduction of free radical formation through the prevention mechanisms or antagonism of redox-active transition metals, such as iron and copper (Bray and Bettger, 1990). The synergetic antioxidant property of zinc and copper in MSE probably contributed to the survival of spermatozoa cryopreserved in this study.

Frozen spermatozoa are susceptible to reactive oxygen species and lipid peroxidation produced in sperm metabolism (Watson, 2000). The protective effect of antioxidants on the plasma membrane of frozen sperm has been observed in both metabolic activity and cellular viability (Cheema et al., 2009). Supplementation of manganese (Mn) in a semen extender during freezing of semen revealed a protective ability and explained the increase for sperm quality characteristics and the decrease in malondialdehyde concentration due to its chain breaking antioxidant property and inhibitory effect on oxidative stress damage (Cheema et al., 2009). The importance of Mn^{2+} has been observed in cryopreserved bull semen, and the supplementation of an egg-yolk-citrate extender with Mn+ glycerol dilutor was found to improve the quality/fertility of cryopreserved semen (Barber et al., 2005). The protective action of extenders supplemented with MSE against reactive oxygen species and lipid peroxidation damage on spermatozoa in this study could further be linked to the antioxidant property of Mn in MSE.

The sustained integrities (acrosome and membrane) in this study could also be attributed to important components of mucuna seeds that are necessary for cell survival in freezing conditions. Mucuna seed is rich in magnesium (Misra and Wagner, 2007). The inclusion of magnesium fumarate in Biosolwens extender improves sperm quality in boar semen (Szcześniak-Fabiańczyk et al., 2003). Magnesium is important for the synthesis of different substances that have energy-rich bonds in form of adenosine triphosphate (Durlach, 1988). Insufficient magnesium makes a cell unable to keep up the appropriate number of high-energy molecules (adenosine triphosphate) to maintain cell function. The sustained viability of the spermatozoa subjected to vitrification in this study could be due to the magnesium stabilizing effect on the membrane and functional integrity of sperm cells (Durlach, 1988).

Conclusion

The findings revealed that MSE in the tris-extenders reduced MDA concentration with congruent elevated acrosin activity and consequently maintained sperm functional integrities of cryopreserved spermatozoa of the WAD goat. The study therefore indicated the protective effect of MSE against oxidative stress damage due to the components of its antioxidant. Vitrified semen specimens of WAD goat bucks with MSE in the tris-extenders would be good enough for a successful artificial insemination programme.

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UTICAJI EKSTRAKTA DOBIJENIH IZ SEMENA STIZOLOBIUMA NA FUNKCIONALNI INTEGRITET I INDEKSE OKSIDATIVNOG STRESA KOD VITRIFIKOVANE SPERME JARČEVA

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R e z i m e

Istraženi su uticaji ekstrakta dobijenih iz semena stizolobiuma (engl. *mucuna seed extract* – MSE) u tris-razređivačima na funkcionalni integritet i indekse oksidativnog stresa kod vitrifikovane sperme jarčeva zapadnoafričkih patuljastih koza (engl. *West African Dwarf* – WAD). Sperma je razređena različitim količinama (0, 0,25, 0,5, 0,75, 1 g/100 mL razređivača) ekstrakta iz semena stizolobiuma u tris-razređivačima. Razblaženi uzorci sperme su vitrifikovani i čuvani u tečnom azotu. Rezultati su pokazali da su funkcionalni integriteti sperme (integriteti akrozoma i membrane) u razređivačima sa ekstraktom iz semena stizolobiuma uporedivi. Međutim, indeksi oksidativnog stresa sperme (aktivnosti malondialdehida [MDA] i akrozina) razlikovali su se ($P<0,05$) među različitim tretmanima i kontrolom. Rezultati su pokazali da je u spermi vitrifikovanoj ekstraktom iz semena stizolobiuma smanjena ($P<0,05$) koncentracija malondialdehida u poređenju sa kontrolom. Više ($P<0,05$) aktivnosti akrozina zabeležene su kod sperme vitrifikovane ekstraktom iz semena stizolobiuma u poređenju sa kontrolom osim kod 0,25g MSE i optimalne vrednosti su bile zabeležene kod 1g MSE. Rezultati su ukazali da ekstrakt iz semena stizolobiuma u tris-razređivačima smanjuje koncentraciju malondialdehida sa kongruentnom povišenom aktivnošću akrozina i kao posledica toga održava se funkcionalni integritet vitrifikovane sperme kod jarčeva zapadnoafričkih patuljastih koza.

Ključne reči: antioksidans, zamrzavanje, stizolobium, oksidativni stres, integritet sperme.

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