VOL. 20 NO. 1
ISSN: 2877-1990
MARCH, 2021

African Scholar Publications & Research International

African Scholar Journal of Agriculture and Agricultural Tech. (JAAT-1)

Antioxidative Effect of Watermelon (*Citrullus Lanatttus)* Juice on Motility and Gross Morphology of Extended Boar Semen

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Abstract

Boar semen preservation is associated with the production of reactive oxygen species which leads to reduction of sperm quality and decrease in fertilizing ability. The aim of this study was to replace conventional semen extender with watermelon juice (WMJ) and to determine the most effective dilution rate that will maintain the viability of the spermatozoa over a 48-hours extension period. Semen was collected from a boar at the piggery unit of the teaching and research farm University of Ibadan, Ibadan using the glove hand method. The experiment was divided into five treatments with Beltsville Thawing Solution being replaced by WMJ at the following rate; 0, 12.5, 25, 37.5 and 50% and designated as T1, T2, T3, T4 and T5 respectively. Diluted semen samples were stored at 17^0C and were evaluated at 0, 24 and 48 hours for progressive motility and morphology. The experimental design was a completely randomised Design; data obtained were analysed using descriptive statistics and ANOVA.

The results showed that 50 % of BTS can be replaced by watermelon juice. However the effect of watermelon juice is inconclusive up to 48 hours of storage.

This evaluation shows that extender developed with WMJ could be an extender of choice for a reliable short term preservation in swine artificial insemination programme.

Keywords: *Boar, Watermelon Juice extension, Semen quality, Spermatozoa.*

Introduction

Successful swine fertility programs can be achieved using extended cooled semen when compared to those using natural service. Although the former is a routine procedure, its use is limited because sperm cells remain viable for about 3 to 5 days (Gadea *et al.,* 2005). Many studies have been carried out on carotenoids, considered an important group of natural antioxidants (Bast *et al*., 1998, Møller *et al*., 2000, Schabath *et al*., 2004and Rao and Rao, 2007). For over a decade, lycopene, a carotenoid present in tomatoes (*Lycopersicon esculentum*) and several ripe fruits and vegetables, such as watermelon, pink grapefruit and carrots (Nguyen and Schwartz, 1999, Perkins-Veazie *et al*., 2003and Tadmor *et al*., 2005), has been considered in some studies related with human and animal health (Bhuvaneswari and Nagini, 2005, Rao *et al*., 2006, Avci and Durak, 2007and Bhom, 2007) and reproductive physiology (Martino *et al*., 2006, Goyal *et al*., 2007, Mangiagalli *et al*., 2007, Turk *et al*., 2007 and Mendiola *et al*., 2010).

In roosters, in particular, Mangiagalli *et al*. (2010) found positive effects of lycopene addition on fertility and qualitative characteristics of semen.

With AI expanding rapidly, the demands for semen increased. The simplest way to meet this demand was to "stretch" each ejaculate further by using fewer spermatozoa per insemination, provided that this could be accomplished without sacrificing fertility.

The need to extend and preserve semen has become more evident due to the increasing use of artificial reproductive technique in animal. Semen extension involves the dilution of high quality semen with a suitable medium.

The main objective of semen preservation is to extend the usefulness of genetically superior males for purposes of expanding the genetic base of breed, repopulating/recreating breeds or lines

and discovering new gene (Waberski *et al.,* 1994; Purdy *et al.,* 2008).
Two major systems (Liquid and Frozen) of storage technologies have been achieved for sperm conservation in the 20th century (Vishwanath *et al.,* 1996).

Liquid storage of semen

For storage of liquid boar semen, two factors are very important: the temperature of collection and storage, and the composition of the storage medium (Johnson *et al*, 2000).
The use of liquid preserved semen for cervical AI in pigs has been widely accepted and accounts for over 80 % of insemination in many regions of the world (Roca *et al*, 2006b; Bailey *et al.,* 2008).

Frozen storage (cryopreservation) of semen

The use of the Tris-egg yolk cryopreservation diluents, such as the one described by Salamon and Ritar (1982) is recommended, as it is easy to use. However, commercial extenders with no biological components have been developed to improve sanitary safety in semen processing (Hinsch *et al*., 1997; Gil *et al*., 2003).
However the adoption of these techniques in the third world countries has been hindered due to high cost of these preparations and poor infrastructural development. This has led to the development of local semen extenders from herbal or plant materials in Nigeria (Umesiobi *et al.,* 1998, 2002; Umesiobi 2004).
The quality of extended semen, to a great extent, depends on the quality of raw ejaculate, provided that the *in vivo* conditions of the semen are maintained *in vivo*. In other words, poor quality ejaculate cannot give extended semen of high quality. Sperm examination is an important tool in estimating the fertilizing capacity of an ejaculate (Frunza *et al.,* 2008). Although several parameters can be investigated, the actual capacity of the spermatozoa to function at the site of fertilization is difficult to assess. Therefore, assays examining morphological or motility characteristics are most routinely performed (Martin-Rillo *et al.,* 1996). Other parameters that can be measured include: concentration, pH and total sperm numbers. However, before one proceeds to do detailed semen evaluation a few parameters can be measured visually at the point of collection, for example, volume, colour, consistency, pH and density

Sperm motility.

Sperm motility has long been considered a major criterion in the assessment of male fertility (Haugan *et al*., 2004); the objective of estimating sperm motility is to determine the motile proportion of spermatozoa and the proportion moving progressively, i.e., actively moving forward. Traditionally, the evaluation has depended on subjective estimates of sperm motility characteristics using a microscope. This method is cheap and simple to use; however, it has the disadvantage that sperm motility estimates can vary among examiners, who can be biased in a number of ways (Malmgren, 1997). For example, the examiner may have prior knowledge of the boar's fertility, or may have viewed its previous ejaculates. Ejaculated boar spermatozoa are vulnerable to cold shock and prolonged storage of boar spermatozoa at low temperatures reduces survival rate resulting in bottleneck for the extension of artificial insemination in pig husbandry (Haugan *et al*., 2004). Sperm motility could be considered as a functional marker in boar sperm analysis, since motility is directly related to the sperm's ability to obtain and process energy (Roldàn, 1998). Sperm motility is an important trait as it is a factor which allows the sperm cell to travel to the zona pellucida. Sperm motility is an important parameter for fertility and the molecular mechanisms of mammalian sperm motility are still largely undefined (Huang *et al*., 1996). However, an accurate determination of boar sperm motility is troublesome (Gadea *et al*., 1998; Sànches, 1991), since the motion characteristics of these cells make an accurate subjective estimation of the samples difficult. Thus, subjective determination of boar sperm motility seems not to be a very useful tool for semen quality analysis (Rigau *et al*., 1996).

The physiological factors regulating sperm motility include protein kinases, phosphatases, calcium ion intracellular pH (Gagnon, 1995; Lanzafame *et al*., 1994). The molecular mechanisms and the signal transduction pathways mediates the processes of capacitation and acrosome reaction are only partially defined, and appear to involve modifications of intracellular calcium and other ions, lipid transfer and phospholipid remodelling in sperm plasma membrane as well as changes in protein phosphorylation (Rigau *et al*., 1996).

Semen morphology

Not all of an individual sperm look exactly alike, abnormalities in sperm size and shape can occur in the head, midpiece and tail. In some cases, these mutation or changes do not impact sperm overall functionality. In other

situations, the sperm may not be able to move properly or quickly enough to reach, puncture or enter the egg membrane. (Jenniffer Huizen, 2017)

Function of antioxidants

The most important and well characterised natural antioxidants in the animal body are vitamin E and C. In the body, all antioxidants are working in concert as a team, the (antioxidant system), responsible for prevention of the damaging effects of free radicals and toxic products of their metabolism. However, the antioxidant (team) acts to control levels of free radical formation as a coordinated system where deficiencies in one component impact the efficiency of others (Peter, 2007). Beta carotene is the most widely studied carotenoid (Alam and Sultan, 2004). Carotenoids are widely distributed natural pigments responsible for the yellow, orange, and red colors of fruits, roots, flowers, fish, invertebrates, and birds (Delia, 1997). Beta-Carotene and lycopenes are the major carotenoids which are mainly composed of carbon and hydrogen atoms. In humans and animals, carotenoids play an important role in protection against photooxidative processes by acting as oxygen and peroxyl radical scavengers (Jacques *et al*., 2008). BetaCarotene is a fat soluble member of the carotenoids which are considered pro vitamins because they can be converted to active vitamin A. Beta carotene is converted to retinol, which is essential for vision. It is a strong antioxidant and is the best quencher of singlet oxygen (Lien *et al*., 2008).

The best dietary sources of beta-carotene are yellow and orange fruits and vegetables. Some of them contain more than 80% of their pro vitamin A in the form of Beta carotene. Only some carotenoids found in nature have pro-vitamin A activity.

Antioxidative Potential of Lycopene in Watermelon Juice

The watermelon fruit has deep green or yellow colored smooth thick exterior rind with gray or light green vertical stripes. Inside the fruit is pink, red or even yellow in color with small black seeds embedded in the middle third of the flesh. Generally, watermelon flesh is the main consumable portion, however, outer rind is also used in some parts of the world (Levi *et al*., 2001; Wehner et al., 2001; Oms-Oliu et al., 2009). Watermelon contributes a plethora of nutritional agents as antioxidants (lycopene, beta-carotene etc.) and some specific amino acids (arginine, citrulline etc.). , it is a good source of potassium and also

contains magnesium, calcium, phosphorus and iron (Quek *et al*., 2007), the aqueous extract of *Citrulus lanatus* has been known to be a good source of glucose, fibre and also an excellent source of vitamin C, lycopene, and beta carotene (Erhardt *et al*., 2003). Lycopene scavenging rate is reported to be higher than beta-carotene and tocopherol. Being a reactive carotenoid, lycopene follows three possible routes to interact as adduct formation, transferring electron to free radical and by allelic hydrogen abstraction. Lycopene mode of action depends on the position within the cell as it lies parallel underneath the surface of cell membrane. Moreover, it significantly inhibits free radicals invasion at membranes surface and serves as primary defense system. Lycopene combinations with other antioxidants have proven synergistic behavior to scavenge reactive oxygen species. Its synergistic role with vitamins E and C and other carotenoids has affirmative impact on human

Crude fiber. For the measurement of crude fiber, fat free health (Huang *et al*., 2007; Skibsted, 2012).

It is important to evaluate the effectiveness of natural antioxidants that are readily available, such as watermelon juice in reducing the oxidative stress in post ejaculated boar semen, to evaluate the effectiveness of antioxidant in watermelon juice on the boar semen quality and determine the proportions of *watermelon juice* that is suitable for preserving boar semen quality.

MATERIALS AND METHODS

Location of Study

Semen was collected from a boar at the piggery unit of the Teaching and Research Farm, University of Ibadan, Ibadan, located in the tropical rain forest zone in Nigeria on latitude 7 20N and 3 50E, with a mean altitude of 277 meters above the sea level. Preparation of water melon juice and semen analyses were carried out in the animal physiology laboratories of the same institution.

Preparation of water melon (*Citrullus lanattus)* juice (WMJ)

Water melon fruits were washed, dried and sliced into small pieces. The flesh was cut after the seeds had been removed, then blended, and sieved using muslin cloth. The juice was further filtered using Whitman filter paper (110mm) to remove remaining fibre in the juice, thus enhancing visibility of spermatozoa during microscopic evaluation (Gibson *et al*, 2000).

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. Once the boar mounts and begins to thrust, the penis was grasped firmly in a gloved hand perpendicular to its body and pressure was applied to the tip of the penis imitating the locking done by the cervix of the sow during natural mating. The grip was sustained until the boar finished ejaculating. Semen was collected into a US bag inserted in a cup such that the pre and post sperm fractions were separated from the sperm rich fraction. Semen and extender were mixed in ratios 1:7, 1:4, 1:3, 1:1.7 and 1:1 as described by Fiser *et al.*, (1993). The mixture was refrigerated at 17^0C. (Althouse *et al.*, 1998).

Experimental treatments and design

A completely randomized design was utilized for the study, such that diluted semen were allotted to five treatments with three replicates per treatments evaluated at 0, 24 and 48 hours:

Treatment 1: Semen + Beltsville Thawing Solution (BTS) Extender
Treatment 2: Semen + BTS + 12.5 % WMJ
Treatment 3: Semen + BTS + 25 % WMJ
Treatment 4: Semen + BTS + 37.5 % WMJ
Treatment 5: Semen + BTS + 50 % WMJ

The composition of BTS used to dilute collected semen is presented in Table 2

Table 1: Composition of Beltsville Thawing Solution (100ml)

Components	g /ml
Glucose	**39.1**
EDTA	**1.32**
Sodium bicarbonate	**1.3**
Potassium chloride	**0.79**
Penicillin	**1.1**
Streptomycin	**1.1**
EDTA = Ethylene diamine tetra acetic acid	

EDTA = Ethylene diamine tetra acetic acid

Semen evaluation

Semen evaluation was carried out on sperm pH, sperm motility, percentage liveability and sperm morphology at 0, 24 and 48 of liquid preservation.

Liveability

This was done by placing a drop of semen on a glass slide, two drops of Eosin-Nigrosin stain was added and mixed gently and smeared on a slide with the edge of another clean slide, airdried and viewed under microscope at magnification of x40. The dead sperm cells absorbed the stain. (Dolt and Foster, 1972).

Morphology

This was done by placing a drop of semen on a glass slide, two drops of Eosin-Nigrosine stain will be added and mixed gently and then smeared on a slide with the edge of another clean slide, air-dried and viewed under microscope at magnification of x400. All sperm cells with coiled or double tail, damaged mid-piece and damaged head were considered abnormal (Dolt and Foster, 1972).

Spermatozoa Motility

Semen was warmed at 37^0C for 3 minutes. It was accessed using a light microscope and viewed under x400 magnification with a warm stage maintained at 37 ^{0}C. A semen mount was made using 5 ul semen sample and was placed directly on a microscope slide the covered with a cover slip. For each samples, five microscope fields were counted to determine sperm cells that are motile.

Statistical analysis

Data obtained was analysed using the analysis of variance procedure of SAS (2003) and means were separated using the Duncan's Multiple Range Test of the same software.

RESULTS

Table 2: Characteristics of semen collected from Boar.

Parameters	Value
Volume (ml)	**50**
Progressive Motility (%)	**95**

Mass activity	++++	
Morphology (%)	**95**	

Characteristics of semen collected

Table 2 shows the results from the initial evaluation of boar semen before extension was done. The result reveals that the sample was suitable for extension as the values fall within acceptable ranges for high quality semen.

Table 3: Effect of water melon juice fortification on extended boar semen quality at 0 hour (Mean ±SD)

Parameters (%)	**T1**	**T2**	**T3**	**T4**	**T5**
Motility	95.00±0.00[a]	95.00±0.00[a]	94.67±0.57[a]	94.33±1.15[a]	93.67±0.57[a]
Morphology	94.33±0.57[a]	90.00±0.00[a]	85.00±5.00[b]	91.67±5.77[a]	86.67±2.88a

abc = mean values in the same row with different superscripts are significantly different (P<0.05); SD = Standard deviation

Effect of water melon juice fortification of boar semen quality at 0 hours

Table 3 shows the mean of motility and morphology of extended boar semen supplemented with graded levels of watermelon juice refrigerated at 17 ^{0}c. There were no significant difference (P>0.05) in motility across the treatments. However, a significant reduction (P<0.05) in morphology was observed in treatment 3 (25 % WMJ) which gave the least mean value.

Table 4: Effect of water melon juice fortification on extended boar semen quality at 24 hours (Mean ± SD)

Parameters	**T1**	**T2**	**T3**	**T4**	**T5**
Motility	90.00±0.00[a]	89.00±1.73[a]	85.00±0.00[b]	84.00±1.00[b]	80.00±0.54[c]
Morphology	87.66±2.51[a]	87.66±0.57[ab]	85.30±0.57[ab]	85.30±0.00[ab]	81.66±0.88[b]

abc = mean values in the same row with different superscripts are significantly different (P<0.05); SD = Standard deviation.

Semen quality of extended (17 ^{0}c) boar semen fortified with water melon juice, (24 hours) Table 4 shows the mean values of motility and morphology of extended boar semen supplemented with graded level of watermelon juice refrigerated at 17^{0}C. Significant differences (P<0.05) in mean values were observed in all the parameters that were evaluated at 24 hours. Significant reduction (P<0.05) in all the parameters were observed with the increase in concentration of watermelon juice extract.

Table 5: Effect of water melon juice fortification on extended boar semen quality at 48 hour (Mean ± SD)

Parameters	T1	T2	T3	T4	T5
Progressive Motility	86.00±1.93^a	82.32±2.50^{ab}	81.00±1.73^{ab}	79.23±1.15^{ab}	78.00±0.00^{ab}
Morphology	83.66±1.15^a	82.00±0.00^{ab}	80.00±0.57^b	80.33±0.57^b	79.33±1.15^b

abc = mean values in the same row with different superscripts are significantly different (P<0.05); SD = Standard deviation

Effect of water melon juice fortification on extended boar semen quality at 48 hours

Table 5 shows the mean values of motility and morphology of extended boar semen supplemented with graded level of watermelon juice refrigerated at 17^{0}C. Significant differences in mean values were observed in all parameters except for the pH that no significant reduction was observed.

In morphology, it was significantly lower (P<0.05) in T5 (50 % WMJ). There was significant improvement (P>0.05) in motility in T2 (37.5 % WMJ).

Discussion

Progressive Motility

Motility in semen was not significantly affected (P<0.05) across the treatments by the watermelon juice in the extender. This indicates that the semen treated with watermelon juice is viable and capable of swimming through the reproductive tract to fertilize the sow or gilt. According to Vyt *et al*, (2008), Levis, (2000) and Roca *et al*, (2006b), motility above 60% was enough to fertilise, if other parameters were good. The author further explained that motility above this figure is sufficient to assist good semen swim through the reproductive tract of receptive sow or gilt which can lead to fertilization. There

was no significant difference (P>0.05) recorded in the motility of the semen extended with watermelon juice even after 24 hours even though, there was a reduction in T5 but this still fall within the range of good semen. Thus, the recorded motility was still adequate to assist semen swimming and fertilization. This supports the report made by Vyt *et al* (2008) that semen that falls within these range of figures are good enough to assist semen swim through the reproductive tract. Notably, the percentage motility recorded in the third day of the experiment was capable of fertilising the oocyte as Britt *et al*, (1999) reported 60% above as the range of sperm quality requirement for motility. The progressive motility recorded on the third day can be linked to the effect of antioxidant present in watermelon juice extract which enhances the sperm cells by giving them a better environment that supports their performance.

Morphology

For sperm morphology, it was observed that significant amount of spermatozoa were normal according to Shipley *et al.*, (1999) that the percentage spermatozoa should be at least 70 %. Also, Cerolini *et al*, (2000) reported that inclusion of antioxidant into storage diluents prevented 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 highest mean value recorded for spermatozoa morphology in treatment 2 can be accrued to inclusion of antioxidant as a supplement in the extender. This is in agreement with the report of Cerrollini *et al*, (2000) that the inclusion of antioxidant into storage diluents prevented deterioration of boar semen quality and provided protection to cells up to 5 days of storage through its prevention of oxidative reduction in levels of major polyunsaturated fatty acid.

CONCLUSION

A study was carried out to investigate the effect of watermelon juice fortification of extended boar semen. Semen was collected from a mature landrace boar and extended with BTS (Beltsville thawing solution). The control treatment contained only BTS with a semen- extender ratio of 1 to 4, while the other treatments contain BTS with different concentrations of watermelon juice. The semen was stored using liquid storage (17^0C). After collection and assessment for odour, colour, volume, contaminant, pH, motility, morphology,

liveability and satisfactory results were obtained, the semen was added to the extender and placed in a regulated refrigerator. Semen evaluation was carried out at every 24 hours for three days. Semen characteristics evaluated include motilityand morphology. There were improvements (P>0.05) in values obtained in semen preserved by watermelon. Mean value for morphology, and motility were reduced but falls within the normal range that can support fertilisation. The results obtained in the study suggest that 50 % of BTS can be replaced by watermelon juice; however the effect of WMJ anti-oxidative properties is inconclusive up to 48 hours of storage.

Watermelon juice extract has proven to have potentials to supply exogenous antioxidant thus inhibiting oxidative stress condition in boar semen. Even at a high level of inclusion in extended semen, it was able to give a percentage motility that was capable of fertilising the oocyte. From this study, extended semen (17^0C) at 12.5% inclusion level of watermelon juice extract gave the best percentage in motility, morphology and liveability throughout the experimental period because all mean values are within acceptable range of normal values indicative of good semen quality.

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