ENHANCING PORCINE SEMEN QUALITY USING GARLIC EXTRACTS AS AN EXOGENOUS ANTIOXIDANT

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ABSTRACT

This study was conducted to investigate the effects of Aqueous Garlic Extracts (AGE) on the quality and spermatozoa fertilizing potential of extended porcine semen. Ejaculate (242.80 mL) was collected from a matured boar using the gloved hand technique. The volume of BTS extender (3mLs) and semen (1mL) was constant across all treatments. The different treatments contained varying volumes of AGE as follows: Treatment 2: 50 mL Treatment 3: 75 mL, Treatment 4: 100 mL and Treatment 5: 125 mL at a semen-to-extender using micropipette at dilution ratio of 1:3, while Treatment 1 contained 3mLs of BTS + 1mL of semen and no AGE. Semen samples were allotted to treatments in a completely randomized design. The semen was analyzed at 0, 24, 48, and 72 hours, for livability (%), normal spermatozoa (%), secondary morphological classification (%), acrosome integrity (%) and pH values remained constant within the accepted range across all treatments. Results showed that AGE inclusion significantly improved ($p<0.05$) livability, normal spermatozoa, acrosome integrity and pH when compared to the control treatment. These improvements were consistent across all AGE concentration levels. Aqueous garlic extracts demonstrated cytoprotective potential and can be used to extend porcine semen for up to 72 hours to improve its quality and fertilizing potential.

Keywords: Garlic extracts, Porcine, Semen quality, Cytoprotection, Spermatozoa fertilizing ability

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INTRODUCTION

One of the most significant reproductive technique that has transformed animal breeding is artificial insemination (AI). Artificial insemination is adjudged to be crucial for livestock producers world-wide which has granted producers access to premier genetics without having to physically own superior sires and one of the cutting-edge technologies to agricultural production. (Sing and Balhara, 2016; Dalen *et al.,* 2021). The success of breeding pigs using artificial insemination (AI) can be attributed to improvements in fertility, labour efficiency, genetics, and production. The establishment of AI centres for the management of boars and the production of semen has allowed for the selection of boars for fertility and spermatozoa production using in vitro and in vivo measures. Today, boars can be managed for the production of 20 to 40 traditional AI doses containing 2.5 to 3.0 billion motile sperm in 75 to 100 mL of extender or 40 to 60 doses with 1.5 to 2.0 billion spermatozoa

in similar or reduced volumes for use in cervical or intrauterine AI (Knox, 2015). Regardless of the spermatozoa dose, in liquid form, extenders are designed to sustain spermatozoa fertility for 3 to 7 days (Knox, 2016).

The use of porcine semen for artificial insemination (AI) using fresh diluted semen has increased considerably during the last decades (Vyt *et al*., 2007; Riesenbeck *et al*., 2015). Compared to natural mating, AI reduces the risk of disease transmission (Maes *et al*., 2008; Maes *et al*., 2016), allowing the introduction of superior genes into sow herds and it additionally leads to better profitability of each boar's ejaculate. Artificial Insemination, therefore, has become a very useful tool in countries with intensive pig production. The extender is a vital component in the cryopreservation process, as it must have an acceptable pH and a buffering capacity, as well as sufficient osmolality and the ability to protect spermatozoa from cryogenic damage (Bustani and Baiee, 2020). Regardless of the extender or storage conditions utilized, semen handling and preservation have a detrimental impact on spermatozoa quality as well as oxidative damage. Due to enhanced lipid peroxidation, mammalian spermatozoa may be more vulnerable to oxidative stress (Aitken and Drevet, 2020).

The inclusion of antioxidants can reduce oxidative damage caused to semen by scavenging harmful molecules. The supplementation of a cryopreservation extender with antioxidant has been shown to provide a cytoprotective effect on mammalian spermatozoa quality (Amrit *et al*., 2011; Sokunbi *et al*., 2020). To prevent oxidative damage during boar semen extension and preservation, semen extender should be reinforced with appropriate components (Patel *et al*., 2016). Plant extracts have recently emerged as a low-cost, natural source of compounds that can help maintain and improve sperm function during storage (Pintus and Ros-Santaella, 2021). Extensive research has shown that herbs and spices rich in antioxidants and phenolic compounds, including flavonoids, carotenoids, and other phenolic compounds, have been utilized in semen processing (Embuscado, 2015; Sokunbi *et al*.,2020; Ros-Santaella, and Pintus, 2021). Most plant species have significant levels of antioxidants, which operate as scavengers of reactive oxygen species (ROS) to minimize the negative effects of oxidative stress on sperm function. Furthermore, these natural chemicals have been shown to improve the activity of a range of antioxidant enzymes that have antibacterial properties and one of such plant species is garlic.

Garlic (*Allium sativum*) is an antioxidant and a detoxifying agent, which scavenges the ROS, enhancing the cellular antioxidant enzymes thus, defending the cells against disease caused by oxidative damage (Banerjee *et al*., 2002). Furthermore, garlic has exceptional biological qualities which include oxidative radical scavenging capabilities and usefulness as an adjuvant in the treatment of number of ailments, as proven by various research studies undertaken in recent decades (Hayat *et al.*, 2018; Alsenosy and Abd El-Aziz, 2019). Garlic has a variety of useful components, including organosulfur chemicals, saponins, and phenolic compounds (Wang *et al*., 2014). Porcine semen extension usually results in the loss of the fertilizing ability of extended spermatozoa. Several cytoprotective agents are being researched to mitigate this problem. Incorporating natural compounds into biological materials injected into animals is encouraged. There is limited knowledge of the cytoprotective potentials of local materials like garlic (*Allium sativum*). Therefore, this research seeks to investigate

the effectiveness of using aqueous garlic extract as a natural antioxidant for preserving sperm in porcine semen.

MATERIALS AND METHODS

***Experimental location*:** Semen collection was carried out at the Piggery Unit of the Teaching and Research Farm, University of Ibadan (7°20'N, 3°50'E;200 - 300 above sea level), while the analyses of semen were carried out at the Animal Physiology and Bioclimatology Laboratories of the Department of Animal Science of the same institution.

Management of boar and semen collection: Semen was collected from a mature boar with proven semen quality and fertility, for this study. The boar was kept in an intensively managed, clean and well-ventilated pen. Feed and clean water were adequately supplied. Semen was collected using a boar semen collection cup; lined with a disposable plastic bag and covered with a disposable milk filter. The gel fraction was separated from the spermatozoa-rich fraction by the milk filter covering the semen collection cup. The gloved-hand technique was used to collect semen from the boar. Before the ejaculate collection, the boar was thoroughly washed to eliminate urine and other materials that could lead to semen contamination during collection.

***Preparation of extracts from garlic (Allium sativum) bulb*:** Fresh garlic was peeled, washed, and 100 grams was chopped into small pieces and blended with 200 mL of distilled water. The blended garlic was filtered using a muslin cloth. The extract was further filtrated using filter paper to obtain the garlic extract.

***Preparation of semen extender*:** Before semen collection from the boar, a conventional extender known as Beltsville Thawing Solution (BTS®) Extender was prepared.

Table 1: Composition of Beltsville Thawing Solution (BTS) extender

Composition	Quantity(g/L)
Glucose	37.00
Sodium citrate	6.00
Potassium chloride	0.75
EDTA	1.25
Sodium bicarbonate	1.25
Penicillin	1.10
Streptomycin	1.10

Source: Johnson (2000);
EDTA = *Ethylenediamine tetra acetate*

***Experimental Treatments and Design*:** Semen samples were allotted to treatments in a completely randomized design.
T1: Control (3 mL BTS + 1 mL semen + 0 µL AGE
T2: 3 mL BTS + 1 mL semen + 50 µL AGE
T3: 3 mL BTS + 1 mL semen + 75 µL AGE
T4: 3 mL BTS + 1 mL semen + 100 µL AGE
T5: 3 mL BTS + 1 mL semen + 125 µL AGE
*AGE = Aqueous Garlic Extract, T = Treatment; BTS = Beltsville Thawing Solution.

***Semen Collection, Processing, and Extension*:** Semen samples collected were immediately evaluated for mass activity, progressive motility, livability, percentage of normal spermatozoa, pH and the temperature were checked 15 minutes after collection. The volume of collected ejaculate was estimated by weighing on a top loader balance. Semen was diluted at a ratio of 1:3 of extender for all the treatments. Diluted semen was preserved in a thermo-regulated fridge at 17 °C and evaluated for livability, normal spermatozoa, pH, acrosome integrity and morphological classification, at 0, 24, 48 and 72 hours.

Semen Quality Evaluation

***Livability*:** A drop of extended semen was placed on a warm microscope slide with a micropipette, and a drop of eosin-nigrosin stain was added, smeared, immediately air-dried, and viewed under the microscope at a

magnification of X400. The proportions of live (eosin-impermeable) and dead (eosin-permeable) spermatozoa in a sample were assessed based on 100 cells counted and expressed in %.

***Normal spermatozoa*:** A drop of the extended semen sample was placed on a glass slide, a drop of eosin-nigrosin stain was added and mixed gently and smeared on a slide with the edge of another clean slide, air-dried and viewed under the microscope at a magnification of X400. From each smear, a total of 100 spermatozoa were examined. All spermatozoa with intact heads, mid-pieces and tails were considered normal, tallied and expressed in %.

***pH*:** A calibrated digital pH meter (Mettler Toledo IP67®) was used to measure the pH of the extended semen samples. The unit was calibrated using a 3-point calibration procedure utilizing buffers with pH of 4.0, 7.0 and 9.0.

***Spermatozoa Fertilizing Potential Assessment*:** Parameters evaluated for spermatozoa fertilizing potential are; acrosome integrity and morphological classification.

***Acrosome integrity*:** Smears of all ejaculates were prepared for confirmation of acrosome integrity condition utilizing an eosin-nigrosin stain. All the slides were assessed under oil immersion at X1000 magnification using a bright field microscope. A total of one hundred spermatozoa per slide were assessed, and normal acrosomes (spermatozoa without apical shifts) were expressed as percentages.

Statistical Analysis: All data collected were analyzed using analysis of variance procedure of SAS (2011) and means were separated using Duncan's New Multiple Range Test of the same software.

RESULTS

Characteristics of semen collected from the selected boar before extension

The results of the initial evaluation of semen before extension is as shown in Table 2. The results reveal that the semen sample collected from the boar before extension showed that the semen volume was 242.80 mL, indicating a relatively high ejaculate volume, which is typical for a healthy boar and appropriate for artificial insemination purposes. The pH value was 7.24, which is slightly alkaline and falls within the normal range (7.0 – 7.5). for boar semen. Meanwhile, this value supports sperm viability and motility. The progressive motility was recorded at 95%, which is excellent this means that 95% of the spermatozoa are actively moving forward in a straight line, indicating high fertility potential. Mass activity was rated as ++++, representing very vigorous and dense swirling movement of spermatozoa when viewed under the microscope. This is a qualitative indicator of good spermatozoa concentration and motility. The percentage of morphologically normal spermatozoa was 95%, which means that the majority of spermatozoa have a normal shape and structure, crucial for successful fertilization.

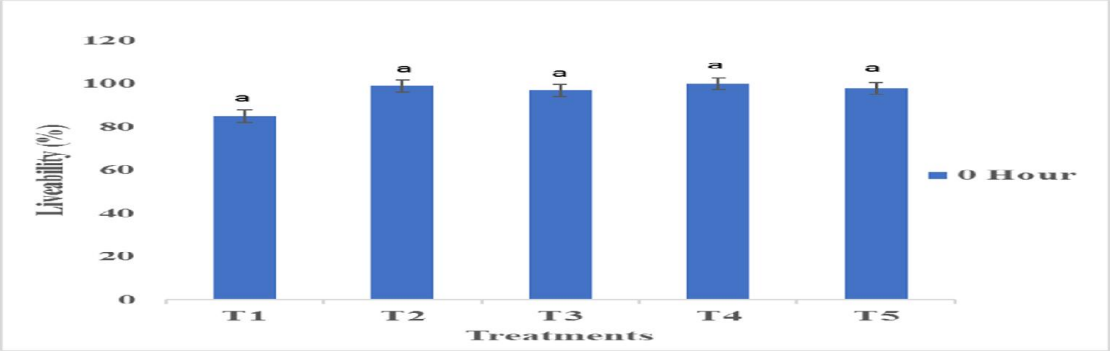
Table 2: Characteristics of fresh boar semen collected before extension

Parameters	Value
Volume (mL)	242.80
Colour	Milky
Ph	7.24
Progressive motility (%)	95.00
Mass activity	++++
Normal Spermatozoa (%)	95.00

Effect of AGE on livability of extended boar semen

Effect of AGE on Livability of extended boar semen refrigerated at 17 ºC is shown in Figures 1 to 4. Results show that at 0 hours, there was no significant difference

(p>0.05) across all treatments. At 24 hours, results obtained show that T1 (80.33) was significantly different (p<0.05) from the rest of the treatments. The result for livability at 48 hours shows that there was no significant difference (p>0.05) across all the treatments, whereas the result for livability at 72 hours shows that T1 (87.10) was significantly different (p<0.05) from T2 (96.13), T3 (96.13), T4 (97.00) and T5 (98.10).



AGE = Aqueous Garlic Extract; a, b = Bars with different superscripts are significantly different (p<0.05).

Figure 1: Effect of AGE on livability of extended porcine semen at 0 hour

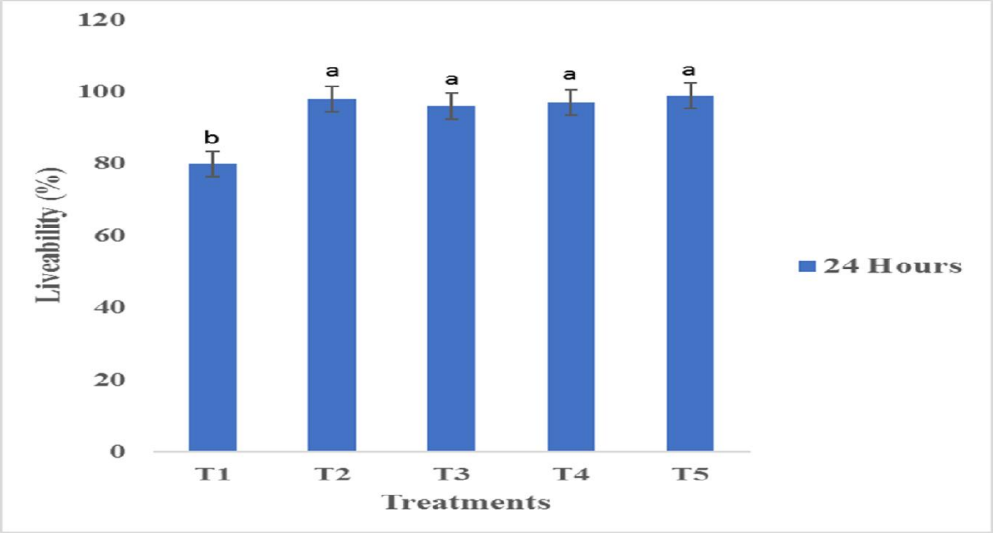


Figure 2: Effect of AGE on livability of extended porcine semen at 24th hour

AGE: Aqueous Garlic Extract; a, b: Bars with different superscripts are significantly different (p<0.05).

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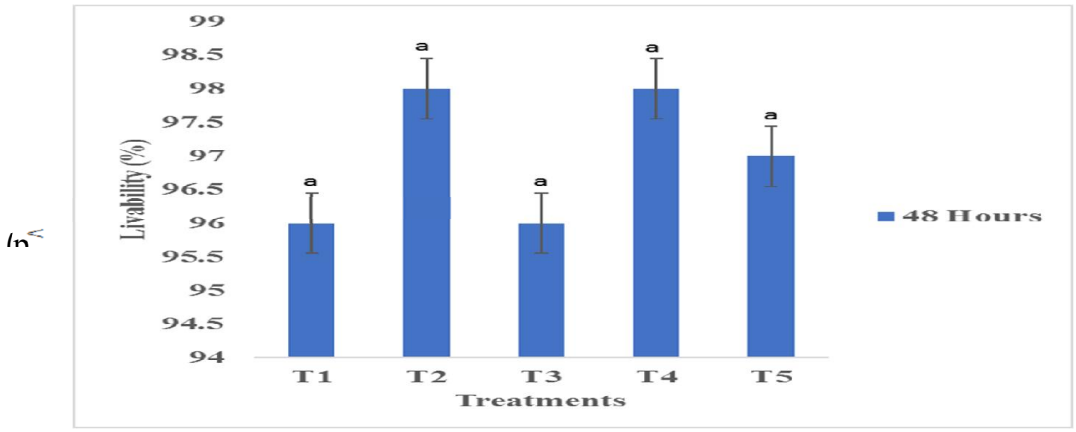
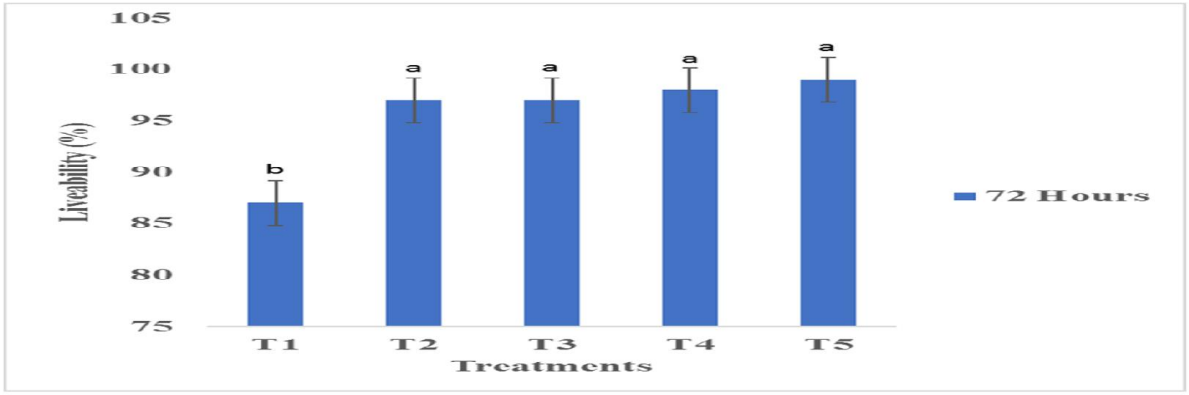


AGE: Aqueous Garlic Extract a, b: Bars with different superscripts are significantly different (p<o.05).

Figure 3: Effect of AGE on livability of extended porcine semen at 48th hour

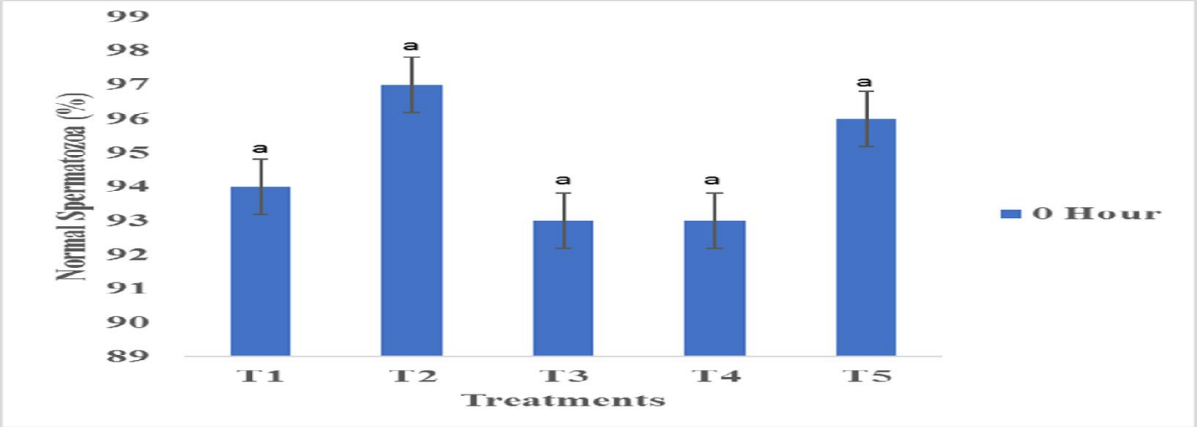


AGE = Aqueous Garlic Extract; a, b; Bars with different superscripts are significantly different (p<0.05).

Figure 4 Effect of AGE on livability of extended porcine semen at 72nd hour

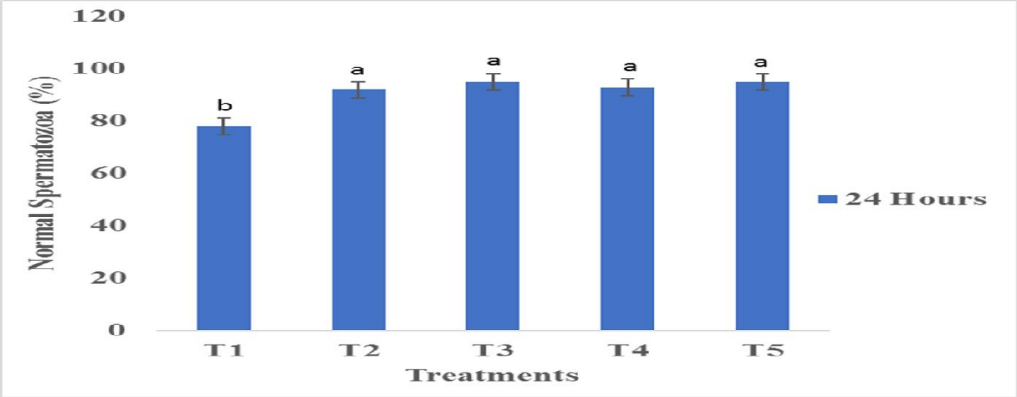
Effect of AGE on normal spermatozoa of extended boar semen

Effect of AGE on normal spermatozoa of extended boar semen refrigerated at 17 ºC is shown on Figures 4.5 to 4.8. The result shows that at 0 hour, there was no significant difference (p>0.05) among all treatments. Result obtained for normal spermatozoa at 24 hours shows that T1 (80.01) was significantly different (p<0.05) from T2 (98.10), T3 (97.00), T4 (97.33) and T5 (98.01). At 48 hours, result obtained shows that there was no significant difference (p>0.05) across treatments. The result obtained for Normal Spermatozoa at 72 hour shows that there was no significant difference (p>0.05) across the treatments.



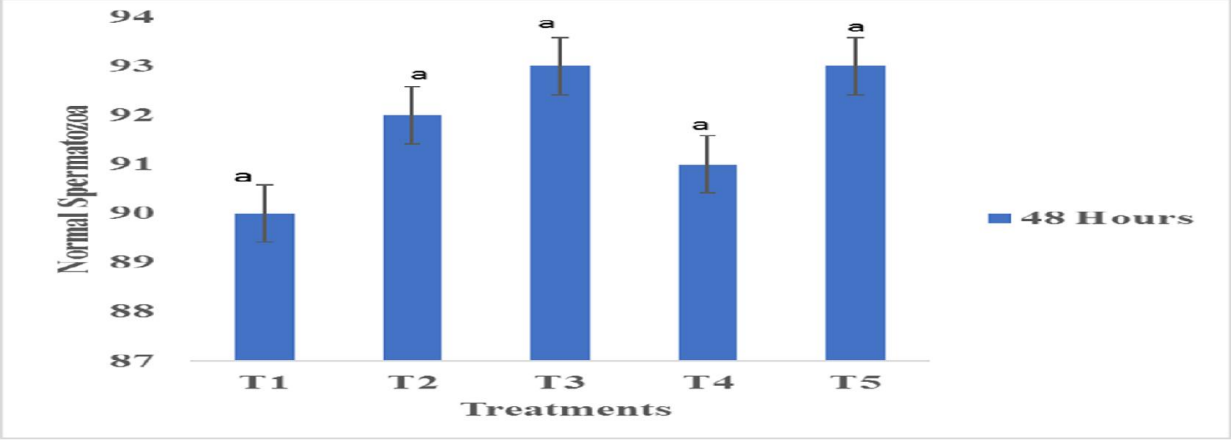
AGE: Aqueous Garlic Extract, a: bars with different superscripts are significantly different (P<0.05)

Figure 5: Effect of AGE on Normal Spermatozoa of Extended Boar Semen at 0 Hour



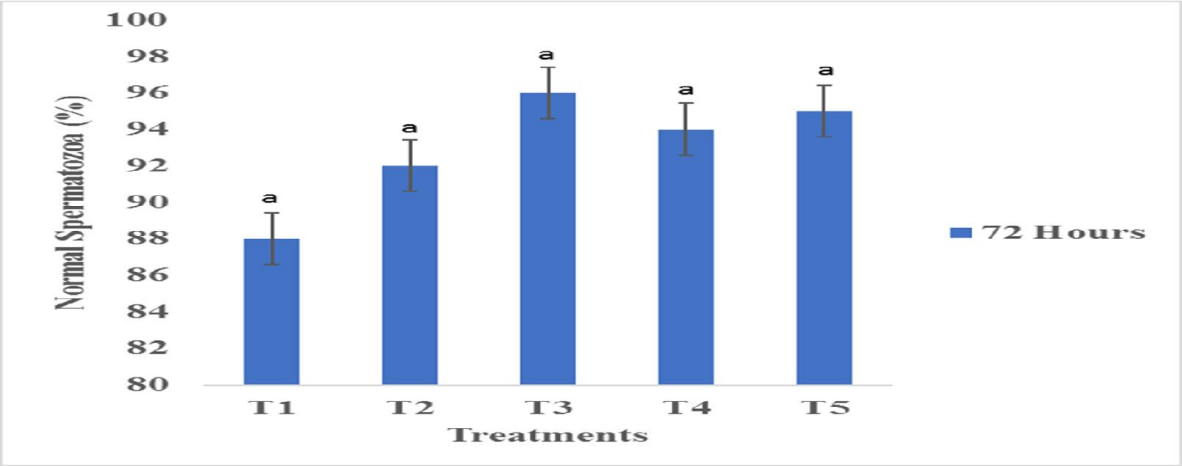
AGE: Aqueous Garlic Extract, a: bars with different superscripts are significantly different (P<0.05)

Figure 6: Effect of AGE on normal spermatozoa of extended boar semen at 24th Hour



AGE: Aqueous Garlic Extract, a: bars with different superscripts are significantly different (P<0.05)

Figure 7: Effect of AGE on normal spermatozoa of extended boar semen at 48th hour



AGE: Aqueous Garlic Extract, a: bars with different superscripts are significantly different (P<0.05)

Figure 8: Effect of AGE on Normal Spermatozoa of Extended Boar Semen at 72nd hour

Effect of AGE on pH of extended boar semen at 0, 24th, 48th and 72nd hour

Table 3 shows the pH values of spermatozoa stored in BTS at varying concentration of AGE and different storage times. At 0 hours, there was no significant difference (p>0.05) across all treatments. Highest value was observed in T1 (7.27) while T4 (7.20) recorded the lowest value. At 24 hours, no significant difference (p>0.05) was observed among the treatments, however, T1 (7.16) recorded the highest pH value while T3 (7.09) recorded the lowest pH value. There was no significant difference (p>0.05) among treatments at 48 hours, however highest pH values were recorded at T2 and T3 (7.16) respectively. The lowest value was recorded in T1 (7.11). Results for pH at 72 hours show significant differences across the treatments. Treatment 1 was significantly different (p<0.05) from, T2, T4, and T5 but not significantly different (p>0.05) from T3. Treatment 2 was significantly different (p<0.05) from T1.

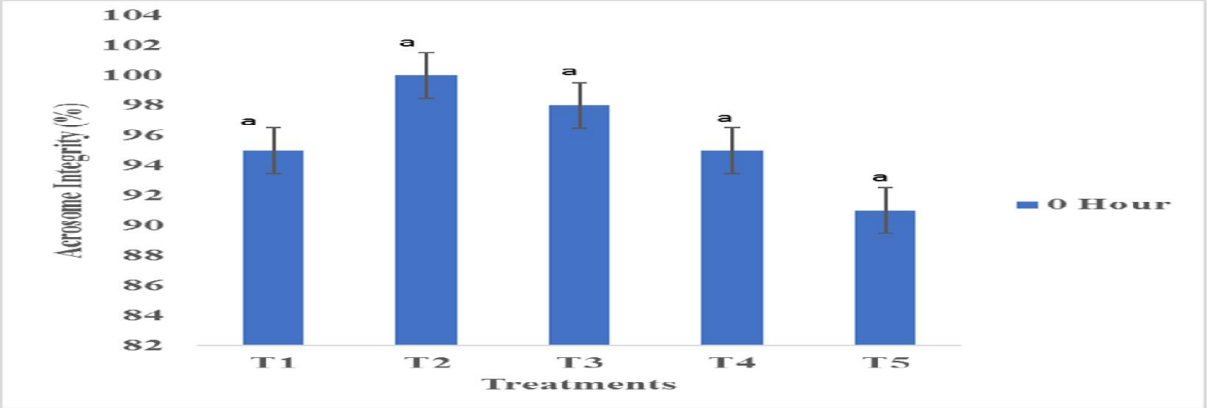
Table 3 Effect of AGE on pH of extended porcine semen

Hours	T1	T2	T3	T4	T5	SEM
0	7.27 ^{a}	7.23^{a}	7.25^{a}	7.20^{a}	7.24^{a}	0.01
24	7.16 ^{a}	7.15^{a}	7.09^{a}	7.12^{a}	7.12^{a}	0.01
48	7.11^{a}	7.16^{a}	7.16^{a}	7.13^{a}	7.13^{a}	0.01
72	7.09^{c}	7.17^{ab}	7.12^{bc}	7.19^{a}	7.23^{a}	0.01

$^{a-c}$Means along the same row with different superscripts are significantly (p<0.05) different.
SEM = Standard error of mean.

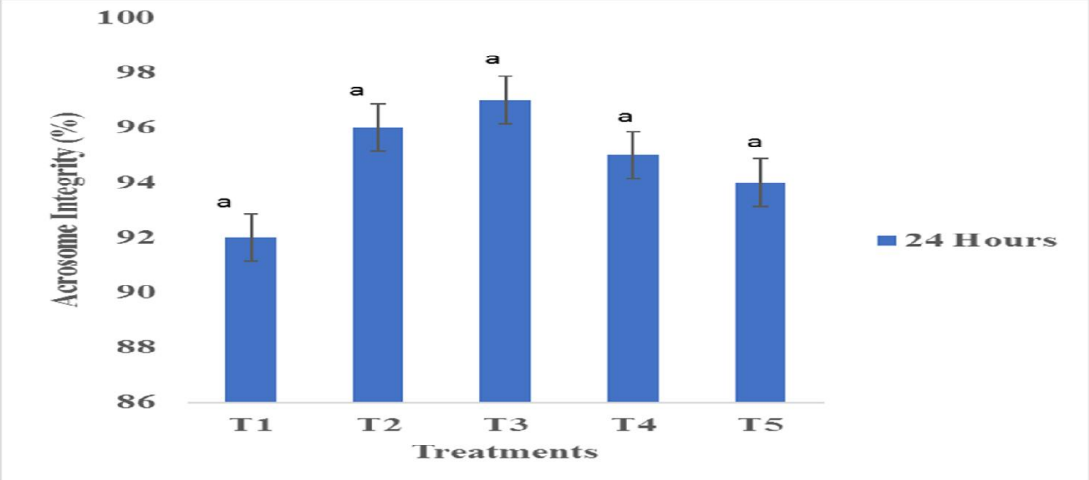
Effect of AGE on Acrosome integrity of extended boar semen

The effect of AGE on the Acrosome Integrity of extended boar semen refrigerated at 17 ºC is shown in Figures 9 to 12. At 0, 24 and 48 hours, there were no significant differences (p>0.05) across treatments. There was a significant difference (p<0.05) across treatments at 72 hours. T1 and T4 were significantly different (p<0.05) from T2, T3 and T5.



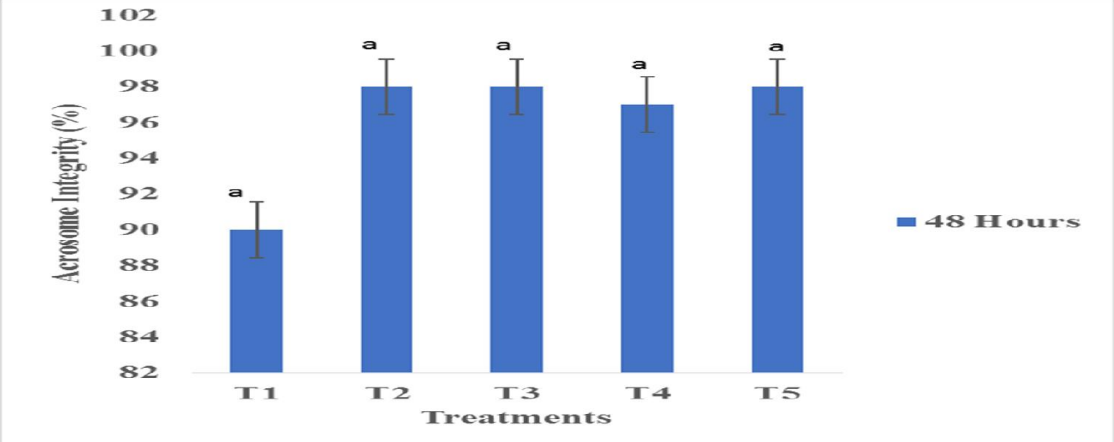
AGE: Aqueous Garlic Extract, a: bars with different superscripts are significantly different (P<0.05)

Figure: 9 Effect of AGE on Acrosome integrity of extended boar semen at 0 hour



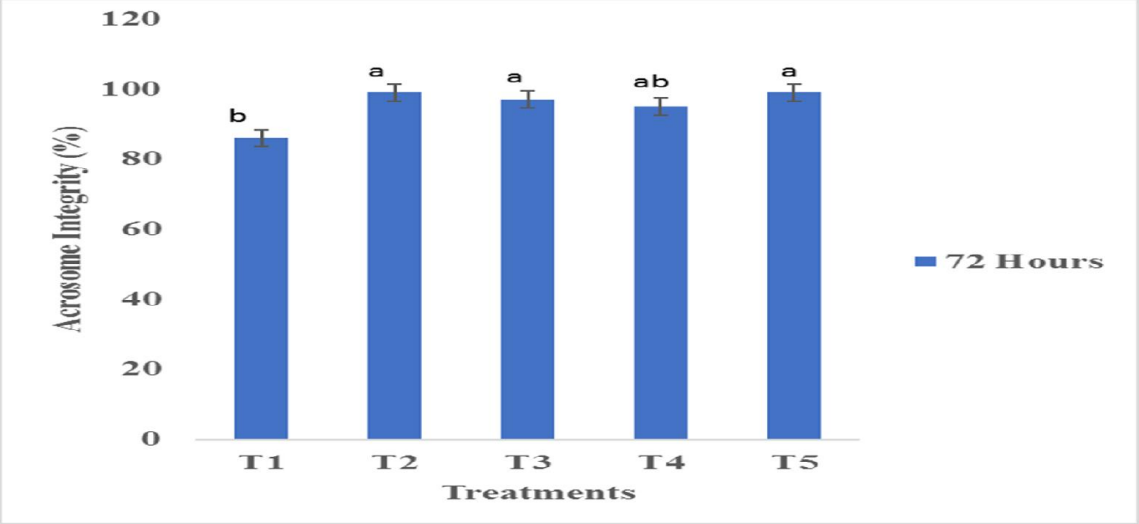
AGE = Aqueous Garlic Extract, a: bars with different superscripts are significantly different (P<0.05).

Figure 10: Effect of AGE on Acrosome integrity of extended boar semen at 24th hour



AGE = Aqueous Garlic Extract, a: bars with different superscripts are significantly different (P<0.05)

Figure 11: Effect of AGE on Acrosome integrity of extended boar semen at 48th hour



AGE: Aqueous Garlic Extract, a: bars with different superscripts are significantly different (P<0.05)

Figure 12 Effect of AGE on Acrosome integrity of extended boar semen at 72nd hour

Effect of AGE on tertiary abnormal spermatozoa of extended boar semen

Table 4 shows values for the tertiary abnormal spermatozoa at different storage time and at different concentration levels of AGE, extended with BTS. From the result obtained, there were no significant differences (p>0.05) for treatments at 0 hour, 48 and 72 hours. However, at 24 hours, T2, T3, T4 and T5 were recorded to be significantly different (p<0.05) from T1.

Table 4: Effect of AGE on tertiary abnormal classification of extended porcine semen

Hour	T1	T2	T3	T4	T5	SEM
0	5.67[a]	3.00[a]	7.33[a]	7.00[a]	3.67[a]	1.09
24	21.67[a]	8.33[b]	5.33[b]	7.00[b]	5.00[b]	2.25
48	10.00[a]	8.33[a]	7.33[a]	8.67[a]	6.67[a]	0.86
72	11.67[a]	8.33[a]	4.33[a]	6.00[a]	5.00[a]	1.23

[a, b]Means along the same row with different superscripts are significantly (p<0.05) different, SEM = Standard Error of Mean

Pearson's Product Correlation Coefficients (r) between Livability, Normal spermatozoa, pH, TMAS and AI at 125 µg/L AGE at 72nd hour

Table 5 shows the Pearson's product correlation coefficients (r) between spermatozoa quality (Livability, Normal Spermatozoa and pH) and spermatozoa fertilizing potential parameters (TMAS and AI) for 125µg/L AGE at 72 hours post preservation. There is a perfect negative correlation (-1.00, and -1.00) between TMAS and both Livability and Normal Spermatozoa, respectively, whereas there was a weak positive correlation (0.371) between TMAS and pH. There is a very strong negative correlation (-0.866, -0.866) between AI and Livability, and Normal Spermatozoa of the extended boar semen at 72 hours post preservation. A strong positive correlation (0.786) occurred between Acrosome Integrity and pH

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Table 5: Pearson's Product Correlation Coefficients (r) between Livability, Normal Spermatozoa, pH, TMAS and AI at 125 µg/L AGE at 72nd hour

SFPP	Livability	NSp	pH
TMAS	r = -1.00^{s**}	-1.00 s**	0.371 ^{ns}
AI	r = -0.866 ^{ns}	-0.866 ^{ns}	0.786 ^{ns}

SFPP = Spermatozoa Fertilizing Potential parameters; TMAS = Tertiary Morphologically Abnormal Spermatozoa; AI = Acrosome Integrity; NSp = Normal spermatozoa, ns = non-significant, ** = P<0.01.

DISCUSSION

High-quality semen is essential for artificial insemination which is essential to obtaining satisfactory fertility rates (Alsenosy and Abd El-Aziz, 2019). This is shown in the quality of fresh semen collected in this study before it was extended and evaluated for quality. The colour of the gel free ejaculates ranged from milky to thick milky colour. The observations of the current study were similar to the findings of Frunza *et al*. (2008). The volume of the fresh semen obtained in this study was 248.80 mL. The observed gel free ejaculate volume was similar to those observed by Kantharaj 2001; Shylesh *et al*., 2019). According to Johnson *et al*., (2000), the pH of boar semen ejaculate was in the range of 7.2-7.5, which is similar to the findings in the current study (7.24). The percentage spermatozoa progressive motility obtained immediately after collection in this study was 95% which agreed with the findings of Kantharaj (2001).

Effects of AGE on quality of extended porcine semen

Livability and Normal Spermatozoa were observed to be high even at 72 hours across treatments. This correlate with the reports of Terry *et al*. (2003) and Sone *et al*. (1992) which stated that extended boar semen can be stored for up to 6 days with no appreciable loss in fertility potential, depending on the extender used. This suggests that at 72 hours of storage, the spermatozoa are still viable enough for a successful artificial insemination. Extended Semen pH mean values across treatments through the storage period were within acceptable range (Johnson *et. al*., 2000).

Effects of AGE on spermatozoa fertilizing potential of extended porcine semen

Acrosomal integrity is one of the determining factors for fertility because, for successful fertilization, sperm must have an intact acrosome and must react on time when they reach the site of fertilization (Buffone *et al*., 2008; Rajabi-Toustani *et al*., 2019). Acrosome reaction is related to sperm fertility and is essential in the process of fertilization. However, its early occurrence during long-term semen storage decreases the viability and fertility of the stored sperm (Lee and Park, 2015). Extended semen samples treated with aqueous garlic extracts indicated higher acrosome integrity at 24 to 72 hours. This observation suggests the cytoprotective potential of AGE. Despite an expected variability among ejaculates, the extender factor did not seem to affect sperm viability in the first 72 hours in the present trial. However, a study also reported an unexpected increase in spermatozoa viability after 72 hours, but did not give a proper explanation for the phenomenon (Boe-Hansen *et al.,* 2005). There were no significant differences found for normal sperm, except in T2 at 24 hours where the value was significantly lower than the rest at 78 %. The morphological percentages of the spermatozoa from the boar ejaculates were within the acceptable range (Almond *et al*., 1998) and therefore suggest acceptability of use in AI programs.

Pearson's Product Correlation Coefficients (r) between Livability, Normal Spermatozoa, pH, TMAS and AI of Extended Porcine Semen

The results of the Pearson's Product Correlation Coefficient between spermatozoa quality and spermatozoa fertilizing potential parameters for 125g/l of AGE at 72 hours showed a perfect negative correlation between tertiary morphologically abnormal spermatozoa (TMAS) and both livability and normal spermatozoa, which signifies that as TMAS increases, livability and normal spermatozoa will also decrease at a rapid rate. The weak positive correlation between TMAS and pH shows that as TMAS increases, pH also increases, leading to low quality spermatozoa. These observations are all in agreement with Wong *et. al*. (2014) who reported, that defects in spermatozoa quality cause fertilization failure.

Tertiary morphologically abnormal spermatozoa

The spermatozoon is a highly structured cell that is streamlined to deliver DNA to the oocyte. To achieve this goal, the DNA is highly condensed and packaged on the nuclear matrix in a very specie specific unique manner. Alterations in the packaging or DNA content of sperm contain the locomotion apparatus and without its proper function, cannot deliver the DNA in the sperm head to the oocyte. Abnormalities to the spermatozoa can cause a decreased ability to reach the site of fertilization, fertilize an oocyte or sustain embryonic development. Some of the abnormalities include; abaxial tail, double mid piece, coiled tail, abnormal heads or nuclei, abnormal acrosomes, abnormal necks, tailless heads, proximal droplets, distal droplets, abnormal mid pieces, abnormal tails and round spermatids (Hafez and Hafez, 2000).

CONCLUSION

- Aqueous garlic extract (AGE) has positive effects on spermatozoa livability, normal spermatozoa, acrosome integrity and pH, regardless of the hour and treatment.
- Aqueous garlic extract has cytoprotective potential, and can be used to extend porcine semen for 72 hours without appreciable decrease in the fertilizing ability of extended spermatozoa.

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