INTRODUCTION

Reactive oxygen species (ROS) production is a normal physiological process; however, an imbalance between ROS generation, scavenging activity, and uncontrolled production of ROS that exceeds the antioxidant capacity of the seminal plasma will lead to oxidative stress and can damage the sperm membrane integrity, DNA, acrosome, metabolic activity, and fertilizing potential of the sperm (Bansal and Bilaspuri 2010, Tariq et al. 2015). In the stages of freezing and thawing, the endogenous defense system of the buffalo semen is not enough to counter oxidative stress due to low concentration of naturally occurring antioxidants in buffalo semen (Ansari et al. 2012). Sperm viability is also decreased by 50% whereas fertilizing capacity is affected after cryopreservation (Lessard et al. 2000). However, the spermatozoa are provided with protection from oxidative damages with the

Influence and Total Antioxidant Capacity of Non-enzymatic Antioxidants on the Quality and Integrity of Extended and Cryopreserved Semen of Murrah Buffalo (Bubalus bubalis)

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The study compared the effect of non-enzymatic antioxidants on the % motility, livability, and plasma membrane integrity of the cryopreserved sperm from four purebred Murrah bulls aged 10–17 years old. The level of antioxidants was also determined. Experiment 1 was analyzed using 4 x 4 factorial design in a randomized complete block design (RCBD) with bulls blocked in four; in Experiment 2, it analyzed the levels of MegaAntiox using PROC ANOVA of SAS software. Results revealed no significant ($P > 0.05$) difference in post-thaw sperm motility (%) between antioxidants but a significant decrease ($P < 0.05$) in the post-thaw sperm motility was observed at Week 3. The addition of non-enzymatic antioxidants did not affect ($P > 0.05$) the live sperm though difference was observed on the livability of sperm cells in different collection weeks. In extended semen, the addition of non-enzymatic antioxidants did not show any significant effect ($P > 0.05$) on post-thaw sperm motility (%) and livability. After six months of cryopreservation, the addition of vitamin E (one of the non-enzymatic antioxidants) had a negative effect on the post-thaw sperm motility. Livability and PMI were not significantly affected ($P > 0.05$) by the different treatments. In conclusion, the addition of 0.1 mg/ml non-enzymatic antioxidants did not improve sperm motility (%) and the livability of the sperm.

Keywords: cryopreservation, Murrah buffalo, non-enzymatic antioxidants, semen, semen extender
presence of various antioxidants and antioxidant enzymes in the seminal plasma (Tariq et al. 2015) and buffalo semen is equipped with anti-oxidative stress system consisting of enzymatic and non-enzymatic antioxidants (Lone et al. 2016). With this, supplementation of antioxidants in extenders was observed to provide a cryoprotective effect on bull, ram, goat, boar, canine, and human sperm quality and minimize the detrimental effect of ROS as well as improve post-thaw spermatozoa (Amidi 2016). Balance measurement of both ROS and total antioxidant capacity (TAC) is essential in the assessment of oxidative stress in sperm and semen (Kashou 2013).

On the other hand, Marai and Habeeb (2010) mentioned that climatic changes affect the reproductive traits of buffaloes and the variation is more marked in buffaloes. Moreover, the quantity and quality of semen vary with season of the year and the degrees of response (to seasonal effects) differ according to species, breed, and locality. The conditions especially temperature and humidity also play vital role in efficient and quality semen production (Sharma et al. 2018).

Understanding the effect of non-enzymatic antioxidants and antioxidant activity on sperm characteristics will help improve the fertilizing ability of the cryopreserved spermatozoa. Therefore, the present study investigated the effects of non-enzymatic antioxidants, week of collection on different seminal qualities and determined the antioxidant activity during the process of cryopreservation (extended and post-thawed semen) and relates it with semen sperm motility, livability, and plasma membrane integrity.

MATERIALS AND METHODS

Experiment 1: Influence of NEA Addition

Experimental animals. Four (4) purebred, matured Murrah buffalo bulls having high genetic traits with ages ranging from 10–17 (mean = 13.5) years old, apparently healthy and regularly used as semen donors were used in the experiments. The animals were selected based on the quality of semen produced and the reproductive performance of the buffalo bulls from the institutional records of the Philippine Carabao Center at the University of the Philippines (PCC at UPLB), Los Baños, Laguna.

Collection of semen was from October to November 2017. The semen used in the study came from the first or second ejaculate, which was collected twice a week from 5:00 AM to 6:00 AM.

Semen Quality Analysis

Semen samples were collected with the aid of an artificial vagina with hot water heated to 45 °C. The ejaculates in the collecting tubes (15 ml capacity) were placed in a pre-heated water bath set at 28–32 °C lowest and highest temperature setpoints for macroscopic and microscopic evaluation using phase-contrast microscope (Nikon, Japan; magnification 100x).

In the analysis of semen quality, microscopic and macroscopic parameters were measured. Macroscopic parameters include semen volume, consistency, and color whereas microscopic parameters include the mass activity, initial motility, and sperm concentration.

Semen volume was measured using calibrated collection tube. The appearance and color were evaluated through visual inspection while sperm concentration was analyzed using spectrophotometer IMV®.

Motility and mass activity scores were estimated by placing a small drop of semen in a glass slide covered with a coverslip on a microscope stage set at 36–39 °C lowest and highest temperature setpoints using phase-contrast microscope. Motility was graded using the percentage of motile sperm: 90–100% (excellent motility), 80–85% (very good motility), 70–75% (good motility), 60–65% (fair motility), 5–55% (unfit to use), and 0 (zero motility); the mass activity movement were scored as follows: – (no movement), + (no forward movement, ++ (weak forward movement), and +++ (progressive forward movement).

Qualified semen ejaculates that passed the initial screening [motility > 60%, volume > 1 ml, concentration > 500 M/ml, semen color (creamy, light creamy and milky white)] were used in the experiment.

Preparation of Buffer Solution

The 1 L Buffer solution containing tris (0.249 mol/L), citric acid (0.087 mol/L), fructose (0.069 mol/L), and raffinose (0.053 mol/L) was placed in a 2 L beaker and filled with distilled water up to 1 L volume. This was prepared one day before collection. The components were mixed thoroughly using magnetic hot plate stirrer for about 5 min at speed of 1,000 rpm.

Preparation of Semen Extender

The extender was prepared the day before semen collection. The composition and preparation of the extender were done by taking 73 ml of the prepared buffer solution added to 20 ml albumen and chalaza free fresh egg yolk. Then 7 ml of glycerol and antibiotics (0.1 g streptomycin and 0.03 g penicillin) were added to the mixture and stirred using magnetic stirrer at 300 rpm under room temperature. Sediments were also removed manually from the extender using forceps.
Dilution Rate
In order to make 100 million/ml sperm concentration on the volume of extender, it was computed using the formula used by Mamuad et al. (2005):

\[
\text{Vol. of semen (ml)} \times \frac{\% \text{ Motility} \times \text{Sperm Concentration (M/ml)}}{100,000,000} - \text{Vol. of semen extender (ml)} = \text{Vol. of semen}
\]

The extender was diluted to the semen with the same temperature at 28–30 °C.

Experimental NEAs
Three kinds of commercially available antioxidants [vitamin C (500 mg), vitamin E (3.44 mg/ml), and mega antioxidants (8.31 mg/ml)] containing minerals, vitamins, plant extracts, and enzymes were procured from a reputable drug supplier. These were dissolved in water (for water-based) or olive oil (for oil-based) and were used in the experiment. NEA was added in the semen extender wherein the semen was diluted according to the treatment, as shown in Table 1. The concentration of the NEA in the semen extender was computed to contain 0.1 mg/ml. Each semen collected from the bulls was divided into four and were randomly assigned into different treatments, then the semen extender with different NEAs were added to the semen.

Steps in Semen Processing Using Slow Freezing Technique
Steps in semen processing were lifted from Artificial Insemination Manual for Water Buffaloes (Mamuad et al. 2005). Semen samples that have more than 60% initial motility were diluted with equal volume of extender (1:1, volume of extender: semen) poured slowly and gently through the sides of the collection tube at temperature of 28–32 °C. Extended semen was placed in a water bath at 28–32 °C. After initial dilution, extended semen was transferred in a pre-cooled water bath at 15–20 °C within 15–20 min. The pre-cooled extended semen was transferred to cold handling cabinet (2–7 °C) for further cooling for about 1.5–2 h at the rate of 0.2–0.3 °C/min. The remaining volume of extender was added when the initially extended semen reaches 2–7 °C in cold handling cabinet. This was computed using the formula:

\[
\text{Final dilution} = \frac{\text{sperm concentration (M/ml) } \times \text{ semen volume (ml) } \times \% \text{ motility}}{80 \text{ M sperm/ml.}}
\]

Extended semen was packed in 0.5 ml capacity properly labeled semen straw. Polyvinyl chloride powder was used in sealing the semen straws. Printed semen straw, sealing powder, and other accessories required for filling and sealing was sterilized by ultraviolet rays. The filling was done using suction pump and air space was created using a bubbler dish and comb which was specifically designed for this purpose. The open-end straw was sealed with polyvinyl chloride /alcohol powder. Sealed straws were kept in cold handling cabinet at 2–7 °C. Goblets with straws were placed in liquid nitrogen tank (LN2) at -196 °C for storage.

Post-thaw Sperm Motility Evaluation (%)
A total of twelve experimental frozen semen straws were used. Three for each treatment were drawn from the LN2 tank after one day of semen cryopreservation and were immediately evaluated for its post-thaw characteristics. Frozen semen was thawed at 39–42 °C for 15–20 s. Examination time from thawing to sampling took 10 s with temperature adjustment at 30 s and samples were examined within 3 min using phase-contrast microscope (Nikon®, magnification of 100x).

Evaluation of the semen was done by an experienced laboratory technician of PCC at UPLB. The average from the three values per treatment of the different parameters

Table 1. The NEA experimental treatments and concentration in the semen extender.

<table>
<thead>
<tr>
<th>Components</th>
<th>T1 (without NEA)</th>
<th>T2 (vit. C)</th>
<th>T3 (vit. E)</th>
<th>T4 (mega antiox.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer, ml</td>
<td>73.0</td>
<td>73.0</td>
<td>73.0</td>
<td>73.0</td>
</tr>
<tr>
<td>Egg yolk, ml</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Glycerol, ml</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Streptomycin, g</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Penicillin, g</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Total volume, ml</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Antioxidants added, ml</td>
<td>0.0</td>
<td>2.0</td>
<td>2.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Concentration of NEA, mg/ml</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
was computed and subjected to statistical analysis.

**Livability Staining of Post-thawed Semen**
The semen used in the livability evaluation also came from the semen straws from each treatment used in the post-thaw motility testing.

The stain used in the livability analysis contained eosin (0.2 g) and nigrosin (1.0 g) dissolved in 20 ml of 0.03% sodium citrate solution. The solution was mixed thoroughly using a magnetic stirrer at 300 rpm. Two to three drops of semen were placed on a clean glass slide then 1–2 drops of stain were added, dried, and evaluated within 24 h. A phase contrast microscope (Leica®, Germany/400x magnification) was used to determine the live-dead semen. From the total of 200 sperm counted, live sperm were those that did not absorb the stain otherwise, dead. Percent live sperm was computed using the formula:

\[
\text{Percent live sperm} = \frac{200 - \text{dead sperm count} \times 100}{200}
\]

The percent dead sperm was computed as the difference of % live sperm to 100%. The averages from the three values in the assessment of the different parameters per treatment were used in the statistical and the mean ± SEM values were taken for the different treatments in the final report.

**Experiment 2: TAC of the Different NEA**
The experimental animals used and the protocol for semen collection, semen evaluation, macroscopic and microscopic examinations and processing of semen, post-thaw motility evaluation, and livability assessment were the same as in experiment 1.

**Sample Preparation of Extended and Post-thawed Semen for TAC Analysis**

**Extended semen.** The collected semen from bulls were divided into two and were assigned the treatments where: T<sub>1</sub> – control and T<sub>2</sub> – with mega antioxidant. The four bulls served as the replication.

Approximately 2 h of equilibration time, evaluation on motility (%), and livability were done. The remaining samples were immediately stored in –20 °C freezer for a day prior to freeze-drying. Samples were then stored in –80 °C freezer for 1 h and freeze-dried for at least 8 h using 0.420 mbar/vacuum and were sent to the Institute of Food Science and Technology (IFST at UPLB) laboratory for TAC analysis using dinotrophenyl picryl hydrazyl radical (DPPH) method. The DPPH method of analysis was used in determining the total antioxidant activity/scavenging activity (%) of each sample.

Antioxidant activity was determined by taking 1.0 ml aliquot from each sample and was added with 4.0 ml distilled water. Prepared samples were added with 1 mL of freshly prepared 1 mM DPPH methanol solution. The sample solutions were left to stand for 30 min. The absorbance readings of the solution were taken at 527 nm. The lower the absorbance at 5 nm represented the higher DPPH scavenging activity (Shimada et al. 1992).

**Post-thaw semen.** The mega-antioxidant was only used because of its highest livability with 98.19% live sperm, as reported in Experiment 1.

The semen coming from the four bulls were divided into four and assigned the treatments. A total of 20 ml of thawed semen straws were used in the analysis of TAC after 6 mo of cryopreservation. Thawing procedures were the same in Experiment 1 and the steps in freeze-drying were the same with extended semen. Freeze-dried samples were sent to the IFST at UPLB for TAC analysis.

**PMI**
Plasma membrane integrity of the sperm was examined by hypo-osmotic swelling (HOS) assay (Andrabi et al. 2008). The HOS solution used contained 0.73 g tri-sodium dehydrate and 1.35 g fructose dissolved in 100 ml distilled water (osmotic pressure ~190 mOsmol/kg). The assay was conducted by mixing 50 µl of frozen-thawed semen sample to 500 µl of HOS solution in a microtube then incubated at 37 °C for 40 min. After incubation, a drop of semen sample was taken out and evaluated under phase contrast microscope (Leica®, magnification 400x). From a pre-counted sperm, swollen sperms with coiled tail indicating an intact plasma membrane (Ahmad et al. 2003 as cited by Andrabi et al. 2008). Percent PMI was computed using the formula:

\[
\% \text{ PMI} = \frac{\text{no. of sperms reacted to HOS} \times 100}{\text{total no. of sperms counted}}
\]

**Data Analysis**

**Experiment 1.** All data were analyzed using 4 x 4 factorial in RCBD (SAS version 9.0) where factor A was the different NEA and factor B was the week of semen collection. Means with statistical significance were separated by LSD (P < 0.05).

**Experiment 2.** All data except TAC and PMI were analyzed in RCBD using SAS software version 9.0 and expressed as mean ± SEM. LSD (P > 0.05), which was
used to compare statistically significant parameters.

RESULTS AND DISCUSSION

Experiment 1: Influence of NEA Addition

Post-thaw sperm motility: The post-thaw sperm motility (%) of Murrah buffalo as affected by NEA addition and week of semen collection is presented in Table 2. For the different treatment combinations, the lowest post-thaw sperm motility was noted in T3 (vit. E) Collection Week 3 (25.00 ± 5.00) while highest in T1 (control) Collection Week 1 (31.75 ± 1.18). Addition of NEA did not increase (P > 0.05) the sperm motility compared with T1 (without NEA) and there is a tendency of lowered sperm motility in the extended sperm with vitamin E and mega antioxidant (T4).

Significantly lowest (P < 0.05) post-thaw sperm motility (%) was obtained in week 3 (27.50 ± 1.07) while no significant differences were found in post-thaw sperm motility in Collection Week 1 (30.65 ± 0.56), 2 (29.00 ± 0.57), and 4 (29.07 ± 0.83). While highest post-thaw sperm motility (30.65 ± 0.56) was obtained in Collection Week 1, no trend was observed in the different week of collection.

Livability, Live Sperm (%)

The live sperm (%) of Murrah buffalo as affected by NEA addition and week of semen collection is presented in Table 3. The highest % live sperm (99.46 ± 0.05) was found in vitamin E addition (T3) in Collection Week 3 while lowest in T1 (without antioxidant) in collection week 4 (95.08 ± 0.11). Live sperms (%) were not significantly affected (P > 0.05) by the addition of NEA although there was a higher number of live sperm observed in T4.

The live sperm (%) obtained in Collection Week 2 (99.06 ± 0.24) and 3 (98.87 ± 0.26) were significantly higher than Collection Week 1 (97.03 ± 0.60) and 4 (95.88 ± 0.29). Significantly lowest (P < 0.05) live sperm (%) was noted in Collection Week 4 and was significantly different from the other treatments.

The addition of NEA did not increase (P > 0.05) the sperm motility at low concentration (0.1 mg/ml) of vitamin C, E, and mega-antioxidant and there is a tendency of lowered sperm motility in the extended sperm with vitamin E and mega antioxidant (T4).

This is in contrast with the results reported by (Andrabi et al. 2008), which was successful in improving the post-thaw motility of Nili Ravi buffaloes sperm using vitamin C (ascorbate) that contained 0.88 mg/ml. Higher motility in extender supplemented with 4.5 mg/ml ascorbic acid was also obtained by Hu et al. (2010). In other species like ram, the incorporation of ascorbic acid in extender having 4.5 mg/ml and preserved at 4 °C significantly decreased

Table 2. Post-thaw sperm motility (%) in NEA-supplemented extender of Murrah buffalo sperm cells collected in different weeks.

<table>
<thead>
<tr>
<th>Collection Week</th>
<th>Treatments</th>
<th>Mean ± SEM (T2–T4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (without NEA)</td>
<td>31.75 ± 1.18</td>
<td>30.65 ± 0.56&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 (vit. C)</td>
<td>30.00 ± 5.48</td>
<td>29.00 ± 5.48</td>
</tr>
<tr>
<td>3 (vit. E)</td>
<td>30.00 ± 3.33</td>
<td>30.00 ± 5.48</td>
</tr>
<tr>
<td>4 (mega)</td>
<td>30.42 ± 5.52</td>
<td>30.00 ± 5.48</td>
</tr>
<tr>
<td>Mean ± SEM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.53 ± 0.41</td>
<td>29.48 ± 0.46</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means in the column followed by a different letter are significantly different at (P < 0.05).
<sup>b</sup>ns – not significant

Table 3. Post-thaw live sperm (%) of Murrah buffalo with NEA addition and week of semen collection.

<table>
<thead>
<tr>
<th>Collection Week</th>
<th>Treatments</th>
<th>Mean ± SEM&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (without NEA)</td>
<td>96.82 ± 1.10</td>
<td>97.03 ± 0.60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 (vit. C)</td>
<td>98.35 ± 0.13</td>
<td>99.06 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 (vit. E)</td>
<td>98.79 ± 0.65</td>
<td>98.87 ± 0.26&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 (mega)</td>
<td>95.08 ± 0.11</td>
<td>95.88 ± 0.29&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean ± SEM&lt;sup&gt;d&lt;/sup&gt;</td>
<td>97.26 ± 0.84</td>
<td>98.19 ± 0.67</td>
</tr>
</tbody>
</table>

<sup>c</sup>Means in the column followed by a different letter are significantly different at (P < 0.05).
<sup>d</sup>ns – not significant
the sperm motility (Rather et al. 2016).

In this study, lower concentrations of NEA were tested because when antioxidants are present at low concentrations, it inhibits the oxidation of a molecule and capable in nullifying the detrimental effects of oxidation brought about by free radicals in the living organism (Jeeva et al. 2015). Inclusion of vitamin C in semen extender provides beneficial effects up to a certain inclusion level and higher concentration may cause negative impact on the motility percentage, while higher concentrations of vitamin E may stimulate oxidation rather act as an antioxidant (Andrabi et al. 2008).

There were limited works of literature on the use of non-enzymatic antioxidants on improving the livability of the sperm. In this study, the addition of NEA did not affect the livability of the sperm but showed a higher number of live sperm, which indicate for the potential of NEA to improve the livability of the sperm. The study of Patel et al. (2016) obtained a significantly higher live sperm (%) in Meshani buffalo using ascorbic acid having 0.2 mg/ml. He also mentioned that there was an improvement in the viability of the sperm with lesser abnormality. In bovine species, sperm viability was significantly enhanced using ascorbic acid having 3 mg/ml. However, a higher concentration of ascorbic acid which contains 4 mg/ml reduced the sperm viability.

Ansari et al. (2012) reported that during the stages of freezing and thawing, the endogenous defense system of buffalo semen is not enough to counter oxidative stress. This weakness can be attributed to low concentrations of naturally occurring antioxidants in buffalo semen, which further decreases during the extension and freezing process.

Although the use of antioxidants during semen cryopreservation is known because of antioxidation reduction, the effectiveness of some antioxidants is proven better while some do not show promising results (Tariq et al. 2015). Different effect of each antioxidant supplementation improving different semen characteristics are attributed to animal species, extender medium, and type of molecule and concentration used for each species (Amidi 2016).

A weekly variation on the semen qualities (semen volume, sperm concentration, and sperm motility) per bull was observed from the records of PCC at UPLB. Significant differences ($P < 0.05$) on the collection of post-thawed sperm motility and livability were obtained. However, factors that caused these variations on the cryopreserved sperm were not identified in this study.

Bhakat et al. (2015) mentioned that sexual activities of different species are highly influenced by the environment and surroundings. The variations in these parameters are manifested with reference to time and place.

Biniová et al. (2017) revealed that collection month had significant effect on bull sperm characteristics (semen volume, motility, and sperm concentration). The maximum motility and sperm concentration were found in March and April while the lowest was obtained in August and September. The highest volumes of ejaculates were collected in July and September, which is significantly different from those collected in November, December, February, and March. Results of their study were significantly influenced by average daily ambient temperature and average daily relative humidity.

Watson (1995) reported that even with the recent preservation techniques, post-thaw survival is restricted to about 50% of the sperm population. The process of cryopreservation covers the entire process from sperm preparation and dilution through to the post-thawing maintenance of functional capability over an extended period. In every stage, spermatzoa can lose their ability to function normally.

**Experiment 2: TAC of the Different NEA**

Results revealed that among the antioxidants, vitamin C had the highest (71.69 ± 1.05) antioxidant capacity (%) followed by mega-antioxidant (48.63 ± 1.19), while the lowest was in vitamin E (23.52 ± 1.05).

**Motility (%), Livability, and TAC (%) of Extended semen**

Percent motility, livability, and TAC (%) of extended semen are presented in Table 2. The addition of NEA did not show any significant effect ($P > 0.05$) on post-thaw sperm motility (%) and livability. Motility did not increase with addition of mega antioxidant but there was trending for motility to increase with NEA addition by 1.25%.

The number of live sperm (%) was 90.08 ± 1.44 in T2 and 90.00 ± 2.02 in T1 while the lowest number of dead sperm (%) of 9.92 ± 2.88 and 10.00 ± 4.04 were observed in T2 and T1, respectively.

**Table 4. Percent motility, livability, and TAC (%) of extended semen (mean ± SEM).**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatments</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>% motility **</td>
<td>1 (w/o NEA)</td>
<td>2 (mega antiox.)</td>
</tr>
<tr>
<td>Livability</td>
<td>59.17 ± 0.00</td>
<td>60.42 ± 1.46</td>
</tr>
<tr>
<td>% live**</td>
<td>90.00 ± 2.02</td>
<td>90.08 ± 1.44</td>
</tr>
<tr>
<td>% TAC, n = 4</td>
<td>21.58 ± 4.64</td>
<td>41.93 ± 9.24</td>
</tr>
</tbody>
</table>

Means in the row followed by a similar letter are not significantly different at ($P < 0.05$).

**ns – not significant**
There was an increase of TAC % in the extended semen from 21.58% in the control compared to 41.93% in semen added with mega antioxidant by as much as 48.53% respectively.

### Motility (%), Livability, PMI (%), and TAC (%) of Post-thawed Semen After 6 mo of Cryopreservation

Percent motility, livability, PMI (%), and TAC (%) of post-thawed semen is presented in Table 5. After six months of cryopreservation, the addition of NEA affected the % motility of the sperm. The sperm with added vit. E obtained the lowest sperm motility (26.25 ± 1.46) and was significantly different (P < 0.05) from the other treatments. The sperm motility of T₁ (31.25 ± 1.66), T₂ (28.75 ± 0.55), and T₄ (31.25 ± 1.66) did not differ from each other. However, the addition of NEA did not affect the livability of the sperm. The values for live sperm (%) ranged from 80.96 ± 1.32 (T₃) to 85.71 ± 2.28 (T₄). Similar values were seen in T₁ (39.38±8.43) and T₂ (39.42 ± 8.09). There is high variation in the values of PMI as affected in the coefficient variation (21.96).

Eghbali et al. (2010) disclosed that TAC was associated with semen characteristics and have an effect on the motility and viability of the spermatozoa after ejaculation. In this study, % TAC and % motility were comparable among treatments in extended semen. The % TAC in post-thawed semen was also comparable among treatments; however, % motility was significantly lower in treatment (T₁) supplemented with vitamin E. The effect of vitamin E may vary depending on the concentration and acts stimulator at higher concentration.

Lone et al. (2016) obtained a significant decrease (P < 0.01) in TAC at pre-freeze and post-thaw stages. A reduction in TAC during equilibration was also observed which may be accounted to the consumption of antioxidants by the ROS.

In this study, there was no literature that could be traced to compare our findings specifically on the TAC of different NEA that were used in this experiment. This may be attributed to the type of antioxidants tested and different species of animals that were used in other experiments. Another factor would be the methods in evaluating the TAC conducted by other researchers.

Most of the studies focused on analyzing the TAC of enzymatic antioxidants (Lone et al. 2016), namely those of Bilodeau et al. (2000) and Kadirve et al. (2014).

### CONCLUSIONS AND RECOMMENDATIONS

The addition of NEA did not improve the sperm motility (%) and livability of the cryopreserved sperm. Likewise, the use of higher concentration may be explored, and other new semen extender in combination with NEA may also be studied.

The DPPH may not be the best assay for TAC; therefore, the study recommends the use of other procedure in measuring TAC. A parallel analysis should be conducted to validate the results of one laboratory to the other using the same methodology.

### ACKNOWLEDGMENT

The authors would like to thank the Department of Science and Technology – Accelerated Science and Technology Human Resource Development Program and the PCC at UPLB for funding this paper.

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