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Evaluation of 8-Hydroxy 2-Deoxy Guanosine, Intact Plasma and Acrosome Membrane of Pig Spermatozoa Diluted using SemenLife® with Addition of Alpha Tocopherol

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ABSTRACT

This research aimed to determine the levels of 8-hydroxy 2-deoxy guanosine (8-OH-dG), Intact Plasma Membrane (IPM), Intact Acrosome Membrane (IAM), and motility of spermatozoa diluted with SemenLife® and Alpha Tocopherol. A completely randomized design was used with eight treatment groups, including semen diluted with SemenLife® diluent stored for 0 hours (control), 24 hours, 48 hours, and 72 hours, as well as the addition of 400 µg/mL alpha-tocopherol diluent with similar storage periods. After dilution, semen was stored in a styrofoam filled with icepack at an internal temperature range of 15-20ºC. Observations were carried out on semen quality, which included 8OH-dG, IPM (%), IAM (%), and progressive motility (%), with each treatment repeated four times. The results showed that the addition of Alpha Tocopherol to SemenLife® diluent significantly ($P<0.05$) improved 8-OH-dG levels, IPM, IAM, and spermatozoa motility. In line with the length of storage, from 0 to 72 hours, there was a decrease in the quality of spermatozoa. Storage for 72 hours produced semen suitable for use with progressive motility of 42.50±1.29% and 48.50±1.29% for SemenLife® and SemenLife® diluent plus Alpha Tocopherol, respectively.

Key words: 8-OH-dG, IPM, IAM, Motility, Shelf life, SemenLife®, Alpha Tocopherol

INTRODUCTION

The demand for pork in Bali Province, Indonesia, is consistently high due to the significance in meeting the meat needs of the community for traditional activities and culinary pork tourism, both locally and internationally. This condition presents an opportunity for farmers to develop a pig farming business by improving farm management to improve production and reproduction performance. Although farmers are aware of the use of artificial insemination (AI) technology for improving the reproductive performance of livestock, in practice, many inseminators still use fresh semen collected directly from a superior male. The use of fresh semen has many disadvantages, including rapid deterioration. According to Foeh et al. (2022), fresh semen can only last up to 6 hours, making the utilization less efficient.

To overcome the problems, fresh semen needs to be diluted using a quality diluent and stored at cold temperatures to preserve quality and availability. An existing commercial diluent commonly used is BTS® (Beltsville Thawing Solution, Minitub, Germany), with a shelf life of up to 76 hours (Bebas et al. 2015; Bebas et al. 2016). SemenLife® (INTEC, Spain) diluent is also commercially available, with the potential to maintain the quality of pig semen for 7-9 days, but information regarding the ability is limited. The optimal temperature for storage of pig semen is 15-20°C (Henning et al. 2022).

Pig semen is susceptible to cold shock during cold storage, compared to ruminant or avian spermatozoa, and more sensitive to reactive oxygen species (ROS) attacks (Azawi and Hussein 2011). During storage at 15-20°C, spermatozoa are subjected to ROS attack, which can produce hydroxyl radicals (-OH) and singlet oxygen ($^1O_2$) (Douard et al. 2003). These ROS peroxidize lipids on the cell membrane, leading to the loss of integrity for both plasma and acrosome (Bebas et al. 2023a, 2023b). Decreased plasma membrane integrity disrupts the process

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of food transport into the cell. In addition, ROS triggers the loss of acrosomal membrane integrity, leading to enzyme leakage and impairing spermatozoa's ability to penetrate the egg's zona pellucida during fertilization (Bebas et al. 2023b). Lipid peroxidation of the plasma and acrosomal membrane produces malondialdehyde (MDA) compounds, which are toxic to cells, reduce motility, and cause damage to Deoxyribonucleic Acid (DNA) (Sanocka and Kurpisz 2004; Hosen et al. 2015).

DNA is composed of the nucleotides adenine (A), thymine (T), guanine (G), and cytosine (C), with guanine being the most sensitive to ROS attacks. Free radicals such as hydroxyl attack guanine, leading to oxidation and forming 8-OH-dG (8-hydroxy 2-deoxy guanosine). Currently, 8OH-dG is used as a marker to determine the occurrence of DNA damage, which harms the fertilization process, embryo development, the transmission of male genetic information during pregnancy, and repeated abortions (Hosen et al. 2015; Rex et al. 2017; Bebas and Agustina 2022).

Alpha Tocopherol, also known as vitamin E, is a fat-soluble antioxidant that mitigates ROS by donating one hydrogen atom (H) in the OH group. ROS becomes a less active radical and loses its destructive power (Traber and Stevens 2011). According to Bebas et al. (2016), the effective vitamin E concentration added to BTS® diluent stored at 15°C is 400µg/mL diluent. Therefore, this research aimed to determine the effect of SemenLife® diluent and Alpha Tocopherol during 0, 24, 48, and 72 hours of storage on 8-OH-dG levels, IPM, IAM, and progressive motility of spermatozoa.

MATERIALS AND METHODS

Ethical approval

All procedures were reviewed by the Experimental Animal Ethics Committee, Faculty of Veterinary Medicine, Udayana University, and received approval Number B/81/UN14.29/PT.01.04/2024.

Research materials

The materials used include 2-year-old landrace pig semen in good health, SemenLife® (INTEC, Spain), crystalline NaCl (1.06404.0500 Merck, Darmstadt - Germany), Sodium citrate (1.06448.0500 Merck, Darmstadt - Germany), Fructose (1.05323 Darmstadt - Germany), Eosin Y (1.15935.0025, Merck), Negrosin (1.15924.0025, Merck), 70% alcohol, 37% Formalin (Merck, Darmstadt- Germany), 8-Hydroxy-desoxyguanosine ELISA Kit (OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG), Alpha Tocopherol/Vitamin E (Santa E®, Sanbe Farma Indonesia), and aquabides.

Research instruments

The instruments used were a binocular microscope (CX23 Olympus, Japan), Counting Chamber Neubauer (Improved Assistant Germany), Micropipette (Eppendorf Research Micropipette 3124000121), Pasteur pipette, 10mL volume pipette, glass object, glass cover, pH paper (Merck pH paper), test tube, measuring cup, Erlenmeyer, test tube rack, water bath (MEMMERT Waterbath 10L, Sumber Aneka Karya Abadi, Indonesia), 1mL and 10mL syringe, pig semen collection device, and Magnetic Stirrer (OAN LAB Magnetic Stirrer HS-12).

Research design

This research used a completely randomized design (CRD) with eight treatment groups, including SemenLife® diluent stored for 0 (control), 24, 48, and 72 hours, as well as SemenLife® plus Alpha Tocopherol 400µg/mL diluent with similar storage periods. After dilution, semen was stored in a styrofoam filled with icepack at an internal temperature range of 15-20°C. Observations on semen quality were made, including 8-OH-dG, IPM (%), IAM (%), and progressive motility of spermatozoa (%), with each treatment repeated four times.

Location and time of research

This research was conducted at the Veterinary Reproduction Laboratory, Faculty of Veterinary Medicine, Udayana University, in April 2023. Male landrace pigs were maintained in Baturiti Village, Baturiti District, Tabanan, Bali. Meanwhile, 8-OH-dG levels were determined at the Joint Laboratory of the Faculty of Medicine.

Research procedures

Semen collection

Semen collection was carried out by massaging with gloved hands on the surface of the collection bottle coated using sterile gauze, which filtered the gel part (Hu et al. 2006).

Semen evaluation

Macroscopic and microscopic examinations were carried out to determine the quality of semen. Specifically, macroscopic examination includes color, volume, viscosity, pH, and odor, while microscopic examination comprises mass and individual movement, as well as concentration of spermatozoa. Semen volume examination was performed by reading the scale on the Erlenmeyer collection tube and pH was measured with pH paper by dripping semen onto the tip, then the results were matched with the standard. Viscosity was measured by tilting the tube to observe semen movement along the tube wall, with poor-quality semen resembling water in flow. The odor was measured by smelling semen, while the color was observed visually. Furthermore, an examination of mass movement was carried out by dripping 0.05ml of fresh semen on a glass object, covering it with a glass cover, and observing with a light microscope at l00x magnification. Mass movement was graded as follows: positive value of 4 (++++) (very good), 3 (+++) (good), 2 (++) (medium), 1 (+) (bad), and negative (-) indicating aspermia or no sperm (Susilawati 2011).

Spermatozoa motility was examined by dropping 0.05ml of fresh semen on a glass object at 37°C, then covering it with a glass cover and observing at 400x magnification. The progressive movement was calculated in percentage, with observations covering five fields of view (Bebas et al. 2023b).

Examination of spermatozoa viability and morphology (normal and abnormal) was carried out using Eosin-Negrosin painting (Kondracki et al. 2017; Bebas et al. 2023b), with 400x observation. In this test, live spermatozoa were transparent, while dead ones were colored red. Spermatozoa with abnormalities in the head, neck, and tail were considered abnormal and calculated in percentage.

Spermatozoa concentration was calculated using a Neubauer hemocytometer (Vrinda and Sneha 2023). Initially, semen from the collection was homogenized, followed by aspirating 0.005ml with a hemocytometer pipette or up to a scale of 0.5. Excess spermatozoa were reduced by attaching filter paper to the pipette's tip, and those attached to the hemocytometer pipette wall were cleaned. About 3% NaCl solution was aspirated until the scale reached 1.01, then the pipette tip was closed with the thumb and middle finger. The solution was homogenized by swinging in the shape of a figure eight. After discarding 4-5 drops, the pipette tip was cleaned with filter paper. The solution was dripped on the hemocytometer object glass at the edge and spread throughout. Spermatozoa contained in five boxes, including four in the corner and one in the middle, were counted using 400x magnification. Assuming the number of spermatozoa in the five boxes is X, the concentration is X x 10^7/mL of semen.

Hypoosmotic swelling test (HOS) is a method used to determine IPM. According to Bebas et al. (2023a), the HOS solution consisted of 0.9g fructose + 0.49g sodium citrate dissolved in aquadest to a volume of 100mL with an osmotic pressure of 100mOsm/kg. A volume of 20mL HOS solution was mixed with 0.2mL semen, followed by homogenization and incubation at 37°C for 45min. Subsequently, a review preparation was evaluated under 400x magnification, with at least 200 cells counted. Spermatozoa with good IPM were characterized by a coiled or inflated tail, while those with damaged IPM had a straight tail.

To examine IAMI, a solution was prepared by dissolving 0.9g of sodium chloride in aquabides up to 100mL. Subsequently, 1mL formalin was added to 99mL NaCl 0.9% solution and shaken until homogeneous. Three parts of the 0.9% NaCl solution and formalin mixture were mixed with one part of semen and allowed to stand for about 3min. About 0.05mL of the solution was dropped on the glass object and covered with glass. Observations were carried out under a microscope with 400x magnification, and the proportion of spermatozoa with intact acrosome membranes was calculated per 100. Spermatozoa with intact acrosome membranes are characterized by black hoods (Susilowati 2010; Zhang et al. 2021).

All reagents were mixed thoroughly before use for the OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation). Each 8-OHdG sample, including unknown and standard, was assayed in duplicate. High-content 8-OHdG urine or serum samples were diluted at least 10-20-fold in Assay Diluent. Subsequently, 50µL of the unknown sample or standard was added to the wells of an 8-OHdG conjugate-coated plate, followed by incubation at room temperature for 10min on an orbital shaker. A 50µL of the diluted anti-8-OHdG antibody was added to each well and incubated at room temperature for 1 hour on an orbital shaker. The microwell strips were washed three times with 250µL 1x Wash Buffer per well with thorough aspiration between each wash. After the final wash, microwell strips were tapped on an absorbent pad or paper towel to remove excess 1X Wash Buffer. About 100µL of the diluted Secondary Antibody-Enzyme Conjugate was then added to all wells, followed by incubation at room temperature for 1 hour on an orbital shaker. The microwell strips were also washed three times according to step four. The substrate solution was warmed to room temperature, and 100 L was added to each well. Incubation was carried out at room temperature on an orbital shaker, with actual incubation time varying from 2 to 30min. The plate was observed, and the reaction was stopped when the color changed rapidly to prevent saturation. The enzyme reaction was stopped by adding 100µL of Stop Solution into each well. The absorbance was read immediately on a spectrophotometer using 450nm as the primary wavelength.

SemenLife® diluent with Alpha Tocopherol was prepared by weighing 60g of SemenLife®, which was dissolved into 1000mL of aquabides, homogenized with a magnetic stirrer, and stored in a water bath at 37°C. SemenLife® diluent with the addition of Alpha Tocopherol 400 µg/ml was prepared by adding 400g of Alpha Tocopherol into 1000mL of SemenLife® diluent, then homogenized with a magnetic stirrer and stored in a water bath at 37°C.

The collected semen and Semen Life® diluent were placed in a 37°C water bath to equalize the temperature. Subsequently, semen that passed the evaluation process was diluted with the diluent prepared using the formula:

$$Total\ Volume\ of\ Diluent = \frac{Semen\ Volume\ x\ Sperm\ Concentration\ x\ Sperm\ Motility\ x\ Dose\ Volume}{Concentration\ Dose}$$

$$Total\ Volume\ of\ Diluent\ added = Total\ Volume\ of\ Diluent - Semen\ Volume$$

Data analysis

The data collected were analyzed using variance analysis of SPSS version 25 for Windows.

RESULTS AND DISCUSSION

Fresh semen of landrace pig

This research used a 2-year-old, healthy landrace pig from a farm in Baturiti, Tabanan, Bali, Indonesia. Semen was collected with a dummy sow as a lure using a gloved hand mash. The sample was immediately taken to a nearby laboratory for macroscopic and microscopic examinations. The results of the examination are presented in Table 1.

Table 1: Results of Macroscopic and Microscopic Examination of Landrace Pig Fresh Semen.

Semen Quality of Landrace Pig		
Macroscopic Examination	Semen viscosity	Medium
	Semen Color	White-beige
	Semen Volume (ml)	180mL
	Acidity/Ph	7.0
	Odor	Typical
Microscopic Examination	Mass Movement	+++
	Concentration (106/ml)	780
	Progressive Movement (%)	78 P
	Live Spermatozoa (%)	88
	Spermatozoa Abnormality (%)	6

Description:+++ = Mass wave movement is good.
P = Individual spermatozoa movement is forward and fast.

The macroscopic and microscopic evaluation results showed that semen was of good quality with a volume of 180mL, 780 x 10^6 cells/ml concentration, and progressive motility of 78%. In contrast, Baku et al. (2022) reported semen quality for landrace pigs with a volume of 230mL, a concentration of 230 x 10^6 cells/ml, and 70% motility. Discrepancies in the results are attributed to differences in semen collection methods. In this research, only the second

fraction of semen was collected, while the first and third fractions were omitted. The second fraction is very rich in spermatozoa, while the first and third are clear with a very low concentration and more gel, increasing the volume. Bebas and Gorda (2016) reported that collecting only the second fraction yielded a volume of 170mL, a concentration of $800x10^6$ cells/mL, with 89% motility, closely resembling the results obtained in this research. The semen obtained was of good quality and feasible for the dilution process according to the research design. The results of semen quality after dilution with SemenLife® diluent, as well as SemenLife® diluent plus Alpha Tocopherol, followed by storage for 0, 24, 48, and 72 hours with the parameters of 8-OH-dG levels, IPM, IAM, and progressive motility of spermatozoa, are presented in Table 2.

Evaluation of 8-Hydroxy-2-Deoxyguanosine (8OH-dG)

Deoxyribonucleic acid (DNA) is composed of the nucleotides adenine (A), thymine (T), guanine (G), and cytosine (C), with guanine being the most susceptible to reactive oxygen species (ROS) attack. Oxidation of guanine leads to the formation of 8-OH-dG (Hosen et al. 2015; Rex et al 2017), currently used as a biomarker of oxidative stress caused by damage to the DNA of the nucleus and mitochondria. The formation of 8-OH-dG in semen samples is associated with increased DNA fragmentation, chromatin retention, as well as decreased spermatozoa motility and fertilization rates, making it a potential biomarker for oxidative stress damage and infertility (Castleton et al. 2022).

The levels of 8OH-dG after diluting semen using SemenLife® with a shelf life of 0, 24, 48, and 72 hours were 3.16±0.73, 3.68±0.39, 5.86±0.72, and 7.67±0.75ng/mL, respectively. Meanwhile, dilution with SemenLife® plus Alpha Tocopherol with a shelf life of 0 hours, 24 hours, 48 hours, and 72 hours yielded 2.92±0.51, 3.69±0.45, 5.18±0.78, and 6.75±0.92ng/mL, respectively. 8-OH-dG levels during 0 and 24 hours of storage in both diluents were not significantly different (P>0.05). Storage for more than 24 hours produced significantly lower 8-OH-dG levels (P<0.05) in diluent with the addition of Alpha Tocopherol. The longer the shelf life, the higher the 8-OH-dG levels. An overview of 8-OH-dG levels during semen storage with SemenLife® as well as SemenLife® diluent plus Alpha Tocopherol is shown in Fig. 1.

DNA damage in spermatozoa during storage can be caused by increased levels of ROS, stemming from normal metabolic byproducts and abnormal, immature, and old spermatozoa. Endogenous sources of ROS include mitochondria, xanthine oxidase, cytochrome P450 metabolism, peroxisomes, and activation of inflammatory cells such as macrophages, neutrophils, and eosinophils (Guindon 2008; Kawai et al. 2018). Therefore, semen diluted with both diluents at 0 hours of storage had 8-OH-dG levels of 3.16±0.73ng/mL and 2.92±0.20ng/mL, respectively. After storage for 24 hours, 8-OH-dG levels for both diluents did not show a significant difference (P>0.05) but beyond 24 hours, there was a significant increase in line with the storage period. Adding Alpha Tocopherol to SemenLife® diluent significantly suppressed the rate of 8-OH-dG expression (P<0.05) compared to only SemenLife® diluent. According to Breininger et al. (2005), Alpha Tocopherol is a fat-soluble antioxidant that counteracts free radicals by breaking the chain of peroxidation reactions. It mitigates ROS by donating one hydrogen atom contained in the OH group,

Table 2: Results of 8-OH-dG Levels, Intact Plasma Membrane, Acrosome Membrane Intact, and Motility of Spermatozoa Diluted with SemenLife® and SemenLife® plus Alpha Tocopherol During Storage

Observations	Diluent							
	SemenLife®				SemenLife®+ Alpha Tocopherol			
	0 Hour	24 Hours	48 Hours	72 Hours	0 Hour	24 Hours	48 Hours	72 Hours
8OH-dG (ng/ml)	$3.16±0.73^{A}$	$3.68±0.39^{A}$	$5.86±0.72^{B}$	$7.67±0.75^{C}$	$2.92±0.51^{A}$	$3.69±0.45^{A}$	$5.18±0.78^{D}$	$6.75±0.92^{E}$
IPM (%)	$73.25±2.21^{A}$	$63.00±0.82^{B}$	$58.75±0.96^{C}$	$53.25±0.96^{D}$	$78.00±2.16^{E}$	$66.50±1.29^{F}$	$63.50±1.29^{G}$	$57.50±1.91^{H}$
AMI (%)	$76.00±1.83^{A}$	$72.50±0.58^{B}$	$66.50±1.29^{C}$	$60.75±0.96^{D}$	$81.25±1.50^{E}$	$76.25±1.50^{F}$	$70.75±0.96^{G}$	$66.00±0.82^{H}$
Motility (%)	$65.50±1.29^{A}$	$56.75±0.96^{B}$	$51.25±1.89^{C}$	$42.50±1.29^{D}$	$65.25±0,96^{A}$	$58.50±0.58^{B}$	$55.75±0.96^{E}$	$48.00±1.29^{F}$

Values (Mean±SD) with different superscripts in a row indicate a significant difference (P<0.05).

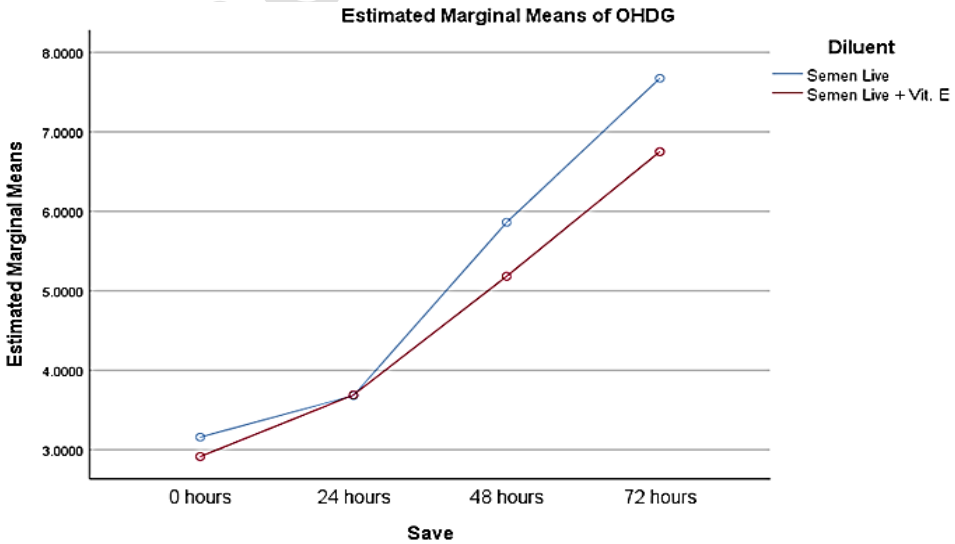

Fig. 1: 8-OH-dG levels of pig semen diluted with SemenLife® (blue line) and SemenLife® plus Alpha Tocopherol (red line) stored for 0, 24, 48, and 72 hours.

making ROS become less active radical and lose destructive power (Traber and Stevens 2011). In the absence of antioxidants, guanine is oxidized to 8-OH-dG which has an adverse effect on the fertilization process, including disruption of embryo development, transmission of male genetic information during pregnancy, and repeated abortions (Hosen et al. 2015; Rex et al. 2017).

Intact Plasma Membrane (IPM)

Intact Plasma Membrane of spermatozoa consists of cholesterol, glycolipids, phospholipids, and proteins. It functions to regulate the entry and exit of electrolytes and substrates needed by cells to maintain survival. In general, glycolipids and phospholipids are free unsaturated fatty acids that are easily peroxidized. Peroxidation of free unsaturated fatty acids in the plasma membrane causes disruption of cell metabolism (Anwar et al. 2015), leading to loss of motility, abnormal cells, as well as damage to the acrosome membrane (Sukmawati et al. 2014).

Based on the results, IPM after semen dilution using SemenLife® with a shelf life of 0, 24, 48, and 72 hours was 73.25±2.21, 63.00±0.82, 58.75±0.96, and 53.25±0.96%, respectively. Meanwhile, semen dilution using SemenLife® plus Alpha Tocopherol with a shelf life of 0, 24, 48, and 72 hours produced values of 78.00±2.16, 66.50±1.29, 63.50±1.29, and 57.50±1.91%, respectively. Storage for 0 to 72 hours in both diluents caused a significant decrease in IPM ($P<0.05$). The addition of Alpha Tocopherol to SemenLife® diluent showed significantly higher IPM ($P<0.05$) compared to only SemenLife® diluent. Alpha Tocopherol mitigates ROS by donating one hydrogen atom contained in the OH group, making ROS less active and losing its damaging ability (Traber and Stevens 2011). Hartono (2008) also reported that the addition of Alpha Tocopherol to egg yolk citrate diluent preserved the quality of Boer goat semen during storage at 4°C. Additionally, Tvrdá et al. (2018) added 50µmol/l curcumin extract to Triladyl diluent (Minitüb GmbH, Germany) as an antioxidant to dilute bovine semen. The results showed significantly higher IPM ($P<0.05$) compared to only Triladyl diluent, with values of 81.60±0.30 and 72.85±0.68%, respectively. An overview of IPM during the storage process of semen with SemenLife® as well as SemenLife® plus Alpha Tocopherol diluents is shown in Fig. 2.

Intact Acrosome Membrane (IAM)

The head of spermatozoa has a hood or membrane known as acrosomal, covering almost two-thirds of the anterior part. The membrane produces corona penetrating enzymes, acrosin, and hyaluronidase, which play crucial roles in fertilization by lysing the zona pellucida. Damage to the acrosome membrane leads to the leakage of these enzymes, rendering spermatozoa incapable of fertilization (Ichwandi 2004).

Semen after being diluted with SemenLive® dilution was then stored for 0, 24, 48, and 72 hours, resulting in intact plasma membranes was 76.00±1.83, 72.50±0.58, 66.50±1.29, and 60.75±0.96%, respectively. Meanwhile, semen diluted using SemenLife® plus Alpha Tocopherol with a shelf life of 0, 24, 48, and 72 hours yielded 81.25±1.50, 76.25±1.50, 70.75±0.96, and 66.00±0.82%, respectively. During semen storage from 0 to 72 hours in both diluents, there was a significant decrease in IAM ($P<0.05$). Adding Alpha Tocopherol to SemenLife® diluent led to a significantly higher IAM ($P<0.05$) than only SemenLife® diluent. Alpha Tocopherol binds ROS by donating one hydrogen atom in the OH group so that it can protect cellular components from oxidative damage (Traber and Stevens 2011). Hartono (2008) also reported that adding Alpha Tocopherol to egg yolk citrate diluent preserved the quality of Boer goat semen during storage at 4°C. According to Breininger et al. (2005), the antioxidant counteracts free radicals by breaking the chain of peroxidation reactions. Tvrdá et al. (2018) added 50 µmol/l curcumin extract to Triladyl diluent (Minitüb GmbH, Germany) as an antioxidant to dilute cow semen. The results showed significantly higher IAM ($P<0.05$) compared to only Triladyl diluent, with values of 82.70±0.52 and 70.80±0.47%, respectively. An overview of AMI during the storage process with SemenLife® as well as SemenLife® plus Alpha Tocopherol diluents is shown in Fig. 4. The microscopic image of spermatozoa with IAM is shown in Fig. 5.

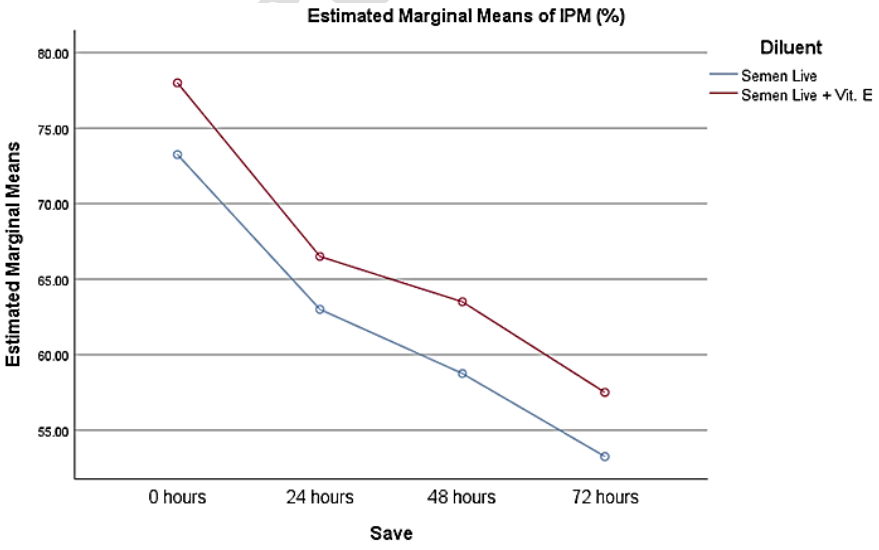

Fig. 2: Plasma Membrane of Pig Semen Diluted with SemenLife® and SemenLife® plus Alpha Tocopherol stored for 0, 24, 48, and 72 hours.

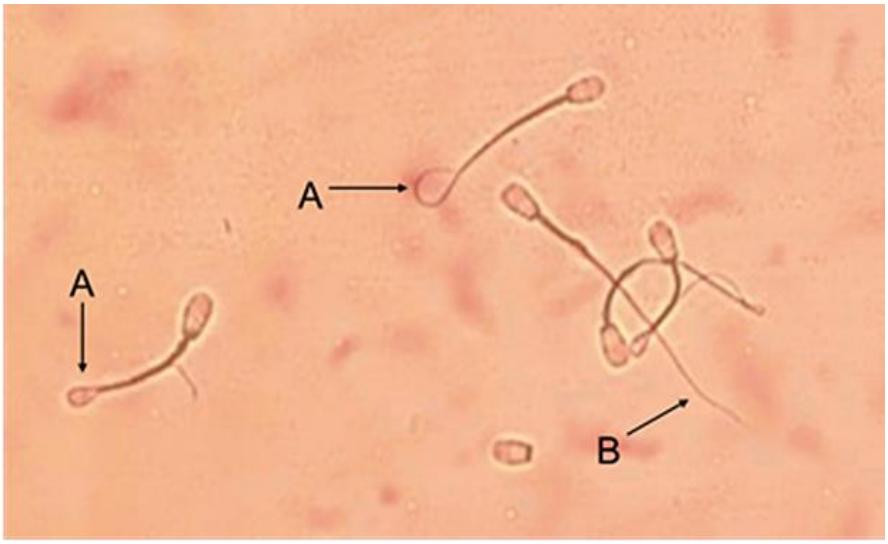

Fig. 3: Spermatozoa with IPM are characterized by a coiled tail and swelling (A); while spermatozoa with incomplete IPM are characterized by a straight tail (B). 400x magnification.

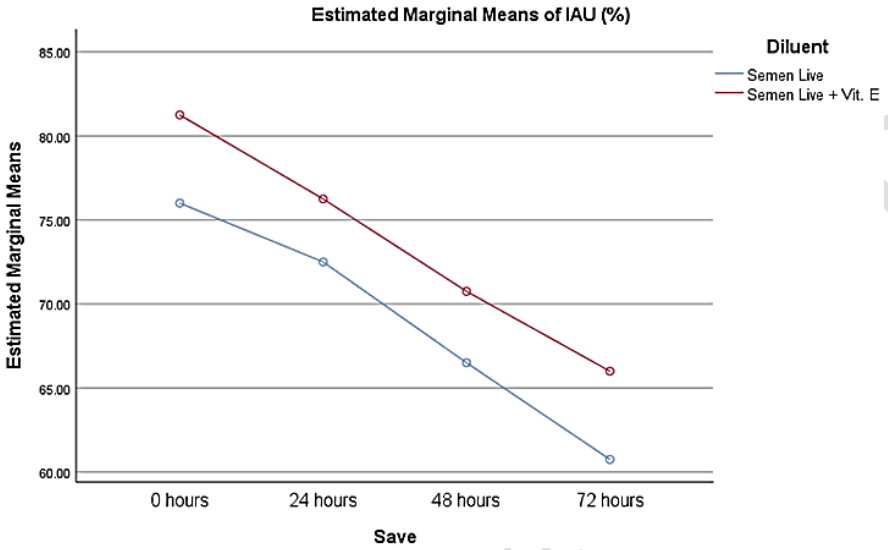

Fig. 4: Whole Acrosome Membrane of SemenLife® Diluted Pig Semen (Red Line) and SemenLife® Plus Alpha Tocopherol (Blue Line) Stored for 0, 24, 48, and 72 Hours.

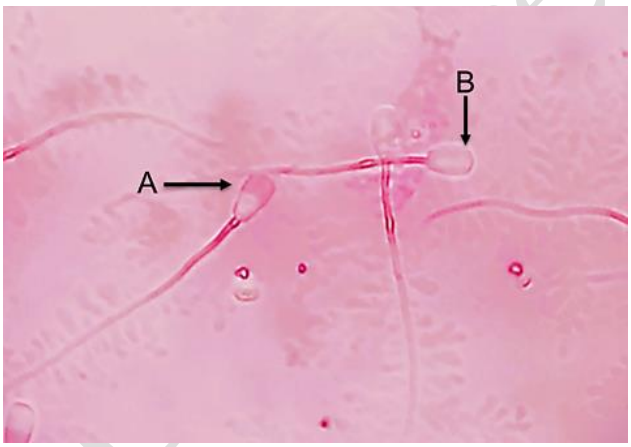

Fig. 5: Spermatozoa with IAM (A) and IAM that is not intact (B) with 400x magnification.

Progressive motility

An important factor in assessing male fertility is the progressive motility of spermatozoa, which plays an important role in moving toward the fertilization site and penetrating the egg wall. Only spermatozoa with progressive motility can move forward and fertilize an egg. Fertilization is enhanced by strong motility and enzymatic assistance produced by acrosoma (Djaelani 2010).

The progressive motility after semen dilution using SemenLife® with a shelf life of 0, 24, 48, and 72 hours was 65.50±1.29, 56.75±0.96, 51.25±1.89, 42.50±1.29%, respectively. Meanwhile, semen diluted using SemenLife® plus Alpha Tocopherol with a shelf life of 0, 24, 48, and 72 hours had progressive motility of 65.50±1.29, 58.50±0.58, 55.75±0.96, 48.50±1.29%, respectively. The motility of spermatozoa during 0- and 24-hours storage in SemenLife® and SemenLife® diluent plus Alpha Tocopherol was not significantly different ($P>0.05$). However, after 24 hours of storage in both diluents, motility was significantly decreased ($P<0.05$). Adding Alpha Tocopherol to SemenLife® diluent caused significantly higher motility ($P<0.05$) than only SemenLife® diluent. This is attributed to the ability of Alpha Tocopherol to donate one hydrogen atom contained in the OH group, causing ROS to become less active and lose destructive power (Traber and Stevens 2011). Hartono (2008) also reported that adding Alpha Tocopherol to egg yolk citrate diluent preserved the quality of Boer goat semen during storage at 4°C. According to Breininger et al. (2005), the antioxidant counteracts free radicals by breaking the chain of peroxidation reactions. Bebas et al. (2016) reported that the use of Alpha Tocopherol at the concentration of 400µg/mL with BTS® diluent enhanced the survival and motility of pig spermatozoa by

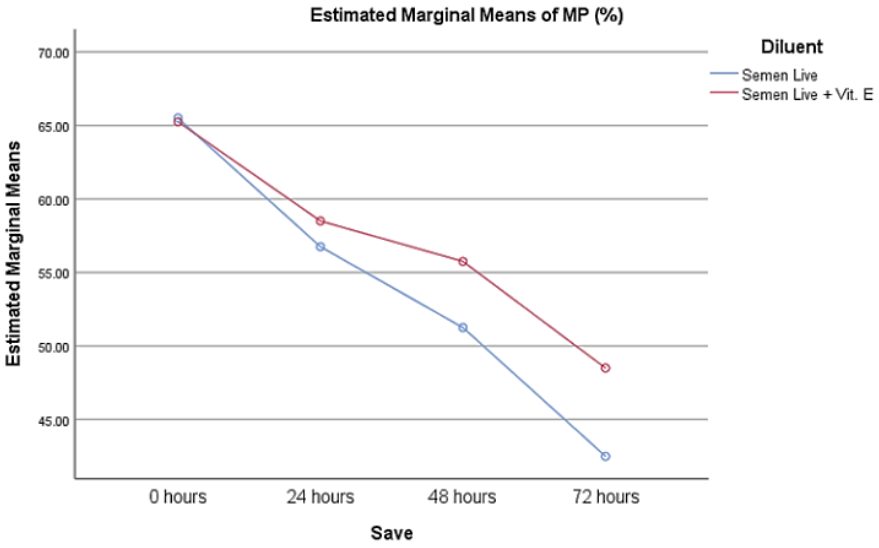

Fig. 6: Motility of Pig Semen Spermatozoa Diluted SemenLife® (Red Line) and SemenLife® Plus Alpha Tocopherol (Blue Line) Stored for 0, 24, 48, and 72 Hours.

50.33±3.77% during 96 hours of storage. Fig. 6 shows an overview of spermatozoa motility during the storage process with SemenLife® and SemenLife® diluent plus Alpha Tocopherol.

Fig. 6 shows that the use of SemenLife® and SemenLife® plus Alpha Tocopherol diluents with a shelf life of 72 hours produces significantly different motility ($P<0.05$) of 42.50±1.29 and 48.50±1.29%, respectively. According to the Indonesian National Standard, liquid semen suitable for the field after the preservation process should have a progressive motility of at least 40%. Pasyah et al. (2021) stated that the percentage of normal individual motility (fertile) was 40-75%, and spermatozoa motility below 40% indicated poor semen value associated with infertility. Based on the results, semen diluted using SemenLife® and SemenLife® plus Alpha Tocopherol with 72 hours of storage was considered the most suitable for artificial insemination services in pigs.

Conclusion

In conclusion, the addition of Alpha Tocopherol to SemenLife® diluent improved the quality of semen, reflected in 8OH-dG, Intact Plasma Membrane, Intact Acrosome Membrane, and progressive motility. Both diluents could be used, as indicated by the progressive motility of 42.50±1.29 and 48.50±1.29% after 72 hours of storage.

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Authors contribution

WB conceptualized and designed the research. WB, IWG, and IMM conducted the research, data collection, laboratory examination, evaluation, and data analysis. WB and IMM conducted a literature review, manuscript writing, and publication.

Conflict of interests

The authors declare no conflict of interest in the publication of this research.

REFERENCES

Anwar P, Ondho YS and Samsudewa D, 2015. Quality of membrane plasma and acrosome integrity of Bali bull preserved at 5°C in extender sugar cane extract and addition of egg yolk. Agromedia 33(1): 53-56. https://doi.org/10.47728/ag.v33i1.103

Azawi OI and Hussein EK, 2011. Study on the effect of adding bovine serum albumin to semen diluent on introduction the viability of Awassi Ram semen preserved at 5°C. Advanced Veterinary Research 1(3): 115-118.

Baku A, Dethan AA and Tahuk PK, 2022. Quality of Landrace Semen in Yolk Citrate Cement which Plus Glucose with Different Concentrations. Journal of Tropical Animal Science and Technology 4(1): 42-55. https://doi.org/10.32938/jtast.v4i1.1268

Bebas W, Budiasa MK and Astuti IK, 2015. Addition of vitamin c in diluent of landrace sperm stored in temperature of 15ºC. Buletin Veteriner Udayana 7(2): 179-185.

Bebas W, Buyona GL and Budiasa MK, 2016. The addition of vitamin e in diluent bts® of viability and mortality of spermatozoa landrace pig stored in temperature 15°c. Buletin Veteriner Udayanan 8 (1): 1-7.

Bebas W and Gorda IW 2016. The addition of astaxanthin on sperm diluentsphosphate eggyolk of various poultry can protect quality of pigsperm during storage. Jurnal Veteriner 17(4): 484-491. https://doi.org/10.19087/jveteriner.2016.17.4.484

Bebas W and Agustina KK, 2022. Short Communication: The use of lactose-astaxanthin to maintain the quality of Green junglefowl frozen semen. Biodiversitas 23(11): 5759-5764. https://doi.org/10.13057/biodiv/d231128

Bebas W, Gorda IW and Agustina KK, 2023a. Spermatozoa quality of Kintamani dogs in coconut water-egg yolk diluent with addition of Moringa leaves and carrot extract. International Journal of Veterinary Science 12(3): 333-340. https://doi.org/10.47278/journal.ijvs/2022.197

Bebas W, Gorda IW, Agustina KK and Merdana IM. 2023b. Effects of adding glutathione to AndroMed diluent on intact plasma and acrosome membranes and progressive motility of cattle spermatozoa during freezing processes. World Veterinary Journal 13(4): 561-570. https://www.doi.org/10.54203/scil.2023.wvj60

Breininger E, Beorlegui NB, O'Flaherty CM and Beconi MT, 2005. Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. Theriogenology 63(8): 2126-2135. https://doi.org/10.1016/j.theriogenology.2004.08.016

Castleton PE, Deluao JC, Sharkey DJ and McPherson NO, 2022. Review measuring reactive oxygen species in semen for male preconception care: a scientist perspective. Antioxidants 11, 264. https://www.doi.org/10.3390/antiox11020264

Djaelani MA, 2010. Peran kuning telur pada medium simpan beku semen TES-Tris yolk citrat terhadap motilitas dan vitalitas spermatozoa manusia post freezing. Buletin Anatomi dan Fisiologi XVIII(1): 1-7.

Douard W, Hermier D, Magistrini M and Blesbois E, 2003. Reproductive Period Affects Lipid Composition and Quality of Fresh and Stored Spermatozoa in Turkeys. Theriogenology 59: 753-764. DOI: https://www.doi.org/10.1016/s0093-691x(02)01086-5

Foeh N, Gaina C and Tophianong T, 2022. Kualitas Semen Segar dan Semen Cair Babi Landrace. Jurnal Kajian Veteriner 10(1): 61-66. https://www.doi.org/10.35508/jkv.v10i1.6701

Guindon KA, 2008. Investigation of the Role of oxidative DNA damage in aflatoxin-b1-induced pulmonary carcinogenesis. Thesis: Queen's. University, Kingston, Ontario, Kanada. Available online at: https://qspace.library.queensu.ca/server/api/core/bitstreams/c8f8b84b-91e1-4c69-95c1-d923ca0b5d98/content

Hartono M, 2008. Optimalization of vitamin E in egg yolk citrate extender to preserve semen quality of boer goat. Journal of the Indonesian Tropical Animal Agriculture 33 [1]: 11-19. https://doi.org/10.25181/jppt.v10i1.246

Henning H, Nguyen OT, Wallner U and Waberski D, 2022. Temperature limits for storage of extended boar semen from the perspective of the sperm's energy status. Frontiers in Veterinary Science 9(953021): 1-12. https://www.doi.org/10.3389/fvets.2022.953021

Hosen MB, Islam MR, Begum F, Kabir Y and Howlader MZH, 2015. Oxidative stress induced sperm DNA damage, a possible reason for male infertility. Iranian Journal of Reproductive Medicine 13(9): 525-532.

Hu JH, Li QW, Gang-Li, Chen XY, Hai-Yang, Zhang SS and Wang LQ, 2006. The Cryoprotective effect on frozen-thawed boar semen of egg yolk low density lipoproteins. Asian-Australasian Journal of Animal Sciences 9(4): 486-494. https://www.doi.org/10.5713/ajas.2006.486

Ichwandi, 2004. Performans motilitas, tudung akrosom utuh dan velositas spermatozoa tanpa dan dengan metode 'swim up' pasca thawing pada semen beku sapi potong. Thesis: Program Pascasarjana Universitas Diponegoro. Semarang. http://eprints.undip.ac.id/12657/1/2004MIT2692.pdf

Kawai K, Kasai H, Li YS, Kawasaki Y, Watanabe S, Ohta M, Honda T and Yamato H, 2018. Measurement of 8-hydroxyguanine as an oxidative stress biomarker in saliva by HPLC-ECD. Genes and Environment 40(5): 1-4. https://doi.org/10.1186/s41021-018-0095-2

Kondracki S, Wysokińska A, Kania M and Górsk K, 2017. Application of two staining methods for sperm morphometric evaluation in domestic pigs. Journal of Veterinary Research 61(3): 345-349. https://www.doi.org/10.1515/jvetres-2017-0045

OxiSelect™ Oxidative DNA damage elisa kit (8-OHdG Quantitation). https://www.cellbiolabs.com/sites/default/files/STA-320-8ohdg-elisa-kit.pdf

Pasyah BI, Rosadi B and Darmawan, 2021. Pengaruh penyimpanan pada suhu 5ºC terhadap motilitas, persentase hidup (viabilitas) dan abnormalitas semen sapi simmental. Jurnal Ilmiah Ilmu-Ilmu Peternakan 24(1): 11-18. https://doi.org/10.22437/jiiip.v24i1.15343

Rex AS, Aagaard J and Fedder J, 2017. DNA fragmentation in spermatozoa: a historical review. Andrology 5(4): 622–630. https://doi.org/10.1111%2Fandr.12381

Sanocka D and Kurpisz M, 2004. Reactive Oxygen Species and Sperm Cells. Reproductive Biology and Endocrinology 2(12): 1-7. https://www.doi.org/10.1186/1477-7827-2-12

Sukmawati E, Arifiantini RI and Purwantara B, 2014. Daya tahan spermatozoa terhadap proses pembekuan pada berbagai jenis sapi pejantan unggul. JITV 19(3): 168-175.

Susilowati S, 2010. Efek waktu sentrifugasi terhadap motilitas, daya tahan hidupdan tudung akrosom spermatozoa kambing. Veterinaria Medika 3(1): 61-64.

Susilawati T, 2011. Spermatology. Universitas Brawijaya Press, pp. 125-134. https://fapet.ub.ac.id/wp-content/uploads/2017/10/Buku-Spermatologi-bu-trinil.pdf

Traber MG and Stevens JF, 2011. Vitamins C and E: Beneficial effects from a mechanistic perspective. Free Radical Biology and Medicine 51(5): 1000–1013. https://doi.org/10.1016/j.freeradbiomed.2011.05.017

Tvrdá E, Greifová H, Mackovich A, Hashim F and Lukáč N, 2018. Curcumin offers antioxidant protection to cryopreserved bovine semen. Czech Journal Animal Science 63(7): 247–255. https://www.doi.org/10.17221/33/2017-CJAS

Vrinda P and Sneha J, 2023. Comparing sperm concentration measurement in human semen using an improved Neubauer hemocytometer and Makler counting chamber. International Journal of Academic Medicine and Pharmacy 5(3): 108-113. https://www.doi.org/10.47009/jamp.2023.5.3.24

Zhang B, Wang Y, Wu C, Qiu S, Chen X, Cai B and Xie H, 2021. Freeze-thawing impairs the motility, plasma membrane integrity and mitochondria function of boar spermatozoa through generating excessive ros. BMC Veterinary Research 17(127): 1-9. https://www.doi.org/10.1186/s12917-021-02804-1