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Determination of Alternative Diluents for Preservation of Duroc Boar Semen Through Modification of Various Semen Diluents

Wilmientje M. Nalley*, Henderiana L.L Belli, Aloysius Marawali, Kirenius Uly, Petrus Kune, and Thomas Mata Hine

Program Studi Peternakan, Fakultas Peternakan, Kelautan dan Perikanan-Universitas Nusa Cendana, Jl. Adisucipto Penfui Kota Pos 104 Kupang 8500
Telp (038) 881580. Fax (0380) 881674

*Corresponding author details: Wilmientje M. Nalley; nalleywm@yahoo.co.id

ABSTRACT

The quality of porcine semen deteriorates very rapidly during the semen preservation process. The aim of this study is to find alternative diluents for Duroc semen preservation by changing different semen diluents. Two Duroc pigs, ± 3 years old, were trained twice weekly for semen collection. Immediately after collection, sperm were examined macroscopically and microscopically. Semen with sperm motility ≥ 70%, sperm concentration ≥200 x 106 cells/ml, and abnormality ≤20%) was diluted with six diluents, namely: Beltsville Thawing Solution® (BTS), Durasperm, Citrate Egg Yolk (CEY), Tris Egg Yolk (TEY), CEY + Olive Oil (CEYO) and TEY + Olive Oil (TEYO) in the ratio of one part semen and four parts diluent. The liquid sperm was stored at 18-20 ^{o}C and examined every eight hours for 80 hours. The result shows that sperm motility, viability and plasma membrane integrity of Duroc semen with CEYO and TEYO are comparable to BTS. No significant difference was found in sperm abnormalities. The results suggest that citrate egg yolk and Tris egg yolk in combination with olive oil are suitable as alternative diluents for the preservation of porcine sperm. Further studies are needed to determine the most suitable olive oil concentration for the preservation of porcine sperm of different breeds.

Keywords: duroc sperm; BTS; citrate; tris diluents; olive oil

INTRODUCTION

The quality of pig semen deteriorates very rapidly during semen preservation. The ability to use liquid-stored semen up to 5 days after collection would significantly improve both production efficiency and economic profit of the artificial insemination industry if the current farrowing rate and litter size are maintained (8). Compared to bovine semen, the survival rate of porcine semen is shorter, only about 1 to 2 days, whereas bovine semen can survive up to 4 to 5 days. This is related to the different composition of the plasma membrane of sperm from these two species. In addition, dilution factors also play a role in determining sperm survival in the in vitro environment. Some diluents commonly used for porcine sperm preservation, such as Beltsville Thawing Solution® (BTS) and Durasperm, failed to improve sperm survival during preservation, likely due to less than optimal availability of nutrients required for sperm motility and viability. In addition, the production of free radicals as a byproduct of sperm metabolism negatively affects the sperm plasma membrane (1). They can cause lipid peroxidation, which in turn unstabilizes and damages the cell membrane and impairs the transport of electrolytes and nutrients into and out of the cell (23)(20). Free radicals can also damage the DNA contained in the cell nucleus so that transcription and translation processes cannot take place and protein synthesis is inhibited, which in turn leads to sperm death.

For this study, a number of diluents that have been shown to be effective in the preservation of bovine semen were used, such as citrate and Tris (3). Citrate and Tris serve as buffers to maintain the pH of the diluent in a range favorable for sperm viability, while egg yolk serves as a nutrient source for sperm, as it contains protein, glucose, and various other nutrients needed by sperm as an energy source, and also helps to regulate the osmolarity of the diluent solution. In addition, the lipoproteins and lecithin contained in the egg yolk help prevent cold shock to the sperm during preservation. The diluents have also been supplemented with local ingredients rich in nutrients and antioxidants that support sperm viability.

Olive oil is one of the ingredients that contain many antioxidants (13)(18). Olive oil contains various components (major and minor) that are very useful for sperm metabolism. The major component of olive oil is unsaturated fatty acids, which play an important role in transport and lipid metabolism and can maintain cell membrane function and integrity. The minor components are composed of α-tocopherol, polyphenols, flavonoids, hydrocarbons, and pigments, which have antibacterial and antioxidant effects and neutralize free radicals produced by lipid peroxidation. The polyphenol content of olive oil consists of oleuropein, tyrosol, and hydroxytyrosol.

Oleuropein and tyrosol can act as antioxidants, while hydroxytyrosol plays a role in protecting cell membranes. Hydroxytyrosol has an amphiphilic structure with a concentration equal to that of the cell membrane. This results in hydroxytyrosol being able to cross the cell membrane easily, allowing it to protect the cell membrane (2).

The specific objective of this study was to find alternative diluents for the preservation of Duroc semen by modifying different semen diluents. The urgency of this research lies in the availability of diluents that can be widely used to increase the success of artificial insemination in pigs, especially Duroc pigs. The specific specifications are: Modification of boar semen diluents by adding various locally available ingredients useful as nutrients and antioxidants to counteract the negative effects caused by free radicals produced as byproducts of sperm metabolism during in vitro storage.

MATERIALS AND METHODS

Animals

Two Duroc pigs, ± 3 years old, trained for semen collection. The pigs were housed in individual pens and fed concentrate (3 kg/head/day); drinking water was given ad libitum.

Methods

The experimental method was laid out in a completely randomized experimental design consisting of six treatments and four replicates, resulting in twenty-eight experimental units. The six treatments were: Beltsville Thawing Solution® (BTS), durasperm, citrate-eggyolk (CEY), tris-egg-yolk (TEY), CEY + olive oil (CEYO), and TEY + olive oil (TEYO).

Preparation of the diluent

Six diluents were use in this study, as follow:

a. Beltsville Thawing Solution® (BTS) 50 g of BTS (Minitub, Germany) dissolved with 1000 mL distilled water, mix well and warm-up at 37°C

b. One packet of Durasperm dissolved in 1000 mL distilled water, mix well and warm-up at 37°C

c. Citrate Egg-yolk (CEY) 29 g of Sodium citrate dissolve with 1000 mL of distilled water, and added with 250 ml of hen egg-yolk (4:1), mix well warm-up at 37°C

d. Tris Egg-yolk (CEY) 38.7 g Tris [hydroxymethyl] aminomethane, 21.7 g citric acid, 15.6 g fructose dissolved in 1000 ml distilled water and added with 250 ml of hen egg-yolk (4:1), mix well warm-up at 37°C

e. Citrate Egg-yolk + olive oil (CEYO) Citrate egg yolk add with olive oil mix well Warm-up at 37°C Tris Egg-yolk + Olive oil (TEYO)

CEY, TEY, CEYO and TEYO diluent were added with antibiotic (1,000 IU penicillin and 1,000 µg streptomycin mL^{-1} diluent. Each solution is stirred until homogeneous. All diluent storage at water bath 34 ° C until use.

Semen collection and evaluation

Sperm collection was performed twice weekly using the glove method. Immediately after collection, sperm were macroscopically examined for: 1) volume (read on a scaled collection tube; 2) color (visually visible in the collection tube; 3) pH (measured with pH indicator paper); 4) consistency (observing the speed of sperm movement in a tilted tube).

Microscopic evaluation includes: 1) sperm motility (subjective comparison of progressive and non-progressive sperm counts from five fields of view); 2) sperm viability (comparison of live and dead sperm counts from ten fields of view) using eosin-nigrosin staining; 3) sperm abnormality (%) (comparison of the number of normal and abnormal sperm from ten fields of view) using eosin-nigrosin staining; 4) sperm with intact plasma membrane (PMI) (comparison of the number of intact and non-intact sperm with plasma membrane from ten fields of view) using hypoosmotic threshold test (3).

Dilution and preservation of semen

Semen with a sperm motility of ≥ 70%, a sperm concentration of ≥200 x 106 cells/ml, and an abnormality of ≤20%) was diluted in the ratio of one-part semen and four parts diluent. The liquid semen was then stored in a cool box at 18-20 °C. To assess sperm motility, a drop of sperm was placed on a microscope slide, covered with a coverslip, and then viewed under a microscope at 400× magnification. Sperm viability, PMI and abnormalities were performed as for fresh semen. The semen is examined every eight hours until sperm motility reaches 40%.

Data Analysis

Data were analyzed by analysis of variance and Duncan's test using SPSS 26.0 software for Windows. Data presented as means ± Standard Error Means (means ± SEM).

RESULTS AND DISCUSSION

The quality of Duroc sperm after preservation is shown in Tables 1-4. In general, sperm quality decreased with increasing preservation time. The rate of decrease in sperm motility in the six diluents up to 80 hours of preservation was 0.48; 0.55; 0.57; 0.49; 0.58; 0.49% per hour for diluents BTS, Durasperm, CEY, TEY, CEYO and TEYO, respectively. The difference in the rate of decrease in sperm motility between the diluents resulted in differences in sperm motility observed in each observation. Sperm motility in diluents BTS, CEYO, and TEYO was slightly better than in the other diluents at 80 hours (Table 1).

TABLE 1: Sperm motility of Duroc in different diluents (means ± SE).

Storage time (hours)	Diluents					
	BTS	Durasperm	CEY	TEY	CEYO	TEYO
0	78.33±2.89	78.33±2.89	78.33±2.89	78.33±2.89	78.33±2.89	78.33±2.89
8	75.67±3.21	73.33±3.06	74.33±2.08	75.33±3.06	73.67±3.21	75.00±3.00
16	72.33±2.52	69.67±4.51	69.33±1.15	72.33±3.79	69.33±1.15	72.00±2.00
24	69.33±1.15	66.00±5.29	65.67±1.15	68.00±3.46	65.33±0.58	69.33±1.15
32	66.33±1.53	61.67±5.86	61.67±2.89	64.67±4.16	62.67±2.31	66.00±1.73

Storage time (hours)	Diluents					
	BTS	Durasperm	CEY	TEY	CEYO	TEYO
40	63.00±2.65	58.00±6.93	55.00±5.00	60.67±5.13	58.33±2.89	60.67±1.15
48	58.33±2.89	53.67±7.51	50.33±5.51	56.67±5.77	54.33±4.04	57.67±2.52
56	53.33±2.89	49.00±7.94	46.67±6.11	52.67±4.04	48.67±2.31	53.00±2.65
64	49.00±3.61	44.33±6.02	42.33±6.66	48.00±3.46	43.67±1.53	48.33±2.89
72	44.33±4.04	38.33±7.64	36.67±5.77	43.33±2.89	38.33±2.89	43.33±2.89
80	40.00±2.00a	34.00±8.54b	33.00±7.00b	39.33±1.15ab	31.67±2.89c	39.33±1.15ab

Different superscripts in the same column indicate significant differences (P<0.05).
BTS= Beltsville Thawing Solution, CEY= citrate Egg-yolk, TEY= Tris-Egg-yolk, CEYO= CEY +olive oil, TEYO= TEY +olive oil

Live sperm are very important because they describe that the sperm membrane still functions as a cell wall, so the organelles in the cell are still functional.

This study shows that sperm viability was not significant in all diluents except after 80 hours. Tris egg yolk with and without olive oil and BTS were higher than the other diluents (Table 2).

TABLE 2: Sperm viability of Duroc in different diluents (means ± SE).

Storage time (hours)	Diluents					
	BTS	Durasperm	CEY	TEY	CEYO	TEYO
0	85.06±2.16	85.06±2.16	85.06±2.16	85.06±2.16	85.06±2.16	85.06±2.16
8	79.73±2.26	78.15±2.33	80.43±2.81	81.53±1.66	80.42±2.70	81.54±3.08
16	77.93±3.24	74.62±3.53	75.94±3.47	78.07±3.40	75.54±0.97	78.49±2.41
24	74.81±1.81	71.80±3.97	72.67±1.79	73.67±3.82	71.56±1.62	76.02±2.27
32	71.12±2.94	66.39±5.20	68.25±2.29	70.35±3.57	68.60±1.80	72.25±2.32
40	69.54±3.21	62.40±6.64	62.09±4.27	67.91±3.95	64.16±2.73	67.51±0.90
48	64.10±1.94	58.94±7.16	57.34±4.52	62.77±3.54	60.56±2.02	63.23±2.67
56	60.35±2.66	54.45±8.11	53.39±7.78	58.46±3.00	54.39±1.99	60.16±1.77
64	54.10±2.07	50.31±7.66	49.25±7.64	54.59±4.22	49.63±1.52	55.18±3.22
72	48.94±2.24	45.00±7.05	43.71±6.34	50.07±3.05	42.61±1.60	50.51±3.87
80	43.15±2.00ab	39.15±9.84b	39.14±8.09b	45.65±3.16a	37.20±2.63b	46.43±1.77a

Different superscripts in the same column indicate significant differences (P<0.05).
BTS= Beltsville Thawing Solution, CEY= citrate Egg-yolk, TEY= Tris-Egg-yolk, CEYO= CEY +olive oil EYO= TEY +olive oil

Sperm morphology is one of the most important parameters in determining sperm quality, as high sperm abnormalities can affect fertility. Morphological observation is used to determine normal and abnormal sperm shape. According to Arifiantini (2012), sperm abnormalities can be divided into two main groups, namely primary abnormalities (head and acrosome spermatozoa) and secondary abnormalities (cytoplasmic droplets in the midpiece and damage to the tail). The amount of porcine semen used in this study was low, less than 5%. Storage of liquid semen in different diluents increased sperm abnormalities, but they were very low and there was no difference between all diluents used (Table 3).

TABLE 3: Sperm abnormalities of Duroc in different diluents (means ± SE).

Storage time (hours)	Diluents					
	BTS	Durasperm	CEY	TEY	CEYO	TEYO
0	4.22±0.59	4.25±0.52	4.23±0.48	4.15±0.64	4.30±0.47	4.43±0.53
8	4.44±0.64	4.34±0.42	4.29±0.45	4.24±0.66	4.37±0.50	4.54±0.45
16	4.60±0.56	4.48±0.38	4.42±0.37	4.36±0.74	4.51±0.53	4.70±0.47
24	4.75±0.53	4.68±0.48	4.49±0.32	4.53±0.78	4.69±0.59	4.78±0.47
32	4.87±0.57	4.90±0.49	4.61±0.32	4.61±0.89	4.83±0.61	4.90±0.46
40	4.96±0.45	5.05±0.48	4.76±0.40	4.75±0.94	5.00±0.70	4.97±0.48
48	5.22±0.30	5.45±0.78	5.54±0.27	5.00±1.11	5.09±0.73	5.11±0.53
56	5.44±0.21	5.59±0.77	5.06±0.50	5.05±1.17	5.17±0.79	5.23±0.47
64	5.66±0.21	5.83±0.87	5.49±0.18	5.29±1.35	5.32±0.85	5.38±0.45
72	5.73±0.01	6.12±0.85	5.65±0.92	5.39±1.37	5.66±1.02	5.62±0.57
80	6.00±0.44	6.32±0.78	5.91±0.32	5.51±1.48	5.81±0.94	5.75±0.56

Different superscripts in the same column indicate significant differences (P<0.05).
BTS= Beltsville Thawing Solution, CEY= citrate Egg-yolk, TEY= Tris-Egg-yolk, CEYO= CEY +olive oil, TEYO= TEY +olive oil

The sperm plasma membrane is a very important structure that serves to protect sperm from extracellular injury and to respond to physiological challenges. It plays a crucial role in sperm capacitation formation, sperm-egg interaction, and finally fertilization (Tapia *et al.* 2012). Plasma membrane integrity was variable in this study after 80 hours of storage, with BTS and TEYO being higher than the other diluents (Table 4).

TABLE 4: Sperm plasma membrane intact of Duroc in different diluents (means ± SE).

Storage time (hours)	Diluents					
	BTS	Durasperm	CEY	TEY	CEYO	TEYO
0	86.74±2.91	86.74±2.91	86.74±2.91	86.74±2.91	86.74±2.91	86.74±2.91
8	83.62±2.76	79.62±1.56	82.20±1.42	82.39±1.12	82.03±2.92	82.43±0.59
16	80.17±2.06	75.96±3.02	79.11±2.48	79.23±2.66	77.13±1.27	79.13±2.64
24	76.67±1.47	73.91±1.76	74.45±1.05	73.70±5.97	75.86±3.66	76.82±2.00
32	74.08±2.87	68.46±2.85	70.29±2.53	71.59±3.45	70.53±0.76	73.53±1.56
40	71.70±2.50	64.74±5.26	64.99±2.81	69.44±3.56	65.47±1.97	70.20±3.06
48	66.12±1.48	60.23±6.14	59.38±2.44	64.41±2.87	61.95±1.70	65.89±3.49
56	63.14±2.04	56.54±6.09	56.85±4.57	59.75±3.47	56.63±0.92	62.76±2.43
64	57.82±2.73	52.29±5.99	52.04±5.30	56.17±3.54	52.07±1.93	56.11±3.19
72	54.47±1.80	47.88±5.05	46.80±3.58	51.27±2.96	45.12±2.91	52.08±3.43
80	47.45±1.90 a	41.81±6.50b	42.39±4.00b	47.35±2.33a	40.78±1.40b	48.56±2.39a

Different superscripts in the same column indicate significant differences (P<0.05).
BTS= Beltsville Thawing Solution, CEY= citrate Egg-yolk, TEY= Tris-Egg-yolk, CEYO= CEY +olive oil, TEYO= TEY +olive oil

The results showed that sperm motility, viability and IPM (P>0.05) were better in BTS, CEYO and TEYO diluents than in other diluents (P>0.05). This result suggests that homemade extenders such as citrate and Tris diluents in combination with olive oil are effective for Duroc sperm. BTS is a commercial diluent and is still imported from other countries, while citrate and Tris are homemade diluents and the materials are readily available. Tris and egg citrate are blended with olive oil so they can be used as alternative diluents for boar seed.

Efforts to maximize the utilization of superior males, especially in swine production, have been intensively pursued by various parties. Maximum utilization of superior males can be achieved through semen collection and preservation, which allow semen collection and extension with various diluents. Sperm storage at a low temperature can suppress the metabolic rate of sperm cells so that the cells survive for a relatively long time.

Dilution and storage of semen is widely used in artificial insemination programs. Sperm preservation can be done either in liquid form at low temperatures to suppress sperm metabolism or in frozen form (at subzero temperatures). Sperm preservation in liquid form can prevent the damage associated with freezing and thus ensure better sperm viability. The success of artificial insemination depends largely on the ability to develop a semen diluent that has the same function as seminal plasma and can maintain sperm fertility for an extended period of time (Jasrotia *et al.* 2022). During cold shock, sperm are permanently damaged. The effects of shock can be minimized by using egg yolk, milk, or soy lecithin (Arief *et al.* 2022). Various homemade diluents such as Tris egg yolk, sodium citrate egg yolk, skim milk, coconut milk, lactose and many other traditional diluents have been successfully used for sperm preservation (Hegedusova *et al.* 2012) The addition of synthetic and natural antioxidants to diluents has been widely reported (Lee *et al.* 2018; Ros-Santaella and Pintus, 2021).

Antioxidants are important to limit harmful oxidative reactions in semen. Olive oil contains large amounts of natural antioxidants that provide oxidative stability during storage (Ros-Santaella and Pintus, 2021).

Antioxidants present in olive oil include tocopherols, sterols, carotenoids, and phenolic compounds (Kabaran, 2018). Kouka *et al.* (2020) state that the protective effect of olive oil is not only due to its high oleic acid content, but also to the antioxidant properties of its polyphenols. Due to its fat solubility, olive oil can penetrate the plasma membrane of sperm and suppress free radical damage (Hazim *et al.,* 2012). Hazim *et al.* (2012) found that diluent supplementation with olive oil at various levels could improve the quality of chicken spermatozoa (motility, viability, membrane integrity, and acrosome) stored at 5 °C and survived up to 72 hours. Silva *et al.* (2016). also showed that olive oil at a concentration of 0.25% can protect pig spermatozoa from freezing damage.

As for sperm technology, possibly the most important factors damaging mammalian sperm membranes are induced by osmotic stress resulting from cell desiccation during refrigeration. These changes rapidly affect plasma and organelle membranes, leading to a gradual loss of membrane architecture, imbalanced production of reactive oxygen species, and increased lipid peroxidation (22). The current study showed that the percentage of spermatozoa with progressive motility decreased significantly with increasing storage time. This may indicate progressive changes associated with defective cellular mechanisms responsible for the efficient functioning of the integrity of the sperm motility apparatus. At the same time, our results suggest a relationship between sperm motility and the ability of sperm mitochondria to oxidize exogenous NADH (nicotinamide adenine dinucleotide hydrogen, an active coenzyme form of vitamin B3), since there was a significant correlation between the percentage of sperm with progressive motility. BTS and durasperm are two commonly used diluents for porcine sperm. Previous studies have shown that both diluents are able to maintain the quality of in vitro preserved porcine sperm, but only for a short period of time (17); (6).

Compared to semen from other species, boar semen is very sensitive to temperatures below 12 °C due to a lower proportion of PUFA in its membrane (15). Banamtuan *et al.* (2021) preserved Duroc sperm with durasperm using palm fruit water and sugarcane nectar.

It was found that the addition of palm fruit water to durasperm preserved sperm longevity better than the addition of sugarcane nectar or durasperm alone.

CONCLUSION

The results suggest that citrate egg yolk and Tris egg yolk in combination with olive oil are suitable as alternative diluents for the preservation of porcine sperm. Further studies are needed to determine the most appropriate olive oil concentration for the preservation of porcine semen of different breeds.

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