

Quality Of Frozen Cement From Landrace Boars In Modified Citrate Diluents With Different Glycerol Concentrations

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ABSTRAK

Tujuan dari penelitian ini adalah untuk mengetahui kualitas semen beku babi landrace dalam pengencer sitrat modifikasi dengan konsentrasi gliserol yang berbeda. Materi penelitian yang digunakan adalah semen segar dari pejantan babi landrace yang berumur 2-3 tahun dari 4 ekor babi. Penelitian ini menggunakan penelitian eksperimental dengan metode rancangan acak lengkap yang terdiri dari 4 perlakuan dan 4 kali ulangan. Adapun perlakuan yang dimaksud yakni P1: S-KT+ G 3%, P2: S-KT+G 5%, P3:S-KT+G 7 % dan P4: S-KT+G 9%. Semen yang telah diencerkan disimpan pada suhu ruang (27-28°C) selama dua jam (holding time), selanjutnya disentrifugasi dengan kecepatan 3000 RPM selama 15 menit. Setelah sentrifugasi supernatan dibuang dan pelet hasil sentrifugasi diencerkan kembali dengan pengencer dasar. Semen yang telah diencerkan dikemas dalam straw 0,5 mL kemudian dipacking disusun dalam rak pembekuan lalu diekuilibrasikan pada suhu 3-5°C selama 2 jam. Kemudian straw dibekukan diatas permukaan N₂ cair dengan jarak 10 cm pada suhu $\pm$ -110°C selama 10 menit. Hasil penelitian ini menunjukkan bahwa penambahan gliserol yang berbeda tidak berpengaruh nyata ($P>0,05$) terhadap motilitas, viabilitas, abnormalitas dan recovery rate spermatozoa. Disimpulkan bahwa penambahan gliserol yang berbeda memberi dampak yang sama namun tidak layak untuk IB.

Kata-kata kunci: babi landrace, gliserol, kuning telur, sitrat

ABSTRACT

The purpose of this study was to determine the quality of frozen semen from landrace boars in modified citrate diluents with different glycerol concentrations. The research material used was fresh cement from 2-3 year old landrace boar studs from 4 boars. This study used an experimental study with a completely randomized design method consisting of 4 treatments and 4 replications. The treatments in question are T1: S-KT + G 3%, T2: S-KT + G 5%, T3: S-KT + G 7% and T4: S-KT + G 9%. The diluted cement was stored at room temperature (27-28°C) for two hours (holding time), then centrifuged at 3000 RPM for 15 minutes. After centrifugation, the supernatant was discarded and the centrifuged pellet was re-diluted with basic diluent. The diluted semen was packed in 0.5 ml straws, then packed, arranged in a freezing rack, and then equilibrated at 3-5°C for 2 hours. Then the straws were frozen above the surface of liquid N₂ with a distance of 10 cm at a temperature of $\pm$ -110°C for 10 minutes. The results of this study indicate that the addition of different glycerols did not have a significant effect ($P>0.05$) on the motility, viability, abnormality and recovery rate of spermatozoa. It was concluded that the addition of different glycerols had the same impact but were not suitable for IB.

Keywords: landrace boars, glycerol, egg yolk, citrate

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INTRODUCTION

In Indonesia, artificial insemination (AI) in pigs has been performed on several large farms using liquid semen. The use of frozen semen for pig AI has only been implemented on a few large farms, using imported frozen semen from Canada at a very high price. The use of frozen semen has advantages,

including its long-term use. Pig semen is known to be susceptible to cold shock due to its fatty acid and phospholipid content, which differs from that of other livestock, namely the composition of *phosphatidylethanolamine* and *sphingomyelin* at 24% and 14%, respectively. The quality of frozen semen is determined by several factors, including the technique and equipment used, the diluent, and the type and concentration of added cryoprotectants. To produce quality frozen semen, a diluent is required that can meet the physical and chemical requirements during the cooling, freezing, and *thawing processes*.

The diluent often used to dilute boar semen is citrate. Citrate is a diluent containing a buffer that has a role as a buffer or maintains pH during the cold temperature storage process in egg yolk diluents for the preservation of spermatozoa survival and fertility (Johnson, 2000) while egg yolk functions as a protector of spermatozoa against cold shock and as a source of energy for spermatozoa movement. The main composition of egg yolk consists of water, protein, fat, carbohydrates, minerals, vitamins and proteins, including perfect because it contains all types of essential amino acids in sufficient quantities, in addition, egg yolk contains lipoproteins from spermatozoa cells and prevents *cold shock* (Kommisrud et al, 2002). However, the presence of egg yolk can cause instability in the membrane and changes in the concentration of the lipid matrix structure due to the hydrolysis of egg yolk lecithin into isolecithidin (Solihati, 2008) and fatty acids catalyzed by the enzyme phospholipase A secreted by the bulbourethral gland. Therefore, it is necessary to add ingredients that function to protect spermatozoa during the freezing process.

Semen cryopreservation also requires the addition of appropriate cryoprotectants, both in type and quantity, to the dilution medium to protect spermatozoa from *cold shock*. Glycerol is an intracellular cryoprotectant because it can enter spermatozoa cells to bind some of the free water, thus preventing the formation of ice crystals (Blanch, 2014). The use of glycerol in the process of freezing boar semen is 3% (Sugiantini, 2023). Meanwhile, sugar is an extracellular cryoprotectant and as a substrate energy source, so it can protect and maintain the quality of spermatozoa during the freezing process. Commonly used sugars are dextrose, raffinose, trehalose, and sucrose in Garut sheep semen ((Zhou, 2004). The purpose of this study was to determine the quality of frozen landrace boar semen in modified citrate diluents with different glycerol concentrations.

RESEARCH METHODS

Research Materials

The research sample used was fresh semen obtained from four landrace pigs aged 2-3 years and in good health.

Research methods

This study was an experimental study with a completely randomized design (CRD) consisting of four treatments and four replications. The research design was as follows: citrate-egg yolk diluent with different glycerol concentration treatments, namely P1: 3%, P2: 5%, P3: 7% and P4: 9%.

Diluent preparation stage

To prepare the egg yolk, clean the eggshell using cotton wool soaked in 70% alcohol. Then, crack the egg at the point, pour out all the egg white, and separate it from the yolk. The yolk, still wrapped in the vitelline membrane, is placed on filter paper and then tilted in a circular motion so that all the egg white is absorbed by the filter paper. Meanwhile, the vitelline membrane on the egg yolk is broken using a pipette, then pour the egg yolk into a measuring cup and cover it with aluminum foil. After that, the egg yolk is ready to use.

Preparation of sodium citrate, sodium citrate was weighed as much as 2.9 grams then put into *an Erlenmeyer* and dissolved in 100 mL of Aquabidest and homogenized using *a stirrer* assisted by *a spin bar*. Citrate solution was taken as much as 80 mL and added 20 mL of yellow then homogenized again until evenly distributed then the diluent was ready to use.

Semen Storage Stage

The process of collecting pig semen uses the massage method.

Evaluate Semen

Fresh semen obtained was evaluated macroscopically including semen volume, semen color, semen odor, semen consistency, and semen pH and evaluated microscopically including motility, viability and abnormalities and concentration.

Semen Dilution and Freezing

The collected semen was divided into four tubes, which were filled with basic diluent with a ratio of 7:7. The diluted semen was stored at room temperature (27-28°C) for two hours (*holding time*), then *centrifuged* at 3000 RPM for 15 minutes. After *centrifugation* , the supernatant was discarded and the centrifuged pellet was diluted again with the diluent and then divided into tubes containing labeled glycerol treatment diluents, namely (P1 = 3% P2 = 5%, P3 = 7% and P4 = 9% glycerol) with a ratio of 1:1.

After dilution, the semen is packed in 0.5 mL *straws* and then *packed*, then the *straws* are arranged on a freezing rack and then put into the refrigerator for equilibration at a temperature of around 3-5°C for 2 hours, to give the spermatozoa cells a chance to equilibrate with the mixed cryoprotectant. After equilibration, *the straws* are evaluated to determine whether or not they are suitable for freezing. Then *the straws* are frozen above the surface of liquid N₂ with a distance of 10 cm at a temperature of $\pm$ -110°C for 10 minutes . After that, *the straws* are dipped into liquid N₂ and then put into a goblet and then the goblet

is put into a canister. Then they are put into a container containing liquid N₂ at a temperature of -196°C for 24 hours to get optimal results.

Post Freezing Evaluation of Cement

Post-freezing evaluation of frozen semen is performed at least 24 hours after storage. The post-freezing evaluation steps include: thawing the frozen semen straw *in a water bath* at 37°C for 30 seconds. Afterward, examinations are performed for sperm motility, viability, and abnormalities.

Research Variables

The parameters observed in this study were motility, viability, abnormality and recovery rate.

1. Motility is a progressive individual movement forward which is assessed immediately after collection by placing one drop of semen on *a glass object* without covering it with *a cover glass* and then observing it under a microscope with a magnification of 10×40 subjectively in ten different fields of view and the value given ranges from 0 to 100% on a 5% scale.
2. Sperm viability is influenced by their nutritional needs. Nutrients used by spermatozoa are converted into energy, so a decrease in their nutritional needs will result in decreased sperm viability (Blegur et al., 2020). Sperm viability is characterized by the length of time they survive during preservation. The formula for calculating spermatozoa viability is:

$$\text{viabilitas} = \frac{\text{Jumlah spermatozoa}}{\text{Total spermatozoa yang dihitung}} \times 100\%$$

3. Sperm abnormalities can be divided into primary and secondary abnormalities. Determining spermatozoa abnormalities is done by comparing abnormal spermatozoa with normal spermatozoa (Manur, 2024). To calculate the number of abnormal spermatozoa:

$$\text{Abnormalitas} = \frac{\text{Jumlah spermatozoa abnormal}}{\text{Jumlah sel spermatozoa keseluruhan}} \times 100\%$$

4. *The recovery rate* (RR) is the ability of spermatozoa after freezing by comparing the percentage of motile spermatozoa in fresh semen with *post-thawing*. According to (Maria G. Mega, Wilmientje M. Nalley, Aloysius Marawali, 2022) the requirements for semen used have the following criteria: motility $\geq 75\%$, concentration $\geq 200 \times 10^6$ cells/mL, and abnormalities $\leq 20\%$. The formula for calculating *the recovery rate* is:

$$\text{Recovery Rate} = \frac{\text{Motilitas pasca thawing}}{\text{Motilitas semen segar}} \times 100\%$$

Data analysis

All data obtained were analyzed using Analysis of Variance (ANOVA) followed by Duncan's test using SPSS 25 for Windows.

RESULTS AND DISCUSSION

Characteristics of Fresh Semen

Fresh semen used in this study came from the collection of 2-3 year old landrace pigs. Semen was evaluated macroscopically and microscopically. The results of macroscopic examination showed that the volume was 137 ± 20.61 mL, milky white color, typical odor of pig semen, medium semen consistency and pH 6.55 ± 0.17 . In general, the characteristics of normal fresh pig semen according to (Dongkot et al., 2022) stated that the volume of pigs without gelatin ranges from 200-250 mL, white color with spermatozoa concentration ranging from $200-300 \times 10^6$ cells/mL of semen (Sumardani et al., 2008) and medium consistency, the percentage of abnormalities is less than 20% while for the average pH of 6.7, (Sumardani et al., 2008) which states that the average pH is 7.40.

The results of microscopic examination showed spermatozoa motility, namely mass motility (+) and individual motility of 70%. These results are not much different from the results of the study by ((Yusuf et al., 2017) who reported that spermatozoa motility ranged from 76.31%. There are several factors that affect spermatozoa, including the amount of ejaculate, collection interval, male condition, and the environment of (Yusuf et al., 2017). The results of the viability examination of live spermatozoa were identified by not absorbing color (transparent) on the head of the spermatozoa, while dead spermatozoa were characterized by absorbing color on the head of the spermatozoa due to increased spermatozoa permeability. The average spermatozoa viability from this study was 91.45

This 5.81% result is in accordance with the statement of (Jhonson, 2000) that the percentage of abnormal boar spermatozoa per ejaculate should not exceed 20%. In general, abnormalities in spermatozoa can be caused by various factors including genetics, stress, environmental temperature, disease. The results of this study are higher than the results of the study by (Lawa, 2021) which was 85.50%.

The results of the study showed that the percentage of spermatozoa abnormalities reached and even semen freezing cryopreservation treatment (Arifiantini, 2012)

Effect of Different Glycerol Concentrations on Frozen Sperm Motility of Landrace Pigs

Motility is the progressive movement of spermatozoa, or their active forward movement. The ability of spermatozoa to move progressively is positively correlated with their fertility. Sperm motility is crucial for their forward movement in the female genital tract, ultimately fertilizing the ovum. The minimum requirement for post- *thawing sperm motility* for insemination is 30% (SNI, 2023).

Table 1. Effect of Different Glycerol Concentrations on Frozen Sperm Motility of Landrace Pigs

Evaluation Stages	Treatment				P-Value
	P1	P2	P3	P4	
PP	76.25 ± 4.33^a	76.25 ± 4.33^a	75.63 ± 5.15^a	75.63 ± 5.15^a	0.99
PE	72.50 ± 2.89^a	72.50 ± 2.89^a	72.50 ± 2.89^a	72.50 ± 2.89^a	1.00

PT	21.75±6.04 ^a	26.22±10.48 ^a	23.01±5.24 ^a	23.69±7.07 ^a	0.85
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Description: ^{a, b} The same superscript in the same row does not indicate a significant difference (P>0.05). PP = Post-Dilution, PE = Post-Equilibration, PT = Post-Thawing

The analysis results showed that the concentration of glycerol in egg yolk citrate diluent had no significant effect (P>0.05) on spermatozoa motility. Table 1 shows that the highest spermatozoa motility value was found in treatment P2, namely 26.22±10.48%, followed by P4 23.69±7.07%, P3 23.01±5.24% and the lowest was found in treatment P1 21.75±6.04%. The results of this study are lower than those of Yusuf *et al.* (2017) who showed that the motility value of boar spermatozoa in BT'SgT diluent was 30.00%. However, the results of this study are higher than those of Sugiantini *et al.* (2023) who showed a spermatozoa motility value of 22% and also in the study of Koelima *et al.* (2022) showed a post-thawing motility value of 15% for Duroc boar semen. This result does not meet the Indonesian National Standard (SNI, 2024), which is a post-thawing motility value of 30%. This is suspected because the diluent used did not perfectly protect the spermatozoa from the effects of *cold shock*, resulting in a very high sperm mortality rate. The low motility value found in P1, namely 21.75±6.04%, is suspected because the spermatozoa have not perfectly adapted to the diluent. This results in the spermatozoa experiencing cold shock during freezing, which causes a drastic decrease in spermatozoa.

The low motility after thawing is likely related to the composition of fatty acids and phospholipids that differ from other livestock, namely the composition of phosphatidylethanolamine and sphingomyelin in the plasma membrane of pig spermatozoa reaches 24% and 14% (Garner and Hafez, 2000). This causes the permeability of the spermatozoa plasma membrane to decrease and result in the death of young spermatozoa. The composition of semen plasma proteins and spermatozoa membranes that vary between individuals also cause loss of motility and viability of spermatozoa due to freezing.

Effect of Different Glycerol Concentrations on the Vulnerability of Frozen Landrace Pig Sperm

Sperm viability is an indicator for assessing semen quality. The higher the percentage of spermatozoa viability, the better the semen quality (Herdis and Rizal, 2008). Viability assessment is carried out objectively using differential staining. Live spermatozoa are characterized by transparent spermatozoa heads because they do not absorb dye (eosin), while dead spermatozoa heads will be red because they absorb dye (eosin). Examination of spermatozoa viability is important because it affects spermatozoa motility. The higher the spermatozoa viability, the higher the spermatozoa motility because only live spermatozoa can move. However, the percentage of spermatozoa viability is higher than motile spermatozoa because the number of live spermatozoa is not necessarily all progressively motile (Kostaman and Sutama, 2006).

Table 2. Effect of Different Glycerol Concentrations on the Vulnerability of Frozen Landrace Pig Sperm

Evaluation Stage	Treatment				P-value
	P1	P2	P3	P4	
PP	91.13±2.82 ^a	90.65±3.24 ^a	90.62±3.51 ^a	90.24±2.95 ^a	0.98
PE	87.05±0.80 ^a	87.60±8.91 ^a	88.09±1.22 ^a	88.13±1.67 ^a	0.56
PT	35.27±6.50 ^a	47.32±19.82 ^a	44.56±10.01 ^a	45.88±11.60 ^a	0.33

Note: The same superscript ^{a,b} in the same row does not indicate a significant difference (P>0.05).

PP = Post Dilution, PE = Post Equilibration, PT = Post Thawing .

The results of statistical analysis showed that the viability of spermatozoa after *the wing was not* significantly different (P <0.05), in Table 2 it was known that the highest spermatozoa viability value was found in the P2 treatment, namely 47.32 ± 19.82% followed by P4 45.88 ± 11.60%, P3 44.56 ± 10.01% and the lowest was found in the P1 treatment 35.27 ± 6.50%. The results of the study were higher than the study (Dongkot., et al 2022) with a viability value of 45.44 ± 3.29% in modified tris diluent with different equilibration times, and lower than the study by Marlize *et al.* (2021) with a value of 58.21 ± 12.70%.

The decrease in the percentage of spermatozoa viability may be caused by the condition of the spermatozoa plasma membrane which has been damaged. The spermatozoa plasma membrane functions as a guard for cell organelles and regulates electrolyte balance in metabolism (Salmah, 2014). If the plasma membrane is damaged, spermatozoa metabolism is disrupted and spermatozoa lose their fertility due to the release of cellular components and the loss of the ability to inactivate important enzyme protein components in the acrosome. This event causes spermatozoa death and will impact spermatozoa viability (Yulnawati and Agus, 2005).

Effect of Different Glycerol Concentrations on Abnormalities of Frozen Sperm of Landrace Pigs

Abnormality is one of the indicators in determining the quality of sperm because abnormal cell structures can cause disturbances and obstacles during fertilization (Afiati *et al.* , 2015). Abnormal forms of spermatozoa in this study were mostly secondary abnormalities such as curled tails, broken necks, broken necks, and double tails. Secondary abnormalities were caused by treatment when making smear preparations and other environmental factors Solihati *et al.* (2008), while according to Arifantini *et al.* (2005) abnormal spermatozoa are usually caused by nutritional and endocrine imbalances.

Table 3. Effect of Different Glycerol Concentrations on Abnormalities of Frozen Landrace Pig Sperm

Evaluation stage	Treatment				P-Value
	P1	P2	P3	P4	
PP	5.56±0.8 ^a	5.70±1.13 ^a	5.67±0.89 ^a	5.37±0.98 ^a	0.96
PE	6.52±1.19 ^a	6.73±0.77 ^a	6.72±0.83 ^a	6.70±1.06 ^a	0.98
PT	11.92±1.99 ^a	10.78±1.75 ^a	10.77±0.74 ^a	11.28±0.90 ^a	0.65

Note: The same superscript ^{a,b} in the same row does not indicate a significant difference (P>0.05).

PP = Post Dilution, PE = Post Equilibration, PT = Post Thawing .

The results of statistical research show that spermatozoa abnormalities after thawing have no significant effect ($P>0.05$), in table 3 it is known that the highest value of spermatozoa abnormalities was found in treatment P1, namely $11.92\pm1.99\%$ followed by P4 $11.28\pm0.90\%$, P2 $10.78\pm1.75\%$ and the lowest was found in treatment P3 $10.77\pm0.74\%$. This is because the administration of glycerol at an optimal level is able to reduce the increase in abnormalities that occur due to lipid peroxidation simultaneously. The cryoprotectant commonly used in freezing mammalian semen is glycerol. This glycerol is known as an intracellular cryoprotectant (Holt, 2000). Glycerol can protect spermatozoa cells on the inside during freezing from ice crystals that damage spermatozoa. The results of this study are higher when compared to the study by Mega et al. 2022 with a spermatozoa abnormality value of $4.85 \pm 0.57\%$ in frozen landrace pig semen in a modified durasperm diluent with palmyra fruit water.

Recovery Rate

The recovery rate (RR) is the ability of spermatozoa after freezing, comparing the percentage of motile spermatozoa in fresh semen with that in post-thawing semen. Statistical analysis showed no significant difference in spermatozoa recovery rates ($P>0.05$).

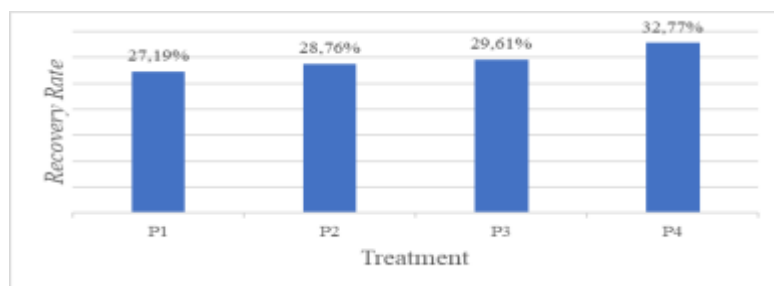


Figure 1. *Recovery Rate* of Landrace Pig Sperm.

The high RR at P4 (32.77%) indicates that the number of motile spermatozoa after thawing is also high. The results of this study differ from those reported by ((Komariah) et al., 2013) who stated that frozen spermatozoa will experience damage of around 40%. (Sulistya et al., 2015) reported that the RR values of frozen semen in three types of TF diluents, Tris Egg Yolk (T-KT) and AndroMed patent diluent (Minitub Germany) were 59.40; 63.48 and 69.66%, respectively. Meanwhile (Sulistya et al., 2015b) reported the RR values of frozen semen in three breeds of Limousin, Simmental and Frisien Holstein cattle were 58.87; 56.27 and 58.87%, respectively. Thus, the highest RR value in this study (P4) is still far below the results of the study by (Arifiantini, 2012). and This is related to the level of integrity of the spermatozoa plasma membrane, which supports the metabolic processes that produce proper sperm motility. If motility decreases, the RR will also decrease. This statement is supported by the statement (Marlize et al., 2021) that a high RR depends on the number of progressively motile spermatozoa.

CONCLUSION

It was concluded that different additions of glycerol had the same effect but were not suitable for IB.

SUGGESTION

Further research is needed using different levels of glycerol to determine the best treatment.

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