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# Frozen Semen Quality of Simmental Cattle in Various Commercial Diluents

*Kualitas Semen Beku Sapi Simmental dalam Berbagai Pengencer Komersial*

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## ABSTRACT

**This study determines the effect of different commercial diluents on the quality of frozen Simmental semen. The extraction of fresh semen from 3 Simmental bulls using an artificial vaginal method was performed twice a week. Fresh semen with motility of >70% was further processed to frozen using a one-step dilution method, and the commercial diluents used were Andromed®, Optixcell®, and Steridyl®. The diluted semen was packed into mini-straws, equilibrated, frozen, and stored for further testing. Data were analyzed by analysis of variance (ANOVA), followed by Duncan Multiple Range Test (DMRT) analysis with a 5% confidence level. The results showed that commercial diluents significantly affected motility, viability, intact plasma membrane, abnormalities, and recovery rate of frozen semen (P<0.05). Commercial diluents with different sources of lecithin reported different results on the quality of frozen semen. Steridyl® has a more optimal ability to maintain the quality of frozen semen of Simmental cattle, followed by Optixcell® and Andromed®.**

**Keywords: commercial diluents, frozen semen, Simmental**

## ABSTRAK

**Penelitian bertujuan untuk menguji pengaruh berbagai jenis pengencer komersial terhadap kualitas semen beku sapi simmental. Semen segar dari tiga ekor sapi simmental dikoleksi menggunakan metode vagina buatan dua kali dalam seminggu. Semen yang menunjukkan motilitas ≥70% diproses menjadi semen beku dengan metode pengenceran satu tahap, menggunakan pengencer komersial dengan sumber lesitin berbeda, Andromed®, Optixcell®, dan Steridyl®. Semen yang telah diencerkan dikemas dalam *mini-straw*, diekuilibrasi dan dibekukan kemudian disimpan dalam kontainer untuk pengujian lebih lanjut. Data dianalisis dengan *analysis of variance* (ANOVA), dilanjutkan dengan analisis *Duncan Multiple Range Test* (DMRT) taraf kepercayaan 5%. Hasil penelitian menunjukkan bahwa pengencer komersial (Steridyl®, Optixcell® dan Andromed®) memberikan pengaruh yang nyata (P<0.05) terhadap motilitas (49.72 ± 0.71a; 43.47 ± 0.69b; dan 36.11 ± 1.67c), viabilitas (80.33 ± 0.20a; 77.92 ± 0.16b; dan 74.54 ± 1.52c) dan membran plasma utuh (MPU) (78.85 ± 0.21a; 75.58 ± 0.28b; dan 71.86 ± 1.47c), abnormalitas (12.17 ± 0.22b; 13.64 ± 0.30a; dan 13.86 ± 0.15a), dan *recovery rate* (64.51 ± 0.92a; 56.40 ± 0.90b; dan 46.85 ± 2.17c) sperma semen beku (P<0.05). Jenis pengencer komersial dengan berbagai sumber lesitin menunjukkan hasil yang berbeda. Pengencer Steridyl® memiliki kemampuan yang lebih optimal dalam mempertahankan kualitas semen beku sapi simmental diikuti Optixcell dan Andromed.**

**Kata kunci: pengencer komersial, simmental, semen**

## INTRODUCTION

Artificial Insemination (AI) is the first biotechnology for reproduction in farm animals with their male counterpart. The advantage of AI over natural mating is getting offspring with superior genetics (Fania *et al.* 2020; Rauthan *et al.* 2022). AI is conducted using frozen semen with longer shelf-life than fresh or liquid semen. The frozen semen used came from the Artificial Insemination Center (AIC) and Regional AIC spread across several regions in Indonesia.

The process of freezing can reduce the level of sperm motility (Borges-Silva *et al.* 2016). Frozen semen produced from AIC should go through a quality and quantity evaluation process and refer to the Indonesian National Standard (SNI) 4869-1:2021 part 1. The quality is influenced by the semen diluent used, which is a nutritious liquid that can protect sperm and functions as an additional liquid volume (Susilawati 2011). The diluents in AIC/ regional AIC are homemade such as skimmed milk-egg yolk, tris-egg yolk (Novita *et al.* 2019) and commercial diluents.

Commercial diluents include Andromed®, Optixcell®, and Steridyl®. Andromed® and Optixcell® are free of animal protein, while Steridyl® still uses animal protein as the essential ingredients. Andromed® contains soy lecithin and tris-buffer, while Optixcell® contains synthetic lecithin (liposome) (Swelum *et al.* 2019). Furthermore, Steridyl® is a commercial diluent based on tris and sterile egg yolk (Nikitkina *et al.* 2020). Andromed® composed of buffer (tris-(hydroxymethyl)-aminomethane, citric acid), sugar (fructose), antioxidants, glycerol, water, and four kinds of antibiotics (tylosin tartrate, gentamycin sulfate, spectinomycin, and lincomycin). Optixcell® diluents are made of the same ingredients but contain lecithin in the form of liposomes. The three diluents have the same composition and characteristics, maintaining a pH between 6.8 and 7.2. They also contain antioxidants to reduce oxidative stress and cold shock (Bustani and Baiee 2021).

Simmental cattle are imported from Europe with a dual function of high milk and meat production. These cattle can also adapt and breed well in the conditions of Indonesia (Agung *et al.* 2014). They have a high growth rate, produce carcasses with little fat, and have a good selling price (Khairi 2016; Muada *et al.* 2017; Kurnia *et al.* 2020). The demand for frozen Simmental semen straws is very high, and individual factors, livestock breeds, and different types of diluents affect the success of freezing (Sukmawati *et al.* 2014). This study aimed to observe the quality of frozen semen in the Andromed®, Optixcell®, and Steridyl® diluents in Simmental cattle.

## MATERIALS AND METHODS

### Research Time and Location

The research was carried out from November 2021 to February 2022 at Ungaran Artificial Insemination Center, Central Java.

### Semen Source

Three to four years old, healthy Simmental bulls named Brian, Leonardo, and Osmond were used. They were housed individually and maintained according to the Ungaran AIC standard. Semen collection was routinely conducted twice a week using an artificial vagina.

### Procedure

The procedure was performed by microscopically evaluating the frozen semen to find post-thawing motility (PTM), sperm viability, intact plasma membrane (IPM), recovery rate (RR), and sperm abnormalities.

**Evaluation of frozen semen sperm motility after thawing**

Motility is assessed by the number of sperms that move progressively and is determined quantitatively by manually calculating the number of cells from five fields of view (Susilawati 2013).

**Evaluation of frozen semen viability after thawing**

The viability was evaluated after thawing using eosin-nigrosine staining, and 200 cells were analyzed. Live cells are recognized by their colorless (transparent) heads, while dead sperms are marked by color-absorbing sperm heads (Arif *et al*. 2020).

**Evaluation of intact plasma membrane (IPM) frozen sperm semen after thawing**

The intact plasma membrane (IPM) was evaluated using the hypoosmotic swelling (HOS) test (7.35 g sodium citrate and 13.51 g fructose per 1 L of aquabidest at 37°C for 60 minutes) (Padrik *et al.* 2012) after thawing.

**Evaluation of frozen semen sperm abnormalities after thawing**

The sperm morphological abnormality of frozen semen was evaluated after thawing using eosin-nigrosine staining (0.2 g of eosin, 2 g of nigrosin, mixed with 100 mL of distilled water) was used to evaluate sperm viability (Pardede *et al.* 2021). The preparations were observed under a 400x magnification microscope for ten fields of view, or at least 200 sperms (Varasofiari *et al.* 2013).

**Evaluation of frozen semen sperm recovery rate after thawing**

The recovery rate of frozen semen was evaluated after thawing in 37 degree celcius for 30 seconds, by comparing the sperm motility with the percentage of fresh semen (Rasad *et al.* 2019).

### Data Analysis

A randomized block design (RBD) was used with three trademarks of commercial diluents and four variable re-measurements (Table 1).

## RESULTS AND DISCUSSION

### Simmental Cattle Individual Profiles

The Simmental cattle used came from Australia, whose body weight ranged from 762 kg to 897 kg. Body length, height, and chest circumference were 163 cm to 185 cm, 144.5 cm to 148 cm, and 200 cm to 211 cm, respectively. Furthermore, the scrotal circumference ranged from 41 cm to 43 cm. The physical characteristics of individual bulls are one of the determining factors for the quality and quantity of semen. Ismaya (2014) explained that the size of the scrotum positively correlates with the testes' weight. The testes are

Table 1. Experimental design of this study

| Codes | Descriptions |
|---|---|
| P1 | Brian's semen diluted with Andromed® diluent |
| P2 | Leonardo's semen diluted with Andromed® diluent |
| P3 | Osmond's semen diluted with Andromed® diluent |
| P4 | Brian's semen diluted with Optixcell® diluent |
| P5 | Leonardo's semen diluted with Optixcell® diluent |
| P6 | Osmond's semen diluted with Optixcell® diluent |
| P7 | Brian's semen diluted with Steridyl® diluent |
| P8 | Leonardo's semen diluted with Steridyl® diluent |
| P9 | Osmond's semen diluted with Steridyl® diluent |

the primary influence on the quality of the sperm produced. However, as the bull ages, the positive correlation between the size of the scrotum and the testes' weights decreases. This is because there is more fatty deposit under the skin of the scrotum.

### Simmental Cattle Fresh Semen Quality

The average quality of fresh semen macroscopically and microscopically is listed in Table 2. Fresh semen from the four collections has good quality. The average sperm motility was eligible for freezing. Fresh semen that can be frozen according to the Indonesian National Standard (SNI) 4869-1:2021 part 1 should have a motility of 70%. Sperm concentration is quite high, with a low percentage of abnormalities. Semen volume, motility, and sperm concentration are important factors, as they are multipliers in semen dilution. The quality of fresh semen from Simmental bulls used in this study was very good and met the requirements for freezing fresh semen. It is caused the animals used are selected bulls kept in a bull rearing system according to the AIC SOPs.

### Sperm Motility of Simmental Frozen Cattle Semen in Various Commercial Diluents

The sperm's mobility during the fertilization of an egg is known as motility (Ratnawati and Luthfi 2020) and is one of the important factors in determining the quality of frozen semen and predicting fertilization ability (Chakraborty and Saha 2022). The type of diluent utilized had a significant impact on the evaluated frozen semen's motility. There are several types of motility in sperm, including total and progressive motility, which is the forward movement of sperm. This movement is needed to reach the site of fertilization in the female genital tract (Yumte *et al.* 2013). The three diluents, namely Andromed®, Optixcell®,

Table 2. Fresh semen quality of Simmental cattle

| Characteristic | Mean ± SE | SNI 4869.1 |
|---|---|---|
| Semen volume (mL) | 7.36 ± 1.32 | |
| pH | 6.47 ± 0.13 | |
| Semen consistency | Thick | |
| Color | Cream | |
| Mass movement | 3.00 ± 0.00 | |
| Sperm motility (%) | 77.08 ± 2.57 | >70% (2021) |
| Sperm viability (%) | 91.64 ± 0.86 | |
| The integrity of the sperm plasma membrane (%) | 90.17 ± 0.76 | |
| Sperm consentration (106) | 1.799.2 ± 279.25 | |
| Sperm abnormality (%) | 4.72 ± 0.61 | <20% (2021) |

and Steridyl® gave different values of frozen semen sperm motility ($P<0.05$). The motility quality requirement in SNI number 4869-1:2021 part 1 is 40% (Badan Standardisasi Nasional, 2021). In this study the results showed that the sperm motility of frozen semen in the Steridyl® diluent was the highest, followed by the Optixcell®, and the lowest in the Andromed® (Table 3). This is presumably because the Steridyl® diluent contains sterile egg yolk as one of its constituent components. Egg yolk contains anti-cold shock compounds because of the lipoproteins and lecithin they contain; glucose and vitamins are readily soluble in water and have a viscosity that can be beneficial for spermatozoa (Arif *et al.* 2020). This is comparable to the results of a study by Cresphilho *et al.* (2012), who evaluated the effectiveness of a diluent with different sources of lecithin in Nellore bulls that egg yolk-based diluent had better progressive motility (40.48%) when compared to soy-lecithin based extender (24.90%) ($P<0.05$). During the cooling process, sperm quality is preserved in part through the use of sperm diluents. Mukhlis *et al.* (2017) stated that post-thawing sperm motility decreases due to the freezing process.

The content of low-density lipoprotein (LDL) derived from egg yolks in the Steridyl® diluent can defend sperm from damage caused by clotting. The cold shock in sperm will decrease flagella activity and sperm motility (Pamungkas and Krisnan 2017). In this study, the value of sperm motility in the Andromed® diluent did not meet the quality requirements of SNI number 4869-1:2021 Part 1. This is thought to be caused by lipid peroxidation during the freezing process until thawing, thereby reducing sperm quality after thawing. This biochemical process inhibits glycolysis and sperm motility (Dasrul *et al.* 2012). Another

Table 3. Sperm motility of frozen semen of Simmental cattle in various commercial diluents

| Simmental individuals | Bull codes | Diluents | | |
|---|---|---|---|---|
| | | Andromed (%) | Optixcell (%) | Steridyl (%) |
| Brian | 61876 | 38.75 ± 2.39 | 44.17 ± 1.08 | 50.00 ± 1.52 |
| Osmond | 61879 | 33.33 ± 4.14 | 44.58 ± 1.25 | 49.17 ± 1.08 |
| Leonardo | 61881 | 36.25 ± 1.72 | 41.67 ± 0.96 | 50.00 ± 1.36 |
| Mean ± SE | | 36.11 ± 1.67c | 43.47 ± 0.69b | 49.72 ± 0.71a |

Means in the same row with different superscript differ significantly ($P<0.05$)

Table 4. Viability of Simmental cattle frozen semen in various commercial diluents

| Simmental individuals | Bull codes | Diluents | | |
|---|---|---|---|---|
| | | Andromed (%) | Optixcell (%) | Steridyl (%) |
| Brian | 61876 | 76.29 ± 0.99 | 78.03 ± 0.26 | 80.27 ± 0.45 |
| Osmond | 61879 | 71.73 ± 4.49 | 78.20 ± 0.33 | 80.33 ± 0.24 |
| Leonardo | 61881 | 75.60 ± 0.54 | 77.52 ± 0.14 | 80.38 ± 0.41 |
| Mean ± SE | | 74.54 ± 1.52c | 77.92 ± 0.16b | 80.33 ± 0.20a |

Means in the same row with different superscript differ significantly (P<0.05)

possibility is the compatibility of the cattle breed with the Andromed® diluent. In another study on Brangus-Hereford cattle, the Andromed® (76.25%) diluent performed better than (P<0.05) the tris-egg yolk based extender (65.58%) (Ghareeb *et al.* 2017). Penggunanaan berbagai sumber lesitin untuk pengencer semen beku sampai saat ini masih mejadi perdebatan yang serius.

**Viability of Simmental Cattle Frozen Semen in Various Commercial Diluents**

Viability is the percentage of live sperm in semen. Live sperm is marked with the head not absorbing color (transparent), while the dead sperm absorbs the red-purple color when it is given eosin-nigrosin staining (Marlize *et al.* 2021). The sperm absorbs color because there is a damage of membrane cells (Mahfud *et al.* 2019). The ability to survive after freezing is an important aspect of males' selection for AI.

The results showed that the diluent factor affected (P<0.05) the viability of frozen semen (Table 4). The highest sperm viability value was found in the Steridyl® diluent, followed by Optixcell® and Andromed®. Furthermore, the viability values obtained from the three diluents were classified as normal. According to Handayani *et al.* (2021), the viability obtained is good because the live semen percentage is still above 50%. The decrease can be explained by the freezing process and reduction in temperature. Membrane damage caused by extreme temperature drops occurs due to the shrinking of the fluid in the membrane (Zenteno *et al*. 2023). Swelling and shrinking are sperm responses to osmotic changes that can cause cell death or significant loss of membrane integrity (Mughal *et al*. 2018). Intracellular cryoprotectants such as glycerol can limit the formation of ice crystals within cells (Anzar *et al*. 2019). Glycerol will enter the cell, replace some of the free water, and push out electrolytes, lowering the intracellular concentration of these electrolytes and reducing damage to sperm (İnanç *et al.* 2018).

Priyanto *et al.* (2015) reported that sperm experienced a decrease in extreme temperatures twice. The first decrease occurred during equilibration, from room temperature to 5 oC. The second decrease occurred in a container containing liquid nitrogen with a temperature of approximately -130 oC. A cold shock can cause cell damage, especially in the plasma membrane.

**Intact Plasma Membrane (IPM) Simmental Frozen Sperm in Various Commercial Diluents**

The integrity of the sperm is the evaluation of the plasma membrane using the hypoosmotic swelling (HOS) test method (Luzardin *et al.* 2020). The plasma membrane regulates the entry and exit of substances and ions needed for metabolism and protects cell organelles from mechanical damage (Arsiwan *et al.* 2014). IPM evaluation has a positive correlation with sperm viability. The high percentage of sperm viability can be caused by low plasma membrane damage. The highest IPM value was found in frozen semen in Steridyl® diluent, followed by Optixcell® and Andromed®. The results showed that the diluent factor affected (P<0.05) the percentage of IPM of frozen semen (Table 5).

The IPM values were quite good, ranging from 71.86% to 78.85%, with the value of frozen semen in Steridyl® diluent was better than Optixcell® and Andromed®. The values resulting from this study are higher than the report by Sukirman *et al.* (2019) used frozen semen of Simmental cattle with different types of extenders (Andromed and skim-egg yolk) which were 70.76% and 66.63%.

The three commercial diluents used in this research could maintain the integrity of the plasma membrane. Raheja *et al.* (2018) lecithin has the membrane coating properties by maintaining the phospholipid bilayer configuration of the spermatozoa. Furthermore, according to İnanç *et al.* (2018), The most common membrane-permeable cryoprotectant is glycerol (G), which limits ice formation inside cells. Glycerol entering the cell will replace some of the free water and push electrolytes out, reducing the intracellular

Table 5. IPM of Simmental cattle frozen semen in various commercial diluents

| Simmental individuals | Bull codes | Diluents | | |
|---|---|---|---|---|
| | | Andromed (%) | Optixcell (%) | Steridyl (%) |
| Brian | 61876 | 73.58 ± 0.69 | 75.61 ± 0.31 | 79.04 ± 0.44 |
| Osmond | 61879 | 68.83 ± 4.18 | 76.19 ± 0.32 | 78.38 ± 0.25 |
| Leonardo | 61881 | 73.16 ± 1.07 | 74.93 ± 0.64 | 79.14 ± 0.38 |
| Mean ± SE | | 71.86 ± 1.47c | 75.58 ± 0.28b | 78.85 ± 0.21a |

Means in the same row with different superscript differ significantly (P<0.05)

Table 6. Abnormalities of frozen semen of Simmental cattle in various commercial diluents

| Simmental individuals | Bull codes | Diluents | | |
|---|---|---|---|---|
| | | Andromed (%) | Optixcell (%) | Steridyl (%) |
| Brian | 61876 | 14.02 ± 0.32 | 13.02 ± 0.39 | 12.44 ± 0.60 |
| Osmond | 61879 | 13.93 ± 0.19 | 14.53 ± 0.53 | 12.12 ± 0.37 |
| Leonardo | 61881 | 13.62 ± 0.26 | 13.38 ± 0.36 | 11.96 ± 0.14 |
| Mean ± SE | | 13.86 ± 0.15a | 13.64 ± 0.30a | 12.17 ± 0.22b |

Means in the same row with different superscript differ significantly (P<0.05)

concentration of these electrolytes and reducing their destructive power to spermatozoa (Grötter *et al.* 2019). Therefore, the IPM values were still within normal values.

**Abnormalities of Simmental cattle frozen semen in various commercial diluents**

Damage can occur during the formation of sperm in the seminiferous tubules and the transport process through the genital tract of male cattle (Manehat *et al.* 2021). The results showed that the diluent factor had a significant effect (P<0.05) on the abnormality of frozen semen as indicated in Table 6. Primary and secondary abnormalities usually occur in sperm cell damage. Primary abnormalities are visible in the head, some of which are genetic, and can cause inertility. Meanwhile, secondary abnormalities in the tail will be easily selected when evaluating sperm motility. The abnormality of sperm to be frozen should not be more than 20%. This is stated in SNI Number 4869-1:2021, part 1, regarding frozen cattle semen and Ministry of Agriculture 10 of 2016. Sperm that exceeds this percentage has low conception power.

The results in frozen semen of Simmental cattle showed that total abnormality is low in the semen in Steridyl® diluent. Sperm abnormality values in Optixcell® and Andromed® diluent were the same, below 20%. Yendraliza *et al.* (2019) showed abnormalities of frozen semen of Bali cattle in Andromed® diluent, with various percentages of carrot juice after thawing, to be around 5.17% to 10.50%. Utami and Tophianong (2014) showed that the highest abnormality of sperm thawed in water at 8 °C and 37 °C was 20% and 23%. Abnormal data in previous studies using frozen semen of non-sexed Limousine cattle was 6.93 ± 1.37% (Mahfud *et al.* 2019). Differences in abnormality values can be caused by various factors, including cattle breeds, individual cattles, freezing techniques, and the examiner's skills.

**Simmental Sperm Recovery Rate of Frozen Cattle Semen in Various Commercial Diluents**

The results showed that the diluent factor affected (P<0.05) the recovery rate (RR) of frozen semen (Table 7). Recovery rate is the ability of sperm to recover after freezing by comparing the percentage of motility after thawing with fresh semen (Fazrien *et al.* 2020). Table 7 indicated that the highest recovery rate values were in Steridyl® diluent, followed by Optixcell® and Andromed®.

These results indicate that each diluent produces sperm with different cell recovery capabilities. The decreased spermatozoa motility from fresh semen to post-thawing motility was due to the plasma membrane condition damaged during the freezing phase. Steridyl® diluent has the highest recovery rate values, which means the ability to recover after freezing is higher. This is comparable to the opinion of Swelum *et al.* (2019) that diluents based on egg yolk lecithin were able to maintain sperm motility after thawing better than diluents based on liposomes or soy lecithin. Other components, such as antioxidants, affect sperm quality after freezing (Miguel *et al.* 2021). It causes antioxidants capable of preventing lipid peroxidation, which can cause loss of motility, a decrease in cell fructolysis and respiration activity and leakage of cell fluid through damage (Haris *et al.* 2020). The presence of antioxidants in the diluent breaks the chain reaction and protects the membrane from damage caused by reactive oxygen species (ROS) (Azura *et al.* 2020).

The recovery rate is also influenced by the diluent and the season when the frozen semen production is carried out. Aisah *et al.* (2017) report showed that the sperm recovery rate of frozen semen for Bali cattle in the rainy and summer season was 61.80% and 66.79%. Other studies on Limousine cattle also showed the same results, where the

Table 7. Recovery Rate of frozen semen of Simmental cattle in various commercial diluents

| Simmental individuals | Bull codes | Diluents | | |
|---|---|---|---|---|
| | | Andromed (%) | Optixcell (%) | Steridyl (%) |
| Brian | 61876 | 50.27 ± 3.11 | 57.30 ± 1.40 | 64.87 ± 1.97 |
| Osmond | 61879 | 43.25 ± 5.37 | 57.84 ± 1.62 | 63.79 ± 1.40 |
| Leonardo | 61881 | 47.03 ± 2.23 | 54.06 ± 1.25 | 64.87 ± 1.77 |
| Mean ± SE | | 46.85 ± 2.17c | 56.40 ± 0.90b | 64.51 ± 0.92a |

Means in the same row with different superscript differ significantly (P<0.05)

recovery rate in the rainy and dry seasons is around 62% and 67% (Sunami *et al.* 2017). This study's recovery rate values are still good, especially for those in Steridyl® diluent.

Different sources of lecithin used have been shown to affect the quality of frozen semen of Simmental cattle. Diluents containing lecithin from egg yolks are still better than other sources. Furthermore, computer-assisted sperm analysis (CASA) tests may give different results. The diluents with egg yolk lecithin and liposomes can be used as alternative diluents for Simmental cattle semen freezing.

## CONCLUSION

In this study, steridyl® diluent for Simmental cattle semen freezing was better than Optixcell® and Andromed®. However, semen frozen in the Optixcell® and Andromed® diluent was also eligible for insemination.

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