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Quality of Sperm Simmental Bulls and Success of Artificial Insemination with the Addition of Nanocalcium Phosphate in Tris Aminomethane Egg Yolk Diluent Using Semen Storage Ampoules from Nanocalcium Silicophosphate Biomaterials

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Abstract: Success in Artificial Insemination (AI) is largely determined by the quality of the stored semen, therefore storage devices, especially straws or ampoules, are an important part that cannot be separated from the overall semen cryopreservation technology. Making a prototype of semen storage ampoules from nanocalcium silicophosphate biomaterials is based on the need for straws or ampoules. Semen storage in spermatozoa cryopreservation technology for the needs of AI and livestock breeding and from the results of an examination of previously patented inventions. The aim of this research was to determine the quality of simental cow sperm and the success of AI with the addition of nanocalcium phosphate to the Tris aminomethane egg yolk diluent using semen storage ampoules made from nanocalcium silicophosphate biomaterial. This research method uses laboratory research with a factorial Completely Randomized Design (CRD) consisting of 2 factors, namely the diluent factor consisting of 2 treatments (tris aminomethane egg yolk and tris aminomethane egg yolk + phosphate 1% w/v) and the shelf life factor. Consisting of 5 treatments (0, 1, 2, 3, 4 days). The second factor is the use of ampoules and without ampoules. Each treatment was repeated 10 times. The results of the research showed that the addition of nanocalcium phosphate to the egg yolk tris aminomethane diluent using semen storage ampoules made from nanocalcium silicophosphate biomaterial had a very significant effect ($p<0.01$) on motility, viability and abnormalities. Likewise, the AI results show the NRR, CR, and S/C values in the good category. This study concluded that the addition of phosphate to the egg yolk tris aminomethane diluent can increase the durability of semen for up to three days at room temperature and with storage at 5°C for up to 3-5 days, likewise, the results of artificial insemination success tests are also good and appropriate standard.

Keywords: Artificial Insemination, Nanocalcium Phosphate, Nanocalcium Silicophosphate Biomaterials, Semen Storage Ampoules, Sperm Quality

Introduction

The manufacture of a prototype semen storage ampoule from nano calcium silicophosphate biomaterial (Karyasa, 2021) was based on the need for semen storage straws or ampoules in spermatozoa cryopreservation technology for the needs of Artificial Insemination (AI) and livestock breeding and from the results of examination of previously patented inventions (Kusumawati, 2021; Kusumawati and Karyasa, 2022; Karyasa, 2018). Success in AI is largely determined by the quality of the stored semen, therefore storage tools,

especially straws or ampoules, are an important part that cannot be separated from the overall semen cryopreservation technology (Kusumawati *et al*., 2019a). Straw material is generally made from thermoplastic material which is very susceptible to damage when cooling at low temperatures and during the thawing of semen after being stored at low temperatures due to biotic and abiotic stress from spermatozoa in the cooled semen. Plastic materials tend to be susceptible to producing microplastics when cooled at high temperatures which can poison or damage the ability of spermatozoa cells (Trifuoggi *et al*., 2019; Tallec *et al*., 2020; Hou *et al*., 2021; D'Angelo and Meccariello, 2021). In addition, inventions related to semen storage ampoules or straws for AI (Gilligan, 2021; Stroud, 2020; Schmitt *et al*., 2019; Ainley *et al*., 2019; Schmitt *et al*., 2020; Watanabe, 2020; Van Kappel-Dufour *et al*., 2021) have weaknesses related to indicators that indicate whether the semen, especially spermatozoa in the stored semen, is still of good quality or it has actually been damaged. This is important because the effectiveness of AI is considered to be still low as stated by several researchers (Rahman *et al*., 2020; Zubor *et al*., 2020; Sutriana *et al*., 2021) such as AI has a pregnancy success of 29.55% compared to natural success rates of 61.28%, even though the resulting calf death from AI only 3.85%, much lower than naturally, with mortality of 10.71% and calf birth weight of 24.5 kg, which is greater than naturally 20.1 kg (Rahman *et al*., 2020).

Therefore, improvements in artificial insemination technology are needed. One of them is a semen storage straw or ampoule which is safe and resistant to cold temperatures and has the ability to absorb water (secretion from damaged cells during storage or during thawing and transfer to animals in AI) by providing a color change so that if the semen is damaged or spermatozoa in the straw or ampoule can be detected. Semen or spermatozoa that are detected as damaged are not used for AI, only healthy or undamaged semen or spermatozoa are used so that the success of AI is better.

Materials and Methods

The material that was the object of observation in the research was fresh semen from a Simmental cow with 70% motility. Simmental cow semen was obtained at the Singosari National Artificial Insemination Center (SNAIC), Malang Regency, Indonesia.

This research method uses laboratory research with a factorial Completely Randomized Design (CRD) consisting of 2 factors, namely the diluent factor consisting of 2 treatments (tris aminomethane egg yolk and tris aminomethane egg yolk + phosphate 1% w/v) and the shelf life factor. Consisting of 5 treatments (0, 1, 2, 3, 4 days). The second factor is the use of ampoules and without ampoules. Each treatment was repeated 10 times.

Research Procedure

a. Collection of fresh semen: Fresh semen was taken from SNAIC and taken to the laboratory at Universitas PGRI Kanjuruhan Malang, Indonesia
b. Observation of motility, viability, and abnormalities: Observations of motility, viability, and abnormalities were carried out at the integrated laboratory of Universitas PGRI Kanjuruhan Malang, Indonesia using a microscope
c. Addition of diluent: The addition of tris Aminomethane egg yolk diluent made by SNAIC was carried out in a 1:1 ratio using 5 mL of fresh semen and 5 mL of egg yolk Tris aminomethane diluent added to the semen container and mixed after mixing it was placed at room temperature. Phosphate was added at 1% w/v
d. Storage at room temperature and 5°C: Storage is carried out at room temperature (25°C) and 5°C by placing the semen in ampoules or semen straws made from nanocalcium silicophosphate biomaterial. The ampoules were made in the Universitas Pendidikan Ganesha of Education Laboratory, Bali, Indonesia. Nano silica gel will change color if it absorbs water from organic preparations such as animal or human semen stored in the ampoule. If damage occurs which causes hydrolysis of the cells of the organic preparation, the water that comes out will be absorbed by this nano silica gel and will show a color change along with the amount of water it absorbs
e. Observation of motility, viability, and abnormalities in sperm

Motility

The motility of individual spermatozoa can be measured by dripping semen in the center of the glass object then covering it with a covered glass and observing under a microscope with a magnification of 400×. Individual motility assessment is carried out by observing the progressive movement which is shared with the total sperm which is counted and expressed as a percentage (%) with the formula:

$$\frac{Progressive\ spermatozoa\ cells}{Total\ spermatozoa\ observed} \times 100\%$$

Viability

Examination of spermatozoa viability begins by dripping 1 drop of eosin negrosine on a glass object, then adding 1 drop of semen mixed with the other end of the glass object then making a smear preparation and heating it over a Bunsen. After drying, the preparation was observed under a light microscope with 400x magnification. Two hundred spermatozoa were evaluated

in at least five different fields, eosin negrosin penetrated dead spermatozoa cells so that they colored red. Viability is expressed in percent (%). Viability can be calculated using the formula:

$$\frac{The\ number\ of\ live\ spermatozoa\ cells}{Total\ spermatozoa\ observed} \times 100\%$$

Abnormalities

Examination of spermatozoa abnormalities begins by dripping 1 drop of eosin negrosine on a glass object, then adding 1 drop of semen mixed with the other end of the glass object then making a smear and heating it over a Bunsen. After drying, the preparation was observed under a light microscope with 400x magnification. Two hundred spermatozoa were evaluated in at least five different fields, eosin negrosin penetrated dead spermatozoa cells so that they colored red. Abnormality is expressed in percent (%). Abnormalities can be calculated using the formula:

$$\frac{An\ abnormal\ number\ of\ spermatozoa\ cells}{Total\ spermatozoa\ observed} \times 100\%$$

Artificial Insemination

Artificial insemination was carried out on 40 Limousin cows which were divided into 4 treatments, treatment 1 namely AI using frozen semen (control); treatment 2 AI using nano calcium silicophosphate ampoules with a Si/P ratio = 1:1 (A1B1); treatment 3 AI using nano calcium silicophosphate ampoules with a Si/P ratio = 1:2 (A1B2); treatment 4 AI used nano calcium silicophosphate ampoules with a Si/P ratio = 2:1 (A2B1). Each treatment used 10 Limousin cattle.

The Success of Artificial Insemination

Measuring the success of AI includes the Non-Return Rate (NRR) Conception Rate (CR) and Service per Conception (S/C).

Non-Return Rate (NRR)

$$\frac{Number\ of\ cows\ in\ AI\ -\ Number\ of\ cows\ in\ re\ -\ AI}{Number\ of\ cows\ in\ AI} \times 100\%$$

Conception Rate (CR)

$$\frac{Number\ of\ Pregnant\ Cows}{Number\ of\ cows\ in\ AI}\ x\ 100\%$$

Service per Conception (S/C)

$$\frac{Number\ of\ AI}{Number\ of\ pregnant\ cows}$$

The higher the CR value obtained, this illustrates that the success rate of artificial insemination in cattle in that area is better and vice versa, if the results obtained are low, the success rate of artificial insemination in that area is not good. A good CR value is at least 45-50%, if it is less than this figure the CR value is considered not good (Priyo Jr *et al*., 2020).

Research Variable

The variables observed in this study included spermatozoa quality (motility, viability, and abnormalities of spermatozoa) and AI success (NRR, CR, and S/C).

Data Analysis

The data obtained were analyzed using analysis of variance. If there is an effect of treatment then proceed with the least significant difference test.

Results

The main aim of this research is to overcome the technological weaknesses of the semen storage process in artificial insemination that previously existed, especially the need for the addition of strengthening and stabilizing agents in the mixture of semen diluent in the semen storage process and the use of ampoules in Fig. 1 which can help strengthen and stabilize the semen from changes caused by the semen storage process, which is characterized by the addition of nano calcium silicophosphate made from nano calcium silicophosphate powder made from bovine bone waste to a diluent solution of tris amino methane and egg yolk and the use of ampoules for semen storage made from nano calcium silicophosphate powder made from bovine bone waste and rice husk ash and semen storage is carried out at room temperature and 5°C without the use of cryopreservation techniques with liquid nitrogen. Another objective of this invention is to contribute to increasing the efficiency of energy use and reducing the use of expensive and environmentally unfriendly materials which contributes to the development of semen storage technology for animal breeding and breeding which contributes to safer, healthier, more efficient, and efficient livestock, environmentally friendly. The use of rice agricultural waste, namely rice husk ash as a source of silica and cow bone waste as a source of calcium phosphate for making nanocalcium silicophosphate ampoules (Fig. 1) and nanocalcium silicophosphate powder, is expected to be able to provide added value to rice agricultural waste, especially providing added value to rice husk ash and especially to cattle farming added value to beef bone waste. Motility, viability, and abnormalities of spermatozoa stored using diluent with added nanocalcium

phosphate and not stored at 5°C and room temperature (without ampoules) in Table 1 and Fig. 2. Motility, viability and abnormalities motility, viability, and abnormalities stored using diluent added with nanocalcium phosphate stored at room temperature with calcium silicophosphate ampoules with various Si/P ratios in Table 2 and Fig. 3. Motility, viability and abnormalities stored using diluent added with nanocalcium phosphate stored at 5°C with calcium silicophosphate ampoules of various Si/P ratios shown in Table 3 and Fig. 4. Spermatozoa viability (400x magnification) in Fig. 5.

To determine the fertility of spermatozoa, a fertility test is carried out using an *in vivo* (AI) application. The fertility of spermatozoa in AI is assessed through the fertilization rate from the Non-Return Rate (NRR), pregnancy rate or Conception Rate (CR), and Service per Conception (S/C). Tests for the fertilization ability of spermatozoa were carried out by AI using semen stored in nanocalcium silicophosphate ampoules with a ratio of Si/P = 1: 1 (A1B1); 1: 2 (A1B2) and 2: 1 (A2B1) and inseminated in 40 cattles ready to become pregnant. The results of NRR and CR observations can be seen in Table 4.

Fig. 1: Semen storage ampoule from nano calcium silicophosphate biomaterial

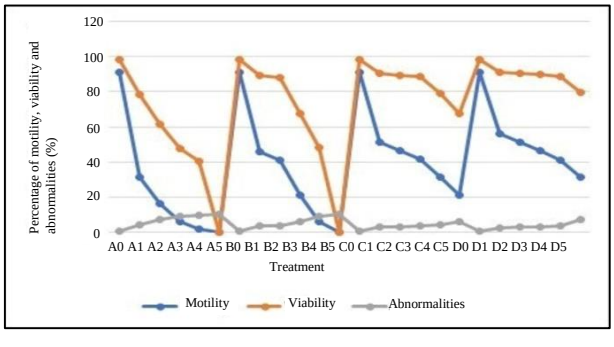

Fig. 2: Motility, viability, and abnormalities of spermatozoa stored using diluent with added monocalcium phosphate and not stored at 5°C and room temperature (without ampoules)

Table 1: Motility, viability, and abnormalities of spermatozoa stored using diluent with added nanocalcium phosphate and not stored at 5°C and room temperature (without ampoules)

Treatment	Motility (%) (Mean ± SD)	Viability (%) (Mean ± SD)	Abnormalities (%) (Mean ± SD)
A0	91,2±1,62^{k}	98,4±0,52^{k}	0,54±0,08^{a}
A1	31,3±1,57^{f}	78,2±2,86^{f}	4,47±0,16^{b}
A2	16,2±1,48^{d}	61,6±1,90^{d}	7,08±0,24^{c}
A3	6,1±1,73^{c}	47,5±3,47^{c}	8,89±0,34^{d}
A4	2,1±1,52^{b}	40,6±2,27^{b}	9,6±0,27^{d}
A5	0±0^{a}	0±0^{a}	9,99±0^{d}
B0	91,2±1,62^{k}	98,4±0,52^{k}	0,54±0,08^{a}
B1	46,1±1,45^{h}	89,4±0,52^{h}	3,325±0,1^{b}
B2	41,3±1,57^{g}	88,4±0,52^{g}	3,61±0,10^{b}
B3	21,2±1,48^{e}	67,5±2,64^{e}	6,23±0,40^{c}
B4	6,1±1,52^{c}	48,2±4,13^{c}	8,9±0,29^{d}
B5	0±0^{a}	0±0^{a}	9,998±0^{d}
C0	91,2±1,62^{k}	98,4±0,52^{k}	0,54±0,08^{a}
C1	51,3±1,57^{i}	90,5±0,53^{i}	3,01±0,1^{b}
C2	46,4±1,51^{h}	89,6±0,52^{h}	3,3±0,01^{b}
C3	41,5±1,43^{g}	88,7±0,48^{g}	3,59±0,09^{b}
C4	31,3±1,57^{f}	79±4,22^{f}	4,31±0,45^{b}
C5	21,2±1,48^{e}	67,5±2,64^{e}	6,23±0,40^{c}
D0	91,2±1,62^{k}	98,4±0,52^{k}	0,54±0,08^{a}
D1	56,1±1,52^{j}	91,4±0,52^{j}	2,665±0,18^{b}
D2	51,4±1,51^{i}	90,6±0,52^{i}	3±0,10^{b}
D3	46,5±1,43^{h}	89,7±0,48^{h}	3,29±0,09^{b}
D4	41,3±1,57^{g}	88,5±0,53^{g}	3,61±0,01^{b}
D5	31,2±1,40^{f}	79,4±22^{f}	7,24±8,7^{c}

Means with different superscripts in a row differ significantly ($p<0.01$). SD = Standard Deviation

Table 2: Motility, viability, and abnormalities motility, viability and abnormalities stored using diluent added with nanocalcium phosphate stored at room temperature with calcium silicophosphate ampoules with various Si/P ratios

Treatment	Motility (%) (Mean ± SD)	Viability (%) (Mean ± SD)	Abnormalities (%) (Mean ± SD)
A0B1-0	91,2±1,62^{s}	98,4±0,52^{o}	0,54±0,08^{a}
A0B1-1	31,3±1,57^{n}	79±4,22^{l}	4,47±0,16^{d}
A0B1-2	18,9±1,37^{j}	65,5±1,58^{j}	6,66±0,26^{g}
A0B1-3	2±0,94^{c}	34,2±8,50^{d}	9,6±0,19^{m}
A0B1-4	0±0^{a}	0±0^{a}	10,4±0,52^{n}
A0B1-5	0±0^{a}	0±0^{a}	10,5±0,53^{o}
A1B0-0	91,2±1,62^{s}	98,4±0,52^{o}	0,54±0,08^{a}
A1B0-1	11,3±1,57^{g}	57,5±2,64^{h}	7,95±0,20^{j}
A1B0-2	3,9±1,37^{d}	40,5±5,50^{e}	9,29±0,21^{l}
A1B0-3	0±0^{a}	0±0^{a}	10,6±0,52^{o}
A1B0-4	0±0^{a}	0±0^{a}	10,7±0,48^{o}
A1B0-5	0±0^{a}	0±0^{a}	10,8±0,42^{o}
A1B1-0	91,2±1,62^{s}	98,4±0,52^{o}	0,54±0,08^{a}
A1B1-1	50,3±1,57^{q}	90,4±0,52^{n}	3,065±0,10^{b}
A1B1-2	37,5±1,43^{o}	86,3±0,95^{m}	3,81±0,07^{c}
A1B1-3	16,7±1,57^{i}	62±1,83^{i}	7,01±0,26^{h}
A1B1-4	1,4±0,52^{b}	27±2,58^{c}	9,72±0,10^{m}
A1B1-5	0±0^{a}	0±0^{a}	10,2±0,42^{n}
A1B2-0	91,2±1,62^{s}	98,4±0,52^{o}	0,54±0,08^{a}
A1B2-1	55,3±1,57^{r}	91,4±0,52^{n}	2,72±0,17^{b}
A1B2-2	41,5±1,43^{p}	88,7±0,48^{m}	3,59±0,09^{c}
A1B2-3	21,6±1,51^{k}	67,1±1,85^{j}	6,14±0,40^{f}
A1B2-4	5,5±0,71^{e}	47,1±2,73^{f}	9,04±0,10^{l}
A1B2-5	0±0^{a}	0±0^{a}	10,1±0,32^{n}
A1B3-0	91,2±1,62^{s}	98,4±0,52^{o}	0,54±0,08^{a}
A1B3-1	21,3±1,57^{k}	66,3±1,57^{j}	6,22±0,41^{f}
A1B3-2	8,9±1,37^{f}	53,9±1,37^{g}	8,35±0,29^{k}
A1B3-3	0±0^{a}	0±0^{a}	10,5±0,53^{o}
A1B3-4	0±0^{a}	0±0^{a}	10,6±0,52^{o}
A1B3-5	0±0^{a}	0±0^{a}	10,7±0,48^{o}

Table 2: Continue

A2B1-0	91,2±1,62^{s}	98,4±0,52^{o}	0,54±0,08^{a}
A2B1-1	40,3±1,57^{p}	87,9±0,99^{m}	3,665±0,10^{c}
A2B1-2	27,5±1,43^{m}	72,6±1,35^{k}	4,85±0,14^{e}
A2B1-3	9,7±1,57^{f}	54,7±1,57^{g}	8,21±0,26^{k}
A2B1-4	0±0^{a}	0±0^{a}	10,3±0,48^{n}
A2B1-5	0±0^{a}	0±0^{a}	10,4±0,52^{n}
A3B1-0	91,2±1,62^{s}	98,4±0,52^{o}	0,54±0,08^{a}
A3B1-1	26,3±1,57^{l}	72,1±2,38^{k}	5,22±0,41^{e}
A3B1-2	13,9±1,37^{h}	58,7±1,70^{h}	7,53±0,31^{i}
A3B1-3	0,6±0,52^{a}	20,6±5,68^{b}	9,88±0,10^{m}
A3B1-4	0±0^{a}	0±0^{a}	10,5±0,53^{o}
A3B1-5	0±0^{a}	0±0^{a}	10,6±0,52^{o}

Means with different superscripts in a row differ significantly ($p<0.01$). SD = Standard Deviation

Discussion

Quality of Spermatozoa with the Addition of Nanocalcium Phosphate Using Ampoules and without Ampoules

Table 1 and Fig. 2, which shows the results of measuring the motility, viability and average abnormalities of 10 repetitions of fresh cow semen spermatozoa ($p<0.01$) with a storage period of 0 days for samples with a diluent solution of tris amino methane and egg yolk without the addition of 1% w/v nanocalcium phosphate powder and storage at room temperature 22-25°C (A0), samples with a diluent solution of tris amino methane and egg yolk without the addition of 1% w/v nanocalcium phosphate powder and storage at temperature 5°C (B0), sample with tris amino methane diluent solution and egg yolk added with 1% w/v nanocalcium phosphate powder and storage at room temperature 22-25°C (C0), sample with tris amino methane diluent solution and egg yolks added with 1% w/v nanocalcium phosphate powder and stored at 5°C (D0), storage time 1 day for samples with tris amino methane diluent solution and egg yolks without added 1% nanocalcium phosphate powder b/v and storage at room temperature 22-25°C (A1), samples with a diluent solution of tris amino methane and egg yolk without the addition of 1% w/v nanocalcium phosphate powder and storage at 5°C (B1), samples with a diluent solution of tris amino methane and egg yolk added with 1% w/v nanocalcium phosphate powder and stored at room temperature 22-25°C (C1), samples with diluent with the addition of 1% calcium phosphate solution and stored at a temperature of 5°C (D1), 2 days storage time for samples with tris amino methane and egg yolk diluent solution without addition of 1% w/v nanocalcium phosphate powder and storage at room temperature 22-25°C (A2), samples with diluent without addition 1% calcium phosphate solution and storage at a temperature of 5°C (B2), samples with a diluent solution of tris amino methane and egg yolk added with 1% w/v nanocalcium phosphate powder and storage at room temperature 22-25°C (C2) , sample with tris amino methane diluent and egg yolk with the addition of 1% w/v nanocalcium phosphate and storage at 5°C (D2), storage time 3 days sample with tris amino methane and egg yolk diluent without the addition of 1% nanocalcium phosphate b/v and storage at room temperature 22-25°C (A3), samples with tris amino methane diluent and egg yolk without the addition of nanocalcium phosphate 1% w/v and storage at 5°C (B3), samples with diluent with the addition 1% calcium phosphate solution and storage at room temperature 22-25°C (C3), samples with diluent with the addition of 1% calcium phosphate solution and storage at 5°C (D3) and storage time 4 days samples with diluent without adding solution calcium phosphate 1% and storage at room temperature 22-25°C (A4), sample with diluent tris amino methane and egg yolk without the addition of nanocalcium phosphate 1% w/v and storage at temperature 5°C (B4), sample with solution tris amino methane diluent and egg yolk added with 1% w/v nanocalcium phosphate powder and stored at room temperature 22-25°C (C4), samples with tris amino methane diluent and egg yolk added with 1% nanocalcium phosphate powder w/v and storage at 5°C (D4). Based on the graph in Fig. 2, the addition of 1% w/v nanocalcium phosphate has a positive effect on semen quality with indicators of average motility and viability levels (%) when stored at room temperature (group B) which are better than without the addition of 1% w/v nanocalcium phosphate (group A), as well as the addition of nanocalcium phosphate 1% w/v has a positive effect on semen quality with indicators of average motility and viability levels (%) when stored at room temperature (group D) which are better than without the addition of nanocalcium phosphate 1% w/v (group C).

Table 2 and Fig. 3, the average level of motility, viability and abnormalities of 10 sperm replications from fresh cow semen ($p<0.01$) using a diluent solution of tris amino methane and egg yolk supplemented with 1% b nanocalcium silicophosphate powder/v at room temperature for consecutive storage periods of 0 (initial), 1, 2, 3, 4 and 5 days in calcium silicophosphate ampoules with a ratio of Si/P = 0:1 with consecutive sample code A0B1-0 , A0B1-1, A0B1-2, A0B1-3, A0B1-4 and A0B1-5; Si/P ratio = 1:0 with consecutive sample codes A1B0-0, A1B0-1, A1B0-2, A1B0-3, A1B0-4 and A1B0-5, Si/P ratio = 1:1 with consecutive sample codes-respectively A1B1-0, A1B1-1, A1B1-2, A1B1-3, A1B1-4 and A1B1-5; Si/P ratio = 1:2 with sample codes A1B2-0, A1B2-1, A1B2-2, A1B2-3, A1B2-4 and A1B2-5; Si/P ratio = 1:3 with sample codes A1B3-0, A1B3-1, A1B3-2, A1B3-3, A1B3-4 and A1B3-5; Si/P ratio = 2:1 with sample code A2B1-0, A2B1-1, A2B1-2, A2B1-3, A2B1-4 and A2B1-5 respectively; and Si/P ratio = 3:1 with sample code respectively A3B1-0, A3B1-1, A3B1-2, A3B1-3, A3B1-4 and A3B1-5; Based on the data in Fig. 3, judging from the average motility, viability and abnormalities and storage time (days), the ampoule is most suitable for storing semen with a diluent solution of tris amino methane and egg yolk which is added with 1% w/v nanocalcium phosphate powder room temperature are ampoules with the composition A1B1 or nanocalcium silicophosphate

ampoules made with a Si/P ratio = 1:1 and ampoules with the composition A1B2 or nanocalcium silicophosphate ampoules made with a Si/P ratio = 1: 2.

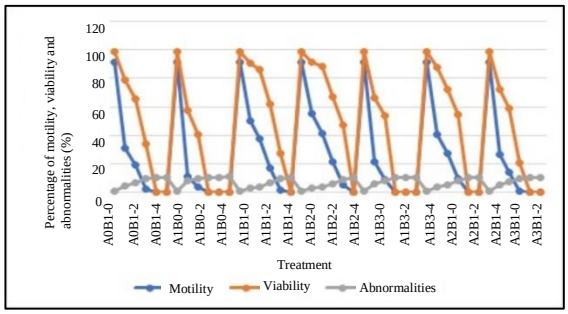

Fig. 3: Motility, viability, and abnormalities motility, viability and abnormalities stored using diluent added with nanocalcium phosphate stored at room temperature with calcium silicophosphate ampoules with various Si/P ratios

Table 3: Motility, Viability, and abnormalities stored using diluent added with nanocalcium phosphate stored at 5°C with calcium silicophosphate ampoules of various Si/P ratios

Treatment	Motility (%) (Mean ± SD)	Viability (%) (Mean ± SD)	Abnormalities (%) (Mean ± SD)
A0B1-0	91,2±1,62^{x}	98,4±0,52^{r}	0,54±0,08^{a}
A0B1-1	51,3±1,57^{q}	90,5±0,53^{o}	3,01±0,10^{h}
A0B1-2	38,9±1,37^{n}	87,5±0,97^{n}	3,75±0,08^{j}
A0B1-3	22±0,94^{h}	71±0,94^{j}	5,9±0,09^{p}
A0B1-4	12,1±0,88^{e}	61,1±0,88^{f}	7,89±0,09$_{s}$
A0B1-5	2±0,94^{b}	25±4,71^{b}	9,6±0,19^{v}
A1B0-0	91,2±1,62^{x}	98,4±0,52^{r}	0,54±0,08^{a}
A1B0-1	31,3±1,57^{k}	77,7±2,95^{l}	4,47±0,16^{m}
A1B0-2	23,8±1,32^{i}	68,8±1,32^{i}	5,67±0,26^{o}
A1B0-3	13,9±1,37^{f}	60,8±2,57^{f}	7,53±0,31^{r}
A1B0-4	3,8±1,23^{c}	35±6,67^{c}	9,3±0,20^{u}
A1B0-5	0±0^{a}	0±0^{a}	10,2±0,42^{w}
A1B1-0	91,2±1,62^{x}	98,4±0,52^{r}	0,54±0,08^{a}
A1B1-1	70,3±1,57^{u}	94,4±0,52^{q}	1,62±0,10^{d}
A1B1-2	57,6±1,35^{r}	91,9±0,32^{p}	2,5±0,12^{f}
A1B1-3	47,5±1,43^{o}	90,2±0,42^{o}	3,225±0,07^{i}
A1B1-4	37,4±1,35^{m}	88,1±0,32^{n}	3,83±0,07^{k}
A1B1-5	27,3±1,25^{j}	73,2±1,03^{j}	4,87±0,13^{n}
A1B2-0	91,2±1,62^{x}	98,4±0,52^{r}	0,54±0,08^{a}
A1B2-1	81,3±1,57^{w}	96,5±0,53^{q}	1,059±0,10^{b}
A1B2-2	75,2±1,40^{v}	95,4±0,52^{q}	1,343±0,07^{c}
A1B2-3	61,5±1,43^{t}	92,7±0,48^{p}	2,255±0,10^{g}
A1B2-4	51,6±1,35^{q}	90,8±0,42^{o}	2,98±0,08^{h}
A1B2-5	41,7±1,25^{o}	88,9±0,32^{n}	3,57±0,07^{j}
A1B3-0	91,2±1,62^{x}	98,4±0,52^{r}	0,54±0,08^{a}
A1B3-1	41,3±1,57^{o}	88,5±0,53^{n}	3,61±0,10^{j}
A1B3-2	28,4±1,43^{j}	75,1±0,32^{k}	4,76±0,14^{n}
A1B3-3	18,3±1,34^{g}	65,2±0,42^{h}	6,77±0,13^{n}
A1B3-4	8,4±1,43^{d}	55,1±0,32^{d}	8,45±0,33^{t}
A1B3-5	0±0^{a}	0±0^{a}	10,1±0,32^{w}
A2B1-0	91,2±1,62^{x}	98,4±0,52^{r}	0,54±0,08^{a}
A2B1-1	59,3±2,54^{s}	92,2±0,63^{p}	2,395±0,20^{g}
A2B1-2	47,5±1,43^{p}	90,1±0,32^{o}	3,225±0,07^{i}
A2B1-3	37,4±1,35^{m}	88,2±0,42^{n}	3,83±0,07^{k}
A2B1-4	27,3±1,34^{m}	72,3±1,34^{j}	4,87±0,13^{n}
A2B1-5	17,5±1,43^{g}	62±1,70^{g}	6,85±0,14^{q}
A3B1-0	91,2±1,62^{x}	98,4±0,52^{r}	0,54±0,08^{a}
A3B1-1	46,3±1,57^{p}	89,5±0,53^{n}	3,31±0,10^{i}
A3B1-2	33,9±1,37^{l}	80±2,31^{m}	4,2±0,16^{l}
A3B1-3	23,8±1,23^{i}	68,8±1,32^{i}	5,72±0,12^{o}
A3B1-4	13,7±1,16^{e}	58,7±1,16^{e}	7,58±0,25^{r}
A3B1-5	3,6±1,17^{b}	34,1±7^{c}	9,33±0,19^{u}

Means with different superscripts in a row differ significantly ($p<0.01$).
SD = Standard Deviation

Table 3 and Fig. 4, the average level of motility, viability and abnormalities of 10 sperm replications from fresh cow semen ($p<0.01$) using a diluent solution of tris amino methane and egg yolk supplemented with 1% nanocalcium phosphate powder b/v at a temperature of 5°C for consecutive storage periods of 0 (initial), 1, 2, 3, 4 and 5 days in nanocalcium silicophosphate ampoules with a ratio of Si/P = 0:1 with consecutive sample code A0B1-0, A0B1-1, A0B1-2, A0B1-3, A0B1-4 and A0B1-5; Si/P ratio = 1:0 with consecutive sample codes A1B0-0, A1B0-1, A1B0-2, A1B0-3, A1B0-4 and A1B0-5, Si/P ratio = 1:1 with consecutive sample codes-respectively A1B1-0, A1B1-1, A1B1-2, A1B1-3, A1B1-4 and A1B1-5; Si/P ratio = 1:2 with sample codes A1B2-0, A1B2-1, A1B2-2, A1B2-3, A1B2-4 and A1B2-5; Si/P ratio = 1:3 with sample codes A1B3-0, A1B3-1, A1B3-2, A1B3-3, A1B3-4 and A1B3-5; Si/P ratio = 2:1 with sample code A2B1-0, A2B1-1, A2B1-2, A2B1-3, A2B1-4 and A2B1-5 respectively; and Si/P ratio = 3:1 with sample code respectively A3B1-0, A3B1-1, A3B1-2, A3B1-3, A3B1-4 and A3B1-5; Based on the data in Fig. 4, in terms of the average motility, viability and abnormalities and storage time (days), the ampoule is most suitable for storing semen with a diluent solution of tris amino methane and egg yolk to which 1% w/v of nanocalcium phosphate powder is added. room temperature is ampoules with the composition A1B1 or calcium silicophosphate ampoules made with a ratio of Si/P = 1:1, ampoules with the composition A1B2 or nanocalcium silicophosphate ampoules made with a ratio of Si/P = 1:2 and ampoules with the composition A2B1 or calcium ampoules silicophosphate is made with a ratio of Si/P = 2:1 Fig. 5 is a picture of the viability of spermatozoa, namely live (transparent) and dead (red).

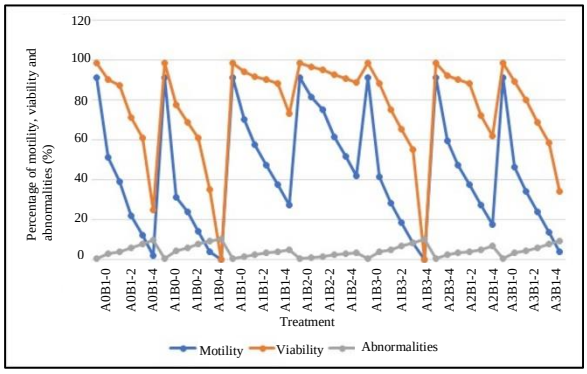

Fig. 4: Motility, viability, and abnormalities stored using diluent added with nanocalcium phosphate stored at 5°C with calcium silicophosphate ampoules of various Si/P ratios

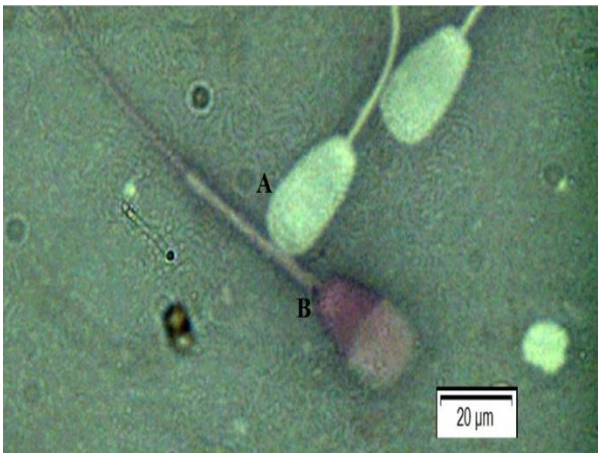

Fig. 5: Spermatozoa viability (400x magnification). Description; (A) Live spermatozoa; (B) Dead spermatozoa

Figures 2-4, the addition of 1% w/v nanocalcium phosphate powder to the retail solution of tris amino methane and egg yolk can increase the shelf life of fresh cow semen in terms of motility, viability, and spermatozoa abnormalities ($p<0.01$) and the use of nanocalcium silicophosphate ampoules with a ratio of Si/P = 1:1 and 1:2 can increase the shelf life of fresh bovine semen stored at room temperature compared to without using the nanocalcium silicophosphate ampoules, as well as the use of nanocalcium silicophosphate ampoules with Si/P ratio = 1:1; 1:2 and 2:1 can increase the shelf life of fresh bovine semen stored at a temperature of 5°C compared to without using the nanocalcium silicophosphate ampoule.

From this description, it is clear that the results of this invention can provide benefits for the development of livestock semen storage technology both at room temperature and low temperature which is more energy-friendly because it does not have to use low-temperature freezing using liquid nitrogen (cryopreservation), but is still able to produce quality semen excellent storage results or very suitable for application in artificial insemination. Thus, the storage process using a diluent solution of tris amino methane and egg yolk with the addition of 1% w/v nanocalcium phosphate powder and using storage ampoules made from nanocalcium silicophosphate provides a guarantee of quality storage results for room temperature storage for up to 2-3 days and with storage at 5°C for up to 3-5 days. The use of ampoules made from calcium silicophosphate biomaterial which is known to have good biocompatibility properties is expected to be able to make semen for artificial insemination or other stored organic preparations safer, especially from exposure to dangerous microplastic substances when using ampoules made of plastic.

Some cryopreservation diluent solutions vary according to the needs and type of animal whose semen is stored, for example when storing sheep/goat semen (Sharafi *et al*., 2022) using a solution with the composition of distilled water, tris-trihydroxymethyl aminomethane, citric acid, sugar, 1 g soy lecithin, resveratrol, penicillin, and streptomycin. This cryopreservation solution is also used for pig, horse, and cattle semen as claimed by Akhtar *et al*. (2022) with the addition of traditional Chinese medicine as a semen protection solution, with the same storage process. In contrast to ruminant animals, fish semen can be cryopreserved using a solution of sperm base diluent, nutrients, antioxidants, and anti-freezing protective agents (Lim *et al*., 2021) where the sperm base diluent is formed by mixing Cortland's solution and hank's solution according to a volume ratio of 2:1-1:3; nutritional substances consist of sucrose and trehalose; antioxidants consisting of bovine serum albumin, melatonin and vitamin E; the anti-freezing protective agent is dimethyl sulfoxide. Improvements to the storage process continue to be made to overcome the weaknesses of storing livestock semen, namely the use of diluent solutions that are cheaper and environmentally friendly but still effective, higher storage temperatures if possible at room temperature so that it does not need to be stored at cold temperatures using liquid nitrogen. The current use of plastic ampoules is not environmentally friendly and does not have a synergistic role in maintaining the quality of stored semen. As an alternative to overcome these various weaknesses, this invention adds a stabilizing agent that also acts as a nutrient, namely nanocalcium phosphate which is added to the semen diluent solution and cooled at room temperature and 5°C using an ampoule of nanocalcium silicophosphate made from rice husk ash and cow bone waste. The addition of calcium phosphate in the process of storing livestock semen in this invention is based on consideration of the results of a study from (Luo *et al*., 2019) which stated that calcium phosphate in the form of monoclonal nano antibodies can maintain the fertility of goat sexing semen, (Siari *et al*., 2022) added dipotassium phosphate in the extender mixture to improve the quality dilution of frozen semen and some recent studies (Yu *et al*., 2022) have reported using phosphate salt buffers in semen dilution. The addition of nanocalcium phosphate to the semen dilution before storage can stabilize the semen solution, provide phosphate and calcium nutrients, and strengthen the spermatozoa cell walls of the stored semen. It is hoped that effective room-temperature storage technology will be able to increase efficiency and ease its application in the field.

Table 4: Observation results of nonreturn rate, conception rate, and service per conception

Treatment	NRR (%) (Mean ± SD)	CR (%) (Mean ± SD)	S/C (Mean ± SD)
Frozen sperm (control)	90±3.16	80±4.22	1.8±1.03
A1B1	90±3.16	80±4.22	1.7±1.06
A1B2	90±3.16	90±3.16	1±0.47
A2B1	80±4.22	70±4.83	2.1±1.19

The success of Artificial Insemination

The NRR value (Table 4) for all treatments was still above 50% ($p>0.05$). The NRR value is in a good category because it is still more than 50% (Priyo Jr *et al*., 2020; Kusumawati *et al*., 2019b). An accurate CR value can only be proven by carrying out a pregnancy check on the 60-90th day after insemination. The diagnosis of pregnancy (CR) carried out in this study was by the rectal palpation method. Rectal palpation is carried out at 90 days (3 months) of pregnancy. There are cows that are not pregnant during rectal palpation examination due to short heat lasting 2-3 h and silent heat, so it is necessary to see directly the quality of the heat by looking at the condition of each cattle's vulva. The signs of lust that are often seen are a slimy, swollen, warm, red, and swollen vulva. Many castles have small cervixes, causing difficulties in semen deposition. When compared with the general benchmark for normal conception rates, namely 45-50% (Sozou and Hartshorne, 2012) the conception rate from this study is classified as above normal because it is more than 50%. According to Swelum *et al*. (2021), livestock that receive good feed will also improve the process of producing reproductive hormones. The normal S/C value is 1.6-2 (Ervandi *et al*., 2020), so the S/C value of this study is considered good because the smaller the S/C value, the better the success rate of artificial insemination.

Factors related to fertility levels include the age of the male and female, season, age of semen, diseases, semen treatment techniques, and other environmental influences. Based on these reasons, the non-return percentage can only be declared significant and accountable if calculated from a large livestock population. The best CR percentage reaches 60-70% (Budiarto *et al*., 2019), whereas for the size in Indonesia considering natural conditions, livestock distribution, and livestock management, a CR percentage of 45-50% is considered normal. The high and low rates of conception or CR are caused by 3 factors, namely male and female fertility and mating techniques. In normal mating, it is rare to find a situation where male and female animals reach 100% fertility capacity. The conception rate is determined based on the results of pregnancy diagnosis within 40-60 days after mating (Yamamoto *et al*., 2018). The success of cattle pregnancy depends on (1) The ability to detect close estrus every day, (2) NRR 5-10% higher than cattle that are truly pregnant, (3) The positive phase often occurs, namely cattle experiencing anestrus and the negative phase occurs in cattle that shows lust, even though the cattle are pregnant (Fernandez-Novo *et al*., 2020).

From research by Meier *et al*. (2021), it is stated that the age factor at first mating influences CR. Cattle that have just reached puberty and are not yet sexually mature when mated will cause low fertility levels, this is because livestock that are not yet sexually mature, the nutrients that enter the body will be used for the main function first, namely for body growth. It is recommended that mating be carried out after sexual maturity (Soliman *et al*., 2014), this is because livestock that have just reached puberty still need a lot of nutrition for body growth. The normal S/C value is 1.6-2.0. If S/C is low, the fertility value of female cows is higher, and if S/C is high, the fertility level of castles (Wathes, 2022). The causes of high S/C values are generally due to (1) Late detection of estrus, (2) Abnormalities in the reproduction of the cattle, (3) Less skilled staff, (4) Limited service facilities, and (5) Lack of smooth transportation (Priyo Jr *et al*., 2020; Depison, 2009; Wulansari *et al*., 2024).

The higher S/C value is caused by several factors, one of which is the age of the parent which is directly related to the physiological status of the animal. Animals that are too young at the time of first mating will find it difficult to become pregnant because the animal's physiological development is not yet perfect. Apart from that, the performance of hormones is still not perfect so usually the detection of estrus is less clear and livestock will experience difficulties when giving birth and have a fairly high risk of reproductive disorders. The fertility level of livestock is also influenced by the age of the livestock, the older the parent, the better reproduction compared to young females (Comizzoli and Ottinger, 2021; Baharun *et al*., 2021).

Conclusion

The study concluded that the addition of phosphate to the tris aminomethane egg yolk diluent can increase the durability of semen for up to three days at room temperature and with storage at 5°C for up to 3-5 days. Likewise, the test results for the success of artificial insemination are also good and according to standards.

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Author's Contributions

Enike Dwi Kusumawati: Designed and coordinated the study.

I Wayan Karyasa: Make ampoules from nanocalcium silicophosphate biomaterials, conceived and designed the analysis.

Yogy Pratama Putra: Collected the data.

Asmad Kari and Connie Fay Komilus: Review and improve the content of the manuscript.

Ethics

This study was approved by Universitas PGRI Kanjuruhan Malang, East Java, Indonesia.

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