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Research Article

Semen Quality and Artificial Insemination Efficacy Using Sericin-Silica-Phosphate-CuO Hydrogel in Thin-tailed Sheep

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Abstract: Artificial Insemination (AI) in livestock is increasingly demanded because it greatly affects the productivity and quality of livestock. AI should be supported by advanced material chemistry, especially organic-inorganic hybrid biomaterials. We aimed to produce a prototype of renewable advanced biomaterial technology in the form of Sericin-Silica-Phosphate (SER-SI-PO) hydrogel reinforced with Copper Nanoparticles (CuNPs) and its application in AI. The testing of the SER-SI-PO-CuO hydrogel prototype design is to enrich the semen diluent in AI technology on experimental sheep in a sheep-goat pen at the Faculty of Animal Husbandry, Universitas PGRI Kanjuruhan Malang so that a prototype of SER-SI-PO-CuO hydrogel that has been tested in a relevant environment is obtained. Semen was obtained from a 2-year-old thin-tailed male sheep of 30 kg. We used a completely randomized factorial design consisting of two factors (the diluent and the storage time factors). The diluent factor consisted of (A) egg yolk tris aminomethane diluent at 5°C, (B) egg yolk tris aminomethane diluent at room temperature, (C) egg yolk tris aminomethane diluent plus SER-SI-PO-CuO (1% w/v) at 5°C, (D) egg yolk tris aminomethane diluent plus SER-SI-PO-CuO at room temperature. The storage time factor consisted of storage times of 0, 1, 2, 3, 4, 5, 6, and 7 days. The variables observed were motility, viability, and abnormality. Each AI treatment used 15 samples of female sheep ready to mate. The AI success variables include Service per Conception (S/C), Non-Return Rate (NRR), and Conception Rate (CR). Semen quality data was analyzed by two-way ANOVA continued by the Least Significant Difference Test. The results showed that the SER-SI-PO-CuO addition to the diluent of tris aminomethane egg yolk using a semen storage ampoule made of nano calcium silico-phosphate biomaterial had a very significant effect ($p<0.01$) on motility, viability, and abnormalities. The NRR, CR, and S/C values were in the good category. The addition of SER-SI-PO-CuO to the tris aminomethane egg yolk diluent increased semen imperishability for up to six days at room temperature and with storage at 5°C for up to seven days, as well as the success of the AI test.

Keywords: Artificial Insemination, Nanocalcium Silicophosphate Biomaterials, Semen Storage Ampoules, Sericin-Silica-Phosphate-CuO, Sperm Quality

Introduction

Livestock productivity is largely determined by the quality of seeds and livestock maintenance. One of the determining factors for the quality of livestock seeds is mating and livestock seeds. Artificial Insemination (AI) is the latest technology to ensure the quality of livestock seeds. The effectiveness of AI continues to be a problem in the world of livestock, so the factors causing the ineffectiveness of AI, such as semen quality evaluation, semen storage technology, supporting materials, and the time of AI implementation (Kowalczyk *et al.*, 2019; Kondracki *et al.*, 2021).

Nanomaterials have now developed their use in animal science, as summarized by Delir *et al.* (2022), namely as a material for delivering nutrients, health care, animal reproduction, and breeding, improving the quality of animal meat, and improving the safety and quality of milk produced.

Some nanomaterials are referred to as biomaterials because of their biocompatibility and ability to interact with biological systems for medical purposes. Biomaterials have been used for a long time because of their superior biocompatibility, biodegradability, low toxicity, and low allergenicity, and they produce degradation products that are less cytotoxic and more easily metabolized by host tissues (Han *et al.*, 2022a). Biomaterials for medical applications can be classified as organic, inorganic, and organic-inorganic hybrid biomaterials.

Organic-inorganic hybrid biomaterials are very potential types of biomaterials because they complement each other in terms of the properties of the materials so that synergy occurs in the final product process (Saveleva *et al.*, 2019). Organic-inorganic hybrid nanomaterials can be based on silica and metal-organic materials (Taylor-Pashow *et al.*, 2010) for example, inorganic-inorganic hybrid biomaterials based on sericin combined with nano-silica and Copper (Cu) nanoparticles.

Sericin, a by-product of silk, has been recognized as an economical glycoprotein extracted during the silk degumming process in the textile industry and has medical properties, including strong biocompatibility and biodegradability in the form of nanoparticles. It functions in the process of delivering cancer drugs and can increase drug efficacy, solubility, and bio-diffusion, which can increase cell stability, cell absorption, and endosomal release (Kumar Dan *et al.*, 2022). The combination of sericin with nano silica and monophosphate can increase its biocompatibility and medical function. Bio-nano-silica from rice husk ash has high biocompatibility properties (Ghaferi *et al.*, 2021), so it is suitable for use in oncology therapy (Huang *et al.*, 2020) and cancer therapy (Li *et al.*, 2019) and nanocalcium phosphate made from bovine bone hydroxyapatite also has medical functional properties such as being able to increase wound healing activity in vitro and has biocompatible properties that are used for the development of biomaterials for wound dressings and implants (Han *et al.*, 2022b). Copper nanoparticles are nanomaterials that have antimicrobial and antiviral properties and have mechanisms such as ion release, Reactive Oxygen Species (ROS), direct contact and immunostimulant effects (Moorthy, 2020).

The application of nanomaterial technology in AI is a very strategic step in increasing the productivity and quality of livestock products (Dwitarizki, 2021). The application of advanced nanomaterials such as renewable organic-inorganic hybrid hydrogels, especially SER-SI-PO-Cu hydrogels, is an effective, efficient and eco-friendly breakthrough with the following scientific basis:

Sericin is a globular protein that is usually waste in the processing of silk cocoons into silk thread because sericin is a wrapper for silk fibroin fibers that must be separated to obtain good quality silk thread and sericin has superior properties as an antimicrobial biomaterial and has very good biocompatibility (Sathyaraj *et al.*, 2023). A life cycle analyses revealed that the sericin extracted from the degumming process of silk fiber exhibited an endpoint impact score for climate change up to 50%, followed by natural resources at 34% and human health impacts at 15% and the application of centrifugation + combined with lyophilization resulted in a 40–50% yield (Capar *et al.*, 2022)

Nanosilica prepared from rice husk ash is a renewable nanomaterial that is environmentally friendly and has high biocompatibility (Ali *et al.*, 2021)

Nanocalcium phosphate prepared from hydroxyapatite from bovine bone waste is a renewable biomaterial that is eco-friendly and has good biocompatibility, bio-absorbability and bioactive properties so that it is often used as a medical biomaterial (Ishikawa *et al.*, 2020), Copper Oxide Nanoparticles (CuO-NPs) are nanomaterials play a very important role in medical biomaterials because of their properties antimicrobial properties and its excellent biocompatibility (Balakrishnan *et al.*, 2019) and hydrogel is a form of biomaterial that can function optimally and practically in medical use and has high stability (Wang *et al.*, 2022). This study predicts that the organic-inorganic hybrid hydrogel of sericin-silica-phosphate-CuO produced by sol-gel synthesis using sericin gel, nano calcium silico-phosphate powder, and CuO-NPs is effective in supporting the improvement of AI quality

Thus, we aimed to formulate a prototype of SER-SI-PO-CuO hydrogel as a medical biomaterial that can be utilized in the field of AI in livestock. The purpose of the findings on the effectiveness of the hydrogel prototype on enriching semen diluents for supporting AI technology tested in relevant environments is expected to be a breakthrough in the world of livestock medicine.

Materials and Methods

Semen was obtained from a 2-year-old thin-tailed male sheep at 30 kg. Sheep were kept in 1 experimental cage in the laboratory of Universitas PGRI Kanjuruhan Malang at a temperature of 28°C with the provision of mixed grass feed and also the same concentrate. The condition of the stage cage with semi-open walls made of wooden slats. Sheep were bathed once a week, and wool was trimmed once every 3 months.

An entirely randomized factorial design was implemented, incorporating two factors: the composition of the diluent (Factor 1) and variations in storage time (Factor 2).

The diluent composition (Factor 1) consisted of (A) egg yolk tris aminomethane diluent at 5°C, (B) egg yolk tris aminomethane diluent at room temperature, (C) egg yolk tris aminomethane diluent plus SER-SI-PO-CuO hydrogel at 5°C, (D) egg yolk tris aminomethane diluent plus SER-SI-PO-CuO hydrogel (1% w/v) at room temperature.

The storage time variation (Factor 2) consisted of storing the semen along respective times of 0, 1, 2, 3, 4, 5, 6, and 7 days. Each treatment was repeated ten times. The experiment was continued by the AI processes, namely using (A) frozen semen with egg yolk tris aminomethane diluent, (B) semen with egg yolk tris aminomethane diluent plus SER-SI-PO-CuO hydrogel at 5°C, (C) semen with egg yolk tris aminomethane diluent plus SER-SI-PO-CuO hydrogel at room temperature.

The variables observed were motility, viability, and abnormalities. Each AI treatment used 15 samples of female sheep ready to mate. The variables associated with the success of AI encompassed Service per Conception (S/C), Non-Return Rate (NRR), and Conception Rate (CR). Semen quality data were subjected to statistical analysis utilizing a two-way ANOVA.

In the event that a treatment effect was identified, further analysis was conducted using the Least Significant Difference Test. A descriptive analysis was performed to describe the AI's success. This approach is implemented with the following stages:

1. Preparation of nanomaterial components of organic-inorganic hybrid hydrogel, namely (a) organic components in the form of sericin proteins that were isolated from *Bombyx mori* silk fiber filaments using the silk fibers degumming method to separate fibroin (water-insoluble protein fibers that will be used as silk thread) and sericin, a globular protein that was dissolved in water through a hot immersing process using soda ash solution (Moorthy, 2020), (b) nano bio-silica powder through the mechano-synthesis process (Lakshmi and Pola, 2020) of rice husk ash powder (obatained from Suwela Amertha Rice Milling Entreprise in Buleleng Regengy, Bali Province of Indonesia), (c) nano calcium phosphate powder prepared from hydroxy-apatite of cow bone waste (obtained from Slaughterhouse RPH Kota Malang, East Java Province of Indonesia) and (d) Copper Oxide Nanoparticles (CuO-NPs) prepared using copper (II) acetate ($Cu(CH_3COO)_2$) powder (Merck) by green synthesis after the Balakrishnan *et al.* method (Balakrishnan *et al.*, 2019).
2. Preparation of SER-SI-PO-CuO hydrogel by conducting a sol-gel synthesis using sericin gel, nano-biosilica powder, nano calcium phosphate powder, and green synthesized CuO-NPs powder with composition of 50: 25: 25: 10 m/m
3. Testing the effectiveness of the hydrogel prototype, namely SER-SI-PO-CuO hydrogel on AI technology application in a relevant environment using sheep as experimental animals in the sheep-goat pen of the Faculty of Animal Husbandry, Universitas PGRI Kanjuruhan Malang
4. Analysing the resulted data descriptively and statistically by conducting ANOVA two-ways analysis with the Least Significant Difference Test. The flow diagram for the manufacture of sericin-silica-phosphate-CuO hydrogel with A-Prototype and its application test is presented in Figure (1).

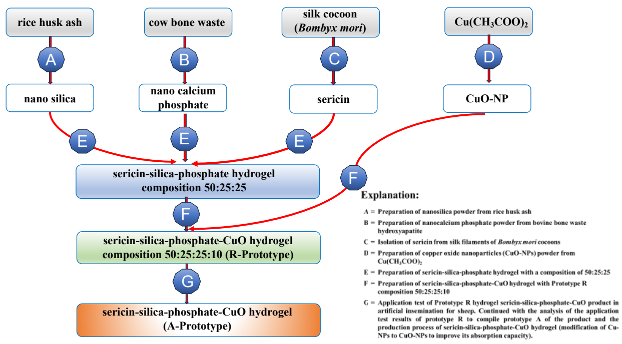

Fig. 1: Flow diagram of the manufacture of sericin-silica-phosphate-CuO hydrogel with Prototype R composition and its application test and analysis to become Prototype A

Procedures for applying the SER-SI-PO-CuO hydrogel on sheep semen preservation for AI technology were conducted in the following steps. Firstly, the collection of fresh semen was taken from Universitas PGRI Kanjuruhan Malang, Indonesia. Secondly, observations of motility, viability, and abnormalities were conducted at the integrated laboratory of Universitas PGRI Kanjuruhan Malang in Indonesia, utilizing a microscope for analysis. Subsequently, a diluent composed of Tris aminomethane and egg yolk, prepared by the Singosari National Artificial Insemination Center (SNAIC) in Malang Regency,

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Indonesia, was added in a 1:1 ratio. Specifically, 2 mL of fresh semen was combined with 2 mL of the Tris aminomethane egg yolk diluent in the semen container, and the mixture was thoroughly blended before being stored at room temperature. The SER-SI-PO-CuO sericin-silica-phosphate-CuO hydrogel was incorporated at a concentration of 1% w/v. Additionally, semen was stored at both room temperature (25°C) and a reduced temperature of 5°C, utilizing ampoules or semen straws constructed from nano calcium silico-phosphate biomaterial for this purpose. The ampoules were made in the Universitas Pendidikan Ganesha of Education Laboratory, Bali, Indonesia, with a bed at one end of the ampoule filled with nano silica gel as a humidity sensor where its color changes when it absorbs water as a result of changing condition of the semen stored in the ampoule, for instance, damage of stored semen occurs because of hydrolysis of the spermatozoa cells of the semen. Ultimately, assessments of sperm motility, viability, and abnormalities were conducted, as outlined in the following brief description.

Motility

The motility of individual spermatozoa can be assessed by placing a drop of semen at the center of a glass slide, which is then covered with a coverslip and examined under a microscope at a magnification of 400×. The evaluation of individual motility involves observing the progressive movement of sperm, which is quantified alongside the total sperm count and expressed as a percentage (%) using the appropriate formula (Kusumawati *et al.*, 2024a):

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

Viability

The assessment of spermatozoa viability commences with the application of a single drop of eosin nigrosine onto a glass slide, followed by the addition of a drop of semen at the opposite end of the slide to create a smear preparation. This preparation is then heated over a Bunsen burner. Once dried, the sample is examined under a light microscope at a magnification of 400×. A total of 200 spermatozoa are evaluated across at least five distinct fields of view. Eosin nigrosine penetrates the cells of non-viable spermatozoa, resulting in a red coloration. Viability is expressed as a percentage (%), which can be calculated using the appropriate formula (Kusumawati *et al.*, 2024b):

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

Abnormalities

The evaluation of spermatozoa abnormalities begins with the application of a drop of eosin nigrosine onto a glass slide, followed by the addition of a drop of semen at the opposite end to create a smear. This smear is then subjected to heating over a Bunsen burner. Once dried, the sample is examined under a light microscope at a magnification of 400×. A total of 200 spermatozoa are assessed across a minimum of five different fields of view. Eosin nigrosine penetrates the cells of non-viable spermatozoa, resulting in a red coloration. The percentage of abnormalities is calculated and expressed as a percentage (%), using the appropriate formula (Kusumawati *et al.*, 2024a):

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

Artificial Insemination

Artificial insemination was carried out on 45 thin-tailed ewes (*Ovis aries*), which were divided into three treatments: T1, namely AI using frozen semen (control); T2 AI using nano calcium silico-phosphate ampoules with egg yolk tris aminomethane diluent plus SER-SI-PO-CuO hydrogel at 5°C; T3 AI using nano calcium silico-phosphate ampoules with egg yolk tris aminomethane diluent plus SER-SI-PO-CuO hydrogel at room temperature. (A) frozen semen with egg yolk tris aminomethane diluent, (B) semen with egg yolk tris aminomethane diluent plus sericin-silica-phosphate-CuO hydrogel at 5°C, (C) semen with egg yolk tris aminomethane diluent plus SER-SI-PO-CuO hydrogel at room temperature. Each AI treatment used 15 samples of ewes ready to mate.

The Success of Artificial Insemination

Measuring the success of AI includes the NRR, CR and S/C (Kusumawati *et al.*, 2024b).

Non-Return Rate (NRR)

$$NRR = \frac{Number\ of\ ewes\ in\ AI\text{-}Number\ of\ ewes\ in\ re\text{-}AI}{Number\ of\ ewes\ in\ AI} \times 100$$

Conception Rate (CR)

$$CR = \frac{Number\ of\ Pregnant\ Ewes}{Number\ of\ ewes\ in\ AI} \times 100$$

Service per Conception (S/C)

$$S/C = \frac{Number\ of\ AI}{Number\ of\ Pregnant\ Ewes} \times 100$$

A higher Conception Rate (CR) indicates a greater success rate of artificial insemination in sheep within a specific region. Conversely, a low CR suggests a poor success rate for artificial insemination in that area. An acceptable CR is typically defined as being at least 45-50%; values falling below this threshold are regarded as unsatisfactory. (Topas Wicaksono *et al.*, 2020).

Semen quality data is evaluated through Analysis of Variance (ANOVA). If treatment effects are identified, further analysis is conducted using the Least Significant Difference (LSD) test. In contrast, the assessment of artificial insemination success is performed using descriptive statistical methods.

Results

The stages of manufacturing the sericin-silica-phosphate-CuO hydrogel product, which is a slight modification of the SER-SI-PO-CuO hydrogel, where nanosilica powder from rice husk ash and nanocalcium phosphate powder from cow bone waste have been produced and are no longer characterized, but the results of green synthesis of CuO-NPs from $Cu(CH_3COO)_2$ using mango leaf extract which was characterized first using SEM-EDX, XRD and PSA. SEM-EDS of CuO-NPs from green synthesis using a 0.1M $Cu(CH_3COO)_2$ solution precursor with mango leaf extract can be seen in Fig. (2).

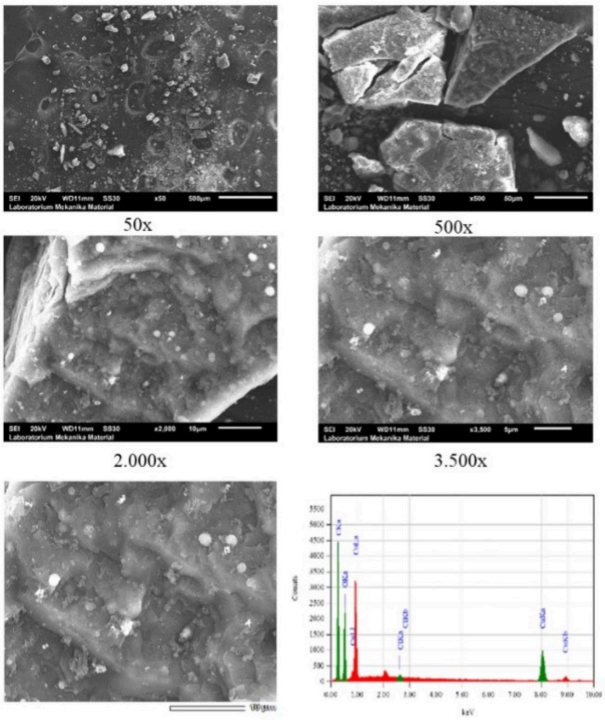

Fig. 2: Results of SEM-EDX analysis of CuO-NPs samples from green synthesis

SEM-EDX results (Figure 2) show that CuO nanoparticles produced from green synthesis re-agglomerate to form CuO aggregates and still with minor impurities of C atoms, which are likely the remaining unreacted mangiferin extract, which is also concluded in the aggregation of CuO nanoparticles. Microgram SEM of this CuO sample, if enlarged up to 500%, will involve homogeneous CuO granules. The results of XRD analysis of CuO-NPs powder samples from green synthesis obtained the following diffractogram as depicted in Figure (3).

The results of XRD (Figure 3) analysis of CuO-NPs samples from green synthesis showed that the diffraction pattern matches the diffractogram of CuO-NPs samples with CuO data bank ICPDS number 01-090-0076. Calculation of crystallite size using HighscorePlus Schrrer Calculator obtained an average crystallite size of 352.6 nm. Meanwhile, grain size measurements using Particle Size Analyzer (PSA) obtained an average particle size of 43.38575±1.00077 nm with a particle distribution as in Figure (4).

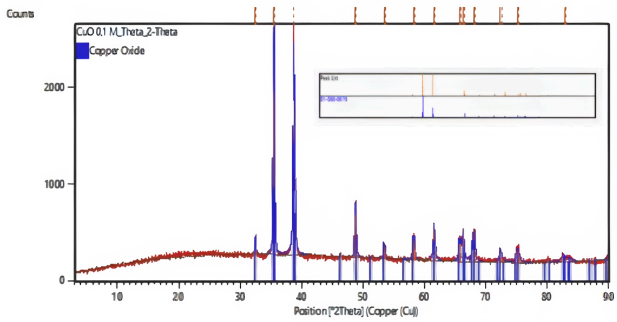

Fig. 3: Results of XRD analysis of CuO-NPs samples from green synthesis

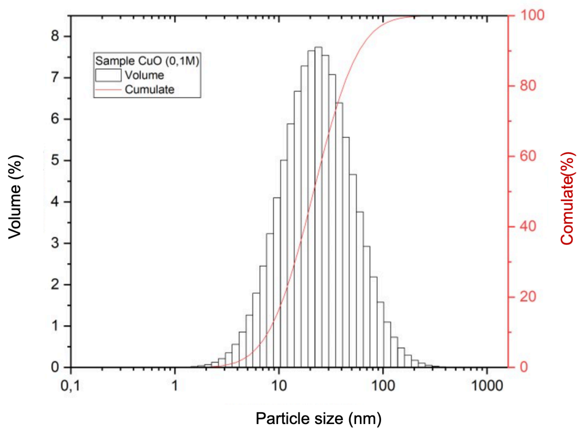

Fig. 4: Results of PSA analysis of the prototype hydrogel sericin-silica-phosphate-CuO

The difference in particle size with crystallite size proves that CuO-NPs aggregate into crystallites during heating calcination of Cu-NPS into CuO-NPs.

Table 1: Physical properties of SER-SI-PO-CuO hydrogel

Sample Code	Properties	
Organoleptic test	Texture	Thick gel
	Color	Greyish cream
	Smell	Typical sericin
Homogeneity test		High
Spread power test (cm)	0 g	8.0
	100 g	8.7
	250 g	9.3
	500 g	9.5
	1000 g	9.5
Adhesion test (seconds)		37
Viscosity (Cps)	H1	44275
	H5	44284
	H15	44310

The results of testing the physical properties of Prototype R SER-SI-PO-CuO hydrogel are in Table (1).

Microstructural characterization of hydrogel to see the distribution of constituent particles, aggregation and surface porosity can be studied by SEM-EDS instrumentation method. The results of scanning of SER-SI-PO-CuO hydrogel samples and EDS analysis to determine the distribution of Si, Cu, P, O elements are presented in Figure (5).

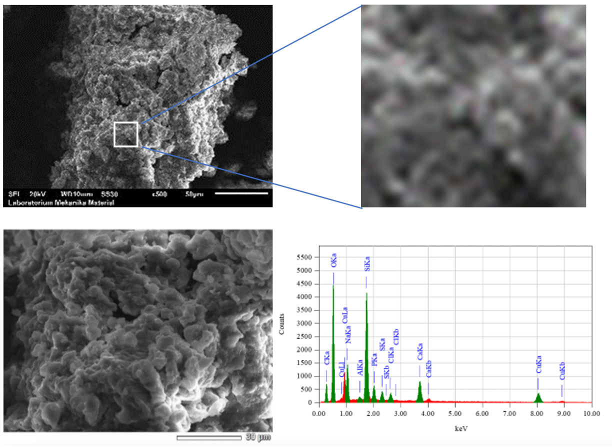

Fig. 5: SEM-EDS micrograms of sericin-silica-phosphate-CuO hydrogel

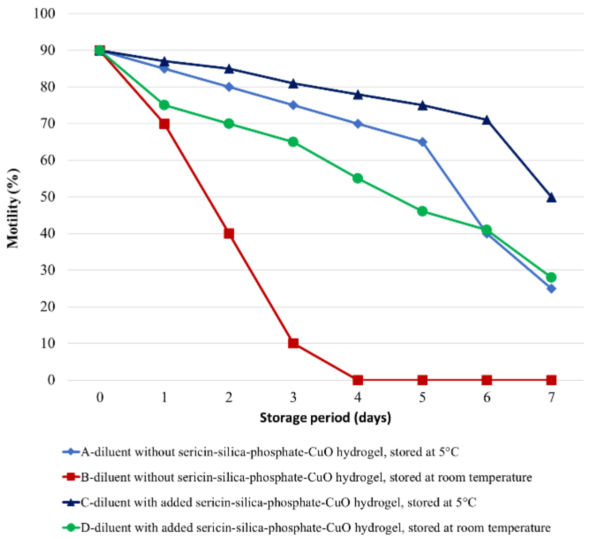

Fig. 6: The motility of spermatozoa stored at 5°C and room temperature, comparing those preserved with a diluent containing SER−SI−PO−CuO to those stored without it

Based on the results of the sperm quality test presented in Figs. (6-8), the quality of sperm (motility, viability, and abnormalities) using egg yolk tris aminomethane diluent added with SER-SI-PO-CuO hydrogel that has been modified into SER-SI-PO-CuO hydrogel and stored at 5°C showed better results and was able to maintain sperm quality until day 7, while without sericin it was only able to survive until day 6. Meanwhile, the quality of sperm using egg yolk tris aminomethane diluent added with sericin-silica-phosphate-CuO hydrogel and stored at room temperature was able to survive until day 6, while without sericin hydrogel, it was only able to survive until day 2. This is seen by the comparison of the percentage of decreased motility and viability of spermatozoa. Likewise, it is also seen by the comparison of the percentage of spermatozoa abnormalities in diluents and storage temperatures.

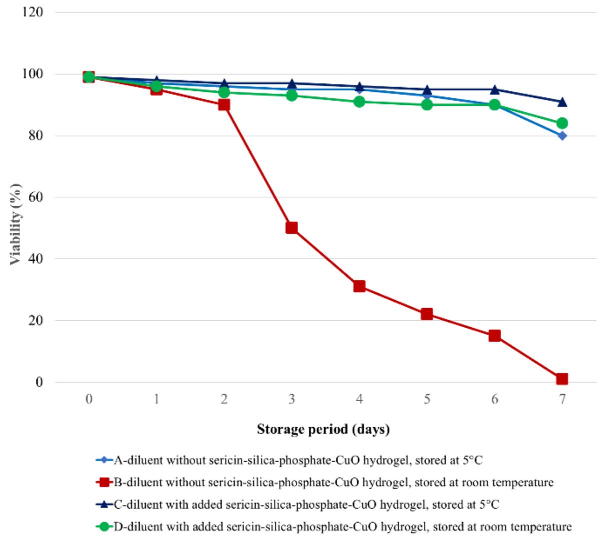

Fig. 7: Viability of spermatozoa stored using diluent with added SER-SI-PO-CuO hydrogel and not stored at 5°C and room temperature

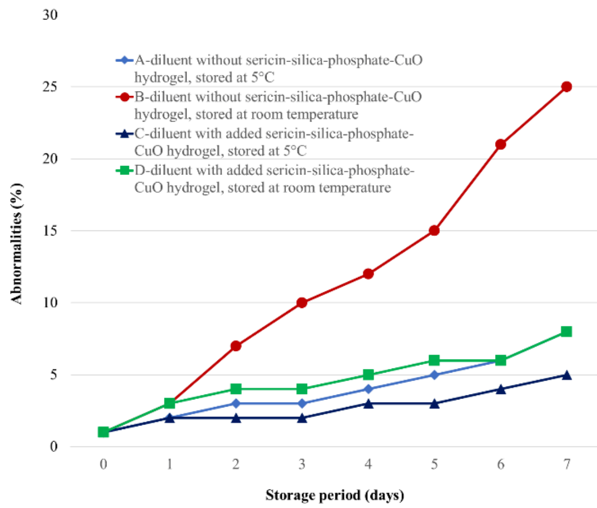

Fig. 8: The abnormalities observed in spermatozoa stored at 5°C and room temperature, both with and without the addition of a diluent containing SER−SI−PO−CuO, are analyzed

The success of AI was tested by implementing AI using (A) Frozen semen with egg yolk tris aminomethane diluent, (B) Semen with egg yolk tris aminomethane diluent plus sericin-silica-phosphate-CuO hydrogel at 5^{o}C, (C) Semen with egg yolk tris aminomethane diluent plus SER-SI-PO-CuO at room temperature. Each treatment was applied to 15 ewes ready for mating. The factors influencing AI success, including S/C, NRR, and CR, are presented in Table (6).

Table 2: Tests of Between-Subjects Effects with Dependent Variable of Motility

Source	Sum of Squares	DF	Mean Square	P	P
Diluent	115293.575	3	38431.192	183248.066	<0.001
Storage Time	129060.300	7	18437.186	87912.409	<0.001
Diluent Storage Time	39069.525	21	1860.454	8871.037	<0.001
Residual	60.400	288	0.210	-	-

Table 3: Tests of Between-Subjects Effects with Dependent Variable of Viability

Source	Sum of Squares	DF	Mean Square	P	P
Diluent	113709.184	3	37903.061	208720.491	<0.001
Storage Time	44916.672	7	6416.667	35334.612	<0.001
Diluent Storage Time	67490.841	21	3213.850	17697.680	<0.001
Residual	52.300	288	0.182		

Table 4: Tests of Between-Subjects Effects with Dependent Variable of Abnormality

Source	Sum of Squares	DF	Mean Square	P	P
Diluent	3892.509	3	1297.503	7037.305	<0.001
Storage Time	3466.247	7	495.178	2685.712	<0.001
Diluent Storage Time	2274.516	21	108.310	587.446	<0.001
Residual	53.100	288	0.184		

Discussion

Quality of Spermatozoa with the Addition of Sericin-Silica-Phosphate-CuO Hydrogel Stored at 5°C and Room Temperature

Tables (2-5) and Figs. (6-8) showed the results that the quality of spermatozoa using tris aminomethane egg yolk diluent added with SER-SI-PO-CuO hydrogel that has been modified into SER-SI-PO-CuO hydrogel and stored at 5°C showed better results and was able to maintain the quality of spermatozoa (motility, viability and abnormalities until the seventh day, while without sericin it was only able to survive until the sixth day. Meanwhile, the quality of spermatozoa using tris aminomethane egg yolk diluent added with SER-SI-PO-CuO hydrogel and stored at room temperature was able to survive until the sixth day, while without sericin hydrogel, it was only able to survive until the second day.

Motility in Figure (6) showed that with increasing storage time, there was a gradual decrease in motility until the seventh day. This also occurs in spermatozoa viability, which continues to decrease until the seventh day, as in Figure (7). This incident was also in line with the increasing abnormalities of spermatozoa shown in Figure (8). A very significant effect ($p<0.01$) of the addition of SER-SI-PO-CuO hydrogel on motility, viability and abnormality of thin-tailed sheep spermatozoa.

Table 5: LSD Results

Treatment	Motility (%) (Mean ± SD)	Viability (%) (Mean ± SD)	Abnormalities (%) (Mean ± SD)
A0	90.30±0.48[b]	99.10±0.32[b]	1.00±0.47[a]
A1	85.40±0.52[b]	97.20±0.42[b]	2.10±0.32[a]
A2	80.20±0.42[b]	96.10±0.57[b]	3.00±0.47[a]
A3	75.10±0.57[b]	95.10±0.57[b]	3.10±0.32[a]
A4	70.20±0.42[b]	95.00±0.47[b]	4.10±0.32[a]
A5	65.10±0.57[b]	93.10±0.32[b]	5.20±0.42[a]
A6	40.30±0.48[a]	90.20±0.42[b]	6.10±0.32[a]
A7	25.20±0.42[a]	80.30±0.48[b]	8.20±0.42[a]
B0	90.30±0.48[b]	99.10±0.32[b]	1.00±0.47[a]
B1	70.40±0.70[b]	95.00±0.47[b]	3.10±0.32[a]
B2	40.20±0.42[a]	90.20±0.42[b]	7.20±0.42[a]
B3	10.30±0.48[a]	50.10±0.57[a]	10.00±0.47[a]
B4	0.00±0.00[a]	31.00±0.67[a]	12.10±0.57[b]
B5	0.00±0.00[a]	22.00±0.47[a]	15.10±0.57[b]
B6	0.00±0.00[a]	15.10±0.32[a]	21.10±0.32[b]
B7	0.00±0.00[a]	1.00±0.47[a]	25.10±0.57[c]
C0	90.30±0.48[b]	99.10±0.32[b]	1.00±0.47[a]
C1	87.10±0.32[b]	98.00±0.47[b]	2.10±0.57[a]
C2	85.20±0.42[b]	97.10±0.32[b]	2.20±0.42[a]
C3	81.10±0.57[b]	97.00±0.47[b]	2.30±0.48[a]
C4	78.10±0.57[b]	96.20±0.42[b]	3.10±0.32[a]
C5	75.00±0.00[b]	95.10±0.32[b]	3.20±0.42[a]
C6	71.10±0.57[b]	95.00±0.47[b]	4.30±0.48[a]
C7	50.40±0.70[a]	91.00±0.00[b]	5.10±0.32[a]
D0	90.30±0.48[b]	99.10±0.32[b]	1.00±0.47[a]
D1	75.30±0.67[b]	96.10±0.32[b]	3.10±0.32[a]
D2	70.10±0.32[b]	94.00±0.47[b]	4.20±0.42[a]
D3	65.20±0.42[b]	93.10±0.32[b]	4.30±0.48[a]
D4	55.00±0.47[a]	91.20±0.42[b]	5.10±0.32[a]
D5	46.30±0.48[a]	90.20±0.42[b]	6.20±0.42[a]
D6	41.10±0.32[a]	90.00±0.47[b]	6.30±0.48[a]
D7	28.20±0.42[a]	84.10±0,32[b]	8.10±0.32[a]

Means with different superscripts in a row differ significantly (p<0.001). SD = Standard Deviation, the same marks of a,b,c mean having no significant difference, but different marks of a,b,c mean having significant difference.

Table 6: NRR, CR, and S/C values

Treatment	NRR (%) (Mean ± SD)	CR (%) (Mean ± SD)	S/C (%) (Mean ± SD)
	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)
Frozen sperm (control)	80.00±4.14	73.33±4.58	1.27±0.46
Diluent with added Sericin-Silica-Fosfat-CuO (5°C)	93.33±2.58	86.67±3.52	1.13±0.35
Diluent with added Sericin-Silica-Fosfat-CuO (room temperature)	86.67±3.52	80.00±4.14	1.20±0.41

The best treatment was the addition of SER-SI-PO-CuO hydrogel to tris aminomethane egg yolk diluent stored at 5°C which showed the best percentage of motility, viability, and abnormality and could still survive until day 7, as summarized in Figure (9).

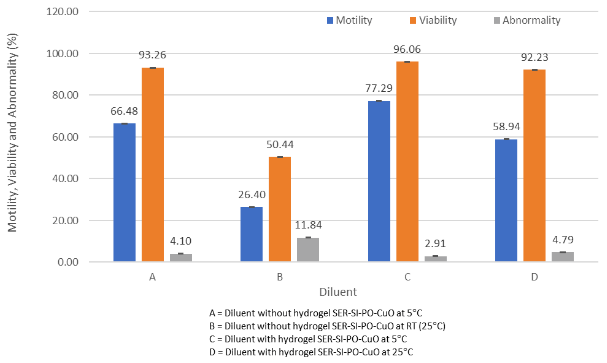

Fig. 9: Summary of diluent and temperature effect on motility, viability, and abnormality

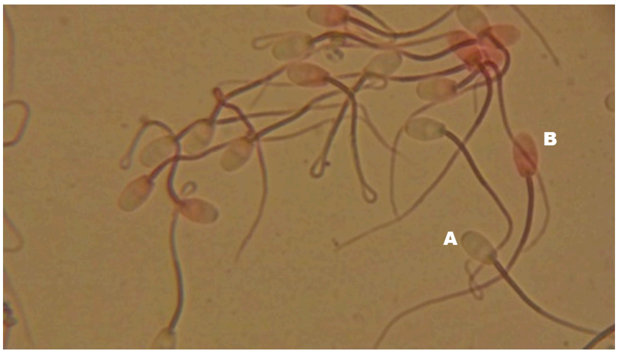

Fig. 10: Spermatozoa viability is assessed at 400x magnification, distinguishing between (A) live spermatozoa and (B) dead spermatozoa

Data summarized in Figure (9) infer that the SER-SI-PO-CuO hydrogel can improve motility and viability as well as reduce abnormality of spermatozoa; thus, it can support the success of AI. The hydrogel contains a lot of -OH functional groups from the Si-OH terminal as well as the P-OH terminal and N-H group from the amino acid terminal of sericin protein. Those -OH and N-H can interact with phospholipid membranes of spermatozoa cells to protect them from biotic (such as viruses and bacteria) as well as abiotic (temperature change, pressure, and acidity) stresses. Until day 7, the motility and viability of thin-tailed sheep spermatozoa were still following the minimum standard for AI. Abnormalities were also still following AI standards. Spanner *et al.* (2024) stated that the minimum standard for spermatozoa motility for AI is a minimum of 40%, while the maximum abnormality is 15%. This is also illustrated in Figs. (9-10), which shows that after being stained with eosin nigrosine, the spermatozoa that are alive have their heads not red, but the spermatozoa that are dead have their heads red. This happens because, in dead spermatozoa, there is damage to the spermatozoa head membrane so that it can absorb the eosin nigrosine color. At the same time, the spermatozoa that are still alive have their head membranes intact so that they cannot absorb the eosin nigrosine color.

The advancement of livestock semen storage technology offers significant advantages, as it allows for preservation at both room temperature and low temperatures without the need for energy-intensive freezing methods using liquid nitrogen. This approach can still yield high-quality semen suitable for AI. Specifically, the use of a tris aminomethane egg yolk diluent solution combined with a 1% b/v SER-SI-PO-CuO hydrogel, along with storage in nano calcium silico-phosphate ampoules, ensures effective storage quality. This method enables preservation at room temperature for up to 6 days and at 5°C for up to 7 days.

The incorporation of ampoules made from nano calcium silico-phosphate biomaterial, recognized for its excellent biocompatibility, is anticipated to enhance the safety of semen storage for artificial insemination and other organic preparations. This approach minimizes the risk of contamination from harmful microplastics associated with traditional plastic ampoules. Various cryopreservation diluent solutions are tailored to meet the specific requirements of different animal species. For instance, the storage of sheep and goat semen utilizes a solution comprising distilled water, tris-trihydroxy methyl aminomethane, citric acid, sugar, 1 g of soy lecithin, resveratrol, penicillin, and streptomycin (Sharafi *et al.*, 2022). This solution is similarly applicable to the semen of pigs, horses, and cows, as noted by Akhtar *et al.* (2022), who also incorporate traditional Chinese medicine as a protective agent during storage. In contrast, fish semen can be cryopreserved using a basic diluent that includes nutrients, antioxidants, and antifreeze agents (Lim *et al.*, 2021). This basic diluent is created by mixing Cortland's solution and Hank's solution in a 2:1-1:3 volume ratio, with nutrients such as sucrose and trehalose, antioxidants like bovine serum albumin, melatonin, and vitamin E, and dimethyl sulfoxide as the antifreeze agent.

Ongoing improvements in the storage process aim to address the challenges associated with sheep semen preservation, focusing on the development of cost-effective and environmentally friendly diluent solutions that can be stored at higher temperatures, ideally at room temperature, thus eliminating the need for liquid nitrogen. The use of plastic ampoules is increasingly viewed as less sustainable and ineffective in preserving semen quality. To mitigate these issues, this innovation introduces a stabilizer that also serves as a nutrient—nano calcium phosphate—into the semen diluent solution, which is then cooled at room temperature and 5 °C in ampoules made from rice husk ash and cow bone waste. The rationale for adding calcium phosphate is supported by research indicating that nano monoclonal antibodies of calcium phosphate can enhance the fertility of goat-sexed semen (Luo *et al.*, 2019). Additionally, Siari *et al.* (2022) found that incorporating dipotassium phosphate into extender mixtures improves the quality of frozen semen, while recent studies (Yu *et al.*, 2022) have highlighted the benefits of phosphate salt buffers in semen dilution. The inclusion of nano calcium phosphate in the semen dilution prior to storage is expected to stabilize the semen solution, supply essential phosphate and calcium nutrients, and reinforce the cell membranes of stored spermatozoa. This effective room-temperature storage technology aims to enhance efficiency and facilitate practical applications in the field.

The Success of Artificial Insemination

Based on Table (6), the highest NRR, CR, and S/C values were in the AI treatment using tris aminomethane egg yolk diluent with the addition of SER-SI-PO-CuO hydrogel 1% b/v stored at 5°C, which was followed by the AI treatment using tris aminomethane egg yolk diluent with the addition of SER-SI-PO-CuO hydrogel 1% b/v stored at room temperature, all of which used nano calcium silico-phosphate ampoules and finally the AI treatment using frozen semen with tris aminomethane egg yolk diluent. The NRR, CR, and S/C values were still in the category according to the AI success standards for sheep. Hydrogel nanoparticles when introduced into the body, their components can be modulated to achieve controlled drug release, thereby increasing the bioavailability of the payload at the site of administration while minimizing systemic toxicity (Jiang *et al.*, 2020). The hydrophilic and microporous structure of hydrogels makes them excellent hosts for nanomaterials (Wahid *et al.*, 2020). The success of nanoparticle technology has been demonstrated in artificial insemination, from the collection to the protection during storage of semen for artificial insemination and to improve sperm-related biotechnologies such as sperm-mediated gene transfer, sperm sorting, sex sorting, and cryopreservation (Feugang *et al.*, 2019). The success rate of pregnancy from artificial insemination in goats using nanomaterials was 71.7% (Kusumawati *et al.*, 2024b). The incorporation of nanocalcium phosphate into a tris aminomethane egg yolk diluent, combined with the use of semen storage ampoules made from nanocalcium silico-phosphate biomaterials, resulted in an artificial insemination success rate of 80-90% in cattle (Kusumawati *et al.*, 2024a). According to Souza-Fabjan *et al.* (2023), pregnancy rates in sheep are from 50-80%. The efficacy of AI is significantly influenced by several factors, including the breed of ewe, the technique and site of semen deposition, the specific type of semen utilized, the number of spermatozoa introduced, and the overall quality of the spermatozoa. In the sheep industry, the success rate of laparoscopic intrauterine artificial insemination with frozen semen is estimated to be around 70% (Spanner *et al.*, 2024). The increase in size and weight of Australian sheep (Sawyer *et al.*, 2019) over the past 10–15 years has affected post-AI fertility.

Semen storage ampoules or straws are typically constructed from thermoplastic materials, which are prone to damage when subjected to low temperatures or during the thawing process following storage. This vulnerability arises from both biotic and abiotic stresses affecting the spermatozoa in cooled semen. Additionally, plastic materials are susceptible to generating microplastics when exposed to low temperatures or subjected to rapid heating or thawing, which can adversely affect the viability of spermatozoa (Trifuoggi *et al.*, 2019; Tallec *et al.*, 2020; Hou *et al.*, 2021; D'Angelo & Meccariello, 2021). Furthermore, existing semen storage ampoules or straws used in Artificial Insemination (AI) exhibit limitations in providing indicators of semen quality, particularly regarding the condition of spermatozoa in stored samples (Gilligan, 2021; Stroud, 2020; Schmitt *et al.*, 2019-2020; Ainley *et al.*, 2019; Watanabe, 2020; Van Kappel-Dufour *et al.*, 2021). This is critical, as the effectiveness of AI remains relatively low, with reported pregnancy success rates of 29.55% compared to a natural conception rate of 61.28% (Rahman *et al.*, 2020; Zubor *et al.*, 2020; Sutriana *et al.*, 2021). Consequently, advancements in artificial insemination technology are essential. One potential improvement involves the development of straws or ampoules that are not only safe and resistant to cold temperatures but also capable of absorbing moisture (from secretions of damaged cells during storage or thawing) and providing a colorimetric change to indicate whether the semen or spermatozoa within the ampoule or straw have been compromised.

Conclusion

The incorporation of SER-SI-PO-CuO hydrogel into the tris aminomethane egg yolk diluent enhanced the longevity of semen, allowing for preservation at room temperature for up to 6 days and at 5°C for as long as 7 days. Additionally, the outcomes of the AI success tests were favorable.

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Author's Contributions

Enike Dwi Kusumawati: Designed and coordinated the study, maintenance of experimental animals, and testing of the application of hydrogel in artificial insemination for experimental sheep.

I Wayan Karyasa: Make ampoules from nanocalcium silico-phosphate biomaterials, preparation of sericin-silica-phosphate-CuO hydrogel and its characterization conceived and designed the analysis.

Duran Corebima Aloysius: Experimental sheep keeping.

Estri Pamungkasih, Diva Cahyo Pradana and Haydar Wafiq Nugke: Collected the data.

Asmad Kari: Review and improve the content of the manuscript.

Ethics

This study was approved by the Animal Care and Use Committee of Brawijaya University, Malang, Indonesia with the number 131-KEP-UB-2024 on July 10, 2024.

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