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Motility and Viability of Nunukan Chicken Spermatozoa in Egg Yolk-Skim Milk Diluent with the Addition of Various Glucose Levels as an Energy Source

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Abstract*. This study aims to determine the effect of adding glucose to skim milk diluent on the motility and viability of nunukan chicken spermatozoa. The materials used are fresh semen from 20 male Nunukan chickens aged 1-2 years, glucose 2%, 3%, and 4%, egg yolk, skim milk, and distilled water. The research design used in this research is a Completely Randomized Design (CRD) with four treatments and five replications. The data obtained will then be analyzed using variance (ANOVA). The treatments were P0 with semen + skim milk without adding glucose, P1 = cement + skim milk + 2% glucose, P2 = cement + skim milk + 3% glucose, P3 = cement + skim milk + 4% glucose. The results showed that the percentage of sperm motility of nunukan chickens from all treatments showed significantly different results (P<0.05). It can be concluded that adding 3% glucose to skim milk diluent provides optimal motility and viability of nunukan chicken spermatozoa.*

Keywords: *Glucose, Motality, Nunukan chicken, Sperm, Viability*

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INTRODUCTION

Optimizing the productivity of livestock commodities is currently being intensified by the local government, especially in several IKN buffer areas, one of which is increasing the population and production of broiler and egg chickens (Zein et al., 2009). Nunukan chicken is a type typical of Kalimantan that results from the domestication of the red jungle fowl (Gallus gallus gallus). Nunukan chickens have high development potential (Aisen et al., 2002). Nunukan chickens have high development potential. This is seen in its usefulness and advantages. This advantage makes Nunukan chickens very suitable for cultivation in East Kalimantan, an area with hot environmental conditions because it is on the equator and an IKN area. The Artificial Insemination (AI) program in Nunukan chickens could be a solution to developing Nunukan chickens however, currently, information regarding the quality of spermatozoa from Nunukan chickens still needs to be made available, so genetic improvement is still generally good quality spermatozoa is not balanced by the survival (viability) of spermatozoa, in fact, the viability of spermatozoa is very short (Sartika et al., 2006). Semen plasma is limited as a source of nutrition for spermatozoa, while post-ejaculation, spermatozoa motility is very active. As a result of these contradictory conditions, namely limited nutritional sources, and active spermatozoa motility, the spermatozoa death rate increases (Nataamijaya, 2010).

In the Artificial Insemination (AI) program, semen collected from males undergoes several processes before being inseminated. The motility or movement power of spermatozoa is assessed immediately after semen collection. Observing motility plays a vital role as a measure of the ability of spermatozoa to fertilize an egg or ovum. Furthermore, besides observing motility, general examinations are needed, such as checking spermatozoa's volume, odor, color, consistency, pH, and concentration (Sonatina, 2007). Viability observations were conducted to determine the percentage of live and dead spermatozoa. Efforts to obtain good-quality semen require a diluent medium that can provide optimum nutrition for spermatozoa. Ingredients that can be added to diluents include fat proteins found in egg yolks (Kartasudjana, 2001). Semen dilution is carried out to reduce density so that the viability of spermatozoa is maintained. The addition of other ingredients in egg yolk skim milk diluent, such as carbohydrates, which are essential for providing energy for spermatozoa. Glucose is an excellent primary

energy source for spermatozoa used in metabolic processes (Bearden & Fukuay, 1997). Apart from egg yolk, milk can also be used as a diluent. Glucose, a carbohydrate, functions as a protective substance for lecithin and a substance for metabolic oxidation processes, including the breakdown of fat components such as glycerol and fatty acids. The function of glucose is to add energy to spermatozoa (Djanuar, 1985). Therefore, research was carried out to determine the Motility and Viability of Nunukan Chicken Spermatozoa in yolk-skimmed milk Diluent with the Addition of Various Levels of Glucose as Energy.

LITERATURE REVIEW

Nunukan chicken is a local type of chicken that is also a germplasm source from East Kalimantan. Nunukan chickens originate from Tarakan Island, East Claimantan, and are dual-purpose chickens that can be used as broilers and laying chickens (Creswell & Gunawan, 1982). Chicken. The original Nunukan is known to be able to reach a weight of 4-5 kg with egg production of around 185 eggs per year under intensive maintenance (Ardhani et al., 2019; Nataamijaya, 2010). The potential for nunukan chicken to be used as a commodity providing animal protein, especially with the presence of the National Capital (IKN) of the archipelago, has excellent potential to be developed. Still, the development of nunukan chicken has not received much attention from the government. This causes obstacles to the development of nunukan chickens in the community.

Macroscopic and microscopic testing is used to determine the quality and quantity of semen and to determine the suitability of semen to support the success of artificial insemination (AI). This testing stage requires a cement dilution process to minimize quality degradation during storage. Cement uses a diluent that contains the appropriate composition with the correct ratio between diluent and cement (Haryanto, 1996). Macroscopic examination of cement consists of volume, odor, color, consistency, and pH, while macroscopic examination includes mass movement, individual movement, and cement concentration (Yildiz et al., 2000).

An average chicken can produce around 0.2-0.5 mL of semen in one ejaculation, with an average of 0.25 mL of semen per ejaculation (Moce & Graham, 2006). Variations in semen production will occur due to the influence of age, nutritional status, ejaculate frequency, libido, and livestock condition (Yandriza et al., 2015). Ejaculation frequency significantly influences semen volume, spermatozoa concentration, and motility in lung

chickens with the best smear collection distance every 3 days. Spermatozoa concentration is vital in determining semen quality because it is a determining factor that describes the nature and number of spermatozoa in it. Apart from that, the smell and color of the cement will physically indicate its quality. Semen with an abnormal color and odor or unpleasant color and odor can indicate an infection in the male's reproductive organs or tract.

RESEARCH METHODS

Research Location and Time

This research was conducted at the Animal Reproduction and Breeding Laboratory, Animal Husbandry Study Program, Faculty of Agriculture, Mulawarman University from June to October 2024.

Research Materials and Tools

The materials used in the research consisted of Nunukan chicken semen, skim milk, egg yolk, glucose 2.5%, 3.5%, and 4.5%, 70% alcohol, Eosin-Negrosin dye solution, physiological NaCl, warm water, and distilled water. The samples used in this research were semen from twenty male Nunukan chickens, which met the requirements for healthy chickens with high mating desire. The tools used in this research include an artificial vagina complete with a measuring tube, measuring cup, Erlenmeyer tube, universal pH indicator paper, tube rack, test tube, object glass, cover glass, stirrer, Pasteur pipette, 1 ml tuberculin syringe, light microscope and micro scales, water baths, mixers, counters.

Experimental Design

The research design used in this research is a Completely Randomized Design (CRD) with four treatments and five replications. The data obtained will then be analyzed using variance (ANOVA).

Research methods

Cement Storage

Semen collection in 20 Nunukan chickens was carried out by massaging the male's back and using an electro-ejaculator. The amount of cement collected using both methods is measured and recorded. The cement obtained is then examined macroscopically and microscopically. Macroscopic examination includes semen volume, color, odor, pH, and consistency (thickness), while microscopic examination includes evaluation of concentration, motility (movement of individual spermatozoa), and viability of spermatozoa.

Preparation of Diluents and Test Samples

Make skim milk-egg yolk diluent by putting 10 grams of skim milk powder into a glass beaker, then adding 100 ml of distilled water, stirring until homogeneous, and heating over a bath to 92-95°C for 10 minutes. The milk is cooled slowly to room temperature (20-32°C). Then, the milk heads are discarded, if any, and filtered using gauze. Fresh eggs are prepared, and the shells are cleaned with 70% alcohol; the eggs are broken in half using sterile tweezers. All egg whites are carefully removed. Egg yolks that are intact and encased in a vitelline membrane are transferred to filter paper or absorbed to remove the remaining egg white liquid. The vitelline membrane is broken with a mixing glass or spatula, and the egg yolk is poured into a measuring glass. Each drop of egg yolk falls directly to the bottom of the tube so that the volume can be measured precisely.

Egg yolk is added to 5% skim milk (5 ml in 100 ml). Then, stir until evenly mixed. Penicillin 1000 IU/ml diluent and streptomycin 1 mg/ml were added and stirred until evenly distributed (Yildiz et al. l, 2000). The diluent that has been made is then used to prepare the test sample. The test samples consisted of three treatments and one control. Testing the diluent's effectiveness is carried out by giving different percentages of glucose to the sample. Glucose, as a food source material for spermatozoa, has a vital role in the survival of spermatozoa after they are accommodated. The treatments in this research are as follows:

P0: semen + (egg yolk skim milk + no glucose)

P1: semen + (egg yolk skim milk + 2% glucose)

P2: semen + (egg yolk skim milk + 3% glucose)

P3: semen + (egg yolk skim milk + 4% glucose)

Macroscopic Examination of Semen

The macroscopic examination is carried out by observing the physical form of the collected cement and grouping it based on standards for each macroscopic test parameter. The variables observed in the macroscopic examination consist of cement volume, color, odor, pH, and viscosity. Volume measurements are carried out during the holding process. The volume measured is the cement collected from each method for each chicken held.

Evaluation of Spermatozoa Motility

Examination of spermatozoa motility is carried out at 37oC to ensure optimal movement. The method of examination is first to stir the semen in the tube until it is homogeneous, then take one drop of the semen on an object glass, then cover it with a cover glass and observe how many spermatozoa are moving progressively (forward) at a breakneck speed using a microscope with a magnification of 400 times and calculating spermatozoa motility is carried out by counting the movement of motile spermatozoa in several fields of view. Data obtained from the visual field will be averaged. Spermatozoa motility according to the direction of movement is divided into four criteria, namely Progressive/P (forward), Reverse/R (backward), Oscilatory/O (rotating or in place), Necrospermia/N (not moving). In contrast, according to speed, it is divided into 0 (no or little movement of spermatozoa), number 1 (slow or slow movement), number 2 (medium movement), number 3 (fast movement), and number 4 (swift movement).

$$\%\ Spermatozoa\ Motility = \frac{\text{Progressive spermatozoa count}}{\text{Total spermatozoa observed}} \text{ x } 100\%$$

Evaluation of Spermatozoa Viability

The number of live and dead spermatozoa determines the quality of the semen produced. Examination of sperm viability is carried out by making smear preparations, which require a clean glass object. The object glass is given one tiny drop of cement and one significant drop of Eosin nigrosine solution next to it. Then, the dye and cement are mixed until homogeneous. After that, a thin preparation is made, and the preparation is dried over a flame (this process must be completed in a maximum of 15 seconds). The finished preparations were observed under a microscope with 400x magnification. Determination of the percentage of live spermatozoa using the formula:

$$\%\ Spermatozoa\ Motility = \frac{\text{Number of live spermatozoa}}{\text{Total spermatozoa observed}} \text{ x } 100\%$$

RESULTS AND DISCUSSION

Fresh Cement Quality

Based on the results of microscopic and macroscopic examination of fresh semen, the following data were obtained:

Table 1. Average value of fresh semen from Nunukan chickens

Characteristics of Spermatozoa	Average
Volume	0,3 ± 0,12
Color	Putih Susu
pH	7,1 ± 0,10
Consistency	Kental
Aroma	Khas
Mass Movement	+++

*source: research data

The Nunukan chicken semen used in the study was of good quality and in the normal range (Table 1). In macroscopic observation, the volume of free-range chicken semen was found to be 0.3 ± 0.12, lower than the volume in research conducted by Ardhani (2019). as much as 0.40 ± 0.13. The pH value in this study was 7.1 ± 0.10, which is lower than Ardhani's (2019) research of 7.34 ± 0.15. However, it is still classified as within the normal pH range, namely 7.0-7.6 (Toelihere, 1993). Variations in the degree of acidity are influenced by the environmental temperature around the maintenance drum (Mulyadi, 2007). The pH value of semen can also be influenced by germ contamination and the number of dead spermatozoa in the semen due to prolonged exposure, which triggers the formation of ammonia (Ardhani, 2014).

Cement is white, like milk. Research conducted by Kusumawati et al. (2020) showed that the color of free-range chicken semen is white and can be said to be very typical because there is no other color mixture that indicates contamination, such as a mixture of feces. Cement has a thick consistency, indicating that the cement is at an average level, and the thickness of the cement is slightly thicker than milk, indicating that the cement is of good quality. Semen has a distinctive smell, like the fishy smell typical of sperm, accompanied by the animal's scent. A semen with a foul odor indicates an infection of the reproductive organs or tract because the semen can contain pus (Woli et al., 2017). The mass movement of spermatozoa is of good quality (+++). According to Junaedi et al. (2016), the mass movement of spermatozoa is assessed based on the thickness of the mass wave and the speed at which the mass wave moves from place to place, with the assessment criteria being perfect (+++/3), good (++/2), fair (+/1), poor (no there are waves).

The effect of adding glucose to skim milk diluent on the motility and viability of nunukan chicken spermatozoa

The motility results in Table 2 show that the treatment with the addition of 3% glucose to skim milk diluent provided optimal motility and viability values for nunukan chicken spermatozoa of 52.00 ± 2.7. Glucose is an energy source that can be used by spermatozoa during the storage period because, during the storage process, glucose will still be metabolized, so spermatozoa will always need energy. Ardiansyah et al. (2018) stated that glucose is a monosaccharide (simple sugar) that functions as an energy source that can be used in metabolic processes as an energy reserve for spermatozoa.

Table 2. Mean and Standard Deviation of Motility and Viability Percentage of Nunukan Chicken Spermatozoa

Treatment	Test	Spermatozoa Motility $\bar{x}$ ± SD	Spermatozoa Viability $\bar{x}$ ± SD
P0	5	$35{,}00^{a}$ ± 3,5	$40{,}20^{a}$ ± 4,1
P1	5	$43{,}00^{b}$ ± 2,1	$58{,}80^{b}$ ± 3,1
P2	5	$52{,}00^{c}$ ± 2,7	$72{,}40^{c}$ ± 2,6
P3	5	$50{,}00^{c}$ ± 5,9	$70{,}20^{c}$ ± 2,7

*Research source

**Note: P0 with cement + skim milk without adding glucose, P1 = cement + skim milk + 2% glucose, P2 = cement + skim milk + 3% glucose, P3 = cement + skim milk + 4% glucose

P0 treatment without adding glucose had the lowest results in this study because spermatozoa only got their energy from skim milk. Irvanto et al. (2018) stated that spermatozoa need energy to carry out their activities. If the availability of ATP is lacking, then the contraction activity of the fibrils in the spermatozoa tail does not function to cause movement (motility) in the spermatozoa. The same opinion was expressed by Hardiyanti and Kurniawan (2020) that the cause of the decrease in motility values was due to the lack of energy that spermatozoa could utilize in the metabolic process, while spermatozoa were unable to re-synthesize energy and were unable to repair the damage that occurred during storage.

The results of the ANOVA test show that there are significant differences at P0, P1, P2, and P3. The Duncan test was conducted as a follow-up test to determine the highest and lowest treatment for spermatozoa motility. Duncan's test shows that P2 and P3, namely egg yolk skim milk with the addition of 3% and 4% glucose with the highest motility, 52% and 50%, show that there is no real difference in the results of the two, egg yolk skim milk with the addition of 2% glucose on P1 43%, and egg yolk skim milk without added glucose at the lowest P0, namely 35%.

The results showed that the percentage of viability of Nunukan chicken spermatozoa was at P0 at 40.20a ± 4.1, at P1 at 58.80b ± 3.1, at P2 at 72.40c ± 2.6, and at P3 at 72.40c ± 2. 6 (Table 2). The highest percentage of spermatozoa viability was in P2 egg yolk skim milk with the addition of 3% glucose (72.4), which was not significantly different from P3 egg yolk skim milk with the addition of 4% glucose (72.4), while the lowest result was in P0 of (40, 2). This proves that adding 3% glucose can optimally maintain the viability of spermatozoa in liquid semen.

The source of group diversity in the semen storage period showed a significant influence ($P<0.05$) on the motility of nunukan chicken spermatozoa. This is to the opinion of Nugroho and Saleh (2016) that the shelter period influences the motility of spermatozoa in Nunukan chickens. When cement collection is carried out, environmental temperature and weather are the influencing factors. The same opinion was expressed by Adnani et al. (2012) that factors that can influence spermatozoa motility and viability are pH, osmotic pressure, electrolytes, temperature and light, and diluent levels.

Examine the percentage of live and dead spermatozoa from smear preparations using eosin negrosine dye. Spermatozoa that do not absorb the dye eosin nigrosine indicate that the number of live and dead spermatozoa increases the permeability of the cell membrane so that they will absorb the eosin nigrosine. Living spermatozoa have good membrane conditions, so the dye has difficulty penetrating the membrane. As a result, the spermatozoa remain unstained (Susilowati et al., 2010).

Based on Table 2, P1 shows lower results than P2 and P3 because the addition of glucose at this level is insufficient for optimal spermatozoa movement (Mayesta et al., 2014). P2 experienced an optimal increase in spermatozoa motility, presumably because the addition of glucose at this level can reduce the speed of destruction of spermatozoa membrane permeability (Hidayaturrahmah, 2007). P3 experienced a decrease in spermatozoa motility than P2. This is thought to be because the addition of large amounts of glucose can cause spermatozoa to move very actively, which has a direct effect on increasing spermatozoa metabolism so that lactic acid, which is a metabolic waste product, accumulates and is toxic to spermatozoa, resulting in a decrease in spermatozoa motility (Hafez, 2000).

CONCLUSION

Based on the research results, the Nunukan chicken semen used is of good quality, both macroscopically and microscopically. According to the literature, spermatozoa's volume, color, consistency, aroma, pH, and mass movement were within the normal range. Adding glucose to egg yolk skim milk diluent significantly affected spermatozoa motility and viability. The best treatment was obtained by adding 3% glucose (P2), with motility of 52.00% ± 2.7 and viability of 72.40% ± 2.6. This shows that a glucose level of 3% can provide sufficient energy to support metabolism and maintain spermatozoa cell membranes during storage. Adding glucose up to 4% (P3) also gave good results, but not significantly different from 3% (P2). On the other hand, adding more than 3% glucose can increase metabolism excessively, producing toxic lactic acid and reducing spermatozoa motility. Thus, adding 3% glucose in egg yolk skim milk diluent can be considered the most optimal treatment to increase the motility and viability of Nunukan chicken spermatozoa during storage.

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