

DETERMINATION OF THE BEST ROSELLA (*Hibiscus sabdarifa* L.) FILTRATE CONCENTRATION IN EGG YOLK CITRATE DILUENT

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ABSTRACT

The aim of this research was to determine if the substitution of rosella filtrate (*Hibiscus sabdarifa* L.) in citrate-based diluent and egg yolks with the ratio 4:1 (16 mL citrate + 4 mL yolk) would help maintain the progressive motility of Kacang goat spermatozoa at room temperature. This study used a male Kacang goat with an average sperm quality of approximately $\geq 205 \times 10^7$ spermatozoa/mL and motility of 81.6%. An electro-ejaculator was used once a week to collect the semen. The treatments were labelled as follows: P0 was the negative control group, treatment P1 was given 5% rosella filtrate, treatment P2 was given 10% rosella filtrate, and treatment P3 was given 15% rosella filtrate. Examination of the motility of the spermatozoa was carried out at room temperature. The examination was carried out once every four hours. It was concluded that the rosella filtrate could maintain the progressive motility of spermatozoa of the Kacang goat at optimal concentrations. The optimal motility percentage was found at a concentration of 15% at a storage life of 0-4 hours.

Key words: Kacang goat, motility spermatozoa, rosella

ABSTRAK

Penelitian ini bertujuan mengetahui substitusi filtrat rosella (*Hibiscus sabdarifa* L.) dalam pengencer berbasis sitrat dan kuning telur dengan perbandingan 4:1 (16 ml sitrat + 4 ml kuning telur) untuk mempertahankan motilitas spermatozoa kambing kacang yang disimpan pada suhu ruangan. Dalam penelitian ini digunakan satu ekor kambing kacang jantan yang memiliki rata-rata kualitas sperma $\geq 205 \times 10^7$ spermatozoa/ml dan motilitas 81,6%. Koleksi semen dilakukan dengan elektroejakulator sekali dalam seminggu. Perlakuan yang diberikan adalah perbedaan dosis filtrat rosella dalam pengencer yakni 0, 5, 10, dan 15% masing-masing untuk P0, P1, P2, dan P3. Pengamatan motilitas spermatozoa dilakukan segera setelah pengenceran (jam ke-0) dan setiap satu jam sampai jam ke-4 setelah pengenceran. Dari hasil penelitian dapat disimpulkan bahwa filtrat rosella mampu mempertahankan motilitas spermatozoa kambing kacang. Konsentrasi rosella terbaik untuk mempertahankan motilitas adalah sebesar 15%.

Kata kunci: kambing kacang, motilitas spermatozoa, rosella

INTRODUCTION

As a superior goat breed, Kacang goats are great candidates for breeding through artificial insemination (AI) technology. The use of the AI technique is closely related to the semen dilution process. In semen processing, a diluent is needed to increase the volume of semen for the purpose of inseminating many females with one ejaculate, and to produce semen that can last for several days or even years at freezing temperatures.

The semen diluent must contain an energy source, buffer, and antibiotics in order for it to remain viable. A common material that can provide energy and protection for spermatozoa is egg yolk (Aboagla and Terada, 2004). A commonly used buffer is citrate which functions as a living medium for the spermatozoa cells, as an isotonic buffer, and is useful for cell metabolism in maintaining the pH and viability of spermatozoa.

Spermatozoa will die at room temperature due to an increase in the toxic quality of free radicals originating from the metabolism of the spermatozoa. Antioxidants can be used to reduce the toxic quality of the free radicals and prevent damage to the spermatozoa cell membrane. Antioxidants are substances that can bind to free radicals in order to prevent cell damage.

Antioxidants that are often used by researchers include vitamin E, vitamin C, and antioxidants that are derived from plants. One of the plants that antioxidants can be extracted from is the rosella (*Hibiscus sabdarifa* L.). Some of the compounds contained in the petals of the rosella are vitamin C and anthocyanin pigments that form flavonoids, which also act as antioxidants (Arellano *et al.*, 2004; Andayani *et al.*, 2008).

Based on the results of a study by Rinjani (2013), it was stated that the substitution of egg yolk tris with rosella filtrate at a concentration of 40-60% causes damage to spermatozoa. This is thought to be related to the high concentration of antioxidants contained within the filtrate that become prooxidants, which cause membrane damage due to lipid peroxidation (Jasda *et al.*, 2014). Therefore, it is necessary to do further research using a lower concentration of rosella filtrate at room temperature storage.

MATERIALS AND METHODS

This study used a healthy two-year-old male Kacang goat that was proven fertile. The semen collection was carried out by electroejaculation once a week. This study used a split-plot design which was divided into 4 treatments. The treatments were labelled

as follows: P0 was the negative control group, treatment P1 was given 5% rosella filtrate, treatment P2 was given 10% rosella filtrate, and treatment P3 was given 15% rosella filtrate. The research treatment pattern is presented in Table 1.

Research Procedure

Preparing egg yolk citrate diluent

A mixture of 2.9 g of Na-citrate and 100 mL of distilled water was heated to a temperature of 92° C, and then cooled to room temperature. Fresh eggs were prepared and cleaned with 70% alcohol cotton. The egg shells were broken up into 1/3-1/2 parts using sterile tweezers, and all of the albumin was removed. The egg yolks were then wrapped in a vitelline membrane and transferred to blotting paper to remove any remaining egg white. The vitelline membrane was broken down and the egg yolk was poured into a measuring cup. The Na-citrate solution was then added at a ratio of 1:4, and stirred until evenly distributed. The antibiotics penicillin 1000 IU and streptomycin 1 mg were added to each mL of the diluent and stirred evenly.

Making rosella filtrate

Fully intact red rosella petals were selected and blended until smooth. The blended petals were divided into 5, 10, and 15 g before being poured into each holding glass. One hundred mL of distilled water was added to each rosella solution and stirred until the mixtures became homogenous, and then they were left to precipitate for 24 hours. After that, the filtrate was separated using a centrifuge at 1500 rpm for 2 minutes. The results obtained were rosella filtrates with concentrations of 5, 10, and 15% (Rinjani, 2013).

Semen storage

The semen samples used were collected from a healthy two-year-old Kacang goat. The semen was collected once a week using an electro ejaculator with a sterile container glass. Immediately after the semen was collected, macroscopic and microscopic examinations of the quality of the semen were carried out. The microscopic examination carried out was to determine the motility of the spermatozoa.

Dilution technique

After evaluating the quality of the semen, it was then immediately diluted with an amount of diluent that was calculated according to the instructions of Umiyasih *et al.* (1999). The expected semen concentration was 10 million spermatozoa/mL:

$$\text{The amount of diluent} = \frac{\text{vol (mL)} \times \text{concentration} \times \text{motility} \times 0.25}{\text{expected semen concentration}}$$

A total of 16 mL of citrate was added with 4 mL of egg yolk and mixed with the semen (mL). The mixture was then divided into four tubes. Tube 1 consisted of 5 mL of a mixture of diluent and semen (P0), tube 2 consisted of 5 mL of a mixture of diluent, semen and 0.2 of 5% rosella filtrate (P1), tube 3 consisted of 5 mL of a mixture of diluent, semen and 0.2 of 10% rosella filtrate (P3), and tube 4 consisted of 5 ml of a mixture of diluent, semen and 0.2 of 15% rosella filtrate (P4).

Examination of sperm motility percentage

Examination of the motility of the spermatozoa was carried out at room temperature. The examination was carried out once every four hours. The movement of the mass of spermatozoa could be observed by dropping semen on an object glass and then observing it under a microscope with a magnification of 10 x 10. Individual movements could be observed by adding 1 drop of physiological NaCl to the semen on the object glass and adjusting the microscope to a magnification of 10 x 40. With a magnification of 10 x 40, 200 spermatozoa can be examined sequentially through 5 fields of view by shifting the field of view from left to right. The categories of movement were labelled as follows: P (progressive movement), C (circular movement/circular movement), N (normospermia, no movement), and R (reverse movement, backwards movement). The percentage of spermatozoa motility was calculated by:

$$\text{Motility} = \frac{\text{progressive spermatozoa}}{\text{total number of spermatozoa}} \times 100\%$$

Data Analysis

The data obtained was analyzed by split-plot analysis of variance (ANOVA) and continued with Duncan's test with a significance level of 5%.

RESULTS AND DISCUSSION

From the examination results of the quality of fresh semen, it was concluded that the research sample had met the requirements and was feasible to be diluted. This was obtained based on the evaluation of semen macroscopically (volume, color, consistency, and pH) and microscopically (mass movement, concentration, and individual motility). Each of the results of the average quality of fresh goat semen is presented in Table 2.

The results of the examination showed that the consistency of the semen was mostly thick. The thicker the semen, the higher the concentration of spermatozoa. This is in line with a study by Evans and Maxwell (1987), which stated that the degree of viscosity of semen has a positive correlation with the content of the

Table 1. Research treatment pattern

Treatment	Spermatozoa motility examination at hour-				
	0 (t0)	1 (t1)	2 (t2)	3 (t3)	4 (t4)
P0 (0%)	P0t0	P0t1	P0t2	P0t3	P0t4
P1 (5%)	P1t0	P1t1	P1t2	P1t3	P1t4
P2 (10%)	P2t0	P2t1	P2t2	P2t3	P2t4
P3 (15%)	P3t0	P3t1	P3t2	P3t3	P3t4

spermatozoa in the semen and that if the semen is found to be too diluted, it can be assumed that the semen has a low concentration of spermatozoa.

The concentration of spermatozoa in each (mL) of semen is one of the parameters of semen quality that is very useful for determining the number of females that can be inseminated using the semen. Goat semen that is considered to have good quality has a concentration of 2.5-5.0 million/mL (Evans and Maxwell, 1987). The concentration of goat semen in this study upon examination was 2.13 million/mL. This indicates that the concentration of spermatozoa in the sample is below normal. The difference in the concentration of spermatozoa in this study was possibly due to differences in the types of experimental animals used, age, animal health status, collection techniques, and nutrition. Male cattle that have been fed with feed that contains a high nutritional content, especially energy and protein, will show an increased production of spermatozoa. Assessment of spermatozoa concentration per millilitre is very important, because this factor is used to determine semen quality and the level of diluent.

The semen obtained was cream in colour. Normal goat semen is generally milky white or yellowish-white. The colour of healthy goat semen is creamy white, milky white, or yellow. Evans and Maxwell (1987) found that the cream colour of semen is caused by the presence of riboflavin which is secreted from the vesicular glands. The semen sample in this study had a distinctive odor but it did not have an abnormal smell, so it was determined to be feasible for dilution.

The degree of acidity of the Kacang goat semen was slightly acidic with a pH of 6.5-7.0. The average pH of fresh semen from the research was 7. The degree of acidity greatly determines the life status of spermatozoa in the semen. If the pH of the semen is lower or higher than normal, then the spermatozoa will die more quickly when outside the body.

Fresh semen is said to be normal if the spermatozoa show active locomotion and wavy mass movements

(Tambing *et al.*, 1999). The results showed that the average mass motility (+++) and the average individual motility was 81.6%. This result was categorized in the normal range based on the opinion of Hafez and Hafez (2000) who stated that good motility is between 60-80%.

The mean percentages of spermatozoa motility after administration of rosella filtrate at various concentration levels are presented in Table 3. From Table 3, it can be seen that the mean percentages of spermatozoa motility of the Kacang goat showed a significant difference ($P<0.05$) between treatment groups. The results of Duncan's multiple test showed that the percentages of spermatozoa motility in the treatment groups P1, P2, P3, and P0 were significantly different ($P<0.05$), but at the second hour of observation, P2 and P3 were not significantly different ($P>0.05$). At P0 the difference was not significant ($P>0.05$). This indicates that there was an effect on the motility of the spermatozoa when adding the rosella filtrate.

The increase in the percentage of spermatozoa motility occurred along with the increase in the dose given. The highest percent of spermatozoa motility was found at hour 0 of storage; with the means of P0, P1, P2, and P3 being 65 ± 2.18 , 65 ± 2.18 , 65 ± 2.18 , and 65 ± 2.18 respectively, while at the first hour of observation the highest motility of the spermatozoa was found in the P3 treatment. This shows that the most effective dose of rosella filtrate is around 15%.

The decrease in spermatozoa motility at each hour of observation in this study was probably due to the length of time the spermatozoa spent being exposed to the temperature of the environment. Temperature and light can affect the viability of semen outside the body. In addition, the decrease in motility was due to the nutritional content of the diluent that decreased due to the metabolic activity of the spermatozoa, which continued to grow in line with storage time at room temperature. The number of dead spermatozoa in the diluent affected the surviving spermatozoa during the

Table 2. Characteristics of fresh semen in three ejaculations of Kacang goat

Parameter	Average
Volume (mL)	0,8
Consistency	Thick
Concentration (million sperm/mL)	213×10^7
Color	Cream
Smell	Distinct
pH	7
Motilitas massa	+++
Individual motility (%)	81.6%

Table 3. Mean (\pm SD) of spermatozoa motility of Kacang goat before and after treatment with rosella filtrate

Rosella filtrate (%)	Examinations of spermatozoa before and after treatment				
	t0	t1	t2	t3	t4
P0 (0%)	$65\pm 2,18^{a,A}$	$56\pm 0,36^{a,C}$	$51\pm 0,33^{a,B}$	$45\pm 0,33^{a,D}$	$39\pm 0,34^{a,E}$
P1 (5%)	$65\pm 2,18^{a,A}$	$57\pm 0,36^{b,C}$	$52\pm 0,68^{b,B}$	$47\pm 1,73^{b,D}$	$40\pm 0,34^{b,E}$
P2 (10%)	$64\pm 2,18^{a,A}$	$58\pm 0,73^{c,C}$	$52\pm 1,18^{b,B}$	$48\pm 0,67^{c,D}$	$41\pm 0,3^{c,E}$
P3 (15%)	$65\pm 2,18^{a,A}$	$59\pm 0,74^{d,C}$	$55\pm 0,70^{c,B}$	$52\pm 0,69^{d,D}$	$43\pm 1,15^{d,E}$

a,A: a,C; b,C; c,C; d,C; a,D; b,D; c,D; d,D: a,E; b,E; c,E; and d,E Different superscripts in the same row and column represent significant differences ($P<0.05$)

storage process. Yulnawati and Setiadi (2005) found that dead spermatozoa are toxic to other living spermatozoa due to the increase in lactic acid and free radicals. An increase in free radicals can cause lipid peroxidation to occur in the plasma membrane so that the plasma membrane becomes damaged. Damaged plasma membranes will cause a disruption in the production of ATP, which is a source of energy used to maintain the life of the spermatozoa (Hammerstedt, 1993).

The high motility at the first and second hours of storage occurred because the nutritional and antioxidant content in each diluent was still complete so that the spermatozoa could utilize them optimally for their metabolic activities. However, at hours three and four of storage there was a decrease in motility because the nutrient and antioxidant content in each diluent started to run out. During this period, reactive oxygen species (ROS) in the cells' membranes began to carry out a number of chain reactions with unsaturated fatty acids, which is known as lipid peroxidation. Over time, the ROS builds to a point where it can no longer be neutralized by antioxidants, resulting in a higher level of cell membrane damage and eventually spermatozoa death.

The role of antioxidants is very important as an inhibitor that works to neutralize oxidative stress and prevent free radical damage to normal spermatozoa. Antioxidants stabilize free radicals by reacting with free radicals to form unreactive or relatively stable free radicals (Hariyatmi, 2004). The roselle filtrate contains vitamin C and anthocyanins which form flavonoids, which also function as an antioxidant (Arellano *et al.*, 2004). In the diluent, vitamin C and flavonoids can maintain the integrity of the plasma membrane so that it can stimulate various oxidative enzymes in carbohydrate and lipid metabolism and inhibit ROS.

The increase in the spermatozoa motility percentages in the dilution of egg yolk citrate that has undergone treatment with roselle filtrate was thought to be due to the antioxidant vitamin C and flavonoids present in the roselle filtrate. These can optimize the rate of fructolization so that the energy requirements for the motility and survival of the spermatozoa can be met. Vitamin C and flavonoids as antioxidants can bind to oxygen radicals so as to prevent the formation of

lipid peroxides which can inhibit glycolysis and the motility of spermatozoa.

CONCLUSION

Based on the results of the study, it can be concluded that the addition of roselle filtrate can maintain the motility of the spermatozoa of Kacang goats. The optimal motility percentage was found at a concentration of 15% at a storage life of 0-4 hours.

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