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The Benefits of Nori Fruit Extract (*Morinda citrifolia Linn*) in Increasing the Storage of Spermatozoa Y Bali Cows Resulting from Sexing at a Temperature of 5°C

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Abstract: The sperm sexing technique is the separation of spermatozoa carrying the X chromosome and the Y chromosome, which allows farmers to choose male births for beef cattle businesses. However, the process of centrifugation and sperm preservation can cause oxidative stress which gives rise to free radicals. Therefore, an antioxidant ingredient is needed in the diluent, including noni fruit extract which contains lots of flavonoids. This research aims to analyze the addition of noni fruit extract in a diluent to improve the quality of Y-chromosome spermatozoa at a storage temperature of 5°C. The results of the study showed the results of analysis of the progressive motility of Y-chromosome spermatozoa, showing a decrease in progressive motility, on D7 and D-9 there was a difference between control vs P1 and P2 (P<.05) respectively 37.63% vs 49.96% vs 48.56%, while on day 11 showed a very real difference between P0 vs P1 vs P2 respectively 24.32% vs 36.77% vs 40.87%. The addition of 3% and 5% noni extract can improve the quality of Y spermatozoa stored at 5°C. This is because noni extract contains amino acids, ascorbic acid, and flavonoids as antioxidants so that it is able to prevent free radicals, scavengers, and damage to the spermatozoa plasma membrane can be prevented. The addition of 5% noni fruit extract in the diluent to improve the quality of Y-chromosome spermatozoa at a storage temperature of 5°C for up to 11 days.

Keywords: Bali cattle; Morinda citrifolia extract; Sexing sperm; Y chromosome

Introduction

Advances in the field of reproduction have made it possible for breeders to obtain certain types of calves according to the livestock business they are developing, for example the selection of female seeds is carried out if the breeder wants a dairy cow. Meanwhile, beef cattle farming requires the birth of male calves (Lukman et al., 2020). Male livestock grow faster and are more expensive than females. Efforts to obtain the birth of a male cattle can be done by sperm sexing, namely separating spermatozoa carrying the X chromosome and Y chromosome before insemination. It was stated by Susilawati (2014) that if the ovum is fertilized by X spermatozoa will be a female and by Y spermatozoa will be a male offspring. The separation technique using egg white albumin is the cheapest and easiest to apply. The success rate for separating X and Y spermatozoa is around 85-95% (Garner et al., 2000). In this way, semen with a certain spermatozoa content can be obtained and used for artificial insemination (AI).

The implementation of AI using sexed semen shows high fertility, namely, the Service per Conception (S/C) value for X sperm is 1.53 and Y sperm is 1.54. The conception rate (CR) figures are for X sperm at 69.25% and Y sperm at 68.29%. Meanwhile, the accuracy of gender insemination using X sperm was 87.01% for female offspring and 89.5% for Y sperm was obtained for

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male offspring (Gunawan, 2015). Yuliani et al. (2013) added that sex ratio regulation can be produced commercially to produce superior male or female offspring as parents for male offspring or seedlings. Therefore, implementing AI using Y sperm is useful for increasing the birth of male livestock so that it can support the government program, namely the prohibition on slaughtering productive female livestock in Law Number 41 of 2014 (Disnakkeswan Provinsi Jawa Tengah, 2023).

AI implementation can use frozen semen or liquid cement. Frozen semen is semen that is stored in a container containing liquid N2 at a temperature of -196^{o}C and can be stored for up to 10 years, however the freezing process results in damage to the spermatozoa membrane resulting in low motility and low AI results (Susilawati et al., 2018). Meanwhile, liquid semen is semen that is stored in a refrigerator at a temperature of 4^{o}C, aged only up to 3-4 days (Susilawati, 2013), but liquid semen has better motility and is more viable (Borges et al., 2015). The implementation of AI using liquid semen shows a high pregnancy rate (Quan et al., 2016). AI using liquid semen is an alternative for storing semen in a refrigerator (temperature 2-4^{o}C) for areas where liquid N_2 is not available (Yekti et al., 2018).

Several studies have been carried out to extend the shelf life of liquid semen by adding several antioxidant ingredients such as vitamin C and vitamin E. Antioxidants are needed to overcome free radicals which increase during the semen processing, sperm sexing and semen preservation processes. This antioxidant ingredient is found in many noni fruits, according to Sjabana et al. (2002) that noni fruit contains vitamin C, scopoletic, nitric oxide and vitamin A.

Based on this description, it is deemed necessary to carry out research on the benefits of adding noni extract (Morinda citrifolia Linn) to semen diluent in an effort to increase the shelf life of spermatozoa Y of Bali cattle resulting from sexing sperm. It is hoped that the results of this research can produce Y-chromosome semen that has high fertility and longer shelf life by utilizing local fruit, namely *Morinda citrifolia Linn.*

Method

Preparation of Extract Morinda citrifolia Linn

The raw *Morinda citrifolia Linn*, washed with water, thinly sliced and dried for a week, then grinded until it becomes flour (simplicia). Simplicia soaked in ethanol 96% for 48 hours, stirred twice daily for two days. After that the liquid is filtered, then separated the filtrate with dregs. Repeated for three times. Store the filtrate in a closed container until it becomes a thick extract.

Preparation of Diluent

Andromed diluent dissolved in distilled water in a ratio of 1:4. Sperm diluent is ready to use. Treatment diluent (Po): without noni extract in the diluent. Treatment diluent (P1): 3% noni extract in diluent. Treatment diluent (P2): 5% noni extract in diluent.

Preparation of Sexing Media

Separation media: free-range chicken eggs aged 1-3 days, the whites are taken and filtered using filter paper to get the runny egg whites. Egg whites were diluted to 10 and 30%, respectively. 2 ml of 10% stock as the upper fraction (A1) was added above 2 ml of 30% stock as the lower fraction (A2) in a reaction tube. Separation process: 1 ml of semen that has been diluted with Andromed diluent 1:1, is put into a separation media tube then incubated for 20 minutes. Semen from the lower fraction (A2), was placed in a test tube containing 3 ml of diluent and centrifuged at 1500 rpm for 5 minutes. Discard the supernatant until 1 ml remains semen resulting from sexing. Next, put it in a test tube containing diluent + Morinda citrifolia Linn extract according to the treatment and stored at 5^{o}C. Observed every day until motility ≥ 40%.

Preparation Semen

This research used semen which was collected from two Bali cattles 3-5 years old. An artificial vagina (AV) is used to collecting semen, which is taken to the laboratory for processing. Semen is evaluated macroscopically (volume, viscosity, consistency, color, degree of acidity (pH), odor) and microscopically (mass and individual movement, spermatozoa viability). The semen used has a motility of ≥70% with a mass movement of 2++. Semen is collected twice a week and checked for motility.

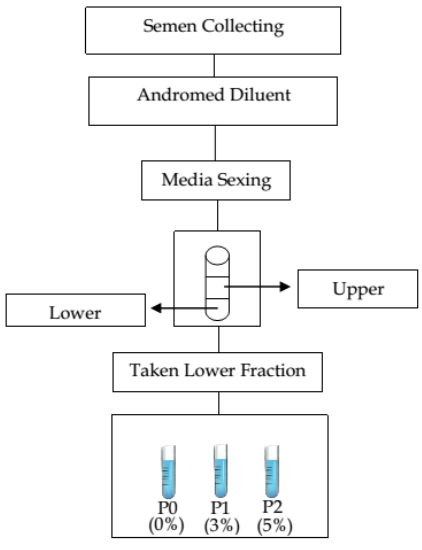


Figure 1. Sperm sexing research flow

Research design

This research is a laboratory experiment consisting of three treatments with 6 repetitions, namely:

P0 = Andromed Diluent + 0% Morinda citrifolia Linn Extract

P1 = Andromed Diluent + 3% Morinda citrifolia Linn Extract

P2 = Andromed Diluent + 5% Morinda citrifolia Linn Extract

Research Variable

Motility and progressive motility were observed using CASA (Computer Assisted Sperm Analysis). Spermatozoa viability is live spermatozoa, carried out by eosin negrosine staining. Live spermatozoa are colorless, while dead ones are purplish pink. Viability calculation uses equation 1.

$$Viabilitas\ spermatozoa = \frac{Viable\ Spermatozoa}{Total\ Spermatozoa} x\ 100\% \quad (1)$$

Evaluation of spermatozoa motility and viability was carried out until the 11th day of storage in a refrigerator at 4°C. Data were analyzed using ANOVA (RAL), if there were differences, the BNT test was continued (Sudjana, 2005) assisted by SPSSv.16 software.

Result and Discussion

Fresh Semen

Immediately after the semen is collected, a macroscopic and microscopic evaluation is carried out (Table 1.) Macroscopic evaluation includes: volume, pH, color, odor and consistency. Meanwhile, microscopic evaluation is mass and individual motility. Evaluation of sperm concentration is carried out using a photometer.

Table 1. Evaluation of Fresh Semen

Parameter	Mean ± SD
Volume	3.98 ± 0.65 ml
pH	6.87±0.03
Color	Cream, milk, yellowish
Smell	Specific
Consistency	Thick
Mass motility	+++
Individual motility	89.34 % ± 1.32%
Concentration	153 x 10^6

Motility

Motility examination uses the CASA tool which can provide objective and automatic data about motility (Verstegen et al., 2002) and characteristics of motility (Ratnawati et al., 2018), in this study shows a motility of 89.34% ± 1.32%, then this semen is suitable for further processing. The minimum requirement for spermatozoa motility for semen processing is ≥ 70% in both cows and buffalo (Michael et al., 2010; Wadood et al., 2022). The pH of fresh semen in this study also showed normal values. The quality of fresh semen shows good and normal because this study used semen derived from the ejaculate of Balinese cows aged between 3-5 years with a body score (BCS) of 3-4 (scale 1-5). According to Nugraha et al. (2019) that 4-year-old bulls have the highest sperm motility and ejaculate volume. There is a correlation between age and body weight on semen quality (Nugraha et al., 2019).

Sexing Sperm

After the semen meets the requirements according to SNI standards, then semen is diluted using Andromed diluent. The function of dilution is to increase the volume of semen and provide a medium and provide nutrition to sperm after ejaculation.

Semen treated with diluent is put into sexing medium for the process of separating X and Y sperm and stored at 4°C. The upper fraction is discarded (containing a lot of X sperm), while the lower fraction (containing a lot of Y sperm) is taken (Susilawati et al., 2013). Next, put it in a test tube containing the diluent according to the treatment and observe for motility and viability.

Motility

Semen containing a lot of Y sperm was stored at 5°C in a refrigerator, motility and progressive motility were observed. Observations were made at 15 minutes, 3 hours, 24 hours, day 3, day 5, day 7, day 9, and 11 of motility (Figure 2.) and progressive motility (Figure 3).

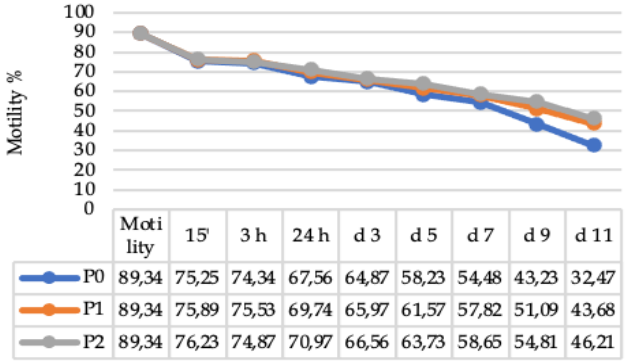


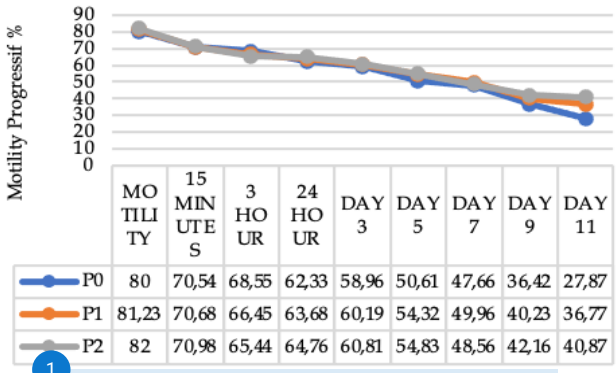
Figure 2. Motility during storage

Information:
P0 = control
P1 = 3% Morinda *citrifolia linn Extract*
P2 = 5% Morinda *citrifolia linn Extract*

Based on Figure 2, it appears that there is a decrease with the length of storage time. During the sperm sexing process and semen preservation, there will

be a decrease in quality due to cold shock during preservation or due to the centrifugation process during sperm separation (Susilawati, 2013). The results of the statistical analysis showed that differences occurred on day 7 of the storage period between control vs P2, while on day 9 there was a real difference between Po vs P1 and P2 (P<.05), and on day 11 there was a very significant difference between P0 vs P1 and P2 (P<.01) but there was no difference between P1 vs P2 (P>.05).

Analysis of the progressive motility of Y-chromosome spermatozoa, showed a progressive decrease in motility, on H7 and H-9 there was a difference between control vs P1 and P2 (P<.05) respectively 37.63% vs 49.96% vs 48.56%, whereas on day 11 shows a very real difference between P0 vs P1 vs P2 respectively 24.32% vs 36.77% vs 40.87%.



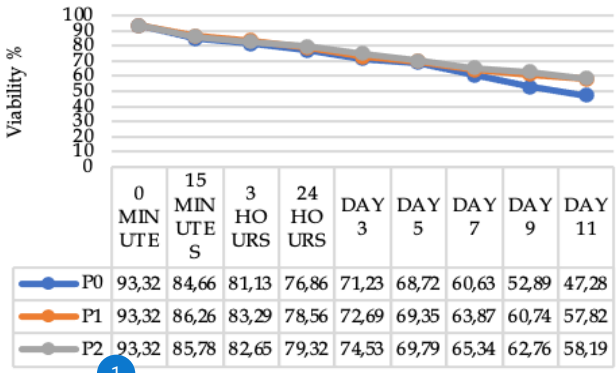
	MOTILITY	15 MINUTES	3 HOUR	24 HOUR	DAY 3	DAY 5	DAY 7	DAY 9	DAY 11
P0	80	70,54	68,55	62,33	58,96	50,61	47,66	36,42	27,87
P1	81,23	70,68	66,45	63,68	60,19	54,32	49,96	40,23	36,77
P2	82	70,98	65,44	64,76	60,81	54,83	48,56	42,16	40,87

Figure 3. Progressive motility of Y spermatozoa

Information:
P0 = control
P1 = 3% Morinda *citrifolia linn Extract*
P2 = 5% Morinda *citrifolia linn Extract*

What is meant by progressive motility is motility that moves nimbly, moves forward, can penetrate the ovum for fertilization, and also experiences a decrease because during the semen processing process there will be a decrease in sperm quality including motility. According to Rizal (2009), semen reservation can cause the death of spermatozoa, due to damage to the spermatozoa plasma membrane due to lipid/fat peroxidation and during the sperm sexing process this can result in damage to the plasma membrane. P2 and P3 treatment on day 7 and P2 can last until day 11 showing progressive motility ≥ 40% so that the semen can still be used for AI purposes, according to SNI standards that semen used for AI has at least progressive motility ≥ 40% (Yekti et al., 2018).

The results of this research have a longer shelf life compared to the research results of Nolasco et al. (2016) which was only 7 days in the diluent tris methane + 20% of egg yolk. 8 days of semen in CEP-2 diluent + 10% egg yolk (Sholikah et al., 2016). The shelf life of up to 11 days in this study is possible because noni extract contains flavonoid antioxidants (Rohman et al., 2017), vitamin C, scopoletic, nitric oxide, and vitamin A (Sjabana et al., 2002) which can protect membrane lipids (Hossain et al., 2006) fights oxidative stress from oxidants and free radicals that occur during semen processing and storage. Apart from that, the content in andromed diluent, namely egg yolk, also acts as a cryoprotectant so that it can maintain membrane integrity which has an impact on sperm motility (Purwoistri et al., 2013; Sugiarto et al., 2014) (see figure 4).



	0 MINUTE	15 MINUTES	3 HOURS	24 HOURS	DAY 3	DAY 5	DAY 7	DAY 9	DAY 11
P0	93,32	84,66	81,13	76,86	71,23	68,72	60,63	52,89	47,28
P1	93,32	86,26	83,29	78,56	72,69	69,35	63,87	60,74	57,82
P2	93,32	85,78	82,65	79,32	74,53	69,79	65,34	62,76	58,19

Figure 4. Viability of Y spermatozoa

Information:
P0 = control
P1 = 3% Morinda *citrifolia linn Extract*
P2 = 5% Morinda *citrifolia linn Extract*

The viability of Y spermatozoa is calculated using formula 1. It shows that on day 9 there is a real difference between P0 vs P2, while on day 11 there is a real difference between P0 vs P1 and P2. This happens because noni contains amino acids, ascorbic acid, beta-carotene, terpenoids, alkaloids, beta-sitosterol, carotene, polyphenols such as flavonoids, flavone glycosides, rutin, and putative proxeronine (Preveen et al., 2007). It was stated by (Simamora, 2009) that flavonoids inhibit the work of enzymes involved in the superoxide anion production reaction, flavonoids also bind trace metals involved in reactions that produce free radicals. Herdis et al. (2005) added that high spermatozoa mortality in the semen processing process can damage the plasma membrane due to lipid peroxidation. The lipid peroxide reaction occurs due to contact between semen and oxygen (O_2) which can produce free radicals and hydrogen peroxide.

The addition of antioxidants in semen diluent is done to minimize or suppress damage to spermatozoa membranes due to free radicals. The increase in vitamin C dosage into skim egg yolk extender was no significant (P>0.05) to sperm motility and life percentage of sperm

(Savitria et al., 2014). On the other hand, the addition of the antioxidant vitamin E as much as 0.134 grams/100 ml extended improvement of post-thawing motility and decreased the percentage of sperm mortality and abnormality (Haris et al., 2020). Simanjuntak (2012) stated that the role of vitamin E can be replaced by flavonoids which have antioxidant activity. These flavonoids are found in many noni fruits.

Conclusion

Adding 3% of noni fruit extract as a source of natural antioxidants to the diluent can maintain the quality of Y-chromosome spermatozoa until the 9th day and 5% until the 11th day.

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Sri Firmiaty: writing-original draft preparation, result, discussion, methodology, conclusion; Bestfy Anitasari: discussion, conclusion. Muhammad Idrus, Hamsina Hamsina: analysis, proofreading, review, and editing.

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Conflicts of Interest
The authors declare that there is no conflict of interest regarding the publication of this paper.

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