Preservation of bovine semen using egg yolk substituted with extract guava in water from yellow coconut on the semen quality and fertility of Bali cattle

A Marawali\textsuperscript{1}, M S Abdullah\textsuperscript{1}, Jalaludin\textsuperscript{1}, W M Nalley\textsuperscript{1}, T M Hine\textsuperscript{1} and Y L Henuk\textsuperscript{2}\textsuperscript{*}

\textsuperscript{1}Faculty of Animal Science, Nusa Cendana University, Kupang, Indonesia.
\textsuperscript{2}Department of Animal Science, Faculty of Agriculture, Universitas Sumatera Utara, Medan, Sumatera Utara, Indonesia.

E-mail: *profesorhenuk@gmail.com

Abstract. The present study used the ratio of 80\% coconut water and 11 - 20\% egg yolk substitution with extract guava ranged from 4 to 9\% in order to determine the quality and fertility of Bali cattle stored at 5°C. This study used a complete factorial design with two factors and six treatments. The first factor was coconut water and egg yolk substituted with extract guava extender at different ratio consisted of 6 treatments. They were: (1) T\textsubscript{0} (control) = 80\% coconut water + 20\% egg yolk without extract guava; (2) T\textsubscript{1} = 80\% coconut water + 16\% egg yolk + 4\% extract guava; (3) T\textsubscript{2} = 80\% coconut water + 15\% egg yolk + 5\% extract guava; (4) T\textsubscript{3} = 80\% coconut water + 14\% egg yolk + 6\% extract guava; (5) T\textsubscript{4} = 80\% coconut water + 13\% egg yolk + 7\%; and (5) T\textsubscript{5} = 80\% coconut water + 12\% egg yolk + 8\% extract guava; and (6) T\textsubscript{6} = 80\% coconut water + 11\% egg yolk + 9\% extract guava. The second factor was the length of storage at low temperature which consisted of 3 treatments, including 1 day, 2 days, 3 days, 4 days, 5 days, 6 days and 7 days. The semen used in this study had motility ranged from 50 to 55\%. The variables measured in this study were intact plasma membrane (IPM) and intact acrosome hood (ICH). The results showed that the percentage of spermatozoa motility for T\textsubscript{1} until the sixth day of storage was 55.51\%, T\textsubscript{2} until the seventh day of storage was 40.14\%, T\textsubscript{0} to the fifth day was 41.73\%, T\textsubscript{3} and T\textsubscript{4} until the fourth day was 43.85\% and 41.11\%, respectively, whereas for T\textsubscript{5} only until the third day were 51.00\% and T\textsubscript{6} until the second day amounted to 48.83\%. Viability of Bali cattle spermatozoa in the six treatment groups where egg yolk substituted with extract guava was higher (T\textsubscript{1}, T\textsubscript{2} and T\textsubscript{6}) compared to T\textsubscript{0} from the first day until the fifth day of storage. It can be concluded that substitution of 15\% egg yolk with 5\% extract guava in 80\% coconut water diluents can maintain IPM and ICH spermatozoa of Bali cattle until the sixth day of stored at 5°C.

1. Introduction

Artificial insemination (AI) is a globally accepted method of breeding cattle and is also effective for other species (Figure 1). AI is widely accepted as a technology that can bring about rapid genetic improvement in breeding cattle. Although AI is widely used in many countries, many factors must be considered in order to achieve the success of AI, such as optimum conception rates will only be achieved if the quality of semen used is good, the insemination is done at the most appropriate time in relation to the oestrous period, and the technicians have adequate training and skills in the procedure. These factors,
together with other socio-economic considerations specific to smallholder production systems and inadequate infrastructure for the efficient delivery of AI services, have often led to poor success rates of AI. If these constraints can be overcome, not only would the cattle farmers and service providers benefit, but the technology would also become more widely adopted [1,2]. Increasing the productivity of Bali cattle needs to be supported by the availability of reproductive technology, especially which is associated with the efficiency and management of reproduction, in order to improve and maintain the fertility. Improvement of the fertility which easily applied is by controlling estrus and insemination time through AI technology by using chilled semen or frozen semen [3]. The method of preserving semen in a liquid form allows an intermediate storage of a few days only while the deep-frozen form permits storage for years without any significant decrease in semen quality. According to the length of storage planned, different semen extenders have been developed. The composition of a semen extender is mainly based on an energy resource (sugars such as glucose and lactose) and a buffer medium of different inorganic or organic salts. Milk and egg yolk, for example, are basic ingredients of most extending media. Egg yolk, especially, is recognized as a protectant against cold shock through its lipoprotein and phosphatidylcholine [4]. Semen extenders should be able to demonstrate the ability to minimize the sperm motility impairment rate to eventually extend the length of storage time. However, not all semen extenders exhibit the same ability to maintain spermatozoa. Therefore, it is necessary to identify the effect of semen extenders and length of storage on the motility and viability of Bali bull fresh semen [2]. The extender ratio of 70% coconut water and 30% egg yolk was able to maintain the sperm viability percentage to survive on storage temperature of 5°C on the third day [3]. The present study used the ratio of 80% coconut water and 11 – 20% egg yolk substitution with extract guava ranged from 4 to 9% in order to determine the quality and fertility of Bali cattle stored at 5°C.

![Figure 1. AI of cow](image)
2. Materials and methods

2.1. Experimental animals and management of experimental extenders

Bovine semen collected from a five year old Bali cattle using artificial vagina. Semen of good quality were kept in six tubes based on the six treatments then stored at 5°C. They were: (1) T₀ (control) = 80% coconut water + 20% egg yolk without extract guava; (2) T₁ = 80% coconut water + 16% egg yolk + 4% extract guava; (3) T₂ = 80% coconut water + 15% egg yolk + 5% extract guava; (4) T₃ = 80% coconut water + 14% egg yolk + 6% extract guava; (5) T₄ = 80% coconut water + 13% egg yolk + 7%; and (5) T₅ = 80% coconut water + 12% egg yolk + 8% extract guava; and (6) T₆ = 80% coconut water + 11% egg yolk + 9% extract guava. The second factor was the length of storage at low temperature which consisted of 3 treatments, including 1 day, 2 days, 3 days, 4 days, 5 days, 6 days and 7 days. The variables measured in this study were intact plasma membrane (IPM) and intact acrosome hood (ICH) of spermatozoa of Bali cattle.

2.2. Data collection and analysis

The variables observed in this study were IPM and ICH of spermatozoa of Bali cattle. Data were collected were subjected to ANOVA’s procedures, where significance difference were observed between treatment means, they were separated by Duncan’s multiple range test [5]. Differences in the average values among treatment groups were examined by Duncan’s multiple range tests. Statistical significance was declared at a probability of p<0.05.

3. Results and discussion

3.1. Effect of treatments on IPM Spermatozoa of Bali Cattle

Percentage of IPM of spermatozoa of Bali cattle where egg yolk is substituted with in coconut water diluent at 5°C storage are presented in Table 1. An IPM is an absolute must-have fertile spermatozoa because the plasma membrane plays a major role in regulating all biochemical processes that occur in cells. The integrity of the plasma membrane largely determines the life and death of spermatozoa, therefore the percentage of IPM should not be much different from the value of live spermatozoa [6]. Evaluation of spermatozoa with an intact plasma membrane can be tested using the hypoosmotic swelling (HOS) test method. Spermatozoa that have the integrity of a intact and living plasma membrane are characterized by swelling of the head followed by a tail rotating with a bright colour beam. While spermatozoa with a damaged and dead plasma membrane are characterized by a straight tail and no swelling of the head accompanied by a red glow.

From the results in Table 1, it can be seen that the IPM of spermatozoa of Bali cattle on storage from the first day to the sixth day is still relatively good is T₁ and T₂, while T₀ until the fourth day, T₃, T₄ and T₅ until the third day of storage, T₆ only until the first day. The percentage of IPM for each treatment from the first day to the sixth day of storage for T₁ was 80.85%, 75.57%, 70.30%, 65.83%, 60.50% and 51.50% with an average of 67.43%; P₂ is 80.75%, 76.57%, 71.30%, 66.84%, 61.50% and 56.11% with an average of 68.85%; T₃ until the fourth day of storage were 74.75%, 67.57%, 53.30% and 50.16% with an average of 61.45%, T₄, T₅ and T₆ until the third day of storage were T₇: 72.63%, 63.97% and 48.80% with an average of 61.80%; P₄ was 70.79%, 56.80% and 47.50% with an average of 58.36%; T₅ was 63.51%, 49.42% and 43.07% with an average of 51.67% and T₆ only until the first day of storage was 54.55%. Statistical analysis showed a significant difference (P <0.05) between T₀ and T₁, T₂, T₃ and T₆; between T₁, T₂ with T₃, T₄, T₅ and T₆; between T₃ and T₄ with T₅ and T₆; between T₃ and T₄ with T₅ and T₆; not significantly different (P> 0.05) between T₀ and T and T₃ and T₄; between T₁ and T₂ with T₃, T₄, T₅ and T₆; between T₁ and T₂ with T₃ and T₄, T₅ and T₆; between T₃, T₄ and T₅ with T₆; between T₃, T₄ and T₅ with
A: T6, not significantly different (P > 0.05) between T0 and T8, between P1 and P2, between P5 and P6 on the third day of storage. Significantly different (P < 0.05) between T0 and T1, T2, T5, T3, T8 and T5; between T2 and T3, T4, T5 and T6; between T3, T4 and T5 with T2; significantly different (P > 0.05) between T1 and T2, between T3, T4 and T5 on the fourth day of storage. Significantly different (P < 0.05) between T5 and T1 and T2 was not significantly different (P > 0.05) between T1 on the fifth day of storage and was significantly different (P < 0.05) between T1 and T2 until the sixth day of storage.

Table 1. Percentage of IPM of spermatozoa of Bali cattle where egg yolk is substituted with in coconut water diluent stored at 5°C

| Treatments | Storage time (days) |
|------------|---------------------|
|            | 0       | 1       | 2       | 3       | 4       | 5       | 6       | 7       |
| T0         | 82.01   | 74.75   | 67.57   | 53.30   | 50.16   | 37.50   |         |         |
| ±0.38      | ±5.19   | ±6.12   | ±6.21   | ±4.01   | ±4.21   |         |         |         |
| T1         | 83.84   | 80.58   | 75.57   | 70.30   | 65.83   | 60.50   | 51.50   |         |
| ±0.38      | ±1.71   | ±1.33   | ±1.48   | ±2.01   | ±1.64   | ±2.09   |         |         |
| T2         | 83.84   | 80.75   | 76.57   | 71.30   | 66.84   | 61.50   | 56.11   | 39.7    |
| ±0.38      | ±1.59   | ±1.33   | ±1.48   | ±2.01   | ±1.64   | ±1.03   |         |         |
| T3         | 83.84   | 72.63   | 69.97   | 48.80   | 33.75   |         |         |         |
| ±0.38      | ±4.74   | ±3.64   | ±7.30   | ±3.10   | ±0.68   |         |         |         |
| T4         | 83.84   | 70.79   | 56.80   | 47.50   | 32.64   |         |         |         |
| ±0.38      | ±1.16   | ±11.66  | ±8.16   | ±7.68   | ±6.80   |         |         |         |
| T5         | 83.84   | 63.51   | 49.42   | 43.07   | 29.93   |         |         |         |
| ±0.38      | ±2.28   | ±7.77   | ±14.36  | ±11.24  | ±7.77   |         |         |         |
| T6         | 83.84   | 54.55   | 36.04   | 26.17   | 14.44   |         |         |         |
| ±0.38      | ±7.00   | ±7.92   | ±7.60   | ±4.39   |         |         |         |         |

Note: Means with same superscript within a column are not significantly different (P > 0.05).

Higher IPM for T1 and T2 compared to T0 provide evidence that egg yolk substitution with guava extract in coconut water diluent in Bali cattle spermatozoa is able to inhibit damage to the integrity of the spermatozoa plasma membrane. This is in accordance with [6] that guava extract is one source of antioxidants that can capture free radicals and prevent chain reactions. Vitamin E and vitamin C contained in guava extracts function as the most powerful intracellular antioxidants in preventing peroxidation of unsaturated fatty acids in and cell walls, so as to avoid peroxidative damage that affects the viability and fertility of spermatozoa [7]. IPM spermatozoa of Bali cattle obtained in this study until the sixth day of storage was not much different compared to the results reported by [8] with IPM spermatozoa of Bali cattle 56.00 ± 1.41% and 55.40 ± 2.41% after six days of storage with basic diluent treatment 80% tris + 20% egg yolk + 0.6 g lactose per 100 ml diluent and 80% tris + 20% egg yolk + 0.6 g maltose per 100 ml diluent.

3.2. Effect of treatments on ICH spermatozoa of Bali cattle

Percentage of ICH spermatozoa of Bali cattle where egg yolk is substituted with extract guava in water of yellow coconut diluent + egg yolk stored at 5°C are presented in Table 2.

The results showed that ICH spermatozoa of Bali cattle at 5°C storage from the first day to the fifth day on T0 was 74.75 ± 5.19%, 67.57 ± 6.12%, 53.30 ± 6.21%, 50.16 ± 4.01% and 37.50 ± 4.21%; T1 until the sixth day of storage were 80.58 ± 1.71%, 75.57 ± 1.33%, 70.30 ± 1.48%, 65.83 ± 2.01%, 60.50 ± 1.64%, and 51.50 ± 2.09%; T2 until the seventh day of storage were 80.75 ± 1.16%, 76.57 ± 1.33%, 71.30 ± 1.48%, 66.84 ± 2.01%, 61.50 ± 1.64%, 56.11 ± 1.03% and 39.7 ± 7.68%; T3 until the fourth day of storage were 72.63 ± 4.74%, 69.97 ± 3.64%, 48.80 ± 7.30% and 33.75 ± 8.31%; T4 until the fourth day of storage were 70.79 ± 6.80%, 56.80 ± 11.66%, 47.50 ± 8.16% and 32.64 ± 7.68%; T5 until the fourth day of storage was 63.51 ± 2.28%, 49.42 ± 7.77%, 43.07 ± 14.36% and 32.64 ± 7.68%; and T6 until the fourth day of storage were 54.55 ± 7.09%, 36.04 ± 7.92%, 26.17 ± 7.60% and 14.44 ± 4.39%.

Based on these results and if related to eligibility for use in the AI program, then T0 is only until the fourth day of storage, T1 and T2 until the sixth day of storage, T3, T4 and T5 until the third day of storage.
and T₆ only until the first day of storage. The success of AI in the field is not only determined by the progressive motility of spermatozoa but also one of the important factors derived from internal spermatozoa is the ICH spermatozoa. The integrity of ICH is very good during the process of capacitation and acrosome reaction during the process of fertilization so that it contributes positively to the fertility of spermatozoa of Bali cattle during renewal. The morphological abnormalities of spermatozoa heads above 10% contributed to the decline in male fertility.

**Table 2.** Percentage of ICH spermatozoa of Bali cattle where egg yolk is substituted with extract guava in water of yellow coconut diluent + egg yolk stored at 5°C

| Treatments | Storage time (days) |
|------------|---------------------|
|            | 0       | 1       | 2       | 3       | 4       | 5       | 6       | 7       |
| T₀         | 82.01 ±4.88a      | 74.75 ±5.19b | 67.57 ±6.12b | 53.30 ±6.21b | 50.16 ±4.01b | 37.50 ±4.21b |        |        |
| T₁         | 83.84 ±3.08a      | 80.58 ±1.71a | 75.57 ±1.33a | 70.30 ±1.48a | 65.83 ±2.01a | 60.50 ±1.64a | 51.50 ±2.09 |        |
| T₂         | 83.84 ±3.08a      | 80.75 ±3.08a | 76.57 ±3.08a | 71.30 ±3.08a | 66.84 ±3.08a | 61.50 ±3.08a | 56.11 ±3.08a | 39.68 ±3.08a |
| T₃         | 83.84 ±3.08a      | 72.63 ±3.08a | 63.97 ±3.08a | 48.80 ±3.08a | 33.75 ±3.08a |        |        |        |
| T₄         | 83.84 ±3.08a      | 70.79 ±3.08a | 56.80 ±3.08a | 47.50 ±3.08a | 32.64 ±3.08a |        |        |        |
| T₅         | 83.84 ±3.08a      | 63.51 ±3.08a | 49.42 ±3.08a | 43.07 ±3.08a | 29.93 ±3.08a |        |        |        |
| T₆         | 83.84 ±3.08a      | 54.55 ±3.08a | 36.04 ±3.08a | 26.17 ±3.08a | 14.44 ±3.08a |        |        |        |

Note: Means with same superscript within a column are not significant different (P>0.05).

The higher percentage of egg yolk substitution with guava extract gave a higher negative effect as indicated by a decrease which was also quite high on IPM and ICH [9]. This indicates that the positive effect of guava extract can only be obtained when the egg yolk is substituted in a level that is not too high, and it will produce a detrimental effect on sperm quality when the yolk is substituted in large quantities. The quality of spermatozoa in coconut water diluents where egg yolks are substituted with 4% (T₁) and 5% (T₂) guava extract is higher ((P <0.05) than treatments with extract guava without substituted yolk (T₀), and T₁, T₄, T₅, T₆) each egg yolk is substituted with extract guava of 6%, 7%, 8% and 9%. Pathological mechanism of cell damage caused by free radicals or oxidants through three things, namely: (1) lipid peroxidation: free radicals can attack unsaturated fatty acids (polyunsaturated fatty acids) on cell membranes causing lipid peroxidation.

The breakdown of fatty acids results in the formation of various oxidative products, which are toxic to cells. Cell membranes contain large amounts of polyunsaturated fatty acids, which serve to maintain their fluidity. Peroxidation of these fatty acids causes loss of membrane fluidity and decreased activity of membrane enzymes and ion channels. As a result, the normal cellular mechanism needed for various physiological processes is inhibited [10]; (2) DNA damage: high levels of free radicals cause DNA fragmentation. Various types of DNA abnormalities such as base modification, base-free site production, DNA cross-links and chromosomal rearrangements; (3) Apoptosis: ROS can also initiate a series of reactions that ultimately cause apoptosis. Apoptosis is a natural process by which the body gets rid of old cells and senescent cells; a programmed cell death process.

Apoptosis can be induced in cell culture by H₂O₂, which supports the theory that ROS is involved in apoptosis. Besides that, the process of apoptosis can be accelerated by free radical damage-induced DNA [11]. Oxidants can react with biomolecules such as lipids, proteins, and DNA. For lipids, lipid membranes and lipids in the circulation of lipoproteins such as low-density lipoprotein (LDL) can interact with free radicals to produce lipid peroxidation. When lipid peroxidation forms, they can interact with oxygen to form highly reactive peroxyl radicals and subsequently undergo radical propagation to
form hydroperoxidation. Ascorbate can reduce the initiation of free radicals so that lipid peroxidation can be inhibited [12]. During in vitro storage, spermatozoa produce high enough free radicals which in turn invade the plasma membrane and DNA [13], damage to the plasma membrane causes the transport of oxygen, various nutrients and ions that enter or exit the cell will be disrupted and cause the survival of degraded spermatozoa. DNA damage will have an impact on the failure of the process of transcription and translation for protein synthesis and lead to the occurrence of cell apoptosis. Antioxidants contained in extract guava can counteract free radical attack, thus cells avoid damage and death.

4. Conclusions
Substitution of 15% egg yolk with 5% extract guava in 80% coconut water diluents can maintain IPM and ICH spermatozoa of Bali cattle until the sixth day of stored aged at 5°C.

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