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Supplementation of Moringa (*Moringa oleifera*) seed extract in an extender on the quality of Bali bull semen

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Abstract. In the processing semen, there is a contact between semen and oxygen (O²) to produce free radicals which can reduce the quality of semen. The decrease in semen quality caused by free radicals can be suppressed by the addition of antioxidants in the extender. This study aimed to prove that the Moringa seed extract can be used as an alternative to natural ingredients that can be added to the semen extender, in which it can maintain the quality of Bali bull sperms at equilibration. This study was used one Bali bull for semen collection with the treatment of Moringa (*Moringa oleifera*) seed extract supplementation into Tris egg yolk (TEY) as an extender; 25% (P1), 50% (P2), 75% (P3). Andromed (P0A) and TEY 100% (P0B) were used as control with three replications. The parameters measured included the individual and progressive motility of the sperms. Each parameter in each treatment was compared using a factorial completely randomized design. The results showed that the use of Moringa seed extract supplemented in the extender resulting both individual and progressive motility of Bali bull sperms decreased slowly in comparison to those without supplementation. The optimum supplementation of Moringa seed extract at 25% could minimize the rate of decrease in sperms motility of Bali bull semen after equilibration.

1. Introduction

The success of Artificial Insemination (AI) is strongly influenced by the quality of the semen to be inseminated in the cows. The quality of semen is an important factor in the success rate of AI [1] and largely depends on good quality semen. The quality of semen can be affected by several factors including handling processes, starting from the time of holding, diluting, equilibrating, and freezing of the semen. Frozen semen of high quality requires a semen extender that can maintain the quality of the sperm during the process of cooling, freezing, and thawing [2]. The importance of high-quality semen as a result of interaction between high-quality bulls and proper handling process of semen reflect the success rate of AI when semen is used. Individual bulls have different abilities to fertilize the oocyte and to develop to be an embryo.

Maxwell and Watson [3] stated that the death of spermatozoa occurs because the plasma membrane of spermatozoa contains a lot of unsaturated fat which is very susceptible to lipid peroxidation reactions. In the processing of semen, there is a contact between semen and oxygen (O₂) to produce free radicals which can reduce the quality of the semen. Excessively high levels of free radicals in the semen may subsequently be associated with the defective sperms function that encountered to the low fertility and finally reduces the conception rate as well as pregnancy rate. This condition would be a great problem



in the planning for increasing cattle population in a certain area; in which the reproductive performance of both bulls and cows decrease and the occurrence of reproductive inefficiency. Therefore, in the problem of bull semen and to achieve high-quality semen before used, decreasing must be avoided. The decrease in semen quality caused by free radicals can be suppressed by adding antioxidants in the extender.

The addition of antioxidants in the extender may improve the quality of semen before use. One of the potential antioxidants is the use of Moringa seed extract that may reduce the presence of free radicals in the semen. This study aimed to prove that the Moringa seed extract can be used as an alternative to natural ingredients that can be added to diluting media, in which it can maintain the quality of Bali bull semen during equilibration.

2. Materials and methods

2.1. Materials

This study was conducted at the Samata Integrated Farming System (SIFS) for collecting the semen and the Laboratory of Animal Reproduction, semen processing unit, Faculty of Animal Science, Hasanuddin University, Makassar. The equipment used consisted of an artificial vagina, a microscope with Computer-Assisted semen analysis (CASA) software, and a spectrophotometer. The main material used was a Bali bull aged six years old. Other supporting materials including a warm water temperature of 40°C, vaseline, 70% alcohol, litmus paper, andromed, aquabidest, egg yolk, object-glass, cover glass, tris (Hydroxymethyl aminomethane), penicillin, streptomycin, glucose, citric acid, glycerol, and the extract from the seeds of *Moringa oleifera*.

2.2. Methods

2.2.1. Moringa seed extract. The process for extracting was carried out by separating the moringa seeds from the skin, then drying them in an oven at 60°C for 48 hours. Furthermore, the Moringa seeds were grounded to make a fine flour. The next step was mixing 100 mg of Moringa seeds flour with 100 ml of aquabidest in a test tube, then homogenizing them with a magnetic stirrer for 15 minutes. The final stage was centrifugation for 10 minutes to precipitate the substances.

2.2.2. Tris egg yolk (TEY). Preparation of TEY was performed by weighing out of 3.634 g of tris (Hydroxymethyl aminomethan), 0.50 g of glucose, and 1.99 g of citric acid. All of them were put in an Erlenmeyer glass and added with aquabidest to reach a volume of 100 mL. The solution was then homogenized for 15 minutes using a magnetic stirrer. All the ingredients were heated to a boil before adding 20 mL of egg yolk to the solution. The solution was then stirred again for 30 minutes until getting homogeneous. Furthermore, 100,000 IU of penicillin and 100 mg of streptomycin were added and then homogenized for 15 minutes. Finally, the addition of 7% glycerol to the solution at equilibration.

2.2.3. Supplementation of Moringa fruit seeds on TEY. The addition of Moringa seed extract into the TEY was supplemented with a ratio of 25% : 75% (P1), 50% : 50% (P2) and 75% : 25% (P3). These ratios were then used as treatments in the study to compare each other for getting the best ratio for effectively used in the future.

2.2.4. Collection and evaluation of semen. Semen of the bull was collected using an artificial vagina twice a week during the morning time. Prior to semen collection, all parts of the artificial vagina were cleaned and sterilized before connecting each part. The artificial vagina was then filled with warm water at a temperature of 40°C and lubricated gently at the front part that will be connected with the bull reproductive organ (penis). A scaled vial was tight at the end of the cone for semen at the time of collection. Obtained semen was then subjected to examination. The quality of semen was examined both macroscopically (volume, color, pH, viscosity) and microscopically (concentration, mass, individual

and progressive motility). The semen that was passed the examination both macroscopically and microscopically and met the required criteria was then subjected to the treatments.

2.2.5. Dilution and equilibration of semen. The Semen dilution was done by slowly pipetting the extender through the Erlenmeyer wall which was already filled with semen. Comparison between extender and volume of semen was carried out using the formula below [4].

$$\text{Amount of diluent (mL)} = \frac{\text{Volume semen} \times \text{concentration} \times \text{motility}}{\text{The desired semen concentration}}$$

The diluted semen was then equilibrated for two hours in a refrigerator with a temperature of 5°C before cryopreservation.

3. Data analysis

The study was arranged with a completely randomized design factorial pattern. Data obtained were analyzed using SPSS version 21. The least significant difference test was carried out to determine the differences in each treatment [5].

4. Results and discussion

4.1. The quality of fresh Semen

The fresh semen collected from Bali bull used in this study is shown in table 1. The quality of fresh semen during the study period can be categorized as good semen and met the requirements to be further processed. This is in line with the previous study [6] in which they stated that the volume of semen collected from the bull from 5 to 8 mL/ejaculation. Normal bull have individual progressive motility of 40% - 75% active sperms, concentrations of more than 2000 million per mL semen and mass movement of ++ / +++ [7].

Table 1. The quality of fresh semen of Bali bull.

Characteristics	Score
Volume (mL)	5.5±0.5
Color	Cream
Motility Mass	Good (++)
Individual Motility (%)	71.16±6.14
Progressive Motility (%)	61.47±2.45
Concentration (10 ⁶)/mL	1,603±315.66

4.2. Individual motility of Bali bull sperms

The percentage of individual motility of Bali bull sperms at different treatments before and after equilibration is shown in table 2. Based on statistical analysis using paired sample t-test, individual motility before equilibration in P2 treatment had significantly ($P < 0.05$) lower than the other treatments. Furthermore, after equilibration, control using Andromed (P0A) and supplementation with 50% Moringa seed extract (P2) had significant different ($P < 0.01$).

There is no significant difference between Andromed (P0A) and TEY 100% (POB) on individual motility of Bali bull sperms before and after equilibration. Supplementation of 25% (P1) and 75% (P3) Moringa seed extract gives an idea that the type of extender used is able to match the components or compounds that are needed by the sperms to move. Moringa seed extract contains antioxidants and several nutrients that are good for preventing oxidation and cool shock of the sperms. TEY contains a buffer found in diluents, which can neutralize metabolic products such as lactic acid so that spermatozoa can survive.

Table 2. Individual motility of Bali bull sperms before and after equilibration.

Treatment	Observation (%)	
	Before equilibration	After equilibration
P0 (A)	75.73 ± 1.43 ^a	64.61 ± 3.52 ^a
P0 (B)	74.45 ± 5.55 ^a	68.26 ± 3.42 ^a
P1	72.48 ± 4.05 ^a	61.80 ± 10.28 ^a
P2	57.18 ± 3.28 ^b	49.22 ± 1.79 ^b
P3	64.92 ± 6.47 ^a	52.47 ± 14.9 ^b

^{a,b} Different superscripts at the same column show significant differences (P<0.05).

4.3. Progressive motility of Bali bull sperms

The percentage of progressive motility of Bali bull sperms is presented in table 3. It shows that in the control, both Andromed (P0A) and TEY (P0B) had high progressive motility values of 57.48% and 56.26%, respectively, but both of them decreased significantly (P<0.01) from 57.48% to 47.07% and from 56.26% to 43.71% before and after equilibration.

In general, both controls (P0A) and (P0B) show a higher percentage of progressive motility compared to those supplemented with moringa seed extract in all treatment groups, but the different percentage decline before and after equilibration was also high. This can be seen 10.41% (P0A), 12.55% (P0B), 8.13% (P1), 4.58% (P2) and 8.03% (P3). The results obtained in the present study are in line with study of Sahiruddin *et al.* [8] that the addition of Arabic bidara leaf extract (*Ziziphus spina christy*) to TEY, can minimize the decrease in spermatozoa motility after equilibration. The decrease in the quality of spermatozoa according to Susilawati [9] occurs due to damage to the membrane structure during cooling so that the metabolic process of spermatozoa is disturbed.

Table 3. Progressive motility of Bali bull sperms before and after equilibration.

Treatment	Observation (%)	
	Before equilibration	After equilibration
P0 (A)	57.48±2.67 ^a	47.07±2.98 ^a
P0 (B)	56.26±6.24 ^a	43.71±4.49 ^b
P1	54.18±3.17 ^a	46.05±9.03 ^a
P2	39.65±3.68 ^b	35.07±2.22 ^b
P3	52.49±11.54 ^a	44.46±15.99 ^b

^{a,b} Different superscripts in the same column show a significant difference (P<0.05).

The results obtained after equilibration show that the progressive motility of Bali bull sperms using Andromed extender and TEY decreased significantly compared to the addition of Moringa seed extract. This indicates that the antioxidant compounds from the Moringa seed extract help in maintaining the sperms to move progressively. According to Rizal and Herdis [10], one of the factors that reduce the quality of frozen semen is the high activity of oxidative metabolism in the semen, as a result of the formation of free radicals that cause lipid peroxidation reactions on the spermatozoa plasma membrane. Lipid peroxidation reactions also occur due to contact between semen and oxygen-containing air during the semen processing process.

5. Conclusion

The use of Moringa seed extract supplemented in the extender at a concentration of 25% into TEY can minimize the rate of decreasing sperms motility of Bali bull after equilibration. This suggests that to minimize the decrease in motility of the sperms after equilibration, it is a good idea to supplement the extender using 25% of Moringa seed extract in the extender.

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