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Fertility Of Chill Thawed Bali Bull Spermatozoa In Synthetic Cauda Epididymis Plasma With Guava Filtrate

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Abstract:
Background: *An Extender Is Needed To Preserve The Fertility Of Spermatozoa During A Period Before Being Used For Artificial Insemination. The Complete Extender Should Be Containing Buffer, Nutrition, Energy, Anti-Cold-Shock, And Antioxidant To Preserve And Protect Spermatozoa From Any Destructive Substances And Free Radicals.This Study Was Performed To Investigate The Fertility Of Bali Bull Spermatozoa During Chilled Storage In Synthetic Cauda Epididymis Plasma Egg Yolk (S-CEPEY) Extender Added By Guava Filtrate (GF).*
Materials And Method: *In Experiment 1, The Spermatozoa That Were Stored In S-CEPEY Without GF (T1), And S-CEPEY With 10% GF (T2) Were Used For Fertilized Of Matured Oocytes By In Vitro Fertilization (IVF). In Experiment 2, The Spermatozoa As Those In Experiment 1 Were Used For Inseminating Synchronized Oestrus Cows By Artificial Insemination (AI). The Cleavage Oocytes At 24h Of IVF, Non-Return Rate (NRR) At 20 – 30 Days, And Gestation Rate At 90 Days After AI Were Recorded.*
Results: *The Cleavage Rate Of Oocytes Fertilized With Spermatozoa In Extender T2 Was 47.51% Compared With 37.04% In T1. As Well As NRR And Gestation Rate Of Cows Inseminated With Semen In Extender T2 Compared With T1 Were 83.3% Vs 75% And 75% Vs 58.3% Respectively.*
Conclusion*: The Addition Of 10% Guava Filtrate In A Synthetic Cauda Epididymis Plasma Extender Is Very Effective In Maintaining Bali Bull Spermatozoa During Chilled Storage.*
Keywords: *Artificial Insemination; Extender; Fertilization; Gestation Rate; Semen*

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I. Introduction

The fertile spermatozoa should be contained a minimum of 50% progressive motility and viability [1]. Progressive motility of less than 60% is not suitable for further process [2]. According to Indonesian National Standard Agency [3], the minimum motility of spermatozoa in frozen semen should be 40% of 50 million spermatozoa in a dose of AI [4]. The viability can be maintained by reducing the metabolic activity and motility in chilled storage [5; 6). However, the percentages of motility and viability may decrease due to cold shock and reactive oxygen species (ROS) activity when stored at low temperatures (5^{o} C). Excessive production of ROS causes membrane damage and oxidative stress [7; 8; 9; 10).

Interaction of spermatozoa with oxygen during storage cause lipid peroxidation in the plasma membrane and produce free radicals [11]. Spermatozoa and seminal plasma has an enzymatic and non-enzymatic antioxidant system that neutralizes free radicals and strives against ROS's negative effects ([12]. However, the addition of an extender into the semen may decrease the concentration the natural antioxidants [13]. Antioxidants are needed to inhibit lipid peroxidation, lowering intracellular protein breakdown and protecting the vitality of spermatozoa (14; 15).

Guava fruit (Psidium guajava Linn) is a natural source of non-enzymatic antioxidants such as vitamins E, C, and A which are efficient to strive against free radicals [16]. It also contains various components with antioxidant activity [17], such as carotene, retinol equivalent, ascorbic acid, lutein, lycopene, and zeaxanthin [18; 19]. The current study was carried out to examine the effect of guava fruit filtrate (GFF) in s-CEPEY-based extenders on maintaining the fertility of Bali bull spermatozoa after chilled storage.

II. Materials And Methods

Research materials

Sexually mature (3.5-4.5 years old) four Bali bulls were selected from the Regional Artificial Insemination of Livestock and Animal Health Office West Nusa Tenggara Province. Fresh semen samples were collected by artificial vagina once a week. The minimum requirements of spermatozoa's progressive motility and viability to be further processed were 60%, concentration 1000 x 10^{6}/ml, and maximum abnormality 20% as used by previous researchers [2; 20; 21; 22].

Treatments extender

The CEP extender was made of 0.877 g NaCl; 0.522 g KCl; 0.441 g $CaCl_2(H_2O)_2$; 0.813 g MgCl; 0.999 g $NaHCO_3$; 1.104 g NaH_2PO_4; 2.722 g KH_2PO_4; 9.91 g fructose; 16.196 g tris; 8.198 g citric acid; 1.0 g sorbitol; 2 g BSA; 0.05 g gentamycine. All materials were mixed in 1.000 ml of deionized water and the pH was arranged at 6.6 and osmolarity 320 mOsm [adapted from 23]. Solution filtered and sterilized by millipore membrane Sartorius Stedim Minisart ® 0.20 μm.

The GFF was prepared by dissolving guava juice in aquabidest 1 : 2 and centrifuged twice at 3000 rpm for 10 min. The supernatant was filtrated or filtered using millipore membranes 0.42 and 0.20 μm respectively, then pasteurized in hot water at 50 – 60° C within 2 – 5 minutes [24]. The treatment extenders were 80% Synthetic CEP + 20% EY without GF as a control (T1) and with 10% GF (T2). The treatment was repeated 10 times. Approximately 0.4 ml semen containing 400 x 10^6 spermatozoa was divided into 2 glass tubes (10 ml in volume) and mixed by each 5 ml treatment extender, cooled gradually from 32 to 5º C for 2 h in a refrigerator, and kept at this temperature [adapted from 25).

Experiment 1

A total of 35 Bali cow's ovaries were collected from the slaughterhouse and brought to the laboratory in 0.9% natrium chloride (NaCl) within 1 – 2 h. The oocytes were aspirated from 3 – 5 mm diameter follicles, washed twice in standard oocytes washing solution (OWS), and evaluated. The oocytes with complexes cumulus then matured in the standard protocol, 50 μl TCM-199 with 10% fetal calf serum (FCS), sodium pyruvate, and gentamycin [modified from 26; 27]. The 3 – 6 of mature oocytes were inhaled and placed in 2 – 3 little wells at the edges of the Petri dish**. S**permatozoa that were chilled storage in both T1 and T2 extenders were arranged in 50 μl containing 100 x 10^3 spermatozoa/oocyte. The spermatozoa are placed in a large well at the center of the Petri dish to fertilize the oocytes by in-vitro fertilization (IVF). The variable recorded was the fertilization rate observed from the number of oocytes cleaved to 2 cells stage in 24 h.

Experiment 2

A total of 36 cows belong to farmers at 5 villages of a subdistrict in West Lombok, West Nusa Tenggara Province used as recipients and the oestrus synchronized by the luteolytic hormone, Estron. The spermatozoa as those in the previous experiment were used for inseminating oestrus cows by artificial insemination (AI). The spermatozoa from both T1 and T2 extenders were arranged at 20 x 10^6/dose (0.25 ml) in plastic straws and inseminated in each of the 12 recipient cows. The variables recorded were non-return rate (NRR) in the day 20 – 30 and conception rate (CR) of cows was groped rectally at day 90 post AI.

Statistical analysis

The data of each variable was counted in mean and percentage, as well as the difference of spermatozoa's fertility preserved in T1 and T2 was tested with a Student t-test using Data Analysis of Exel Program, Windows 2007.

III. Results

The motility and viability of spermatozoa are the two fertility factors that influence the success of AI. The fertility of spermatozoa can be protected by the level of antioxidant content in the extender, namely *Superoxide Dismutase* (SOD), but can be damaged by the level of radical materials, namely *Malondialdehyde* (MDA). The motility and viability of spermatozoa, as well as SOD and MDA were evaluated before being used in each treatment. Table 1 shows the quality of chill-thawed spermatozoa and extender during optimum storage (eight days) in both s-CEPEY with and without GF such as motility and viability, level of *superoxide dismutase* (SOD), and *malondialdehyde* (MDA).

Table no 1. Quality of chill-thawed spermatozoa and extender in both s-CEPEY with and without GF

Variables	Treatments	
	T1	T2
Motility of spermatozoa on day 8th (%)	37.65 ± 3.5[a]	45.70 ± 1.70[a]
Viability of spermatozoa on day 8th (%)	44.0 ± 3.2[b]	60.8 ± 6.0[b]
Level SOD (u/mL) in extender	27.1 ± 12.5	35.1 ± 3.9
Level MDA (ng/mL)	207.3 ± 69.1	201.5 ± 83.5

T1 = s-CEPEY without GF; T2 = s-CEPEY with GF

Different superscripts within each row indicate significant differences (P <0.05)

In experiment 1: A total of 54 matured oocytes were in Vitro fertilized by spermatozoa after chilled storage in the control extender. As many as 20 or 37.04% of oocytes were fertilized after 24 hours incubated in a CO_2 incubator. A total of 61 oocytes were fertilized by spermatozoa stored in the T2 extender, those 29 or 47.51% oocytes were fertilized after 24 hours. The results showed, that IVF by spermatozoa stored in a s-CEPEY extender added by 10% GF was significantly ($P < 0.05$) higher as compared to spermatozoa stored in a control extender (without GF).

In experiment 2: The spermatozoa as those used in experiment 1 were applied in-vivo by AI to confirm IVF results. A total of 36 cows were inseminated by chill-thawed spermatozoa stored in both s-CEPEY with and without GF. The results of experiments 1 and 2 are presented in Table 2.

Table no 2. The fertility of spermatozoa obtained in both experiments 1 and 2

Fertilization test by	Perlakuan	
	T1	T2
IVF: Total Oocytes	54	61
Cleavage to 2 sel at 24h	20	29
Percentage (%)	$37,04 \pm 10,7^{a}$	$47,51 \pm 7,5^{b}$
AI: Total of recipients of AI (head)	12	12
NRR up to 30 days of AI (%)	75% (9)	83,3% (10)
CR on 90 days (%)	58,3% (7)	75% (9)

Different superscripts within each row indicate significant differences ($P < 0.05$)

Table 1 shows, that the percentages of motility and viability of spermatozoa in s-CEPEY with GF were significantly ($P < 0.05$) higher than in s-CEPEY without GF. Meanwhile, the SOD and MDA levels in extender s-CEPEY extender with 10% GF were higher than CEP-EY without GF, but not significantly different ($P > 0.05$). Table 2 shows, that the percentage of cleavage oocytes fertilized by spermatozoa stored in s-CEPEY with 10% GF were significantly ($P < 0.05$) higher in comparison to spermatozoa stored in s-CEP-EY without GF. However, the NRR and CR cows inseminated by spermatozoa stored in s-CEPEY with 10% GFF was 83.3% (10 of 12 cows), higher compared to s-CEPEY without GF, 75% (9 of 12 cows). The NRR was observed at 20 – 30 days, and the CR was observed by grope rectally at 90 days after AI. Nine of 12 cows (75%) were recipients in T2, and 7 of 12 cows (58.3%) recipients in T1 were conceived.

IV. Discussion

The fertility of spermatozoa is affected by various factors, including the preservation method and extender. Using GF in various extenders was done to preserve spermatozoa of bull, buck, and cock. It was found that the quality of spermatozoa including motility, viability, and abnormality was better than without GF [24]. In a recent study, the fertility of spermatozoa stored in a s-CEPEY extender with and without GF was examined by IVF and AI.

The results of IVF showed that the cleavage rate of oocytes fertilized by spermatozoa from an extender containing 10% GF was higher than without GF. Overall, the result of this study was higher than previous studies with an average of 34.8 ± 3.6% in the pre-morula and 38.71% in the cleavage phase [28], 37.3% of 4- to 7-cell stage [29], 22.5% to 27.9% in cleavage [30]. The embryos produced were 46% and 26% when adult-derived embryos and blastocyst [31], 31.91% in 2 cells, 32.97% in 4 cells, 24.46% in 8 cells, and 10.63% in 16 cells after in-vitro culture of 48 hours [32].

Embryos production in vitro has not the same as in vivo due to the influence of various factors such as the quality of oocytes and spermatozoa preparation after being preserved. The quality of embryos produced in-vivo has better than in-vitro. In-vivo embryos production has better quality after freezing, and lower chromosomal abnormalities than in-vitro, in which only 30- 40% of oocytes develop into blastocysts after in-vitro fertilization [33; 34]. Development of cleavage embryos that failed to reach blastocyst between the 2 and 8 cells stage was 67% in 6 to 8-month-old heifers, significantly higher than in adults, 18% [35].

Various physical and biochemical damage can occur on spermatozoa and oocytes during preparation for IVF caused by ROS. The excessive ROS level results in lipid peroxidation, apoptosis, and DNA fragmentation, causing infertility of spermatozoa. On the other hand, small amounts of ROS are required for increasing the intracellular c-AMP to activate protein kinase, nuclear maturation, regulation, and condensation to fertilize the oocyte, hyperactivation, capacitation, acrosome reaction, and fertilization of spermatozoa [36; 37].

Oxidative stress is caused by excessive production of ROS causing spermatozoa injury, due to an increase in the membrane's unsaturated fatty acids and the lack of cytoplasmic antioxidant enzymes [38]. A deficiency in antioxidants can lead to oxidative stress (OS), nuclear and mitochondrial DNA damage, shortening of telomere, epigenetic alterations, and Y chromosomal microdeletions [39]. In follicular fluid (in-vivo) and

seminal plasma, there are various enzymatic antioxidant that captures ROS such as SOD, catalase (CAT), and glutathione peroxidase [40; 41]. Therefore, the IVF results need to be confirmed with in-vivo application to predict the fertility of spermatozoa after storage [42].

The results of AI showed, that the NRR of recipients inseminated by spermatozoa in synthetic s-CEP-EY-based extender added by 10% GF was better than without GF (control), as well as the gestation rate. The NRR obtained in the recent study was 83.3%, higher compared to 71.68% [43], 61.5% [44], and 43.3% [45]. Likewise, the pregnant cows when groping rectally at 90 days after AI was 75%, higher compared to 41.5% obtained by Anzar et al. [46], 60% [47], and 58%, 50%, and 57% in January, February, and March respectively [48]. The combination of s-CEP-EY and GFF in the extender may prevent membrane damage and maintain viability and motility to preserve the fertility of spermatozoa during cooling and chilled storage. Therefore, the percentages of fertile spermatozoa in the s-CEPEY extender added by 10% GFF was higher than that of control in both IVF and AI tests.

The s-CEPEY extender contains fructose, citric acid, sorbitol, and bovine serum albumin (BSA) to preserve the motility and membrane integrity of spermatozoa [49; 50]. Lecithin (phospholipids) and low-density lipoprotein (LDL) of egg yolk (EY) may prevent cold shock and protect the plasma membrane [51; 52]. Phospholipid fraction of LDL can form a thin layer on the surface of spermatozoa or replace the lost membrane phospholipids or be damaged during a cooling process [53; 54]. Using soy-lecithin in an extender can mitigate the efflux of phospholipids and reduce the intracellular ice crystals formed during the cryopreservation of spermatozoa [55].

Various antioxidants in both enzymatic and non-enzymatic, such as SOD and CAT, vitamins E and C, and melatonin are needed to preserve and protect the spermatozoa to reduce the detrimental effect [56]. Guava containing non-enzymatic such as vitamin C (ascorbic acid) can stabilize superoxide dismutase (SOD) to neutralize free radicals by accepting or donating one or more electrons to reduce unpaired free radical conditions. The protection system of antioxidants may suppress malondialdehyde (MDA) and increase the fertilizing ability of spermatozoa. Vitamin C may also neutralize the radical material of other antioxidants such as glutathione and vitamin E radical, as well as regenerate these antioxidants [57].

Both enzymatic and non-enzymatic antioxidants maintain redox homeostasis by scavengers the ROS [58]. A deficiency of available antioxidant protection may cause oxidative stress, by which the generations of ROS are at an excessive level [59]. The mechanism of non-enzymatic antioxidants can be exemplified at reduced glutathione and alpha-tocopherol (vitamin E). Antioxidants can protect against free radicals by prevention to avoid the initiation of a chain reaction of free radicals, interception, or restrain activities to break the developing reaction by forming a non-radical end product and reparations to repair the unpair electrons by donating one or more electrons as stated by Rao et al. [60].

V. Conclusion

Guava fruit filtrate has a very significant effect on maintaining the fertility of Bali bull spermatozoa. The addition of 10% guava fruit filtrate significantly increases the preservation effect of the synthetic-Cauda Epididymis Plasm Egg Yolk extender for maintaining spermatozoa fertility of Bali bull for 8 days.

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