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The potency of guava filtrate (*Psidium guajava* Linn) for preservation of Bali bull spermatozoa

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Abstract: *The aim of this research was to investigate the potency of guava filtrate (GF) in synthetics cauda epididymal plasma (CEP-2) based diluent to maintaining the quality of Bali bull spermatozoa during chilled storage (5º C). Ten ejaculates from Bali bulls were collected using an artificial vagina once a week. Each ejaculate was divided into seven aliquots, each aliquot diluted with CEP-2 diluents containing 0%, 4%, 6%, 8%, 10%, 12% and 14% GF (v/v), respectively, to replace 20% egg yolk (EY) in the diluent. All samples were placed in the refrigerator and cooled gradually to 5º C. Once reached 5º C, they were kept at this temperature and stored for 8 days. The mean percentages of progressive motility and viability of spermatozoa stored for 8 days in diluent containing 10% GF was significantly higher ($p<0.05$) than other six diluents containing different levels of GF. Conversely, the percentage of abnormal spermatozoa was significantly lower ($p<0.05$) in the previous diluent compared with the latter ones. In conclution, guava filtrate is potential when included in CEP-2 diluent for maintaining the quality of Bali bull spermatozoa at 5º C. It seems that 10% GF is the best level for substituting 20% EY.*

Keywords: *Bali bull, chilled storage, preserve, guava diluent, semen quality*

I. Introduction

Bali cattle as one of the indigenous cattle breeds raised in many villages of Indonesia have good adaptability and high fertility. However, the genetic performance of Bali cattle is still low, so that their productivity have not maximum yet. Utilization of reproductive technologies such as artificial insemination (AI) is important for improving genetic and productivity of Bali cattle rapidly [1]. In order AI to be successful, this technology should be supported by the use of good quality sperm after being preserved in appropriate time of storage and proper extender [2]. Preservation of semen in chilled conditions (5° C) is an alternative method to maintain the quality of sperm [3] because it might reduced metabolic activity of sperm, so their viability and motility can be prolonged [4]. The type of extender have to appropriate, as the source of nutrients, energy, buffers and antioxidants for spermatozoa during storage [5].

Egg yolk (EY) is an extracellular protectant in the extender [6], it contains protein, lipids, vitamins and minerals, phospholipids (lecithin) and low-density lipoprotein (LDL) to prevent cold shock and protects sperm plasma membrane [7]. Addition of 10% EY in CEP-2 can preserve bovine semen for 6 days at 5° C [8] and CEP-2 with 20% EY may preserve motility and membrane integrity of sperm for 8 days [9]. However, EY extender is often contaminated with bacteria and mycoplasma that harmful to sperm [10; 11]. Several substances in EY may inhibit respiration, reduce motility and disrupt biochemical testing and metabolic examination [12]. Therefore, the use of EY in the extender should be reduce or substituted by non-animal materials.

Synthetic CEP-2 is an extender containing fructose and citric acid as the energy and buffer sources, sorbitol to increase osmolarity and bovine serum albumin (BSA) as macro-molecular. This extender does not contain an antioxidant to protect sperm from free radicals or reactive oxygen species (ROS) that may damage DNA, carbohydrate, lipid, nucleic acid and protein in the nucleus and cell membrane [13]. Antioxidants are need to break the chain of oxidative reactions during formation of radicals [14], inhibit oxidative damage of proteins, nucleic acid and lipid [15]. The level of antioxidant in both seminal plasma and sperm can be reduced by diluting semen with extender that can generate excessive ROS molecules. Addition of exogenous antioxidants could reduce the impact of ROS during storage, defense functioning mechanism against lipid peroxydation (LPO) and thus improve the quality of chilled semen [16].

Non-enzymatic antioxidants such as vitamin E, C and A can be found abundantly in various fruits and efficient in combating against free radicals [17]. Guava (*Psidium guajava* Linn) is one of the fruits that having

very rich antioxidants, such as carotene, lycopene, zeaxanthine, vitamin B1and B2 and vitamin C (ascorbic acid) [18]. However, the use of guava as an alternative natural antioxidant in semen extender has not been investigated yet. Therefore, the present study was undertaken to examine the potency of guava filtrate (GF) in maintaining quality of Bali bull sperm during chilled storage in synthetic CEP-2 based extender.

II. Material And Methods

2.1. Preparation of extenders

Synthetic CEP-2 base extender was made of 0.88 g NaCl; 0.52 g KCl; 0.44 g $CaCl_2(H_2O)_2$; 0.81 g MgCl; 1.00 g $NaHCO_3$; 1,10 g NaH_2PO_4; 2.72 g KH_2PO_4; 9.91 g fructose; 8.20 g citric acid; 1.0 g sorbitol; 0.05 g gentamycin (all : Merck, Germany); 16.2 g tris and 2 g BSA (Sigma, USA), mixed in 1.000 ml of destilated water with pH 6.6 and osmolarity of 320 mOsm. The solution was filtered and sterilized with millipore membrane 0.20 µm [19].

The EY was prepared from chicken egg one day laid by removing the eggshell and poured out the albumin, then the yolk was centrifuged at 300 rpm for 10 minutes. The GF was prepared by dissolving guava juice in aquabidest (1:2 ratio), then centrifuged twice at 3500 rpm for 10 minutes. Once finished, the GF solution was filtered using millipore membrane (Sartorius stedim Minisart ®) 0.42 µm and continued with 0.20 µm. The liquid filtrate was pasteurized in warmth water at 60° C for 3 minutes. The level of GF to be used was formulated based on vitamin C requirement, 0.5 mM/mL (v/v) [20; 1].

The study was performed using complete randomized design (CRD), comprised 7 extenders as treatments and replicated 10 times. Experimental extenders were prepared in seven glass tubes (1 to 7) volume 10 ml, each contain of 8 ml CEP-2 based extender. Thereafter, into each glass tube of CEP-2 was added consecutively with 2 ml EY without GF (control), 0.4 ml, 0.6 ml, 0.8 ml, 1.0 ml, 1.2 ml and 1.4 ml of GF (v/v), respectively, for substituting 20% EY in the extender. All the treatment extenders then stored at 5° C until used.

2.2. Semen collection and evaluation

Ten ejaculates were collected from four Bali bulls at Regional Artificial Insemination that belongs to Livestock and Animal Health Office West Nusa Tenggara Province, using an artificial vagina. The fresh ejaculated semen was immediately brought to laboratory for macroscopic and microscopic evaluation to determination of its quality. The semen to be used had a minimum mass motility of +2, individual motility and viability ≥ 70%, and abnormality ≤ 20% [5; 21; 22].

2.3. Semen dilution and preservation

Approximately 1.4 ml ejaculate from each bull with about 2000 x 10^6 progressive motile sperm was divided into 7 aliquots (0.2 ml/aliquot). Each aliquot was mixed slowly with 0.2 ml treatment extenders, then homogenized. The remaining treatment extenders was added gradually to semen solution until the volume attained 10 ml/treatment. Thereafter, all seven treated diluted semen were placed in a 15 x 20 x 7 cm plastic box contain of 300 ml water, and cooled gradually from 32 to 5º C within 1.5 - 2 hours in refrigerator. Having reached this temperature, all diluted semen samples stored for 8 days for further evaluation as modification of Menchaca methods [23].

2.4. Sperm evaluation

Evaluation of all diluted semen was performed daily in samples stored for 8 days. A drop of diluted sample was placed on the object glass, covered with a cover glass and then placed on heat stage at 37° C for 10 – 15 seconds. Progressive motilility of sperm was examined under a light microscope at 400x magnification. The percentages was assessed by comparing sperm which has straight forward movement with those inmotile sperm, vibrates, backwards and rotates [24]. Sperm viability and abnormality were observed by putting a drop of diluted semen on object glass using a micro-pipette, then solution of eosin-negrosin was pipetted and dropped near diluted semen and homogenized. Smear was made with the tip edge of cover glass at 30° slope along the surface of object glass, then dried over a bunsen flame lights or air-dried. Minimum of 100 sperms were observed using light microscope at 400x magnification. The sperm cells that absorbed color was defined as dead, while disordered or damaged head or tail was considerd as abnormal sperm [25; 22].

Fluorescein isothiocyanate (FITC-con A) staining was performed to examine the intact acrosome (IA) as well as sperm capacitation and acrosome reaction (CAR). Sperm samples were fixed in 4% formaldehyde, then washed by adding 3 ml PBS and centrifugated at 4000 G for 30 minutes. The supernatant was discarded and put into 0.1 ml of FITC-con A that contain 10 µl/ml in PBS Dulbecco's. Staining was kept for 25 minutes at room temperature, then washed twice by centrifugation at 4000 G for 10 minutes. Supernatant were discarded and a drop of the sediment was placed on a clean glass slide, put a drop of 90% glycerol on the sediment and homogenized, then examined using fluorescent microscope at 400x magnification. Every speciment was examined using epifluorescence ellumination with excitation B (490 nm excitation and 525 nm emission) to

observe the fluorescent on the sperm as FITC-con A staining result (Nishikima, 1997) adapted by Susilawati [25]. The FITC staining was conducted in Biology Laboratory, University of Brawijaya, Malang.

Scanning clectrone microscopy (SEM) analysis was performed to identify ultrastructure damage of sperm. This analysis was conducted in Bioscience Laboratory, University of Brawijaya, Malang, according to standard operational procedure of the laboratory.

2.5. Extencder alteration

Alteration of the extender during chilled preservation of semen is important to evaluate. For this purpose, superoxide dismutase (SOD) and malondialdehide (MDA) level of extender herewith the semen on the D-1 and D-8 of storage were measured to know the antioxidant activity for sperm protection. All analysis process was done in Faali Laboratory, Faculty of Medicine, University of Brawijaya, Malang, according to standard operational procedure of the laboratory.

2.5. Statistical analysis

Data for sperm quality were analyzed by one way analysis of variance (ANOVA) and least significant difference (LSD) test was further done for comparation of each means using SPSS for windows statistics software (Version 20 – 32 bits). All data were presented as Mean ± SEM. Significantly different among the treatments was decided at $P<0.05$.

III. Results And Discussion

The semen samples from Bali bulls used in this study were evaluated both macroscopic and microscopically. The mean characteristics of ten ejaculates with regard to volume, color, pH, consistency, sperm mass motility, progressive motility, live, abnormal and concentration were 5.94 ± 1,22 ml, milky white, 6.2 ± 0.85, thick, 2^+ to 3^+, 70 ± 0%, 93.9 ± 2.42, 5.1 ± 1.91% and $1.89 \pm 0.3 \times 10^9$/ml, respectively. The effects of substitution of EY with GF in CEP-2 based extender during 8 days chilled storage for progressive motility, viability and abnormality of sperm are shown in Tabel 1.

The mean percentages of both progressive motility and viability of sperm were significantly highest ($P<0.05$) in extender which substituted 10% EY with GF as compared to 0, 4, 6, 8, 12 and 14% GF. Conversely, the mean percentage of abnormality was significantly lowest ($P<0.05$) in the previous diluent compared with the latter ones. The mean of intact acrosome (IA) was higher on sperm diluted with 10% GF as compared to control (0% GF) either for D-4 or D-8, whereas the mean of CAR was higher for D-4 and lower for D-8 on sperm diluted with 10% GF as compared to control (0% GF). However, no significant differences ($P>0.05$) was observed for overall mean percentages of semen characteristics (see Table 2). The effect on alteration of SOD and MDA levels in extender treated with 0% and 10% GF were also measured. It was found that the mean of MDA level in 10% GF to be decrease at D-1 to D-8, whereas in 0% GF, it was found to be increase.

There is an effect of substitution or replacement of EY with GF in CEP-2 based extender for sperm motility, viability, abnormality and membrane integrity. The mean percentages of motility for D-1 to D-5 preservation were not significantly different among all treatments, however, 10% GF and 4% GF showed the highest and the lowest of means for all samples treated (Tabel 1). During D-6 to D-8, the use of 10% GF was significantly different ($P<0.05$) with the use of 12 and 14% GF, and very significantly different with that of 8, 6, 0 and 4% GF ($P<0.01$).

Using of 10% GF and 10% EY in CEP-2 based extender may support vitamin C and various enzymes as an antioxidant and nutrient source. Egg yolk is a macro-protectant or extracellular protectant agent, which contain protein, lipid, vitamins and minerals that can prevent cold shock and maintain sperm motility [7]. These results are not much different with the research of Ducha *et al*. [9], that CEP-2 with 20% egg yolk was able to maintain sperm motility up to 41% after D-8 storage. Previous studies explained that CEP-2 with 10% egg yolk may prolong the preservation of fresh bovine semen up to 6 days at 5° C [8; 19]. In Awassi ram semen, spermatozoa motility declined gradually in vitamins C Tris containing diluents when preserved at 5˚C up to 120 hr (54.7 ± 1.0%), compared with Tris diluent without vitamins (35.9 ± 1.9%) [27].

The results of the study indicated that the formulation of extender containing 80% CEP-2 + 10% EY + 10% GF is the most effective in maintaining quality of Bali bull sperm during storage, as compared with all other formulations. It could be that the extender containing sufficient substances that are required by sperm such as nutrients, energy, buffer, macro-molecular and antioxidant. The CEP-2 extender containing the same biochemical and ionic components as liquid epididymis, so it may comply with the energy and buffers required for sperm. Fructose and citric acid in CEP-2 are source of energy, sorbitol to increase osmolarity and bovine serum albumin (BSA) as a macro-molecular. Thereby, the CEP-2 is a good alternative extender to preserve bovine semen in the chilled form, as stated by Verberckmoes et al. [19], and capable in maintaining motility and membrane integrity of bovine sperm during chilled or liquid storage [11].

Table 1. Mean±SEM of sperm progressive motility, viability and abnormal sperm during chilled storage in CEP-2 based extender containing GF (n=10)

Semen characteritics	GF (%)	Day of preservation at 5° C								Overall Mean ± SEM
		D-1	D-2	D-3	D-4	D-5	D-6	D-7	D-8	
Progressive motile sperm	0	70.0 ± 0.0	68.7 ± 1.2	66.0 ± 2.7	62.6 ± 3.6	59.4 ± 4.2	53.2 ± 3.1^{c}	47.6 ± 4.9^{c}	37.7 ± 3.5^{ce}	58.2 ± 2.9^{c}
	4	69.5 ± 1.0	67.6 ± 1.9	64.2 ± 3.6	60.3 ± 4.3	57.5 ± 4.7	49.8 ± 3.6^{c}	43.8 ± 5.6^{c}	34.1 ± 4.1^{c}	55.9 ± 3.6^{d}
	6	70.0 ± 0.0	68.1 ± 1.2	65.8 ± 2.4	63.0 ± 3.3	59.8 ± 4.1	52.9 ± 4.1^{c}	47.4 ± 5.3^{c}	39.4 ± 3.5^{c}	58.3 ± 3.0^{c}
	8	70.0 ± 0.0	69.0 ± 1.3	66.7 ± 1.5	63.4 ± 3.6	61.0 ± 3.9	55.1 ± 2.4^{ce}	49.4 ± 4.7	42.1 ± 3.4^{ce}	59.6 ± 2.6^{b}
	10	70.0 ± 0.0	69.6 ± 1.7	67.9 ± 1.8	65.7 ± 2.1	63.2 ± 3.2	58.3 ± 3.4^{a}	52.8 ± 4.6^{a}	45.7 ± 1.7^{a}	61.7 ± 2.3^{a}
	12	69.9 ± 0.2	68.6 ± 1.7	66.4 ± 2.2	63.6 ± 3.3	61.9 ± 3.5	55.5 ± 4.2^{e}	50.7 ± 4.9^{e}	42.6 ± 3.1^{a}	59.9 ± 2.9^{b}
	14	69.9 ± 0.3	68.6 ± 2.0	66.3 ± 2.5	63.6 ± 3.8	61.5 ± 4.1	55.3 ± 3.5^{e}	50.1 ± 5.0^{e}	42.4 ± 4.4^{e}	59.7 ± 3.2^{b}
Sperm viability	0	91.1 ± 1.9^{c}	86.9 ± 1.7^{c}	82.9 ± 1.7^{c}	71.9 ± 2.1^{c}	66.1 ± 4.0^{c}	61.6 ± 2.1^{c}	53.6 ± 3.7^{c}	44.0 ± 3.2^{c}	69.8 ± 2.6^{c}
	4	89.4 ± 2.4^{a}	84.6 ± 2.2^{a}	79.7 ± 2.6^{a}	68.2 ± 1.8^{b}	62.7 ± 4.5^{a}	53.6 ± 2.7^{a}	48.4 ± 4.6^{a}	37.5 ± 4.7^{a}	65.5 ± 3.2^{a}
	6	90.9 ± 2.3^{c}	86.5 ± 2.1^{cd}	81.5 ± 1.7^{cb}	74.1 ± 3.8^{ad}	65.4 ± 3.3^{c}	59.7 ± 2.0^{be}	52.2 ± 3.7^{c}	42.6 ± 2.8^{c}	69.1 ± 2.7^{c}
	8	91.5 ± 2.1^{c}	87.8 ± 2.3^{c}	83.3 ± 1.4^{c}	76.6 ± 3.3^{ad}	69.8 ± 2.3^{ae}	62.5 ± 1.7^{ce}	$55.8 \pm 4.0a^{a}$	45.3 ± 1.9^{cb}	71.6 ± 2.4^{c}
	10	93.4 ± 1.8^{ae}	90.6 ± 1.7^{ae}	86.0 ± 1.8^{ae}	82.2 ± 2.4^{ae}	76.6 ± 2.8^{ae}	73.6 ± 2.6^{ae}	66.6 ± 3.0^{ae}	60.8 ± 6.0^{ad}	78.7 ± 2.8^{ad}
	12	89.7 ± 2.3^{ba}	86.9 ± 1.9^{ce}	81.9 ± 2.9^{c}	77.3 ± 2.5^{ae}	68.3 ± 2.5^{cc}	64.5 ± 1.8^{ae}	53.5 ± 4.1^{ce}	46.3 ± 1.8^{ca}	71.1 ± 2.5^{c}
	14	91.2 ± 2.3^{c}	88.3 ± 2.0^{be}	83.2 ± 1.6^{cd}	76.8 ± 5.3^{ad}	68.0 ± 3.2^{cc}	66.6 ± 2.5^{ae}	55.5 ± 3.4^{be}	47.6 ± 3.4^{ac}	72.2 ± 3.0^{a}
Sperm abnormality	0	1.2 ± 0.6	1.5 ± 0.5	2.0 ± 0.7^{b}	2.5 ± 0.9^{b}	3.3 ± 0.7^{b}	4.3 ± 1.0^{a}	5.3 ± 1.4^{d}	6.6 ± 1.4^{b}	3.4 ± 0.9^{b}
	4	1.8 ± 1.1	2.1 ± 0.7	3.0 ± 1.2^{c}	3.7 ± 1.0^{d}	4.8 ± 1.0^{d}	5.9 ± 1.6^{c}	7.2 ± 1.9^{c}	9.1 ± 1.0^{c}	4.7 ± 1.2^{c}
	6	1.3 ± 0.8	1.9 ± 1.0	3.0 ± 1.2^{c}	3.8 ± 1.0^{d}	4.5 ± 1.0^{c}	5.7 ± 1.6^{c}	6.8 ± 1.7^{c}	8.2 ± 1.7^{c}	4.4 ± 1.3^{c}
	8	$1. 0 \pm 0.7$	1.8 ± 0.9	2.8 ± 0.8^{c}	3.8 ± 1.0^{b}	4.4 ± 1.0^{c}	5.6 ± 1.0^{c}	6.5 ± 1.6^{d}	7.7 ± 1.5^{c}	4.2 ± 1.1^{c}
	10	1.0 ± 0.8	1.1 ± 0.7	1.5 ± 0.7^{a}	2.1 ± 0.7^{a}	2.8 ± 0.9^{a}	3.6 ± 0.8^{a}	4.4 ± 1.0^{a}	5.3 ± 1.0^{a}	2.8 ± 0.7^{a}
	12	1.5 ± 0.7	1.8 ± 0.6	2.3 ± 0.7	2.8 ± 0.8^{c}	3.7 ± 1.1^{b}	4.8 ± 1.2^{b}	6.0 ± 1.3^{c}	7.3 ± 1.3^{b}	3.8 ± 1.0^{d}
	14	1.3 ± 0.7	1.7 ± 0.7	2.2 ± 0.6	2.7 ± 0.8^{c}	3.4 ± 1.0^{b}	4.2 ± 1.2^{a}	5.0 ± 1.6^{a}	6.1 ± 1.0^{a}	3.3 ± 1.0^{b}

Values in the same column with different superscripts are differ significantly ($P<0.05$)

Table 2. Mean±SD of sperm with intact acrosome and capacitation or acrosome reaction during chilled storage in CEP-2 based extender containing GF (n=10).

Variables	Day of storage (D)	GF concentrations (%)		Mean ± SD
		0	10	
Intact acrosome (%)	D4	28.8 ± 4.9	31.3 ± 2.8	30.0 ± 3.8
	D8	27.5 ± 2.7	29.5 ± 4.2	28.5 ± 3.4
Capacitation and acrosome reaction (%)	D4	84.0 ± 5.4	85.3 ± 3.2	84.6 ± 8.6
	D8	86.8 ± 4.2	85.8 ± 3.3	86.3 ± 3.8

The values among treatments are not significantly different ($P>0.05$)

Based on scanning electron microscopy (SEM) analysis, the ultrastructure of sperm in 10% GF are found to be normal during storage for 8 days at 5° C as shown in Figure 1. This might indicate that the protective mechanism for sperm diluted in extender containing vitamin C directly suppress the generation of ROS and inhibit capacitation [26].

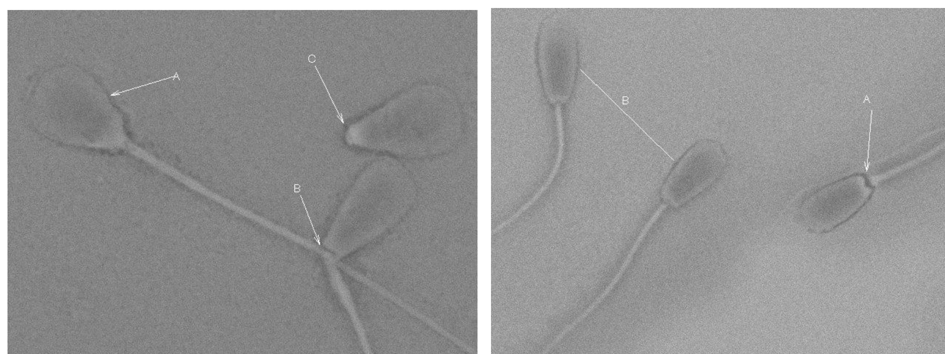

Figure 1. Left (0% GF) : A. membrane damaged, B. Brocken neck, C. Tailed loose. Right (10% GF.) : A. loose of midpiece, B. normal structures.

In the present study, the highest mean percentages of viability of Bali bull sperm stored for 8 days was found when 10% EY was replaced with 10% GF, while the lowest was found in 4% GF (Tabel 1). The lowest abnormal sperm was also observed in 10% GF, conversely, the highest was observed in 4% GF, which are very significantly different with the other treatments (P<0.01). In addition, sperm abnormality was recorded less than 20%. This is in agreement with Ax et al. [28], who reported that abnormality is one of the establish factors that affected sperm quality, when its value above 20%, this indicates poor semen quality and may influence the fertilizing spermatozoa. Duration of preservation decreased the mean percentage of abnormal spermatozoa from 5.88% on day 0 to 10.24% on day 4 [29]. Sperm abnormality may occurred at every parts of sperm and the largest amount was in the tail compared with mid-piece and head [1]. This result indicated, vitamin C conten of 10% GF in CEP-2 base extender is the effective formulation in preserving viability and morphology of sperm. A significant (P<0.05) higher sperm abnormalities values (37.6 ± 1.3%) was found after 120 hr incubation in vitamin C free Tris (Control) at 5 ˚C compared with those obtained in Tris diluent containing vitamin C (18.8 ± 1.8%) [27].

Previous study [2] stated that in-vitro viability and normal sperm both are affected by type of extender, their interaction with extender, method and duration of storage. Substitution of 10% EY with GF in CEP-2 based extender was the most ideal extender composition to preserve and protect bovine sperm for cool temperature storages. The equilibrium concentration of three ingredients mentioned are estimated capable of providing energy intake, nutrients and antioxidants needed by sperm, thereby maintaining the motility, viability, normal sperm and intact acrosome during chilled storage. Results of the study are parallel with that of Azawi and Hussein [27], who reported that viability sperm stored at 5 ˚C for 120 hr with Tris diluent containing vitamins C was 59.7 ± 1.3% which is significantly different (P<0.05) as compared with control (40.9 ± 1.8%).

Enzymatic antioxidant may provide protection action of sperm from CAR, so that the viability of sperm can be prolonged. Fluorescein isothiocyanate (FITC) test indicates that Bali bulls sperm with IA was higher (P<0.05) in 10% GF than that of 0% GF for D-4 to D-8 semen samples, and the sperm with CAR is lower in 10% GF at D-8 samples. The effect of treated extender on alteration of SOD and MDA levels were also showed that the antioxidan activity of SOD was found to be higher in 10% GF than in 0% GF as well as higher at D-8 than D-1 (35.1 u/mL at D-8 vs 31.7 u/mL at D-1 in 10% GF; 27.1 ± 12.5 u/mL at D-8 vs 23.7 ± 10.3 u/mL at D-1 in 0% GF).

The mean of MDA level in 10% GF was found to be decrease from 201.5 ± 83.5 ng/mL at D-1 to 183.2 ± 90.7 ng/mL at D-8. Conversely in 0% GF, the mean of MDA level was found to be increase from 207.3 ± 69.1 ng/mL at D-1 to 229.8 ± 90.2 ng/mL at D-8. The high SOD level measured in 10% GF at the present study might indicate that the activity of an antioxidant in extenders is existed. This is indicated that substitution of 10% GF may increased the antioxidant activity of SOD which blocked the MDA to generate reactive oxygen species (ROS) and prevented oxidative stress (OS) that finally prolonged the preserveability of sperm in the extenders. Previous study [30] explained that antioxidant may pressure MDA level, prevent the oxygen activity to promote free radical and ROS generation, that resulted in prolonged the preserveability of extenders.

Radical scavenging activity of antioxidant could reduce the ferrous-ascorbate mediated lipid peroxidation and increase the SOD level, so may protect the sperm [30]. Oxidative damage of sperm is resulted from an improper balance between ROS generation and scavenging activities of antioxidant. Glutathione peroxidase, catalase, superoxide dismutase, vitamin C and E are among the major antioxidants naturally present in mammalian semen. They might be functioning against ROS to protect the sperm from lipid peroxidation and to maintain its integrity. Antioxidant level of seminal plasma and sperm decreased during the preservation by

dilution with extender and excessive generation of ROS molecules. Antioxidants vitamin and intracellular enzymes are possible to prevent MDA production and protect sperm parameters during preservation [16].

Damaged of sperm in diluent with 0% GF occurs mostly on the head region, whereas in 10% GF the damages only a few, especially on the tail. This can be explained that extender containing GF and EY may preserve and protect sperm from damages caused by free radical or ROS. Previous studies [6; 10] demonstrated that phospholipid (lecithin) and low-density lipoprotein (LDL) in the extenders are considered as protective components that attached on sperm membrane to formed interfacial layer during cooling processes. Thereby they enhance the binding of phospholipids and cholesterol in the membranes. In addition, formation of a complex with seminal plasma proteins might cause the lipids had no function in the cell membrane, as a result the occurrence of cold shock can be prevented. The LDL component is also able to stabilize the membrane, reduce the loss of sperm membrane phospholipids and grab toxins from proteins of seminal plasma [31]. Aerobic organisms have developed complex antioxidant systems that can counteract ROS and free radicals. This system can prevent and/or reduce ROS and free radical-induced oxidative damage to tissues and cells and is composed of different antioxidant substances, including enzymes, non enzymatic antioxidants, and an array of small molecules [32].

The present study shows that substitution of 10% EY with GF in CEP-2 based extender may protect sperm from free radicals and ROS disruption, thus prolonging survival and motility during chilled storage. This was supported by previous study which indicated that fruits and vegetables are major sources of dietary antioxidants [33]. Their benefits as a source of tocopherols, carotenoids and ascorbic acid are well known as described in the literatures [34]. Guava is one of the fruits that seems to be useful as a source of exogenous antioxidants to protect sperm during chilled storage. Guava is very rich in antioxidants, vitamins and enzymes. There is a 151.4 mg or about 50-300 mg of vitamin C (ascorbic acid) in 100 grams of fresh fruit beside lutein, lycopene, zeaxanthine and high polyphenols [18; 35; 36]. The content of ascorbic acid in the skin, pulp and middle part of pulp reaches 350-450 mg (50-600 mg) in nearly ripe fruits [37].

Vitamin C is the critical biological antioxidant in cells, plays a role in the redox response tagging. Its main function is as a co-factor in enzymatic reactions and as an antioxidant to scavenge free radicals or capture. Vitamin C is transported into the cell in the oxidized form, i.e. dehydroascorbic acid (DHA) through the facilitative glucose transporters (gluts), then rapidly reduced in the cell and accumulate as AA [38]. Vitamin C or ascorbic acid (AA) indirectly regulate and maintain antioxidant α-tocopherol in the active state to reduce the sensitivity of sperm against lipid peroxidation. This mechanism has been sustained by high dehydroascorbate reductase. If lack of vitamin C or E, it will cause oxidative stress [26].

IV. Conclusion

Guava filtrate is potential when included in the extender for preservation of Bali bull spermatozoa. Substitution of 10% egg yolk with guava filtrate in CEP-2 based extender seems to be very effective in maintaining motility, viability, normality and intact acrosome of Bali bull spermatozoa during chilled storage for 8 days.

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