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THE ROLE OF GUAVA FRUIT FILTRATE ON MAINTAINING THE PRE-FREEZING AND POST-THAWED QUALITY OF BALI BULL SPERMATOZOA

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ABSTRACT

This study aimed to determine the role of guava fruit filtrate (GFF) on maintaining the pre-freezing and post-thawed quality of Bali bull spermatozoa in tris-egg yolk (TEY) extender. The bovine semen was collected using artificial vagina twice a week (n= 10). Ejaculate samples were divided into four tubes and each tube was respectively added the following diluents: TEY, TEY + 5% GFF, TEY + 10% GFF, and TEY + 15% GFF. The diluted semen was filled into the straws (0.25 mL) and cooled in refrigerator at 4° C for 2.5 hours. The samples were kept on vapor phase of liquid nitrogen (N_2) (-120° C) for 10 minutes prior to be stored in liquid phase (-196° C). The quality of pre-freezing and post-thawed spermatozoa in straw sample (i.e. motility, viability, and abnormality) was evaluated under light microscopy at 400x magnification. Data were analysed statistically. The percentage of motility and viability both on pre-freezing and post-thawed process were significantly higher in semen diluted with TEY + 10% GFF compared to control (TEY), TEY + 5% GFF, and TEY + 15% GFF. Addition of 10% GFF into tris-egg yolk extender play role for maintaining the quality of pre-freezing and post-thawed Bali bull spermatozoa.

Key words: bovine, guava filtrate, pre-freezing and post-thawed, spermatozoa

ABSTRAK

Penelitian ini bertujuan mengetahui peran filtrat buah jambu biji (FBJB) dalam mempertahankan kualitas spermatozoa sapi Bali sebelum dan setelah pembekuan di dalam pengencer tris-kuning telur (TKT). Semen sapi ditampung dengan vagina buatan dua kali sehari (n= 10). Sampel semen dibagi dalam empat tabung reaksi, kemudian masing-masing ditambahkan pengencer TKT, TKT + 5% FBJB, TKT + 10% FBJB, dan TKT + 15% FBJB. Semen yang telah diencerkan dimasukkan ke dalam straw (0,25 ml), kemudian didinginkan di dalam kulkas suhu 4° C selama 2,5 jam. Selanjutnya dilakukan prapembekuan di atas uap nitrogen (N_2) cair suhu -120° C selama 10 menit. Pembekuan dilakukan dengan cara dicelupkan ke dalam N_2 cair suhu -196° C dan disimpan di dalam kontainer berisi N_2 cair. Kualitas spermatozoa pada sampel prapembekuan dan setelah thawing dievaluasi di bawah mikroskop cahaya pembesaran 400x meliputi motilitas, viabilitas, dan abnormalitasnya. Data dianalisis secara statistik. Hasil penelitian adalah persentase motilitas dan viabilitas spermatozoa tertinggi dan abnormalitas terendah terdapat pada pengencer TKT + 10% FBJB jika dibandingkan dengan perlakuan kontrol (TKT), 5% FBJB dan 15% FBJB, baik pada proses pra-pembekuan maupun setelah thawing. Penambahan 10% FBJB di dalam pengencer tris-kuning telur berperan dalam mempertahankan kualitas spermatozoa sapi Bali sebelum dan setelah pembekuan.

Kata kunci: sapi, filtrat jambu biji, sebelum dan setelah pembekuan, spermatozoa

INTRODUCTION

Washing, diluting, cooling, freezing, and thawing can change the surface protein of spermatozoa, causing instability and decreasing fertility (Gillan *et al.*, 2004; Leahy and de Graaf, 2012). Lipid peroxidation during cryopreservation can increase the sensitivity of spermatozoa toward reactive oxygen species (ROS) and reduce motility and viability up to 50% (Vishwanath and Shannon, 2000; Andrabi *et al*., 2008). Spermatozoa mortality rate reached more than 50% during frozen sperm making and thawing process (Delgado *et al*., 2009; Kaeoket *et al*., 2011).

Death and damage of spermatozoa during handling produce ROS including hydroxyl radical, anion superoxide radical, hydrogen peroxide, singlet oxygen, hypochlorite, nitrate oxide and peroxynitrite radical. Free radicals play role as oxidant or reductant and damage DNA, protein, carbohydrate, lipid, acid, and nuclear protein by delivering or receiving electron from other molecules in cell nucleus or cell membrane (Lobo *et al*., 2010).

Seminal plasma has antioxidant system for protection against ROS activity. However addition of diluents to the semen can reduce antioxidant level and ruin the balance of ROS and endogenous antioxidants leading to spermatozoa damage (Rao and Rao, 2007; Kothari *et al*., 2010). Antioxidants supplementation in diluents is needed to protect spermatozoa (Asadpour *et al*., 2011; Bansal and Bilaspuri, 2011), reduce damage caused by free radical and ROS, as well as to protect sperm vitality (Tavilani *et al*., 2008; Stevanov *et al*., 2011).

Antioxidant can be produced both endogenously by the body and exogenously from daily foods and beverage (Ibrahim *et al*., 2008). There are two types of antioxidants, specifically enzymatic and non-enzymatic. Enzymatic (superoxide dismutase, catalase, glutathione, and alpha lipoic acids) and non-enzymatic antioxidants (vitamin E, C, and A) are generally efficient against free radical. Vitamin E and C protects against free radicals by maintaining structural and functional integrity of the spermatozoa. Vitamin C is a hydrophilic vitamin which cannot be stored endogenously and must be consumed or supplemented. Low concentration of vitamin C (ascorbic acid) acts as oxidant, while high concentration (±5 mM) acts as antioxidant (Andrabi *et al*., 2008).

Adding antioxidant to the semen diluents is necessary to prevent cell damage by breaking the oxidation reactions chain and protect molecules from oxidation activity by oxidizing their own molecules

(Norshazila *et al*., 2010; Bansal and Bilaspuri, 2011). Antioxidant in diluents can act as electron receiver (Funahashi and Sano, 2005). Antioxidant molecules reduce oxidative stress effect, while internal compound protect cells from oxygen radicals that can increase the spermatozoa quality (Kulaksiz and Daskin, 2010).

Currently, there are a lot of attentions about the use of natural antioxidant to inhibit lipid peroxide and prevent sperm cell damage (Hui-Yin and Gow-Chin, 2007). This new innovative diluents are expected to preserve and protect the quality of spermatozoa, further improving its fertility capability.

Guava fruit (*Psidium guajava* Linn) contains many antioxidants including polyphenol, vitamin C, A, B, and E, and carotenoid. It was found a good result when added guava fruit filtrate (GFF) to the CEP-2 diluents for cow spermatozoa preservation at 5° C (Sumadiasa *et al*., 2015). Addition of GFF in tris-egg yolk (TEY) to maintain the quality of Bali bull pre-freezing and post-thawing spermatozoa needs to be evaluated to increase semen post-thawing quality. The combination of TEY and GFF is expected as source of nutrition, energy, macro-protectant, and antioxidant.

MATERIALS AND METHODS

Semen samples were collected from 4 cows at Regional Artificial Insemination that belongs to Livestock and Animal Health Office West Nusa Tenggara Province which were taken in rotation using artificial vagina, 2 times a week for 5 weeks (n= 10). The ejaculated semen was immediately examined macroscopically and microscopically to determination of its quality.The criteria for semen used in this study were having spermatozoa with >70% motility and viability, less than 20% abnormality, and had concentration of ± 1000 million/mL. This was a randomized-group experimental study which involved 4 groups of treatment i.e. P0, P1, P2, and P3.

Guava Fruit Filtrate (GFF) Production

Guavas were blended and dissolved in double distilled water with 1:2 ratios. The solution was then centrifuged at 3500 rpm for 10 minutes. The supernatant was infiltrated using millipore membrane (Sartorius stedim Minisart ®) 0.42 µm continue with 0.2 µm to obtain the filtrate. The filtrate was pasteurized in boiling water at 60° C for 3 minutes, then put in microtube 2.5 mL and stored in refrigerator at 5° C (Sumadiasa *et al*., 2015).

Tris-Egg Yolk (TEY) Production

Tris buffer contained 3.634 g tris (hydroximethyl) aminomethane, 0.5 g glucose, and 1.99 g citric acid, and was boiled at 90° C in 100 mL double distilled water for 10 minutes and then left out until 32° C. After that, the buffer was added by 0.1 g streptomycin and 0.06 g penicillin, then homogenized. Finally, the buffer added by 10 mL egg yolk and 10% glycerol. Buffer solution was put in reaction tube P_0 (TEY only), P_1 (TEY + 5% GFF), P_2 (TEY + 10% GFF), and P_3 (TEY + 15% GFF) (modification from Toelihere, 1993; Sumadiasa, 2015).

Dilution, Straw Filling, and Equilibration

Stored semen were diluted by each media with concentration of spermatozoa as much as 80 million/mL and then filled in straw (0.25 mL) so that every straw contain at least 20 million spermatozoa. The filled straw from room temperature was equilibrated in refrigerator to reach 5° C for ±2.5 hours. After that, pre-freezing method was conducted by putting the straw on liquid nitrogen (N_2) vapor for 10 minutes and then dipped into liquid N_2 and stored as frozen semen.

The Evaluation of Spermatozoa Quality Before and After Freezing Treatment

The preservation and protection ability of GFF towards spermatozoa quality was evaluated. After pre-freezing period, straw samples were warmed up in water bath at 42° C and then evaluated (motility, viability, and abnormality) under microscope with 10x40 magnifications. The quality evaluation of post-thawing spermatozoa was performed on frozen-straw semen after being stored for 15-30 minutes and warmed up (thawing) in water bath at 42° C.

Data Analysis

Data was analysed using amalysis of variance (ANOVA) continued with Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

The bulls at Regional Artificial Insemination that belongs to Livestock and Animal Health Office West Nusa Tenggara (NTB) Province were intensively maintained for more than 3-5 years and were a semen producer to supply all districts in NTB. The evaluation toward semen quality showed that this study used a good and worth processing semen.

The mean semen volume from 10 times collection were 7.5±2.35 mL and similar to semen volume found in exotic cows ranged from 5-8 mL (Garner and Hafez, 2008); 4.84±0.01 mL (Patel and Siddiquee, 2013); 10.5 mL (Hossain *et al.*, 2012). The color and consistency of semen were in normal range with pH 6.2 which was between normal range of the bull semen ranged from 5.15-6.53 (Rehman *et al*., 2012), 6.2-6.4 (Yavas and Daskin, 2012), and 6.4-6.88 (Patel and Siddiquee, 2013). The condition was presumably due to differences in bull types, temperature or season when the semen was collected, or the type of feed, whereas the weather at the study was quite good and normal rain intensity.

Spermatozoa mass motility was ++, progressive motility was 70±0%, the number of lives spermatozoa was quite high at 79.8±4.39%, and the abnormality was very low at 8.6±3.06%. Macroscopic examination showed spermatozoa concentration more than 1000 million cell/mL (121.36±32.88 x 10^7), which was higher than reported by Andrabi *et al*. (2002), Hossain

et al. (2012), and Abavisani *et al*. (2013) i.e. 0.81 x 10^9/mL and was close to the average concentration of 1.32 x 10^9/mL (Sarder, 2007) and 1.25 x 10^9 (Patel and Siddiquee, 2013).

We found a good average of pre-freezing spermatozoa that was feasible to be frozen. Table 1 showed the average value of motility, viability, and abnormality of pre-freezing Bali bull spermatozoa dissolved in TEY + GFF. The average quality of spermatozoa dissolved in 10% GFF + TEY was quite good and there were different quality in every treatment. Addition of 10% GFF to the TEY gave highest mean percentage of motility and viability as well as lowest abnormality of spermatozoa. The motility of spermatozoa would be very low by adding 15% GFF into TEY. In addition, control group (TEY only) showed lowest viability and highest abnormality of spermatozoa.

The mean motility of spermatozoa in group P2 was statistically higher than group P3 (P<0.01), but was not statistically different with group P0 and P1. Generally, the best treatment for maintaining the pre-freezing Bali bull sperm was by adding 10% of GFF into TEY. It was expected that the combination of tris, egg yolk, and guava fruit filtrate could be synergized for preserving and protecting the spermatozoa during pre-freezing process.

The quality of spermatozoa can be maintained everlastingly after being frozen and stored in liquid N_2 at -196° C as long as the liquid N_2 was still available in the containers. It mean that there was no quality difference between 15 minutes and 1 year or even several years of storage, unless the amount of liquid N_2 was reduced which cause decreasing storage temperature of lower than -196° C. After being thawed, the spermatozoa still showed a high mean percentage of motility and viability above the average quality of sperm that is suitable for artificial insemination specifically 50% of minimum motility and viability and 20% maximum of abnormality.

Table 2 provided the average value of motility, viability, and abnormality of post-thawing Bali bull spermatozoa in all groups. In post thawing condition, spermatozoa dissolved in TEY + 10% GFF had highest mean percentage of motility and viability as well as lowest mean percentage of abnormality, whereas addition of 15% GFF to the TEY provided lowest mean percentage of motility and viability. In addition, highest abnormality of spermatozoa was found in control group. The mean motility of post-thawing spermatozoa in group P2 was statistically higher than all groups (P<0.01), but the viability and abnormality were not statistically different.

The best quality of spermatozoa in both pre-freezing and post-thawing was found in group P2 by adding 10% GFF into TEY. The P2 diluents had good combination because GFF provide antioxidant properties to protect spermatozoa from ROS damage, while egg yolk provides nutrition, energy, and low density lipoprotein (LDL) as membrane cell protector.

The abnormality of spermatozoa begins as membrane damaged by ROS. The ROS has an ability in adhere at the ovum to ruin fertilization process and damage the DNA material inside spermatozoa. LDL in egg yolk could reduce the membrane phospholipid loss, stabilized membrane, and seized toxins from seminal plasma proteins (Akhter *et al*., 2010).

Phospholipid (lecithin) and LDL contained in egg yolk prevent the cold shock phenomenon. Both of the components build an interfacial cell layer during freezing or equilibration process thus causing an increase in phospholipid and cholesterol bonds to the plasma membrane of spermatozoa. Further, it builds up a complex with seminal protein plasma which make the lipid cannot work inside the membrane cell (Chanapiwat *et al*., 2011; Trout, 2012).

Antioxidant is an active material which is very important to protect spermatozoa during storage. Antioxidant system such as glutathione (GSH), GPX, catalase (CAT) and SOD can neutralize ROS. Moreover, addition of several non-enzymatic molecule including glutathione, thioredoxin, vitamin D, E, and C in semen work against lipid peroxide, maintain the motility and viability of spermatozoa (Zeitoun and Al-Damegh, 2015).

Guava fruit (*Psidium guajava* Linn) contains several antioxidant and important component to protect the cell i.e. carotene, vitamin B1 and B2, niacin, fiber, vitamin C, lutein, lycopene, and zeaxanthine (Rahmat *et al*., 2006). Vitamin C content in guava was 50-300 mg/100 g fresh weight (Thaipong *et al*., 2005), even reaching 350-450 mg before ripe (Rao and Rao, 2007). Therefore, GFF could significantly maintain the quality of spermatozoa pre-freezing and post-thawing process.

Table 1. The average of pre-freezing spermatozoa quality (n= 10)

Variable	Treatment			
	P0	P1	P2	P3
Motility	63.0±4.4^{b}	60.3±5.5^{b}	65.8±6.1^{b}	52.8±7.0^{a}
Viability	79.2±3.1^{a}	81.0±2.7^{a}	87.4±3.5^{b}	80.9±3.8^{a}
Abnormality	21.1±4.3^{b}	20.0±3.6^{ab}	17.4±2.8^{a}	20.4±3.5^{ab}

^{a,b}Different superscripts in the same row indicate significant different (P<0.01)

Table 2. The average of post-thawing spermatozoa quality (n= 10)

Variabel	Treatment			
	P0	P1	P2	P3
Motility	52.8±4.2^{a}	51.0±5.9^{a}	61.0±3.2^{b}	49.3±7.6^{a}
Viability	76.8±4.8^{ab}	76.7±6.1^{ab}	81.4±4.7^{b}	76.1±4.9^{a}
Abnormality	23.1±3.0^{b}	22.4±3.1^{b}	19.5±3.3^{a}	22.6±2.8^{b}

^{a,b}Different superscripts in the same row indicate significant different (P<0.01)

Vitamin C content in GFF could neutralize toxins in semen, suppress the rate of cholesterol catabolism, and reduce spermatozoa attachment to the cluster and make them move and swim easily. High antioxidant intake from vitamin C, E, and β-carotene could significantly maintain the motility of spermatozoa. Supplementation of 5 mM vitamin C or vitamin E into diluents could decrease the percentage of spermatozoa abnormality after storage (Mittal *et al.*, 2014).

CONCLUSION

Guava fruit filtrate (GFF) in tris-egg yolk (TEY) dilution has important role in maintaining the quality of Bali bull spermatozoa before and after frozen. The addition of 10% GFF into TEY is very effective in preserve and protect the spermatozoa quality both before and after frozen.

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