# Additional Freeze Drying Fig Fruit (Ficus carica L) Filtrate into Tris Egg Yolk Extender and Its Effect on Sperm Membrane Integrity and Acrosome of Kacang Buck

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# Additional Freeze Drying Fig Fruit (*Ficus carica L*) Filtrate into Tris Egg Yolk Extender and Its Effect on Sperm Membrane Integrity and Acrosome of Kacang Buck

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**ABSTRACT.** A study was designed to determine Kacang buck sperm membrane integrity and acrosome reaction as to the effect of different concentration of freeze-drying fig fruit filtrate in tris egg yolk based extender. This study used 5 proven fertility Kacang goats aged 3-4 years, maintained by the Faculty of Animal Sciences, Mataram University, Indonesia. Semen was collected by artificial vagina at every five days. The collected semen was divided into four aliquots in accordance to the treatments extender such as Control (0 gr), T1 (0.02 gr), T2 (0.04 gr) and T3 (0.06 gr) freeze-drying fig fruit filtrate in tris egg yolk based extender (gr/v), respectively. Plasma membrane integrity and intact acrosome after re-concentration and preserved at 5ºC were assessed visually at 0 and every 24 hours for 5 consecutive days. The statistical significance of the result was evaluated by a one way ANOVA for completely randomized design analysis of variance. Data were presented as Mean±SD. Results suggest that the mean percentages of sperm membrane integrity in T0, T1,T2 and T3 at 96 h post extended and preserve at 5ºC were 34.3±5.3, 40.6±4.7, 44.8±5.4 and 42.1±5,1, respectively. The mean percentages of sperm acrosome intact were 16.4±4.8, 18.5±1.9, 21.6±3.1 and 19.6±2.8, respectively. The results of the study suggested that additional 0.04gr freeze drying fig fruit filtrate into tris egg yolk based extender have a significant preservation effect on both spermatozoa membrane integrity and acrosome intact of kacang buck.
Key Words: Buck, Spermatozoa, Acrosome, membrane, fig fruit extract, freeze-drying

**ABSTRAK. P**enelitian bertujuan untuk mengetahui pengaruh suplementasi sari buah tin yang di freeze drying kedalam pengencer berbasis tris kuning telur terhadap keutuhan membran dan status akrosom spermatozoa kambing kacang yang disimpan pada suhu 5°C. sampel semen diperoleh dari 1 ekor kambing kacang dewasa berumur 3 tahun. Koleksi semen dilakukan setiap 5 hari menggunakan vagina buatan. Semen yang terkumpul dibagi menjadi 4 bagian sesuai dengan perlakuan yaitu berturut-turut kontrol (0 gr), T1 (0.02 gr), T2 (0.04 gr) and T3 (0.06 gr) freeze drying sari buah tin didalam pengecer berbasis tris kuning telur (gr/v). Integritas plasma membran dan intak akrosom setelah pengenceran dan penyimpanan pada suhu 5°C diperiksa secara visual menggunakan mikroskop phase kontras pada 0 dan setiap 24 jam setelah pengenceran dan penyimpanan pada suhu 5°C selama 5 hari berturut-turut. Data dianalisis menggunakan one way ANOVA dan dipersentasikan dalam bentuk Mean±SE. Hasil penelitian menunjukkan bahwa, rataan persentase spermatozoa dengan plasma membran utuh pada pengencer T0, T1,T2 dan T3 pada 96 jam setelah pengenceran dan penyimpanan pada suhu 5°C berturut-turut 34.3±5.3, 40.6±4.7, 44.8±5.4 dan 42.1±5,1. Sedangkan rataan persentase spermatozoa dengan acrosom intak adalah 16.4±4.8, 18.5±1.9, 21.6±3.1 dan 19.6±2.8. hasil penelitian ini menyimpulkan bahwa, penambahan 0,04 gr freeze drying sari buah tin kedalam pengencer berbasis tris kuning telur meningkatkan daya preservasinya terhadap keutuhan membran dan keutuhan akrosom spermatozoa kambing kacang.
Kata Kunci: kambing jantan, Spermatozoa, Acrosom, membran, ekstrak buah tin, kering beku

## Introduction

Early capacitation of spermatozoa *in vitro*, followed by acrosome reaction results in rapidly energy loses and quickly dies (Zamfirescu et al. 2003). Integrity and functional activity of spermatozoa and acrosome play an important role in the process of fertilization. Therefore, the integrity of the membrane and acrosome is an indicator to estimate the spermatozoa fertility rate (Zamfirescu et al. 2003 and Hashida et al. 2005) and the necessities for the occurrence of attachment and the joining of spermatozoa with zona pellucida and acrosome reactions (Hashida et al. 2005).

Extenders were one of the most decisive factors to maintain the quality of both liquid and frozen semen. Many studies have been

carried out to investigate the preservation effect extender containing organic materials. Papa et al. (2010), reported that substitution of egg yolk using soybeans lecithin tends to increase the preservation effect of extender in retaining mass motility, progressive motility and membrane integrity of the post-thawing frozen horse semen. Sumadiasa et al. (2015) reported that the substitution of 10% of 25% egg yolk using guava filtrate on CEP-2-based extender, had a very positive influence on the motility, viability, morphology and acrosomes of Bali cow spermatozoa. Bahmanpour et al. (2006) also reported that date fruit pollen also proved to improve the quality parameters of spermatozoa in mice.

Three previous studies (Zaenuri et al. 2013; Zaenuri et al. 2014a and Zaenuri et al. 2014b) carried out research to investigate the effect of fresh fig fruit ((*Ficus carica L and Ficus carica R*) filtrate in tris egg yolk based extender in preserving buck semen quality. These studies noted that to some extent, additional fresh fig fruit filtrate in tris egg yolk based extender was able to maintain progressive motility, viability, sperm membrane integrity and acrosome of goat spermatozoa. However, preparation of freeze drying fig fruit filtrate is concurrently cheaper and prolonged, it expires quickly than fresh filtrate. This research was performed in order to know the preservation effect of freeze drying fig fruit filtrate in tris egg yolk based extender in maintaining the membrane integrity and acrosome of Kacang buck spermatozoa.

## Materials and Methods

### Experimental Extender

Preparation of fig fruit filtrate is carried out according to the method described by Zaenuri et al. (2014a). The fig fruit filtrate underwent freeze drying for 2 x 24 hours, then it was kept at room temperature. Preparation of standard extender was as described by Evans and Maxwell (1989). The treatment extender comprising a standard extender (Evans and Maxwell 1989) was added to freeze-dried fig fruit extract 0 gr (TO), 0.02 gr (T1); 0.04 g (T2) and 0.06 gr (T3) per 1000 ml (v/v) of extender, then homogenous using magnetic stirrer prior to use.

### Semen Collection, Evaluation and Extended

This study used 5 proven fertility Kacang goats aged 3-4 years, maintained by Faculty of Animal Sciences, Mataram University, Indonesia. Semen was collected by artificial vagina at every five days. Initial evaluation for fresh semen was immediately performed in the laboratory including volume, colour, and consistency, followed by microscopic assessment including mass movement, wave motion (scale of 0-5), concentration and *percentage of motile spermatozoa*. The quality of each ejaculates to be suitable for a further process, were not less than 0.8 ml in volume, more than 70% motile and 2.5 to 3 x $10^9$/ml in concentration (Evans and Maxwell 1989).

The concentration of spermatozoa for each treatment was 75 million / 0.25 ml treatment extender. Semen dilution was done by inserting the diluent gradually and shaking gently to homogeneous. Extended semen was incubated in water bath at 37ºC for 30 minutes for antibiotics to function optimally. To prevent cold stress, the tube containing the semen was retained in the quilled water jacket so that the semen temperature will decrease from 30ºC to 5ºC within 1 hour (Hafez 2008).

### Plasma Membrane Integrity

First of all, the prepared Hypo-osmotic solution was made with fructose (8.72 g/l) and sodium citrate (4.74 g/l) at *p*H of 8.05 (Susilawati, 2013). A volume of 10 µl of semen was added to 1 ml of the solution and incubated at 37°C for 40 min. Immediately after the incubation, 1 drop of the semen was placed on a glass slide covered with a cover slide and evaluated under a phase-contrast microscope

(*CarlZeis*, Germany) at 400 magnificent. Microscope fields were selected randomly. At least 200 spermatozoa were evaluated per slide and percentages of swollen tail spermatozoa were calculated. The percentage of spermatozoa with the intact membrane is the number of spermatozoa whose tail is circular, divided by 200 (Susilawati 2013; Ahmad et al. 2003).

### Intact Acrosome

Acrosome intact was evaluated using the method as described by Tamuli and Watson (1994) as follows. First, the spermatozoa staining material comprises a stock *tartrate phosphate buffer* consisting of 50 mM *Disodium hydrogen anhydrous orthophosphate*, 25 mM *Potassium dihydrogen orthophosphate*, 77 mM *Potassium sodium tartrate*. Secondly, these three ingredients were mixed together, stored in the refrigerator until the time of use. Thirdly, 50 mM glucose with volume as needed was made. After that, it was continued by making stock Nigrosin Eosin consisting of 10 g of Nigrosin dissolved into 60 ml of aquabides and boiled. After cooling, it was poured into a 100 ml reaction tube that already contains 0.7 g eosin using a glass filter paper. 7.5 ml of 50 mM glucose and 7.5 ml *Tartrate Phosphate Buffer* (TPB = 50 mM *Disodium hydrogen orthophosphate anhydrous*, 25 mM *Potassium dihydrogen orthophosphate*, 77 mM *Potassium sodium tartrate*) was added. aquabides up to 100 ml volume was added and then kept in the refrigerator. Fifthly, make Stock Giemsa by 0.7 g Giemsa added 75 ml of absolute methanol and 25 ml of glycerol and homogenized, store at 37ºC for 1 week, shake giemsa bottle daily. Stock giemsa will be ready after one week. Lastly, a stock fresh Giemsa Stain every day just prior to use was prepared. The fresh giemsa stain consists of 4.5 ml of giemsa stock being mixed with 3 ml of TPB (50 mM disodium hydrogen anhydrous orthophosphate, 25 mM potassium dihydrogen orthophosphate, 77 mM *potassium sodium tartrate*) and 32.5 ml of aquabides. This solution was only made for single use, so the volume was made as needed.

To evaluate acrosome intact, mix a drop of semen and 3 drops of Nigrosin-Eosin in warm object glass and then leave for 30 seconds. Make a smear and dry on a warm plate. The smear that has been made was fixed on the TPB (TPB = 50 mM disodium hydrogen anhydrous orthophosphate, 25 mM potassium dihydrogen orthophosphate, 77 mM potassium sodium tartrate) with 4% formaldehyde for 10 min. After that, the smear was lifted and dried at room temperature then washed in tap water for 10-15 minutes, then rinsed with aquabides and left at room temperature a few minutes to dry. Next, soak the smears in fresh Giemsa Stain for 60 min, dry it at room temperature. Wash the smears by putting it in a container of tap water for 10-15 minutes then rinsed with aquabides, lift the smears up and allow it for a few minutes at room temperature to dry. Calculate 200 spermatozoa for each treatment using a 1000x magnificent phase contrast microscope. This study only identifies intact acrosomes without distinguishing live and dead spermatozoa with intact or damaged acrosome.

### Statistical Analysis

The statistical significance of the result was evaluated by a one way ANOVA completely randomized design analysis of variance using CoStat for windows statistical software (version 6.303). Data was presented as Mean±S.E. Probability $P<0.05$ was considered significantly different.

## Results and Discussions

### Plasma Membrane Integrity

Percentages and standard deviation of spermatozoa membrane integrity are presented in Table 1. The percentages of spermatozoa membrane integrity of this study showed that since 72 hours the percentage of plasma spermatozoa membrane integrity in extender

containing 0.04 gr of fig fruit filtrate tended to be higher (55.2±7.1) and there were statistically significant differences as compared to Spermatozoa plasma membrane integrity at 0.02 gr and 0.06 gr which are 40.6±4.7 and 42.1±5.1 except on control, respectively. The same trend occurred at 96 hours after being extended and stored. Membrane integrity and spermatozoa viability are the main requirements for fertilization (Mocé and Graham 2008).

The integrity of the goat spermatozoa plasma membrane integrity in extender containing 0.04 gr of fig fruit filtrate is higher to that reported by some researcher. Anghel et al. (2010) found that, in addition to 0.1 and 1 mM α-tocopherol in Tris-based extenders, the percentage of plasma integrity of post-thawing goat spermatozoa membranes was 52.72±2.08 and 55.00±1.65 compared to controls (49.75±2.6). Ari and Daskin (2010) reported that the integrity of goat spermatozoa plasma membrane in Tris extender + 20% egg yolks was 38.0±3.4%. Saraswat et al. (2012) reported that the addition of 1.5; 2.5 and 3.5 mM of vitamin E in extenders and stored at 5ºC which was examined after 96 hours, to obtain a percentage of plasma integrity of spermatozoa membranes 23.42±10.0; 24.07±10.09 and 14.05±7.85. Hu et al. (2009) reported that, addition of 1.25; 2.5; 3.75 and 5 mg / ml of vitamin B12 in post thawing cattle semen obtained a percentage of plasma integrity of spermatozoa membrane consecutively 50.69±2.50; 42.34±2.36; 39.45±2.31 and 37.46±1.76.

Physiologically, the integrity of the spermatozoa membrane plays a vital role in the physiological processes of spermatozoa, including protecting and maintaining the motility of spermatozoa in the female reproductive tract, also the capacitation and fertilization so that the integrity of the membrane is one indicator that determines the fertility rate of spermatozoa (Mocé and Graham 2008 ; Zamfirescu et al. 2003).

Table 1: Percentage of Sperm Membrane Integrity in Tris Egg Yolk Based Extender Supplemented by Different Concentration of Freeze Drying Fig Fruit Filtrate at 5ºC.

| Freeze Drying Fig Fruit Extract (gr) | Preservation (hours) | | | | |
|---|---|---|---|---|---|
| | 0 | 24 | 48 | 72 | 96 |
| 0 | 67.1±6.2 | 63.7±4.2$^{ab}$ | 57.8±9.6$^{a}$ | 52.3±7.9$^{a}$ | 34.3±5.3$^{c}$ |
| 0.02 | 68.8±6.4 | 64.1±5.0$^{ab}$ | 56.1±5.5$^{a}$ | 50.3±5.6$^{a}$ | 40.6±4.7$^{b}$ |
| 0.04 | 68.1±7.2 | 61.4±6.1$^{b}$ | 57.9±9.9$^{a}$ | 55.2±7.1$^{b}$ | 44.8±5.4$^{b}$ |
| 0.06 | 74.8±5.6 | 69.3±5.3$^{a}$ | 54.0±9.1$^{a}$ | 51.5±5.2$^{a}$ | 42.1±5.1$^{a}$ |

The values in the same column with different superscripts differ significantly ($P<0.05$)

Table 2: Percentage of Sperm With Intact Acrosome in Tris Egg Yolk Based Extender Supplemented by Different Concentration of Freeze Drying Fig Fruit Filtrate at 5ºC.

| Freeze Drying Fig Fruit Extract (gr) | Preservation (hours) | | | | |
|---|---|---|---|---|---|
| | 0 | 24 | 48 | 72 | 96 |
| 0 | 40.6±4.9$^{a}$ | 36.2±4.4$^{a}$ | 21.8±5.4$^{a}$ | 19.5±4.4$^{a}$ | 16.4±4.8$^{a}$ |
| 0.02 | 34.6±2.6$^{ab}$ | 32.0±2.1$^{b}$ | 24.9±3.6$^{b}$ | 21.1±3.2$^{ab}$ | 18.5±1.9$^{ab}$ |
| 0.04 | 34.5±1.3$^{b}$ | 30.3±1.1$^{b}$ | 28.1±4.7$^{b}$ | 24.2±2.4$^{b}$ | 21.6±3.1$^{b}$ |
| 0.06 | 32.7±3.1$^{b}$ | 30.9±3.1$^{b}$ | 26.4±2.5$^{b}$ | 22.6±2.8$^{b}$ | 19.6±2.8$^{b}$ |

The values in the same column with different superscripts differ significantly ($P<0.05$)

**Intact Acrosome**

The percentage of mean and standard deviation of spermatozoa with intact acrosome is presented in Table 2. This study found that, the percentage of spermatozoa with intact acrosome in extender containing 0.04 gr of fig fruit filtrate up to 96 hours post extended and stored at 5ºC was 21.6 ± 3.1 and was significantly ($P<0.05$) higher than control extender (16.4±4.8), 0.02 gr (18.5±1.9) and 0.09 gr (19.6±2.8) fig fruit filtrate, respectively. The results of this study are not much different from those reported by Saraswat et al. (2012) that, the percentage of intact acrosomes of Sirohi goat spermatozoa with extenders containing 2.5; 3.5; 5 and 7 mM of vitamin E at 96 hours post extended and stored at 5ºC were 22.1±3.1; 28.5±4.2; 26.4±4.9 and 24.4±3.1. Hu et al. (2009) reported that, the addition of 1.25; 2.5; 3.75 and 5 mg / mL of vitamin B12 in cattle frozen semen post-thawing obtained the percentage of intact acrosomes were 67.25±2.74; 60.61±2.85; 54.06±2.27 and 48.59±2.56, respectively.

Acrosome has a greater influence than the spermatozoa motility rate on the result fertility rate (Sacke 1972). An important role of acrosome reactions is in the release of proteolytic enzymes that is necessary for sperm penetration via zonna pelucida in mammals (Talbot et al. 2003). Thus, an assessment of the integrity of the acrosome is necessary in order to determine the spermatozoa fertility rate, because spermatozoa with incomplete acrosome are negatively correlated with spermatozoa fertility (Garner and Hafez 2008). Therefore, preservation ability of extender should be the main concern so that the acrosome and fertility rate of spermatozoa could be preserved. Zaenuri et al. (2014) found that, the optimum concentration of fresh fig fruit filtrate in tris egg yolk based extender for optimum preservation ability is to preserve incapacitated intact acrosome and acceptable pregnancy rates that are found at fix time insemination of buck sperm when tris egg yolk based extender was supplemented with 6% fresh fig fruit filtrate (v/v).

Increased preservation of tris egg yolk based extender supplemented by fig fruit filtrate may be due to three main reasons. First, Fig fruit filtrate is not only a high concentration of exogenous antioxidants but also fructose and micro minerals, and it may be beneficial to the longevity of sperm quality preserved in tris egg yolk based extender (Zaenuri et al. 2014a). Secondly, every 100 gr of fresh fig fruit contains 0.81, 20.40, 266.34, 181.50 and 0.46 mg α-tocopherol, total tocopherol, total phenol, vitamin C and β-carotene, respectively. Fig fruits are also rich in energy sources (g/100 g) such as protein (1.26), carbohydrate (24.27), glucose (17.58) and fructose (18.20) and finally, rich in minerals (mg/1000 g) including Cu (0.15), Mg (223.53) and Ca (0.044). Lastly, all of these microelements are indispensable for spermatozoa to neutralize the negative effects of free radicals from the residual metabolism of spermatozoa (Zaenuri et al. 2014b).

## Conclusions

The addition of freeze-drying fig fruit extract at a definite concentration into the tris egg yolk based extender showed a positive impact on goat spermatozoa during preservation at 5ºC. The concentration of fig fruit extract in the most optimized tris egg yolk based extender to preserve goat sperm plasma membrane integrity and acrosomes is 0.04 gr in 100 ml tris egg yolk based extenders.

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