



# THE EFFECT OF SEMEN WASHING AND SOYBEAN LECITHIN LEVEL ON MOTILITY AND VIABILITY OF RAM SPERMATOZOA STORED AT 5°C

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## ABSTRAK

Tujuan penelitian ini adalah untuk mengetahui pengaruh pencucian semen dan kadar lesitin kacang kedelai terhadap motilitas dan viabilitas sperma domba yang disimpan pada suhu 5°C. Semen tampung dari tiga ekor domba jantan dewasa dengan vagina buatan. Semen dievaluasi secara makroskopis dan mikroskopis. Semen dibagi dalam sepuluh tabung, semen dari lima tabung pertama tidak dicuci (W0) dan diencerkan dengan extender 0%, 1%, 2%, 3% dan 4% lesitin kacang kedelai (L0, L1, L2, L3 dan L4). Semen dari lima tabung lainnya dicuci (W1) kemudian masing-masing diencerkan dengan kadar extender yang sama. Sampel semen yang telah diencerkan disimpan pada suhu 5°C, kemudian motilitas dan viabilitas sperma dievaluasi setiap hari. Interaksi dari pencucian semen dan kadar lesitin kacang kedelai tidak berbeda nyata ( $P>0.05$ ) tetapi secara tunggal faktor kadar lesitin kacang kedelai berpengaruh nyata ( $P<0.05$ ) dan faktor pencucian semen tidak berpengaruh nyata ( $P>0.05$ ) terhadap motilitas progresif dan viabilitas sperma domba. Ekstender terbaik adalah 3% lesitin kacang kedelai (L3) dengan persentase motilitas progresif dan viabilitas spermatozoa masing-masing  $63.18 \pm 3.61\%$  dan  $71.76 \pm 2.32\%$ .

*Kata kunci: domba, motilitas, lesitin kacang kedelai, pencucian semen, spermatozoa, viabilitas*

## ABSTRACT

The objectives of this research were to study the effect of semen washing and soybean lecithin level on the motility and viability of ram spermatozoa stored at 5°C. Semen was collected from three mature rams by artificial vagina. Semen was evaluated by macroscopic and microscopic. Semen was divided into ten tubes, the semen of first five tubes was unwashed (W0) and diluted with extender 0%, 1%, 2%, 3% and 4% soybean lecithin (L0, L1, L2, L3 and L4). Semen of another five tubes were washed (W1) then each diluted with similar extender levels. The diluted semen samples were stored at 5°C and the sperm motility and viability were evaluated each day. The interaction of the semen washing and soybean lecithin levels were no significant differences ( $P>0.05$ ) but the single factor of soybean lecithin level was significant differences ( $P<0.05$ ) and the single factor of semen washing were no significant differences ( $P>0.05$ ) on progressive motility and viability of ram sperm. The best extender was 3% soybean lecithin (L3) with the percentage of ram sperm progressive motility was  $63.18 \pm 3.61\%$  and viability was  $71.76 \pm 2.32\%$ .

*Keywords: motility, ram semen, semen washing, sperm, soybean lecithin viability*

## INTRODUCTION

The problems of cold shock in sperm preservation can be partly overcome by the use of protective agent in the extender. The popular protective agent used universally for sperm preservation or cryopreservation are glycerol and

lecithin (Salamon and Maxwell, 1995). The main source of lecithin that has been long time used is the egg yolk, but the use of such materials as lecithin source to prevent the effects of cold shock has the risk on microorganisms contamination that jeopardize the spermatozoa and the female reproductive tract (Aku *et al.*, 2007). These

microorganisms are bacteria (Bousseau *et al.*, 1998), especially *Salmonella thyphimurium* (Froning, 1998). Some other weakness of egg yolk as the protectant of spermatozoa are: (a) causing turbidity in the sperm dilution that lead to complicate the observation process under microscope when evaluating the spermatozoa quality (Aku *et al.*, 2007), (b) only effective for the sperm preservation at temperature of 5°C so that it is not optimal in protecting spermatozoa from cooling effect under freezing point, and (c) at high concentrations it can decrease post thawing viability of ram spermatozoa (Fukui *et al.*, 2008). Based on these considerations, it is necessary to develop alternative protectant that has a high quality than lecithin derived from egg yolk.

One of the potential protectant developed in protecting spermatozoa from detrimental preservation effect is soybean lecithin. This material is one of the vegetable lecithin that has most important phospholipids content to protect the spermatozoa membrane in preservation process. Naturally, the lecithin content in soybean is from 1.48 to 3.08%. The main component of soybean lecithin is phospholipid composed of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositols and glycolipids (Shurtleff and Aoyagi, 2007). In the soybean lecithin was not found any microorganisms that can damage the spermatozoa or female reproductive tract (Bousseau *et al.*, 1998).

The effectiveness of using soybean lecithin as component of the ram semen extender to protect spermatozoa on cold shock is hindered by some seminal plasma components that the bulbourethral glands produces. The bulbourethral glands secrete enzyme, called phospholipase A (Chemineau *et al.*, 1991). Phospholipase A in the ram semen (Scott and Dawson, 1968; Roldan and Fragio, 1993) may catalyse the hydrolysis of lecithin in fatty acid and lysolecithin that are toxic for spermatozoa (Chemineau *et al.*, 1991). Particularly in the buck semen, removal of plasma seminal by washing and centrifugation is routine procedure if diluted with extender containing egg yolk lecithin (Evans and Maxwell, 1987; Chemineau *et al.*, 1991). In the ram semen, the effectiveness of removal of seminal plasma in this way before diluted with soybean lecithin was not exists its explanation. The accurate information on the effect of semen washing and soybean lecithin levels to the ram sperm quality is needed.

## MATERIALS AND METHODS

Three mature rams were used as semen donors. Semen was collected by artificial vagina once every three days. The semen volume, semen colors, semen consistency, mass activity, sperm concentration, sperm morphology, sperm motility were measured. Only ejaculates with a concentration of greater than  $250 \times 10^7$  sperm/mL, having >75% progressively motile sperm and <15% of the sperm with abnormal morphology were selected for this research.

### Media Preparation

All chemicals were obtained from Merck, Germany. The Tris extender consisted of 3.634 g Tris (hydroxymethyl) aminomethane, 1.99 g citric acid, and 0.50 g glucose diluted in 100 mL aquabides (Evans and Maxwell, 1987). Its extender is prepared in five tubes then added each as much as 0%, 1%, 2%, 3% and 4% (v/v) soybean lecithin (CENTROL 3 flub, certificate number: TSC 04020, USA), and antibiotics (1,000 IU penicillin and 1 mg streptomycin per mL). A total of five extender were prepared i.e. L0 (100% Tris extender + 0% soybean lecithin), L1 (99% Tris extender + 1% soybean lecithin), L2 (98% Tris extender + 2% soybean lecithin), L3 (97% Tris extender + 3% soybean lecithin) and L4 (96% Tris extender + 4% soybean lecithin). The Krebs-Ringer phosphate glucose solution was prepared for semen washing (Chemineau *et al.*, 1991).

### Semen Preparation, Washing and Dilution

Fresh semen was equally divided into two tubes, both were unwashed (W0) and washed semen (W1). Each semen from both tubes of unwashed and washed semen was equally divided into five tubes, the five tubes of unwashed semen were immediately diluted with extender tested (L0, L1, L2, L3, and L4). The five tubes of washed semen were diluted after semen washing process. The semen were washed with the *Krebs-Ringer phosphate glucose* solution (Chemineau *et al.*, 1991).

### Semen Evaluation

The macroscopic evaluation included semen volume, pH (using Sentron 501 pocket FET®, Netherlands), consistency and color. The microscopic evaluation of semen before and after washed was conducted under microscope 100-400x magnification [using a camera (Optilab-pro

viewer ®)] included sperm concentration using Neubauer counting chamber (Kirkman-Brown and Björndahl, 2009), mass activity and percentage of progressive motility (0 to 100%), viable spermatozoa using eosin-negrosin (Bearden *et al.*, 2004; Toelihere, 1985).

The percentage of motile and viable sperm was daily evaluated (24 hours) for five days of storage. The calculation of sperm motility based on the percentage of progressive motile sperm (Salmin, 2000) using formula:

$$\text{Sperm motility (\%)} = \left[ \frac{\text{Total of sperm observed} - \text{Total of not progressive motile sperm}}{\text{Total of sperm observed}} \right] \times 100\%$$

The criteria of not progressive motile sperm are moving backwards, vibrating in place, moving back, moving in circles and not moving at all or die.

The calculation of viable sperm was based on the percentage of live sperm through differential staining with eosin-negrosin stain (Bearden *et al.*, 2004) using formula:

$$\text{Sperm viability (\%)} = \left[ \frac{\text{Total of live sperm}}{\text{Total of sperm observed}} \right] \times 100\%$$

### Data Analysis

The data were statistically analyzed for differences among the means by two way analysis of variance. The Duncan's Multiple Range Test was used to compare the treatment means using statistical software SPSS 17 version.

## RESULTS AND DISCUSSION

Fresh semen (Table 1) used in this research were found eligible for further processing, especially for dilution and preservation due to its in the normal range recommended by some experts. Macroscopically, the normal standards of ram fresh semen is 0.5 to 2.0 ml in volume (Ax *et al.*, 2000; Rizal and Herdis, 2008), thin to thick creamy in consistency, milky-white or pale cream in color (Toelihere, 1985; Evans and Maxwell, 1987; Ax *et al.*, 2000), 5.9 to 7.3 in pH (Bearden *et al.*, 2004). Microscopically, the normal concentration ranges from 1500-3800 million sperm/mL, percentage of sperm motility and viability were 75.0 to 89.8% and 87.33 to 94.2% (Rizal and Herdis, 2008) and percentage of sperm abnormality were 8 to 10 % (Bearden *et al.*, 2004). The sperm quality after semen washing

(Table 2) was also still in good condition for being further processed. The success of semen washing process have been reported by many researchers with a variety of methods (Somfai *et al.*, 2002; Councel *et al.*, 2004; Lee *et al.*, 2009; Marcus, 2010).

The interaction of semen washing and soybean lecithin levels in this research found no significant differences ( $P > 0.05$ ) on the sperm progressive motility and viability (Table 3 and Table 4) but the single factor of the soybean lecithin level was significantly ( $P < 0.05$ ) affects the motility and viability of the ram sperm for five days of storage at temperature 5°C. The semen washing treatment had no significant effect ( $P > 0.05$ ) on the sperm progressive motility and viability of ram for five days of storage. This phenomenon was indicated that ram semen diluted with soybean lecithin was not necessary being washed to remove the plasma seminal.

The use of soybean lecithin as extender did not give effect that can disrupt the function, motility and viability of ram spermatozoa. This was presumably because the phospholipase A enzyme in the plasma seminal of ram semen can tolerate soybean lecithin levels used in this research, so it did not catalyse the hydrolysis of soybean lecithin in fatty acids and lysolecithin that has caused coagulation of extender medium and toxic for spermatozoa. Most spermatozoa was still survival in motion and activity to maintain its viability in the extender medium enriched with soybean lecithin. This fact similar to the existence of egg yolk as a source of lecithin component in the ram semen extender that does not cause the spermatozoa death due to toxicity and coagulation of the extender medium. This phenomenon is supported by Ritar and Salamon (1982) that the viability of ram sperm was still not change when there is exist or no egg yolk in the ram semen extender. The opinion was also in line with several other researchers who use egg yolk as a source of lecithin component in extender medium of ram semen to against cold shock in preservation and cryopreservation (Herdiawan, 2004; Fukui *et al.*, 2008; Yulnawati and Herdis, 2009). Unlike with the buck semen, the phospholipase A in its plasma seminal can catalyse the hydrolysis of egg yolk lecithin in fatty acids and lysolecithin which in large quantities was causes coagulation of extender medium and toxic for spermatozoa (Chemineau *et al.*, 1991; Rizal *et al.*, 2008). Although in the small levels, i.e. 2.5% egg yolk in the buck

Table 1. The Macroscopic and Microscopic Quality of Ram Fresh Semen

Variables	Group of Ram Semen		
	1	2	3
Volume of semen (mL)	0.80± 0.14	0.82± 0.18	0.83± 0.16
Color of semen	Pale creamy	Pale creamy	Pale creamy
pH of semen	5.99± 0.12	6.01± 0.13	5.99± 0.11
Consistency of semen	Thick creamy	Thick creamy	Thick creamy
Mass activity of spermatozoa	4.22 ± 0.44	4.44 ±0.53	4.33± 0.50
Concentration of spermatozoa (x10 <sup>7</sup> sperm/mL)	297.56±37.45	285.22±25.51	281.67±46.55
Motility of spermatozoa (%)	77.22± 7.95	78.33 ± 9.01	77.78± 7.55
Viability of spermatozoa (%)	89.93± 1.64	90.38± 1.78	90.03± 1.43
Abnormality of spermatozoa (%)	8.63± 1.47	9.68± 1.77	9.35± 1.44

Table 2. The Quality of Ram Washed Semen

Variables	Group of Washed Ram Semen		
	1	2	3
Concentration of spermatozoa (x10 <sup>7</sup> sperm/mL)	295.56±42.06	280.89±37.99	280.11±41.62
Motility of spermatozoa (%)	75.00± 3.54	77.22± 3.63	75.56± 4.64
Viability of spermatozoa (%)	89.07± 1.30	89.60± 1.23	88.59± 1.61
Abnormality of spermatozoa (%)	9.62± 1.21	9.74± 1.13	9.55± 1.26

plasma seminal has most negatively affect on spermatozoa (Ritar and Salamon, 1982) so that it needs to be washed firstly (Evans and Maxwell, 1987; Chemineau *et al.*, 1991). The ram semen diluted with egg yolk or other lecithin sources include soybean lecithin was not recommended for being washed. In contrast, the ram plasma seminal is often used by many researchers as substitution of the buck plasma seminal that has been removed to avoid toxicity and coagulation (Ritar and Salamon, 1982; Rizal *et al.*, 2008; Souhoka *et al.*, 2009; Ari and Daskin, 2010).

The positive effects of soybean lecithin in the ram semen extender appeared from the data trend of ram sperm motility and viability for five days of storage at temperature 5°C (Table 3 and Table 4). This study indicated that 3% soybean lecithin level in extender was optimal level to increase the progressive motility (63.18 ± 3.61%) and viability (71.76±2.32%) of ram sperm for five days of storage at 5°C.

The high of progressive motility and

viability of ram sperm at the 3% soybean lecithin levels (L3) was presumed because at the levels of the soybean lecithin are relatively effective to protect sperm membrane against cold shock at temperature 5°C. The motility and viability was maintained, mortality can be reduced, morphology undisturbed and sperm membrane remained intact (data not shown). At these levels, the spermatozoa avoided from bad influences of temperature refrigeration 5°C in the form of cold shock. The sperm are experiencing cold shock resulted in curled tail, swirling motion and backward motion so disturbing and decreasing its progressive motility (Evans and Maxwell, 1987). In addition, the cold shock also resulted in disruption of function and cell membrane structure which causes the sperma death (Souhoka *et al.*, 2009; Rizal, 2006). In morphology, spermatozoa experiences cold shock was characterized by coiled tail or curled tail which became one of parameters of the sperm abnormality. The shape of sperm tail is like coiled

Table 3. The Ram Sperm Progressive Motility Based on Semen Washing and Soyben Lecithin Level for Five Days Stored at 5°C

Semen Washing (W)	Soybean Lecithin Level (L)					Means of Semen Washing (W)
	L0	L1	L2	L3	L4	
W0	52.22 ± 6.37	58.46 ± 4.66	60.93 ± 5.69	63.16 ± 4.10	54.92 ± 7.63	57.94 ± 6.98
W1	51.04 ± 8.07	58.46 ± 5.47	60.97 ± 3.76	63.21 ± 3.13	54.98 ± 5.83	57.73 ± 6.96
Means of Soy Lecithin (L)	51.63 ± 8.18 <sup>a</sup>	58.46 ± 5.03 <sup>b</sup>	60.95 ± 4.78 <sup>c</sup>	63.18 ± 3.61 <sup>d</sup>	54.95 ± 6.72 <sup>e</sup>	57.83 ± 6.96

W0=Unwashed semen; W1=Washed semen; L0=0% soybean lecithin; L1=1% soybean lecithin; L2=2% soybean lecithin; L3=3% soybean lecithin; L4=4% soybean lecithin.

a, b, c, d, e = Different superskript in the same row indicates significantly different (p<0.05)

Table 4. The Ram Sperm Viability Based on Semen Washing and Soyben Lecithin Level for Five Days Stored at 5°C

Semen Washing (W)	Soybean Lecithin Level (L)					Means of Semen Washing (W)
	L0	L1	L2	L3	L4	
W0	70.62±1.88	71.61±2.89	71.94±2.23	72.28±2.36	72.04±2.12	71.70±2.36
W1	70.64±2.10	71.57±2.43	71.85±2.47	72.12±2.14	71.48±2.52	71.53±2.36
Means of soy lecithin (L)	70.63±1.97 <sup>a</sup>	71.59±2.64 <sup>b</sup>	71.89±2.47 <sup>b</sup>	72.2±2.23 <sup>c</sup>	71.76±2.32 <sup>bc</sup>	71.61±2.35

Explanation is similar to Table 3

or curled tail were categorized as secondary abnormalities (Partodihardjo, 1992). Most abnormal spermatozoa in the part of tail cannot motile (Bearden *et al.*, 2004).

### CONCLUSION

Based on this research results it was concluded that use of soybean lecithin as extender does not give effect that can disrupt the motility and viability of ram sperm. The ram semen diluted with soybean lecithin was not necessary washed to remove the plasma seminal. The suitable extender for the best quality of ram semen stored at 5°C was 3% soybean lecithin as protectant agent.

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