# The Effects of Inclusion of Exogenous Phospholipid in Tris-Diluent Containing a Different Level of Egg Yolk on the Viability of Bull Spermatozoa

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**ABSTRAK**

SITUMORANG, P**.** 2002. Pengaruh penambahan eksogenous phospholipid ke dalam pengencer Tris dengan tingkat kuning telur yang berbeda pada daya hidup spermatozoa sapi. *JITV* 7(3): 181-187.

Penelitian ini dilakukan untuk mengevaluasi pengaruh penambahan phospholipid ke dalam pengencer Tris yang mengandung tingkat kuning telur yang berbeda, terhadap daya hidup spermatozoa sapi setelah pendinginan dan pembekuan. Semen ditampung dengan menggunakan vagina buatan, diencerkan pada pengencer Tris untuk mendapatkan konsentrasi spermatozoa 100 x $10^6$ spermatozoa/ml. Semen encer didinginkan ke $5^0$C selama 45-60 menit, ekuilibrasi selam 4 jam dan dibekukan dengan meletakkan straw 5 cm di atas permukaan nitrogen cair selama 10 menit. Rancangan penelitian adalah faktorial dengan dua tingkat phospholipid (0 dan 0,5 mM) dan 4 tingkat kuning telur (0, 5, 10 dan 20% v/v). Daya hidup spermatozoa dinilai dengan mengevaluasi persentase motil, hidup dan kondisi dari *apical ridge* setelah semen didinginkan ke $5^0$C, disimpan pada suhu tersebut selama 3 dan 7 hari dan setelah dicairkan kembali. Penambahan phospholipid pada pengencer Tris dengan berbagai tingkat kuning telur nyata meningkatkan daya hidup spermatozoa sapi baik setelah pendinginan maupun setelah pembekuan. Rataan persentase motil, hidup dan *intact apical ridge* setelah penyimpanan selama 3 dan 7 hari pada suhu $5^0$C nyata lebih tinggi (P<0,05) pada pengencer dengan 0,5 mM phospholipid dibandingkan dengan tanpa phospholipids. Setelah pencairan kembali, rataan persentase motil, hidup dan *intact apical ridge* pada perlakuan 0,5 mM phospholipid (49,9; 60,2 dan 60,0) nyata lebih tinggi (P<0,05) dibandingkan dengan tanpa phospholipid (39,1; 54,1 dan 51,5). Tidak didapat pengaruh interaksi yang nyata antara phospholipid dan tingkat kuning telur terhadap daya hidup spermatozoa baik setelah pendinginan maupun pembekuan. Pengaruh tingkat kuning telur nyata mempengaruhi daya hidup spermatozoa dimana tingkat yang optimal untuk semen dingin ($5^0$C) adalah 10% v/v dan 20% v/v untuk semen beku. Dapat disimpulkan perlindungan terbaik spermatozoa sapi terhadap pengaruh buruk pendinginan dan pembekuan didapat dari pengencer Tris dengan 0,5 mM *phosphatidyl choline* dan 10 atau 20% kuning telur.

**Kata kunci**: Spermatozoa, daya hidup, phospholipid, kuning telur

**ABSTRACT**

SITUMORANG, P. 2002. The effects of inclusion of exogenous phospholipid in Tris diluent with different level of egg yolk on the viability of bull spermatozoa. *JITV* 7(3): 181-187.

This study has been conducted to evaluate the effect of inclusion of phospholipid in Tris-diluent containing different level of egg yolk on the viability of bull spermatozoa after chilling and freezing. Semen was collected by means of artificial vagina, diluted in Tris-diluent to get a final concentration 100 x $10^6$ spermatozoa/ml. Diluted semen was cooled to $5^0$C for 45-60 minute, equiliberated in those temperature for 4 hours and frozen by placing the straw 5 cm above surface of liquid nitrogen for 10 minutes. The experiments was factorial designed with two level of phospholipid (0 and 0.5 mm) and 4 concentration of egg yolk (0, 5, 10 and 20% v/v). The viability of spermatozoa was assessed by evaluating the percentage of motile, live and condition of apical ridge after the temperature reduced to $5^0$C, stored at those temperature for 3 and 7 days and after thawing. Inclusion of phospholipid and level of egg yolk in Tris diluent significantly increased the viability of spermatozoa for both chilling and deep-freezing. The mean percentage of motile, live and intact apical ridge for 3 and 7 days of storage times at $5^0$C, were significantly higher (P<0.05) in diluent containing phospholipid than without phospholipid. After thawing, the mean percentage motile, live and intact apical ridges were significantly higher (P<0.05) in a diluent containing phospholipid (49.9; 60.2 and 60.0) than those without phospholipid (39.1; 54.1 and 51.5). The effects of interaction between phospholipid and level of egg yolk on the viability of spermatozoa was not significant for both chilling and freezing. Level of egg yolk significantly (P<0.05) affected the viability of spermatozoa where the optimal level of egg yolk was 10% v/v for chilled semen and the higher level (20% v/v) was needed for frozen semen. In conclusion, Tris diluent containing 0,5 mM phosphatidyl coline with 10 or 20% egg yolk gave a best protection for chilled and frozen semen respectively.

**Key words**: Spermatozoa, viability, phospholipid, egg yolk

## INTRODUCTION

Technology of artificial insemination (AI) has been widely used particularly in dairy cattle to increase and improve the genetic quality of their stock using a semen from bulls of high genetic merit and the selective rearing of calves of high breeding merits as replacements. The preservation of semen is though to be very necessary since the viability of sperm is very limited post-ejaculate. Frozen semen technology makes the sperm remain viable for long periods and can be used in a different place or time from originate place of collection. Nevertheless, frozen semen is not without problems because a substantial portion of bull sperm are killed during freezing procedure and sperm that have been frozen do not survive after thawing as long as those that have been cooled to $5^0$C or fresh semen. GOLDMAN *et al.* (1991) showed that more than 30% of spermatozoa were killed during freezing and majority of those survive after thawing were sensitive to environment and have a low fertility (PARKS and GRAHAM, 1992 ). Low fertility of post-thawed sperm has a correlative with the damages of the membranes during freezing. Loss of viability of sperm after cold shock or freezing undoubtedly involves disruption of the sperm membranes. As membranes components, phospholipid and cholesterol play an important role in maintaining the structural integrity of the various highly organized membrane system (PARKS and EHRENWALD, 1990; WHITE, 1993). There is substantial loss of total phospholipid when bull, ram and boar spermatozoa are cooled, and even greater loss after freezing and thawing (JOHNSON and PURSEL, 1972; DARIN-BENNETT and WHITE, 1977; SIMPSON *et al*., 1986). The nature of these phospholipid has been determined for a number of sperm, including ram (EVANS and SETCHELL, 1979b), bull (PURSEL and GRAHAM, 1967), boar (GROGAN *et al*., 1966; EVANS and SETCHELL, 1976a) and human (WHITE *et al*., 1976). The major components are phosphatidyl choline (lecithin), phosphatidyl ethanolamine, phosphatidyl serine, choline plasmalogen, ethanol plasmalogen, sphingomeyline and cardiolipin. In protecting the sperm from the effects of cooling and freezing, the semen was generally diluted in extender containing protective substrates such as cryoprotectants and macromolecules. Egg yolk has been included in diluents for protection spermatozoa against cold shock and the fraction of egg yolk which is responsible for this protection was the low density protein (WATSON and MARTIN, 1975). Although the mechanism of protection was not very clear however its phospholipid is thought have a significant contribution for those protection (PACE and GRAHAM, 1974). A comparable results was reported by many research workers who found that phospholipids of egg yolk was able to protect spermatozoa from the bed influence of cooling and freezing (WATSON and MARTIN, 1975; QUINN *et al*., 1980; WATSON, 1981). SIMPSON *et al*. (1987) evaluate the relation between phospholipid in egg yolk and calcium uptake, motility, oxygen consumption and structure of membranes of ram and boar sperm that has been previously given a cold shock treatment. Furthermore, the protective ability of phospholipid was affected by the composition of lipid and the mechanism of protection was still not clear (PARKS *et al*., 1981; GRAHAM and FOOTE, 1987). Since all previous study not yet explore the effects of phospholipids and egg yolk in the same time on the ejaculate bull semen, therefore this present study will investigate the effects of inclusion of phospholipids in Tris-citrate diluents containing different level of EY on the survivability of bull spermatozoa.

## MATERIALS AND METHODS

The study conducted at Indonesian Research Institute for Animal Production (IRIAP) Ciawi from April to December 2000, using three of 3-4 years old dairy bull, with body weight 500-600 kg. Bulls were individually kept in 2x3 meter penned in all day and there was no physical exercise except when bringing those bulls from animal pens to site of semen collection. Elephant grass and drinking water was offered *ad lib.* and 8 kg commercial concentrate (16% crude protein) was given as supplementation.

Semen was collected by means of artificial vagina (AV), twice a week using cow as a teaser. Immediately after collection the semen was transferred from site of collection to laboratory for within 15 minutes, and the quality of semen was evaluated by observing a volume, concentration, motility, percentage of motile, percentage of live and condition of apical ridge. Semen with a good quality was used for experiment. The experiment was a 2 x 4 factorial design with two level of phosphatydilcholine (Sigma, P 0579) 0 and 0.5 mM and four levels of egg yolk (EY) 0, 5; 10 and 20% v/v.

Briefly after evaluation, semen was diluted in Tris-Citrate diluent part A and the composition of these extender is shown in Table 1, containing 0.5; 10 and 20% v/v egg yolk with and without phospholipid to get concentration of spermatozoa 200 x $10^6$/ml. The diluted semen was then cooled from $35^0$C to $5^0$C using a cooling machine for 45-60 minutes. During cooling, the extender part B which containing the respective treatments was added in three times after the temperatures has been reduced to 15, 10 and $5^0$C and the total volume of extender part B was similar as total volume extender part A to get a final concentration of spermatozoa 100 x $10^6$/ml. The semen samples then divided into 2 sub-sample, which is the first sample was stored at $5^0$C and the second sample was equilibrated at $5^0$C for 4 hour and filled into straw following a feezing.

Freezing was performed by placing the rack containing straws 5 cm above surface of liquid nitrogen for 10 minutes.

The parameter recorded was the survivability of spermatozoa by evaluating the percentage of motile, live and condition of apical-ridge immediately after the temperature reduced to $5^0$C, stored at $5^0$C for 3 and 7 days and after thawing at $35^0$C for 30 seconds. All data obtained were analyzed according to STEEL and TORRIE (1991).

## RESULT AND DISCUSSION

The characteristics of semen collected in this experiments was normal and the means of volume, concentration, %M, %L/D and intact apical ridge sperm (% IAR) were 8.40 $\pm$ 1.67 ml, 1.60 $\pm$ 0.16 x $10^9$; 78.5 $\pm$ 4.5; 80.6 $\pm$ 4.7 and 80.7 $\pm$ 5.3. This is in agreement with the results reported earlier by HAFEZ (1987) who found that the volume and concentration of spermatozoa per-ejaculates was 5-10 ml and 1-2 x $10^9$.

The effect of phospholipid and level of egg yolk on the viability and the condition of apical ridge of sperm after the temperature had been reduced to $5^0$C and stored at $5^0$C for 3 and 7 days are shown in Tables 2 and 3.

There was a tendency that inclusion of phospholipid in Tris diluent containing a different level of egg yolk, improve the viability of spermatozoa immediately after reduction of temperature to $5^0$C but this improvement was not significant.

**Table 1.** The composition of Tris-Citrate diluent

| Ingredients | Part A | Part B |
|---|---|---|
| Tris (Hydroxymethyl) amino methane, g | 2.422 | 2.422 |
| Citric acid, g | 1.34 | 1.34 |
| Fructose, g | 1.0 | 1.0 |
| Streptomycin, mg/ml | 1000 | 1000 |
| Benzylpenicillin, IU /ml | 500 | 500 |
| Glycerol, % (v/v) | 2.4 | 12.4 |
| Egg Yolk, % (v/v) | 0; 5; 10; 20 | 0; 5; 10; 20 |
| Phospholipid, mM | 0; 0.5 | 0; 0.5 |

**Table 2.** The effects of phospholipid, level of egg yolk on the percentage of motile and live sperm after $5^0$C and stored at this temperature for 3 and 7 days

| Time of evaluation | Viability | Phospholipid (mM) | Egg yolk (%) | | | | Mean |
|---|---|---|---|---|---|---|---|
| | | | 0 | 5 | 10 | 20 | |
| $5^0$C | % M | 0.0 | 38.3 | 62.3 | 67.8 | 68.0 | 59.1 |
| | | 0.5 | 40.0 | 65.8 | 68.5 | 72.0 | 61.6 |
| | Mean | | $39.2^a$ | $64.0^b$ | $68.2^b$ | $70.0^b$ | |
| | % L/D | 0.0 | 45.0 | 67.5 | 72.5 | 72.5 | 64.4 |
| | | 0.5 | 47.0 | 72.0 | 75.5 | 77.5 | 68.0 |
| | Mean | | $46.0^a$ | $69.8^b$ | $74.0^b$ | $75.0^b$ | |
| Day 3 | % M | 0.0 | 25.0 | 37.5 | 40.0 | 36.5 | $34.8^a$ |
| | | 0.5 | 27.0 | 41.0 | 42.0 | 40.5 | $38.6^b$ |
| | Mean | | $26.0^a$ | $38.3^b$ | $40.0^b$ | $38.5^b$ | |
| | % L/D | 0.0 | 30.8 | 52.0 | 55.0 | 52.0 | $48.4^a$ |
| | | 0.5 | 32.5 | 61.5 | 60.0 | 59.7 | $53.9^b$ |
| | Mean | | $31.7^a$ | $56.8^b$ | $57.5^b$ | $55.4^b$ | |
| Day 7 | % M | 0.0 | 15.0 | 21.3 | 26.3 | 25.3 | $21.5^a$ |
| | | 0.5 | 19.0 | 25.3 | 30.0 | 30.5 | $29.3^b$ |
| | Mean | | $17.0^a$ | $23.3^b$ | $28.2^b$ | $27.9^b$ | |
| | % L/D | 0.0 | 26.3 | 38.0 | 42.5 | 38.3 | $36.3^a$ |
| | | 0.5 | 30.0 | 42.0 | 46.0 | 47.3 | $43.3^b$ |
| | Mean | | $18.2^a$ | $30.0^b$ | $34.3^b$ | $32.8^b$ | |

%M: Percentage of motile sperm %L/D: Percentage of live sperm
Values with different superscripts within rows or columns shows a significant value ($P<0.05$)

**Table 3.** The effects of phospholipid, level of egg yolk on the condition of apical ridges of spermatozoa after $5^0$C stored at this temperature for 3 and 7 days

| Time of evaluation | Viability | Phospholipid (mM) | Egg yolk (%) | | | | Mean |
|---|---|---|---|---|---|---|---|
| | | | 0 | 5 | 10 | 20 | |
| $5^0$C | % IAR | 0.0 | 37.9 | 58.8 | 62.1 | 56.9 | 54.5 |
| | | 0.5 | 36.8 | 60.0 | 63.9 | 60.9 | 56.9 |
| | | Mean | $37.4^a$ | $59.4^b$ | $63.5^b$ | $58.9^b$ | |
| | % BAR | 0.0 | 47.1 | 21.0 | 17.6 | 19.1 | 25.2 |
| | | 0.5 | 46.1 | 14.5 | 14.6 | 17.8 | 23.8 |
| | | Mean | $46.6^a$ | $17.8^b$ | $16.1^b$ | $18.5^b$ | |
| Day 3 | % IAR | 0.0 | 32.1 | 35.0 | 39.5 | 33.5 | $35.8^a$ |
| | | 0.5 | 32.8 | 39.0 | 44.7 | 39.3 | $41.7^b$ |
| | | Mean | $32.5^a$ | $37.0^b$ | $42.2^b$ | $36.4^b$ | |
| | % BAR | 0.0 | 58.8 | 51.3 | 47.0 | 46.3 | 50.9 |
| | | 0.5 | 58.0 | 42.7 | 42.7 | 43.3 | 46.7 |
| | | Mean | $58.5^a$ | $47.0^b$ | $44.9^b$ | $44.8^b$ | |
| Day 7 | % IAR | 0.0 | 28.9 | 30.2 | 30.6 | 28.2 | $29.5^a$ |
| | | 0.5 | 29.8 | 34.5 | 37.9 | 35.1 | $35.8^b$ |
| | | Mean | $28.4^a$ | $32.4^b$ | $34.2^b$ | $31.7^b$ | |
| | % BAR | 0.0 | 65.4 | 60.2 | 57.1 | 60.1 | 60.7 |
| | | 0.5 | 66.7 | 57.8 | 53.2 | 56.5 | 56.1 |
| | | Mean | $66.1^a$ | $59.0^b$ | $55.2^b$ | $58.4^b$ | |

% IAR: Percentage of *intac apical-ridge*; % BAR: Percentage of *broken apical ridge*
Values with different superscripts within rows or columns shows a significant value ($P<0.05$)

**Table 4**. The effects of phospholipid, level of egg yolk on the survivability of spermatozoa following thawing at $35^0$C for 30 seconds

| | Phospholipid (mM) | Egg yolk (%) | | | | Mean |
|---|---|---|---|---|---|---|
| | | 0 | 5 | 10 | 20 | |
| % M | 0.0 | 16.3 | 42.5 | 45.0 | 52.5 | $39.1^a$ |
| | 0.5 | 20.0 | 52.5 | 56.5 | 66.7 | $49.9^b$ |
| | Mean | $18.1^a$ | $47.5^b$ | $50.8^b$ | $59.6^c$ | |
| % L/D | 0.0 | 22.5 | 50.8 | 65.8 | 77.3 | $54.1^a$ |
| | 0.5 | 32.5 | 55.0 | 70.0 | 82.5 | $60.2^b$ |
| | Mean | $27.5^a$ | $52.9^b$ | $67.9^c$ | $80.0^d$ | |
| % IAR | 0.0 | 34.0 | 48.3 | 59.3 | 64.3 | $51.5^a$ |
| | 0.5 | 38.3 | 53.7 | 64.7 | 72.3 | $60.0^b$ |
| | Mean | $36.2^a$ | $51.0^b$ | $56.0^c$ | $68.3^d$ | |
| % BAR | 0.0 | 59.3 | 42.7 | 39.7 | 33.3 | $43.8^a$ |
| | 0.5 | 52.7 | 40.3 | 34.3 | 27.0 | $36.6^b$ |
| | Mean | $56.0^a$ | $42.0^b$ | $37.0^c$ | $30.2^d$ | |

% M: Percentage of motile sperm; % L/D: Percentage of live sperm; % IAR: Percentage of intact apical ridges
% BAR: Percentage of broken apical ridge
Values with different superscripts within rows and columns shows a significant value ($P<0.05$)

The discernible effects of phospholipid was found after storage at $5^0$C for 3 and 7 days, where the % M and % L/D spermatozoa was significantly higher ($P<0.05$) in diluent containing 0.5 mM phospholipid than without phospholipid. This results is in agreement to results those reported by previous workers who found that addition of phospholipids, particularly those present in egg yolk, protect spermatozoa as judged by observations of motility (WATSON and MARTIN, 1975; QUINN *et al*., 1980; WATSON, 1981). SIMPSON *et al*. (1986; 1987) have examined the extent to which the addition of egg yolk phosphatidylcholine to the medium maintains the normal calcium flux as well as motility, oxygen uptake and ultra structural integrity of ram and boar sperm on cold shocking. Although the effects of phospholipid was not significant immediately after reducing the temperature to $5^0$C, however, it appears the phospholipid somehow, have already interact in membranes of spermatozoa to make the respectively spermatozoa more fit to the following treatment such as storage time or freezing. Although the precise mode action of phospholipid in protecting sperm against cold shock is not yet clear, it is generally believed that they must interact with membranes to stabilize them (WATSON, 1976; 1979; 1981; QUINN *et al.,* 1980). The study of SIMPSON *et al*. (1986) suggest that phosphatidylcholine is bound to membranes, because its beneficial action persisted after most of the phospholipid had been removed from the sperm medium. Those founding supports the result we found in this presents study where the percentage of spermatozoa with intact apical ridge was significantly higher ($P<0.05$) in diluent containing phospholipid following storage at $5^0$C for 3 and 7 days. As the consequence the percentage of spermatozoa with broken apical ridge was consistent lower in diluent containing phospholipid although this different was not significant. The ability of phospholipid in protecting spermatozoa from the harmful effect of freezing was more pronounced (Table 4). These protecting effects was detected immediately after thawing where the percentage of motile, live spermatozoa was significantly higher ($P<0.05$) in diluents containing phospholipid. The good effects of phospholipid was also detected on the condition of membrane where the percentage of spermatozoa with intact apical ridges was significantly higher ($P<0.05$) and therefore in contrast the percentage of spermatozoa with broken apical ridge was significantly lower ($P<0.05$). Against the explanation can be drawn from this results, the phospholipid had been interacted to membranes of spermatozoa, stabilize and maintain the fluidity of its lipid contents and become less susceptible to freezing treatments. Transmission electron microscope studies showed that cold shock or freezing inflicted severe structural damage on ram and bull semen (SITUMORANG, 1983; 1985), however the sperm that were exposed to phosphatidylcholine before cold shock were well preserved (SIMPSON *et al.,* 1986).

Level of egg yolk significantly affect ($P<0.05$) the viability and the condition of apical ridge of spermatozoa immediately after reduction of temperature to $5^0$C or after storage at $5^0$C for 3 and 7 days. Percentage of motile, live sperm and spermatozoa with intact apical ridge was significantly higher ($P<0.05$) in diluents containing egg yolk than without egg yolk. This results is in agreement to many results have been reported earlier. Firstly, egg yolk has been included in diluents for preservation of bull sperm (PHILIPS and LARDY, 1940). Since that time, many workers reported of the beneficial effects of egg yolk as the components of diluents for ram and bull sperm (DAVIS *et al*, 1963; FOOTE, 1970; SHANNON *et al.*, 1984; JONES and MARTIN, 1973, PACE and GRAHAM, 1974; WATSON and MARTIN, 1976). The optimal level of egg yolk for protecting sperm has been varied from species to species. Fraction of egg yolk which function to preserve spermatozoa from the harmful effect of cooling and freezing was lipoprotein and phospholipid. (WATSON and MARTIN, 1975; SITUMORANG, 1985; SIMPSON *et al*., 1986). Although there were no significant differences observed between level of egg yolk from 5 to 20%, however there was a tendency that level of egg yolk 10% to be an optimum level. Addition of egg yolk to 20% seem to reduce the viability of chilled semen where the percentage of motile and live sperm was lower than those 10% but this difference was not statistically different. This results is in consistent to the earlier worker who found that egg yolk contains both inhibitory (toxic) and protective "conserving" substances. WATSON and MARTIN (1976) showed that fertility of ram semen diluted in a buffered -glucose saline decreased when the level of egg yolk was increased from 1.5% v/v to 6% v/v. The effects of level of egg yolk on the survivability of spermatozoa is shown in Table 4. The egg yolk significantly improved the survivability of spermatozoa where the percentage of motile and live sperm significantly higher ($P<0.05$) by increasing the level of egg yolk from 0 to 20%. Different from chilled spermatozoa, the optimum level of egg yolk in protecting spermatozoa during freezing was found higher (20%). The percentage motile and live sperm was significantly higher ($P<0.05$) in diluents containing 20% than 10% or 5% of egg yolk. The condition of apical ridge was correlated with the percentage of motile and live spermatozoa, where the percentage of spermatozoa with intact apical ridge was significantly increased by increasing the level of egg yolk and an optimum level of egg yolk was 20%, but in contrast, the percentage of spermatozoa with broken apical ridge was significantly reduced. A comparable results reported by SITUMORANG (1985) for ram semen

where increasing level of egg yolk from 10 to 15% v/v in the diluents did not give an additional benefit for chilled semen, but for deep frozen semen 15% egg yolk was superior in protecting percentage of motile and morphology of ram spermatozoa.

The effects of the interaction between phospholipid and level of egg yolk, on the percentage of motile, live and condition of apical ridges spermatozoa following chilling, freezing or after stored at $5^0$C for 3 and 7 days was not significant. This is in consistent that phosphatidylcoline we added to diluents was also found as the substances in egg yolk itself. The failure of addition of 0.5 mM phospholipid did not protect spermatozoa as there was no significant different in percentage motile, live and condition of acrosome as judged by condition of apical ridges from without phospholipid can be drawn in two possibility of explanation. Firstly the concentration of phospholipid was not sufficient, and these in a comparable with study by SIMPSON *et al*. (1986) who found that the optimal concentration of phosphatidylcholine to be 2.0 mg/ml and the higher concentration needed for freezing. Secondly, although egg yolk has been included in synthetic diluents for protection against cold shock which is provided by its phospholipid, however the maximum protection obtained by a combination with protein (WATSON and MARTIN, 1976). SITUMORANG (1985) showed that the protective activity of egg yolk was associated with having high molecular weight and these substances were soluble in water and therefore, the substances must be related to protein. The evidence that phospholipids and egg yolk were needed to give a maximum protection is supported by the present study, where a significant benefit was found when the phopholipid was included in diluents containing a different level of egg yolk.

## CONCLUSION

Inclusion of 0.5 mM phospholipid, phosphatidylcholine in Tris diluents containing different level of egg yolk for both chilling and freezing significantly increased the percentage of motile, live and intact apical ridge of bull spermatozoa. The better protection observed, assessed by percentage of motile, live and intact apical ridges after both the temperature reduced to $5^0$C and thawing was given when the Tris diluents containing egg yolk. The best survival obtained after thawing was given when the level of egg yolk was 20% v/v. However, for chilled semen only a lower level of egg yolk of about 10% v/v was needed. There was no significant interaction between phospholipid and level of egg yolk, and the best protection obtained, when the Tris diluent containing 0.5 mM phospholipid and 10% egg yolk v/v for chilling and 20% egg yolk v/v for freezing.

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