

## THE EFFECT OF EXTENDERS AND GLYCEROL LEVEL TO THE QUALITY OF AFTER FREEZING PRIANGAN RAM SPERM

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### Abstract

A study on the effect of five extenders (Whole Milk, Skim Milk, Egg Yolk Citrate and Egg Yolk Tris) and three different level of glycerol (7.5 %, 10 %, and 12.5 %) has been conducted at Animal Reproduction Laboratory, Faculty of Animal Husbandry, Padjadjaran University, Jatinangor. The objectives of this study were to find out the effect of semen extenders, glycerol level, and the combination of both to the semen quality of after freezing of Priangan ram sperm. This study used five - 1.5 years old - of Priangan rams. They were kept in individual pen and fed by field grass and dairy cattle concentrate. Semen were collected by artificial vagina and evaluated macroscopic and microscopically. Semen was extended in two stages, and glycerol was added gradually in 5° C. Semen was frozen manually in liquid nitrogen on – 196° C. Evaluation of after thawed semen quality has been done 24 hours later. The evaluated variables were sperm motility and sperm abnormality. The study used Completely Randomised Design with 5 x 3 factorial pattern. The collected data were analyzed with Analysis of Variance and followed by Duncan's New Multiple-Range Test. The result of this study shows that factors of extenders, glycerol level, and the combination of both are highly significant ( $P < 0.01$ ) affect the quality of after freezing Priangan ram semen. The combination between Egg Yolk Tris and 7.5 % or 10 % of glycerol level is the best semen extender on maintaining the quality (sperm motility and sperm abnormality) of after freezing Priangan ram semen.

Key words: Semen, sperm, Priangan ram, Semen extender, Glycerol level

### Introduction

Priangan sheep is a indigenous plasma (*plasma nutfah*) which has good potency to be bred in other region in Indonesia. This livestock is not only producing meat but it can also as fancy ram or fighting ram (*domba aduan*), which can support the tourism sector.

The productivity and ability of priangan sheep's bio-reproduction must be keeping increasing since the genetic quality of this sheep is getting decreased. It can be seen from the weight of male Priangan sheep in the pass could reach 80 – 100 kg

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and adult female was 60 kg. Such condition is now rarely to be found. The effort to get those sheep's performance can be done through the breeding by mating a big strong male sheep with preferable female sheep. However the number of available superior male sheep is very limited. Since they must copulate with many female sheep to support the increasing of population, so that it is necessary to do an effort to maximize the used of this superior male sheep. The above problem can be solved by an alternative action: artificial insemination. Sheep's artificial insemination is the same as cows: can use liquid or frozen semen. Some researches stated that the live expectancy of sheep's frozen semen is shorter than cow's, so that liquid semen is more used in the field. But according to Evans and Maxwell (1987), frozen semen has the same fertilizing ability as liquid semen if it is inseminated intra uterine. The freezing of sheep's semen is also necessary to conduct since the frozen semen can be stored longer and can solve the problem of place and time's obstacle. The used of dilution semen is to increase volume and to be a buffer which can maintain the pH caused by accumulation of the lactate acid from sperm metabolism, also it is able to supply necessary nutrition and energy for sperm. Diluter, which can be used to dilute sheep's semen, is i.e. full milk, skim milk, yolk, yolk coconut water, and Tris yolk. Normally, during the frozen process, it will occur a big amount of death sperm caused by cold shock and the changes of osmotic (hyper tonic shock). Those two accidents can be solved by increasing yolk, which can cover sperm membrane plasma, and also can add cryoprotectant agent (like glycerol), which can decrease sperm defect caused by the pressure of osmotic solution. The glycerol level used as cryoprotectant agent on the freezing semen is 7.5%, 10% and 12.5%. The aim of this research is to know the influence of dilution type and glycerol level on the Priangan sheep's semen quality after freezing, and also to know the best combination of solution and level of glycerol. The result of this research is expected to give benefit for handling the decreasing of semen quality and quantity, especially on Priangan sheep, which is then can improve fertility of frozen semen. This research is also expected to be a reference for further research.

### **Material and Method**

Semen were collected by artificial vagina and evaluated macroscopic and microscopically. Semen was extended in two stages, and glycerol was added gradually in 5° C. Semen was frozen manually in liquid nitrogen on -196° C. Evaluation of after thawed semen quality has been done 24 hours later. The evaluated variables were sperm motility and sperm abnormality. The study used Completely Randomised Design with 5 x 3 factorial pattern. The collected data were analysed with Analyses of Variance and followed by Duncan's New Multiple-Range Test.

## Result and Discussion

### Fresh semen characteristic

Fresh semen used in this research has macroscopic and microscopic characteristic such as displayed on Table 2.

Table 2. Macroscopic and microscopic characteristic of fresh semen used in this research

| Parameter                                      | Sheep (Number)                   |      |      |      |      | Rates |
|--|----------------------------------|------|------|------|------|-------|
|  | 1                                | 2    | 3    | 4    | 5    |       |
| Volume   | 0.90                             | 1.10 | 1.05 | 0.95 | 1.10 | 1.02  |
| Colour   | Cream                            |      |      |      |      |       |
| Odour  | Specific, mixed with sheep odour |      |      |      |      |       |
| Viscosity                                      | Thick                            |      |      |      |      |       |
| PH   | 6.7                              | 7.1  | 7.0  | 6.7  | 6.8  | 6.89  |
| Sperm concentration (x10 <sup>7</sup> cell/ml) | 294                              | 306  | 297  | 312  | 328  | 307.4 |
| Sperm Motility                                 | 82                               | 88   | 86   | 89   | 85   | 86    |
| Sperm Abnormality                              | 3.7                              | 4.2  | 3.4  | 3.9  | 3.8  | 3.8   |

Based on its' performance of macroscopic and microscopic, sheep's fresh semen in this research is included good semen category and suitable to be further processed, both in the form of liquid or frozen.

### The effect of dilution type and glycerol level, and its interaction on sperm motility of Priangan sheep post freezing

Research data about the effect of dilution semen and glycerol type and its interaction on the rate of Priangan sheep's sperm motility displayed on Table 3.

Nominally, Tris Yolk diluter shows highest sperm motility rate (43.00%) followed in order by yolk extract (40.47%), skim milk (39.22), full milk (38.21%) and coconut water yolk (30.89 %). While the level of glycerol of 7.5% shows sperm motility rate of 39.72%, followed by glycerol level of 10% (35.35%) and glycerol level 12.5% (36%). Statistic analysis showed that type of semen dilution and glycerol level, both interactions give a very significant effect ( $P < 0.01$ ) on Priangan sheep's sperm motility. Test result with Duncan Multiple Range Test stated that Tris Yolk solution make a very significant effect ( $P < 0.01$ ) better than skim milk, full milk and coconut water yolk solution on Priangan sheep' sperm motility.

Table 3. Rate of Priangan sheep's sperm motility post freezing  
The effect of combination treatment of dilution semen type and glycerol level

| Glycerol Level | Dilution Type      |                    |                    |                    |                    |
|----------------|--------------------|--------------------|--------------------|--------------------|--------------------|
|                | SP                 | SS                 | AKT                | SKT                | TKT                |
| 7.5%           | 38.98 <sup>F</sup> | 40.19 <sup>D</sup> | 32.69 <sup>I</sup> | 42.34 <sup>B</sup> | 44.41 <sup>A</sup> |
| 10.0%          | 39.11 <sup>F</sup> | 39.58 <sup>E</sup> | 32.30 <sup>J</sup> | 41.19 <sup>C</sup> | 44.60 <sup>A</sup> |
| 12.5%          | 36.53 <sup>H</sup> | 37.90 <sup>G</sup> | 27.69 <sup>K</sup> | 37.88 <sup>G</sup> | 40.00 <sup>D</sup> |

Note: \*) bold superscript letters indicated very significant different result of level (P<0.01).

SP : Full milk

SKT : Egg Yolk Citrate

SS : Skim milk

TKT : Tris egg yolk

AKT : Egg Yolk Coconut water

The effect of skim milk and full milk solution is also very significant (P<0.01) better than coconut water yolk solution. While skim milk solution make a significant effect (P<0.05) better than full milk diluter. However between Tris Egg Yolk and yolk extract solution, between yolk extract and skim milk, statistically, do not show different effect. Based on Duncan Multiple Range Test on glycerol's role shows that the effect of glycerol level 7.5% and 10% is very significant (P<0.01) better than glycerol level of 12.5%. Tris Egg Yolk solution is a semen diluter containing Tris substance (hidroksimetilaminometan) and citrate acid, which is characterized as a buffer on the changes of semen pH caused by lactate acid produced from sperm metabolism. In addition, Tris Egg Yolk diluter also contains fructose, which will be a ready stock supplement for sperm. The egg yolk extract solution only has one substance, which can maintain pH solution is natrium citrate. While full milk, skim milk and Egg Yolk coconut water are organic diluter, which do not contain substance, which can maintain pH so decreasing pH can be occurred. Those diluter only contain chemical substance which play role in supplying supplement for sperm, either in the form of monosaccharide, disaccharide or trisaccharide. Woodroff (1979) explained that Coconut water has buffer characteristic in acid media, in this research the ability is not able to maintain the sperm motility of Priangan sheep post thawing. Chemical elements containing in Tris Egg Yolk diluter is able to maintain and as a buffer to the sperm motility, both in liquid and frozen semen (Maxwell and Evans, 1987). Those elements play a role in maintaining osmolarity solution, PH solution, and energy supplying for sperm (Djaman Hedah, 1992). Adding egg yolk into semen diluter that will be frozen must be conducted. Fresh yolk contains lecithin and lipoprotein known to be able to protect sperm lipoprotein membrane in cold stress condition (Salisbury and Van Demark, 1985). By such protector, sperm cell is able to minimize the negative effect of environmental cold temperature so that sperm will not be cold shock. Sperm saves from cold shock will be able to face the next frozen process. On semen cryopreservation process, sperm

cells will be preserved by storing in low temperature far from water frozen point. During the process, the change of osmolarity solution will make the water inside the cell force to move outside. Expulsion process of intra cellular water has velocity influenced by cell membrane permeability (of sperm). This process will be occurred on un-perfect membrane permeability cell. This condition will excite electrolyte concentration increasing in cell, the increasing of electrolyte sperm occurred suddenly will poison the sperm. The existing of yolk in semen diluter is able to maintain spermatozoa membrane permeability, therefore, negative effect of frozen process can be minimized. The egg yolk can not work alone, but depend on other materials in the diluter. It can be seen from the low phenomena of Priangan sheep's sperm motility, which is diluted with Egg Yolk Coconut Water. Coconut water is biological solution consisting carbohydrate. The osmolarity, relatively, is not really different from blood plasma. Coconut water does not consist of material that able to maintain solution electrolyte equilibrium. The coconut water only consists of material having a role energy reservation for spermatozoa. The absence of the elements above causes the disturbance of sperm motility due to unsuitable media before lecithin and lipoprotein elements from yolk can work. Glycerol is one of the cryoprotectan agents the most commonly used in biological cell frozen process including spermatozoa. The material can protect spermatozoa against the shock hyper tonic phenomenon. On the process of a decreasing temperature, liquid solution changes to frozen (thick, dry and cold). The solution osmotic pressure on this phase will be changed and intra cellular will be come out, then electrolyte concentration in cell will be higher drastically. The contain water in freeze condition will become ice crystal that can damage sperm cell physically. The existence of glycerol in media can arrange velocity of intra cellular waters came out process so that intra cellular electrolyte concentration change will not be occurred suddenly. In addition, glycerol will come in cell as a substitute of intra cellular water that lost. Intra cellular electrolyte increasing phenomena will not occurred relatively on this condition and sperm cell is avoided from the toxicity of electrolyte (Toelihere, 1985). Beside has the advantage characteristic in semen frozen process, the glycerol has also alcoholic characteristic that can be toxic if over dosages. The increasing of glycerol also should be conducted on low temperature to make sperm cell, as consumption is not in active metabolism condition. The previous researcher used the glycerol in any kind of livestock semen frozen process in range 0 – 14.0 % and good sperm motility after thawing, generally, derived from glycerol increasing about 7 – 8 % (Graham, *et al.*, 1978). In case of sheep semen, glycerol content in semen diluter in range 7.0 to 12 % are dosages often used in some research and optimal dosages are about 7 – 10 % (Fachoerozi, 1997; Maxwell and Evans, 1987)

#### **The influenced of thinner, glycerol content, and interaction to Priangan sheep sperm abnormality after frozen**

The average of Priangan sheep sperm abnormality after frozen caused by combination between diluter types and glycerol content are shown in Table 4.

Table 4 shown that sperm of Priangan sheep frozen semen that diluted by coconut waters egg yolk has the highest abnormality average value (21.72 %) and followed by full milk (17.70 %), skim milk (16.85 %), egg yolk (16.12 %), and Tris egg yolk (14.83 %). Based on cryoprotectan agent, the use of glycerol content 12.5 % in all type of diluter produces the highest Priangan sheep sperm abnormality after thawing (19.92 %), and followed by glycerol content 10 % (16.90 %), and glycerol content 7.5 % (15.52 %).

Table 4. The average of Priangan sheep sperm abnormality after frozen caused by combination between thinner type and glycerol content

| Glycerol content | Type of thinner     |                     |                     |                     |                     |
|------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                  | SP                  | SS                  | AKT                 | SKT                 | TKT                 |
| 7.5 %            | 15.46 <sup>DE</sup> | 15.06 <sup>DE</sup> | 20.81 <sup>BC</sup> | 13.63 <sup>EF</sup> | 12.62 <sup>F</sup>  |
| 10.5 %           | 17.13 <sup>CD</sup> | 16.5 <sup>CD</sup>  | 20.50 <sup>B</sup>  | 15.95 <sup>DE</sup> | 14.41 <sup>DT</sup> |
| 12.5 %           | 20.52 <sup>B</sup>  | 18.98 <sup>BC</sup> | 23.90 <sup>A</sup>  | 18.77 <sup>BC</sup> | 17.45 <sup>CD</sup> |

Remark : \*) The bold superscript letters is the significant different result on level (P<0.01)

SP : Full milk                      SS : Skim milk    SKT : Egg Yolk Citrate  
 TKT : Tris egg yolk              AKT : Egg Yolk Coconut water

Statistically conclude that combination between type of diluter and glycerol content that used in this research showed very strong influenced (P<0.01) to Priangan sheep spermatozoa abnormality after thawing. Duncan multiple range test showed that combination between diluter of Tris egg yolk and egg yolk citrate with glycerol content 7.5 % and 10 % are combinations that produce lower sperm abnormality. Its mean the combination between diluter and glycerol content are able to maintain Priangan sheep frozen semen quality better than the others. Combination between diluter of coconut waters egg yolk and any glycerol content produce higher sperm abnormality. The diluter of Tris egg yolk and egg yolk citrate can be very influenced to sperm motility compare to others diluters and also able to reduce Priangan sheep sperm abnormality in this research. This result shown that chemical composition of Tris diluter has a role in case of maintain sperm life in vitro. Diluter that have chemical element that able to keep osmotic pressure and solution PH will give a possibility to produce the best frozen semen quality. All of diluter that used in this research has chemical content function to prepare energy for sperm. The advantages of Tris egg yolk and egg yolk citrate solution are that they have chemical element more complete than three others solution. Glycerol content 7.5 % and 10.5 % is a better content than glycerol content 12.5 % in case to reduce of increasing of Priangan sheep sperm abnormality presentation. This is a good agreement with some researcher who used glycerol in range 7 – 8 % (Evans and Maxwell, 1987; Toelihere,

1985; Graham. *et al.*, 1978). Observation of sperm abnormality in research is secondary sperm abnormality. Secondary sperm abnormality is sperm abnormality that occurred when sperm leaving a testis, and when handling cement is outside. Primary sperm secondary that occurred along spermatogenesis process is not be concerned because that abnormality will not be influenced by animal ejaculation behaviour (Hafez, 1993; Toelihere, 1985).

### Conclusion

1. Combination of semen diluter (Full Milk, Skim Milk, Egg Yolk Coconut Water, Egg Yolk Citrate and Egg Yolk Tris) and gliserol level used in this research (7,5%, 10% and 12,5%) are significantly affect sperm motility and abnormality of Priangan ram semen after thawing.
2. Diluter of Egg Yolk Tris and Glycerol level of 7,5% and 10% are the best combination to maintain sperm motility and abnormality of Priangan ram semen after thawing, compare to the other diluter and glycerol level.

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