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Developing of Tris Soy Milk Diluent for Frisian Holstein Bull Frozen Semen

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Commercial artificial insemination (AI) companies in the beginning of year 2000, introduced new generation of cryoprotectans with lecithin based diluents such as Biociphos (IMV, L'Aigle, France) and Andromed® (Minitub, Germany). Since the commercial diluents were imported, they were often not readily available. This research aimed to develop Tris-soy modified diluent, and investigated its effects on the quality and fertility of Frisian Holstein (FH) bulls frozen semen. This research consists of two experiments. At first, we compared the FH Bull frozen semen quality in modified tris soy milk (TSM), Andromed® and Tris-eggyolk (TEY) diluents, the second was the fertility trials of the frozen semen. Results of the experiment demostrated that post thawed sperm motility in the semen preserved with TEY (49.10%) or Andromed® (50.21%) was significantly higher (P = 0.037) than that preserved with TSM (41.53%). In contrast, the conception rates in cows inseminated with semen preserved with TSM, TEY, and Andromed® were 53.84% (7/13), 38.88% (5/13), and 38.46% (7/19), respectively. We are optimistic that TSM diluents will have similar qualities as TEY and Andromed® on preserving frozen semen by doing future intensive studies.

Key words: soy milk, bull semen, cryopreservation

INTRODUCTION

Artificial insemination (AI) was the first great biotechnology utilised to improve reproduction and genetics of farm animals. It has given an enormous impact worldwide in many species, particularly in dairy cattle. The widespread use of AI in cattle can partly be attributed to the availability of proper diluents. Egg yolk and milk containing low density lipoproteins have been the most common cryoprotectants to preserve bull semen for many years. In European society the perceived health risk of a biological material in diluents has elicit the efforts to replace egg yolk and milk in cryoprotectants. Since, alternative substances had been used by many researchers, but so far no appropriate subtances have yet been reported (van Wagtendonk-de Leeuw *et al*. 2000).

At the beginning of year 2000, commercial AI companies introduced the new generation of cryoprotectans, i.e. lecithin based diluents (Gill *et al.* 2000; Aires *et al.* 2003). Few available brands include Andromed® (Minitub, Germany) and Biociphos (IMV, L'Aigle, France), both were one-step diluents containing soy bean extract replacing egg yolk as a functional ingredient in cryoprotectants. Since they contain no egg yolk or milk, the issue of biological and bacteriological properties of egg yolk altering the quality of cryopreserved sperms is avoided.

According to Gill *et al.* (2000) and Aires *et al.* (2003) a field trial comparing cryoprotectants with or without egg yolk with sperm numbers of 20 millions/insemination demonstrated similar fertility results. Since the commercial diluents were imported, stocks were often not readily available. The requirement of keeping the diluents at 5 °C often creates a new problem. The problem is due to the temperature change during transportation may affect the quality of the diluents. These facts encourage us to create a more readily, accessible alternative diluents. Soy powder is widely available and can be found in most domestic public markets. Information and ingredients of tris-buffer diluents are widely available as well.

This research aimed to develop Tris soy modified diluents, and to be compared with Tris eggyolk and commercial diluents for their effects on the quality and fertility of frozen semen of Frisian Holstein (FH) bulls. We expect that the Tris soy modified diluent can be used as a proper alternative to preserve bull semen for artificial insemination.

MATERIALS AND METHODS

Sources of Semen. Three healthy (Progeny selected) FH bulls of 3-4 years old belong to Lembang Artificial Insemination Centre were used as sources of semen. All bulls were individually cage, and manage with the standard practices of the centre.

Media Preparation. All chemicals were obtained from Merck, Germany. Three diluents used in this experiment, i.e. modified Tris soy milk (TSM), Andromed®, and Tris egg yolk (TEY). Tris buffer was prepared by mixing 30.28 g Tris-hydroxymethyl-aminomethane with 17.8 g monohydrate citric acid and 12.5 g D-fructose, dissolved in 100 ml

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distilled water (Arifiantini *et al.* 2006). TSM diluent was prepared by homogenizing 2.5 g soy milk (Melliea) in 100 ml Tris buffer (2.5%) using a stirrer. The diluents were centrifuged to collect 94 ml of the supernatant, which then added with 6 ml glycerol (6%). Andromed® diluents was prepared by mixing 1 part Andromed® with 4 parts of distilled water. TEY diluent was composed of 74% Tris buffer, 20% egg yolk, and 6% glycerol (Table 1).

Semen Evaluation and Processing. Raw semen from three FH bulls was collected using artificial vagina, based on Lembang Artificial Insemination Centre standard protocols. Only the first ejaculates of each semen collection were used in the experiment. Following the collection, individual semen sample was macroscopically and microscopically evaluated. The macroscopic evaluations included semen volume, pH, consistency and colour. The microscopic evaluation conducted under microscope 100-400x magnification (Olympus CH 20) included mass activity and percentage of progressive motiliy [0 (not motile) – 100% (100% motile], velocity [1 (very slow) -5 (very fast)], viable sperms using eosin-negrosin (Barth & Oko 1989), sperm number using Neubauer counting chamber (Kirkman-Brown & Björndahl 2009) and percentages of sperms with normal and abnormal morphology (carbolfuchsin-eosin) (Al-Makhzoomi A *et al.* 2008). Semen samples with >70% progressive motile and containing <20% sperms with abnormal morphology were used in the experiment.

After the evaluation, each of raw semen was equally divided into three tubes, and diluted in one of three extender (TSM, Andromed®, TEY) to reach the total semen concentration of 100×10^6 per ml (25×10^6 per straw). The diluted semen was individually packaged in 0.25 ml straws, and equilibrated at 4 °C for 4 hours. The straw was frozen in a Styrofoam box at 5 cm above the liquid nitrogen level for 10 minutes. The frozen semen was stored for 24 hours in liquid nitrogen for further evaluations.

Frozen semen was thawed using warm water (37 °C) for 30 seconds. Semen evaluation was mainly focused on the percentage of sperms with progressive motility and the percentage of viable sperms. Recovery rate of semen samples from each bull, extended with different diluents were recorded according to Hafez (1993).

The *in vivo* fertility trials were performed at Cibungbulang Dairy Farm in Bogor. Forty four cows were inseminated by an experience inseminator with frozen semen diluted in TSM, Andromed®, or TEY 12 hours after the onset of oestrus. Pregnancy was evaluated at two months after the insemination. Conception rate was calculated by dividing the number of pregnant cows with the total number of inseminated cows, and multiplied by 100%.

Statistical Analysis. The data were expressed as means ± SD. All data were statistically analyzed for differences among the means by one way analysis of variance (ANOVA). The Tukeys test was used to compare treatment means using Minitab ver. 14.

Table 1. Frozen semen diluent composition

Ingredient	Diluents		
	TSM	Andromed®	TEY
Tris buffer (ml)	100 (ad)	-	74
Egg yolk (ml)	-	-	20
Soy milk (g)	2.5	-	-
Andromed ® concentrate	-	20	-
Glycerol (ml)	6	-	6
Distilled water (ml)	-	80	-
Antibiotic			
Penicillin (IU)	100,000	100,000	100,000
Streptomycin (mg)	100	100	100

TSM: Tris soy milk, TEY: Tris egg yolk.

RESULTS

The semen volume was 5.0 ± 0.76 ml, creamy white in colour; pH was 6.22 ± 0.05 and moderate to thick in consistency. The mass activity was 2.33 ± 0.58 with the sperm motility was 77.08 ± 4.73%, and the average of individual scoring was 4.42 ± 0.52. The viable sperms were 84.06 ± 4.49% and sperm concentration was 1075.00 ± 313.94×10^6 ml^{-1}. The sperms of all bulls used in the study demonstrated an excellent morphology with the average of normal sperms was 94.93 ± 0 .69%.

The percentage of motile sperm in raw semen, post-dilution and in those post equilibration was similar; while that in the frozen semen preserved with TEY (49.10%) or Andromed® (50.21%) was significantly higher (P = 0.037) than that preserved with TSM (41.53%) (Table 2). Recovery rate (RR) is a number of recovered sperms after thawing. The recovery rates of frozen semen preserved with TEY and Andromed® were 64.04 and 65.49%, respectively; these were higher (P = 0.027) than frozen semen preserved with TSM (54.17%).

The viable sperms were observed on raw and post-thawed semen samples. Significant decreased on viable sperms vere observed in all diluted semen samples. The viable sperms were significantly higher (P = 0.02) in semen preserved with Andromed® (65.06%) or TEY (65.10%) than that preserved with TSM (58.30%) (Table 3).

Table 2. Mean (± SD) percentage of progressive motility of bull frozen using TSM, TEY, and Andromed®

Semen freezing step	Semen diluents		
	TSM	TEY	Andromed®
Raw semen	76.67 ± 4.92a	76.67 ± 4.92a	76.67 ± 4.92a
Post-dilution	76.25 ± 5.28a	76.67 ± 4.92a	76.67 ± 4.92a
Post-equilibration	75.42 ± 4.50a	76.67 ± 4.92a	76.03 ± 4.33a
Post-thawing	41.53 ± 4.92b	49.10 ± 3.16c	50.21 ± 3.89c
Recovery rate (%)	54.17	64.04	65.49

Different letters superscript in the same column and row show significant differences (P < 0.05); TSM: Tris soy milk; TEY: Tris egg yolk.

Table 3. Mean (± SD) percentage of viable sperms of bull frozen using TSM, TEY, and Andromed®

Semen freezing step	Semen diluents		
	TSM	TEY	Andromed®
Raw semen	84.22 ± 3.81a	84.22 ± 3.81a	84.22 ± 3.81a
Post-thawing	58.30 ± 4.14b	65.10 ± 4.26c	65.06 ± 5.86c

Different superscript letters in the same column and row demonstrate significant differences (P < 0.05); TSM: Tris soy milk; TEY: Tris egg yolk.

Forty four cows were inseminated with semen diluted with TSM, TEY or Andromed®. The conception rates of the cows inseminated with semen preserved with the diluents were 53.84% (7/13), 38.88% (5/13), and 38.46% (7/19), respectively.

DISCUSSION

Problems associated by using traditional egg yolk-based extenders, including bacterial or xenobiotic contamination and variability in composition can be avoided by using chemically defined animal protein-free extenders for cryopreservation of semen. In this study, the quality of frozen semen preserved with modified TSM diluents was inferior to that preserved with TEY or Andromed® (soy extract diluents commercial brand; Minitub Germany). In our preliminary study the percentage of viable sperms in liquid semen extended with TSM did not differ with semen extended with TEY. However, in the frozen semen, the percentage of viable sperms in semen preserved with TSM was lower than that preserved with TEY or Andromed®.

The beneficial of using lecithin base extender have been reported by several authors; in bovine (van Wagtendonk 2000; Thun *et al.* 2002; Aires *et al.* 2003; Arifiantini *et al.* 2005, 2006), Buffalo (Bard 2008), human (Reed *et al.* 2009) and ram semen (Gilab *et al.* 2003; Fukui *et al.* 2008). According to Bard (2008), the use of Bioxcell® and the Biociphos® extenders (soy extract diluents commercial brand; IMV, France) in buffalo bull semen significantly increased the post-thaw sperm motility and the viability index compared to semen extended in the TRIS-egg yolk extender. The same result reported by Gilab *et al.* (2003) that in ram frozen semen, the subjective motility evaluation was slightly higher in Bioexcell® than that in the milk extender. In human, no significant differences observed between before and after cryopreservation supplemented with egg yolk or soy lecithin for recovery of sperm motility, sperm cell morphology, maintenance of sperm DNA integrity and the ability of sperms to bind to hyaluronate *in vitro* (Reed *et al.* 2009).

Sperm motility and viability in TSM extender was lower than those in the other extenders. Soy milk (Millilea brand) used in this study, did not contain carbohydrates; while egg yolk contains 0.6% of carbohydrate (Manjunath *et al.* 2002). This might explain the higher sperm motility in semen extended with TEY than that extended with modified soy milk diluents. Both egg yolk and soy milk contain phospholipids, which protect sperms during cold shock.

Viable sperms percentage was lower in frozen semen preserved with TSM than those preserved with Andromed® or TEY. This was presumably associated with centrifugation effects on the soy milk. The effects might not be that remarkable on liquid semen maintained at 4-5 °C. On the other hand, in frozen semen during freezing and thawing the sperm cells must change twice into intermediate zone of temperature (freezing point between 0-60 °C) that extreme temperature might shock the sperms. According to Amann and Graham (1993), cold shock alters normal configuration of sperm plasma membrane to hexagonal configuration which in turn will damage the sperm plasma membrane. When the sperm plasma membrane damage is located at the mid piece, aspartate aminotransferase enzyme (AspAT), which is the main mitochondrial enzyme in ATP production, is released from the cell and enters the seminal plasma. The loss of AspAT interrupts ATP production and disturbs sperm motility (Colenbrander *et al.* 1992).

Although post-thaw sperm motility percentage in semen preserved with TSM was lower than that preserved with TEY or Andromed®, but the conception rate after *in vivo* fertilization of TSM was higher than TEY and Andromed®. These results differ with the field trials conducted in ram fertility rates (Gilab 2003). According to Bard (2008) there were no significant differences detected between the *in vitro* fertilization of buffalo sperms extended with Bioxcell®, Biociphos® or TEY. On the other hand Thun *et al.* (2002) reported that bull semen processing using TEY extender produced the best semen quality and field fertility compare to Biociphos®.

Reproduction success was determined by several factors, including the cow's reproductive health, the inseminator, timing of the insemination, and the quality of frozen semen. Frozen semen preserved with TSM had quite fertile sperms and can be used in artificial insemination.

Further studies are needed to improve the modified soy milk diluents in order to replace egg yolk (an animal product) and to be free from the dependency on foreign commercial brand (Andromed®). Some future studies include an improvement on buffer composition, and carbohydrate supplementation in semen diluents; on frozen semen studies, efforts to find a perfect speed and time of centrifuge needs to be done, in order to preserve phospholipids which protect sperms during freezing.

We are optimistic about modified soy lecithin diluents will have similar qualities with TEY and Andromed® on preserving frozen semen by doing future intensive studies. This plan will contribute to the artificial insemination program, thus indirectly will increase cattle population in Indonesia.

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