THE POTENTIALITY OF USING BULL SEMEN AT ROOM TEMPERATURE AFTER SHORT TERM PRESERVATION IN DIFFERENT EXTENDERS

Mmbaga, A.F.N., R. Nkya * and L.R. Kurwijila Sokoine University of Agriculture, P.O. Box 3000, Morogoro, Tanzania.

SUMMARY

Bull semen quality was evaluated following short term preservation in four extenders and at different ambient temperatures and cold storage. Three extenders were formulated using locally available diluents and were compared to one commercial extender used at the National Artificial Insemination Centre (NAIC), Usa River, Arusha. The four extenders were coconut milk (CME), egg yolk citrate (EYCE), skim milk (SME) and Laisphos®. Extended semen samples were stored at five different temperatures namely deep freezer (-18°C), refrigeration (5°C), and ambient (20-22°C), or incubation (25°C and 27°C) to simulate warmer environments. Semen quality was evaluated for percentage motility and live sperm cells. A 4 x 4 x 5 factorial experiment was used in the study. Extended semen samples from eight dairy bulls were evaluated every 24 hours for 96 hours.

Storage temperature had a highly significant effect on the quality of sperm cells ($P<0.001$). Storage at -18 °C and 27 °C had significantly lower sperm cell quality compared to samples that were stored at 20-22°C, 25°C and 5°C. Refrigeration temperature however, had intermediate values. The type of extender did not have significant effect ($P>0.05$) on spermatozoa quality during the entire storage period at different temperatures. Sperm quality decreased with storage period. Laisphos® had figures comparable to CME and EYCE while SME was the least efficacious in the preservation of spermatozoa viability. Temperatures between 20-25 °C gave the best results compared to the other temperatures under study. The study showed that both CME and EYCE could be used in preservation of room temperature semen for use within 72 hours provided the ambient temperatures did not exceed 25 °C. This was enough to enable the operation of artificial insemination (AI) based on room temperature semen within a small radius area with high population of dairy cows.

INTRODUCTION

Tanzania's large and small scale dairy farms rely on the use of bull alone at the farm/bull centre/neighbour, or centralised AI service run by the government extension service or donor funded dairy development projects, or both for breeding of their cows. Experience in Tanzania has shown that both centralised AI service based on liquid nitrogen frozen semen and bull centres have not worked well, partly because of the high costs, shortage of liquid nitrogen caused by frequent breakdown of nitrogen plants and mismanagement (Mchau et al., 1985). The use of room temperature semen could strengthen implementation of AI at community level, and especially in supplementing frozen semen based AI (Malmberg and Israelsson, 1972; Norman and Rao, 1972; NZDB, 1972). Also this can make it possible establishment of nuclei of AI service by dairy farmers organisations. For room temperature semen based AI service to succeed appropriate locally available semen extenders must be used and area specific conditions for preservation must be determined. Several locally available natural substances may be used for this purpose.

Percentage sperm motility and live sperm cells are widely used to assess semen quality and therefore fertility (Arthur et al., 1989; Senger, 1986). Spermatozoa are normally considered living when they exhibit motility although under certain conditions sperm may become immobile and still retain potential for motility and fertility (Liu and Foote, 1998). A low incidence of live spermatozoa is associated with poor motility and low fertility.

Sperm viability can be influenced by many exogenous factors, including storage temperature and the extender used (Hafez, 1993). Spermatozoa are markedly affected by change of storage temperature. Low temperature cools causing gradual loss of motility until near immobility is reached and death at sub-zero temperature from moving of water out of cells and crystallisation pattern (Schwartz and Diller, 1983). Elevated temperature is destructive to sperm viability due to increased rate of metabolism leading to paralysis of the contractile mechanism before the actual death (Nashed et al., 1964). Sperm metabolic rate tends to be proportional to absolute temperature, so reduced temperatures of about 5 °C and -196 °C for unfrozen and frozen semen respectively have been the chief means of slowing down chemical reaction and prolonging life. But in areas where refrigeration and liquid nitrogen are not widely available or are expensive, as is the case with most of Tanzania, consideration has to be given to preserving bull semen at ambient temperatures.

Extenders used for preservation of semen and solutions that are similar in composition to body fluids or are naturally produced liquids, such as egg yolk, milk and coconut water, appear to be compatible with bull sperm (Sane et al., 1982). Extenders have to ideally be approximately isotonic to semen to minimise effects of sperm metabolism and survival (Foote, 1988). Extenders for ambient temperature semen storage should contain a combination of antibacterial agents to prevent bacterial growth and be free from toxic factors or products during storage. For that purpose milk must be heated beyond the pasteurisation point to destroy lactenin, a spermicide (Jones, 1968) and extenders must be prepared from only pure reagents (Foote, 1964).

Staining of semen is used for differentiating live from dead spermatozoa in which dead allow the stain through the membrane thus get stained while live spermatozoa do not as observed under high power bright field or under phase contrast microscopes with preheated stage (Hafez, 1993; Hunter, 1980).

Successful use of room temperature semen based AI in Tanzania depends on the availability of information on performance of different extenders formulated from locally available natural diluents for preservation of bull semen. Limited studies have been done on the use of such extenders in Tanzania. This study was therefore designed to assess motility and proportion of live spermatozoa following short term storage of bull semen in locally prepared extenders and at different temperatures that simulate different Tanzanian environments. This would determine the extenders suitable for preservation of sperm viability for the purpose of establishing room temperature semen based AI.

MATERIALS AND METHODS

Study area

The study was undertaken at the National Artificial Insemination Centre (NAIC), that lies in northern Tanzania at an altitude of 1200 metres above sea level, and has annual rainfall of 800-2000 mm. The rainfall spreads over two months (November to December) of short rains and three months (March to May) of long rains. The rest of the year forms the dry period. Mean monthly humidity is 65 % whilst minimum average temperature is 16.5 °C in June to July and maximum temperature is 22 °C in January to February.

Experimental animals and their management

The experiment comprised of two groups of randomly picked bulls from those kept at NAIC. Group one was made up three Friesian and one Ayrshire bulls. They were more than four years old. Group two comprised of three Ayrshire and one Jersey bulls. They were 18 months of age. The bulls are kept individually in sheds and are stall fed on cut fodder mainly of improved pastures and are normally used for routine collection of semen.

Semen collection

Using artificial vagina semen was collected once weekly at 7.30 am before feeding. The artificial vagina was warmed to bring the inner temperature to 37°C before being put to use. Bulls were allowed three false mounts before semen was collected. Two ejaculates were collected from each bull and pooled together to get 5 ml which were put in a water bath at 32°C.

Preparation of semen extenders

The extenders were prepared from coconut milk, egg yolk citrate and skim milk. A commercial extender, Laisphos® was also used as a control. Only pure chemicals, double distilled water and sterile equipment were used. The pH and osmolality of the extenders were controlled through proper balance of the electrolytes. Freshly prepared extender had the pH adjusted to near neutral (6.7-7.2) using pH-meter (pH meter 3305, Wagtech International Ltd, UK).

Coconut Milk Extender (CME)

Its formulation was as described by Norman et al. (1962) and Grove and North (1965). Into 17 ml boiled for 10 min. and filtered coconut milk was added 2.2g sodium citrate dihydrate; 300mg sulphanilamide powder; 60mg penicillin G. sodium; 135mg dihydrostreptomycin sulphate; 10mg mycostatin sterile powder (one vial); 7ml egg yolk, and distilled water to make 100ml. The pH was adjusted by adding drops of 10% Sodium hydroxide solution.

Egg Yolk Citrate Extender (EYCE)

This extender was prepared according to Salisbury et al. (1941). It consisted of 80 ml citrate buffer (2.9 mg sodium citrate dihydrate, 60 mg penicillin G. sodium, 100 mg dihydrostreptomycin sulphate, 300 mg sulphanilamide dissolved in 100 ml distilled water) and 20 ml egg yolk.

Skim Milk Extender (SME)

Preparation was according to the method described by Almquist et al. (1954). Milk heated to 95°C for 10 min was cooled to room temperature then filtered and 60 mg penicillin G. potassium and 100 mg dihydrostreptomycin sulphate were added per 100 ml milk.

Laisphos®

Preparation of this extender was according to manufacturer's instructions. Detailed descriptions are given by Mmbaga (1994): 478 g Laisphos® powder and 50 ml egg yolk were dissolved in 500 ml distilled water.

Extenders were prepared one day before semen collection and stored in a refrigerator. Before processing of the semen extenders were put in thermostatically controlled water bath at 37°C.

Semen processing

Ejaculates that showed initial motility below 70% were discarded. Spermatozoa concentration for each bull was determined using a haemocytometer. Polyvinyl chloride straws of 0.25 ml were used for semen packaging.

Storage of semen samples

Straws of diluted semen samples were stored at 20-22, 5, -18°C or in an incubator maintained at 25 or 27°C to simulate temperatures in warm lowland areas of Tanzania. To avoid handling of many samples at a time, for each storage condition, semen was collected, processed and evaluated separately.

Semen quality evaluation

i) Staining for differential live-dead spermatozoa count was done according to Swanson and Bearden (1951).

ii) Percentage motility. A droplet preparation under a coverslip on a preheated stage (37°C) was observed under high power (1000x) bright field microscope. The best possible fields in the preparations were examined. Grading scale of 0-5 was adopted in a descending order where 5 is excellent (85-100%), 4 very good (75-85%) and 3 good (65-75%), 2 satisfactory (50-65%) and 1 poor (<5%).

Experimental design and statistical analysis

A 4 x 4 x 5 factorial experiment was used to evaluate the quality of semen at 24, 48, 72 and 96 hours storage after dilution in the four extenders (CME, EYCE, SME and Laisphos®) and at five storage temperatures (-18, 5, 20-22, 25 and 27°C). Bulls were considered to have random effect. The data obtained were analysed using the general linear models procedure (GLM) of SAS (1990) using statistical model elaborated according to Snedecor and Cochran (1980).

RESULTS

Table I shows the least squares means of the effect of storage temperature on the quality of semen stored for up to 96 hours.

Analysis of variance for semen quality during storage for 24 to 96 hours at various temperatures in the four extenders showed that storage temperatures had a highly significant effect (P<0.001) during the entire storage period. Type of extender and extender x storage temperature interaction did not have a significant effect on the semen quality (P>0.05). It is evident that storing semen at - 18°C or 5°C lowered quality significantly (P<0.05) within 24 hours compared to storage at 20-22 or 25°C. Elevated ambient temperature (27°C) did affect more adversely the quality of the semen stored in either of the four extenders used.

Analysis further showed that at 48 hours EYCE was significantly better than both CME and SME but comparable to Laisphos®. At 72 hours, Laisphos® was the best extender while EYCE and CME were comparable, i.e not significantly different (P>0.05). At 96 hours the quality of the semen had deteriorated to less than 10% motility and 20% live spermatozoa for all extenders and there was no significant difference between them in their inability to preserve the quality of semen beyond 72 hours at the temperatures studied.

Table I: Least square means of percentage motility and percentage live spermatozoa in semen stored at different temperatures for up to 96 hours.

	Storage period (hours)			
% Motility	**24**	**48**	**72**	**96**
-18 ^{0}C	49.7 ± 2.5^{b}	29.6 ± 2.9^{b}	13.7 ± 2.7^{c}	4.1 ± 2.5^{ab}
5 ^{0}C	57.7 ± 2.5^{ab}	38.9 ± 2.9^{b}	20.7 ± 2.7^{b}	7.4 ± 2.5^{ab}
20 - 22 ^{0}C	63.7 ± 1.5^{a}	50.1 ± 1.6^{a}	32.6 ± 1.6^{a}	12.9 ± 1.4^{a}
25 ^{0}C	62.5 ± 2.5^{a}	52.7 ± 2.8^{a}	36.9 ± 2.7^{a}	8.3 ± 2.4^{a}
27 ^{0}C	51.5 ± 2.5^{b}	37.9 ± 2.8^{b}	18.5 ± 2.7^{cb}	0.5 ± 2.4^{b}
% Live spermatozoa				
-18 ^{0}C	59.1 ± 2.1^{c}	40.4 ± 2.7^{b}	23.8 ± 2.8^{c}	11.3 ± 2.4^{c}
5 ^{0}C	71.5 ± 2.1^{a}	54.4 ± 3.7^{ab}	34.5 ± 2.8^{b}	16.7 ± 2.4^{bc}
20 - 22 ^{0}C	71.5 ± 1.2^{a}	63.9 ± 1.5^{a}	47.2 ± 1.6^{a}	25.5 ± 1.4^{a}
25 ^{0}C	72.3 ± 2.1^{b}	60.6 ± 2.6^{a}	46.5 ± 2.7^{a}	19.8 ± 2.4^{b}
27 ^{0}C	64.4 ± 2.0^{c}	47.9 ± 2.7^{b}	27.2 ± 2.7^{bc}	9.5 ± 2.3^{c}

Note: Means in the same column having the same superscript within same parameter do not differ significantly (P>0.05).

DISCUSSION

The poor performance of deep frozen semen may be attributed to water moving out of cells and crystallisation pattern - a phenomenon called 'cold shock' (reduction in temperature that has a marked depressing effect on motility). This results from freezing that encourages formation of large ice crystals in the cell cytoplasm leading to cell damage (Schwartz and Diller, 1983; Salisbury et al., 1978; Bishop et al., 1954). This also explains why semen stored at 5°C was of better quality than that kept at -18°C. On the other hand semen stored in liquid nitrogen is able to retain its quality for many years because the very cold temperatures ensure rapid freezing of cell contents without crystallisation taking place and metabolic activity is completely arrested (Rasbech, 1993; Roberts, 1986). The relatively better performance of Laisphos® and EYCE may be attributed to their superior cryprotective properties against cold shock and subsequent thawing as compared to CME and SME (Salisbury et al., 1978; Sahni and Roy, 1972; Rao and Panday, 1977; Tuli et al., 1985). Semen preserved at 20-25°C retained the highest activity within 72 hours for all extenders but deteriorated within 48 hours when storage temperature was raised to 27°C. This trend was also observed by Yeates et al. (1974) and may be explained by the high metabolic activity which leads to rapid depletion of monosaccharide sugars and subsequent paralysis of contractile mechanism before actual death of the cells (Nashed et al., 1964).

It may be inferred from these results that the four extenders used provided a more or less similar environment under which the storage temperature determined their specific influence on the spermatozoa. The observed non significant differences in percentage live spermatozoa may be explained by the fact that some spermatozoa which have lost motility may not yet be dead and will therefore not pick up the stain. This implies that lack of motility is not necessarily an absolute indicator of cell death, and unstained sperms overestimate sperm viability (Liu and Foote, 1998). In this study the extender pH was controlled. Provided that the cell membrane has not ruptured due to extreme change in the extender osmolality, normally observed during cryopreservation due to increasingly hyperosmotic conditions as water freezes, most immotile spermatozoa can be reversed following return to physiologic conditions, since sperms behave as osmomoters (Hafez, 1993; Salisbury et al.,1978). However, at 96 hours storage all inactive cells had died by then irrespective of the type of extender used, most probably due to interactions between extender osmolality and pH and storage temperature (Salisbury et al., 1978). More studies on the effect of these interactions on the spermatozoa quality at ambient temperature storage and under Tanzanian conditions would be necessary.

The results of this study show that EYCE and CME may easily be used to extend the shelf life of room temperature semen for use in the AI of cows provided the storage temperature is maintained between 20-25°C. Short term storage of bull semen in a refrigerator (5°C) or deep freezer (-18°C) was detrimental to the quality of the semen.

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