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Research Article

Liquid and Frozen Storage of Agouti (*Dasyprocta leporina*) Semen Extended with UHT Milk, Unpasteurized Coconut Water, and Pasteurized Coconut Water

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This study evaluated the effects of semen extension and storage on forward progressive motility % (FPM%) in agouti semen. Three extenders were used; sterilized whole cow's milk (UHT Milk), unpasteurized (CW) and pasteurized coconut water (PCW), and diluted to 50, 100, 150, and 200 $\times$ 10^6 spermatozoa/ml. Experiment 1: 200 ejaculates were extended for liquid storage at 5°C and evaluated every day for 5 days to determine FPM% and its rate of deterioration. Experiment 2: 150 ejaculates were extended for storage as frozen pellets in liquid nitrogen at −195°C, thawed at 30° to 70°C for 20 to 50 seconds after 5 days and evaluated for FPM% and its rate of deterioration. Samples treated with UHT milk and storage at concentrations of 100 $\times$ 10^6 spermatozoa/ml produced the highest means for FPM% and the slowest rates of deterioration during Experiment 1. During Experiment 2 samples thawed at 30°C for 20 seconds exhibited the highest means for FPM% (12.18 $\pm$ 1.33%), 85% rate of deterioration. However, samples were incompletely thawed. This was attributed to the diameter of the frozen pellets which was 1 cm. It was concluded that the liquid storage method was better for short term storage.

1. Introduction

The agouti (*Dasyprocta leporina*) is a Neotropical edible rodent [1] which belongs to the order Rodentia and the family Dasyproctinae. The female agouti produces up to three parturitions per year in captivity with litter sizes ranging between 1 and 6 [2]. The meat of the agouti is very popular in the Republic of Trinidad and Tobago and is considered a delicacy [1, 3]. As estimated 80,000 agoutis are harvested from the local forests annually [3]. Due to harvesting and depletion of the agoutis' habitats, anxieties have arisen concerning the survival of the species on this twin island state. This paper can address this problem by providing information that can be used by animal production personnel to produce agoutis [4] for consumption and release into the wild.

There is little information in the area of preservation of semen from Neo-tropical species although semen from other mammalian species has been extended and stored for decades [5]. Semen has been preserved from domesticated animals [6] using various extenders including cow's milk [7] and coconut milk. Semen was also collected from nondomesticated animals, such as hares (*Lepus europaeus*) [8]. After storage for 48 hours in liquid nitrogen the 0.25 mL plastic straws were thawed in a water bath heated to 37°C for 30 seconds. The motility in the thawed semen was reduced by 52.63%. However, they concluded that semen collected from the hare by electroejaculation was adequate for cryopreservation.

This study was conducted to evaluate the effects on spermatozoa forward progressive motility percentage in agouti semen extended with UHT milk, unpasteurized coconut water, and pasteurized coconut water, after storage in liquid form at 5°C and in frozen form in liquid nitrogen at −195°C.

2. Methodology

2.1. Semen Collection. Animals were anaesthetized with a combination of xylazine and ketamine (dosage 40 mg/kg live body weight) before electro-ejaculation with an AC-1 Electroejaculator (manufactured by Beltron Instruments, Blanco Lane Bryan, TX). Anesthetics were administered by intramuscular injection in the hind quarter using 1 mL syringes and twenty-two gauge needles.

Semen was collected by electro-ejaculation (EE). Animals were EE after they were overcome by the drug. The stimuli sequence began by applying 2 volts. Voltage was applied for 3 seconds (on period) and halted for 3 seconds (rest or off period). The voltage was then incrementaly increased by 1 volt until the maximum of 12 volts was attained. At each increase the "on" and "off" sequence was repeated. At this point the entire sequence was repeated until the animal ejaculated. Ejaculates were collected in 15 mL centrifuge tubes. These procedures were approved by the Animal Welfare Committee. This was a modification of the electro-ejaculation protocol developed by Mollineau et al. [9].

2.2. Criteria for Ejaculate Selection. The forward progressive motility % (FPM%) had to be ≥50% or more, and this was calculated from ten viewing fields for each sample. Spermatozoa concentration had to be $\geq 100 \times 10^6$ spermatozoa/mL. Ejaculate volume had to be ≥0.2 mL. All microscopic semen parameters were observed at a magnification of 40X.

2.3. Animals. Animals were caged reared in an intensive agouti production unit at the university's farm and were proven sires between 2 to 3 years of age. Two weeks prior to the experiments animals were randomly selected and housed in individual cages (width = 46 cm, length = 60 cm, and height = 46 cm), which allowed for easier handling. This served to reduce the stresses associated with capturing the animals. Agoutis were fed *ad lib* with rabbit pelleted feed (17% crude protein, 2.5% crude fat, and 15% crude fibre) (manufactured by Master Mix of Trinidad Limited, Republic of Trinidad and Tobago) and given clean, fresh water daily. Animals were fasted for 12 hours prior to electroejaculation.

2.4. Semen Extenders. Ultrahigh temperature (UHT) whole cow's milk, unpasteurized coconut water (CW) and pasteurized coconut water (PCW) were used as semen extenders. The coconut water was first strained by running it through a piece of Indian cotton cloth. It was then used to extend the semen either pasteurized or unpasteurized. Coconut water was pasteurized by boiling for 15 minutes at 65°C. Extenders were frozen in 25 mL aliquots in plastic bottles. Ejaculates were diluted with the particular semen extender to 50, 100, 150, and 200×10^6 spermatozoa/mL at 18–21°C. Fir,st one half of the required volume of semen extender was slowly added to the ejaculate. After 20 minutes the other half was added. This was done to minimize osmotic shock to the spermatozoa.

2.5. The Two Experiments

Experiment 1. Two hundred agouti ejaculates met the quality requirements, representing 71.4% of all ejaculates collected from 20 males over a 20-week period. Two ejaculates were collected daily from two males and each animal was electroejaculated every 7 days. Ejaculates were sequentially assigned an extender treatment until the desired quota (320 extended samples) for each extender treatment was reached. Ejaculates were extended to 50, 100, 150, or 200×10^6 spermatozoa/mL treatments until the desired quota (80 extended samples) for each concentration treatment was reached depending on the initial volume and spermatozoa concentration of the ejaculates. Nine hundred and sixty samples were extended from the 200 ejaculates collected and stored at 5°C. The 960 samples included 320 samples extended with each of the three extenders. Each of these 320 samples included 80 samples with each of the four spermatozoa concentrations. Ejaculate, semen extender, and all equipments used were cooled to 18–21°C. The aliquots were cooled to 5°C over one hour by placing them into a 200 mL beaker containing 50 mL of cooled water (18–21°C), which was then placed into a 5°C refrigerator. Samples were filled into 0.25 mL safe lock microtubes and stored at 5°C. The FPM% and the rate of deterioration of FPM% of samples were evaluated every 24 hours for 5 days.

Experiment 2. This followed the same format as Experiment 1 however the sample size per treatment was lower, 50 samples were used, and samples were stored in frozen pellets in liquid nitrogen (−195°C). The FPM% and the rate of deterioration of FPM% of samples were evaluated after 5 days.

2.6. Thawing. Samples were thawed in a water bath heated to temperatures of 30°, 40°, 50°, 60°, or 70° C for 20, 30, 40, or 50 seconds.

2.7. Statistical Analysis. The experimental design was a 3 (extenders) by 4 (spermatozoa concentrations) factorial design. Statistical analysis was performed with Microsoft Excel 2007. Analysis included means, standard error of the means, and ANOVA (analysis of variances). ANOVA is a statistical method for making simultaneous comparisons between two or more means. P-values less than .05 were considered significant; liquid storage $n = 960$; frozen storage $n = 600$. The rate of deterioration for FPM% over time was calculated based on the initial FPM% of the ejaculate. The data for the various spermatozoa concentrations for each extender was pooled.

3. Results

3.1. Experiment 1. Initial ejaculate means and ranges during Experiment 1 for ejaculate volume were 0.26 ± 0.01 mL, range

Table 1: Data table for Figure 1 listing means ± SD for FPM% over time.

Extenders/concentrations	Time Day 1	Day 2	Day3	Day 4	Day 5
UHT 50	55.5 ± 6.99	42.5 ± 18.08	32.86 ± 16.17	22.86 ± 10.55	12.14 ± 4.83
UHT 100	59.5 ± 7.75	50.5 ± 6.75*	40.5 ± 6.75	29.5 ± 7.78	22.0 ± 6.24
UHT 150	55.0 ± 16.41	45.63 ± 21.21	34.38 ± 15.32	25.63 ± 10.49	13.75 ± 9.19
UHT 200	52.5 ± 10.06	44.55 ± 4.12	33.5 ± 6.58	18.0 ± 8.43	20.0 ± 3.5
CW 50	39.5 ± 4.92	32.0 ± 17.35	29.0 ± 12.74	15.0 ± 9.72	8.3 ± 6.58
CW 100	54.0 ± 12.35	40.0 ± 22.9	26.25 ± 17.87	20.0 ± 11.65	5.0 ± 8.16
CW 150	53.5 ± 7.91	46.5 ± 17.91	35.0 ± 16.87	18.0 ± 10.66	5.0 ± 13.13
CW 200	40.0 ± 18.59	34.0 ± 17.55	29.0 ± 9.56	20.0 ± 9.66	10.0 ± 6.32
PCW 50	56.5 ± 14.99	41.86 ± 14.68	28.75 ± 16.53	8.7 ± 10.66	5.0 ± 6.85
PCW 100	59.5 ± 16.17	45.5 ± 20.01	29.0 ± 17.2	13.0 ± 11.05	5.0 ± 6.26
PCW 150	45.0 ± 12.65	32.1 ± 23.69	26.0 ± 17.03	15.0 ± 14.49	5.0 ± 10.07
PCW 200	40.0 ± 14.94	37.0 ± 20.45	25.0 ± 16.84	10.0 ± 10.75	5.0 ± 7.47

Table 2: lists the mean rates of deterioration and SD for FPM% where the various spermatozoa concentrations of the various extenders over time are pooled.

Extenders	Time Day 1	Day 2	Day 3	Day 4	Day 5
UHT Milk	15.70 ± 3.72	36.84 ± 3.90	54.36 ± 3.28	71.39 ± 2.74	90.21 ± 2.32
	SD = 23.51	SD = 24.64	SD = 20.72	SD = 17.35	SD = 14.70
CW	18.96 ± 3.30	52.51 ± 5.83	64.55 ± 4.70	79.24 ± 3.06	89.59 ± 2.54
	SD = 20.88	SD = 36.90	SD = 27.71	SD = 19.35	SD = 16.07
PCW	19.47 ± 4.53	50.22 ± 5.60	67.85 ± 4.73	81.25 ± 3.08	92.02 ± 1.87
	SD = 28.64	SD = 35.43	SD = 29.90	SD = 19.45	SD = 11.84

0.10 to 0.60 mL; sperm concentration was 635.90 ± 42.20 × 10^6 spermatozoa/mL with a range of 130.0 to 2,440.0 × 10^6 spermatozoa/mL. The rates of deterioration and the standard deviations (SDs) in FPM% over time for samples extended with the various extenders are listed in Table 1.

Samples extended with UHT milk recorded the slowest rate of deterioration for FPM% over time (Table 2). The survival of spermatozoa was more dependent on the UHT milk than the other extenders. Such samples showed the best survival rates over time. There were significant differences among the various stored concentrations (P = .004), where samples diluted to concentrations of 100 × 10^6 spermatozoa/mL and extended with UHT milk produced the best results over time (Figure 1).

3.2. Experiment 2. Initial ejaculate means and ranges during Experiment 2 for ejaculate volume were 0.29 ± 0.04 mL, range 0.10 to 1.00 mL; and spermatozoa concentration was 697.7 ± 12.1 × 10^6 spermatozoa/mL, with a range of 140.0 to 1,410.0 × 10^6 spermatozoa/mL, and FPM% was 61.92 ± 0.32%, with a range of 50 to 85%.

Interactions of thawing temperatures and thawing times also had significant effects on the frozen-thawed FPM% (P = .000). Higher temperatures and longer exposure times increased the rate of deterioration of FPM%. The

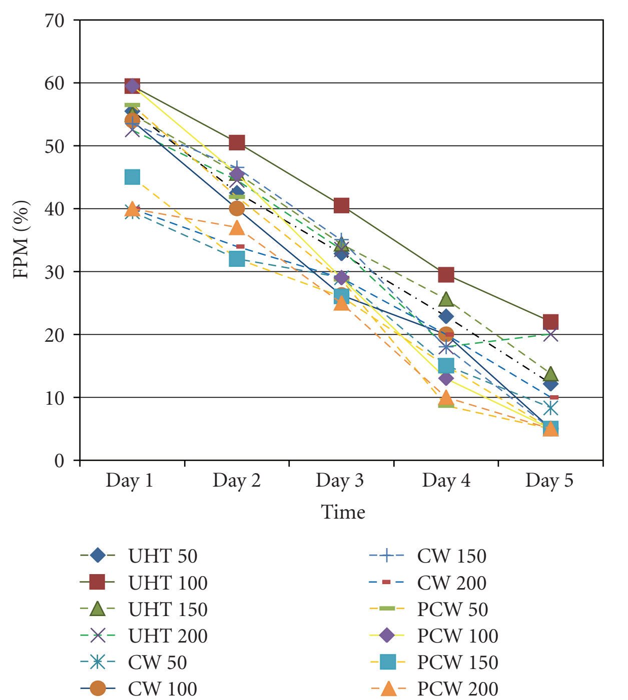

Figure 1: displays means and SD for FPM% for the various extenders at the various concentrations over time.

highest mean frozen-thawed FPM% was 12.18 ± 1.33 and this was obtained when samples were exposed to a thawing temperature of 30°C for 20 seconds. However, these pellets were incompletely thawed. The rate of deterioration in FPM% was 85% after thawing.

4. Discussion

There was significant ($P < .05$) variation among the various extenders used in Experiment 1. Sharma and Tomar [10] also observed a significant difference among the various semen extenders used to dilute Murrah buffaloes semen which were stored at 5°C. The reason that samples extended with UHT milk showed the slowest rate of deterioration over time (Figure 1) could be attributed to possible physiological differences between the effects of the various extenders on the survival of the spermatozoa as suggested by Sharma and Tomar [10].

During Experiment 1 it was observed that the stored spermatozoa concentration had significant ($P < .05$) effects on the survival of spermatozoa (Table 1). Ashworth et al. [11] observed that spermatozoa death was strongly dependent on spermatozoa concentration. At the higher stored spermatozoa concentrations of 150×10^6 spermatozoa/mL and 200×10^6 spermatozoa/mL the energy source (fructose) in the stored volume of extended semen may not have been sufficient to provide adequate energy supplies to the spermatozoa at these concentrations for the longer periods of time. Cerovsky [12] and Čerovský [13] also indicated lower survival rates for semen samples containing higher spermatozoa concentrations. It was also indicated by Čerovský [13] that higher spermatozoa survival rates were recorded at lower concentrations. Thus higher FPM% means were observed for spermatozoa concentrations of 100×10^6 spermatozoa/mL.

Spermatozoa were also observed forming rosettes. This may act as a means of spermatozoa survival [14]. Lower spermatozoa concentrations may affect the formation of effective rosettes. Joyal et al. [15] also observed that motility percentage declined as the spermatozoa concentrations decreased. Hence this is the reason why at 50×10^6 spermatozoa/mL the mean FPM% was less than at 100×10^6 spermatozoa/mL.

The means for frozen-thawed FPM% were low. Thus the rate of deterioration for FPM% for frozen-thawed agouti semen was higher (85%) than the frozen-thawed motility rate of 52.63% for hare semen as reported by Kozdrowski et al [8]. However the samples in Experiment 2 were not completely thawed. Only at 70°C and the longer periods (40 and 50 seconds) did complete thawing occur, but the FPM% was below 1%. Failure of samples to have thawed completely could be attributed to the diameter (1 cm) of the storage pellets. Nakagata et al. [16] successfully thawed wild mice semen stored at −195°C in 0.25 mL plastic straws in a water bath maintained at 20–24°C for five minutes. Kozdrowski et al. [8] also stored extended hare semen in 0.25 mL plastic straws. These researchers did achieve complete thawing in a water bath of 37°C for 30 seconds. The larger diameter of the frozen pellets could have presented more resistance to heat penetrating throughout the sample than with the 0.25 mL straws. Therefore the samples were incompletely thawed, and only spermatozoa at the periphery were thawed and possessed very low motility. Therefore leaving the samples for longer periods only destroyed more of the viable spermatozoa. The spermatozoa at the periphery moving inwards were exposed to longer heat periods. It is recommended that more research be conducted in this area to develop a protocol for thawing extended agouti semen stored in frozen pellets.

5. Conclusion

The liquid storage method for storing extended agouti semen is recommended for short-term storage over the frozen storage method. Frozen storage should be better for long-term storage. However more research in this area is required to identify more effective protocols.

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