Broad-nosed Caiman [*Caiman latirostris*] Semen Collection, Evaluation, and Maintenance in Diluents

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Abstract

Spermatozoa were collected from 8 live male *Caiman latirostris* during December in Piracicaba, Sao Paulo, Brazil. Sperm cells were collected by aspiration from the penile urethra or "groove". In one animal spermatozoa were not found. Two animals produced over one billion sperm cells. To study maintenance of spermatozoa in semen extenders and assess the toxicity of glycerol, six diluents were prepared. These were: 1) BEST - BES (n,n - bis - (2 hydroxyethyl)-2-aminoethane sulfonic acid), TRIS (hydroxymethyl aminomethane), NaCl, glucose, and egg yolk; 2) NaCl with egg yolk; 3) Glucose with egg yolk; 4) BEST with 2% glycerol; 5) BEST with 6% glycerol; 6) BEST with 10% glycerol. All extenders were titrated to pH 7.0 and 327 milliosmoles (before addition of glycerol in diluents 4,5, and 6). Glycerol at 10% caused immediate loss of motility. Glycerol at 6% caused more rapid loss of motility than did 2%. BEST maintained motility at a higher level than did NaCl or Glucose for five days of storage at 5C.

Introduction

The broad-nosed caiman (*Caiman latirostris*) is an endangered species, uncommon in zoos in Europe and North America (Honegger and Hunt, 1990). The native range of this species is the tributaries of the Parana and Sao Francisco Rivers in southern Brazil. A number of *C. latirostris* have been born in captivity in Brazil (Verdade and Lavorenti, 1990) and some observations on reproduction in the species have been made at zoos in North America (Widholzer et al, 1986; Honegger and Hunt, 1990). A colony of *C. latirostris* is maintained at the University of Sao Paulo, Piracicaba, Brazil, for investigations in captive management (Verdade and Lavorenti, 1990).

The purpose of this study was to attempt semen collection in *Caiman latirostris*, assess the numbers of spermatozoa available for artificial insemination and sperm cell preservation efforts, and compare the efficacy of different diluents in maintaining motility of spermatozoa.

Materials and Methods

The *Caiman latirostris* colony maintained at Piracicaba, Sao Paulo, Brazil was utilized for male subjects. Eight males, in apparent good health, were captured by noose from drained concrete ponds where they were maintained either alone or with other animals. Animals #1 and #2 were kept in individual pens. Animals #3 #4, #5, and #6 were housed together in one pond. Animals #7 and #8 were each maintained as the lone male in a pond with three females. Animals were captured without tranquilization by neck snare, the jaws taped shut, and weighed and measured. They were held in dorsal recumbency for exposure of the cloaca.

Semen Collection: The highly concentrated spermatozoa present in the penile urethra and penile groove of males were collected by aspiration and scraping of the groove. The penis was manually exteriorized and held in place by one operator. A second operator passed a soft plastic catheter (size: 04) into the groove and moved it proximally to the fully enclosed urethra. Gently aspiration was applied with a 3 cc syringe. Before placement the catheter was filled with BEST diluent to prevent loss of cells from wetting the interior of the catheter. Spermatozoa in the more distal open groove of the penis were scraped from the inner surface of the groove using the smooth end of a scalpel handle as a spatula. The spermatozoa and mucus were mixed with a drop of BEST diluent.

After collecting all retrievable spermatozoa the cells were diluted in different extenders at approximately 10 x 10^6/cc. Pooled samples of the remaining cells from all animals evaluated in a single day were reconstituted in BEST at 200 x 10^6/cc.

Semen Diluents: Stock solutions of BES (n, n-bis-(2 hydroxyethyl)-2-aminoethane sulfonic acid), TRIS (hydroxymethyl aminomethane), NaCl, and glucose were prepared at 350 mOsM. 1) BEST solution contained approximately 50% NaCl, 25% glucose, and 25% BES and TRIS to titrate the mixture to pH 7.0. Table 1 lists the relative concentrations of components required to produce a final solution with pH 7.0 and osmolality of 327 mOsM after 20% egg yolk v/v is added. The concentrations listed are for the clear solution prior to addition of the yolk. A freeze-point osmometer was used to assess osmolality so all solutions could be adjusted to 327 mOsM after yolk was added. The concentrations listed in Table 1 include the addition of BES, TRIS, and water to titrate the solutions to pH 7.0 and 327 mOsM. 2) NaCl solution was primarily NaCl with 20% egg yolk v/v and

sufficient BES and TRIS to titrate pH to 7.0. 3) Glucose solution contained primarily glucose with 20% egg yolk v/v with TRIS titration to pH 7.0. 4) BEST with 2% GLYCEROL was BEST with 2% glycerol added v/v. 5) BEST with 6% GLYCEROL and 6) BEST with 10% GLYCEROL were prepared similarly.

TABLE 1

Composition of semen extenders used for dilution of Caiman latirostris spermatozoa. Concentrations of BES, TRIS, NaCl and Glucose are calculated for solution before addition of yolk

Constituent	BEST	NaCl	Glucose
BES	1.192 g %	0.143 g %	
TRIS	0.430 g %	0.091 g %	0.120 g %
NaCl	0.515 g %	0.980 g %	
Glucose	1.590 g %		5.507 g %
Na Pen	1000 u/cc	1000 u/cc	1000 u/cc
Yolk	20% v/v	20% v/v	20% v/v
mOsM	327	327	327
pH	7.0	7.0	7.0

Motility evaluation: Diluted spermatozoa were maintained at 5C in capped airtight plastic tubes. Once daily, a drop of diluent with spermatozoa was placed on a glass slide, coverslipped, and examined at room temperature by two observers. Motility was estimated to the nearest 5 percent. Because no more than three males were handled on each day, some samples were evaluated for longer periods than others.

Results

Two of eight males stored sufficient semen in the penile groove that over one billion cells were collected. One male was not apparently producing spermatozoa. Of six attempts to collect the maximum number of cells from the penile urethra, mean yield was 576 $\pm$ 384 cells. This included a second attempt on a male subjected to collection two days earlier. For all collection attempts in which sperm cells were obtained, motile spermatozoa were present in the material evaluated (Table 1).

Spermatozoa obtained by aspiration were highly concentrated in a volume less than 0.1 cc. These cells were sometimes in clumps when viewed by microscopy, but dispersed into solution as motility was acquired, evidently from the dilution effect. Semen obtained by scraping of the penile groove contained mucus.

In some cases, the catheterization of the penile groove lumen caused bleeding and most of the samples had a slight pink tinge due to contamination with blood. The tissues lining the groove are fragile with a highly vascular tissue, possibly erectile, lining the lumen.

Table 3 summarizes the motility of sperm cells diluted in BEST, NaCl, and Glucose over a five day period. Animals 1, 2 and 3 are included in every observation as spermatozoa were obtained from them on the first day of collection efforts. Other considerations made it impossible to treat subsequent samples from other animals in exactly the same way, but means for all samples available for the specific time period are calculated. BEST maintained a slight advantage over NaCl and Glucose for 3 days. At 4 and 5 days the ability of BEST to maintain motility of spermatozoa was markedly superior to NaCl or glucose.

TABLE 2

Weight and length of animal, total sperm cells recovered, and initial motility and 24-hour motility of sperm cells recovered from the penile groove of *Caiman latirostris*.

Animal	Weight (Kg)	Length (meters)	Total Cells	Initial Motility	24-Hr Motility
1 (238)	44.9	1.96	$1,025 \times 10^6$	90	85
2 (2568)	41.3	1.97	360×10^6	90	90
3 (3478)	32.2	1.80	220×10^6	90	90
4 (378)	31.3	1.89	No sperm cells	--	--
5 (28)	29.8	1.79	$1,100 \times 10^6$	70	80
6 (267)	20.3	1.59	430×10^6	60	80
7 (57)	30.0	1.76	NA	70%	40%
8 (3467)	38.8	1.95	NA	80%	40%
1 (2nd collection)	--	--	320×10^6	40%	70%

NA: No attempt made to collect maximum number of cells

TABLE 3

Mean motility (± s.d) of spermatozoa recovered from 7 *Caiman latirostris* (one animal was used twice) over time in semen extenders BEST, NaCl, and Glucose (n = number of samples available at each time period)

TIME	BEST	NaCl	GLUCOSE
Initial	75.0 ± 17.5 (8)	71.4 ± 15.7 (7)	52.8 ± 29.3 (7)
24 h	73.0 ± 24.6 (8)	66.6 ± 28.5 (7)	65.7 ± 25.9 (7)
2 d	57.5 ± 22.5 (8)	58.0 ± 27.7 (5)	36.0 ± 33.6 (5)
3 d	49.1 ± 30.7 (8)	56.0 ± 23.0 (5)	39.0 ± 30.5 (5)
4 d	63.3 ± 5.8 (3)	20.0 ± 17.3 (3)	20.0 ± 10.0 (3)
5 d	66.7 ± 5.8 (3)	13.3 ± 11.5 (3)	0.67 ± 1.2 (3)

TABLE 4

Motility of *Caiman latirostris* spermatozoa diluted in BEST semen extender with glycerol added at concentrations of 2,6, and 10%

		Glycerol concentration		
Time	Animal	2%	6%	10%
24 h	1	90	70	0
	2	80	10	0
	3	80	5	0
2 d	1	70	20	0
	2	70	10	0
	3	70	0	0
3 d	1	70	0	0
	2	50	5	0
	3	60	0	0
4 d	1	30	20	0
	2	20	2	0
	3	40	0	0

Table 4 presents the results of incubation of spermatozoa from animals 1, 2 and 3 in BEST with 2, 6 or 10% glycerol. Glycerol at 10% eliminated sperm cell motility. Glycerol at 6% was detrimental but allowed some motility. Glycerol at 2% did not cause a diminution in motility over that seen in the same diluent without glycerol until the fourth day.

Discussion

Results from this pilot study in short-term maintenance of liquid cooled semen in *Caiman latirostris* are similar to those obtained in work with *Alligator mississippiensis* (Larsen et al., 1984). The method of collection used is not completely atraumatic in either insult to the tissues involved or in stress to the animal. A semen sample obtained two days after the first collection from one male was decidedly inferior in both quality and quantity to the first.

Motility of cells was maintained better in a solution with a mixture of NaCl and glucose and strong buffering capacity than in a solution with only yolk constituents providing buffering elements. Glycerol was toxic at 6% and 10%, though at 2% it did not markedly inhibit motility for the first three days of liquid cooled storage.

The optimum number of cells for oviductal artificial insemination is unknown in this and other crocodilian species. Work with *Alligator mississippiensis* suggests that artificial insemination can be successful with 300 to 1000 x 10^6 spermatozoa deposited in each oviduct (Cardeilhac et al., 1982; Larsen et al., 1982). The number required for artificial insemination in *Caiman latirostris* may well be smaller but no successes have been recorded.

Freeze preservation of spermatozoa would be helpful in building up the numbers of stored sperm cells for an effort to inseminate multiple females. The studies in cryopreservation could be carried out with spermatozoa collected in the manner described here. Even younger, smaller males would serve to provide 200-300 x 10^6 spermatozoa for investigations of this sort. For studies in artificial insemination with fresh semen, it would appear that larger males providing in excess of one billion cells per collection attempt are necessary to build a pooled sample of spermatozoa adequate for ventures utilizing multiple females.

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