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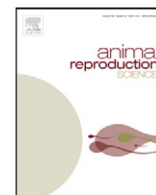

Semen preservation and artificial insemination in domesticated South American camelids☆

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ABSTRACT

Semen preservation and artificial insemination in South American camelids are reviewed giving emphasis to work done in Peru and by the authors. Reports on semen evaluation and the preservation process indicate that semen of alpacas and llamas can be manipulated by making it liquid first. Collagenase appears to be the best enzyme to eliminate viscosity. Tris buffer solution maintains a higher motility than egg-yolk citrate, phosphate buffered saline (PBS), Triladyl, and Merck-I extenders. Cooling of semen took 1 h after collected, and equilibrated with 7% glycerol presented a better motility and spermatozoa survival at 1, 7, 15 and 30 days after being slowly frozen in 0.25 mL plastic straws. Trials of artificial insemination with freshly diluted semen and frozen–thawed semen are encouraging and needs to be tested extensively under field conditions. Recently, fertility rates varied from 3 to 67%. Semen preservation and most important, artificial insemination appear to be a reality, and could be used to improve the genetic quality of alpacas and llamas.

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1. Introduction

Semen extension, preservation and artificial insemination (AI) in alpacas and llamas are developing technologies. Semen collection by the use of an artificial vagina has increased the knowledge of semen physiology of alpacas and llamas (Bravo et al., 1996, 1997a, 2002; Urquieta et al., 2005). Artificial insemination is used on small number of animals mostly in research stations, but its commercial application is still hampered by the lack of interest of most breeders in South America and the regulation of registering the AI-produced offspring (crias) in other countries.

This paper includes a short review on semen evaluation, extenders and preservation as well as experimental trials using AI.

2. Semen collection and initial preparation for dilution

The techniques to collect semen in alpacas and llamas have evolved throughout the years. The use of vaginal sacs (condoms), sponges placed inside the vagina (San Martin et al., 1968), did not result in adequate samples and is not used anymore. Semen collection by electro-ejaculation, introduced by Fernandez-Baca and Calderon (1968), was not used for many years due to the contamination of semen with urine and the painful effects of applying electrical discharge inside the rectum of the male; however this collection method was reintroduced by Director et al. (2007). Semen collected using an artificial vagina (Sumar and Leyva, 1981) has been improved by the simulation of a cervix to trigger ejaculation, and the wrapping of the artificial vagina by an electric blanket (Bravo et al., 1997b,

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2002; Von Bear and Hellemann, 1998), and currently is the method of choice to obtain ejaculates that are suitable for laboratory manipulation and mimic the natural time of breeding (20–30 min, San Martin et al., 1968).

The use of an artificial vagina for semen collection presents several advantages including reliability and ease once the males are trained, and does not require general anesthesia as in the case of electro-ejaculation. An artificial vagina is the most common method used to obtain a semen sample for the use of artificial insemination trials in alpacas and llamas.

The first step in semen handling is the removal of its viscosity (Garnica et al., 1993; Bravo et al., 1997c, 2000). Various enzymes have been used to eliminate semen viscosity including trypsin, hyalurodinase, amylase, and collagenase; the latter was most effective as liquefaction was rapid and irreversible (Bravo et al., 2000). Once the ejaculate is liquid, seminal parameters are determined according to standard techniques used in other livestock species. These include: motility, concentration, percentage of live spermatozoa, and morphology of spermatozoa. Semen quality may reflect individual variation as well as management factors. A compilation of semen collection data produced at La Raya research station is shown in Table 1. Nearly, 92% of ejaculates collected are suitable for preservation and artificial insemination, i.e., spermatozoa concentration greater than 80 million spermatozoa/mL, 90% live spermatozoa, and 80% motile spermatozoa. However, factors such as size of the herd and numbers of males available for a reliable semen sample must be considered in the success of the technique.

3. Extenders and semen dilution

Several of the commonly used extenders for ruminant semen preservation have been applied to alpaca and llama semen, and only one commercial extender specifically labeled as a camelid semen extender is available (Camel Buffer, IMV technologies). In addition to the results of extenders tested in alpaca and llama semen listed in Table 2, results of a rural development project in Australia (Vaughan et al., 2003) showed that of 15 extenders tested, only 5 resulted in motility > 30% after 24 h (Sheep Red, Green Camel, Triladyl, Biladyl, Andromed).

Viability and motility of semen after dilution varies from one study to another and few studies have compared the effect of extender and storage conditions on semen viability. However a general evaluation of these results suggests that Tris-buffered extenders may be more suitable than others. More viable alpaca spermatozoa was obtained in egg-yolk citrate (36.4%) than with PBS (22.2%) or skim milk (15.6%) when semen samples were maintained for 2 h at 35 °C (Fig. 1; Bravo et al., 1998). In another study, extended alpaca semen maintained at 4 °C for up to 72 h showed better viability with egg-yolk glucose citrate than with skim milk or glucose citrate extenders. The percentage of live spermatozoa was nearly 70% in egg-yolk glucose citrate for up to 48 h (Fig. 2). Other studies (Baca, 1998; Tito, 2003) have shown Tris-buffered extenders to be superior to Triladyl® and egg-yolk citrate or to skim milk and Merck-I® extenders in alpacas and llamas, respectively (Fig. 3).

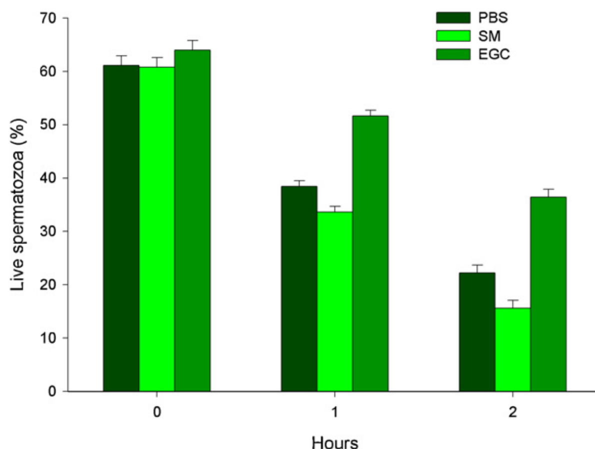

Fig. 1. Mean (±SEM) for alpaca live spermatozoa of 5 males evaluated after incubation at 35 °C for 2 h in three extenders: phosphate buffer saline (PBS), skim milk (SM), and egg-yolk glucose citrate (EGC). [abc] Values with no common superscript are different ($P < 0.05$).
Adapted from Bravo et al. (1997c).

Recently, live spermatozoa percentages have also been evaluated in samples maintained at 4 °C for up to 72 h in the alpaca and 24 h in the llama. In alpaca semen samples maintained for 24 h at 4 °C, and extended with lactose egg-yolk, the progressive motility and live spermatozoa did not change and were 62% (Morton et al., 2007).

4. Semen preservation

Although there have been some studies in the last decade on the preservation of alpaca and llama semen, there are very few detailed studies on the effect of each preservation factor (i.e., dilution rate, extender and type of cryoprotectant). Except for treatment to eliminate viscosity, the protocols used for alpaca and llama semen freezing mimic those used for bull semen. These steps can be summarized as follows: collection of a good quality ejaculate,

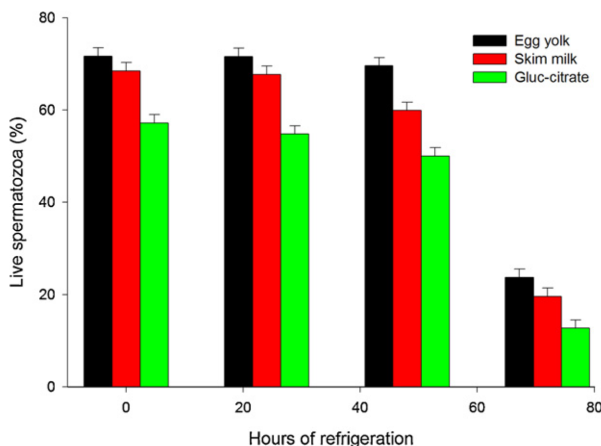

Fig. 2. Mean (±SEM) for alpaca live spermatozoa incubated at 4 °C up to 72 h. Extenders used are egg-yolk glucose citrate (EGC), skim milk, and glucose citrate (gluc-citrate). [abc] Values with no common superscript are different ($P < 0.05$).

Table 1
Semen characteristics of single alpacas (A) and llamas (L) used for freezing at La Raya, Peru (1997–2009).

Male	Volume (mL)	Motil. (%)	Concentration[a]		Live (%)	Normal (%)	Abnormal (%)		
			Mean	Range			Head	Droplet	Tail
A1	0.9	77.1	127	9–344	75.8	67.8	4.5	6.6	21.1
A2	1.8	57.1	54	8–191	65.8	58.4	8.3	20.9	12.4
A3	1.2	73.8	210	22–656	69.5	63.8	1.8	5.2	29.2
A4	1.0	69.1	90	38–156	69.4	65.9	4.1	11.7	18.3
A5	1.4	53.3	56	13–134	53.7	62.8	7.6	13.7	15.9
A6	0.9	79.3	145	31–397	79.5	79.7	7.1	3.4	10.6
A32	2	80	180	90	90	70	5	0	10
A69	2	80	200	100	83	83	4	3	10
A11	1	80	95	95	78	70	6	4	20
A28	3	75	332	115	70	60	10	7	23
A7	2	90	420	145	96	86	4	4	6
L1	2	80	200	100	87	89	5	0	10
L2	3	75	285	95	84	84	4	1	11
L3	2	70	230	115	82	79	10	2	9
L4	2	80	209	105	78	81	5	2	12
L17	1.7	62.5	64	10–96	ND	66.7	ND	ND	ND
L21	1.6	52.5	57	9–80	ND	69.4	ND	ND	ND
L22	1.5	52.9	58	15–64	ND	65.3	ND	ND	ND
L24	1.5	49.2	56	10–80	ND	63.2	ND	ND	ND
L25	1.7	52.1	57	10–82	ND	65.2	ND	ND	ND

ND, not determined.
[a] In millions/mL.

elimination of viscosity, dilution, cooling, glycerolation, equilibration and freezing (Huanca and Gauly, 2001; Aller et al., 2003; Santiani et al., 2005).

Following collection and treatment to minimize viscosity, semen is diluted and cooled slowly from 37 °C to 4 °C over 1 h. Semen was then extended to a final concentration of 8–10 million spermatozoa. There is no difference in motility and live spermatozoa between 1 and 2 h of cooling, thus a 1 h cooling period is adequate (Baca, 1998). The addition of a cryoprotectant is performed at the end of the cooling period. Although ethylene glycol and DMSO have been used for alpaca and llama semen, respectively (Raymundo et al., 2006), glycerol (Salinas, 1999) remains the most used cryoprotectant for semen preservation in both species. A concentration of 7% glycerol was superior, in terms of viability and motility, to concentrations below 4% (Fig. 4). In general, glycerol is added in three equal volumes given in 15 min intervals.

Table 2
Percentage of motile spermatozoa of South American camelids using different extenders and for different times (in parenthesis).

Species	Extender	Motility at 0 h	Motility after *X* h	References
Alpaca	PBS	61.1	22.2 (2 h)	Bravo et al. (1997)
	Skim milk	60.8	15.6	
	Egg-yolk citrate	64.0	35.4	
Alpaca	Egg-yolk citrate	71.7	69.6 (48 h)	Bravo et al. (1998)*
	Skim milk	68.5	59.9	
	Glucose citrate	57.2	50.0	
Alpaca	Tris buffer	76.9	72 (3 h)	Baca (1998)
	Triladyl	76.9	65	
	Egg-yolk citrate	76.9	58	
Alpaca	Tris	72	65 (3 h)	Salinas (1999)
Alpaca	Tris gluc	72	56.6 (3 h)	Santiani et al. (2005)
	Skim milk	72	49.2	
Alpaca	Tris gluc	30–90	ND (5.8 h)	Raymundo et al. (2006)
	Tris fruct	30–90	ND (6.1 h)	
	Continental (pig)	30–90	ND (5.5 h)	
Alpaca	BSA-gluc	60.7	ND (2 h)	Torres, 2006
Alpaca	Androhep	33.5	27 (3)	Morton et al. (2007)
	Tris	58.0	27	
	Skim milk	28.2		
Llama	BSA-gluc	57	9 (72 h)	Huanca and Gauly (2001)
Llama	Tris buffer	62.1	60 (3 h)	Tito (2003)
	Skim milk	62.1	59	
	Merck-I	62.1	33	
Llama	Citrate-DMSO	54	20 (3 h)	Aller et al. (2003)
Llama	Lactose egg-yolk	62	24	Giuliano et al. (2003)

* Semen samples were kept refrigerated at 4 °C.
ND, not determined.

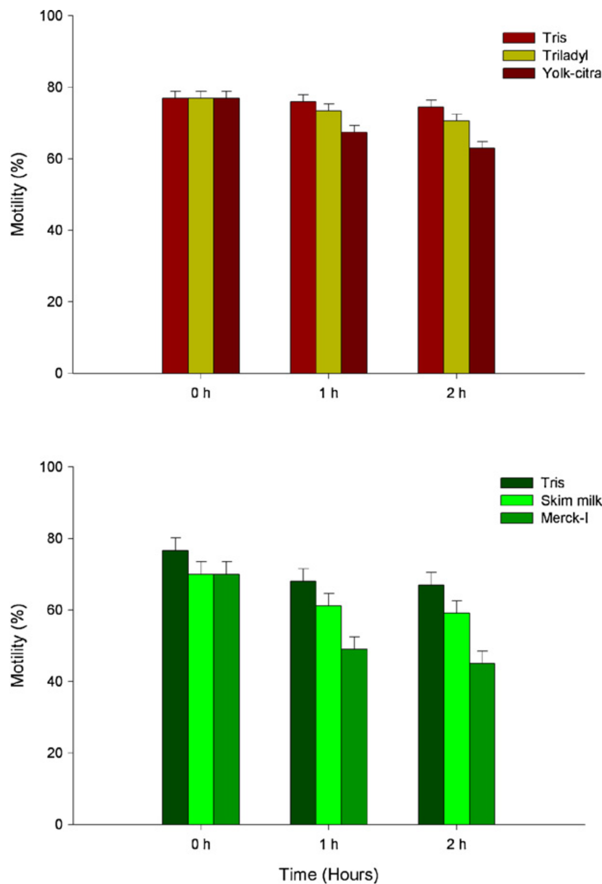

Fig. 3. Mean (±SEM) for alpaca (n = 6 males) spermatozoa motility (upper panel) and for llama (n = 5 males) spermatozoa motility (lower panel) extended in three different extenders. For the alpaca, the extenders are: Tris buffer (Tris), Triladyl, and egg-yolk citrate (yolk-citra). For the llama, extenders are: Tris buffer (Tris), skim milk, and Merck-I. [abc] Values with no common superscript are different (P < 0.05).

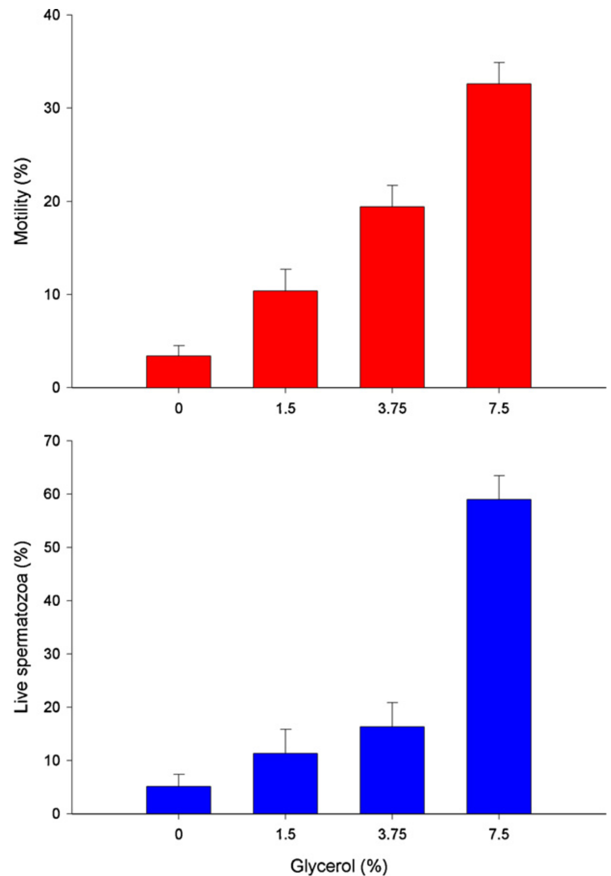

Fig. 4. Mean (±SEM) motility (upper panel) and live spermatozoa (lower panel) for alpaca spermatozoa (n = 6 males) glycerolated with three different glycerol concentrations at time of thawing. [abc] Values with no common superscript are different (P < 0.05).

Using spermatozoa motility, as the end point, different times of equilibration have been evaluated (Baca, 1998; Tito, 2003). One hour of equilibration is appropriate for alpaca and llama semen. Spermatozoa motility should be determined at each step of semen preservation, and should be close to the initial motility. Fig. 5 depicts the motility of alpaca semen extended with Tris buffer and during the process of semen preservation.

5. Freezing and thawing

Freezing of South American camelid (SAC) semen was first reported in 1978. In most studies, following equilibration at 4 °C, alpaca and llama semen is packaged in 0.25 mL or 0.5 mL plastic straws and frozen in liquid nitrogen vapors (Bravo et al., 1996). There are no controlled studies comparing the freezing rates on post-thaw quality. Semen is loaded into plastic straws and placed on a rack at 12 cm above liquid nitrogen level then lowered slowly, 1 cm for each minute has resulted in good frozen–thawed semen motility and live spermatozoa (Cuba, 2000).

Thawing protocols consist of placing straws in a water bath at 35 °C for 8–10 s (Salinas, 1999; Cuba, 2000), or at 37 °C for 20–60 s (Tito, 2008). In a study on alpaca semen, post-thaw spermatozoa motility was similar (50%) when straws were thawed for 1 min at 35 or 37 °C but were significantly lower (38%) when straws were thawed for 5 or 10 min at 37 and 40 °C (Tito, 2008). Post-thaw motility and viability of alpaca semen (Cuba, 2000) was not different following storage in liquid nitrogen for 1, 7, 15, or 30 days (Fig. 6). The motility of thawed spermatozoa is also variable; reports of 5% spermatozoa motility to around 40–50% have been reported (Raymundo et al., 2006).

Freezing of epididymal spermatozoa using three different extenders have been also reported. Thawed sperm motility was better using lactose egg-yolk, than Tris, and citrate–glucose (18.2, 11.3 and 6.9%, respectively). In addition, the acrosome integrity was 83.4% when sperm was frozen into Tris, 80% for lactose egg-yolk, and 79% for citrate–glucose extender (Morton et al., 2007).

6. Artificial insemination

Although the first report of the use of AI appeared 40 years ago (Fernández-Baca and Novoa, 1968), the use of

Table 3
Results of different methods of artificial insemination used in alpacas and llamas (in chronological order).

Species	Semen	Extender	Ovulation Induction	Females inseminated	Pregnant (%)	Authors
Alpaca	Fresh	Undiluted	Male[a]	42	2.4	Fernandez-Baca et al. (1968)
Alpaca	Fresh	Undiluted	Male, hCG	96	46.5	Calderón et al. (1968)
Alpaca	Fresh	Undiluted	Male, hCG	94	30.8	Leyva et al., 1979
Alpaca	Fresh	Undiluted	hCG	40	67	Bravo et al. (1994)
Alpaca	Fresh	Egg-yolk gluc citrate	hCG	80	57	Bravo et al. (1995)
Alpaca	Fresh	PBS	Male	133	40	De la Vega (1996)
Alpaca	Thawed	Egg-yolk gluc citrate	hCG	19	26	Bravo et al. (1996)
Alpaca	Fresh	Tris buffer	GnRH	25	69.6	Castillo
	Refrigerated	Tris buffer	GnRH	25	66.7	(1997)
	Thawed	Tris buffer	GnRH	25	68	
Alpaca	Thawed	Tris buffer	hCG, GnRH	90	25	Bravo et al. (1999)
Alpaca	Fresh	BSA-gluc	GnRH	205	50.7	Apaza et al. (2001)
Alpaca	Fresh/Chilled	Triladyl	GnRH	24	0	Vaughan
	Thawed	Biladyl				et al. (2003)
		Green camel				
Alpaca	Thawed		Male	59	13.6	Pacheco (2004)
Alpaca	Fresh	BSA-gluc	GnRH	155	39	Torres (2006)
Alpaca	Thawed	Tris	GnRH	104	65	Bravo et al. (2008)
Alpaca	Fresh	Undiluted	Male	41	20.9	Tito (2008)
Llama	Thawed	Citrate	GnRH	38	3	Aller et al.
		DMSO				(2003)
Llama	Fresh	Citrate-egg yolk	GnRH	23	5	Aller
Llama	Fresh	Tris	GnRH	23	22	et al.
Llama	Thawed	Tris	GnRH	38	8	(2003)
Llama	Thawed	Tris	hCG	28	50	Tito (2003)

[a] Vasectomized male.

this technology is still not widely used. The reasons for this slow progress are mainly technical and include problems with method of semen collection, handling and freezing. Artificial insemination trials using undiluted, diluted, refrigerated, and thawed semen with the application of different AI protocols and semen extenders are currently under investigation (Table 3).

The first report of AI used undiluted semen from 2 male vicunas and 4 paco-vicunas collected by electro ejaculation (Fernandez-Baca et al., 1968). Forty-two female alpacas were induced to ovulate by copulation with vasectomized males, and insemination was performed immediately after breeding. Semen was deposited at the bifurcation of the two uterine horns. A single cria was born after 340 days of gestation.

The second attempt of AI used alpaca semen collected by electroejaculation (Calderón et al., 1968). Ninety-six female alpacas were artificially inseminated at different intervals from induction of ovulation. A 75% fertilization rate (fertilized ova recovered 3 days after insemination) was obtained when insemination was performed between 35 and 45 h of induction of ovulation (Table 4; Calderón et al., 1968). A third account in AI used 83 female alpacas and 11 female llamas and vicuna and paco-vicuna semen was collected by electroejaculation. Ovulation was induced by the administration of hCG or copulation with a vasectomized male (Leyva et al., 1979). Undiluted fresh semen was deposited into the uterine horns via the cervix. The overall percentage of pregnant females was 31%. For hCG-treated females, pregnancy rate was 48% and 11% for vasectomized male induced ovulation (Leyva et al., 1979). The pregnancies generated by AI using frozen–thawed alpaca semen are variable, 29–35% (7 crias out of 22 females inseminated) of live crias born have been reported. Recently, a 67% pregnant females at 21 days was achieved in females inseminated 24 h after being induced to ovulate with hCG in receptive females. Insemination was done with fresh undiluted semen deposited inside the uterine horn by laparoscopy and fixing the cervix through the rectum as used in cattle (Castillo, 1997).

In llamas, artificial insemination trials with fresh and frozen–thawed semen have been reported with pregnancy rates of 21.7% with fresh and 7.9% with frozen–thawed semen (Aller et al., 1997). Currently, trials of AI in alpacas and llamas in Peru consistently yield a birthing rate of 50% following a single insemination with fresh extended semen. It should be pointed out that a pregnancy rate of 50% is common in other species, especially considering that alpacas are inseminated once (Bravo et al., 1997b). Recently, a single trial of repeated insemination in alpacas with fresh semen has been reported (Torres, 2006). In this last case, the first insemination yielded 50% of females pregnant; however, fertility increased to 67% after 3 inseminations in females of those not becoming pregnant after the first and second time.

The number of spermatozoa per straw used in AI protocols has been also explored, and the results are variable (De la Vega, 1996; Bravo et al., 1999). In one study, the pregnancy rate at 30 days (61%) was obtained using 8–12 million spermatozoa per straw. By contrast, a 53% fertility rate was found when 4 million spermatozoa per straw were used. In a subsequent study, there was no difference in fertility when using 4–12 million spermatozoa (De la Vega, 1996).

The use of GnRH (10 μg, Fertagyl, Intervet, Amsterdam, The Netherlands) in the uterus and deposited immediately after insemination has been also investigated (Bravo et al.,

Table 4
Fertilized ova recovered 3 days after artificial insemination of alpacas at different intervals after induction of ovulation (n = number of alpacas inseminated; from Calderón et al., 1968).

Ovulation induction (n)	Interval from induction (h)	Fertilized ova/total collected (%)	Fertilized ova per time interval (%)
Male[a] (3)	0–6	0/2 (0)	0/4 (0)
LH (3)		0/2 (0)	
Male[a] (3)	7–18	1/4 (25)	1/8 (12)
LH (3)		0/4 (0)	
Male[a] (3)	19–26	3/6 (50)	10/19 (53)
LH (3)		7/13 (54)	
Male[a] (3)	27–34	2/4 (50)	3/7 (43)
LH (3)		1/3 (33)	
Male[a] (3)	35–45	4/4 (100)	6/8 (75)
LH (3)		2/4 (50)	
Male[a] (3)	46–52	5/6 (83)	7/12 (58)
LH (3)		2/6 (33)	
Total			27/58 (46)

[a] Vasectomized male.

2004). GnRH (10 μg Fertagyl, Intervet) was deposited in the uterine horn immediately after AI (Bravo et al., 2008). Pregnancy rate was 10% higher ($P < 0.05$) in females treated with GnRH than in non-treated females.

Finally, it is noteworthy to mention that the first cross between a guanaco, a SAC, and an old world camel, dromedary, was achieved using AI. Crosses between SAC are possible, and the may occur naturally and/or intentionally; however, this cross was done in the Arab Emirates and used semen from a 450 kg male dromedary, "Musehan" and deposited into a 75 kg guanaco female "Smokey".

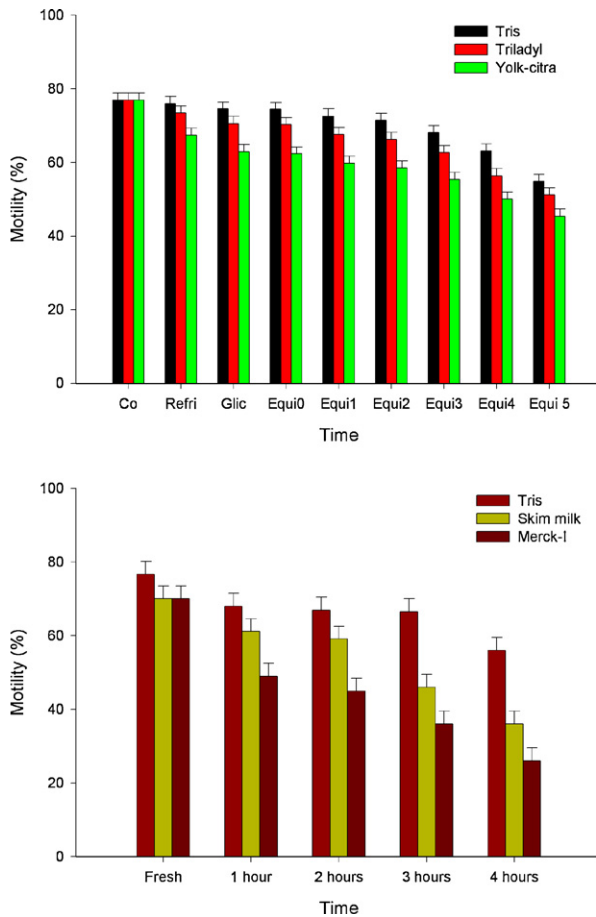

Fig. 5. Mean (±SEM) for alpaca (n = 6 males) spermatozoa motility during the process of semen preservation into three different extenders, Tris, Triladyl, and egg-yolk citrate (yolk-citra). The times of evaluation are: fresh (Co), cooling (Refri), glycerolation (Glic), equilibration (Equi0), and 1–5 h of equilibration (Equi1, Equi2, Equi3, Equi4, Equi5, respectively). [abc] Values with no common superscript are different ($P < 0.05$).

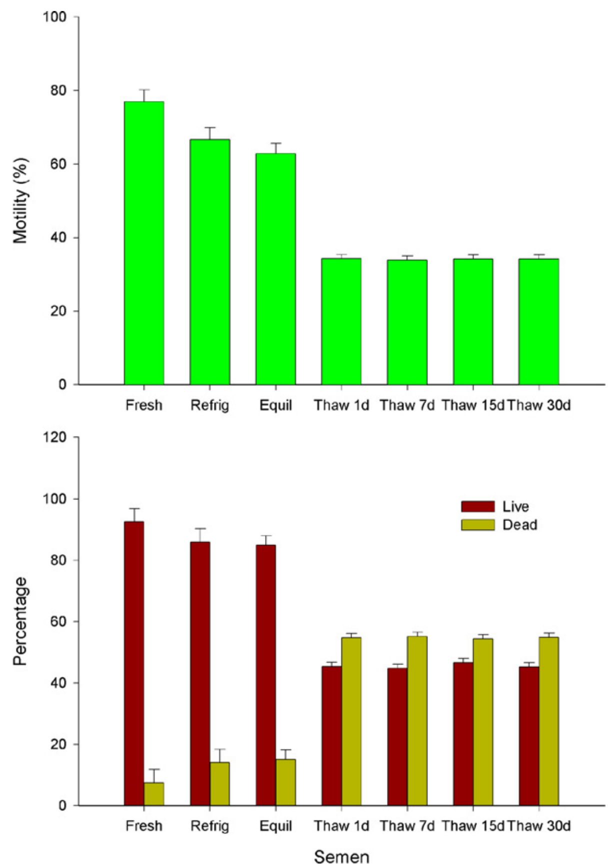

Fig. 6. Mean (±SEM) for alpaca (n = 6 males) motility (upper panel) and live/dead spermatozoa (lower panel) at different times of semen preservation, and thawing at different days after being frozen. [abc] Values with no common superscript are different ($P < 0.05$).

The offspring is a male called Rama, who was born in 1998. Interestingly, Rama's appearance is between a guanaco and a camel. He does not have a hump, but have short ears like camel, and his feet have two toe nails like a guanaco.

7. Conclusions

Progress in AI of domesticated South American camelids is slow. However, results of AI generated in Peru and other countries are encouraging. More research is needed to fine tune the technique of the process of semen freezing and controlled studies of thawing semen. In the years to come, AI will make an impact on the way that alpacas and llamas are genetically improved.

Conflict of interest statement

There is no conflict of interest.

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