Animal Reproduction Science 173 (2016) 8–12

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Comparison of differents methods of sperm selection of llama raw semen

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ARTICLE INFO

Article history:
Received 6 March 2016
Received in revised form 25 July 2016
Accepted 2 August 2016
Available online 4 August 2016

Keywords:
Sperm selection
Androcoll
Percoll
Swim up
Llama

ABSTRACT

The objective of this study was to compare the efficiency of different sperm selection methods applied to the same llama ejaculate. Four treatments were compared: two variants of the swim up technique (with and without seminal plasma), and two different colloids, Androcoll-E-Large and Percoll®. Using electroejaculation, 21 semen samples were obtained from 7 llama males (n = 7, r = 3). The ejaculates were incubated in a solution of 0.1% collagenase, to decrease thread formation, and then split into 4 aliquots: one aliquot was layered over a column of Androcoll-E-Large (SLC) and the second over a column of Percoll (45%). The third aliquot was deposited in a tube with culture medium and was incubated at a 45° angle for 30 min at 37 °C (SU1). The last aliquot was centrifuged to separate the spermatozoa and seminal plasma. The sperm pellet obtained was resuspended, and transferred to a tube with culture medium which was incubated at an angle of 45° for 30 min at 37 °C (SU2). Both aliquots SLC and P showed higher proportions of progressive motility and plasma membrane functionality ($p \leq 0.05$) than raw semen. There were no significant differences ($p > 0.05$) in sperm viability and in normal spermatozoa between raw semen and treatments. Nevertheless, only SLC did not have a significant increase of bent tails. In conclusion SLC centrifugation would be the method of choice for selecting llama spermatozoa.

1. Introduction

Selection of potentially fertile spermatozoa is an event that occurs during sperm migration through the female reproductive tract. The application of assisted reproduction techniques (ART) by-pass, some of these *in vivo* sperm selection mechanisms (intrauterine insemination, *in vitro* embryo production) or may even increase the percentage of non-fertile spermatozoa (damage by cryopreservation),

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http://dx.doi.org/10.1016/j.anireprosci.2016.08.001
0378-4320/

thus decreasing pregnancy rates and healthy offspring. Therefore the use of *in vitro* sperm selection techniques is needed to obtain a high percentage of motile spermatozoa, with normal morphology, free from cell debris and dead spermatozoa. In addition, the sperm separation technique should isolate as many motile spermatozoa as possible and should not cause sperm damage or physiological alteration (Henkel and Schill, 2003).

Swim-up and density centrifugation techniques are the most common methods used to select sperm in different species, including humans. Swim-up requires progressive motility of the sperm, because the selection is based on their ability to move from the semen into fresh medium. With this technique, it is possible to obtain a high percentage of motile and morphologically normal sperm. It is a low-cost method, with easy preparation and there is no need to expose spermatozoa to substances that could be toxic. The disadvantage is that the ejaculates should have a high sperm concentration and good motility.

The method of colloid centrifugation relies on highly motile spermatozoa that can penetrate the different layers and pass to the bottom of the tube. Its main advantage is that it can be used with ejaculates of low sperm concentration. Disadvantages are the high cost and the difficulty in preparing density gradients.

As the semen of South American camelids (SACs) characteristically has a high viscosity (Casaretto et al., 2012) is not possible to separate sperm from seminal plasma without performing centrifugation at high speeds (Giuliano et al., 2010). In addition, the sperm of these species do not have progressive oscillatory motility in the native ejaculates (Giuliano et al., 2010). Due to these unique characteristics of the semen, most studies on *in vitro* embryo production and sperm cryopreservation have been done with epididymal spermatozoa (Trasorras et al., 2013; Carretero et al., 2014). Therefore, the development of protocols to prepare spermatozoa from fresh ejaculates is needed. Currently there are no reports about comparative experiments of different sperm selection methods in camelids using the same ejaculate and under the same experimental conditions. The objective of this study was to compare the efficiency of different sperm selection methods applied to the same llama ejaculate. Two variants of the swim up technique (with and without seminal plasma) were evaluated, and a comparison between two different colloids Androcoll-E-Large and Percoll® was done.

2. Materials and methods

2.1. Animals and location

The study was carried out at the Faculty of Veterinary Sciences of the University of Buenos Aires, in Buenos Aires, Argentina. The city is situated at sea level, latitude 34° 36′ and longitude 58° 26′.

For the study, 7 male *Lama glama* ranging between 6 and 10 years of age and weighing 154.67 ± 19.20 kg (mean ± SD) were used. Animals were kept at pasture in pens and supplemented with alfalfa; they also had free access to fresh water throughout the study. All males were shorn during the month of November.

2.2. Semen collection

A total of 21 ejaculates (7 males, 3 ejaculates per male) was collected, processed and evaluated. Semen collections were carried out using electroejaculation (EE) under general anesthesia according to the technique described by Director et al. (2007). All procedures were approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol 2010/24).

2.3. Semen evaluation

The sperm characteristics studied were: sperm motility, membrane function, viability and sperm morphology. Sperm motility was evaluated on a warm stage (37 °C) using a phase contrast microscope (100×); motility was classified as either oscillatory or progressive (Giuliano et al., 2010).

The HOS test for evaluating membrane function was conducted according to Giuliano et al. (2008). Sperm showing the characteristic swelling of the tail were classified as HOS positive, having a functional plasma membrane according to Jeyendran et al., 1992. Morphology was evaluated using phase contrast microscopy (1000×). The staining techniques, using fluorochromes 6-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) for evaluating membrane integrity (viability), were conducted according to Giuliano et al. (2008). Spermatozoa that fluoresced green throughout their length were classified as being viable (intact membrane) while sperm nuclei that fluoresced red were classified as non-viable (damaged membrane) (described by Harrison and Vickers, 1990). Morphology was evaluated using phase contrast microscopy (1000×) according to Giuliano et al. (2008). In all cases 200 sperm per sample were evaluated. In addition, semen rheological characteristics were evaluated by determining thread formation with a Pasteur pipette, both in the whole ejaculates and in the supernatant of each treated aliquot according to Giuliano et al. (2008).

2.4. Semen treatment

Each ejaculate was diluted 4:1 in 0.1% collagenase in H-TALP-BSA medium (Parrish et al., 1986) and incubated 4 min at 37 °C according Giuliano et al. (2010) with the objective of decreasing thread formation and facilitating manipulation of the samples. Afterwards, the ejaculates were divided into four aliquots (between 750 μl and 1 ml each): two aliquots were used for swim up (SU1 and SU2), and two were centrifuged with two different colloids: Percoll® (P) and Androcoll-E-Large (A).

2.4.1. Swim-up techniques (SU)

2.4.1.1. SU1. An aliquot of semen was deposited in a 15 ml centrifuge tube and 2 ml of culture medium (Hepes-HAM with of 0.3% m/v bovine serum albumin: H-HAM-BSA) were

layered on top. The tube was placed at a 45° angle and incubated for 30 min at 37 °C (adapted from WHO, 2010).

2.4.1.2. SU2. An aliquot of semen was centrifuged for 8 min at 800*g* to separate the spermatozoa and seminal plasma. The sperm pellet obtained was resuspended in 1 ml of H-HAM-BSA and placed in the bottom of a tube. Two millilitres of H-HAM-BSA were carefully layered on top. The tube was placed at an angle of 45° and incubated for 30 min at 37 °C (adapted from WHO, 2010). After the incubation, 1 ml of the sample from the upper layer was aspirated and centrifuged at 600*g* for 10 min. The supernatants were discarded and the pellets resuspended in H-HAM-BSA. This procedure was done for both variants SU1 and SU2.

2.4.2. Centrifugation with colloids

2.4.2.1. Percoll® (P). An aliquot of semen was deposited gently on the top of 1 ml of 45% Percoll colloid H-HAM-BSA, without disturbing the interface between layers and afterwards the tube was centrifuged at 600*g* for 20 min (Conde et al., 2008). After centrifugation, the pellet obtained was resuspended in 2 ml of medium H-HAM-BSA and centrifuged at 600*g* for 10 min. The pellet was resuspended in the same medium according to Conde et al. (2008).

2.4.2.2. Androcoll-E-Large (A). An aliquot of semen was deposited gently on the top of 1 ml of Androcoll, without altering the interface between layers (Santa Cruz et al., 2010). After centrifugation, the pellet obtained was resuspended in 2 ml of medium H-HAM-BSA and was centrifuged at 600*g* for 10 min. The pellet was resuspended in the same medium.

2.5. Statistical analysis

Data obtained were analyzed using the statistical software Rv2.2.1 using One way ANOVA block design with the main effect of treatment (unselected, Androcoll, Percoll, SU1 and SU2). Male identity was considered to be a blocking variable. A Friedman test was used when normality of data distribution was not achieved.

3. Results

The results of various assays are summarized in Tables 1 and 2. With regard to thread formation, all 21 ejaculates formed a thread when pipetted with a Pasteur pipette before incubation. No semen sample incubated with 0.1% collagenase H-TALP- medium formed a thread when pipetted.

3.1. Influence of treatment on sperm progressive motility

Significant differences ($p \leq 0.05$) were found in the percentage of progressive motility between four methods of sperm selection. The percentages were significantly higher ($p \leq 0, 05$) in both methods using colloids in comparison with unselected semen. There were no significant differences ($p > 0.05$) between swim-up 1 and swim-up 2; however the treatment with Androcoll had significantly higher total and progressive motility than SU2 ($p \leq 0.05$).

3.2. Influence of treatment on sperm membrane functionality, viability and morphology

The percentage of sperm with endosmosis were significantly higher ($p \leq 0.05$) in treatments with Percoll and Androcoll than in SU, and no significant differences were found between them.

In the percentage of sperm with intact membranes (live), no significant differences ($p > 0.05$) between the selected aliquots and unselected semen were observed. The treatment with Androcoll had significantly better viability ($p \leq 0.05$) compared to swim up (SU1 and SU2).

Epithelial cells were observed in unselected semen. There were fewer cells in the Percoll treatment group than in the unselected group, and no cells at all in the Androcoll group.

Table 2 shows a descriptive analysis of sperm morphology of unselected semen and different treatments.

4. Discussion

The majority of the studies on *in vitro* production of embryos and sperm selection techniques have been done with epididymal sperm from SACs due to the difficulty in handling fresh semen (Trasorras et al., 2013). The rheological characteristics of the semen make it difficult to separate the spermatozoa from the seminal plasma and to extend the ejaculate and homogenize samples. In addition, SAC spermatozoa show only oscillatory motility and do not show progressive motility in their ejaculates (Tibary and Vaughan, 2006; Giuliano et al., 2010; Carretero et al., 2015). For these reasons, it was necessary to development protocols to reduce semen viscosity and to improve progressive motility from raw semen (Giuliano et al., 2010). Some previous studies have used Percoll to prepare SAC semen (Conde et al., 2008; Huanca et al., 2009, 2010; Condori et al., 2010; Berland et al., 2011).However, it has been suggested that Percoll might adhere to sperm membranes and can damage them (Henkel and Schill, 2003). Consequently, it is recommended to wash and centrifuge the spermatozoa after using this colloid, although this additional procedure prolongs the preparation time (Henkel and Schill, 2003) and can be detrimental to the spermatozoa because of the action of reactive oxygen species (Aitken and Clarkson, 1988). Thus, a new colloid has been tried with llama sperm (Santa Cruz et al., 2010). Androcoll is a species- specific colloid based on silane-coated silica particles in a ready-to-use formulation. It has been used to select spermatozoa from fresh llama semen sample (Santa Cruz et al., 2010; Trasorras et al., 2012, 2014) and fresh or cryopreserved stallion semen samples (Morrell et al., 2009, 2014; Bucci et al., 2013; Dorado et al., 2013; Urbano et al., 2013).

To our knowledge, this is the first study comparing the efficiency of two semen selection techniques applied to the same ejaculate in SAC. The results for all raw semen parameters were within the normal range reported for raw ejaculates in this species (Tibary and Vaughan, 2006; Carretero et al., 2014). When comparing the two selec-

Table 1
Characteristics of the spermatozoa in unselected semen and four different selection methods (Androcoll, Percoll and two variants of swim up: SU1 and SU2). N = 21 ejaculated. Values are averages ± SD. Values with different letter (a, b, c) in the same row have significant difference ($p < 0.05$).

	Fresh semen	Androcoll	Percoll	Swim Up 1	Swim Up 2
Progressive motility (%)	1.43 ± 4.78^{c}	23.55 ± 16.07^{a}	20.60 ± 16.09^{ab}	7.50 ± 7.55^{abc}	6.74 ± 9.48^{bc}
Oscillatory motility (%)	25.47 ± 16.27^{a}	16.75 ± 11.39^{ab}	12.55 ± 9.32^{abc}	7.00 ± 6.32^{bc}	6.53 ± 7.06^{c}
Total motility (%)	26.90 ± 16.77^{abc}	40.30 ± 18.44^{a}	32.15 ± 21.85^{ab}	14.50 ± 12.35^{bc}	13.26 ± 13.99^{c}
Concentration ($\times 10^6$ sperm/ml)	19.07 ± 19.53^{a}	22.83 ± 24.38^{a}	21.77 ± 28.20^{a}	2.51 ± 3.45^{b}	3.95 ± 3.35^{b}
Total number of sperm$\times 10^6$)	71.81 ± 7.88^{a}	8.28 ± 9.70^{b}	7.39 ± 10.83^{b}	1.64 ± 2.78^{c}	1.58 ± 2.68^{c}
Total number of motile sperm ($\times 10^6$)	0.43 ± 1.73^{c}	2.42 ± 3.35^{a}	2.02 ± 3.18^{ab}	0.28 ± 0.43^{abc}	0.19 ± 0.34^{bc}
Percentage of sperm with endosmosis	31.29 ± 12.09^{b}	44.26 ± 11.31^{a}	43.57 ± 15.97^{a}	38.14 ± 12.00^{ab}	40.16 ± 13.28^{ab}
Percentage of sperm with endosmosis	49.13 ± 13.03^{ab}	57.18 ± 17.92^{a}	45.32 ± 13.03^{ab}	35.36 ± 13.46^{b}	42.38 ± 15.93^{b}

Table 2
Sperm Morphology of the spermatozoa from unselected semen samples (non-selected) and four different selection methods (Androcoll, Percoll and two variants of swim up). N = 21 ejaculated. Values are averages ± SD. Values with different letter (a, b, c) in the same row have significant difference ($p < 0.05$).

	Fresh semen	Androcoll	Percoll	Swim Up 1	Swim Up 2
Normal spermatozoa	72.73 ± 10.84^{a}	76.33 ± 10.66^{a}	70.81 ± 12.10^{a}	67.19 ± 12.45^{a}	68.89 ± 9.14^{a}
Loose heads	3.05 ± 2.60^{a}	2.74 ± 2.62^{a}	2.47 ± 2.36^{a}	2.19 ± 2.08^{a}	4.68 ± 3.53^{a}
Abnormal heads	2.32 ± 1.53^{a}	1.78 ± 1.45^{a}	1.77 ± 1.50^{a}	1.07 ± 1.37^{a}	1.78 ± 1.79^{a}
Broken neck	4.26 ± 5.29^{a}	2.90 ± 3.69^{a}	2.36 ± 2.58^{a}	6.59 ± 5.50^{a}	2.61 ± 2.26^{a}
Midpiece abnormalities	1.06 ± 1.75^{a}	0.71 ± 1.16^{a}	0.47 ± 0.87^{a}	0.21 ± 0.63^{a}	0.85 ± 1.15^{a}
Proximal cytoplasmic drop	8.24 ± 7.02^{a}	6.57 ± 8.87^{a}	3.99 ± 3.78^{abc}	6.45 ± 6.45^{a}	6.86 ± 4.44^{a}
Distal cytoplasmic drop	2.74 ± 5.06^{a}	2.44 ± 4.29^{a}	1.97 ± 3.58^{a}	2.28 ± 5.80^{a}	2.63 ± 4.09^{a}
Bent tails	5.16 ± 5.68^{a}	6.20 ± 6.96^{a}	15.50 ± 11.90^{b}	13.52 ± 2.81^{ab}	10.80 ± 5.96^{b}
Broken tail	0.40 ± 0.80^{a}	0.28 ± 0.71^{a}	0.63 ± 1.44^{a}	0.43 ± 0.86^{a}	0.51 ± 0.81^{a}

tion techniques, better progressive motility and membrane functionality were obtained with both colloids, in comparison with Swim- up. Henkel and Schill (2003) reported that the concentration and motility of the sample should be high in order to get good results with this technique. This is not the case with the semen of SAC, which has low sperm concentration and low progressive motility in raw ejaculates (Tibary and Vaughan, 2006; Giuliano et al., 2010). In previously reports there were disparate results between both techniques. Liu et al. (2013) studied the effects of different techniques on yak sperm quality. They determined that swim-up produced higher percentage of sperm with intact plasma and acrosome membrane than Percoll gradient centrifugation. Monqaut et al. (2011) compared the quality of human sperm samples obtained after density-gradient centrifugation and swim-up and concluded that Swim-up produces samples with better quality, but the recovery rate is also lower. Amiri et al. (2012) demonstrated benefits of the gradient methods in the separation of normal and motile human spermatozoa while Santiago-Moreno et al. (2014) compared the effectiveness of two methods of sperm selection – Caprigure® density-gradient centrifugation (DGC) and dextran swim-up (DSU) in semen samples from Iberian ibex (Capra pyrenaica) and European mouflon (Ovis musimon). They concluded DGC would seem the most useful method for selecting the best spermatozoa from both species than DSU. Luppi et al. (2015) suggested that DC technology induced a better sperm capacitation potential than Swim-up technology. The disparity in results among reports about spermatozoa prepared by density gradient centrifugation or swim-up could be due to different species and laboratories.

In this study centrifugation with Androcoll and Percoll, significantly increased the proportion of sperm with progressive motility and decreased the proportion with oscillatory sperm motility in comparison with raw semen. These results were similar to those reported by Santa Cruz et al. (2010) who observed an increase in progressive motility from 0% in raw semen to 20- 60% in samples selected with Androcoll, and with those published by Trasorras et al. (2012) showing an similar increase from 0 to 37%. Likewise, these results were in agreement with Conde et al. (2008) using Percoll, who showed an increase from 0% to 100%. Stallion sperm motility was also improved by colloid centrifugation compared to control samples (Morrell et al., 2008).

With respect to sperm morphology, there were no significant differences between the unselected semen samples and the four selection methods. However, it is interesting to note that a significant increase in bent sperm tails ($p \leq 0.05$) was observed with Percoll. To our knowledge, there are no reports in other species showing an increase of bent tails.

5. Conclusions

The swim up technique would not be efficient in the recover of adequate number of progressively motile sperm from Llama semen for reproductive biotechnologies. The semen selection method using Androcoll was superior to Percoll, selecting a higher percentage of motile and morphologically normal sperm and therefore SLC centrifugation would be the method of choice for selecting llama spermatozoa.

Declaration of interest

The authors declare there is no conflict of interest, actual or perceived, that could be perceived as prejudicing the

impartiality of the research reported. JMM is inventor and patent holder of Androcoll-E.

Conflicts of interest

None.

Acknowledgement

This research was supported by a grant from the Universidad de Buenos Aires, Argentina (20020120200270BA).

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