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Stallion Sperm Selection by Density Gradient Centrifugation Involving a Double Layer Colloid: Effects on Sperm Subpopulation Dynamics in Fresh and Stored Semen

Research Article

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ABSTRACT

Sperm selection techniques have became an important tool to improve sub fertile ejaculates. The main aim of this study was to assess the dynamics of motile sperm subpopulations of ejaculates from subfertile stallions subjected to density gradient centrifugation (DGC) involving a double layer colloid [DGC2], before and after storage for 24 and 48 hours. Ejaculates from eight stallions with fertility problems were subjected to the different treatments: 1) control [C]; 2) centrifugation [CT]; 3) density gradient centrifugation involving a double layer colloid [DGC2] and 4) post storage density gradient centrifugation involving a double layer colloid DGC2 post]. In the C, CT and DGC2 treatments, sperm variables were analysed at 0 h and after 24 and 48 h of storage at 4-7 ˚C. In the DGC2 post treatment, the semen was stored at 4-7 ˚C and sperm samples selected by DGC2 at 24 and 48 h. Viability, sperm abnormalities and motility were then examined using a computerized system. Four motile sperm subpopulations with different motility characteristics were observed in the all treatments. The DGC2 sperm had faster, straighter moving and more active cells at 0 h. At 24 and 48 h, however, the motility variable values fell and the slower spermatozoa subpopulations increased in size. The DGC2 post treatment returned better overall sperm motility variable values at 24 and 48 h; a larger percentage of spermatozoa fell into the faster subpopulations. In conclusion, DGC2 could be used to improve semen from subfertile stallions as well as semen showing poor fertility after storage.

KEY WORDS density gradient centrifugation, EquiPure™, sperm selection, sperm subpopulations, subfertile stallions.

INTRODUCTION

Sperm selection ensures that the best sperm cells are collected for use in the assisted reproduction (ART). Selection techniques have been used to improve the semen of humans (Hammoud *et al.* 2007; Fleming *et al.* 2008), equids (Macías García *et al.* 2009; Miró *et al.* 2009), bovines (Thys *et al.* 2009), pigs (Morrell *et al.* 2009a) and wild animals (Crosier *et al.* 2008). A technique of proven worth is that of colloidal centrifugation through a density gradient (DGC) (Sieme *et al.* 2003; Varner *et al.* 2008; Macías García *et al.* 2009; Johannisson *et al.* 2009; Morrell *et al.* 2009b; Morrell *et al.* 2009c).

Subpopulations of motile sperm have been identified in several mammalian species (Abaigar *et al.* 1999; Abaigar *et al.* 2001; Quintero-Moreno *et al.* 2003; Miró *et al.* 2005; Martínez-Pastor *et al.* 2005). There is no consensus regarding the physiological role of these subpopulations, but their proportional distribution is strongly related to semen quality (Abaigar *et al.* 2001) and the maintenance of an overall

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subpopulation structure may be important for maintaining ejaculate function (Flores *et al.* 2008). Quintero-Moreno *et al.* (2005) suggest that the sperm subpopulation structure may influence the fertilizing capacity of stallion ejaculates.

Morrel *et al.* (2011) described improvements in large volumes of stored (24 h) high-sperm-count stallion semen after DGC with a single layer colloid (DCC1). The same may be true for small amounts of stallion semen with low sperm counts, especially if DGC with a double layer colloid is used (DGC2).

The present work assesses the motile sperm subpopulation dynamics in subfertile stallion ejaculates, subjected to DGC2, after 24 and 48 h of storage at 4-7 ˚C.

MATERIALS AND METHODS

Semen samples were obtained from the eight clinically healthy stallions with proven fertility problems (pregnancy rates ≤ 20% in the previous two breeding seasons). Ejaculates (n=8) were collected using a Hannover artificial vagina with an in-line nylon mesh filter.

Semen samples were evaluated for volume, sperm concentration (using a Neubauer haemocytometer), viability and morphological abnormalities (by eosin-nigrosin staining) [21] and motility (using a ISAS V1.0 CASA system [Proiser SL, Valencia, Spain]). Gel-free semen was diluted 1:5 (v:v) with pre-warmed (37 ˚C) Kenney extender [20] in air-free 50 mL Corning tubes. Aliquots were then taken and subjected to one of the following treatments:

1) control (C). Sperm samples diluted 1:5 (v:v) with Keeney (1975) extender.

2) centrifugation (CT). The diluted semen samples were centrifuged at 660 g for 15 min at 20 ˚C. The resulting pellet was re-suspended in Keeney (1975) extender, and adjusted to a final concentration of 200×10^6 spermatozoa/mL in order to homogenise the samples conditions and following analyses.

3) DGC involving a double layer colloid (EquiPure™) before sperm storage (DGC2). A double layer density gradient was prepared using the EquiPure™ kit following the manufacturer's instructions. Two millilitres of the higher density (bottom) layer was placed in 15 mL corning tubes and 2 mL of the lower density (top) layer placed on top. A 4 mL aliquot of diluted semen (maximum 500×10^6 spermatozoa/mL) was carefully pipetted onto the surface of the top layer. After centrifugation at 300 g for 20 min at 20 ˚C, the formed pellet was re-suspended in 1.0 mL Keeney (1975) extender.

4) post sperm-storage DCG involving a double layer colloid (EquiPure™) (DGC2 post). Fifty millilitre corning tubes with sperm samples diluted 1:5 (v:v) with Kenney extender were stored at 4-7 ˚C for 24 and 48 h. At these times the spermatozoa were gently homogenised and DCG performed as in the DGC2 treatment. In the C, CT and DGC2 treatments, the samples were analysed for sperm concentration, viability, morphological abnormalities and motility at 0 h. Two millilitre sperm aliquots representing each treatment were then stored at 4-7 ˚C for 24 and 48 h. They were then gently shaken, placed in water bath at 37 ˚C for 10 min and re-analysed.

DNA fragmentation was assessed in the C and DGC2 treatments at 0 h using the Sperm-Halomax™ kit (Chroma Cell SL, Madrid, Spain).

This methodology is based on the sperm chromatin dispersion test (SCD) and has been validated for use with a range of mammalian species, including humans (López-Fernandez *et al.* 2007).

In the DGC2post treatment, sperm concentration, viability, morphological abnormality and motility analyses were performed after DCG2 at 24 h and 48 h. The obtained data were analyzed using the SAS® for Windows v.6.1 statistical package (SAS, 1996). Means and standard deviations for all variables were determined using the Proc means procedure. The Proc GLM routine was used to identify differences between times/treatments, and the LSMEANS procedure to determine their significance. The limit for statistical significance was set at P < 0.05.

The FASTCLUS disjointed cluster analysis procedure was used to separate motile spermatozoa into subpopulations. FASTCLUS performs a disjointed cluster analysis based on Euclidean distances computed taking into account one or more quantitative variables. In this case, these variables were the different sperm motility features measured by the CASA system. Sperm cells that shared similar motility characteristics were assigned to the same cluster; spermatozoa that differed in motility characteristics were assigned to different clusters.

RESULTS AND DISCUSSION

No differences were seen between treatments at 0 h in sperm viability, abnormalities, total or progressive motility. There were no differences in DNA fragmentation between the C and DGC2 treatments at 0 h. At 24 h of refrigeration storage, sperm viability in the DGC treatment was significantly better than in the C treatment (P<0.001); no significant differences were seen between the CT, DGC2 or DGC2 post treatments. At 48 h, no significant differences were observed between any treatments in measured variables (Table 1).

Major differences between the treatments were observed after storage time, however, in terms of CASA-assessed sperm motility variables (Table 1). At 0 h, the DGC2 sperm presented significantly higher (P<0.0001) VCL, VAP, VSL, LIN, STR and WOB values (P<0.05) than the C and CT sperm (Table 2).

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Table1 Sperm concentration, viability, total and progressive motility, abnormalities and DNA fragmentation in fresh and storage stallions sperm samples in C, CT, DGC2 and DGC2 post treatments

Treatment	Sperm count ($\times 10^6$/mL)	Viability (%)	Total motility (%)	Progressive motility (%)	Abnormalities (%)	DNA fragmentation (%)
C (0 h)	191.50±167.45	66.2±13.3	67.5±22.5	31.0±13.3	43.3±20.2	33.4±17.2
CT (0 h)	366.69±132.57	73.9±8.5	67.7±13.7	34.7±6.4	36.8±8.9	-
DGC2 (0 h)	98.04±50.23	74.6±14.4	83.9±10.6	53.3±15.8	38.1±9.5	36.9±22.9
C (24 h)	191.50±167.45	52.7±14.8^{a}	56.7±24.5	24.1±16.4	41.1±12.3	-
CT (24 h)	200.00±0.00	65.6±4.8^{a,b}	33.2±17.2	16.7±11.8	45.6±11.1	-
DGC2 (24 h)	98.04±50.23	74.2±6.1^{b}	46.6±18.7	21.2±17.5	39.2±9.2	-
DGC2 (post 24 h)	88.67±44.98	60.2±20.5^{a,b}	62.7±26.6	34.9±17.9	41.5±12.6	-
C (48 h)	191.50±167.45	47.9±13.1	39.1±23.2	18.9±15.5	41.9±14.8	-
CT (48 h)	200.00±0.00	66.5±6.4	22.2±8.4	6.7±5.1	43.7±5.9	-
DGC2 (48 h)	98.04±50.23	64.1±8.7	53.0±15.7	26.4±15.9	44.6±7.2	-
DGC2 (post 48 h)	88.67±44.98	56.0±15.3	53.0±26.0	38.2±27.4	43.3±15.9	-

Table 2 Results of sperm motility parametersin fresh and storage stallions' sperm samples in C, CT, DGC2 and DGC2 post treatments at 0, 24 and 48 hours

Treatment	SPZ (n)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALHmed (μm)	BCF (Hz)
C (0 h)	1094	80.5±46.1^{a}	39.8±29.1^{a}	53.7±34.1^{a}	48.3±22.8^{a}	71.6±21.5^{a}	65.1±17.8^{a}	2.9±1.6^{a}	9.3±4.3^{a}
CT (0 h)	1266	68.8±40.8^{b}	32.7±24.3^{b}	41.8±27.3^{b}	44.5±19.8^{b}	72.8±20.4^{a}	59.4±15.3^{b}	2.6±1.5^{b}	9.4±4.4^{a}
DGC2 (0 h)	1068	95.5±39.9^{c}	52.7±27.5^{c}	66.5±29.7^{c}	56.5±22.0^{c}	77.7±19.5^{b}	70.9±15.9^{c}	3.3±1.5^{a}	9.7±3.6^{a}
C2 (4 h)	436	84.5±53.6^{a}	37.4±29.1^{a}	56.6±38.6^{a}	44.4±23.6^{a}	66.3±23.8^{a}	64.8±18.2^{a}	3.1±1.9^{a}	8.7±4.5^{a}
CT2 (4 h)	243	69.0±45.7^{b}	32.5±28.5^{a}	40.3±30.6^{b}	41.9±21.6^{a}	71.5±22.2^{a,c}	56.1±16.9^{b}	2.6±1.5^{b}	9.0±5.1^{a}
DGC2 (24 h)	245	55.3±39.7^{c}	32.6±26.5^{a}	38.0±28.8^{b}	55.7±26.5^{b}	79.6±20.3^{b,c}	66.6±21.1^{a}	2.0±1.4^{b}	8.9±4.4^{a}
DGC2 post (24 h)	424	97.6±45.3^{d}	52.5±29.1^{b}	68.1±33.0^{c}	55.3±24.2^{b}	76.7±20.7^{c}	69.6±18.1^{c}	3.3±1.7^{c}	10.6±4.3^{b}
C (48 h)	181	77.2±52.3^{a}	39.1±31.0^{a}	50.1±36.9^{a}	47.5±22.9^{a}	73.4±21.9^{ab}	62.4±18.4^{a}	2.7±1.8^{a}	9.8±5.1^{a}
CT (48 h)	205	53.2±41.3^{b}	20.8±20.9^{b}	28.6±24.6^{b}	36.2±18.4^{b}	66.8±21.0a	52.3±15.3^{b}	2.3±1.6^{b}	7.9±4.5^{b}
DGC2 (48 h)	423	70.7±42.9^{c}	37.9±26.8^{a}	46.2±29.6^{a}	51.0±22.9^{c}	76.7±20.5^{b}	64.0±17.3^{a}	2.6±1.5^{b}	9.4±4.3^{a}
DGC2 post (48 h)	205	95.4±51.8^{a}	54.5±38.4^{c}	69.2±40.6^{c}	55.4±25.1^{d}	75.1±21.9^{b}	71.1±18.4^{c}	3.2±1.8^{c}	9.2±4.2^{a,b}
Total	5790	-	-	-	-	-	-	-	-

SPZ: spermatozoa; VCL: sperm curvilinear velocity; VSL: sperm linear velocity; VAP: mean velocity; LIN: linear coefficient; STR: straightness coefficient; WOB: wobble coefficient; ALH med: mean lateral head displacement and BCF: frequency of head displacement.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

After 24 h incubation, the VCL, VAP and ALHmed values were lower in CT and DGC2 treatments than in C treatment (Table 1). At 48 h, the CT treatment showed significant reductions in almost all sperm motility variables compared to the C treatment (P<0.05; Table 2).

After refrigeration for 24 h, the DGC2 post sperm presented significantly higher VCL, VAP, VSL, WOB, ALH med and BCF values than any other treatment (P<0.05; Table 1). After refrigeration for 48 h, the same treatment returned significantly higher VSL, VAP, LIN, WOB and ALH med values than any other treatment (P<0.05; Table 2).

The FASTCLUS procedure identified four sperm subpopulations: SP1 to SP4, in increasing order of sperm velocity values (Table 3). At 0 h, the CT and C sperm had similar sized SP1 and SP2 subpopulations. However, at 24 and 48 h, large increases (P<0.05) were seen in the size of SP1 in the CT sperm. At 0 h the DGC2 sperm had a smaller SP1 and larger SP4 than the C sperm (P<0.05). However, at 24 and 48 h, SP4 and SP1 were respectively smaller and larger in the DGC2 treatment than in the C treatment (P<0.05) (Figure 1). Finally, the DGC2 post sperm showed an increase in the size of SP4 at 24 h (P<0.05), and an increase in the size of SP3 at 48 h compared to the C sperm (P<0.05; Figure 1). Obtained results showed that DGC2 filtration of subfertile semen results in an improving of sperm motility patterns just after semen collection, but not after this semen refrigeration (4-7 ˚C) during 24 or 48 h.

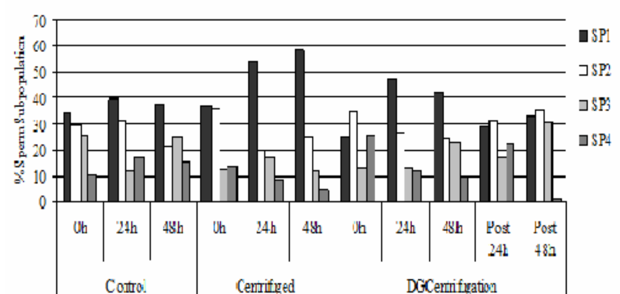

Figure 1 Distribution of sperm subpopulations in each time / treatment

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Table 3 Identification of sperm subpopulations based on the assessment of sperm motility by ISAS

Subpopulation	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
SP1	27.2±0.9	10.6±0.7	15.1±0.6	37.2±0.0	66.0±0.0	57.5±0.0	1.3±0.0	6.1±0.2
SP2	80.9±0.9	41.1±0.7	52.7±0.6	53.9±0.0	80.3±0.0	68.5±0.0	2.9±0.0	10.1±0.9
SP3	125.0±1.0	58.6±0.8	85.1±0.7	50.0±0.0	72.2±0.0	71.4±0.0	4.2±0.1	11.6±0.2
SP4	156.5±2.2	103.7±1.8	120.8±1.6	68.7±0.0	87.9±0.1	79.9±0.0	4.6±0.1	11.4±0.5

SPZ: spermatozoa; VCL: sperm curvilinear velocity; VSL: sperm linear velocity; VAP: Mean velocity; LIN: linear coefficient; STR: straightness coefficient; WOB: wobble coefficient; ALH med: mean lateral head displacement and BCF: frequency of head displacement.

SP1: was characterised by having the lowest values for all sperm velocity values (VCL, VSL and VAP); SP2: was characterised by having medium values for these values; SP3: was characterised by having high values for VCL, VSL and VAP and SP4: was characterised by having very high values for all sperm velocity values.

However, after 24-48 h of refrigeration DGC2 filtration showed maintenance of sperm motility patterns. In this work, DGC2 was associated with faster, straighter-moving and more active sperm at 0 h (Table 2). This agrees with DGC1 results reported by Morrel *et al.* (2009d). However, at 24 and 48 h the DGC sperm showed lower motility variable values (Table 2) and an increase in the size of SP1. Johannisson *et al.* (2009) and Morrel *et al.* (2009d) reported that DGC1 maintains sperm quality over short-term refrigerated storage. In the present work, despite reduction in the motility values in the DGC treatment at 24 and 48 h, sperm quality remained acceptable; the quality of the CT sperm, however, had fallen strongly by these times (Table 2). The DGC2 post treatment would appear to return the best results for low-sperm-count semen stored for up to 48 h (Table 3). Morrel *et al.* (2011) reported similar findings for DGC1 after storage for 24 h when using high-sperm-count semen. The maintenance of an overall subpopulation structure may be important in maintaining ejaculate function (Abaigar *et al.* 2001; Quintero-Moreno *et al.* 2003; Miró *et al.* 2005; Martínez-Pastor *et al.* 2005; Flores *et al.* 2008). As reported by Quintero-Moreno *et al.* (2003), four sperm cell subpopulations (SP1-SP4) were detected (Table 3) in all treatments. As reported for other treatments, e.g., freezing-thawing (Flores *et al.* 2008), sperm selection by DGC led to changes in sperm motility variable values related to changes in the sizes of these sperm subpopulations. Macias Garcia *et al.* (2009) reported a larger SP3 in frozen / thawed stallion sperm after DGC1. At 24 h and 48 h in the present DGC2post treatment, SP3 and SP4 were much larger than in the DGC treatment. Indeed, at these times, the DGC sperm showed a large increase in the size of SP1; the CT sperm showed very large increases in the size of SP1 at these times. Thus, for stored, cooled sperm samples, DGC may be the best sperm selection option.

In heterologous *in vitro* fertilization assays, our group (De Souza Soares *et al.* 2011; Morató *et al.* 2013) reported penetration rates of 24.16% for frozen-thawed stallion sperm selected by DGC, but only 6.36% for non-selected sperm. This supports the idea that DGC induces changes in sperm subpopulations affecting fertilization capacity. In summary, after 24 and 48 h of storage, DGC was associated with better overall sperm motility (Table 2).

The DGC2post treatment also best preserved the sperm subpopulation structure, yet was associated with larger SP3 and / or SP4 subpopulations (faster spermatozoa with higher VCL, VAP LIN, VSL ALH med and BCF values).

CONCLUSION

As a result, we assume that it may therefore be a good way of preparing low-sperm-count semen for artificial insemination (AI) or intracytoplasmatic sperm injection, or for preparing semen that habitually shows poor post-storage fertilization capacity.

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چکیده

تکنیک‌های انتخاب اسپرم یکی از ابزارهای مهم در بهبود انزال‌های کم بارور است. هدف اصلی در این مطالعه، دستیابی به دینامیک زیرجمعیت‌های اسپرم متحرک از انزال‌های اخذ شده از نریان‌های کم بارور با استفاده از سانتریفیوژ گرادیان چگالی (DGC) و یک کلویید دو لایه (DGC2)، پیش و پس از ۲۴ ساعت و ۴۸ ساعت ذخیره است. انزال‌های اخذ شده از ۸ نریان دارای مشکلات باروری در تیمارهای مختلف مورد استفاده قرار گرفتند. تیمارها شامل کنترل (C)، سانتریفیوژ (CT)، سانتریفیوژ گرادیان چگالی همراه با کلویید دولایه (DGC2) و سانتیرفیوژ گرادیان چگالی همراه با کلویید دو لایه پس از چگالی (DGC post) بودند. در تیمارهای C، CT و DGC2، متغیرهای اسپرم در ۰، ۲۴ و ۴۸ ساعت پس از ذخیره در ۴ تا ۷ درجه سلسیوس مورد آنالیز قرار گرفت. در تیمار DGC2 post منی در ۴ تا ۷ درجه سلسیوس ذخیره، و نمونه‌های اسپرم با کمک DGC2 در ۲۴ و ۴۸ ساعت انتخاب شدند. سپس زنده‌مانی، نقایص و تحرک اسپرم با کمک یک سامانه رایانه‌ای مورد سنجش قرار گرفت. ۴ زیرجمعیت اسپرم متحرک با خصوصیات تحرک متفاوت در کلیه تیمارها مشاهده شدند. اسپرم DGC2 سریع‌تر بوده و مستقیم‌تر حرکت کرده و دارای سلول‌های فعال‌تر در ساعت صفر بودند. ولی در ساعت ۲۴ و ۴۸، مقادیر متغیر تحرک کاهش یافته و اندازه زیرجمعیت‌های اسپرم کُندتر افزایش یافتند. تیمار DGC2 post مقادیر متغیر تحرک بهتری را در ساعت ۲۴ و ۴۸ نشان داده و درصد بیشتری از اسپرم‌ها در زیرجمعیت‌های سریع‌تر قرار گرفتند. به طور کلی می‌توان نتیجه گرفت که DGC2 می‌تواند برای بهبود منی اخذ شده از نریان‌های کم بارور و منی‌هایی که باروری پایینی پس از ذخیره‌سازی نشان می‌دهند، به‌کار گرفته شود.

کلمات کلیدی سانتریفیوژ گرادیان چگالی، EquiPure^{TM}، انتخاب اسپرم، زیرجمعیت‌های اسپرم، نریان‌های کم بارور.

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