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Animal Reproduction Science 77 (2003) 213–221

ANIMAL REPRODUCTION SCIENCE

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Seasonal changes of semen quality and freezability in Franches–Montagnes stallions

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Received 9 April 2002; received in revised form 26 November 2002; accepted 20 December 2002

Abstract

The objective of this study was to investigate seasonal changes of semen quality parameters in Franches–Montagnes stallions and to compare the freezability of ejaculates collected in autumn and winter. Experiments were performed using 15 stallions from the National Stud Farm in Avenches (Switzerland). Ejaculates were collected and evaluated every month during 1 year as well as cryopreserved in autumn and winter (September to February). In fresh semen the gel-free volume, concentration, motility and morphology (normal sperm, major defects, vacuoles and acrosome defects) were evaluated and in frozen-thawed semen the motility as well as the viability (SYBR-14/PI) were performed. To analyse seasonal differences four periods of 3 months each were defined as autumn (September, October, November), winter (December, January, February), spring (March, April, May) and summer (June, July, August). During the 1-year experiment all fresh semen quality parameters demonstrated a clear seasonal and individual pattern. The gel-free volume was significantly ($P < 0.05$) higher in spring and summer compared to autumn and winter while sperm concentration was significantly ($P < 0.05$) lower in spring than at any other time of the year. Total sperm number was significantly ($P < 0.05$) higher and sperm motility significantly ($P < 0.05$) lower in summer than in other seasons. Regarding sperm morphology, normal sperm was significantly ($P < 0.05$) higher in autumn than in winter and summer and major defects were lowest ($P < 0.05$) in autumn. In frozen-thawed semen motility was significantly ($P < 0.05$) improved in the ejaculates collected in autumn compared to winter, while viability showed no obvious differences. Our results clearly demonstrate that individual and seasonal differences occurred in semen quality of Franches–Montagnes stallions. Ejaculates collected in autumn (September, October, November) demonstrated good quality, especially regarding sperm morphology, and were more suitable for cryopreservation because of better motility in frozen-thawed semen collected during autumn than in winter.

Keywords: Horse; Seasonal dynamics; Semen quality; Sperm morphology; Cryopreservation

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0378-4320/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0378-4320(03)00039-3

1. Introduction

The use of artificial insemination (AI) in equine reproduction has been increasing worldwide. In Switzerland, more than 60% of mares are bred by AI, 30% of them using frozen semen. Although pregnancy rates have been shown to be equal or even higher after AI with fresh or chilled semen compared to natural service, lower fertility occurs when mares are inseminated with frozen semen (Samper, 2000, 2001). One of the prerequisites for successful preservation of stallion semen is high initial quality that has been shown to differ considerably between individual stallions (Pickett et al., 1976; Magistrini et al., 1987; Dowsett and Knott, 1996). In the northern Hemisphere, the physiological breeding season lasts from April to October and during this time sperm production (Johnson and Thompson, 1983; Johnson, 1985, 1991) and endocrine testicular function (Harris et al., 1982; Johnson and Thompson, 1983; Clay and Clay, 1992; Byers et al., 1883; Nagata et al., 1998; Hoffmann and Landeck, 1999; Gerlach and Aurich, 2000) are increased. Semen for cryopreservation, however, is usually collected during the non-breeding season (November to February) when sexual activity is low, possibly influencing semen quality. In general, only little information is available about seasonal changes of stallion semen quality and freezability and in the Franches–Montagnes, the only indigenous Swiss horse breed, such data are completely lacking. Therefore, the objective of this study was to investigate seasonal changes of semen quality in Franches–Montagnes stallions and to compare the freezability of ejaculates collected in autumn and winter.

2. Material and methods

2.1. Animals

Experiments were performed using 15 healthy Franches–Montagnes stallions of proven fertility from the National Stud Farm in Avenches. The animals were fed hay, oats and pellets supplemented with minerals. All animals were daily exercised for at least 1 h. Before starting the experiment the stallions were trained to mount the phantom and extragonadal sperm reserves were equilibrated by five daily semen collections to reduce the chance to obtain aged spermatozoa. Semen was collected and evaluated every month during 1 year from September 1997 to August 1998 as well as cryopreserved in autumn and winter (September to February). During the breeding season (March to July) all stallions were used in natural service and the ejaculatory frequency could therefore not be standardised. In the non-breeding season (August to February), ejaculatory frequency was standardised by one weekly semen collection.

2.2. Semen processing and examination

After filtration of the ejaculate, the volume, the concentration and motility (Cell Motion Analyser SM-CMA-1074, MTM, Switzerland) were determined. For morphological examination five drops of fresh semen were fixed in 2 ml Hancock solution and smears prepared according to the method of Hancock (1957). For classification of sperm cells in major and

minor defects (Blom, 1973; Jasko et al., 1990) at least 200 spermatozoa were evaluated by phase contrast microscopy (Olympus BX50, UplanFl 100×/1.30). The gel-free semen was diluted with centrifugation medium (INRA 82-Hepes + 2% egg yolk) to a concentration of 100×10^6 spermatozoa/ml and centrifuged at $1000 \times g$ for 2 min. After centrifugation, the supernatant was removed and the sperm pellet resuspended in freezing medium (lactose solution 11%, egg yolk 20%, glycerol 5%) to a final concentration of 300×10^6 spermatozoa/ml. Thereafter, semen was cooled to 4 °C during at least 20 min, packaged into 0.5 ml straws and frozen in an automatic freezer (Minidigitcool 700 ZB 290, IMV, France) at a cooling rate of 60 °C/min from +4 to −100 °C and 30 °C/min from −100 to −140 °C. The straws were then stored in liquid nitrogen until examination.

From each ejaculate three straws were thawed (37 °C, 30 s), pooled and diluted with 3 ml centrifugation medium to determine the motility and viability. Viability was performed by dual DNA staining (LIVE/DEAD® Sperm Viability Kit, Molecular Probes Europe, Leiden, NL) using SYBR-14 in combination with propidium iodide (PI). SYBR-14 (component A) was diluted with anhydrous dimethyl sulfoxide (DMSO) 1:10 (SYBR-14 working solution), while PI (component B) was used in original concentration. One micrometer of the SYBR-14 working solution was added to 1 ml of diluted thawed semen. After incubation of 10 min at 37 °C, 5 μl of component B were added. Five minutes later, 5 μl of stained semen were placed on a slide, covered with a coverslip (24 mm × 24 mm) and examined using fluorescence microscopy (Olympus BX50, UPlanApo 40×/0.85, FITC filter U-MWIB, high pressure Hg-lamp). Different sequences of fluorescence stained spermatozoa were monitored by connecting a video camera (SANYO VCC-2972) to the microscope. At least 500 sperm cells were counted on the screen and the percentage of green fluorescing spermatozoa was defined as viability (Garner et al., 1994).

2.3. Statistical analyses

Data were analysed using StatView 5.0 and JMP 2.0 software program (both SAS Institute, Switzerland). A multivariate analysis of variance (ANOVA) with repeated measures was carried out to analyse the effects of stallion (within-effect) and time (between-effect) of semen collection. To determine seasonal changes in semen quality parameters, four periods of 3 months each were defined as autumn (September, October, November), winter (December, January, February), spring (March, April, May) and summer (June, July, August). Seasonal means were compared with Fisher's post hoc test. Correlations between pre- and post-freeze motility as well as viability were calculated by Fishers *r*- to *z*-test. Values were considered to be statistically significant at $P < 0.05$.

3. Results

The results from ANOVA (Table 1) clearly demonstrate that the time of semen collection significantly ($P < 0.05$) influenced all quality parameters except the viability of thawed-frozen semen. The influence of stallion on semen quality was highly significant ($P < 0.05$) for all parameters examined. The large individual variation of fresh semen motility and major defects during 1 year are shown in Figs. 1 and 2.

Table 1
Effect of stallion ($n = 15$) and time of semen collection ($n = 12$) on quality parameters of ejaculates obtained monthly during 1 year

Parameter	Stallion-effect (P)	Time-effect (P)
Volume (ml)	<0.0001	<0.0001
Concentration ($\times 10^6$/ml)	<0.0001	<0.0001
Total sperm ($\times 10^9$)	<0.0001	0.0003
Motility fresh semen (%)	<0.0001	<0.0001
Normal spermatozoa (%)	<0.0001	<0.0001
Major defects (%)	<0.0001	<0.0001
Acrosome defects (%)	<0.0001	0.0002
Vacuoles (%)	<0.0001	<0.0001
Motility frozen-thawed semen (%)	<0.0001	<0.0001
Viability (%)	<0.0001	0.8042

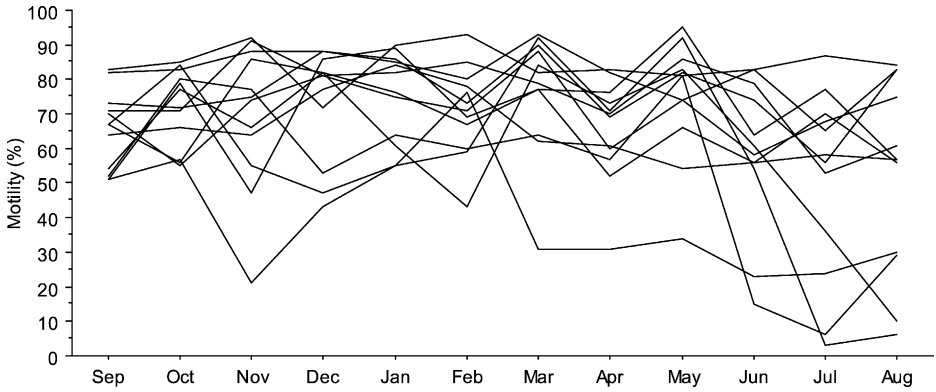

Fig. 1. Individual variation of fresh semen motility in 12 stallions during 1 year.

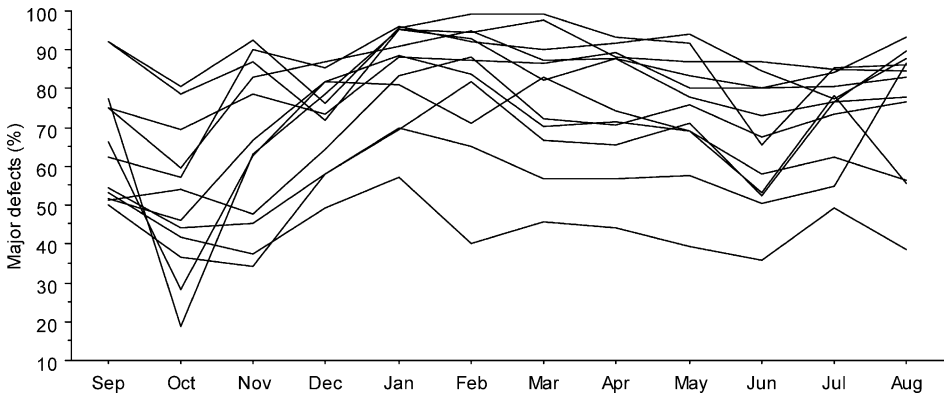

Fig. 2. Individual variation of major sperm defects in 12 stallions during 1 year.

Table 2
Means ($m \pm$ S.E.M.) of fresh semen characteristics from ejaculates collected monthly during autumn, winter, spring and summer in Franches–Montagnes stallions ($n = 12$)

Parameter	Autumn ($m \pm$ S.E.M.)	Winter ($m \pm$ S.E.M.)	Spring ($m \pm$ S.E.M.)	Summer ($m \pm$ S.E.M.)
Volume (ml)	27.6 ± 2.6 a	20.5 ± 2.2 a	45.5 ± 3.1 b	40.3 ± 3.5 b
Concentration (10^6/ml)	270.5 ± 17.2 ac	297.0 ± 14.4 a	120.3 ± 9.4 b	231.9 ± 18.0 c
Total sperm ($\times 10^9$)	6.5 ± 0.5 a	5.4 ± 0.4 a	5.6 ± 0.7 a	8.3 ± 0.7 b
Motility fresh semen (%)	69.1 ± 2.5 a	73.2 ± 2.3 a	72.4 ± 2.8 a	53.9 ± 4.2 b
Normal spermatozoa (%)	22.3 ± 2.3 a	11.8 ± 1.6 b	18.5 ± 2.0 ac	16.9 ± 2.4 bc
Major defects (%)	63.4 ± 2.9 a	81.0 ± 2.2 b	77.4 ± 2.2 bc	73.8 ± 2.3 c
Acrosome defects (%)	8.7 ± 1.3 a	11.8 ± 1.7 b	12.6 ± 1.6 b	9.7 ± 1.4 ac
Vacuoles (%)	25.5 ± 2.6 a	36.9 ± 2.8 b	32.8 ± 2.5 b	27.4 ± 2.5 ac

Means with different letters (a, b, c) are significantly different ($P < 0.05$).

Seasonal differences of fresh semen quality parameters are shown in Table 2. During the 1-year experiment all parameters showed a distinct seasonal pattern. The gel-free volume was significantly ($P < 0.05$) higher in spring and summer compared to autumn and winter while sperm concentration was significantly ($P < 0.05$) lower in spring than at any other time of the year. Total sperm number was significantly ($P < 0.05$) higher and sperm motility significantly ($P < 0.05$) lower in summer than in other seasons. Regarding sperm morphology, normal sperm was significantly ($P < 0.05$) higher in autumn than in winter and summer and major defects were significantly ($P < 0.05$) lower in autumn than in winter, spring and summer.

Comparing means of frozen-thawed semen parameters from ejaculates obtained in autumn and winter, motility was significantly ($P < 0.05$) improved in autumn, while viability showed no difference (Table 3). The individual changes of motility in frozen-thawed semen during the months of September to February are shown in Fig. 3.

Looking at interrelationship, post-freeze motility as well as viability were significantly correlated ($P < 0.0001$) with pre-freeze motility (Table 4).

Table 3
Means ($m \pm$ S.E.M.) of motility and viability of frozen-thawed semen from ejaculates collected monthly during autumn and winter in Franches–Montagnes stallions ($n = 13$)

Parameter	Autumn ($m \pm$ S.E.M.)	Winter ($m \pm$ S.E.M.)
Motility frozen-thawed semen (%)	39.0 ± 2.3 a	32.1 ± 1.6 b
Viability (%)	64.2 ± 1.5 a	65.8 ± 1.4 b

Means with different letters (a, b) are significantly different ($P < 0.05$).

Table 4
Correlation values between motility and viability in pre- and post-freeze semen

	Post-freeze motility (r)	Post-freeze viability (r)
Pre-freeze motility	0.56[a]	0.63[a]
Post-freeze motility		0.60[a]

[a] Significant difference ($P < 0.0001$).

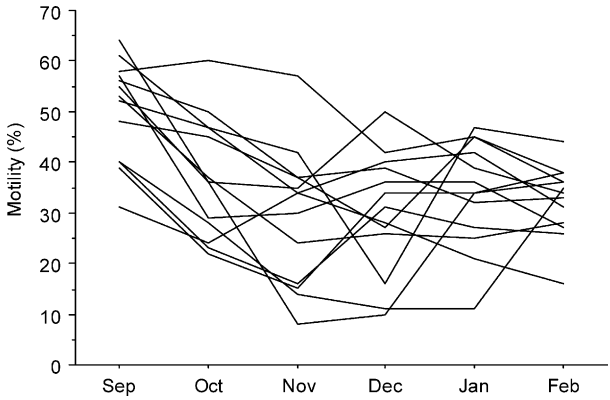

Fig. 3. Individual variation of post-freeze motility in 13 stallions during autumn and winter.

4. Discussion

Results of our study demonstrate the presence of significant seasonal differences of all fresh semen quality parameters investigated in Franches–Montagnes. A high total sperm number was observed during summer which agrees with reports from the USA (Pickett et al., 1976; Jasko et al., 1991) also showing highest values in July and August, but disagrees with those from Europe (Magistrini et al., 1987) where a clear annual pattern was absent. Regarding sperm concentration, significantly higher values were seen in winter than in spring and summer. Our results contrast earlier findings in which highest and lowest concentrations were found in autumn and summer (Magistrini et al., 1987) or in August and December (Pickett et al., 1976; Jasko et al., 1991), respectively. The gel-free volume was negatively related to semen concentration as previously reported (Pickett et al., 1976; Magistrini et al., 1987). The ejaculate volume was clearly increased in spring and summer compared to autumn and winter which was also observed by Magistrini et al. (1987), while Pickett et al. (1976) and Jasko et al. (1991) reported highest values in July and March and lowest in August and December, respectively. These deviating results obtained by various researchers may either be caused by differences in latitude, stallion management or frequency of semen collection. In addition, the number of experimental animals as well as breed differences (Colenbrander et al., 1992; Dowsett and Knott, 1996) must be considered. The increase of total sperm and ejaculate volume during summer might be caused by changes in photoperiodicity. In the stallion testicular endocrine activity (Harris et al., 1982; Johnson and Thompson, 1983; Hoffmann and Landeck, 1999; Gerlach and Aurich, 2000) and the secretion of accessory glands are stimulated by increasing daylength. Increased sperm production during the breeding season has been explained by an elevated population of spermatogonia (Johnson, 1985, 1991).

Progressive sperm motility together with total sperm and sperm morphology are known to be important parameters for breeding soundness evaluation (Hurtgen, 1992). It has been shown that subfertile stallions had clearly lower sperm motility than normal fertile stallions (Jasko et al., 1992). Sperm motility measured in our Franches–Montagnes stallions showed

significantly lower values in summer than in other seasons. These results differ from earlier investigations where seasonal motility changes were either absent (Pickett et al., 1976) or showed only a tendency of lower values in winter (Jasko et al., 1991) or a significantly better motility in summer (Magistrini et al., 1987). In a recent study, Blottner et al. (2001) found a higher motility in December than in May. To what extend seasonal changes in sperm motility will be influenced by breed and ambient conditions (temperature, humidity, etc.) needs further investigation. In this context, seasonal changes in seminal plasma constituents (Gebauer et al., 1976) may also play an important role. Moreover, it has also been shown that small and high seminal plasma volumes will negatively affect sperm motility (Pickett et al., 1975).

A clear seasonal pattern was obvious in sperm morphology. The percentage of normal spermatozoa was higher in autumn than in winter and summer and major defects were lowest in autumn. These findings differ from those obtained at breeding soundness evaluation in the Netherlands (Van der Holst, 1975) showing a higher incidence of abnormal spermatozoa in autumn and winter. This contrasts to results of Blottner et al. (2001) reporting a higher percentage of morphological intact spermatozoa in December than in May. According to Hurtgen (1992), highly fertile stallions have more than 60% morphologically normal spermatozoa and less than 5% acrosome and mid-piece abnormalities. Our stallions did not fulfil these requirements, as the percentage of abnormal spermatozoa was surprisingly high during the whole year. Sperm morphology is known to be an important criterion in breeding soundness evaluation and a negative correlation between the percentage of spermatozoa with major defects and fertility was reported by Jasko et al. (1990) and Love et al. (2000). Our stallions however had normal foaling rates between 61–89% in natural service despite of a high number of sperm abnormalities. This may be explained by the large number of ejaculated spermatozoa compensating for the high proportion of defects. On the other hand, conception will most likely be compromised when reducing the insemination dose in AI. Although the Franches–Montagnes are known to be a highly fertile breed, improvement of semen quality should be an important goal in future breeding strategies.

During autumn and winter all ejaculates were cryopreserved and in all frozen-thawed samples the motility as well as the viability (SYBR-14/PI) were measured. A discrepancy in motility was noticed between fresh and frozen-thawed semen. Motility from frozen-thawed semen in autumn was significantly better than in winter although in fresh semen an opposite tendency was apparent. This observation is not in agreement with Magistrini et al. (1987) who found best motility after freezing thawing in winter when the motility of fresh semen was lower. In contrast to motility, viability of frozen-thawed semen showed no obvious changes whether measured with SYBR-14/PI as in this study ore using eosin-nigrosin staining (Magistrini et al., 1987).

The highly significant correlations between post- and pre-freeze motility observed in our study confirms the assumption that semen with higher motility will freeze better. Therefore, fresh semen motility represents an important criterion for the selection of stallions intended to be used for frozen-semen artificial insemination. As considerable variation exists not only between but also within stallions the best time for freezing semen must individually be found.

In conclusion, our results demonstrate the existence of individual and seasonal differences in semen quality of Franches–Montagnes stallions. Ejaculates collected in autumn

(September, October, November) demonstrated good quality, especially regarding sperm morphology, and were more suitable for cryopreservation because of better motility in frozen-thawed semen collected in autumn than in winter.

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