NOTE *Theriogenology*

Spermatozoa Morphology during the Breeding Season in Thoroughbred Stallions in Japan

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(Received 19 September 2007/Accepted 30 May 2008)

ABSTRACT. The morphology of spermatozoa of modern Thoroughbred stallions in Japan was investigated during the breeding season. A total of 299 semen samples were collected from the penises of 16 stallions immediately after service. The rate of abnormalities in sperm heads and tails, spermatozoa with cytoplasmic droplets and slides with medusa cells to total observed slides in each stallion were 3.9 ± 2.1%, 11.5 ± 5.9%, 2.4 ± 2.6% and 20.1%, respectively. The values for the area, length, width and aspect ratio of the stallion sperm head were 12.54 ± 1.34 μm^2, 5.93 ± 0.40 μm, 2.69 ± 0.21 μm and 0.46 ± 0.05, respectively. With the exception of medusa cells, the features were significantly different among the stallions ($P<0.05$).

KEY WORDS: morphology, spermatozoa, stallion.

J. Vet. Med. Sci. 70(10): 1121–1124, 2008

The breeding stallion for Thoroughbred racehorses is prized for the chance of siring future champions. The number and quality of his prodigy depend, however, on the quality of his spermatozoa. Morphological features of the spermatozoa provide useful criterion for assessing the quality of semen and clinical value for informing veterinary decisions [6]. Jasko *et al.* reported that the rate of spermatozoa with normal morphology correlates positively with the rate of fertility [16]. Wide variation in the size and shape of spermatozoa occurs in virtually all mammalian species, however, and an abundance of abnormalities can be found. The sperm head has been reported to be larger in semen taken from subfertile stallions than in that from fertile stallions [6, 14]. The converse has also been reported [17], and variations in size of the sperm head have been attributed to the staining methods of the studies [4, 5, 15, 22]. The staining method has also influenced the ratio of morphologically abnormal spermatozoa to the total sperm count [18, 21]. These problems remain unsolved.

For a popular stallion, the number of services may exceed 200 in a single breeding season. A previous study showed in Thoroughbreds engaged in five services every hour that, in spite of decreases in volume, number or concentration of spermatozoa, sperm viability was not different in the ejaculation repeats [23].

The morphology of the spermatozoa has not been investigated in Thoroughbred stallions during the breeding season in Japan. We hypothesized that if the ejaculated semen could be examined from time to time throughout the breeding season, the semen would exhibit morphological differences and allow clarification of abnormalities in the spermatozoa produced during that critical period in the work of the stallions. Morphological data on semen ejaculated throughout the breeding season would provide a reference index for judging the normal morphology of spermatozoa to estimate reproductive activity at copulation. To clarify the morphology of spermatozoa from Thoroughbred stallions servicing a large number of mares during the breeding season, in the present study we examined spermatozoa collected from the semen remaining in the urethra of the penis immediately after the stallion dismounted from servicing since sperm head measurements from dismount semen are representative of those of the ejaculate [13].

The study was comprised 16 Thoroughbred stallions in Hokkaido within age range of 4 to 15 (mean ± SD, 8.9 ± 3.2) years and a servicing range of 69 to 412 (215 ± 104) times per breeding season. Immediately after a service each week during the breeding season, February to July 2001, semen was collected from the flaccid penis and mounted on a glass slide for examination by light microscopy. Briefly, 3 μl of semen were smeared onto the slide, air-dried and kept at room temperature until staining with hematoxylin and eosin, and each slide preparation was completed with addition of mounting medium and a cover glass.

The spermatozoa were examined with a light microscope (Olympus, BX51TF, Tokyo, Japan) at magnification of 1,000 ×. Abnormal spermatozoa were divided according to criteria of Dott [9], and the spermatozoa displaying cytoplasmic droplets were not categorized as having abnormal morphology but were grouped separately. Medusa cells were counted if two or more were found in 100 spermatozoa.

Micrographs of the stained spermatozoa were taken with a camera (Olympus, C-5060 Wide Zoom, Tokyo, Japan) fitted on the light microscope, transferred to a computer and edited with Photoshop (Adobe, Photoshop 6.0, San Jose, CA, U.S.A). The areas, lengths and widths of the heads of 20 normal spermatozoa were measured with Scion Image

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(Scion Image ver. 2, Yodosha, Tokyo, Japan), a semi-automated software for analyzing morphology, and the aspect ratio (width/length) was calculated.

One-way analysis of variance (ANOVA) was used to compare averages, and the Chi-square test was used to compare the presence and absence of medusa cells among the stallions. *P*-values of less than 0.05 were considered significant.

Significant ($P<0.05$) anomalies among the stallions were found in sperm heads at a rate of 3.9 ± 2.1 (range 0–11), in sperm tails at 11.5 ± 5.9 (2–36) and in spermatozoa with cytoplasmic droplets at 2.4 ± 2.6 (0–17) (Table 1). Medusa cells were present at a rate of 20.1% (range: 0–42.9%) but this feature was not significantly different (Table 1).

Significant anomalies ($P<0.05$) were also found in the areas (12.54 ± 1.34 μm^2; range: 8.06–17.01), lengths (5.93 ± 0.40 μm; range: 3.99–7.65), widths (2.69 ± 0.21 μm; range: 1.98–3.55) and aspect ratios (0.46 ± 0.05; range: 0.29–0.89) of sperm heads among the stallions, respectively (Table 2).

The results of this study support our hypothesis that dur-

Table 1. The rates of the abnormalities in sperm heads and tails spermatozoa with cytoplasmic droplets and numbers of slides with medusa cells in stallions

Stallion No.	Slides[a)]	Abnormality		Droplets[b)]*	Medusa[c)] (%)
		Head*	Tail*		
1	22	4.9 ± 1.2	10.6 ± 2.5	3.0 ± 1.9	4 (18.2)
2	20	2.0 ± 1.4	9.0 ± 4.6	1.3 ± 2.2	6 (30.0)
3	17	2.9 ± 1.8	7.8 ± 2.2	0.4 ± 0.6	3 (17.6)
4	20	3.4 ± 1.5	7.4 ± 3.0	0.7 ± 1.1	0 (0)
5	17	2.3 ± 1.8	9.6 ± 3.9	1.2 ± 1.1	4 (23.5)
6	15	4.2 ± 1.5	15.8 ± 3.5	3.8 ± 2.5	4 (26.7)
7	21	3.6 ± 1.3	13.0 ± 3.0	3.9 ± 2.6	5 (23.8)
8	21	5.3 ± 2.2	21.1 ± 5.3	1.6 ± 1.4	9 (42.9)
9	15	5.9 ± 2.0	8.5 ± 2.3	4.7 ± 3.3	4 (26.7)
10	21	4.8 ± 2.5	15.4 ± 6.7	4.1 ± 4.3	3 (14.3)
11	15	1.5 ± 1.2	5.3 ± 2.5	2.7 ± 1.8	3 (20.0)
12	21	6.1 ± 1.9	6.0 ± 3.1	2.8 ± 2.1	3 (14.3)
13	22	3.7 ± 1.3	12.7 ± 3.0	3.4 ± 3.2	7 (31.8)
14	14	3.6 ± 1.5	13.9 ± 3.5	2.0 ± 1.9	1 (7.1)
15	21	3.8 ± 2.0	7.7 ± 3.0	1.0 ± 1.2	4 (19.0)
16	17	3.1 ± 1.7	19.8 ± 3.8	2.8 ± 2.8	0 (0)
Total	299	3.9 ± 2.1	11.5 ± 5.9	2.4 ± 2.6	60 (20.1)

a) The number of slides investigated.
b) The rate of spermatozoa with cytoplasmic droplets.
c) The number of slides showing medusa cells. (%) : Percentage of slides showing medusa cells among the total slides examined.
* : Significantly different among the stallions ($P<0.05$, ANOVA).

Table 2. Characteristics of sperm heads in the 16 Thoroughbred stallions

Stallion No.	Sperm[a)]	Area (μm^2) *	Length (μm)*	Width (μm)*	Aspect ratio[b)]*
1	440	12.49 ± 1.31	6.03 ± 0.40	2.64 ± 0.20	0.44 ± 0.05
2	400	11.98 ± 1.14	5.69 ± 0.32	2.68 ± 0.16	0.47 ± 0.03
3	340	13.03 ± 1.10	5.85 ± 0.31	2.83 ± 0.15	0.49 ± 0.03
4	400	11.87 ± 1.01	6.05 ± 0.31	2.50 ± 0.15	0.41 ± 0.03
5	340	12.51 ± 1.10	5.99 ± 0.31	2.66 ± 0.17	0.44 ± 0.04
6	300	11.17 ± 1.00	5.42 ± 0.30	2.62 ± 0.17	0.49 ± 0.04
7	420	12.95 ± 1.38	6.09 ± 0.37	2.70 ± 0.19	0.44 ± 0.04
8	420	11.63 ± 1.21	5.95 ± 0.33	2.49 ± 0.18	0.42 ± 0.03
9	300	12.80 ± 1.21	5.93 ± 0.35	2.75 ± 0.20	0.47 ± 0.04
10	420	12.96 ± 1.11	6.12 ± 0.33	2.70 ± 0.18	0.44 ± 0.04
11	300	13.63 ± 1.08	5.71 ± 0.28	3.04 ± 0.16	0.53 ± 0.03
12	420	12.73 ± 1.21	6.02 ± 0.32	2.69 ± 0.18	0.45 ± 0.03
13	440	13.00 ± 1.32	5.96 ± 0.34	2.77 ± 0.19	0.47 ± 0.03
14	280	11.58 ± 1.01	5.35 ± 0.27	2.76 ± 0.17	0.52 ± 0.03
15	420	13.43 ± 1.09	6.28 ± 0.32	2.72 ± 0.18	0.43 ± 0.04
16	340	12.62 ± 1.19	5.97 ± 0.32	2.69 ± 0.16	0.45 ± 0.03
Total	5980	12.54 ± 1.34	5.93 ± 0.40	2.69 ± 0.21	0.46 ± 0.05

a) The number of spermatozoa measured.
b) Length / width of sperm heads.
* : Significantly different among the stallions ($P<0.05$, ANOVA).

ing the breeding season the semen ejaculated by Thoroughbred stallions at a service does at times contain morphologically abnormal spermatozoa. The rate of abnormalities in sperm tails was almost five times as great as the rate of abnormalities in sperm heads. Collectively, the results of the present study provide a starting point toward establishing a reference index for informing future studies and improving management of stallions in case that overwork has any bearing on the morphological quality of spermatozoa.

The rate of abnormalities in sperm heads and tails and spermatozoa with cytoplasmic droplets per 100 spermatozoa in the present study were different from those in previous reports [10, 11, 16, 21, 24]; on the other hand, there are some reports with the same results [2, 20]. This paper also showed that there are more abnormalities in sperm tails than in sperm heads [2, 10, 11, 20, 21]. Some sperm tails generally have a morphological curve and refraction [19]. The rate of spermatozoa with cytoplasmic droplets was lower in the present study [10, 11, 16, 21, 24]. However, the rate of those in the ejaculate collected at 1–6 hr between services was nearly the same as the rate in the present study [11]. The number of medusa cells was investigated in a large number of ejaculates, especially those of stallions with a large number of services, to determine whether they contained two or more cells during observation of each sample. In the horse, the rate of appearance of the medusa cell is normally greater than that in bulls, with about one per 10,000 spermatozoa [3]. Therefore, we decided that the number of services was related to the appearance of medusa cells.

The values for area, length, width and aspect ratio of sperm heads in the stallions were determined using H.E. staining. Previous studies have reported that the values depend on the staining method [4, 5, 15, 22]. Therefore, our values were similar to or different from those in some previous studies [1, 7, 8, 12, 14]. The aspect ratios reported in the previous data were, however, independent of the staining method [1, 6, 7, 14]. However the aspect ratio in our study was lower than in previous reports. The stallions used in present study had run and trained as racehorses from two years of age until retirement. After that, the horses that had achieved excellent results were selected as breeding stallions and were assigned a large number of mares to service. These stallions have been kept under special care. A large number of services might lead to a change in the aspect ratio of sperm heads in stallions. Further study is needed to shed light on the reason for this.

In conclusion, the morphological quality of the ejaculated spermatozoa of Thoroughbred racehorse champions used for breeding purposes in Japan varies considerably from service to service during the breeding season. The results of our study suggest that a heavy schedule of multiple services in active breeding stallions may have no degenerating effect on the morphological quality of spermatozoa. Further study is necessary to elucidate whether morphological quality affects infertility.

REFERENCES

1. Ball, B. A. and Mohammed, H. O. 1995. Morphometry of stallion spermatozoa by computer-assisted image analysis. *Theriogenology* **44**: 367–377.
2. Bielanski, W. 1950. Characteristics of the semen of stallions. Macro- and microscopic investigations with estimation of fertility. *Mem. Acad. Pol. Sci.* **16**: 1–58.
3. Blom, E. 1950. Interpretation of spermatic cytology in bulls. *Fertil. Steril.* **1**: 223–238.
4. Boersma, A., Braun, J. and Stolla, R. 1999. Influence of random factors and two different staining procedures on Computer-assisted sperm head morphometry in bulls. *Reprod. Dom. Anim.* **34**: 77–82.
5. Boersma, A., Ra?hofer, R. and Stolla, R. 2001. Influence of sample preparation, staining procedure and analysis conditions on bull sperm head morphometry using the morphology analyzer integrated visual optical system. *Reprod. Dom. Anim.* **36**: 222–229.
6. Casey, P. J., Gravance, C. G., Davis, R. O., Chabot, D. D. and Liu, I. K. M. 1997. Morphometric differences in sperm head dimensions of fertile and subfertile stallions. *Theriogenology* **47**: 575–582.
7. Davis, R. O., Gravance, C. G. and Casey, P. J. 1993. Automated morphometric analysis of stallion spermatozoa. *Am. J. Vet. Res.* **54**: 1808–1811.
8. Deka, N. N., Batabyal, A. K. and Singh, R. P. 1985. Biophysical characteristics of Murrah buffalo spermatozoa. *Livestock Adviser* **10**: 56–59.
9. Dott, H. M. 1975. Morphology of stallion spermatozoa. *J. Reprod. Fertil. (Suppl.)* **23**: 41–46.
10. Dowsett, K. F. and Knott, L. M. 1996. The influence of age and breed on stallion semen. *Theriogenology* **46**: 397–412.
11. Dowsett, K. F. and Knott, L. M. 1997. The effect of service frequency and season of year on stallion semen. *Aust. Equine Vet.* **15**: 16–26.
12. Garner, D. L. 2006. Flow cytometric sexing of mammalian sperm. *Theriogenology* **65**: 943–957.
13. Gravance, C. G., Champion, Z., Liu, I. K. M. and Casey, P. J. 1997. Sperm head morphometry analysis of ejaculate and dismount stallion semen sample. *Anim. Reprod. Sci.* **47**: 149–155.
14. Gravance, C. G., Liu, I. K. M., Davis, R. O., Hughes, J. P. and Casey, P. J. 1996. Quantification of normal head morphometry of stallion spermatozoa. *J. Reprod. Fertil.* **108**: 41–46.
15. Hidalgo, M., Rodriguez, I., Dorado, J. and Soler, C. 2005. Effect of sample size and staining methods on stallion sperm morphometry by the Sperm Class Analyzer. *Vet. Med. (Prague)* **50**: 24–32.
16. Jasko, D. J., Lein, D. H. and Foote, R. H. 1990. Determination of the relationship between sperm morphologic classification and fertility in stallion: 66 cases (1987–1988). *J. Am. Vet. Med. Assoc.* **197**: 389–394.
17. Kodagali, S. B., Bhavsar, B. K. and Deshpande, A. D. 1973. Biometrics of Surti buffalo spermatozoa. *Indian Vet. J.* **50**: 50–54.
18. Kustritz, M. V. R., Olson, P. N., Johnston, S. D. and Root, T. K. 1998. The effects of stains and investigators on assessment of morphology of canine spermatozoa. *J. Am. Anim. Hosp. Assoc.* **34**: 348–352.
19. Nishikawa, Y. 1972. General character of semen. pp. 198–222. *In*: Spermatozoa of Mammalian Animal (Nishikawa, Y. supervision. and Iida, I. eds.), Gakusosha, Tokyo (in Japanese).
20. Nishikawa, Y. 1959. pp. 188–193. Morphology of spermato-

zoa. *In*: Studies on Reproduction in Horses. Singularity and artificial control in reproductive phenomena. Japan Racing Association, Tokyo (in Japanese).
21. Nowakowska, I. and Pozor, M. A. 2003. Sperm morphology of stallions using four different methods. *Medycyna Weterynaryjna* **59**: 351–353.
22. Soler, C., Gadea, B., Soler, A. J., Fernandez-Esteso, M. R., Esteso, M. C., Nunez, J., Moreira, P. N., Nunez, M., Gutierrez, R., Sancho, M. and Garde, J. J. 2005. Comparison of three different staining methods for the assessment of epididymal red deer sperm morphometry by computerized analysis with ISAS-Reg. *Theriogenology* **64**: 1236–1243.
23. Squires, E. L., Pickett, B. W. and Amann, R. P. 1979. Effect of successive ejaculation on stallion seminal characteristics. *J. Reprod. Fertil. (Suppl.)* **27**: 7–12.
24. Voss, J. L., Pickett, B. W. and Squires, E. L. 1981. Stallion spermatozoal morphology and motility and their relationship to fertility. *J. Am. Vet. Med. Assoc.* **178**: 287–289.