Magnetic Orientations of Bull Sperm Separated into Head and Flagellum Treated by DTT or Heparin

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ABSTRACT : This paper describes the magnetic orientation of bull sperm separated into the head and the flagellum treated by DTT or heparin in a 5,400G static field. Semen samples collected from four bulls (*Japanese Black*) were mixed to the same sperm density. One percentage triton X-100 was used to extract the plasma membrane. The intact and demembranated sperm suspensions were treated with 20, 200, 2,000 mM DTT, 100, 1,000 or 10,000 units heparin solutions at 4℃ for 6 days. The decondensation of the sperm nuclei treated by DTT or heparin was examined by measuring the head area at 1, 3 and 6 days. After measuring the area, each sample was exposed to a 5,400G static magnetic field generated by Nd-Fe-B permanent magnets for 24 hours at room temperature. Results showed that the sperms were separated into the head and the flagellum through the DTT treatment. Almost of the separated heads showed that their long axis oriented perpendicularly to the magnetic lines of force, and most of the long axis perpendicularly oriented heads showed that their flat plane oriented perpendicularly in a 5,400G magnetic field. Also, the demembranation of the head tended to increase those perpendicular orientations, while those perpendicular orientations of the head declined with the decondensation of the sperm nuclei. These findings suggest that strong magnetic anisotropy for the perpendicular orientation of the long axis and the flat plane of the head occurs in the sperm nuclei in a 5,400G magnetic field. The separated flagellum showed lower parallel orientation, and the separated and demembranated flagellum showed parallel orientation to the magnetic lines of force in this magnetic field. These findings suggest that weak magnetic anisotropy of the parallel orientation of the flagellum occurs in the inside components in a 5,400G field. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 2 : 167-175*)

Key Words : Bull Sperm Nucleus, Head, Flagellum, DTT or Heparin, Magnetic Field, Orientation

INTRODUCTION

Several papers have reported the effects of weak magnetic fields on the activation and the viability of salmon sperm (Formicki et al., 1990), on the viability and motility of bull sperm (Shinjo and Utimura, 1992; Suga et al., 1995), on the freezing of boar sperm (Masuda, 1995) and bull sperm (Suga and Shinjo, 1997), but those mechanisms were not clear. Experiments exposing sperm to strong magnetic fields and the sperm orienting to the magnetic lines of force have also been conducted.

Magnetic orientations of sperm have been reported for human sperm (Kamei and Yokoyama, 1990) and bull sperm (Ashida et al., 1996; Suga et al., 1998a, b; Suga et al., 2000). Bull sperms orient on themselves each with its long axis perpendicular and the flat plane of its head perpendicular to the magnetic lines of force in strong magnetic fields. It has been thought that the mechanism of the orientation stemming from the magnetization of bull sperm head occurs in the chromatin, and then, the magnetic anisotropy induces the sperm to orient with its long axis perpendicular (Suga et al., 1998b). Therefore, it was considered that maintaining the structure of protamine in the chromatin is necessary for the head of the sperm to be oriented by magnetic fields with its flat plane perpendicular to the magnetic lines of force, while maintaining the crosslinks of DNA-protamine in the chromatin is necessary for the long axis of the sperm to be oriented perpendicularly to the magnetic lines of force (Suga et al., 2000).

The present investigation examines the different aspects of the direction of the magnetic orientation of the head and the flagellum, and the effects of the magnetic orientation on the disulfide bond or protamine of the sperm nuclei in the sperm head through the treatment with dithiothreitol or heparin alone.

MATERIALS AND METHODS

Semen preparation and treatment with DTT or Heparin

Materials and methods were similar to our previous reports (Suga et al., 2000). Semen samples were collected from four bulls (*Japanese Black*) held in the Okinawa Prefectural Livestock Experimental Station. Semen samples with the same sperm density were mixed in a plastic vial. The collection and mixing of semen samples was repeated six times. The mixed semen sample was carefully placed and settled in an incubator (CR-32c, Hitachi Ltd., Tokyo, Japan) at 4℃

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for about 1 day. After the settling of the semen, 100 μl of sperm suspensions were added to 2 ml of a salt solution (consisting of 150 mM NaCl, pH approx. 7.0 adjusted by small volumes of HCl and NaOH) or 2 ml of the salt solution containing 1% triton X-100 at room temperature (25℃) both for the intact sperm and the demembranated sperm. These two sperm suspensions were incubated under weak rocking in a water bath (BT-25, Yamato Scientific Co. Ltd., Tokyo, Japan) at 38℃ for 1 hour. After incubation, 100 μl of the intact and demembranated sperm suspensions were added to 2 ml of each of the salt solutions containing 20 mM dithiothreitol (DTT), 200 mM DTT, 2,000 mM DTT, 100 units heparin sodium (heparin), 1,000 units heparin, or 10,000 units heparin. These samples treated with DTT or heparin were incubated at 4℃ for 6 days.

Measurement of the sperm head area

The examinable method for nuclear-decondensed sperm was undertaken according to Motoishi et al. (1996). The decondensation of the intact sperm head and the demembranated sperm head treated with DTT or heparin alone was examined at 1, 3 and 6 days, before exposing the sperm samples to a static magnetic field. Fifty μl of these treated samples were mixed with 100 μl of 2% eosin B solution at room temperature (approximately 25℃). The mixture was applied to a slide and was covered with a cover glass. The length and width of the flat plane of the sperm head was randomly selected from 10 places on the slide. Sperm heads were examined in wet preparations under a differential interference contrast microscope equipped with a micrometer at 1,000× magnification. The surface area of the flat plane of each sperm head was calculated from the formula: area = {the long axis of sperm head × the short axis of sperm head × (π/4)} according to an ellipsoidal formula.

Exposure of bull sperm to a magnetic field

After measuring the head area, each sample was exposed to a 5,400G static magnetic field for 24 hours at room temperature (approximately 25℃). Methods of exposing bull sperm to a magnetic field were carried out according to our previous reports (Suga et al., 2000). Fifty μl of each of the treated samples were dropped on the slide chamber, and covered carefully with a cover glass in order that air bubbles did not occur in the slide chamber, and sealed with nail enamel to keep the sample fluid from flowing and evaporating.

The magnetic fields were generated by Nd-Fe-B permanent magnets. Wood stage was linked with two magnets between N and S poles. Mean and standard deviation of magnetic flux density and magnetic flux gradient were measured by the gauss meter (type TM-201, Kanetsu Kogyo Co., Ltd., Tokyo, Japan) at the center of the putting point of the chambers on the stages; the values were 5,487.5±83.45 G and 49.6±3.30 G/mm respectively. The slide chambers were put on the stages for 24 hours at room temperature (25℃) to expose the sperm to the magnetic field. Both of the control (non-exposed) chambers of the intact sample and the demembranated sample were put in a place unaffected by the artificial magnetic field in the room.

Measurement of magnetic oriented sperm

Classification of oriented sperm heads and flagella to the magnetic lines of force was similar to our previous reports (Suga et al., 1998b) and is shown in figure 1.

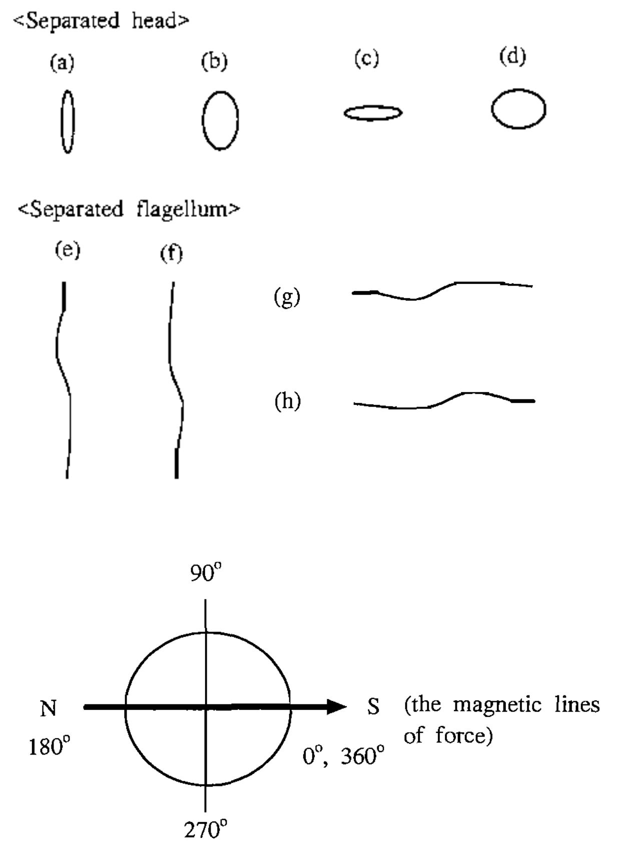


Figure 1. Classification of oriented sperm
(a) perpendicular, with sperm head perpendicular (Perp-Hperp)
(b) perpendicular, with sperm head parallel (Perp-Hpara)
(c) parallel, with sperm head perpendicular (Para-Hperp)
(d) parallel, with sperm head parallel (Para-Hpara)
(e) upward (Up), (f) downward (Down), (g) leftward (Left), (h) rightward (Right)

After exposing the sperm to the magnetic field for 24 hours, the slide chamber was put on a stage of a differential interference contrast microscope equipped with an angle micrometer. The orientations of the heads and the flagella were examined at 200× magnification at random fields of vision in a slide chamber. Measurement in the field of vision ended when the total number of sperms and either the heads or flagella became equal to or more than 1,000.

The orientations of the heads were classified into four sub-categories, which were (a) perpendicular, with the head perpendicular (Perp-Hperp), long axis of the head oriented on +45° <long axis< +135° and +225° <long axis< +315° with the flat plane of the head perpendicular to the magnetic lines of force; (b) perpendicular, with the head parallel (Perp-Hpara), long axis oriented on the same as (a) with the flat plane of its head parallel; (c) parallel, with the sperm head perpendicular (Para-Hperp), long axis oriented on +135° ≤long axis≤ +225 , +315° ≤long axis≤ +360° and 0° ≤long axis≤ +45° with the flat plane of its head perpendicular; (d) parallel, with the sperm head parallel (Para-Hpara), long axis oriented on the same as (c) with the flat plane of its head parallel.

The orientations of the flagella were classified into four sub-categories, which were (e) upward (Up), long axis of the flagellum where the upward point is the point separating head from the sperm, and downward is the end piece of flagellum, oriented on +45° < long axis< +135° to the magnetic lines of force; (f) downward (Down), long axis of the flagellum oriented on +225° <long axis< +315° ; (g) leftward (Left), long axis of the flagellum oriented on +135° ≤long axis≤ +225° ; (h) rightward (Right), long axis of the flagellum orients on +315° ≤long axis≤ +360° and 0° ≤long axis≤ +45°.

Statistical analysis

All data were analyzed using SAS (1990). With regard to the data of the head area, Duncan's multiple range test of GLM procedure was used to examine the difference among treatment levels by treatment lengths of time, or among treated lengths of time by treatment levels. The Chi-square test of FREQ procedure for equal population was used to analyze the orientation data, which was classified into two categories: percentages of the perpendicular orientation and percentages of the parallel orientation. Contrasts of CATMOD procedure were used to examine the difference among treatment levels of DTT or heparin by treatment lengths of time, or among treatment lengths of time by treatment levels. The total numbers of heads or flagella were divided by the number of replications, and then the values obtained (weighted means of the number of heads or flagella) were analyzed (Suga et al., 1998a, b; Suga et al., 2000).

RESULTS

Sperm head area

Means, standard errors (SE) and ranges of head area of separated sperm head are shown in table 1. Means and ranges of head area of the intact sample and the demembranated sample for the control and non-treated with DTT or heparin, and means and ranges of the intact and demembranated samples treated with heparin did not show remarkable change because of the treatment time. However, highs of maximum values or lows of minimum values were shown with treatment of DTT. Separated sperm head was decondensed with DTT (figure 2). Means of head area for the intact and demembranated samples treated with 2,000 mM DTT showed the smallest values in all treatment lengths of time (table 1), while highs of maximum values of the head area were shown with treatment of 20 mM and 200 mM DTT (table 1). These results were similar to the head area of non-separated sperm (Suga et al., 2000). Based on our results, it could be said that a small decondensed head similar to an atrophy of its short axis occurred through a treatment of a very high concentration of 2,000 mM DTT, and small and large (crumbly) decondensed heads occurred through treatment of 20 mM DTT and 200 mM DTT. The demembranation of the head slightly enhanced these small or large (crumbly) decondensations.

Magnetic orientation of sperm

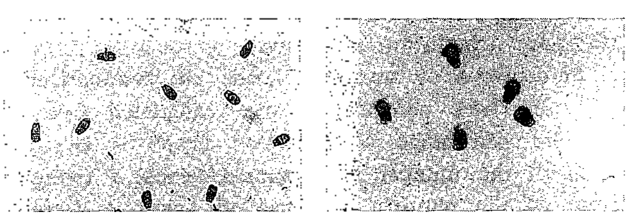
Percentages of separated heads are summarized in table 2. These percentages were calculated from the formula: percentage of separated heads = (weighted mean of the number of separated heads) / (weighted mean of the number of separated heads + weighted mean of the number of sperms)×100, which represented a percentage of separated heads to the total observation number (the number of sperms plus the number of separated heads). Weighted means of the number of sperms are quoted from our previous report (Suga et al., 2000, in table 2). The percentages of separated heads in the intact sample and the demembranated sample treated with DTT increased with an increase in treatment time, because the sperms were separated into the heads and the flagella with the DTT treatment. In particular, the low concentration of 20 mM DTT induced this separation. However, a clear separation of the sperm was not induced by the non-treatment or the heparin treatment.

Percentages of perpendicular (Perp) and parallel (Para) oriented heads are summarized in table 2. This table reclassified the sperm from four sub-categories

Table 1. Means and standard errors (n=60) of head area (μm^2) of intact or demembranated bull sperm heads after treatment with dithiothreitol (DTT) or heparin sodium for various lengths of time (days) before exposure to a magnetic field

Condition of the head	Concentration of DTT (mM) or heparin (units/ml)	Treatment time (days) of DTT or heparin					
		1		3		6	
		Mean±SE	Range	Mean±SE	Range	Mean±SE	Range
Intact							
Control		33.8 ± 0.58^{ab}	(25.1~39.3)	33.6 ± 0.62^{a}	(25.1~39.3)	32.9 ± 0.63^{ab}	(25.1~39.3)
Non-treated		33.5 ± 0.57^{ab}	(28.3~39.3)	33.2 ± 0.63^{a}	(25.1~39.3)	32.9 ± 0.55^{ab}	(25.1~39.3)
Treated with DTT							
	20 mM	33.2 ± 0.57^{ab}	(28.3~39.3)	32.9 ± 0.70^{a}	(25.1~51.8)	33.4 ± 0.87^{bc}	(16.5~51.8)
	200 mM	32.9 ± 0.63^{ab}	(25.1~39.3)	33.5 ± 0.63^{a}	(28.3~47.1)	33.1 ± 0.71^{ab}	(25.1~47.1)
	2,000 mM	32.3 ± 0.63^{a}	(21.2~39.3)	32.2 ± 0.63^{a}	(21.2~43.2)	31.1 ± 0.53^{a}	(21.2~39.3)
Treated with heparin							
	100 units	33.2 ± 0.61^{ab}	(28.3~39.3)	33.5 ± 0.59^{a}	(25.1~43.2)	34.2 ± 0.55^{bc}	(28.3~39.3)
	1,000 units	32.7 ± 0.61^{ab}	(28.3~39.3)	33.5 ± 0.59^{a}	(28.3~39.3)	33.6 ± 0.58^{bc}	(28.3~39.3)
	10,000 units	32.8 ± 0.60^{ab}	(28.3~39.3)	33.4 ± 0.59^{a}	(25.1~39.3)	33.5 ± 0.59^{bc}	(28.3~39.3)
Demembranated							
Control		33.9 ± 0.59^{ab}	(28.3~39.3)	33.6 ± 0.55^{a}	(28.3~39.3)	34.5 ± 0.58^{bc}	(25.1~39.3)
Non-treated		33.4 ± 0.58^{ab}	(28.3~39.3)	33.5 ± 0.56^{a}	(28.3~39.3)	33.3 ± 0.57^{bc}	(28.3~39.3)
Treated with DTT							
	20 mM	32.5 ± 0.58^{a}	(25.1~43.2)	33.9 ± 0.68^{a}	(28.3~47.1)	33.2 ± 0.97^{ab}	(16.5~55.0)
	200 mM	34.6 ± 0.68^{b}	(25.1~47.1)	36.0 ± 0.88^{b}	(21.2~56.5)	35.6 ± 1.22^{c}	(21.2~60.5)
	2,000 mM	32.5 ± 0.63^{a}	(21.2~39.3)	31.9 ± 0.57^{a}	(28.3~39.3)	31.2 ± 0.54^{a}	(21.2~39.3)
Treated with heparin							
	100 units	34.0 ± 0.60^{ab}	(28.3~39.3)	34.0 ± 0.59^{a}	(28.3~39.3)	34.7 ± 0.53^{bc}	(28.3~39.3)
	1,000 units	33.9 ± 0.64^{ab}	(28.3~39.3)	33.8 ± 0.57^{a}	(28.3~39.3)	34.7 ± 0.56^{bc}	(28.3~39.3)
	10,000 units	34.6 ± 0.58^{b}	(28.3~39.3)	34.0 ± 0.55^{a}	(28.3~39.3)	35.1 ± 0.55^{bc}	(28.3~39.3)

^{a,b,c} Means of head area in the same column with different superscripts differ ($p<0.01$~0.05).



(a) (b)

Figure 2. Conditions of the sperm nuclei of the separated head treated with DTT (original magnification 400×)

(a) Non-decondensed head

(b) Crumbly decondensed head

(figure 1) into two categories. Category Perp, consisting of Perp-Hperp and Perp-Hpara sub-categories, showed that the heads oriented to the magnetic lines of force, each with its long axis perpendicular. The Para category, consisting of Para-Hperp and Para-Hpara sub-categories showed that the heads oriented to the magnetic lines of force, each with its long axis parallel. The separated heads showed very high percentages for long axis perpendicular orientation. As for the non-treated and exposed samples, there were very low numbers in the observation (weighted means of the number of heads), but the Perp category showed high values from 73.7% of the intact sample at 1 day, to 88.7% of the intact sample at 3 days. Similarly, for the heparin treatment, there were very low numbers in the observation, but Perp category showed high values from 66.7 to 89.5%.

As for the DTT treatment, there were remarkable differences of the number of observations among each concentration of the DTT treatment, but Perp oriented percentages showed very high values from 70.2% of the 2,000 mM treatment at 1 day on the intact sample, to 92.5% of the 20 mM treatment at 1 day on the demembranated sample. The demembranation of the head tended to increase Perp oriented percentage. However, the Perp oriented percentage tended to decline with concentration and length of time of the DTT treatment increase.

Table 3 presents the oriented percentages of the heads that were classified into four sub-categories (see figure 1). The treatment of DTT and the treatment of heparin presented the different conditions of the

Table 2. Oriented percentages (%) of intact or demembranated bull sperm heads each with its long axis perpendicular (Perp) or parallel (Para) to the magnetic lines of force after treatment with dithiothreitol (DTT) or heparin sodium for various lengths of time (days) and exposure to a magnetic field of 5400 G for 24 hours

Condition of the head	Concentration of DTT (mM) or heparin (units/ml)	Treatment time (days) of DTT or heparin											
		1				3				6			
		% of separated heads[2]	n[3]	Orientation[4] Perp	Orientation[4] Para	% of separated head	n	Orientation Perp	Orientation Para	% of separated head	n	Orientation Perp	Orientation Para
Intact[1]													
Control		0.9	9	50.8^{ab}	49.2	0.8	9	56.1^{ab}	43.9	1.3	14	56.3^{ab}	43.7
Non-treated, and exposed		0.8	9	73.7^{abcd}	26.3	0.8	8	88.7^{abc}	11.3*	1.1	12	74.7^{abc}	25.3
Treated with DTT, and exposed													
	20 mM	18.1	209	88.6^{cd}	11.4**	36.9	445	86.7^{bc}	13.3**	48.0	583	83.7^{c}	16.3**
	200 mM	2.7	30	84.0^{bcd}	16.0**	5.0	56	81.3^{abc}	18.7**	13.2	156	79.7^{bc}	20.3**
	2,000 mM	1.6	17	70.2^{abc}	29.8	1.7	19	79.7^{abc}	20.3**	2.1	23	76.9^{abc}	23.1**
Treated with heparin, and exposed													
	100 units	1.4	15	77.7^{abcd}	22.3*	1.6	17	89.5^{bc}	10.5**	1.5	17	66.7^{abc}	33.3
	1,000 units	1.2	12	70.1^{abcd}	29.9	1.4	15	83.3^{abc}	16.7*	1.2	13	72.3^{abc}	27.7
	10,000 units	1.5	16	77.3^{abcd}	22.7*	2.1	23	86.4^{abc}	13.6**	1.8	20	84.8^{bc}	15.2**
Demembranated[1]													
Control		1.0	11	47.8^{a}	52.2	0.9	10	55.6^{a}	44.4	1.1	12	50.0^{a}	50.0
Non-treated, and exposed		1.1	12	86.3^{bcd}	13.7*	1.1	12	76.7^{abc}	23.3	1.3	14	78.8^{abc}	21.2*
Treated with DTT, and exposed													
	20 mM	19.8	223	92.5^{d,B}	7.5**	28.7	334	90.5^{c,B}	9.5**	64.4	767	82.9^{c,A}	17.1**
	200 mM	10.8	118	92.0^{cd}	8.0**	20.0	226	89.9^{c}	10.1**	45.0	561	86.5^{c}	13.5**
	2,000 mM	2.0	22	76.7^{abcd}	23.3*	2.2	24	79.6^{abc}	20.4**	3.0	34	78.2^{abc}	21.8**
Treated with heparin, and exposed													
	100 units	1.0	11	77.1^{abcd}	22.9	0.9	10	79.0^{abc}	21.0	1.0	11	80.0^{abc}	20.0*
	1,000 units	1.1	12	77.3^{abcd}	22.7	1.1	12	77.0^{abc}	23.0	1.1	12	73.3^{abc}	26.7
	10,000 units	1.4	15	78.9^{abcd}	21.1*	1.7	18	79.5^{abc}	20.5*	1.6	18	74.1^{abc}	25.9*

[1] Intact, non-treated with Triton X-100. Demembranated, treated with Triton X-100.

[2] (Weighted mean of the number of separated heads)/(weighted mean of the number of separated heads + weighted mean of the number of sperms) × 100.

[3] Weighted means of the number of separated heads.

[4] Perp, oriented heads each with its long axis perpendicular to the magnetic lines of force. Para, oriented heads each with its long axis parallel to the magnetic lines of force.

, * Significant differences between perpendicular and parallel oriented percentages ($p<0.01$, * $p<0.05$).

a,b,c,d Oriented percentages in the same column with different superscripts differ ($p<0.01$~0.05).

A,B Oriented percentages in the same row with different superscripts differ ($p<0.01$).

magnetic orientation. For Perp percentages using the DTT treatment, Hperp oriented percentages were much higher than Hpara oriented percentages, except for the intact sample treated with 2000 mM at 3 days. The demembranation of the head tended to increase Hperp oriented percentages; 79.6 and 75.2% of the demembranated sample treated with 20 mM at 1 day and 3 days, respectively; 78.4, 74.7 and 70.5% of the demembranated sample treated with 200 mM at 1 day, 3 days and 6 days, respectively. However, with the 2000 mM treatment, Perp-Hperp oriented percentages showed low values, while Para-Hperp oriented percentages showed high values compared with samples using concentrations of DTT. Also, crumbly decondensed heads using the DTT treatment tended to show Perp-Hpara orientation rather than Perp-Hperp (figure 3). Oriented percentages using the heparin treatment showed very low numbers in the observation, but the Perp-Hperp oriented percentages were much lower than the Perp-Hpara oriented percentages. Also, Para-Hpara (containing non-oriented heads) percentages were increased with the heparin treatment.

Percentages of the magnetic orientations of the flagella are summarized in table 4. This table reclassified the sperm from four sub-categories (figure 1) into two categories. Category Perp, consisting of Up and Down sub-categories showed that the flagella oriented to the magnetic lines of force, each with its long axis perpendicular. The Para category, consisting of Left and Right sub-categories showed that the flagella oriented to the magnetic lines of force, each with its long axis parallel.

The flagellum was different from the perpendicular orientation of the head and oriented to the magnetic lines of force with its long axis parallel. The non-treatment of the demembranated flagella, and the treatment of heparin of the intact and the demembranated flagella tended to show parallel orientations but these Para percentages were not significant differences to Perp percentages due to the low numbers in the observation. The treatment of DTT showed parallel orientation and these Para percentages showed significant differences to Perp percentages; 58.9% vs. 41.1% and 55.9% vs. 44.1% of the intact flagella treated with 20 mM at 3 days and 6 days, respectively; 62.4% vs. 37.6%, 59.9% vs. 40.1% and 58.3% vs. 41.7 % of the demembranated flagella treated with 20 mM at 1 day, 3 days and 6 days, respectively; 61.1% vs. 38.9%, 60.2% vs. 39.8% and 58.5% vs. 41.5% of the demembranated flagella treated with 200 mM at 1 day, 3 days and 6 days, respectively. However, these parallel oriented percentages of the flagella were much lower than perpendicularly oriented percentages of the heads in a 5,400G magnetic field. Also, in the 2000 mM treatment, Para percentages were not significant differences to Perp percentages due to the low numbers in the observation. The parallel oriented percentages of the 20 mM and 200 mM DTT did not show any variation between the leftward orientation and the rightward orientation (table 5).

DISCUSSION

The magnetic orientations of bull sperm separated into the head and the flagellum were investigated in this study. Almost of the separated heads (at least 80%, see table 2) showed that their long axis oriented perpendicularly to the magnetic lines of force, and most of the long axis perpendicularly oriented heads showed that their flat plane oriented perpendicularly (see table 3) in a 5,400G magnetic field. Also, the demembranation of the head tended to increase those perpendicular orientations. These findings suggest that strong magnetic anisotropy for the perpendicular orientation of the long axis and the flat plane of the

Table 3. Oriented percentages (%) of intact or demembranated bull sperm heads after treatment with dithiothreitol (DTT) or heparin sodium for various lengths of time (days) and exposure to a magnetic field of 5,400 G for 24 hours

Condition of the head	Concentration of DTT (mM) or heparin (units/ml)	Treatment time (days) of DTT or heparin											
		1				3				6			
		Perp		Para		Perp		Para		Perp		Para	
		Hperp	Hpara	Hperp	Hpara	Hperp	Hpara	Hperp	Hpara	Hperp	Hpara	Heprp	Hpara
Intact													
Control		16.9	33.9	13.6	35.6	15.8	40.4	8.8	35.1	12.6	43.7	6.9	36.8
Non-treated, and exposed		40.4	33.3	10.5	15.8	47.2	41.5	7.5	3.8	32.0	42.7	6.7	18.7
Treated with DTT, and exposed													
	20 mM	69.2	19.4	2.1	9.3	64.5	22.2	3.0	10.3	59.0	24.7	4.0	12.3
	200 mM	54.1	29.8	6.6	9.4	46.9	34.4	5.3	13.4	58.0	21.7	8.0	12.3
	2,000 mM	39.4	30.8	16.3	13.5	36.4	43.2	7.6	12.7	50.3	26.6	10.5	12.6
Treated with heparin, and exposed													
	100 units	31.9	45.7	3.2	19.1	27.6	61.9	1.9	8.6	13.7	52.9	2.0	31.4
	1,000 units	29.9	40.3	5.2	24.7	25.6	57.8	3.3	13.3	16.9	55.4	6.0	21.7
	10,000 units	26.8	50.5	10.3	12.4	22.9	63.6	5.0	8.6	22.4	62.4	4.8	10.4
Demembranated													
Control		11.6	36.2	11.6	40.6	11.1	44.4	7.9	36.5	8.3	41.7	11.1	38.9
Non-treated, and exposed		49.3	37.0	5.5	8.2	45.2	31.5	5.5	17.8	48.2	30.6	5.9	15.3
Treated with DTT, and exposed													
	20 mM	79.6	12.9	1.6	5.8	75.2	15.3	2.6	6.9	58.1	24.8	3.7	13.4
	200 mM	78.4	13.6	3.5	4.5	74.7	15.1	2.4	7.8	70.5	16.0	3.5	10.0
	2,000 mM	53.4	23.3	16.5	6.8	56.5	23.1	15.6	4.8	53.9	24.3	11.7	10.2
Treated with heparin, and exposed													
	100 units	25.7	51.4	2.9	20.0	27.4	51.6	6.5	14.5	34.3	45.7	8.6	11.4
	1,000 units	16.0	61.3	8.0	14.7	33.8	43.2	9.5	13.5	17.3	56.0	9.3	17.3
	10,000 units	21.1	57.8	5.6	15.6	16.1	63.4	3.6	17.0	15.7	58.3	3.7	22.2

Perp, oriented heads each with its long axis perpendicular to the magnetic lines of force.

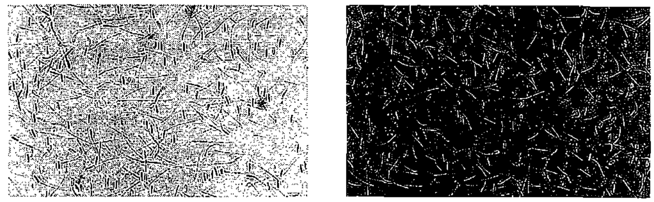
Para, oriented heads each with its long axis parallel to the magnetic lines of force.

Hperp, oriented heads each with its flat plane perpendicular to the magnetic lines of force.

Hpara, oriented heads each with its flat plane parallel to the magnetic lines of force.

head occurs in the sperm nuclei in a 5,400G field. However, the flagellum showed lower parallel orientation (about 60%, see table 4), and also the separated and demembranated flagellum showed parallel orientation to the magnetic lines of force in this magnetic field. These findings suggest that weak magnetic anisotropy for the parallel orientation of the flagellum occurs in the inside components in a 5,400G magnetic field.

As for the perpendicular orientation of the heads, Para-Hperp percentages tended to be increased by the treatment of 2,000 mM DTT, and Perp-Hpara and Para-Hpara percentages tended to be increased by the treatment of heparin. These results are similar to the sperm (non-separated into the head and the flagellum) treated by 2,000 mM DTT and heparin (Suga et al., 2000). These findings suggest that maintaining the structure of protamine in the chromatin is necessary for the head to orient with its flat plane perpendicular, while maintaining the crosslinks of DNA-protamine in the chromatin is necessary for the long axis of the head to orient perpendicularly, if heparin only attacks the protamine for its strong affinity (Chargaff and Olson, 1938), and if DTT reduces on disulfide bonds crosslinking the DNA- protamine only.



(a) (b)

Figure 3. Magnetic orientations of the flagellum and the separated head treated with DTT (original magnification 200×)
(a) The flagellum and non-decondensed head
(b) The flagellum and crumbly decondensed head

Table 4. Oriented percentages (%) of intact or demembranated bull sperm flagella each with its long axis perpendicular (Perp) or parallel (Para) to the magnetic lines of force after treatment with dithiothreitol (DTT) or heparin sodium for various lengths of time (days) and exposure to a magnetic field of 5,400 G for 24 hours

Condition of the flagellum	Concentration of DTT (mM) or heparin (units/ml)	Treatment time (days) of DTT or heparin: 1				3				6			
		% of separated flagella[1]	n[2]	Orientation[3] Perp	Para	% of separated flagella	n	Orientation Perp	Para	% of separated flagella	n	Orientation Perp	Para
Intact													
Control		0.7	8	47.9	52.1	0.7	7	57.8	42.2	1.0	11	50.0	50.0
Non-treated, and exposed		0.8	9	50.9	49.1	0.7	7	56.5	43.5	0.9	9	46.6	53.4
Treated with DTT, and exposed													
	20 mM	18.8	218	43.8	56.3	38.3	472	41.1**	58.9	53.6	730	44.1**	55.9
	200 mM	2.6	29	42.5	57.5	5.6	63	44.9	55.1	17.4	217	43.5	56.5
	2,000 mM	1.5	16	50.0	50.0	1.6	18	49.1	50.9	2.6	30	43.2	56.8
Treated with heparin, and exposed													
	100 units	1.1	11	47.8	52.2	1.2	12	46.8	53.2	1.4	15	48.4	51.6
	1,000 units	1.2	13	56.4	43.6	1.2	13	52.5	47.5	1.5	16	49.0	51.0
	10,000 units	1.1	11	52.9	47.1	1.5	17	46.1	53.9	1.8	20	50.4	49.6
Demembranated													
Control		0.7	8	54.2	45.8	0.8	9	53.4	46.6	0.8	9	53.7	46.3
Non-treated, and exposed		1.0	10	46.9	53.1	1.1	12	49.3	50.7	1.0	11	44.1	55.9
Treated with DTT, and exposed													
	20 mM	19.7	221	37.6**	62.4	28.6	332	40.1**	59.9	64.3	764	41.7**	58.3
	200 mM	10.6	116	38.9*	61.1	20.1	228	39.8**	60.2	46.0	582	41.5**	58.5
	2,000 mM	1.9	21	49.2	50.8	2.3	25	46.4	53.6	2.7	31	47.4	52.6
Treated with heparin, and exposed													
	100 units	1.1	11	43.7	56.3	1.1	12	44.6	55.4	0.9	10	49.2	50.8
	1,000 units	1.1	12	48.0	52.0	1.1	12	40.8	59.2	1.0	11	49.3	50.7
	10,000 units	1.2	13	52.4	47.6	1.4	15	48.4	51.6	1.5	17	46.1	53.9

[1] (Weighted mean of the number of separated flagella)/(weighted mean of the number of separated flagella + weighted mean of the number of sperms) × 100.

[2] Weighted means of the number of separated flagella.

[3] Perp, oriented flagella each with its long axis perpendicular to the magnetic lines of force. Para, oriented flagella each with its long axis parallel to the magnetic lines of force.

, * Significant differences between perpendicular and parallel oriented percentages ($p<0.01$, * $p<0.05$).

However, crumbly decondensed heads treated with 20 mM or 200 mM DTT alone tended to decrease the Perp-Hperp percentage (see figure 2, figure 3 and table 2) and increase Perp-Hpara, and Para-Hpara percentages, because it is considered that the anisotropic magnetic susceptibility (Higashi et al., 1995) for the perpendi- cular orientation of the flat plane of the head was decreased by destroying the three-dimensional structure of the DNA-protamine complex through these DTT treatments.

In our previous report (Suga et al., 2000), for bull sperm (non-separated into the head and the flagellum) treated by 2,000 mM DTT, the decline of long axis perpendicularly oriented percentages was caused by the increase of long axis parallel orientation with the flat plane of the head perpendicular to the magnetic lines of force. However, flat plane perpendicular percentages (Perp-Hperp plus Para-Hperp) tended to be stable using the 2,000 mM DTT treatment. As the cause of those observations, most of the three-dimensional structure of DNA-protamine complex may be maintained, because large (crumbly) decondensed sperm was not observed through the 2,000 mM DTT treatment. However, it is considered that the anisotropic magnetic susceptibility for the perpendicular orientation of the flat plane of the head was low declined by cutting disulfide crosslinkages of the DNA-protamine complex through the DTT treatment, while, the flagellum occurs weak anisotropic magnetic susceptibility for parallel orientation.

That is, the sperm treated with 2,000 mM DTT tries to orient its long axis perpendicularly with the flat plane of its head perpendicular to the magnetic lines of force; but the sperm treated with 2,000 mM DTT orients its long axis parallel maintaining the flat plane of the head perpendicularly by declining the strong anisotropic magnetic susceptibility of the head for the perpendicular orientation of its long axis through the DTT treatment, and by weak anisotropic magnetic susceptibility for the parallel orientation of the flagellum.

It is difficult to estimate the anisotropic magnetic susceptibilities of bull sperm head, bull sperm flagellum, and bull sperm at the present time, because the structure of the chromatin in bull sperm proposed by the primary model of Balhorn (1982), has not been

Table 5. Oriented percentages (%) of intact or demembranated bull sperm flagella after treatment with dithiothreitol (DTT) or heparin sodium for various lengths of time (days) and exposure to a magnetic field of 5,400 G for 24 hours

Condition of the flagellum	Concentration of DTT (mM) or heparin (units/ml)	Treatment time (days) of DTT or heparin: 1				3				6			
		Perp		Para		Perp		Para		Perp		Para	
		Up	Down	Left	Right	Up	Down	Left	Right	Up	Down	Left	Right
Intact													
Control		27.1	20.8	16.7	35.4	28.9	28.9	20.0	22.2	28.8	21.2	24.2	25.8
Non-treated, and exposed		26.3	24.6	17.5	31.6	26.1	30.4	19.6	23.9	25.9	20.7	24.1	29.3
Treated with DTT, and exposed													
	20 mM	23.2	20.6	30.7	25.5	21.3	19.7	28.7	30.3	22.0	22.1	24.0	31.9
	200 mM	20.7	21.8	29.9	27.6	24.3	20.6	25.6	29.5	20.6	22.9	26.2	30.4
	2,000 mM	24.0	26.0	22.9	27.1	25.9	23.1	29.6	21.3	23.5	19.7	29.0	27.9
Treated with heparin, and exposed													
	100 units	23.2	24.6	23.2	29.0	18.2	28.6	23.4	29.9	25.8	22.6	23.7	28.0
	1,000 units	35.9	20.5	23.1	20.5	30.0	22.5	21.3	26.3	28.0	21.0	22.0	29.0
	10,000 units	32.4	20.6	19.1	27.9	27.5	18.6	30.4	23.5	28.8	21.6	27.2	22.4
Demembranated													
Control		31.3	22.9	20.8	25.0	24.1	29.3	24.1	22.4	20.4	33.3	24.1	22.2
Non-treated, and exposed		21.9	25.0	20.3	32.8	30.7	18.7	18.7	32.0	23.5	20.6	27.9	27.9
Treated with DTT, and exposed													
	20 mM	18.9	18.6	31.1	31.3	19.5	20.6	30.3	29.6	21.0	20.7	28.9	29.4
	200 mM	19.5	19.4	29.8	31.3	19.4	20.4	30.4	29.8	20.8	20.7	29.6	28.9
	2,000 mM	27.8	21.4	28.6	22.2	24.8	21.6	24.8	28.8	21.1	26.3	26.8	25.8
Treated with heparin, and exposed													
	100 units	25.4	18.3	38.0	18.3	21.6	23.0	28.4	27.0	20.6	28.6	28.6	22.2
	1,000 units	29.3	18.7	28.0	24.0	22.4	18.4	31.6	27.6	26.1	23.2	31.9	18.8
	10,000 units	25.6	26.8	25.6	22.0	24.2	24.2	23.2	28.4	20.6	25.5	26.5	27.5

Up, oriented flagella each with its long axis upward to the magnetic lines of force.

Down, oriented flagella each with its long axis downward to the magnetic lines of force.

Left, oriented flagella each with its long axis leftward to the magnetic lines of force.

Right, oriented flagella each with its long axis rightward to the magnetic lines of force.

introduced for the second and the third models, nor for the masses and the specific gravities of the head and the flagellum. It is now necessary to prove experimentally the inside components of the flagellum which cause the magnetization of a parallel orientation.

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REFERENCES

Ashida, N., T. Higashi and T. Takeuchi. 1996. Orientation of bull sperm in static magnetic field. The Journal of Japan Biomagnetism and Bioelectromagnetics Society. 9:29-34 (in Japanese).

Balhorn, R. 1982. A model for the structure of chromatin in mammalian sperm. Journal of Cell Biology. 93:298-305.

Chargaff, E. and K. B. Olson. 1938. Studies on the chemistry of blood coagulation. VI. Studies on the action of heparin and other anticoagulants. The influence of protamine on the anticoagulant effect *in vivo*. The Journal of Biological Chemistry. 122:153-167.

Formicki, K., A. Sobocinski and A. Winnicki. 1990. Motility of spermatozoa of danube salmon (*HUCHO HUCHO* L.), exposed to magnetic field prior to activation. Pol. Arch. Hydrobiol. 37:439-447.

Higashi, T., A. Yamagishi, T. Takeuchi and M. Date. 1995. Effects of static magnetic fields on erythrocyte rheology. Bioelectrochemistry and Bioenergetics. 36:101-108.

Kamei, H. and H. Yokoyama. 1990. Seisi no ziba ni yoru haikou. The Journal of Japan Biomagnetism and Bioelectromagnetics Society. 3:93-94. (In Japanese).

Masuda, H. 1995. Effect of magnetic field on the motility of frozen-thawed boar spermatozoa. The Japanese Journal of Swine Science. 32:203-205. (In Japanese).

Motoishi, M., K. Goto, K. Tomita, S. Ookutsu and Y. Nakanishi. 1996. Decondensation of bull and human sperm nuclei by dithiothreitol and/or heparin. Journal of Reproduction and Development. 42:7-13.

SAS. 1990. SAS/STAT User's Guide. Version 6, Fourth Edition. SAS Institute Inc., SAS Campus Drive, Cary NC 27513.

Shinjo, A. and M. Utimura. 1992. Effect of magnetic field on the viability of the cattle sperm. Japan Journal of Veterinary Informatics. 29:13-15. (In Japanese).

Suga, D., A. Shinjo, S. Nisikawa and Y. Chiba. 1995. Effect of a weak static magnetic field on the viability, motility, and velocity of cattle sperm. Journal of the Magnetics Society of Japan. 19:872-874. (In Japanese).

Suga, D. and A. Shinjo. 1997. Effects of 600 gauss static magnetic field on the freezing of cattle sperm. Journal of the Magnetics Society of Japan. 21:1134-1137. (In Japanese).

Suga, D., A. Shinjo, K. Inamine, T. Nakada and O. Makishi. 1998a. Effects of cell surface sialic acid on the orientation of motile and killed cattle sperm in a 5,400-G static magnetic field. Journal of the Magnetics Society of Japan. 22:194-198. (In Japanese).

Suga, D., A. Shinjo, K. Inamine, T. Nakada and H. Nikadori. 1998b. High and low magnetic orientations of demembranated and nuclear-decondensed cattle sperm in a 5,400-Gauss static magnetic field. Journal of the Magnetics Society of Japan. 22:1080-1084. (In Japanese).

Suga, D., A. Shinjo, E. Kurnianto and T. Nakada. 2000. Magnetic orientations of bull sperm treated by DTT or heparin. Asian-Aus. J. Anim. Sci. 13(1):10-18.