Magnetic Orientations of Bull Sperm Treated by DTT or Heparin

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ABSTRACT : This paper describes the magnetic orientation of the intact and demembranated bull sperm treated by DTT or heparin in a 5,400 G 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 sperm head area at 1, 3, and 6 days. After measuring the area, each sperm sample was exposed to a 5,400 G static magnetic field generated by Nd-Fe-B permanent magnets for 24 hours at room temperature. Results showed that the decondensation of bull sperm nuclei was not induced by the heparin treatment, however, incomplete decondensation was induced by the DTT treatment. During the magnetic orientation, bull sperms treated by DTT or heparin had low percentages of long axis perpendicular to the magnetic lines of force. However, different aspects were obtained for long axis perpendicular orientations following treatment of DTT or heparin. Through the DTT treatment, the decline of long axis perpendicularly oriented percentages was due to the increase of long axis parallel orientation with the head of the flat plane perpendicular to the magnetic lines of force, whereas, using the heparin treatment, the decline of long axis perpendicular orientation was due to the increment of long axis parallel orientation with the head of the flat plane parallel to the magnetic lines of force. Also, percentages of the head of the flat plane perpendicular were decreased by the heparin treatment. These findings suggest that maintaining the structure of protamine in the chromatin is necessary for the sperm head to orient with its flat plane perpendicular, and maintaining the disulfide bond in the chromatin is necessary for the long axis of sperm to orient perpendicularly. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 1 : 10-18*)

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

INTRODUCTION

Several papers have reported on the magnetic orientation of normal erythrocytes in Tesla (T, 1 T= 10,000 G) -ordered strong fields (Higashi et al., 1993a; Higashi et al., 1993b; Higashi et al., 1995; Higashi et al., 1996), since the sickled erythrocytes orienting themselves each with its long axis perpendicular to the magnetic lines of force in a 3500 Gauss (G) magnetic field was observed by Murayama (1965) and this observation was confirmed by Brody et al. (1985). The magnetic orientation of other cells or biological tissues have been reported on the chlorella (Geacintov et al., 1971; Geacintov et al., 1972) and on the retinal rod outer segment (Becker et al., 1978).

Recently, the magnetic orientation of several organic compounds, e.g., the fibrin, fibrinogen, collagen, gelatin and the lipid tubules have been observed (Torbet et al., 1981; Freyssinet, et al., 1983; Freyssinet et al., 1984; Torbet and Ronziere, 1984; Murthy, 1984; Rosenblatt et al., 1987; Yamagishi et al., 1990; Ueno et al., 1991), obtaining the experimental environments of 10 T (-ordered strong magnetic fields) introduced by the superconducting magnet. It is thought that the mechanism of this magnetic orientation is caused by the magnetic anisotropy occurring in the molecular structure (Maret and Dransfeld, 1977; Worcester, 1978; Pauling, 1979; Freyssinet et al., 1984; Yamagishi et al., 1989; Iwasaka et al., 1994; Higashi et al., 1995).

As for the orientation of the sperm, Kamei and Yokoyama (1990) observed that human sperms orient on themselves each with their long axis perpendicular to the magnetic lines of force in a 55 mT field, and reported that non-motile sperm previously exposed did not orient in the field. Ashida et al. (1996) reported on bull sperms, that their orientations were affected by a 0.1 T field already, and it was observed that approximately 100 percent of bull sperms oriented on themselves, each with its long axis perpendicular and the flat plane of its head perpendicular to the magnetic lines of force in a 1 T field.

With regard to the magnetic orientation of the sperm, Kamei and Yokoyama (1990) stated that the effect of a like dynamic power called as the Lorentz force occurs in the motile of a negatively-charged sperm in the magnetic field. Ashida et al. (1996) stated that it is difficult to consider that the magnetic orientation of the sperm is only affected by the magnetic anisotropy in the plasma membrane. The magnetic anisotropy of DNA, which is the main

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component in the sperm head, may induce the orientation.

Suga et al. (1998a, b) reported on the magnetic orientations of ejected bull sperm (*Japanese Black*) in a 5400-G static field, and found that (1) the live sperm and the dead (non-motile) sperm oriented on themselves each with its long axis perpendicular and the flat plane of its head perpendicular or parallel to the magnetic lines of force; (2) percentages of long axis perpendicular orientations differed in each bull, the value from approximately 65% to 90%; (3) a negatively-charged cell surface with sialic acid inhibited bull sperm from orientating because neuraminidase (a glycosidase known to liberate sialic acid) treatment enhanced the orientation of the sperm; (4) plasma membrane of bull sperm also inhibited the sperm orientating because the demembranation of the sperm by triton X-100 (a detergent known to extract plasma membrane) enhanced the orientation of sperm; (5) nuclear-decondensed sperms and decondensed heads treated using both methods of dithiothreitol (a disulfide-reducing agent) and heparin sodium (a polyanion known to dissociate DNA-protamine complex of sperm nuclei) had very low percentages of long axis perpendicular orientation, while the flagella showed low long axis parallel orientation. These findings suggest that the magnetization of bull sperm head perpendicularly oriented to the magnetic lines of force occurs in the chromatin.

The present investigation examines the effects of the magnetic orientation on the disulfide bond or protamine of bull sperm nuclei by the treatment with dithiothreitol or heparin separately.

MATERIALS AND METHODS

Semen preparation and treatment with DTT or Heparin

Semen samples were collected from four bulls (*Japanese Black*) held in the Okinawa Prefectural Livestock Experimental Station, which gave a higher response of orientation to the magnetic field in our preliminary experiment. These four 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℃ for about 1 day. After the settling of the semen, 100 μl of sperm suspensions were added to 2ml 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, 2000 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 and demembranated sperm 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 eosin B solution (2% in the salt solution) at room temperature (approximately 25℃). The mixture was applied to a slide and was covered with a cover glass. The sperm was randomly selected from 10 places on the slide. The length and width of the flat plane of the head was examined in wet preparation 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.

Chemicals

Triton X-100, heparin sodium salt and eosin B were purchased from Sigma Chemical Co., MO, USA. Dithiothreitol was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Other chemicals were reagent grade from Kanto Chemical Co., Inc., Tokyo, Japan.

Exposure of bull sperm to a magnetic field

After measuring the head area, each sample was exposed to a 5400 G 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., 1998a, b) and are shown in figure 1.

The sample dropping area of a parafilm (a laboratory film, American National Can., WI, USA) was clipped out and the remaining parafilm was stuck onto a slide with heating. Fifty μl of each of the treated samples were dropped at the clipping area on the slide. In our previous reports (Suga et al., 1998a, b), when the sample was injected by capillarity into a chamber which had been covered with a cover glass on the slide, non-motile sperms oriented on themselves

each with its flagellum towards the flow of the fluid. After dropping the sample on the slide, the slide was covered carefully with a cover glass in order that air bubbles did not occur in the slide chamber. The cover glass was moved to any direction, because the directions of the sperms were random in the slide chamber before exposure to the magnetic field. The slide chamber was sealed with nail enamel to keep the sample fluid from flowing and evaporating.

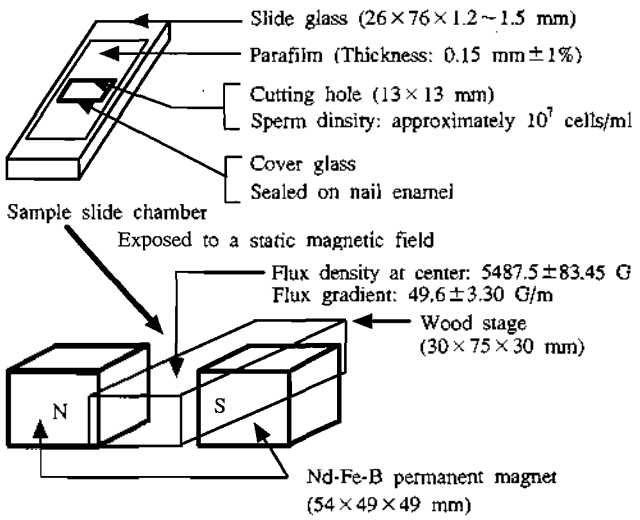


Figure 1. Method of exposing bull sperm to a 5,400 G static magnetic field

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 5487.5±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 and the demembranated sperm samples were put in a place unaffected by the artificially magnetic field in the room.

Measurement of magnetic oriented sperm

Classification of oriented sperms to the magnetic lines of force was similar to our previous reports (Suga et al., 1998a, b) and is shown in figure 2. 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. Approximately one thousand of the sperms were examined at 200×magnification at random fields of vision in the slide chamber.

Our preliminary experiment showed that the sperm was separated into head and flagellum by DTT treatment over lengths of time. Therefore, measurement in the field of vision ended when the total number of sperms and either the separated heads or the separated flagella became equal to or more than 1,000.

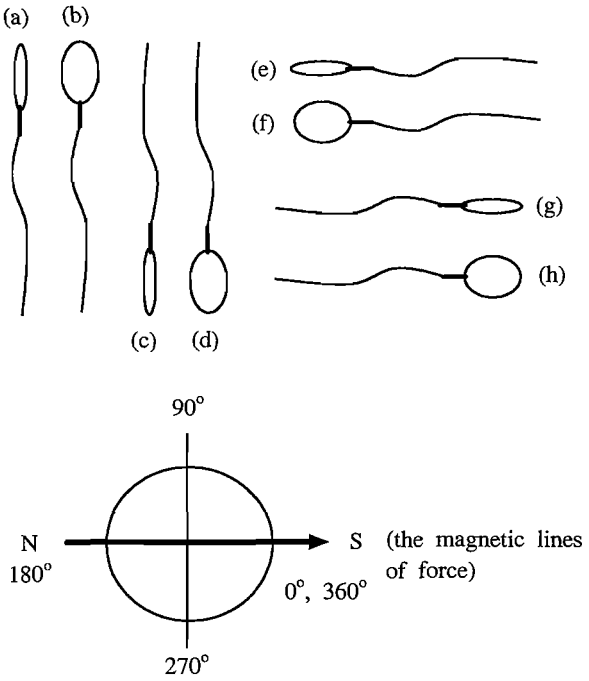


Figure 2. Classification of oriented sperm
(a) Upward, with sperm head perpendicular to the magnetic lines of force (Up-Hperp)
(b) Upward, with sperm head parallel (Up-Hpara)
(c) Downward, with sperm head perpendicular (Dow- Hperp)
(d) Downward, with sperm head parallel (Dow-Hpara)
(e) Leftward, with sperm head perpendicular (Lft-Hperp)
(f) Leftward, with sperm head parallel (Lft-Hpara)
(g) Rightward, with sperm head perpendicular (Rit-Hperp)
(h) Rightward, with sperm head parallel (Rit-Hpara)

The orientations of the sperms were classified into eight sub-categories, which were (a) upward, with sperm head perpendicular (Up-Hperp), long axis of sperm where the upward point is the top point of the head and downward is the end piece of the flagellum, oriented on +45° <long axis <+135° with the flat plane of its head perpendicular to the magnetic lines of force; (b) upward, with sperm head parallel (Up-Hpara), long axis oriented on the same as (a) with the flat plane of its head parallel; (c) downward,

with sperm head perpendicular (Dow-Hperp), long axis oriented on +225° < long axis < +315° with the flat plane of its head perpendicular; (d) downward, with sperm head parallel (Dow-Hpara), long axis oriented on the same as (c) with the flat plane of its head parallel; (e) leftward, with sperm head perpendicular (Lft-Hperp), long axis oriented on +135° ≤ long axis ≤ +225° with the flat plane of its head perpendicular; (f) leftward, with sperm head parallel (Lft-Hpara), long axis oriented on the same as (e) with the flat plane of its head parallel; (g) rightward, with sperm head perpendicular (Rit-Hperp), long axis oriented on +315° ≤ long axis ≤ +360° and 0° ≤ long axis ≤ +45° with the flat plane of its head perpendicular; (h) rightward, with sperm head parallel (Rit-Hpara), long axis oriented on the same as (e) with the flat plane of its head parallel. Incidentally, figure 2 and other tables show the orientation of the sperm in the real direction, because, upward and downward, or leftward and rightward in an eyesight of the microscope are the reverse of actual direction, respectively.

Statistical analysis

All data were analyzed using SAS (1990). With regard to the data of the head area of the sperm, Duncan's multiple range test of GLM procedure was used to examine the differences among treatment levels by treatment lengths of time, or among treatment 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 the parallel orientation. Contrasts of CATMOD procedure were used to examine the differences among treatment levels of DTT or heparin by treatment lengths of time, or among treatment lengths of time by treatment levels. The total number of sperms in each treatment level amounted to approximately 6,000, because the sixfold replication was repeated in all treatment levels. The total number of sperms was divided by the number of replications, and then the value obtained (weighted mean of the number of sperms) was analyzed (Suga et al., 1998a,b).

RESULTS

Sperm head area

Means, standard errors (SE) and ranges of the sperm head area are shown in table 1. Means and ranges of head area of the intact sperm sample and the demembranated sperm sample for the control and the non-treated with DTT or heparin; means and ranges of the intact sperm samples treated with heparin, and ranges of the demembranated sperm samples treated with heparin did not undergo remarkable change because of the treatment time. Means for the demembranated sperm samples treated with heparin on 1,000 units and 10,000 units for 6 days showed 35.4 μm, but these were not significantly different to the demembranated samples of the control and the non-treated.

Bull sperm nuclei was decondensed by DTT (figure 3), and the sperm showed remarkable separation into the sperm head and the flagellum by 20 mM and 200 mM DTT. Means of head area for the intact and demembranated sperm samples treated by DTT showed small values compared with the non-treated and treatment of heparin, and means for treatment with 2000 mM DTT showed the smallest values in all treatment lengths of time. However, highs in maximum values of the head area were shown with treatment of 20 mM and 200 mM DTT; 42.4 μm in the intact sample treated with 20 mM DTT for 1 day; 47.1 μm and 51.8 μm in the demembranated sample treated with 20 mM DTT for 1 day and for 3 days, respectively; 51.8 μm and 43.2 μm in the demembranated sample treated with 200 mM DTT for 3 days and 6 days, respectively.

Based on the results above, it could be said that a small decondensed sperm, like an atrophy of its short axis, resulted from a treatment of a very high concentration of 2000 mM DTT, and small and large decondensed sperms occurred through treatments of 20 mM DTT and 200 mM DTT (figure 3). The demembranation of the sperm slightly enhanced these small or large decondensations (see table 1).

Magnetic orientation of sperm

Percentages of perpendicular (Perp) and parallel (Para) oriented sperms are summarized in table 2. This table reclassified the sperms from eight sub-categories (figure 2) into two categories. Category Perp, consisting of Up-Hperp, Up-Hpara, Dow-Hperp and Dow-Hpara sub-categories showed that the sperms oriented to the magnetic lines of force, each with its long axis perpendicular. The Para category, consisting of Lft-Hperp, Lft-Hpara, Rit-Hperp and Rit-Hpara sub-categories, showed that the sperms oriented to the magnetic lines of force, each with its long axis parallel.

The numbers of sperms (weighted means) of the intact sperm sample or the demembranated sperm sample treated with DTT decreased because of the separation of the sperms into heads and flagella.

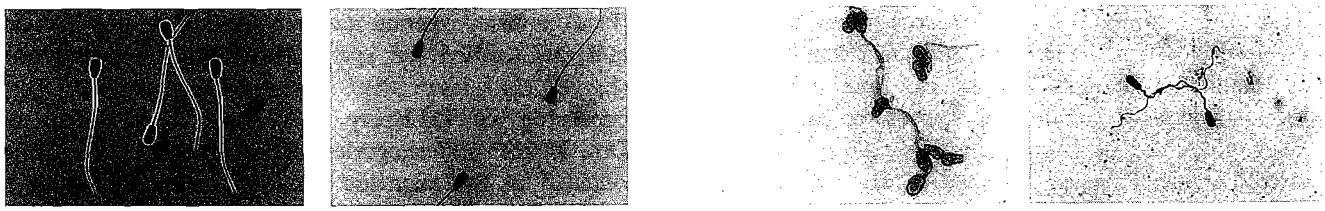
All perpendicular (Perp) oriented percentages of the intact sperm or the demembranated sperm exposed to a magnetic field were significantly higher than parallel (Para) oriented percentages. The Perp oriented percentages of the intact sperm non-treated with DTT or heparin and exposed to a magnetic field showed

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

Condition of the sperm	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 sperm							
Control		33.7 ± 0.62^{ab}	(25.1~39.3)	33.7 ± 0.61^{c}	(28.3~39.3)	33.0 ± 0.60^{bcde}	(25.1~39.3)
Non-treated		33.9 ± 0.56^{ab}	(25.1~39.3)	33.5 ± 0.59^{c}	(28.3~39.3)	33.3 ± 0.58^{bcde}	(25.1~39.3)
Treated with DTT							
	20 mM	$33.6 \pm 0.58^{ab,B}$	(28.3~42.4)	$32.6 \pm 0.59^{bc,AB}$	(25.1~39.3)	$31.7 \pm 0.72^{b,A}$	(16.5~39.3)
	200 mM	33.4 ± 0.66^{ab}	(25.1~39.3)	32.8 ± 0.62^{bc}	(25.1~39.3)	31.7 ± 0.62^{b}	(21.2~39.3)
	2,000 mM	$32.3 \pm 0.62^{a,B}$	(23.6~39.3)	$31.2 \pm 0.52^{ab,AB}$	(25.1~39.3)	$29.9 \pm 0.63^{a,A}$	(21.2~39.3)
Treated with heparin							
	100 units	33.4 ± 0.62^{ab}	(28.3~39.3)	33.4 ± 0.57^{c}	(25.1~39.3)	34.4 ± 0.55^{def}	(28.3~39.3)
	1,000 units	33.4 ± 0.59^{ab}	(28.3~39.3)	33.6 ± 0.61^{c}	(28.3~39.3)	33.8 ± 0.60^{cdef}	(28.3~39.3)
	10,000 units	33.5 ± 0.57^{ab}	(28.3~39.3)	33.3 ± 0.56^{c}	(28.3~39.3)	33.6 ± 0.57^{bcdef}	(28.3~39.3)
Demembranated sperm							
Control							
Non-treated		33.5 ± 0.59^{ab}	(28.3~39.3)	34.1 ± 0.58^{c}	(28.3~39.3)	33.6 ± 0.63^{bcdef}	(28.3~39.3)
Treated with DTT		33.4 ± 0.61^{ab}	(28.3~39.3)	33.9 ± 0.60^{c}	(28.3~39.3)	33.5 ± 0.61^{bcdef}	(28.3~39.3)
	20 mM	32.9 ± 0.62^{ab}	(28.3~47.1)	32.9 ± 0.67^{bc}	(28.3~51.8)	32.1 ± 0.71^{bc}	(22.0~39.3)
	200 mM	32.6 ± 0.64^{ab}	(21.2~39.3)	33.9 ± 0.73^{c}	(28.3~51.8)	32.4 ± 0.68^{bcd}	(21.2~43.2)
	2,000 mM	$32.6 \pm 0.61^{ab,C}$	(21.2~39.3)	$30.4 \pm 0.56^{a,B}$	(21.2~39.3)	$28.4 \pm 0.58^{a,A}$	(18.8~39.3)
Treated with heparin							
	100 units	34.4 ± 0.61^{b}	(28.3~39.3)	34.0 ± 0.59^{c}	(28.3~39.3)	34.6 ± 0.60^{ef}	(28.3~39.3)
	1,000 units	34.3 ± 0.62^{ab}	(28.3~39.3)	33.7 ± 0.59^{c}	(28.3~39.3)	35.4 ± 0.54^{f}	(28.3~39.3)
	10,000 units	34.1 ± 0.64^{ab}	(28.3~39.3)	34.3 ± 0.60^{c}	(28.3~39.3)	35.4 ± 0.57^{f}	(28.3~39.3)

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

^{A,B,C} Means of head area in the same row with different superscripts differ ($p<0.01$~0.05).



(a) (b) (c) (d)

Figure 3. Conditions of the sperm nuclei of bull sperm treated with DTT or heparin (original magnification 400×) (a) Non-treated; (b) Treated with 10,000 units heparin; (c) Treated with 20 mM DTT; (d) Treated with 2,000 mM DTT

75.9, 75.8 and 74.8% at 1 day, 3 days and 6 days, respectively, and these percentages were significantly higher than the control (non-exposed) of the intact sperm. The Perp oriented percentages of the non-treated demembranated sperm exposed to a magnetic field showed 75.7, 76.5 and 76.8% at 1 day, 3 days and 6 days, respectively, in which these percentages were significantly higher than the control of the demembranated sperm, but were not significantly higher than the non-treated and exposed intact sperm at 3 days and 6 days.

Perp oriented percentages of the demembranated sperm treated with 20 mM and 200 mM DTT at 1 day showed 77.0% and 76.0%, respectively, and these were not significantly higher than the non-treated intact or demembranated sperm. However, Perp oriented percentages of the intact sperm treated with all concentrations of DTT used at all treatment lengths of time, of the demembranated sperm treated with 20 mM and 200 mM DTT at 3 days and 6 days, and of

Table 2. Oriented percentages (%) of intact or demembranated bull sperm 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 sperm	Concentration of DTT (mM) or heparin (units/ml)	Treatment time (days) of DTT or heparin								
		1			3			6		
		n[3]	Orientation[4]		n	Orientation		n	Orientation	
			Perp	Para		Perp	Para		Perp	Para
Intact sperm[1]										
Control[2]		1,136	50.2^{a}	49.8	1,136	50.8^{a}	49.2	1,126	50.0^{a}	50.0
Non-treated, and exposed		1,116	75.9^{fg}	24.1**	1,081	75.8^{f}	24.2**	1,111	74.8^{gh}	25.2**
Treated with DTT, and exposed										
	20 mM	947	74.3^{fg}	25.7**	761	74.2^{ef}	25.8**	631	71.2^{fg}	28.8**
	200 mM	1,083	72.8^{ef,B}	27.2**	1,072	71.7^{de,B}	28.3**	1,034	66.5^{cde,A}	33.5**
	2,000 mM	1,086	68.9^{cd,B}	31.1**	1,118	68.8^{cd,B}	31.2**	1,130	63.1^{bc,A}	36.9**
Treated with heparin, and exposed										
	100 units	1,080	74.6^{fg,B}	25.4**	1,087	73.2^{ef,B}	26.8**	1,101	69.3^{def,A}	30.7**
	1,000 units	1,072	67.9^{cd}	32.1**	1,080	68.4^{cd}	31.6**	1,114	65.1^{bc,A}	34.9**
	10,000 units	1,049	65.9^{bc}	34.1**	1,079	63.8^{b}	36.2**	1,132	62.2^{b}	37.8**
Demembranated sperm[1]										
Control										
Non-treated, and exposed		1,106	50.0^{a}	50.0	1,130	49.9^{a}	50.1	1,128	49.5^{a}	50.5
Treated with DTT, and exposed		1,104	75.7^{fg}	24.3**	1,109	76.5^{f}	23.5**	1,105	76.8^{h}	23.2**
	20 mM	903	77.0^{g,B}	23.0**	831	73.7^{ef,B}	26.3**	423	64.1^{bcd,A}	35.9**
	200 mM	982	76.0^{fg,B}	24.0**	907	68.5^{cd,A}	31.5**	685	63.9^{bc,A}	36.1**
	2,000 mM	1,081	70.2^{de,B}	29.8**	1,077	67.1^{bc,B}	32.9**	1120	62.9^{bc,A}	37.1**
Treated with heparin, and exposed										
	100 units	1,103	69.0^{cd}	31.0**	1,095	69.0^{cd}	31.0**	1102	69.7^{ef}	30.3**
	1,000 units	1,094	65.8^{bc}	34.2**	1,102	63.2^{b}	36.8**	1189	63.5^{bc}	36.5**
	10,000 units	1,086	63.5^{b}	36.5**	1,096	64.2^{b}	35.8**	1086	61.1^{b}	38.9**

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

[2] Control, non-treated with DTT or heparin and non-exposed to a magnetic field.

[3] Weighted means of the number of sperms.

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

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

^{a,b,c,d,e,f,g,h} Oriented percentages in the same column with different superscripts differs ($p<0.01\sim0.05$).

^{A,B} Oriented percentages in the same row with different superscripts differ ($p<0.01\sim0.05$).

the demembranated sperm treated with 2,000 mM DTT at all times were lower than non-treated and exposed on the intact sperm or the demembranated sperm, respectively. Moreover, Perp oriented percentages significantly declined with increasing the lengths of the treatment time as can be seen in the intact sperm treated with 200 mM DTT, 2,000 mM DTT and the demembranated sperm treated with all concentrations of DTT.

Similarly to the DTT treatment, Perp oriented percentages of the intact or the demembranated sperms treated with all concentrations of heparin at all lengths of time were lower than non-treated and exposed intact or demembranated sperms, and the declines of these Perp oriented percentages for the treatment of heparin were greater than the treatment by DTT. However, Perp oriented percentages of the treatment of heparin tended to be stable except for the intact sperm treated by 100 units.

Table 3 presents the oriented percentages that were reclassified into four categories. Categories Perp-Hperp and Perp-Hpara, consisting of Up-Hperp and Dow-Hperp or Up-Hpara and Dow-Hpara sub-categories, respectively, showed that the sperms were oriented to the magnetic lines of force, each with its long axis perpendicular (Perp) and its head of the flat plane perpendicular (Hperp) or parallel (Hpara), respectively. Categories Para-Hperp and

Table 3. Oriented percentages (%) of intact or demembranated bull sperm 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 sperm	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	Hperp	Hpara
Intact sperm													
Control		7.8	42.4	7.8	42.0	8.5	42.3	8.1	41.1	8.1	41.9	8.0	42.0
Non-treated, and exposed		34.2	41.8	4.8	19.3	35.1	40.7	4.8	19.4	35.5	39.2	4.7	20.6
Treated with DTT, and exposed													
	20 mM	36.7	37.6	5.1	20.6	31.7	42.5	4.4	21.4	37.0	34.2	6.1	22.7
	200 mM	33.9	38.9	6.3	20.9	33.3	38.4	6.5	21.9	31.1	35.4	10.2	23.3
	2,000 mM	32.2	36.7	10.0	21.1	32.4	36.4	11.4	19.8	28.3	34.8	13.7	23.3
Treated with heparin, and exposed													
	100 units	30.8	43.8	5.0	20.4	30.3	42.9	5.5	21.3	28.2	41.1	5.3	25.5
	1,000 units	25.6	42.3	5.7	26.4	23.2	45.3	5.5	26.1	20.1	44.9	5.9	29.0
	10,000 units	22.9	43.0	5.7	28.4	20.6	43.1	5.4	30.9	18.1	44.2	5.8	32.0
Demembranated sperm													
Control		7.2	42.8	7.4	42.6	7.4	42.5	7.3	42.8	8.0	41.5	8.2	42.4
Non-treated, and exposed		34.2	41.5	4.7	19.6	35.1	41.4	4.9	18.6	35.4	41.5	5.7	17.4
Treated with DTT, and exposed													
	20 mM	38.5	38.5	4.8	18.1	35.5	38.2	8.4	17.9	26.3	37.8	10.0	25.9
	200 mM	37.2	38.8	6.2	17.8	32.4	36.1	9.4	22.1	29.6	34.3	8.8	27.3
	2,000 mM	33.7	36.4	11.3	18.5	30.2	37.0	13.5	19.3	30.0	32.9	16.5	20.6
Treated with heparin, and exposed													
	100 units	25.6	43.4	5.5	25.5	25.8	43.2	5.8	25.2	26.1	43.6	5.6	24.8
	1,000 units	21.0	44.7	5.0	29.3	20.5	42.7	6.7	30.1	20.2	43.3	7.2	29.3
	10,000 units	20.3	43.2	4.9	31.7	21.2	43.0	5.3	30.5	20.5	40.6	6.3	32.6

Perp, oriented sperms each with its long axis perpendicular to the magnetic lines of force.
Para, oriented sperms each with its long axis parallel to the magnetic lines of force.
Hperp, oriented sperms each with its head of the flat plane perpendicular to the magnetic lines of force.
Hpara, oriented sperms each with its head of the flat plane parallel to the magnetic lines of force.

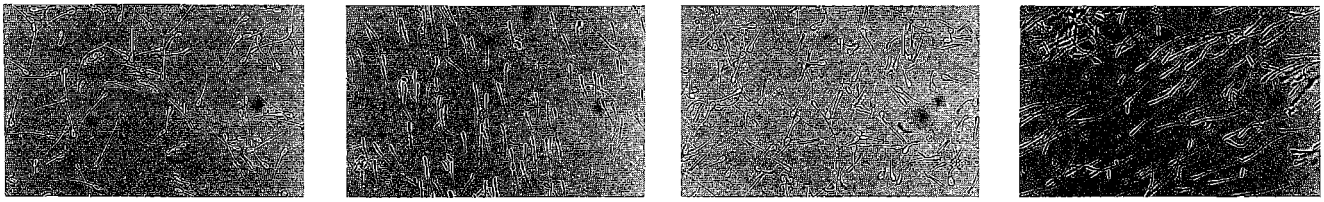
Para-Hpara, consisting of Lft-Hperp and Rit-Hperp or Lft-Hpara and Rit-Hpara sub-categories, respectively, showed that the sperms were oriented to the magnetic lines of force, each with its long axis parallel (Para) and its head of the flat plane perpendicular (Hperp) or parallel (Hpara), respectively.

Different conditions of the magnetic orientation were presented by treatment with DTT and heparin. For Perp oriented percentages, Hperp oriented percentages treated by DTT were higher than by heparin at all treatment lengths of time, while Hpara oriented percentages treated with heparin were higher than with DTT. Similarly, for Para oriented percentages, Hperp oriented percentages treated with DTT were higher than with heparin at all treatment lengths of time, while Hpara oriented percentages (containing non-oriented sperm) treated with heparin were higher than with DTT (table 3 and figure 4). It can be concluded that the decline of Perp oriented percentages for the DTT treatment in table 2 was caused by the increase of Para-Hperp percentages, whereas the decline of Perp oriented percentages for the heparin treatment was caused by the increment of Para-Hpara percentages. Also, Hperp percentages (Perp-Hperp plus Para-Hperp) were decreased by the heparin treatment, whereas, the percentages tended to be stable by the DTT treatment compared with non-treated.

DISCUSSION

The magnetic orientation of bull sperm is a physical phenomena differing from biochemical or biological taxis of the sperm since ejected bull sperm regardless of its life or death (non-movement) is induced to the orientation by the magnetic field (Suga et al., 1998a). It has been thought that the mechanism is caused by the magnetic anisotropy occurring in the molecular structure of bull sperm.

The negatively charged cell surface sialic acid and the plasma membrane of bull sperm inhibit the sperm orientation, because the neuraminidase treatment and the demembranation of bull sperm enhance the orientation (Suga et al., 1998a, b). However, the nuclear-decondensed bull sperms and decondensed heads treated using both methods (Motoishi et al., 1996) of DTT and heparin have very low percentages



(a) (b) (c) (d)

Figure 4. Magnetic orientations of bull sperm treated with DTT or heparin (original magnification 200×)
(a) Non-treated and non-exposed; (b) Non-treated and exposed; (c) Treated with 10,000 units heparin and exposed; (d) Treated with 2,000 mM DTT and exposed

of long axis perpendicular orientation (Suga et al., 1998b). Therefore, the non-decondensed heads show high long axis perpendicular orientation with these flat planes perpendicular to the magnetic field, while the flagella show low long axis parallel orientation (Suga et al., 1998b; Suga et al., 2000). These findings suggest that the magnetization of bull sperm oriented perpendicularly to the magnetic lines of force occurs in the chromatin of the head.

A model for the primary structure of the chromatin in bull sperm is proposed by Balhorn (1982), which consists of DNA-protamine complex crosslinked by disulfide bond. Motoishi et al. (1996) suggested that disulfide bond reduction caused by pre-treatment with DTT (a disulfide-reducing agent) was required for the bull sperm nuclei to decondense in response to heparin (which has a strong affinity for protamine molecules, (Chargaff and Olson, 1938), because the decondensation of bull sperm nuclei was not induced by DTT or heparin treatment alone.

In the present experiment, the decondensation of the bull sperm nuclei was not induced by the heparin treatment. However, the decondensation or incomplete decondensation (as with atrophy) of the sperm nuclei was induced by DTT treatment at low concentrations (20 mM and 200 mM) or a very high concentration (2000 mM) for a long time (several days).

Bull sperms treated with DTT or heparin showed low percentages of long axis perpendicularity to the magnetic lines of force. Different aspects were obtained for long axis perpendicular orientations following treatment of DTT or heparin. For treatment with DTT, the decline of long axis perpendicularly oriented percentages was caused by the increase of long axis parallel orientation with the head of the flat plane perpendicular to the magnetic lines of force. While, for treatment with heparin, the decline of long axis perpendicular orientation was caused by the increment of long axis parallel orientation with the head of the flat plane parallel to the magnetic lines of force. Moreover, percentages of the head of the flat plane perpendicularity (Perp-Hperp plus Para-Hperp) were decreased by the heparin treatment, whereas the percentages tended to be stable with the DTT treatment.

It is inversely hypothesized, the results of this experiment suggest that maintaining the structure of protamine in the chromatin is necessary for the sperm head to orient with its flat plane perpendicular, if heparin only attacks the protamine for its strong affinity. Meanwhile, maintaining the disulfide bond in the chromatin is necessary for the long axis of the sperm to orient perpendicularly, if DTT reduces disulfide bonds crosslinking the DNA-protamine only.

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