Doctoral Dissertation (Shinshu University)

Study on the mechanism of thermotaxis and chemotaxis of bull spermatozoa

March 2017

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学位論文

Study on the mechanism of thermotaxis and chemotaxis of bull spermatozoa
ウシ精子の走温性と走化性の作用機構に関する研究

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Chapter I

General Introduction

The breeding program of livestock may use either artificial insemination (AI) or natural service. AI has been commercially available for more than 60 years; it is widely used in dairy cattle but is used much less in beef cattle because of handling and labor costs. In cattle, AI is used primarily for genetic improvement of livestock and to facilitate high health replacement strategies. The worldwide adoption of AI for genetic improvement in dairy cattle was made possible by development of a progeny test system and subsequent use of milk production records as an objective measure of performance on which to select superior bulls, techniques for freezing semen and cryopreservation. Semen cryopreservation is a worldwide practice for dairy and beef cattle production. In south Asian countries like Bangladesh, Pakistan, Nepal and India, AI is a popular assisted reproductive technology (ART) with cryopreserved (frozen-thawed) semen for dairy cattle production.

Genetic improvement of livestock could be made by planned AI, multiple ovulation and embryo transfer (MOET) and in vitro produced (IVP) embryo transfer. Among these technique AI is the most widely used tool in modern animal breeding and thereby facilitating extensive utilization of cryopreserved spermatozoa. Due to the development of cryopreservation technique of semen for AI have been popularized all over

the world. But fertilizing capacity of sperm has always been regarded as one of the most important factor for successful AI program. However, it is well established that cryopreservation procedure reduces the efficiency of sperm fertility, which need to be compensated by inseminating greater amount of spermatozoa (Shannon and Vishwanath, 1995) [1]. Thus, after thawing, a decrease in sperm motility and viability, and the acceleration of capacitation is the main undesirable consequences of cryopreservation (Curry, 2000) [2].

Therefore, it is needed to verify whether frozen-thawed cattle spermatozoa chemotactically respond to the homologous follicular fluid and experimental conditions were used to achieve a thawed acceptable sperm population of motile capacitated spermatozoa with few head-to-head associations. Spermatozoa can find out the egg by following an increasing gradient of attractant molecules. This mechanism is known as sperm chemotaxis (Eisenbach and Giojalas, 2006) [3]. In the past 20 years, several features of mammalian sperm chemotaxis have been studied, including the size and physiological state of the chemotactic sperm population, the biological sources of attractants, the identity of a physiological attractant candidate, the species specificity of the phenomenon and some signal transduction pathways by which chemotaxis is induced. In species with internal fertilization such as mammals, spermatozoa are transported inside the oviduct, from the sperm reservoir in the isthmus towards the site where the ovum resides before fertilization. It has been postulated that spermatozoa may navigate this distance by at

least two mechanisms, thermotaxis and chemotaxis (Eisenbach and Giojalas, 2006) [3].Thermotaxis is a cellular mechanism where the spermatozoon detects a temperature gradient, and in consequence, orients its directional movement towards the place where the temperature is comparatively higher as 2°C higher temperature been reported in the ampulla than that of the isthmus of rabbit and pig. Instead, in a chemotactic response the spermatozoon follows an attractant concentration gradient in the direction of the chemoattractant source, e.g., the egg and/or its surrounding cells (Eisenbach and Giojalas, 2006) [3]. Chemotaxis was found in human, mice and rabbit spermatozoa towards follicular fluid in mice and oviductal fluid in humans as well as to conditioned medium from eggs and cumulus cells, and in humans and rabbits to minute concentrations of primary steroid progesterone (Teves et al., 2006) [4].

In mammals, sperm chemotactic response seems not to be species specific because rabbit and human spermatozoa are able to chemotactically respond towards follicular factors from each other species and also to cattle follicular fluid (Sun et al., 2003) [5]. For the evaluation of a dynamic phenomenon such as sperm chemotaxis, a single cell movement analysis in a sperm population with great motility is required. Spermatozoa must be capacitated to demonstrate the chemotactic response (Cohen-Dayag et al., 1995 [6], Fabro et al., 2002 [7], Giojalas et al., 2004 [8]). However, incubation under capacitating conditions with the presence of bovine serum albumin, calcium and bicarbonate also induces

a head-to-head sperm agglutination (Harayama and Kato, 2002) [9], phenomenon that is displayed by most eutherian mammals. Contrary to a prevalent believe there appears to be no competition in the mammalian female genital tract between large numbers of sperm cells racing towards the egg. Instead, small numbers of the ejaculated sperm cells enter the fallopian tube and these few must be guided in order to make the remaining long, obstructed way to the egg.

However, the homologous chemotactic response in bull spermatozoa has not yet been determined. Two active guidance mechanisms, chemotaxis and thermotaxis so far been assumed and both mechanisms might be restricted to capacitated sperm cells, namely to cells that reached a maturation stage at which they can penetrate the egg and fertilization occurs. It was also reported that both the egg and its surrounding cumulus cells secrete sperm chemoattractants, and that a temperature difference is established at ovulation in the female's oviduct as a consequence of a temperature drop at its lower part of isthmus. It was thought that, in vivo, thermotaxis is a long-range mechanism, guiding sperm cells in the fallopian tube towards the fertilization site, and chemotaxis is a short-range mechanism that is mainly functional at close proximity to the egg as shown in Fig.1.

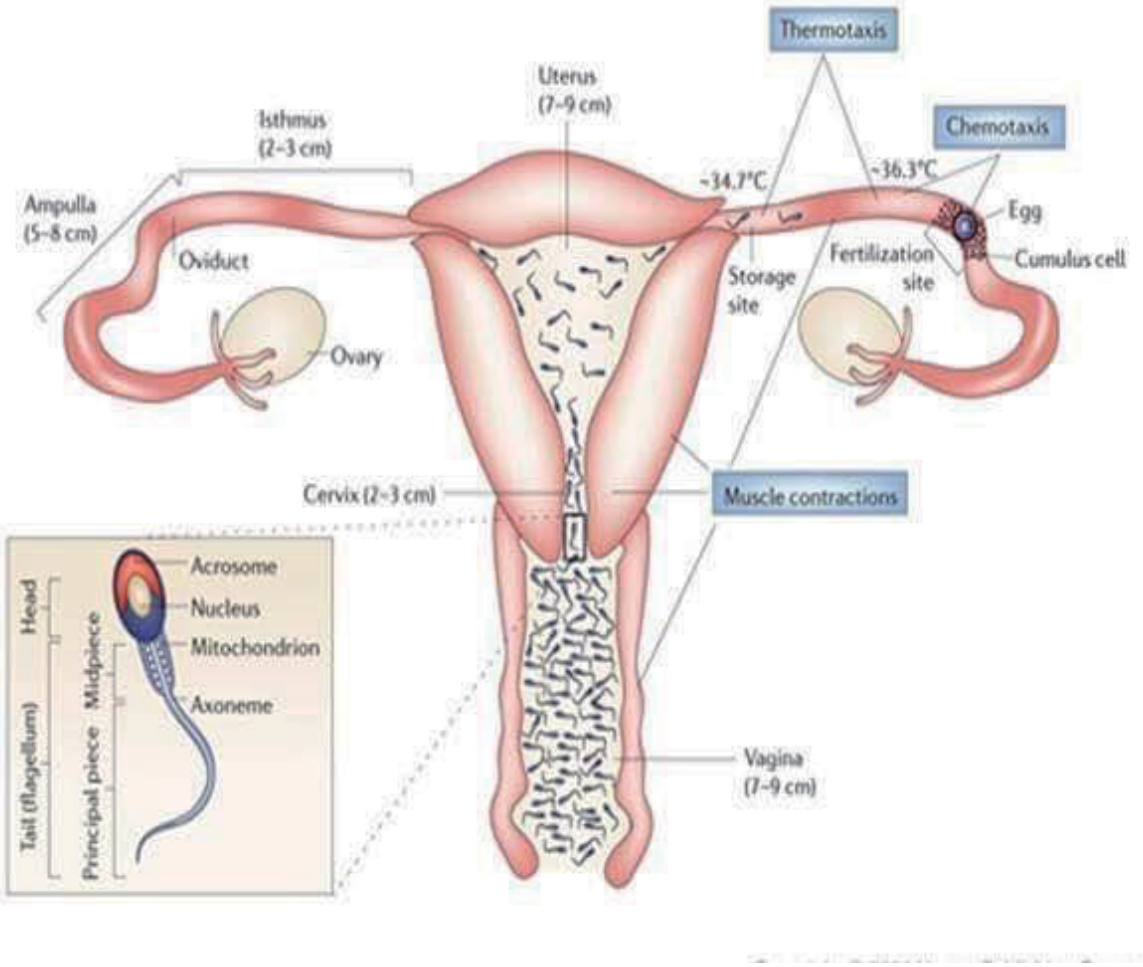

Fig.1

A scheme of the female genital tract demonstrating the location of sperm thermotaxis and chemotaxis in female reproductive tract (Molecular cell Biology, 2006).

It was also demonstrated that hyperactivated motility a vigorous motility type with large amplitudes of head displacement whose function had been unknown at large is part of the chemotactic response, causing sperm cells to change their swimming direction. They further found that human sperm cells detect the chemical gradient of the chemoattractant over time rather than over space, meaning that they have kind of a primitive memory. Additionally, they proposed a model for the behavioral mechanism of human sperm cells in a spatial chemoattractant gradient. Few of the molecular components involved as thermotaxis in human sperm cells. It was also reported that human sperm cells can respond thermotactically within a wide temperature range (at least 29-41°C), that within this range they preferentially accumulate in warmer temperatures rather than at a single specific, preferred temperature, that they can respond to both ascending and descending temperature gradients, and that they can sense and thermotactically respond to temperature gradients as low as <0.014°C/mm. This temperature gradient is astonishingly shallow because it means that as a spermatozoon swims through its entire body length (0.046 mm) it can sense and respond to a temperature difference of <0.0006°C.

From that standpoint the present research was undertaken to study the mechanism of thermotaxis and chemotaxis of bull spermatozoa and present research work consisted of two experiments described in Chapter II and III.

Chapter II

Effect of calcium ion channel and intracellular calcium on sperm motility and thermotaxis

II-1. Introduction

Artificial insemination is a method which is efficient in calf production. To date, before cryopreservation of bull semen, motility, concentration and morphology of sperm has been used as parameters for evaluation of semen fertility. Mammalian sperm ejaculated into a female reproductive tract migrate to the fertilization site due to the effects of multiple factors. Mammalian sperm migrate in the female reproductive tract in order to penetrate and fertilize an ovulated egg in the ampulla. Recently, it was reported that the migration of sperm in the female reproductive tract associated with fertilization is at the very least regulated by chemotaxis and thermotaxis [10-11]. Furthermore, the involvement of rheotaxis has been confirmed [12], and the mechanisms of taxis in sperm migration associated with fertilization have been elucidated [13]. Before arriving at the ampulla, which is the fertilization site, sperm motility is important for passing the utero cervix and tubal junction, and is essential for fertilization. The involvement of chemotaxis, thermotaxis, and rheotaxis in the migration of sperm has been previously reported [13]. David et al. [14] and Hunter and Nichol [15] reported that the difference in temperature between the isthmus and ampulla of the oviduct at ovulation were approximately 2 and 0.7 ºC in rabbit and boar, respectively.

These findings raise the possibility that difference of temperature in the reproductive tract induces a sperm to seek the fertilization site. Bahat et al. [16] reproduced temperature difference of 2 ºC in the rabbit reproductive tract, and reported positive thermotaxis of capacitated rabbit sperm migration to the high-temperature area. These same researchers also confirmed positive thermotaxis in human sperm with a similar experiment [17]. Bahat et al. theorized that thermotaxis and chemotaxis were involved complementarily in the fallopian tubes site [11, 16]. Because the chemoattractants are spread by oviduct peristalsis at ovulation, sperm migration to the fertilization site by chemotaxis alone is difficult. Thermotaxis is effective for creating the temperature difference between the isthmus and ampulla of oviduct; around the egg, the sperm is primarily induced by chemotaxis and is not regulated by thermotaxis [16].

Bahat et al. [17] reported the involvement of IP^3R and PLC on thermotaxis from human sperm analysis. Also, they confirmed the involvement of the calcium ion, but not the transient receptor potential (TRP), which is one of the calcium ion channels. Kawanishi et al. [18] demonstrated the positive thermotaxis of mouse sperm and suggested involvement of the calcium ion channel TRPV4 in mouse sperm thermotaxis. Hamano et al. [19] examined thermotaxis in Trpv4 gene-deficient mouse sperm, and confirmed the involvement of the TRPV4 calcium channel. Thermotaxis is thought to be an extremely important physiological function in mammalian sperm fertilized in the female reproductive tract. However, the regulation and the mechanism of

thermotaxis in bull sperm have not yet been established.

The functional changes such as sperm migration and penetration ability have been studied, because conventional sperm examination techniques (number, morphology and viability of sperm) cannot evaluate fertilizing ability directly. As examination of in vitro sperm migration might predict migration ability within the female reproductive tract, such migration has been examined to evaluate fertility [20]. Easy and more precise detection of sperm quality would be of great assistance in evaluating bull fertility. To date, there was no evaluation method has yet been established to predict the conception rate of artificially inseminated bovine females.

In this chapter, bull sperm migration and the expression of thermotaxis in vitro using a temperature gradient were examined to elucidate the thermotaxis of sperm and its involvement in the fertilization mechanism. Also examined the involvement of calcium and related substances were examined to clarify the expression mechanism of bull sperm thermotaxis.

II-2. Materials and Methods

Media and chemicals

The basic medium (BO) (Brackett & Oliphant [21]) containing 1% bovine serum albumin (BSA) (Wako, Tokyo) for washing, dilution of sperm, and column construction. The sperm capacitation-inducing medium (HC) contained BO mixed with both 15 μg/ml heparin (Mochida, Tokyo) and 5-mM caffeine (SIGMA-Aldrich, St Louis, MO, USA). Sperm pre-incubation was conducted in a microdrop of BO or HC in a CO_2 incubator with 38.5 ºC, 5% CO_2/95% air. To examine the effect of calcium on thermotaxis, I used BO without calcium (Ca-) and BO containing double concentration: 4.5 mM calcium (2Ca). I added 1-mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and 0.5μM 1,2-bis (o-aminophenoxy) ethane-*N,N,N‘,N’*-tetraacetic acid (BAPTA-AM) (SIGMA) of the calcium chelating agent into the BO. To inhibit a calcium channel, I added 0.1-mM lanthanum (general calcium channel inhibitor; SIGMA), 1-mM verapamil (voltage-dependent L-type calcium channel inhibitor; SIGMA), 40-μM mibefradil (voltage-dependent T-type calcium channel inhibitor; SIGMA), 10-μM ruthenium red (TRP calcium channel inhibitor; SIGMA), 10-μM SKF96365 (TRP calcium channel inhibitor; Wako), and 0.25-mM 2-Aminoethyl diphenylborinate (2APB) (intracellular calcium-releasing inhibitor; SIGMA) to the BO.

Semen

Holstein Friesian bulls A, B and C had conception rates of 65% (324/495

heads), 57% (585/1029 heads), and 34% (95/277 heads), respectively. Semen was obtained from three bulls. Evaluation of thermotaxis was conducted using the semen from bull B, and I evaluated sperm thermotaxis of the three bulls, A, B, and C.

Chamber preparation

Manufactured analysis chambers were used for evaluation of sperm motility and thermotaxis (Fig.2). Each chamber were constructed with a slide glass (S2215: Matsunami, Kishiwada) (Fig.2: a) and a cover glass (22 ×40: Matsunami) (Fig.2: b) adhered by double-stick tape (100-μm thick; No. 7046; Teraoka, Tokyo) (Fig.2: c1-c4) to create a cross-type column (Fig.2: d). The cross-type column was 10-mm long vertically (width: 5 mm), 40-mm broad (width: 3 mm) and connected along 13-mm vertically (width: 1 mm) to the slide glass with double-stick tape. The glass cover glass was adhered and the chamber was filled with BO (Fig. 2). Both ends of the wide column were covered with mineral oil once the sperm was introduced.

Sperm preparation

Frozen bull semen was thawed in a water bath at 38.5 ºC and diluted with an equal volume of BO. Diluted semen was washed three times by centrifugation at 1500 rpm for 5 min. Sperm concentration was adjusted to 1×10^7/ml.

Evaluation of sperm motility and thermotaxis

The specially manufactured “Thermo Plate” (Tokai Hit, Shizuoka) was used for temperature gradient device. The sperm motility and thermotaxis were evaluated by examining sperm migration ability in a temperature gradient. In the no-temperature-gradient condition, I set the three ends of the vertical column and wide column to 38 ºC. In the temperature-gradient condition with a 1 or 2 ºC difference, the low-temperature end (Fig.3: C) at the wide column was more than 34 ºC, the high temperature end (Fig.3: B) was less than 42 ºC.

10^4 sperm was introduced at the end of the vertical column of the cross column (Fig.3: A), and allowed to incubate for 20 min. Sperm motility was evaluated by examining the ratio of motile sperm that arrived at the 10-mm ends of both wide columns (Fig.3: B,C). After immobilizing the sperm by warming to 60 ºC, thermotaxis was evaluated by measuring the number of sperm that had arrived at both ends of the wide columns.

Statistical analysis

Each experiment was replicated at least three times. The significance of the differences in sperm motility and migrated sperm numbers were determined with Student’s t-test and the Welch’s F test. For this part of the analysis, multiple range tests were adapted.

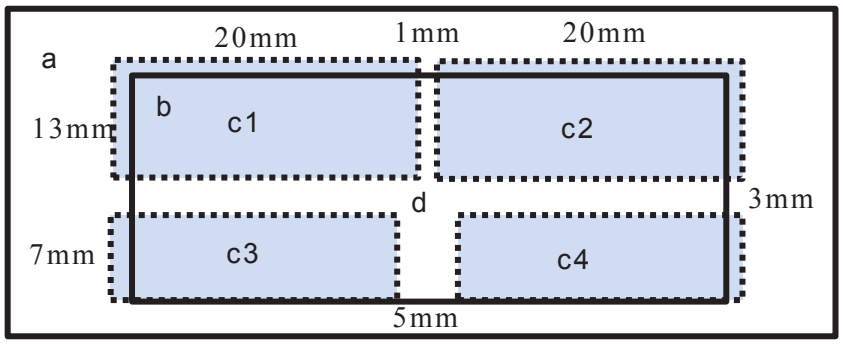

Fig. 2

Sperm motility and thermotaxis were estimated using analysis chambers (Fig. 2). The chamber was constructed with a slide glass (Fig. 2: a) and a cover glass (22 × 40) (Fig. 2: b; a solid-line square) adhered by double-stick tape (100-μm thick) (Fig. 2: c1–c4; dotted-line squares) to create a cross-type column (Fig. 2: d). The cross-type column was 10-mm vertical long (width: 5 mm), 40-mm broad (width: 3 mm), and connected vertically for 13 mm (width: 1 mm) onto the slide glass with double-stick tape; the cover glass was then adhered, and the column was filled with BO (Fig. 2). Both ends of the wide column were covered with mineral oil after the sperm was introduced.

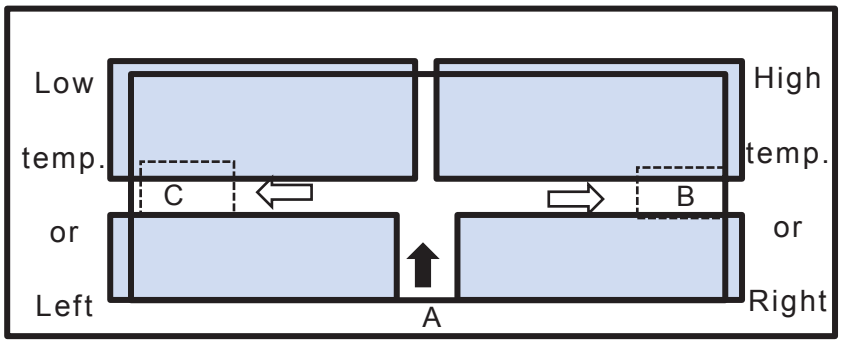

Fig. 3

A total of 10^4 sperm were introduced at the end of the vertical column of the cross-type column (Fig. 3: A), and allowed to incubate for 20 min. Sperm motility was then evaluated by examining the ratio of motile sperm that arrived at the 10-mm ends of both wide columns (Fig. 3: B,C). After immobilizing the sperm by warming to 60 ºC, thermotaxis was evaluated by measuring the number of sperm that had arrived at the ends of both wide columns (Fig. 3: B, C)

II-3. Results

Sperm motility and migration at various temperatures (Table 1)

Table 1 presents the results for sperm motility under different temperatures, and migration to both wide column ends. Motility at 38 ºC was 52.7%, significantly higher than at 34 ºC (41.3%). There was no significant difference in number or ratio of migrated sperm to the right and left ends of the wide column.

Sperm migration in columns with temperature gradients (Table 2)

Table 2 shows sperm migration in 1 ºC or 2 ºC temperature-difference gradients. In the 2 ºC difference gradient, the migrated sperm ratio in the high-temperature area of 40 ºC was 62.5%, significantly higher than that in the low-temperature area (38 ºC). In the 1 ºC difference gradient, the migrated sperm ratio in the high-temperature area (39 ºC) was 59.7%, significantly higher than that in the low-temperature area (38 ºC).

Effect of Ca on bull sperm motility and migration (Table 3)

Table 3 presents the effects of calcium on sperm motility and migration. The sperm migration ratio to the high-temperature area of 39 ºC in both BO (58.9%) and 2Ca (59.4%) were significantly higher than that to the low-temperature area. Sperm migration ratio toward the high-temperature area of 39 ºC in Ca- (56.7 %), BO containing EDTA (56.6%) and BO containing BAPTA-AM (57.9%) were also higher than to the to the low-temperature area, but the difference was not significant.

Effect of Ca channel inhibitor on bull sperm motility and migration (Table 4)

Table 4 presents the effects of the calcium channel inhibitors on sperm migration. In the BO with verapamil and 2APB, sperm motility was significantly lower than others, 43.6% and 41.8%, respectively. Similar to the control, in BO with Mibefradil, sperm migration to the high-temperature area of 39 ºC was significantly higher, at 59.0%. In the BO with verapamil and SKF, there was a tendency toward more sperm migration to the high-temperature area, but it was not significant. In the BO with lanthanum, ruthenium red, and 2APB, the ratio of migrated sperm to the high temperature area was almost same, 53.7%, 55.9%, and 53.5%, respectively.

Sperm motility and migration of non-capacitated and capacitated bull sperm (Table 5)

Table 5 shows the thermotaxis of capacitated sperm. The motility and total number of migrated sperm to both high- and low-temperature areas was lower than for pre-capacitation. Capacitated sperm migration to the high-temperature area of 40 ºC and 39 ºC were 60.4% and 59.1%, respectively, significantly higher than for the low-temperature area.

Sperm motility and migration of fertility confirmed bulls (Table 6)

Table 6 shows the thermotaxis of the sperm from the three bulls with their fertility rates. There was no difference in sperm motility between the

three bulls. The migrated sperm ratio to the high-temperature area of 39 ºC of the high-fertility bulls A and B were 60.6%, 59.5%, significantly higher than for the low-temperature area. Sperm migration to the high-temperature area of low fertility bull C was 52.4%, similar to the migrated sperm to the low-temperature area.

Table 1. Bull sperm motility and migration at various temperatures (34–42°C)

Temp. (°C)	Motility (%)		Left			Right	
			No. sperm	%		No. sperm	%
34	41.3 ± 2.1	a	179.4 ± 14.9	51.1		171.9 ± 22.4	48.9
36	46.6 ± 3.4		189.6 ± 25.2	47.6		208.5 ± 25.9	52.4
38	52.7 ± 5.2	b	280.3 ± 28.1	46.5		322.4 ± 29.5	53.5
40	49.8 ± 3.2		288.7 ± 24.9	52.9		257.2 ± 33.5	47.1
42	44.0 ± 2.7		203.1 ± 25.5	47.7		222.6 ± 22.6	52.3

Each value represents the mean ± SEM of sperm from 3-5 experiments.

a, b : Indicates a significant difference ($P<0.05$).

Table 2. Bull sperm migration in columns with temperature gradients (34–42ºC)

Temp. (ºC)	Low temp			High temp		
	No. sperm	%		No. sperm	%	
38	276.9 ± 36.4	45.2		335.7 ±27.7	54.8	
34-36	318.2 ± 39.5	46.9		360.3 ± 33.2	53.1	
36-38	286.5 ± 35.7	44.5		357.2 ± 30.4	55.5	
38-40	276.5 ± 26.3	37.5		461.7 ± 31.8	62.5	*
40-42	284.2 ± 34.2	44.3		357.1± 35.6	55.7	
36-37	256.1± 30.7	45.9		301.4 ± 33.2	54.1	
37-38	286.1 ± 37.3	45.6		341.7 ± 31.5	54.4	
38-39	294.1 ± 25.3	40.3		435.3 ± 31.2	59.7	*
39-40	297.3 ± 28.5	46.1		347.1 ±30.1	53.9	
40-41	268.41 ± 33.5	43.9		342.6 ±29.5	56.1	

Each value represents the mean ± SEM of sperm from 3-5 experiments.

*The number of sperm was significantly different between low and high temperature of the same temperature gradient ($P<0.05$).

Table 3. Effect of Ca on bull sperm motility and migration in the column with a temperature gradient

Medium	Motility (%)	Low temp		High temp		
		No. sperm	%	No. sperm	%	
BO	52.1 ± 6.1	293.1 ± 22.5	41.1	419.5 ± 32.4	58.9	*
Ca-	48.4 ± 3.0	265.7 ± 27.8	43.3	347.5 ± 25.9	56.7	
2Ca	52.8 ± 6.3	269.3 ± 30.4	40.6	434.2 ± 33.9	59.4	*
ET	46.5 ± 4.2	253.8 ± 39.6	43.4	331.2 ± 41.3	56.6	
BT	43.9 ± 5.8	221.7 ± 33.4	42.1	304.9 ± 41.3	57.9	

Each value represents the mean ± SEM of sperm from 3-5 experiments.

Ca-; Ca free, 2Ca; double concentration of Ca, ET; BO containing EGTA, BT; BO containing BAPTA-AM

Sperm motility was evaluated by the ratio of migrating sperm.

*The number of sperm was significantly different between low and high temperature of the same temperature gradient ($P<0.05$).

Table 4. Effect of Ca channel inhibitor on bull sperm motility and migration in columns with a temperature gradient (38–39°C)

Medium	Motility (%)		Low temp			High temp		
			No. sperm	%		No. sperm	%	
BO	51.7 ± 3.3	a	261.4 ± 25.1	39.6		398.5 ± 31.4	60.4	*
La3+	45.1 ± 3.5	ab	261.5 ± 38.2	44.9		320.9 ± 40.9	55.1	
verapamil	41.5 ± 5.8	b	200.8 ± 41.5	42.5		271.3 ± 44.3	57.5	
mibefradil	47.1 ± 6.4	ab	262.3 ± 25.4	40.5		385.1 ± 31.5	59.5	*
SKF	47.9 ± 5.1	ab	275.3 ± 29.3	43.1		362.8 ± 44.1	56.9	
RR	45.8 ± 3.9	ab	261.5 ± 37.8	44.1		331.7 ± 47.3	55.9	
2APB	40.7 ± 4.8	b	211.5 ± 41.9	46.6		242.3 ± 39.5	53.4	

Each value represents the mean ± SEM of sperm from 3-5 experiments.

La3: lanthanum; RR: rutenium red.

Sperm motility was evaluated as the ratio of migrating sperm.

a, b : The sperm motility was significantly different between the medium of sperm treatment ($P<0.05$).

*The number of sperm was significantly different between low and high temperature of the same temperature gradient ($P<0.05$).

Table 5. Sperm motility and migration of non-capacitated and capacitated bull sperm in various temperature gradients (37-40°C)

Sperm capacitation	Temp. (°C)	Motility (%)		Low temp			High temp		
				No. sperm	%		No. sperm	%	
non-capacitated	37-38	50.3 ± 5.9	a	280.5 ± 38.1	45.9		331.2 ± 29.6	54.1	
	38-39	52.4 ± 5.0	a	284.3 ± 22.5	39.9		428.7 ± 35.3	60.1	*
	39-40	49.2 ± 4.9	a	273.6 ± 25.4	44.8		337.1 ± 28.7	55.2	
capacitated	37-38	41.3 ± 3.9	b	213.9 ± 34.5	43.2		291.7 ± 36.5	57.7	
	38-39	42.1 ± 4.8	ab	197.4 ± 38.5	41.2		281.9 ± 37.9	58.8	*
	39-40	38.9 ± 4.5	b	169.1 ± 41.2	39.2		262.2 ± 34.3	60.8	*

Each value represents the mean ± SEM of sperm from 3-5 experiments.

Sperm capacitation was induced with treatment of incubation of sperm with media containing with heparin, caffein.

a, b : The sperm motility was significantly different with/without sperm capacitation ($P<0.05$).

*The number of sperm was significantly different between low and high temperature of the same temperature gradient ($P<0.05$).

Table 6. Sperm motility and migration of fertility confirmed with temperature gradient (38-39°C)

Bull	Fertility(%)	Motility (%)	Low temp		High temp		
			No. sperm	%	No. sperm	%	
A	65	51.5 ± 3.5	215.1 ± 41.5	38.8	338.9 ± 48.5	61.2	*
B	57	49.8 ± 4.9	264.3 ± 23.2	40.6	387.4 ± 31.9	59.4	*
C	34	53.5 ± 5.7	331.5 ± 33.1	48.4	353.6 ± 40.9	51.6	

Each value represents the mean ± SEM of sperm from 3-5 experiments.

Fertility of bull A, B and C revealed with conception ratio after AI were 65%, 57%, 34%, respectively.

*The number of sperm was significantly different between low and high temperature of the same temperature gradient ($P<0.05$).

II-4. Discussion

There are few studies regarding thermotaxis and chemotaxis in bull sperm, and the relation between migration ability of sperm and fertility of bulls is uncertain. In this chapter, thermotaxis in bull sperm was confirmed and examined its mechanisms, particularly the involvement of calcium and the relation between thermotaxis and sperm capacitation and bull fertility. Bull sperm showed significantly higher motility and more migrated sperm at 38 ºC. This suggests that bovine body temperature supports migration, fertilization, and involvement of thermotaxis in fertilization.

Temperature at the fertilization site of rabbits at ovulation is higher than the sperm storage site and increases after ovulation [24]. In particular, it has been confirmed that the difference in temperature at the fertilization site significantly increases from 0.8 ºC at pre-ovulation to 1.6 ºC post-ovulation [16, 24]. In this study, many bull sperm migrated from a low 38 ºC to high 40 ºC or from a low 39 ºC to high 40 ºC. It has been reported that at the time of ovulation, the temperature gradient from the isthmus to the ampulla of the oviduct is 0.5-2 ºC higher than body temperature [15, 16, 17]. It has also been confirmed that the intravaginal temperature of bovines was elevated 0.4 ºC at estrus [25, 26]. Therefore, a temperature gradient of 39–41 ºC, higher than body temperature (38-39 ºC) [26, 27], is formed in the reproductive tract of the estrus bovine, and thermotaxis may be involved in supporting the migration of fertilizing sperm. In this study, the thermotaxis of bull sperm toward the 1–2 ºC higher temperature was confirmed and suggested the involvement of thermotaxis in the

fertilization mechanism of bull sperm.

The effect of calcium on bull-sperm thermotaxis was examined to investigate the expression mechanism of thermotaxis. Calcium plays an important role in sperm hyperactivation [28], acrosome reaction [29] and chemotaxis [30]. In this chapter, firstly the effect of two kinds of chelating agents; EGTA acts outside of sperm, were examined while BAPTA-AM acts specifically on the intracellular storage of calcium, which is associated with calcium ion decrease both internal and external to the cell. Human sperm thermotaxis regulated by internal stored Ca^{2+} was reported by Bahat et al. [17], however it was not confirmed that either intracellular or extracellular storage-type calcium was involved in bull-sperm thermotaxis. Calcium influx is regulated by several types of calcium channels. The TRP channel is an important temperature-related calcium channel [31, 32], but it has been determined that it is not involved in human-sperm mechanisms [17]. Kawanishi et al. and Hamano et al. confirmed the involvement of TRPV4 in sperm thermotaxis using Trpv4 gene-deficient mice [18, 19]. Recently Kumar et al. [33] showed that TRPV4 induced calcium influx into human sperm, and was strongly associated with fertilization. These findings suggest that the different function of calcium channels by animal species and the multiple calcium channels involvement in thermotaxis [17, 34].

In the calcium ion channel analysis in this study, thermotaxis was not confirmed in either lanthanum; general calcium ion channel inhibitor and SKF, or Ruthenium red; TRP calcium ion channel inhibitor. Involvement

of these calcium channels in sperm thermotaxis has been suggested. Because verapamil; voltage-dependent L-type calcium ion channel inhibitor and 2APB; intracellular calcium-releasing inhibitor significantly inhibited motility as compared with other treatments, sperm migration ability by thermotaxis might be inhibited indirectly. Mibefradil ; voltage-dependent T-type calcium ion channel, which also inhibits the CatSpar channel involved in sperm hyperactivation, did not affect sperm thermotaxis.

From the above, it was surmised that thermotaxis was regulated not only by single effects, but also by mechanisms such as the involvement of the calcium ion, calcium ion channel, and intracellular calcium release.

In this chapter, the different results were demonstrated from those of humans [17, 34] in terms of expression of thermotaxis of bull sperm pre- and post-capacitation. The thermotaxis of bull sperm was confirmed as sperm migrated to higher temperature areas pre-capacitation with a lower ratio compared to that of post-capacitation. Bull sperm shows positive thermotaxis in the female reproductive tract before and after ovulation. In other words, bull sperm injected into the reproductive tract at pre-ovulation expresses thermotaxis, by which sperm can recognize slight differences of temperature, and may support migration ability in the uterus and oviduct. In the temperature gradient of 38–39 ºC, the sperm migration ability of bull C, which showed conception rates of 34% after AI, was lower than that of bulls A and B, which showed higher rates of 65% and 57%, respectively. More definitive conclusions will require evaluation of

a large number of bulls. Because thermotaxis of pre- and post-capacitation was involved in the migration of sperm in the female reproductive tract, it may be that bull fertility could be estimated by increasing the sample size to a large number of bulls.

In conclusion, bull sperm thermotaxis was confirmed. The involvement of specific calcium channels and intracellular storage calcium in the bull sperm thermotaxis were suggested. Furthermore, the relation between thermotaxis and bull fertility may be surmised. Therefore thermotaxis may be a potential predictor of bull fertility.

II-5. Summary

In this study, the migration ability of bull sperm in a temperature gradient was examined to confirm thermotaxis and elucidate the involvement of calcium in such thermotaxis, as well as the relation between sperm capacitation and bull fertility. Thermotaxis was evaluated in a temperature gradient of 34–42 ºC using analysis chamber with cross-type column. Significantly more sperm migrated to 39 ºC than 37 ºC temperature gradient, and to 40 ºC than 39 ºC in the temperature gradient. The migrated sperm ratio in the two temperature areas was almost the same in calcium-free, BAPTA containing, and EGTA containing media. Thermotaxis were not confirmed in media containing lanthanum, ruthenium red, and 2APB. Pre- and post-capacitated sperm migrated to the high-temperature area, expressing thermotaxis. The sperm from high-fertility bulls showed clear thermotaxis. Based on these results, thermotaxis of bull sperm was confirmed and the involvement of both calcium channels and intracellular stored calcium in thermotaxis was suggested. The relation between thermotaxis and bull fertility was confirmed, and bull fertility diagnosis and improvement of cow conception rate by sperm thermotaxis evaluation were suggested.

Chapter III

Motility and migration analysis of chemotactic reacted sperm

III-1. Introduction

Mammalian sperm migrate in the female genital tract and fertilize in the ampulla of the uterine tube. To date, the sperm motility, migration ability, and capacitation which are essential for fertilization have been studied [24, 27, 29]. Chemotaxis is the regulation of the direction of movement of migrating sperm up a concentration of extracellular chemoattractant gradient. In vivo sperm motility and migration are strongly influenced by the physiological state of contraction of and fluid secretion in the female genital tract. Sperm chemotaxis up to the gradient of follicular fluid has thus far been reported and distinguished from other processes that may cause sperm accumulation in only two mammalian species: humans and mice. Sperm chemotaxis was confirmed in species fertilized in vitro [10, 35]. Based on a detailed kinetic analysis of migrated sperm in a female genital tract, it was found that chemotaxis [7] thermotaxis [20, 23], and rheotaxis [16] are involved in the sperm migration ability. In mammals, swine sperm chemotaxis toward the leukocytes [36] and chemotaxis to the follicular fluid in rabbit and human sperm have been reported [7, 37]. The follicular fluid includes fluid secreted from the ovary and follicular cells and influences the physiological function and motility of the sperm. Sperm chemotaxis has been conventionally analyzed using the needle method and the chamber method [10]. In the study of the chemotactic movement of

marine invertebrates' sperm, mainly the sperm trajectory in vitro has been examined. Ascidian sperm swims in a straight line at the time of access to the chemoattractant, and the sperm makes sudden quick changes in the direction of the swimming paths to the chemoattractant when leaving from the chemoattractant [35, 38]. Calcium bursts induced by the concentration of the chemoattractant SAAF trigger a sequence of flagellar responses comprising quick turns to direct the sperm toward the eggs [39]. There have been few studies on the motility mechanism of mammalian sperm chemotaxis. Although the trajectory of the sperm head has been examined [7, 40-42], there is little detailed analysis of the flagellar movement. Sperm motility is analyzed objectively and exactly using CASA.

In this chapter, the migration and motility of the sperm toward the bovine follicular fluid (BF) were examined for the purpose of confirming bull sperm chemotaxis and to elucidate the motility mechanism of chemotaxis using CASA.

III-2. Materials and Methods

Media and chemicals

Media were prepared as explained detail in chapter II, and BO containing 1% BSA was used for washing, dilution of sperm, and column construction. HC was contained BO mixed with both heparin and caffeine. Sperm pre-incubation was similarly conducted as explained detailed in chapter II, and in BO or HC with 38.5 ºC, 5% CO_2/95% air.

BF was obtained from bovine follicles (2-5 mm in diameter) of the ovary and frozen at -80 ºC. Frozen BF was thawed and diluted with BO from 100 to 10^{-4}. The media used for the washing and migration examination of the sperm was BO either with or without BF.

Semen

Semen was obtained from three bulls, A, B and C had conception rates of 65%, 57%, and 34%, as explained detail in chapter II. Evaluation of thermotaxis was conducted using the semen from bull B, and I evaluated sperm thermotaxis of the three bulls, A, B, and C. The evaluation of chemotaxis was conducted using semen from bull B, and I evaluated the fertility of all three bulls.

Chamber preparation

Chemotaxis was estimated using manufactured analysis chambers, as explained detail in chapter II (Fig. 2). Both ends of the wide column were covered with mineral oil after the sperm was introduced.

Sperm preparation

Frozen bull semen was thawed in a water bath at 38.5 ºC and diluted with an equal volume of BO. The diluted semen was washed three times by centrifugation at 1500 rpm for 5 min. The sperm concentration was adjusted to 10^7/ml.

Evaluation of sperm motility and chemotaxis

The sperm suspension (10 µl) was examined motile sperm ratio using an examination plate (Sekisui, Tokyo) at 38.5 ºC. The motility was shown as a ratio of the total sperm (%). Sperm movements were recorded by phase-contrast microscope with power LED stroboscopic illumination system. Sperm swimming paths and flagellar waveforms were analyzed by CASA using Bohboh software. The sperm head image and flagellar waveform could be tracked automatically, and the swimming velocities, path curvatures, and flagellar curvature could be calculated from the tracking data as follows: straight-line velocity (VSL), the distance between the first- and last-tracked points divided by the time elapsed, curvilinear velocity (VCL), the sum of the distances between adjacent points divided by the time elapsed, linearity (LIN), an index of the straightness of the path, given by VSL/VCL×100, flagellar curvilinear ratio (FCR), an index of the curvilinearity of the flagella, calculated from points corresponding to around 5 µm in flagellar length after 11 points are averaged, and flagellar beat frequency (FBF), an index of vigor, given by the frequency

with which the cell track crosses the smoothing path in either direction.

The sperm chemotaxis was evaluated by examining the sperm's migration ability in a chemoattractant BF gradient in the cross-type column (Fig. 2). In the no-BF gradient condition, BO was set at both ends of the wide column. In the BF gradient condition, diluted BF (diluted with BO from 100 to 10^{-4}) was set at one end of the wide column, and BO was set at the other end (Fig. 4). Sperm were introduced into the end of the vertical part (Fig. 4 black arrow) of the cross-type column and observed for 20 min. I evaluated the sperm's chemotaxis by measuring the number of sperm that arrived at the ends (Fig. 4; D, E) of both columns after I immobilized the sperm by warming it to 60ºC. Sperm swimming paths and flagellar waveforms were analyzed at pre- (Fig. 4; A), during (Fig. 4; B, C) and post- (Fig. 4; D, E) winding and migration in the ends of the vertical and broad columns of the chamber.

Statistical Analysis

Each experiment was replicated at least four times using sperm from different males. The significance of the differences in sperm motility and in the migrated sperm numbers was determined with Student's t-test and the Mann-Whitney U-test.

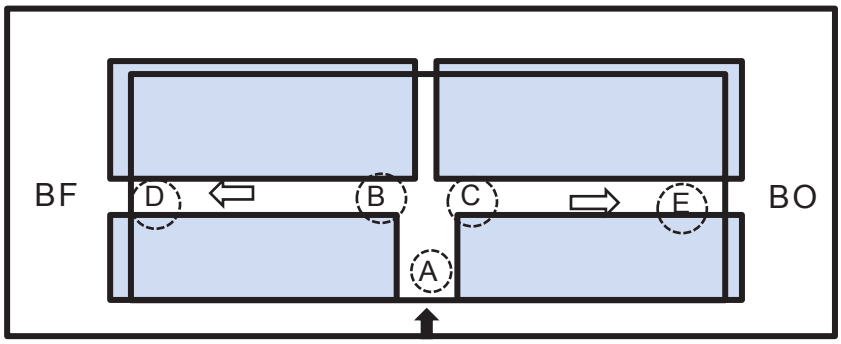

Fig. 4

Motility and swimming paths evaluation of chemotactic sperm in the cross-type column. The analysis areas are selected for pre- (A), during (B, C) and post- (D, E) winding and migration in the ends of the vertical and broad columns of the chamber.

III-3. Results

Effect of different concentration of BF on the sperm attraction (Table 7)

Table 7 shows bull sperm attraction to the different concentrations of BF. The ratio of sperm migration to 0.1% BF was 61.6%, which was significantly higher than that to BO. The ratios of sperm migration to BF and 10% BF were 26.3% and 32.8%, respectively, and were significantly lower than that to BO. The ratios of sperm migration to 1% and 0.01% BF showed no remarkable difference with that to BO.

Attraction of pre- and post- capacitated bull sperm in BF gradient (Table 8)

Table 8 represents Pre- and post-capacitated bull sperm motility and migration. The ratios of the migration of pre- and post-capacitated bull sperm to BF were 62.1% and 59.6% and were significantly higher than that to BO. VCL, VSL and LIN of post-capacitated sperm were lower than those of pre-capacitated sperm.

Bull fertility and its sperm attraction (Table 9)

Table 9 shows sperm motility and migration of 3 bulls. The ratios of sperm aattraction to BF of bulls A, B, and C were 63.9%, 61.8%, and 59.2% and were significantly higher than those to BO. There was no remarkable difference in the mobility and LIN of the sperm from bulls A, B, and C. The VCL and VSL of the migration bull A's sperm to BF were significantly higher than those of bull C.

Motility of attracted migrating sperm (Table 10)

Table 10 represents the motility of the sperm pre- (Fig. 4; A), during (Fig. 4; B, C) and post- (Fig. 4; D, E) winding and migration to BF or BO. The area (Fig. 4; A – E) of the cross-type column used for sperm motility analysis is shown in Fig. 4. From the analysis of the trajectory of the sperm that migrated to BF, I determined that the sperm wind more than 45 degrees and migrate as "winding sperm". The number of times that the “winding sperm” engaged in winding was one or more. The sperm that migrated to BO showed the trajectory of a major gentle arc, or migrated straight in the vertical column and then along the wide column. FCR of the sperm (Fig. 4; B) as it wound to the BF was significantly higher than pre- and post-winding. The VCL and VSL of pre-winding sperm (Fig. 4; A) in the vertical column tended to be high, but there was no remarkable difference in the LIN and FBF of these sperm The VCL, VSL, and FBF of the sperm (Fig. 4; B) winding to the BF tended to be higher than the others, but the difference was not significant.

Table 7. Effects of different concentrations of bovine follicular fluids (BF) on the attraction of bull sperm

Chemoattractant	Migrating sperm	BF	10% BF	1% BF	0.1% BF	0.01% BF	BO
BO	No	188.9 ± 15.7	235.8 ± 19.4	176.1 ± 15.3	105.7 ± 11.4	129.5 ± 12.8	131.6 ± 14.9
	%	73.7 ± 5.9^{a,A}	67.2 ± 5.1^{b,A}	57.8 ± 7.9	38.4 ± 5.8^{c,A}	44.9 ± 7.6	46.4 ± 7.1^{B}
BF	No	67.5 ± 10.3	114.6 ± 15.2	128.4 ± 14.8	169.3 ± 12.9	159.1 ± 18.3	151.9 ± 14.7
	%	26.3 ± 5.8^{a,C}	32.8 ± 6.2^{b,C}	42.2 ± 8.1	61.6 ± 6.0^{c,C}	55.1 ± 5.8	53.6 ± 6.8^{D}

Each value represents the mean ± SEM of results (the number and ratio of sperm) of 8-10 experiments.

Bull B semen was used in this experiment.

BF (3 µl) and BO (3 µl) were set at the opposite ends of the horizontal column of the manufactured analysis chamber.

There were significant differences between values marked with the same letters (a, b and c) within the same columns, $p<0.05$.

There were significant differences between values marked with A and B and between values marked with C and D within the same lines, $p<0.05$.

Table 8. Pre- and post- capacitated bull sperm motility and migration in the BF gradient

Sperm capacitation treatment*	Chemoattractant	Migrating sperm					
		No	%	Motility (%)	VCL(μ m/s)	VSL(μ m/s)	LIN (%)
Before	BO	111.4 ± 13.3	37.9 ± 5.5[a]	51.4 ± 6.1	138.4 ± 19.5	78.1 ± 8.3	56.5 ± 6.2
	BF	182.6 ± 17.4	62.1 ± 6.3[a]	52.3 ± 7.5	171.3 ± 21.9	93.5 ± 10.2	54.4 ± 5.3
After	BO	89.7 ± 15.7	40.4 ± 5.9[b]	40.6 ± 4.9	105.7 ± 14.8	48.6 ± 5.1	45.7 ± 4.5
	BF	132.3 ± 12.4	59.6 ± 4.8[b]	43.7 ± 5.1	123.6 ± 17.1	59.4 ± 6.3	48.0 ± 5.1

*Sperm were treated in the capacitation media containing heparin and caffeine for 4 h.

Bull B semen was used in this experiment.

BF (0.1%, 3 μl) and BO (3 μl) were set at the opposite ends of the horizontal column of the manufactured analysis chamber.

Each value represents the mean ± SEM of results of 3-5 experiments.

There were significant differences between values marked with the same letters (a and b) within the same columns, $p<0.05$.

Table 9. Sperm motility and migration of fertility-confirmed bulls in the BF gradient

Bulls	Chemoattractant	Migrating sperm					
		No	%	Motility (%)	VCL(μ m/s)	VSL(μ m/s)	LIN (%)
A	BO	116.6 ± 13.5	36.1 ± 4.4[a]	55.5 ± 6.4	144.5 ± 16.2	85.2 ± 9.2	59.0 ± 7.2
	BF	206.4 ± 19.4	63.9 ± 6.8[a]	54.8 ± 7.2	195.8 ± 20.9[d]	109.4 ± 11.3[e]	55.9 ± 6.4
B	BO	112.7 ± 10.3	38.2 ± 5.6[b]	50.3 ± 7.1	129.6 ± 18.1	71.9 ± 8.7	55.0 ± 4.7
	BF	182.3 ± 20.3	61.8 ± 6.1[b]	53.4 ± 5.6	166.1 ± 22.2	88.2 ± 6.6	53.0 ± 5.5
C	BO	112.2 ± 14.1	40.8 ± 4.5[c]	49.1 ± 5.3	102.3 ± 9.5	48.1 ± 5.9	47.1 ± 4.9
	BF	162.8 ± 15.2	59.2 ± 5.3[c]	51.7 ± 6.5	111.7 ± 13.8[d]	52.7 ± 7.1[e]	46.8 ± 5.4

The fertility levels of bulls A, B and C in terms of the conception ratios of cows after AI were 65%, 57%, and 34%, respectively.

BF (0.1%, 3 μl) and BO (3 μl) were set at the opposite ends of the horizontal column of the manufactured analysis chamber.

Each value represents the mean ± SEM of results of 3-5 experiments.

There were significant differences between values marked with the same letters (a, b, c, d and e) within the same columns, $p<0.05$.

Table 10. Kinetic parameters of chemotactically winding bull sperm in the BF gradient						
Chemoattractant	Winding trajectory	VCL(μ m/s)	VSL(μ m/s)	LIN(%)	FCR(rad/um)	FBF(Hz)
pre	pre-winding	202.6 ± 19.7	107.3 ± 9.8	50.2 ± 6.3	0.03 ± 0.01^{a}	10.1 ± 1.8
BO	winding	165.5 ± 17.6	79.2 ± 6.9	47.9 ± 5.1	0.05 ± 0.02^{a}	12.5 ± 2.3
	post-winding	131.9 ± 15.4	75.4 ± 5.7	57.3 ± 5.9	0.05 ± 0.01^{a}	11.3 ± 2.3
BF	winding	189.6 ± 14.9	97.8 ± 8.8	51.3 ± 6.2	0.09 ± 0.02^{b}	13.4 ± 1.8
	post-winding	169.3 ± 17.3	91.2 ± 8.7	53.3 ± 5.7	0.06 ± 0.02^{ab}	12.2 ± 2.7
Each value represents the mean ± SEM of sperm from 3-5 experiments.						
BF (0.1%, 3 μl) and BO (-3μl) were set at the opposite ends of the horizontal column of the manufactured analysis chamber.						
There were significant differences between values marked with different letters (a and b) within the same column, $p<0.05$.						

III-4. Discussion

In this chapter, the existence of bull sperm chemotaxis to BF was confirmed and examined the relation between chemotaxis and capacitation, fertility as well as the motility-regulating mechanism of the chemotaxis-expressing sperm.

Sperm chemotaxis long known in marine species, because of the general agreement about the completion of a large number of sperm to fertilize the egg. Sperm chemotaxis to follicular fluid and egg-related material has been examined in human [42, 43-45], rabbit [7] and mouse [41] sperm, but the motility mechanism of chemotactic sperm is not sufficiently understood. In rabbits and humans, the most strongly chemotactic sperm responded and migrated toward 10^3- and 10^4-times-diluted rabbit follicular fluid [7] and 10^3-times-diluted human follicular fluid [37]. A subpopulation of rabbit sperm showed the high chemotactic response at follicular fluid dilutions of 10^3- and 10^4-times, which is in agreement with the results of humans [42] and mice [41].

In this chapter, the chemotaxis of bull sperms from 1- to 10^4-times-diluted BF were compared, and found that 10^3-times-diluted (0.1%) BF revealed the strongest response. Because similar results have been found in other species, there may be some materials that inhibit sperm motility and migration in follicular fluid. Furthermore, it was speculated that the physiological function of the follicular fluid to attract sperm was increased by dilution with culture media, but the material was not identified. BF may enhance the VSL and VCL of bull sperm and then

increase the motility and swimming velocity of the sperm. These results are supported by reports [40, 41] showing that materials derived from oocytes enhance the VAP and VSL of human and mouse sperm.

Rabbit sperm did not exhibit hyperactivation when exposed to follicular fluid; instead, they maintained a rather linear and progressive migration, becoming increasingly transitional with follicular fluid concentration. At least for human sperm, progesterone is the factor in follicular fluid that causes hyperactivation. Capacitated mammalian sperm induces hyperactivation. Fabro [7] and Ralt [37] reported that chemotaxis is effective for sperm selection and that only capacitated sperm express chemotaxis. It is suggested that the hyperactivation is involved in the direction-changing of chemotaxis-expressing human sperm [42]. Although the involvement of calcium ions in capacitation and in the chemotactic mechanism is known, the hyperactivation of ascidian sperm showing calcium bursts has not been confirmed [39].

In this chapter, chemotaxis was confirmed in both pre- and post-capacitated bull sperm. Furthermore, post-capacitated bull sperm which expressed chemotaxis and migrated to BF did not show hyperactivation. Different species-specific mechanisms may be involved in the relation between capacitation and chemotaxis. In the chemotactic examination using the cross-type column of this study, the ratio of the sperm of the three bulls which migrated to BF was significantly higher than that to BO. Therefore, bull sperm chemotaxis toward BF was confirmed. Also, chemotaxis varied according to bull fertility, and the chemotaxis to BF of

highly fertile bull sperm showed a tendency to be higher, but the difference was not significant. As for bull fertility and sperm capacitation, it seems likely that other factors is guessed in addition to chemotaxis are involved.

In this chapter, the motility and kinetic trajectory of the head and flagella of chemotactic bending sperm to BF were analyzed using CASA for elucidating the motility mechanism of chemotactic sperm. It was speculated that chemotaxis-expressing bull sperm showed increased VSL and VCL, and then increased swimming velocity, because the VCL and VSL of the sperm winding to BF tended to be higher than those to BO. Most of the sperm confirmed to have wound to the BF were confirmed to be "winding sperm" which bent more than 45 degrees. The FCR of the winding and bending sperm that showed chemotaxis to BF was significantly higher than that of other sperms. Therefore, I concluded that chemotaxis-expressing bull sperm changed their direction of migration to a chemoattractant by changing their FCR. Miller [35] and Yoshida et al. [46] reported that the change in the swimming direction toward a chemoattractant is induced by the quick turning of the swimming path with asymmetric waveforms. The involvement of the flagellar movement mechanism in direction-changing toward a chemoattractant may be different between marine animal sperm expressing chemotaxis in vitro in seawater and mammalian sperm expressing it in vivo in the female genital tract. The sliding of dynein motor-driven microtubules during sperm chemotaxis was reported by Mizuno et al. [47]. To elucidate the swimming direction-changing mechanism of chemotactic sperm, a general

investigation of the chemical characteristics of the chemoattractant, the signal transduction mechanism of the calcium ion, and the correlation of dynein and microtubules should be performed. In this chapter, the involvement of BF in both the increase of the bull sperm velocity and in the changing of the swimming direction toward BF by chemotactic sperm were suggested. The results of this study contribute to the elucidation of the bull sperm motility.

In conclusion, bull sperm chemotaxis was confirmed. The involvement of BF in both the increase of sperm velocity, VSL and VCL and in the swimming direction, changing of sperm migrating direction were suggested. Furthermore, the relation between chemotaxis and thermotaxis, chemotaxis and bull fertility may be surmised. Therefore chemotaxis may be a potential predictor of bull fertility.

III-5. Summary

The chemotaxis was evaluated for examining the sperm migration to one end BF of the wide column using a cross column chamber. Movement of head and flagellar of chemotaxis expressing sperm were analyzed by CASA. I confirmed bull sperm chemotaxis by recognizing of 61.6% of sperm migration toward 0.1% BF. Both large number of pre and post capacitated bull sperm migrated toward BF. VCL and VSL of the sperm of the high fertility confirmed bull were significantly higher than low bull. FCR of the sperm winding to BF was more significantly higher than that of pre and post winding sperm. On the basis of these results, the chemotaxis of bull sperm was confirmed, and chemotactic response to BF made increasing of VSL and VCL of bull sperm. It was suggested that chemotaxis expressing bull sperm toward BF changed the swimming direction by the change of FCR.

Chapter IV

General Discussion

The present research was undertaken to elucidate the mechanism of thermotaxis and chemotaxis of bull spermatozoa and present research work was consisted of two experiments. In first experiment it has been examined the migration ability of bull sperm in a temperature gradient to confirm thermotaxis and elucidate the involvement of calcium in such thermotaxis, as well as the relation between sperm capacitation and bull fertility. Thermotaxis was evaluated with setting in a temperature gradient at 34-42°C using a cross-type column. Significantly higher sperm migrated to the high-temperature area of 39 ºC in a 2 ºC temperature gradient, and to 40 ºC in a 1 ºC temperature gradient. In calcium-free, BAPTA and EGTA containing medium, the migrated sperm ratio in the two temperature areas found almost same. Pre- and post-capacitated sperm migrated to the high-temperature area, expressing thermotaxis. The sperm from high-fertility bulls showed clear thermotaxis and based on these results, thermotaxis of bull sperm was confirmed and the involvement of both calcium channels and intracellular stored calcium in thermotaxis further suggested. The relation between thermotaxis and bull fertility was confirmed, and bull fertility diagnosis and improvement of cow conception rate by sperm thermotaxis evaluation were concluded.

Sperm chemotaxis during fertilization is a widely observed phenomenon across most species. Although sperm chemoattractants released by egg and

accessory organs are species specific and differ among species, they induce similar behaviors, in the swimming direction of swimming migration. Mammalian sperm ejaculated into a female reproductive tract migrate to the fertilization site due to the effects of multiple factors. Chemotaxis has also been described in marine animals and bacteria [30], and in mammals the chemotaxis of sperm that migrated toward chemical attractants in the cumulus cells and follicular fluid was reported [7]. Mammalian sperm must migrate in the female reproductive tract in order to penetrate and fertilize an ovulated egg in the ampulla.

Recently, it was reported that the migration of sperm in the female reproductive tract associated with fertilization is at the very least regulated by chemotaxis and thermotaxis [10-11]. Furthermore, the involvement of rheotaxis has been suggested [12], and the mechanisms of taxis in sperm migration associated with fertilization have been elucidated [13]. Before arriving at the ampulla, sperm motility is important for passing the utero cervix and tubal junction, and is essential for fertilization. The involvement of chemotaxis, thermotaxis, and rheotaxis in the migration of sperm has been previously reported [13].

Thermotaxis in mammals is a transport-supporting mechanism for sperm penetration and egg fertilization in the female reproductive tract. The motility function of migrating sperm in relation to female conception rate and male fertility have been studied, and physiological conditions such as components of secreting fluid, temperature, and contraction of the reproductive tract are known to strongly affect sperm migration. However,

there are few studies regarding thermotaxis and chemotaxis in bull sperm, and the relation between migration ability of sperm and fertility of bulls is uncertain.

In this study, the presence of thermotaxis in bull sperm so far been confirmed and also able to established the mechanisms, particularly the involvement of calcium and the relation between thermotaxis and sperm capacitation and bull fertility. Temperature at the fertilization site of rabbits at ovulation is higher than the sperm storage site and increases, depending on a time course, after ovulation [24]. At the time of ovulation, the temperature gradient from the isthmus to the ampulla of the oviduct is higher than body temperature [15, 17]. A temperature gradient, higher than body temperature is formed in the reproductive tract of the estrus bovine, and thermotaxis may be involved in supporting the migration of fertilizing sperm [26, 27]. The thermotaxis of bull sperm toward the 1–2 ºC higher temperature was confirmed and present findings further suggested the involvement of thermotaxis in the fertilization mechanism of bull sperm.

In second experiment chemotaxis was evaluated in order to examine the sperm migration to BF using a cross-column chamber. After analysis of movement of chemotaxis-expressing sperm, bull sperm chemotaxi was noted that s by recognizing of the migration of 62.7% of sperm to 0.1% BF. Both pre- and post-capacitated bull sperm migrated toward the BF. The high-fertility-confirmed bull sperm velocity were significantly higher than the low-fertility bull.

Finally it was concluded that the chemotaxis of bull sperm was

confirmed, and the chemotactic response to BF increased bull sperm velocity and that chemotactic bull sperm toward BF changed their swimming direction through a change in FCR. Present study was confirmed the existence of bull sperm chemotaxis to BF and examined the relation between chemotaxis and capacitation and fertility as well as the motility-regulating mechanism of the chemotaxis-expressing sperm. In this study, the chemotaxis of bull sperms was found and revealed the strongest response to 10^3-times-diluted BF. Because similar results have been found in other species. Furthermore, it was speculated that the physiological function of the follicular fluid to attract sperm was increased by dilution with culture media, but the material was not identified. These results are supported by other reports of human [40] and mouse [41] sperm. The chemotaxis is known to be effective for sperm selection and that only capacitated sperm express chemotaxis. After apacitation, mammalian sperm induces hyperactivation [42]. But the hyperactivation of ascidian sperm showing calcium bursts has not been confirmed [39]. In this study, post-capacitated bull sperm which expressed chemotaxis and migrated to BF did not show hyperactivation. Different species-specific mechanisms may be involved in the relation between capacitation and chemotaxis. In this study, the ratio of the sperm migrated to BF was significantly higher than that to BO. Chemotaxis also varied according to bull fertility, and the chemotaxis to BF of highly fertile bull sperm showed a tendency to be higher, but the difference was not significant. For sperm capacitation and bull fertility, it seems likely that

other factors might be involved in addition to chemotaxis.

In this study, the motility mechanism of chemotactic sperm was also elucidated using CASA. The velocity of the sperm winding to BF tended to be higher than those to BO. Therefore, it was speculated that chemotaxis-expressing bull sperm showed increased VSL and VCL, and then increased swimming velocity. The FCR of the winding and bending sperm that showed chemotaxis to BF was significantly higher than that of other sperms. The chemotactic bull sperm changed their direction of migration to a chemoattractant by changing their FCR. The change in the swimming direction toward a chemoattractant is induced by the quick turning of the swimming path with asymmetric waveforms was reported [35, 46]. The involvement of the flagellar movement mechanism in direction-changing toward a chemoattractant may be different between marine animal and mammalian sperm.

The results of present study indicate that both female fluids enhance sperm motility and migration, and induce a chemotactic response, although the significance of this in vivo is unknown. A hypothesis involving a relay mechanism with the steps is proposed to explain the results. When sperm reach the oviduct, the oviductal fluid enhances their motility and directs them chemotactically towards the isthmus storage site. When capacitated sperm detach from the reservoir, follicular fluid enhances their speed and directs the sperm chemotactically towards the ampulla area to contact the egg. Future study should reveal the identity and the physiological origin of the chemoattractant secreted in the female reproductive tract, as well

as the identity and of their respective receptors on the sperm.

In the future, thermotaxis and chemotaxis may be exploited as a diagnostic method for sperm quality and male infertility and that it may be used as a biological sperm selection procedure before AI, IVF and ICSI. Sperm responsiveness to a temperature and chemoattractant shift could be a new parameter for determining sperm quality, which is easy to measure and evaluate. For being applicable, however, further research is required.

Acknowledgment

All glorifies are due to the Almighty “Allah” who has given the author to pursue higher education and complete this piece of research work successfully and immense indebtedness.

I would like to express my heartfelt gratitude, indebtedness, profound appreciation to my honorable supervisor, Professor Dr. Koh-ichi Hamano Department of Bioscience and food production science, Faculty of Agriculture, Shinshu University for his sagacious innovative suggestions, constant inspiration, cordial support and fruitfull discussion on all phases of research work invaluable advice, suggestions, punctuality and constructive criticism throughout the course of this research work and immense help in preparing the thesis manuscript.

I wish pleased to express his immense gratitude and profound respect grateful thanks to my co-supervisor Professor Dr. Soichiro Nakamura, Faculty of Agriculture, Shinshu University and vice-president of Shinshu University for recommend me for scholarship. I also grateful to Professor Dr. Hiroshi Fuji, Professor Dr. Tamao Ono, Faculty of Agriculture, Shinshu University and Professor Dr. Tetsuya Kohsaka, Faculty of Agriculture, Shizuoka University for their valuable comments and suggestion.

I especially thanks to all present and past laboratory members for their friendly and nice co-operations I am also grateful to academic authority of Shinshu University for providing me academic supports throughout the

study period.

Special thanks to the Ministry of Education, Science, Sports and Culture of Japan for awarding me scholarship in Japan.

A special debt goes to my teachers Dr. K. M. Nassiruddin, Professor, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh and special thanks to Dr. M. A. M. Yahia Khandoker, Professor, Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

I would like pleasure to extent her heartiest respect, deepest gratitude and cordial thanks to Abdul Gaffar Miah, Professor, Department of Genetics and Animal Breeding, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University Dinajpur-5200, Bangladesh.

I would like pleasure to extent her heartiest respect, deepest gratitude and cordial thanks to Md. Atikur Rahaman for his helping me in various way to stay in Japan.

I wish to express my profound gratitude to my beloved parents, my brother, uncle cousins and relatives for their encouragement spiritual support and prayers offered during the long absence from Bangladesh.

I am thankful to my beloved wife and daughter for their encouragement, patience, sacrifice and supportive help in smooth completion of my research work.

Reference

1. Shannon P, Vishwanath R. The effect of optimal and suboptimal concentrations of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. *Animal Reprod Sci* 1995; 39: 1-10.
2. Curry MR. Cryopreservation of semen from domestic livestock. *Reviews Reprod* 2000; 5: 46-52.
3. Eisenbach M, Giojalas LC. Sperm guidance in mammals –an unpaved road to the egg. *Nat Rev Mol Cell Biol* 2006; 7: 276-285.
4. Teves ME, Barbano F, Guidobaldi HA, Sanchez R, Miska W, Giojalas LC. Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. *Fertil Steril* 2006; 86: 745-749.
5. Sun F, Giojalas LC, Rovasio RA, Tur-Kaspa I, Sanchez R, Eisenbach M. Lack of species-specificity in mammalian sperm chemotaxis. *Dev Biol* 2003; 255: 423-427.
6. Cohen-Dayag A, Tur-Kaspa I, Dor J, Mashiach S, Eisenbach M. Sperm capacitation in humans is trasient and correlates with chemotactic responsiveness to follicular factors. *Proc Natl Acad Sci USA* 1995; 92: 11039-11043.
7. Fabro G, Rovasio RA, Civalero S, Frenkel A, Caplan SR, Eisenbach M, Giojalas LC. Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay. *Biol Reprod* 2002; 67: 1565-1571.
8. Giojalas LC, Rovasio RA, Fabro G, Gakamsky A, Eisenbach M. Timing

of sperm capacitation appears to be programmed according to egg availability in the female genital tract. *Fertil Steril* 2004; 82: 247-249.

9. Haramaya H, Kato S, Relationship between bicarbonate and cyclic nucleotide in the promoting effects on head-to-head agglutination in boar spermatozoa. *Asian J Androl* 2002; 4: 87-96.Eisenbach M. Sperm chemotaxis. *Rev Reprod* 1999; 4: 56-66.
10. Eisenbach M, Sperm chemotaxis. *Rev Reprod* 1999; 4: 56-66.
11. Bahat A, Eisenbach M. Sperm thermotaxis. *Mol Cell Endocrinol* 2006; 252: 115-119.
12. Miki K, Clapham DE. Rheotaxis guides mammalian sperm. *Curent Biol* 2013; 23: 443-452.
13. Cerezales SP, Boryshpolets S, Eisenbach M. Behavioral mechanism of mammalian sperm guidance. *Asian J Andol* 2015; 17: 628-632.
14. David A, Vilensky A, Nathan H. Temperature changes in the different parts of the rabbit's oviduct. *Int J Gynaecol Obstet* 1972; 10: 52-56.
15. Hunter RH, Nichol R. A preovulatory temperature gradient between the isthmus and ampulla of pig oviducts during the phase of sperm storage. *J Reprod Fertil* 1986; 77: 599-606.
16. Bahat A, Tur-Kaspa I, Gakamsky A, Giojalas LC, Breitbart H, Eisenbach M. Thermotaxis of mammalian sperm cells: a potential navigation mechanism in the female genital tract. *Nat Med* 2003; 9: 149-150.
17. Bahat A, Eisenbach M. Human sperm thermotaxis is mediated by phospholipase C and inositol triphosphate receptor Ca2+ channel. *Biol*

Reprod 2010; 82: 606-616.

18. Kawanishi T, Takahashi H, Funai S, Suzuki M, Mizuno A, Goto C, Roh S-G, Sasaki S, Tsujii H, Hamano K. The Possibility of the Involvement of Transient Receptor Potential Vanilloid (TRPV) 4 in Mouse Sperm Thermotaxis. *Hokushinetsu J Anim Sci* 2007; 94: 35-41.
19. Hamano K, Kawanishi T, Mizuno A, Suzuki M, Takagi Y. Involvement of Transient Receptor Potential Vanilloid (TRPV) 4 in mouse sperm thermotaxis. *J Reprod Dev* 2016; 62: in press.
20. Hamano K, Tanaka S, Kawana Y, Tsujii H, Sasada H, Sato E, Takahashi T, Miyawaki K, Arima H. Evaluation of bull fertility by migration of frozen-thawed and washed sperm in medium containing cervical mucus. *J Reprod Dev* 2001; 47: 393-398.
21. Brackett BG, Oliphant G. Capacitation of rabbit spermatozoa in vitro. *Biol Reprod* 1975; 12: 260-274.
22. Bahat A, Caplan SR, Eisenbach M. Thermotaxis of human sperm cells in extraordinarily shallow temperature gradients over a wide range. *PloS One* 2012; 7: e41915.
23. Hossain AM, Barik S, Rizk B, Kulkarni PM, Thorneycroft IH. Analysis of in vitro migration patterns of human spermatozoa by a petri dish-based horizontal column. *Biol Reprod* 1999; 61:406-410.
24. Bahat A, Eisenbach M, Tur-Kaspa I. Periovulatory increase in temperature difference within the rabbit oviduct. *Hum Reprod* 2005; 20: 2118-2121.
25. Redden KD, Kennedy SD, Ingalls JR, Gilson TL. Detection of estrus

by radiotelemetric monitoring of vaginal and ear skin temperature and pedometer measurements of activity. J Dairy Sci 1993; 76: 713-721.

26. Kyle BL, Kennedy AD, Small JA. Measurement of vaginal temperature by radiotelemetry for prediction of estrus in beef cows. *Theriogenology* 1998; 49: 1437-1449.
27. El-Sheikh AH, Kitahara G, Tamura Y, Kobayashi I, Hemmi K, Torisu S, Samashima H, Horii Y, Zaabel S, Kamimura S. Presence of temperature gradient among genital tract portions and the thermal changes within these portions over the estrus cycle in beef cows. J Reprod Dev 2013; 59: 59-65.
28. Ho H-C, Granish KA, Suarez SS. Hyperactivated motility of bull sperm is triggered at the axoneme by Ca2+ and not cAMP. *Dev Biol* 2002; 250: 208-217.
29. Yanagimachi R. Mammalian fertilization. *2nd edition Raven Press* New Yolk, 1994; 189-317.
30. Shiba K, Marian T, Krasznai Z, Baba SA, Morisawa M, Yoshida M. Na+/Ca2+ exchanger modulates the flagellar wave pattern for the regulation of motility activation and chemotaxis in the ascidian spermatozoa. *Cell Motil Cytoskeleton* 2006; 63:623-632.
31. De Blass GA, Darszon A, Ocampo AY, Serrano CJ, Castellano LE, Hernandez-Gonzalez EO, Chirinos M, Larrea F, Beltran C, Trevino CL. TRPM8, a versatile channel in human sperm. *PloS One* 2009; 4: e6095.
32. Castellano LE, Trevino CL, Rodriguez D, Serrano CJ, Pacheco J, Tsutsumi V, Felix R, Darszon A. Transient receptor potential channels

(TRPC) in human sperm: expression cellular localization and involvement in the regulation of flagellar motility. *FEBS Lett* 2003; 541: 69-74.

33. Kumar A, Kumar R, Majhi K, Swain N, Gin SC, Kar S, Samanta L, Goswami C. TRPV4 is endogenously expressed in vertebrate spermatozoa and regulates intracellular calcium in human sperm. *Biochem Biophys Res Commun* 2016; 473: 781-788.
34. Brokaw CJ, Josslin R, Bobrow L. Calcium ion regulation of flagellar beat symmetry in reactivated sea urchin spermatozoa. *Biochem Biophys Res Commun* 1974; 58: 795-800.
35. Miller R.L. Sperm chemo-orientation in metazoa, In: Biology of Fertilization C.B.Metz and A. Monroy eds., Vol. 2, Academic Press, New York, 1985.p.275-337.
36. Rozeboom KJ, Troedsson MH, Rocha GR, Crabo BG. The chemotactic properties of porcine seminal components toward neutrophils in vitro. J Anim Sci. 2001; 79: 996-1002.
37. Ralt D, Manor M, Cohem-Dayag A, Tur-Kaspa I, Makler A. Chemotaxis and chemokinesis of human spermatozoa to follicular factors. Biol Reprod. 1994; 50: 774-85.
38. Yoshida M, Inaba K, Morisawa M. Sperm chemotaxis during the process of fertilization in the ascidians Ciona savignyi and Ciona intestinalis. Dev. Biol. 1993; 157: 497-506.
39. Shiba K, Baba SA, Inoue T, Yoshida M. Ca2+ bursts occur around a local minimal concentration of attractant and trigger sperm

chemotactic response. Proc Natl Acad Sci USA. 2008; 105: 19312-17.

40. Caballero CP, Buffone MG, Benencia F, Conejo GJR, Rinaudo PF, Gerton GL. A role for the chemokine receptor CCR6 in mammalian sperm motility and chemotaxis. J Cell Physiol. 2014; 229:68-78.
41. Oliveira RG, Tomasi L, Rovasio RA, Giojalas LC. Increased velocity and induction of chemotactic response in mouse spermatozoa by follicular and oviductal fluids. J Reprod Fert. 1999; 115: 23-27.
42. Armon L, Eisenbach M. Behavioral mechanism during human sperm chemotaxis: involvement of hyperactivation. PLoS One. 2011; 6: e28359.
43. Tacconis P, Revelli A, Massobrio M, Battista LaSalaG, Tesarik J. Chemotactic responsiveness of human spermatozoa to follicular fluid is enhanced by capacitation but is impaired in dyspermic semen. J Assist Reprod Genet. 2001; 18:36-44.
44. Spehr M, Gisselmann G, Poplawski A. Identification of a testicular odorant receptor mediating human sperm chemotaxis. Science. 2003; 299: 2054-58.
45. Blengini CS, Terves ME, Unates DR, Guidobaldi HA, Gatica LV, Giojalas LC. Human sperm pattern of movement during chemotactic re-orientation towards a progesterone source. Asian J Andrology. 2011; 13: 769-73.
46. Yoshida M, Ishikawa M, Izumi H, De Santis R, Morisawa M. Store-operated calcium channel regulates the chemotactic behavior of ascidian sperm. Proc Natl Acad Sci USA. 2003; 100:149-54.

47. Mizuno K, Shiba K, Okai M, Takahashi Y, Shitaka Y, Oiwa K, Tanokura M, Inaba K. Calaxin drives sperm chemotaxis by Ca^{2+}-mediated direct modulation of a dynein motor. Proc Natl Acad Sci USA. 2012; 109: 20497-502.