Study on effects of acrosomal conditions of mammalian spermatozoa on the results of artificial insemination and in vitro fertilization

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Study on effects of acrosomal conditions of mammalian spermatozoa on the results of artificial insemination and *in vitro* fertilization

(人工授精および体外受精の成績に及ぼす
哺乳類精子の先体性状の影響に関する研究)

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CHAPTER 1

General introduction

Studies on mammalian artificial insemination (AI) (Hashizume, 2000) have a long history since the 18th century. In 1780, Spallanzani reported the first success in AI of the dog. After the 1930s, researches on the practical application of AI for the cattle were made actively in the United States and Denmark. In the United Kingdom of the 1950s, Polge and his colleagues (Polge, 1952) established cryopreservation techniques for bull spermatozoa, and thereafter their AI techniques with bull frozen spermatozoa were rapidly spread to many countries. In Japan (Hashizume, 2000), the practical use of AI for the cattle began in the 1950s, then utilization rates of AI were beyond 90% in 1955, and finally frozen spermatozoa have been used for AI in almost all farms since 1970s. The development of this biotechnology enabled efficient production of the offspring of sires with the prominent heredity. Consequently, animal breeding was greatly advanced mainly in consideration of heredity from sires. In addition, the dam contributes less effectively to the bovine breeding than the sire in the calf production using AI. It is because a cow usually gives birth to only single calf from each pregnancy. However, the superovulation (SOV)-AI/embryo transfer (ET) techniques enabled efficient production of many calves from cows with the prominent heredity (Baracaldo *et al.*, 2000). In the SOV treatments, the cows are given a series of hormonal injections and then used for AI with frozen spermatozoa. After these treatments, multiple early embryos can be non-surgically recovered from the uteri of the cows and then each of them can be non-surgically transplanted into the uteri of other recipient cows by ET techniques (Sugie, 1965; Cristser *et al.*, 1980).

Studies on mammalian *in vitro* fertilization (IVF) have a relatively shorter history compared with those on mammalian AI. However, IVF has currently been well-established as a research tool for fertilization and early embryo development in mice, a biotechnological method to produce early embryos for ET in the cattle, and a clinical treatment for infertile couples in the human. The

development of IVF techniques has been promoted by the important discoveries in the reproductive biology. Specifically, Chang (1951) found that rabbit ejaculated spermatozoa were capable of penetrating oocytes in the oviduct after their preservation in the uterus for approximately 5 h. Austin (1951) also reported a similar result on rat spermatozoa. These reports implied that a variety of changes in the female reproductive tract (termed “capacitation”) might be necessary for mammalian spermatozoa to fertilize oocytes (Austin, 1952). In 1963, Yanagimachi and Chang (1963) observed that spermatozoa could undergo the capacitation during the culture in an appropriate environment after removal of seminal plasma. Dan (1952) found an event that was indispensable to sperm fertilization in the sea urchin. This is called acrosome reaction and defined as the exocytosis of acrosomal contents before fertilization. In mammalian spermatozoa, this reaction usually occurs after the accomplishment of the capacitation. Furthermore, Yanagimachi (1970) reported that the capacitated spermatozoa exhibited an entirely vigorous movement (hyperactivation) and gained a stronger driving force to pass through the cumulus oophorus and zona pellucida before fertilization. In the livestock, one of breakthrough researches was the success of the penetration of bull spermatozoa into bovine oocytes *in vitro*, as observed by Iritani and Niwa (1977). Subsequently, Brackett *et al.* (1982) produced the first calf from *in vivo*-matured oocytes, and Hanada *et al.* (1986) first obtained the offspring from *in vitro*-matured oocytes which were recovered from the ovaries of slaughtered cows. Such improvement of IVF techniques makes it possible to produce early embryos efficiently and consequently to provide a number of calves for the beef industry. For the scientific researches, additionally, IVF also provides a laboratory assay for the evaluation of sperm penetrability into the oocytes and development of fertilized eggs to blastocysts *in vitro* (Graham and Moce, 2005).

In the human, meanwhile, the conventional IVF techniques which were originally devised for the laboratory animal and livestock were applied as the clinical treatments for infertile couples. The production of the IVF baby was first reported by Steptoe and Edwards in 1978 (Steptoe and Edwards, 1978) and followed by Japanese researchers in 1983 (Suzuki *et al.*, 1983). The

conventional IVF is currently one of the primary options of clinical treatments to produce babies of infertile couples. Causes of female infertility include impaired follicular development, ovulation disorder, anovulation (Buckler *et al.*, 1991), endometriosis (Toma *et al.*, 1992) and corpus luteum malfunctions (Weström, 1980; Soules *et al.*, 1989). Causes of male infertility include varicocele (Bonvadi *et al.*, 2013; Agarwal *et al.*, 2015), ejaculatory duct obstruction (Meza-Vázquez *et al.*, 2008), antisperm antibodies (Check, 2010) and azoospermia (Chen *et al.*, 2014). Some of infertile couples occasionally suffer from the multiple diseases of above-mentioned examples. Furthermore, there are various kinds of infertility owing to the gamete dysfunctions; precocious hardening of zona pellucida (Loret De Mola *et al.*, 1997), severe oligospermia, immotility of spermatozoa (Chulkwunvwew *et al.*, 2015), inability of spermatozoa to penetrate into and fuse with oocytes (Hoshi *et al.*, 1988). For these cases, an alternative option of the clinical treatment "intracytoplasmic injection of single spermatozoon into oocyte (ICSI)" is available (Hoshi *et al.*, 1994).

As stated above, AI and conventional IVF were widely spread as reproductive biotechnologies through farms and medical clinics, respectively. However, there are recently increasing problems concerning these biotechnologies. Specifically, in the AI program of the cattle, the conception rates are decreasing in Japan and other countries (Dochi *et al.*, 2010; Barbat *et al.*, 2010; see "http://liaj.or.jp/giken/gijutsubu/seieki/jyutai.htm"). The reduced AI conception rates of the cattle are likely due to female factors such as breeding disorder of feeble estrus and depression of uterus functions as well as to environmental factors such as heat stress and abnormal climate (Tani *et al.*, 2010; Flamenbaum and Galon, 2010). The AI subfertility of females results in the extension of the calving interval and increase of product costs which have bad influences on the business management in the farms (Dochi *et al.*, 2010; Nabenishi *et al.*, 2011; Kadokawa *et al.*, 2012). On the other hand, male factors for the decrease of AI conception rates were less understood (Kuroda *et al.*, 2007; Karoui *et al.*, 2012). Moreover, AI program faces a specific problem on the validity of the routine examination of the spermatozoa. Specifically, several

samples from each lot of the frozen sperm straws were used for the examination of sperm motility and then remaining frozen sperm straws of the qualified lots are used for AI. However, AI using frozen spermatozoa with high motility from a certain bull results in relatively low conception rates. In human IVF, similarly, motility is considered as the most important parameter of sperm characteristics. Indeed, the spermatozoa are usually used for the IVF treatments after the swim-up method to recover highly motile ones. However, there are large variations in the results of IVF even if highly motile spermatozoa are used for all treatments. In summary, it should be noted that the sperm motility examination (in other words, the examination of sperm flagellar functions) is not always valid for the estimation of the results of cattle AI and human IVF.

Mammalian spermatozoa are composed of three parts; head, neck and flagellum (Eddy, 2006). The sperm head is regionally sorted into two parts (acrosomal and postacrosomal regions) and is characterized by the protamine-dependently condensed nucleus in the inside and acrosomal cap in the anterior. Moreover, the acrosomal region is structurally divided into three domains (marginal, principal and equatorial segments). The marginal and principal segments have unique membrane structures composed of plasma, outer acrosomal and inner acrosomal membranes. A variety of hydrolytic enzymes [serine protease (acrosin) and glycosidase (hyaluronidase)], which are contained between outer and inner acrosomal membranes in these segments, are released by the exocytosis and then digest the extracellular matrix of oocytes. The equatorial segment also has three membranes. The subdomain (equatorial sub-segment), of which surficial structures are crude and heterogeneous, is observed in the central part of this segment (Ellis *et al.*, 2002; Jones *et al.*, 2008). As the equatorial segment is a site where spermatozoa start membrane adhesion and fusion with oocytes after the passage through zona pellucida, fertilization-indispensable molecules are localized in this part (Yanagimachi, 1994). Therefore, functions of the acrosomal region are very important for the achievement of sperm fertilization with oocytes. However, the exact examination of the acrosomal conditions is scarcely adopted as the method of sperm assessment in bovine AI program and human conventional IVF treatments.

The aim of this study was to investigate whether the exact examination of the acrosomal conditions is valid for the prediction of the results of bovine AI program and human conventional IVF treatments. In the second chapter, I demonstrated the existence of large individual differences in the acrosomal conditions of frozen-thawed spermatozoa among Japanese Black bulls by the lectin-staining and indirect immunofluorescence of acrosomal tyrosine-phosphorylated proteins and then examined effects of their acrosomal conditions of frozen-thawed spermatozoa on the results of AI. In the third chapter, I identified acrosomal tyrosine-phosphorylated proteins (which were used as molecular markers for the assessment of acrosomal conditions) as sperm acrosome associated 1 (SPACA1) proteins in the spermatozoa from Japanese Black bulls. In the fourth chapter, I demonstrated the existence of large individual differences in the sperm acrosomal conditions by the lectin-staining and indirect immunofluorescence of SPACA1 proteins among male patients of infertile couples and discussed possible their impacts on outcomes of conventional IVF.

CHAPTER 2

Effects of acrosomal conditions of frozen-thawed spermatozoa on the results of artificial insemination in Japanese Black cattle

2-1. Introduction

For the purpose of maintaining the safety and increasing the satisfaction in the dietary life, it is important to improve the production systems of clean and delicious foods. Beef of Japanese Black cattle is a representative of Japanese delicious foods and world-famous exports, though it is rather expensive for the ordinary people. As higher prices of this livestock product may weaken its competitiveness in the markets, it is likely preferable to consider that high-quality beef of Japanese Black cattle is appropriately supplied at affordable prices to the markets. Previous efforts to decrease the prices include intensive use of AI with frozen-thawed spermatozoa for the production of bovine offspring. Specifically, this reproductive technique is indispensable for wide use of the spermatozoa collected from high-performance sires, and it has enabled us to produce a large number of high-performance offspring of the cattle. Moreover, it has also allowed us to limit the number of sires and to reduce large costs for the feeding and transportation of sires.

As described above, the sperm cryopreservation technique is essential to AI of the cattle. In Japan, this technique was established several decades ago and almost all of bovine AI are currently made using frozen-thawed spermatozoa. However, the conception rates of AI are gradually decreasing (Dochi *et al.*, 2010; Barbat *et al.*, 2010). The decrease may be due partially to disorder of feeble estrus in cows, inbreeding depression (Karoui *et al.*, 2012) and inappropriate breeding environment including heat stress (Tani *et al.*, 2010). Low conception rates of AI are accompanied by the extension of calving interval in cows and consequently by the decline in the production of offspring which makes large damages to the farm management.

The reproductive performance of bulls is conventionally predicted by the routine

examination of motility and morphological shape of frozen-thawed spermatozoa. However, a severe problem has recently been caused for the validity of this examination. Specifically, AI using frozen-thawed spermatozoa with high motility and normal shape from a certain bull resulted in relatively low conception rates. Kuroda *et al.* (2007) reported the increase of bulls which were recognized as such AI-subfertile males. In the urgent experiments using only limited number of Japanese Black bulls (Harayama *et al.*, 2010) to address this severe problem, it was found that frozen-thawed spermatozoa from AI-subfertile bulls frequently had severely-damaged acrosomes and showed abnormal distribution of tyrosine-phosphorylated proteins in the acrosomal principal segment. These suggest the possibility that morphologically and molecularly defective acrosomes in frozen-thawed spermatozoa may be related to the occurrence of AI-subfertility in Japanese Black bulls.

In the first part of this chapter, I made additional investigations to confirm the statistical significance in the relationship between AI subfertility and acrosomal defects of frozen-thawed spermatozoa in Japanese Black bulls. In the second part, moreover, for the purpose of finding possible causal factors for the low conception rates in the AI using frozen-thawed spermatozoa with the defective acrosomes, I conducted AI tests and IVF tests using frozen-thawed spermatozoa with different acrosomal conditions and then obtained results regarding development of *in vivo* fertilized eggs after AI and sperm penetration rates in the IVF tests.

2-2. Materials and methods

Animals and experiment outlines

Procedures of this chapter were approved in the research project plan "Improvement of fertility assay for Japanese Black bull spermatozoa" by the General Technological Center of Hyogo Prefecture for Agriculture, Forest and Fishery (General Technological Center).

This chapter was composed of (1) assessment of acrosomal conditions in frozen-thawed spermatozoa, (2) investigation of the reproductive performance (assessed by the conception rates) of bulls by the routine AI tests using frozen-thawed spermatozoa, (3) evaluation of *in vivo* fertilizing ability of frozen-thawed spermatozoa by the SOV/AI-EC tests, and (4) evaluation of *in vitro* fertilizing ability of frozen-thawed spermatozoa by the IVF tests. All of the cattle which were used for the investigations of this study were Japanese Black cattle. Bulls (37 males, >12 months old) were fed as candidates of sires in the General Technological Center and used for the semen collection using the artificial vagina to produce frozen spermatozoa. Frozen-thawed spermatozoa were used for the assessment of acrosomal normality by the fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA)/propidium iodide (PI) staining (for the samples from 37 bulls; A1-A9, B1-B9, C1-C9, D1-D9 and E1) and immunodetection of acrosomal tyrosine-phosphorylated proteins (for the samples from 34 bulls; A1-A9, B1-B9, C1-C9 and D1-D7). The AI tests (total number of tests: 1413 times, the number of tests per bull: 30-129 times) for frozen-thawed spermatozoa from 19 bulls (A2, A4-A9, B1-B9, D8, D9 and E1) were conducted in the General Technological Center and farms near the Center. The SOV/AI-EC tests were carried out using delivered females (57 females, >34 months old) of the General Technological Center and frozen-thawed spermatozoa from 16 bulls (A1, A3-A9, B1-B5 and C1-C3). In SOV/AI-EC tests, embryos (total number: 358 embryos, the number per bull: 13-39 embryos) were collected and evaluated. Moreover, the IVF tests were done using frozen-thawed spermatozoa from 5 bulls (A1-A5) and ovaries obtained in the local meat center.

All reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan), unless otherwise specified.

Preparation of sperm samples

Frozen spermatozoa were produced using an egg yolk-Tris-citrate extender by a straw method in the Northern Center of Agricultural Technology of the General Technological Center. The spermatozoa were then stored in liquid nitrogen. Frozen spermatozoa were rapidly thawed in a warm water (38.5°C) immediately before use and then aliquots of the samples were used for the subjective assessment of sperm motility on a heated stage at 37 or 38.5°C under a bright-field microscope. The balance of the samples were washed in a phosphate-buffered saline (PBS) containing 0.1% (wt/vol) polyvinyl alcohol (PVA, Sigma-Aldrich, Co., St. Louis, MO, USA) (PBS-PVA) by centrifugation (700 g, three times for 5 min).

Assessment of acrosomal normality in frozen-thawed spermatozoa by FITC-PNA/PI staining

Washed spermatozoa were fixed in 3% (wt/vol) paraformaldehyde (PFA) in PBS for 15 min and subsequently washed twice with blocking buffer [1% (wt/vol) bovine serum albumin (BSA) and 100 mM glycine in PBS] by centrifugation. The resultant sperm pellets were treated with 1% (wt/vol) Triton X-100 (Sigma-Aldrich) in PBS for 5 min, washed three times in blocking buffer and then stained with FITC-PNA (20 μg/ml, Sigma-Aldrich) in PBS for 30 min. After washing three times in blocking buffer, the sperm nuclei were counterstained with 25 μg/ml PI in PBS for 5 min. After washing in PBS, the spermatozoa were put on glass slides, and covered with VECTASHIELD Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA) and coverslips. Approximately 100 spermatozoa on each preparation were classified into seven categories according to the fluorescent state of FITC-PNA in the acrosome (I pattern: intact acrosome, II pattern: slightly disordered acrosome, III pattern: severely disordered acrosome with highly bright fluorescence, IV pattern: acrosome with less fluorescence in the whole part, V pattern: severely

disordered acrosome with less fluorescence in the anterior region, VI pattern: acrosome with fluorescence only along its outline, and VII pattern: acrosome with almost no fluorescence, Harayama *et al.*, 2010). In this chapter, the acrosomes classified into FITC-PNA staining patterns I and II were considered as normal ones.

Indirect immunofluorescence

Washed spermatozoa were gently smeared on a glass slide and fixed in methanol for 10 min. The slides were gently rinsed with PBS twice, blocked with 5% (wt/vol) BSA in PBS (PBS-BSA) for 60 min and then treated with the diluted primary antibody overnight at 4°C. After rinsing twice with PBS, the slides were treated with the diluted secondary antibody for 60-90 min. After being rinsed twice with PBS, the slides were covered with VECTASHIELD Mounting Medium and coverslips. The antibodies used in this chapter were mouse anti-phosphotyrosine monoclonal antibody (the primary antibody, clone 4G10, Upstate Cell Signaling Solutions, Charlottesville, VA, USA, 1:1,000) and FITC-conjugated goat anti-mouse immunoglobulin polyclonal antibody (the secondary antibody, Dako Cytomation Denmark A/S Glostrup, Denmark, 1:100). On each preparation, approximately 100 spermatozoa were observed under a differential interference microscope equipped with epifluorescence (mirror unit U-MWIB2: excitation filter BP 460-490, dichroic mirror DM 505, emission filter BA510IF, Olympus Optical Company Ltd., Tokyo, Japan). The distribution of acrosomal tyrosine-phosphorylated proteins was assessed according the criteria shown in Fig. 1.

Routine AI tests

Japanese Black cows were subjected to AI at natural estrus or estrus induced with an intramuscular injection of 150 μg of prostaglandin F 2alpha ($PGF_{2\alpha}$) analog (d-cloprostenol, Dalmazin, Kyoritsu Seiyaku Corporation, Tokyo, Japan) at random stages of the estrous cycle. Cows that did not exhibit any estrous signs for more than 40 days after AI were examined for

pregnancy by ultrasound examination and/or rectal palpation. Data were obtained in routine AI during the progeny tests at the General Technological Center and farms near the Center.

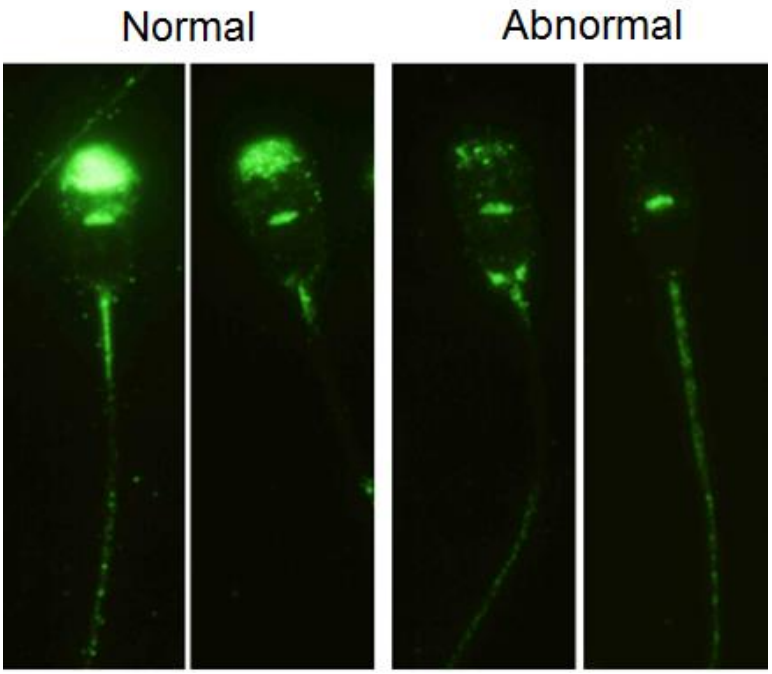

Fig. 1. Typical examples of indirect immunofluorescence patterns of acrosomal tyrosine-phosphorylated (pY) proteins of Japanese Black bull spermatozoa.

SOV/AI-EC tests

Japanese Black cows received a controlled internal drug release (CIDR) containing progesterone (1.9 g, Zoetis Japan K.K., Tokyo, Japan) in combination with intramuscular injection of gonadotropin-releasing hormone analog (Conceral injection containing 100 μg fertirelin acetate, Intervet K.K., Tokyo, Japan) at random stages of the estrous cycle (Day 0) at the General Technological Center. The SOV treatments were performed during the period between Day 7 and Day 9 with six intramuscular injections of gradually decreasing doses of porcine follicle-stimulating hormone (FSH, total dose of 20 AU, Antrin R10, Kyoritsu Seiyaku) at 12 h intervals. On the third day after the first FSH injection, the CIDR was removed, and 225 μg of $PGF_{2\alpha}$ analog was intramuscularly administered. Two AIs were conducted using frozen-thawed spermatozoa, one at 12 h and one at 24 h after the onset of standing estrus. On Day 7.5 after the onset of standing estrus, embryos were collected non-surgically, as described previously (Kawate *et al.*, 2007). Recovered embryos were morphologically evaluated to determine rates of transferable embryos [embryos developed up to the stages between late morulae and expanded blastocysts with code 1 (excellent or good) according to the IETS manual] (Robertson and Nelson, 1998).

IVF tests

Collection and culture of oocytes for in vitro maturation

Collection and culture of oocytes for *in vitro* maturation were performed according to procedures described previously with minor modifications (Imai *et al.*, 2002). Bovine ovaries were obtained from heifers in the local slaughterhouse and immediately transported to our laboratory. They were washed once in 0.2% (wt/vol) cetyltrimethylammonium bromide and then three times in PBS-PVA. Small follicles (3 to 5 mm in diameter) of the ovarian surface were punctured with an 18-gauge needle connected to a 5 ml disposable syringe (Terumo Co., Tokyo, Japan) and the cumulus-oocyte complexes were aspirated into the syringe. Oocytes with compact cumulus oophorus were selected under the stereoscopic microscope, washed once with PBS-PVA and then washed twice with TCM 199 (Medium 199 Earle's, liquid buffered with 25 mM Hepes,

Life Technologies, Carsbad, CA, USA) supplemented with 5% (vol/vol) fetal bovine serum (FBS, INC Biomedicals Inc., Aurora, OH, USA), 100 IU/ml penicillin G potassium (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and 100 μg/ml streptomycin sulfate (Meiji Seika) (a maturation medium). Oocyte-cumulus complexes (20 to 30) were cultured for 21-22 h in a droplet (100 μl) of the maturation medium covered with mineral oil (Sigma-Aldrich) at 38.5°C at 100% relative humidity and 5% (vol/vol) CO_2 in air.

Sperm preparation and insemination

Frozen-thawed spermatozoa (one lot for each bull), which were evaluated for the acrosomal conditions (acrosomal morphology and distribution of acrosomal tyrosine-phosphorylated proteins), were used for the IVF tests. One 0.5-ml straw was thawed in a warm water at 37°C. The frozen-thawed spermatozoa were washed in an isotonic Percoll [2 ml 90% (vol/vol) and 2 ml 45%, GE Healthcare UK Ltd., Buckinghamshire, UK] to remove egg yolk-based extender (at 700 g for 10 min) and then washed twice in the IVF100 medium (Research Institute for the Functional Peptides Co., Ltd., Yamagata, Japan) (at 300 g for 4 min for the first washing and at 200 g for 3 min for the second washing). The final concentration of the spermatozoa was adjusted to 5×10^6/ml with the IVF100 medium (the insemination medium). Droplets (100 μl) of the sperm suspension were put on the plastic dishes. Finally, approximately 20 cumulus-oocyte complexes after the culture were transferred to each of droplets for the sperm insemination, followed by 5-h culture at 38.5°C, 5% CO_2 in air. After the insemination, they were cultured for early development.

In addition, aliquots of the cumulus-oocyte complexes after the maturation culture were used for the prolonged insemination culture for 19 h to determine the *in vitro* fertilizing ability of the spermatozoa. After the prolonged insemination, the fertilized (or unfertilized) eggs were separated from the cumulus by gentle pipetting with a small-bore pipette and put on a slide glass. The eggs on the glass slides were fixed with the mixture of glacial acetic acid and ethanol (3 vol

and 1 vol, respectively) for more than 48 h and stained with 1% (wt/vol) aceto-orcein solution for 15 to 30 min. Successful fertilization was determined by the presence of two pronuclei.

Culture for early development of fertilized eggs

After the 5-h insemination culture, cumulus-fertilized (or unfertilized) eggs complexes were washed in the fresh TCM199 supplemented with 5% FBS and 20-30 complexes were transferred to each of droplets (100 µl) of the fresh medium (Day 0). On Day 2, Day 4 and Day 6 of the culture at 38.5°C, 5% CO_2 in air, a half volume of the culture medium was replaced by new one. On Day 7 to Day 9 of the culture, developmental stages of the embryos were observed by microscopy to determine the rates of formation of transplantable blastocysts. Recovered embryos were morphologically evaluated to determine rates of transferable embryos [embryos developed up to the stages between late morulae and expanded blastocysts with code 1 (excellent or good) according to the IETS manual] (Robertson and Nelson, 1998).

Statistical analyses

Spearman rank-order correlation coefficients (McDonald, 2014) between the obtained results were computed with Ekuseru-Toukei 2010 (Social Survey Research Information Co., Ltd., Tokyo, Japan) that was an add-in software for the Microsoft Excel 2010 Japanese version (Microsoft Japan Co., Ltd., Tokyo, Japan). Differences in the obtained results between two groups were analyzed using the two-tailed unpaired *t*-tests after arcsine transformation (McDonald, 2014) with the above-mentioned computer software.

2-3. Results

Relationship between acrosomal conditions of frozen-thawed spermatozoa and results of AI tests using frozen-thawed spermatozoa

I observed individual differences in the morphologically and molecularly acrosomal conditions of frozen-thawed spermatozoa (Fig. 2). The percentages of frozen-thawed spermatozoa with morphologically normal acrosomes were largely varied among different bulls in the range between 22% and 79% (mean ± standard error; 51 ± 3%, n=34). Similar individual differences were observed in the percentages of frozen-thawed spermatozoa with normal distribution of acrosomal tyrosine-phosphorylated proteins (the range between 14% and 95%, mean ± standard error; 67 ± 4%, n=34). Moreover, there was a significantly positive correlation between these parameters ($P<0.01$, $r=0.743$, n=34).

For bulls which had different conception rates of routine AI tests [Fig. 3 left panel, n=19 including three AI severely-subfertile bulls (conception rates, < 25%), the range between 4% and 75%, mean ± standard error; 46 ± 4%, (cf. n=16 excluding three AI severely-subfertile bulls, mean ± standard error; 52 ± 3%) and right panel, the range between 4% and 74%, mean ± standard error; 44 ± 5%, n=16 including three AI severely-subfertile bulls], I assessed acrosomal conditions of frozen-thawed spermatozoa. As expected, both the percentages of frozen-thawed spermatozoa with morphologically normal acrosomes (Fig. 3 left panel, $P<0.05$, $r=0.495$, n=19) and the percentages of frozen-thawed spermatozoa with normal distribution of acrosomal tyrosine-phosphorylated proteins (Fig. 3 right panel, $P<0.01$, $r=0.702$, n=16) had significantly positive correlations with the conception rates of routine AI tests.

Possible male reproductive dysfunctions causing low conception rates in the AI using frozen-thawed spermatozoa with acrosomal poor conditions

For bulls which had different rates of transplantable embryos in the SOV/AI-EC tests (Fig. 4,

n=16, the range between 0% and 100%, mean ± standard error; 46 ± 8%), I assessed acrosomal conditions of frozen-thawed spermatozoa. Both the percentages of frozen-thawed spermatozoa with morphologically normal acrosomes (Fig. 4 left panel, $P<0.01$, $r=0.838$, $n=16$) and the percentages of frozen-thawed spermatozoa with normal distribution of acrosomal tyrosine-phosphorylated proteins (Fig. 4 right panel, $P<0.01$, $r=0.721$, $n=16$) had significantly positive correlations with the rates of transplantable embryos of SOV/AI-EC tests. In addition, the percentages of frozen-thawed spermatozoa exhibiting rapidly progressive movement had only small variations among bulls examined in the SOV/AI-EC tests and did not have any significant correlations with the rates of transplantable embryos of the SOV/AI-EC tests (Fig. 5, $P=0.69$, $r=-0.11$, $n=16$).

In the IVF tests for bulls which had different acrosomal conditions in frozen-thawed spermatozoa, I assessed fertilization rates and rates of formation of transplantable blastocysts (Fig. 6). The average fertilization rate of bulls A4 and A5 (relatively acrosomal poor conditions) (mean ± standard error; 36 ± 3%) was approximately half the value of bulls A1-A3 (relatively acrosomal good conditions) (mean ± standard error; 69 ± 3%). Moreover, the average rate of transplantable blastocysts of bulls A4 and A5 (mean ± standard error; 6 ± 1%) was a third value of that of bull A1-A3 (mean ± standard error; 19 ± 2%).

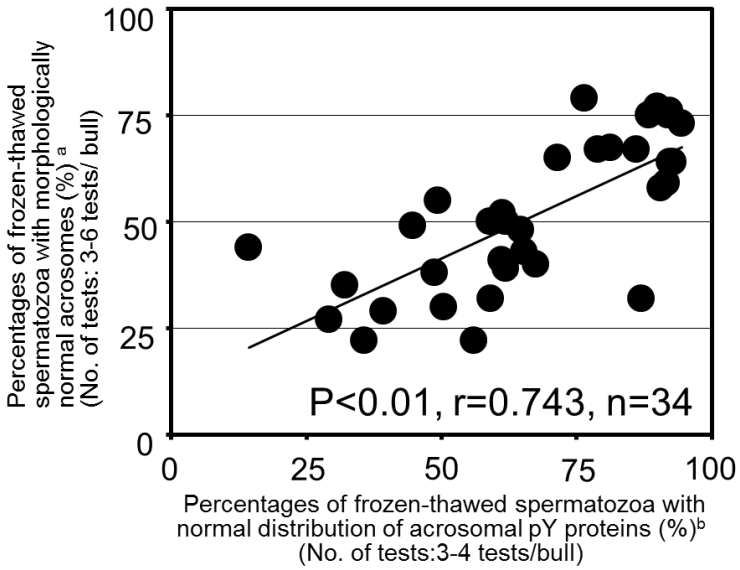

Fig. 2. Relationship between acrosomal integrity and distributive normality of acrosomal pY proteins in frozen-thawed spermatozoa from Japanese Black bulls.

● A spot indicates results obtained in each of 34 bulls (A1-A9, B1-B9, C1-C9 and D1-D7).

[a] Acrosomal integrity was examined by the fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA)/ propidium iodide (PI) staining.

[b] Distributive normality of acrosomal pY proteins was examined by indirect immunofluorescence.

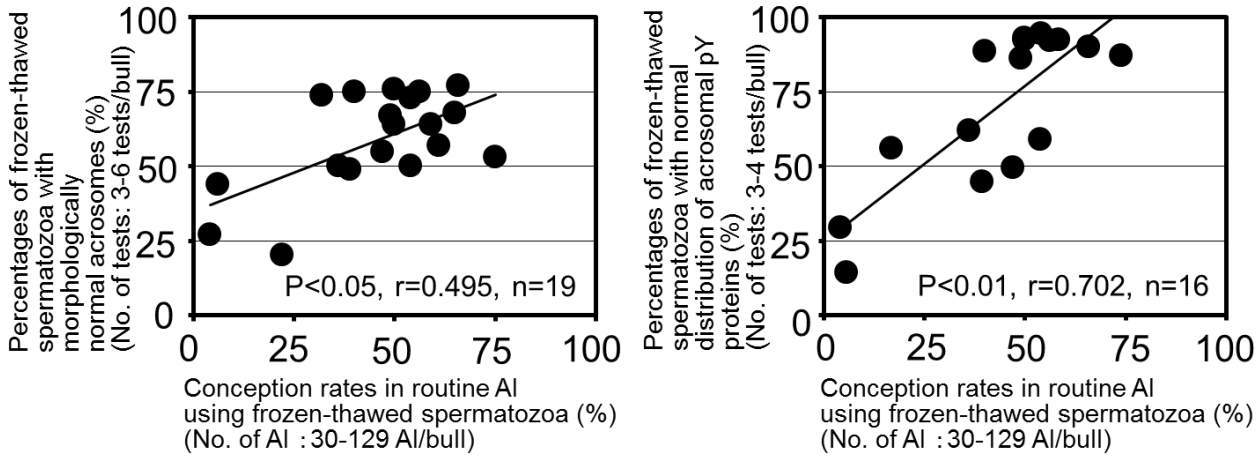

Fig. 3. Relationship between acrosomal conditions of frozen-thawed spermatozoa and results of artificial insemination (AI) using frozen-thawed spermatozoa in Japanese Black bulls.

● A spot indicates results obtained in each bulls (left panel, 19 bulls, A2, A4-A9, B1-B9, D8-D9 and E1; right panel, 16 bulls A2, A4-A9 and B1-B9).

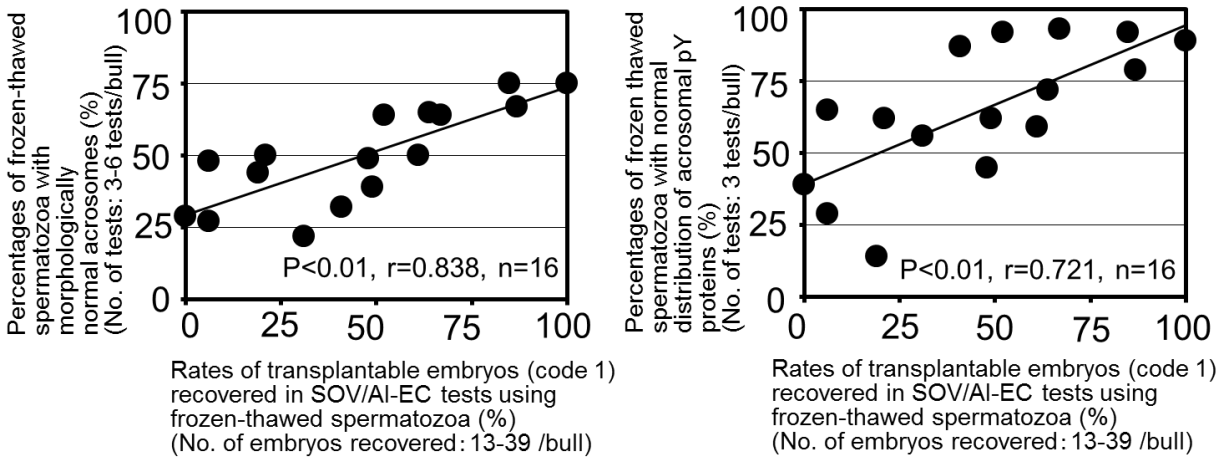

Fig. 4. Relationship between acrosomal conditions of frozen-thawed spermatozoa and results of superovulation/artificial insemination-embryo collection (SOV/AI-EC) tests using frozen-thawed spermatozoa in Japanese Black bulls.

● A spot indicates results obtained in each of 16 bulls (A1, A3-A9, B1-B5 and C1-C3).

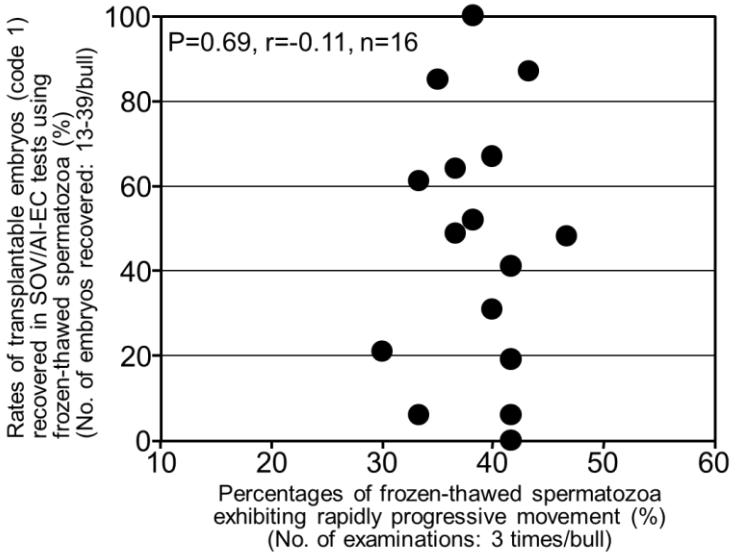

Fig. 5. Relationship between rapidly progressive movement of frozen-thawed spermatozoa and results of SOV/AI-EC tests using them in Japanese Black bulls.

● A spot indicates results obtained in each of 16 bulls (A1, A3-A9, B1-B5 and C1-C3).

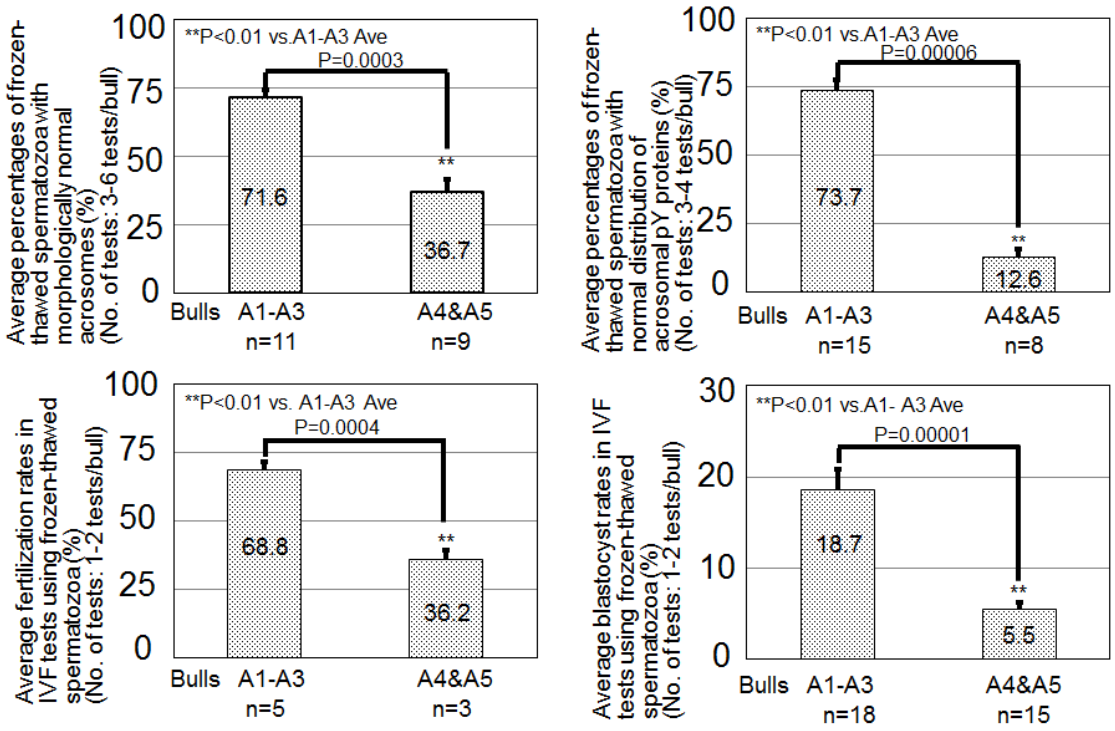

Fig. 6. Results of *in vitro* fertilization (IVF) tests using frozen-thawed spermatozoa with different acrosomal conditions.
Values are means ± SEM.

2-4. Discussion

Validity of distribution of acrosomal tyrosine-phosphorylated proteins in frozen-thawed spermatozoa as an AI fertility-associated marker

Among 34 Japanese Black bulls examined in this study (Fig. 2), the percentages of frozen-thawed spermatozoa with normal distribution of acrosomal tyrosine-phosphorylated proteins (the range between 14% and 95%) showed similar individual differences as those with morphologically normal acrosomes (the range between 22% and 79%). Moreover, there was a significantly positive correlation between these parameters ($P<0.01$, $r=0.743$), indicating possible linkages between acrosomal tyrosine-phosphorylated proteins and morphological normality of the acrosomes. In the investigations of freshly ejaculated spermatozoa from 15 Japanese Black bulls which were fed in the same AI center (Arai *et al.*, 2014), there were large individual differences in the percentages of spermatozoa with normal distribution of acrosomal tyrosine-phosphorylated proteins (22-99%). However, most of the freshly ejaculated spermatozoa (>80%) had normal acrosomes in the ejaculates from these bulls. These findings suggest that abnormal distribution of acrosomal tyrosine-phosphorylated proteins observed in frozen-thawed spermatozoa is probably detectable in freshly ejaculated spermatozoa, and that morphological damages of the acrosomes occurred during the handing process for the sperm freezing-thawing.

Next, I analyzed the relationship between sperm acrosomal conditions and routine AI results (Fig. 3). The conception rates of routine AI were significantly positively correlated with the percentages of frozen-thawed spermatozoa with normal distribution of acrosomal tyrosine-phosphorylated proteins as well as those with morphologically normal acrosomes. These results support the suggestion previously made with only limited number of bulls (Harayama *et al.*, 2010) that acrosomal poor conditions of frozen-thawed spermatozoa are related to the occurrence of AI subfertility in Japanese Black bulls. Moreover, I indicate that the distribution of acrosomal tyrosine-phosphorylated proteins in frozen-thawed spermatozoa is an AI fertility-associated marker which is valid for the prediction of the AI results.

Possible reproductive dysfunctions causing low conception rates in the AI using frozen-thawed spermatozoa with acrosomal poor conditions

Researches using genetically modified mice demonstrated that successful IVF of spermatozoa with oocytes requires active serine proteases (acrosin and testisin) of the acrosomal contents (Kawano *et al.*, 2010), but that the acrosome-reacted spermatozoa without intact acrosomes can penetrate into zona pellucida and then ooplasm *in vitro* (Jin *et al.*, 2011, Inoue *et al.*, 2011). In the first part of this study, however, I showed that AI using frozen-thawed spermatozoa with acrosomal poor conditions (namely without intact acrosomes) resulted in low conception rates (Fig. 2). This indicates that the acrosomal normal conditions of frozen-thawed spermatozoa are important in the maintenance of high conception rates in the AI program. Thus, I attempted to determine possible reproductive dysfunctions causing low conception rates in the AI using frozen-thawed spermatozoa with acrosomal poor conditions by the analyses on the relationships between sperm acrosomal conditions and results of SOV/AI-EC tests in the second part of this study (Figs. 3 and 4). The obtained data indicate that AI of frozen-thawed spermatozoa with acrosomal poor conditions is often accompanied by the reproductive dysfunctions in the processes before embryo development up to blastocysts, possibly by failed fertilization of frozen-thawed spermatozoa *in vivo* and/or *in vivo* development of early embryos. Moreover, the IVF tests of the final part of this study showed that *in vitro* fertilization rates were significantly lower in the frozen-thawed spermatozoa with acrosomal poor conditions (Fig. 5). This result suggests that acrosomal poor conditions reduce the penetrability of bull frozen-thawed spermatozoa into zona pellucida and ooplasm *in vitro*.

In conclusion, to my knowledge, this is the first report that indicates that low conception rates in the AI using frozen-thawed spermatozoa with acrosomal poor conditions in Japanese Black cattle result from reproductive dysfunctions in the processes between sperm insemination into females and early embryo development, probably failed fertilization of frozen-thawed spermatozoa with oocytes. The investigations of the reproductive dysfunctions causing male AI subfertility would be contributory to the recovery of high conception rates in the AI program of Japanese Black

cattle.

CHAPTER 3

Relationship of acrosomal tyrosine-phosphorylated proteins with SPACA1 proteins in the spermatozoa from Japanese Black bulls

3-1. Introduction

There are a number of sperm-specific proteins in the acrosomal region which are potentially involved in the fertilization (Eddy, 2006). For instance, Hao *et al.* (2002) reported that sperm acrosome associated 1 (SPACA1) proteins are 32-34 kDa membrane antigens of human spermatozoa, mRNAs of which are specifically expressed in the testes. These membrane antigens are phosphorylated at the serine residue and localized in the equatorial segment and along the inner acrosomal membrane of the acrosomal principal segment. In addition, they remain in the equatorial segment of the acrosome-reacted spermatozoa and the antiserum to SPACA1 proteins suppresses binding and fusion of capacitated human spermatozoa with the zona-free hamster oocytes. These observation suggest possible involvement of human SPACA1 proteins in the fertilization, especially, the interaction between spermatozoa and oocytes. In addition, the serum from an infertile man with the anti-sperm antibody was reacted to the SPACA1 proteins, indicating that disturbance of the function of SPACA1 proteins may cause of reproductive dysfunction in men. Moreover, they observed that immunological detection of the SPACA1 proteins in human testicular germ cells is limited to the acrosome at all developmental stages of spermatids. Recently, Fujihara *et al.* (2012) reported that *Spaca1* gene-disrupted male mice are infertile with abnormally shaped sperm heads reminiscent of globozoospermia, and that deficiency of SPACA1 proteins leads to the disappearance of the nuclear plate, a dense lining of the nuclear envelope facing the inner acrosomal membrane. These observations demonstrated indispensability of SPACA1 proteins for acrosomal formation during the spermiogenesis.

The results of the chapter 2 demonstrated that there are large individual differences in the

distribution of acrosomal tyrosine-phosphorylated proteins in frozen-thawed spermatozoa among Japanese Black bulls, and that the immunodetection level of these acrosomal proteins may be valid as a molecular biomarker to predict the AI results. Moreover, Harayama *et al.* (2010) showed by the immunoprecipitation-Western blotting techniques that at least aliquots of acrosomal tyrosine-phosphorylated proteins in frozen-thawed spermatozoa from Japanese Black bulls were SPACA1 proteins. However, there are only limited data regarding relationship between acrosomal tyrosine-phosphorylated proteins and SPACA1 proteins.

In this chapter, I confirmed the existence of the tyrosine-phosphorylated form of SPACA1 proteins in the unfrozen spermatozoa of Japanese Black bulls by the immunoprecipitation-Western blotting techniques. And I investigated the acrosomal distribution patterns of these proteins in the unfrozen spermatozoa by the double immunostaining with the anti-phosphotyrosine antibody and anti-SPACA1 protein antibody, and then compared distributive normality of SPACA1 proteins in the frozen-thawed spermatozoa with different results of the IVF tests.

3-2. Materials and methods

Reagents and preparation of sperm samples

All reagents were purchased from Wako, unless otherwise specified. Frozen-thawed spermatozoa were prepared as described in the chapter 2. Unfrozen spermatozoa were freshly ejaculated spermatozoa (from three mature bulls) and cauda epididymal spermatozoa (from three mature bulls) which were collected and prepared as described by Mizuno *et al.* (2015).

Immunoprecipitation-Western blotting

The detailed procedures were described previously (Harayama *et al.*, 2010).

Indirect immunofluorescence

Indirect immunofluorescence was performed as described in the chapter 2. The antibodies used in this chapter were primary antibodies [mouse anti-phosphotyrosine monoclonal antibody (1:1,000) and guinea pig anti-SPACA1 (SAMP32) protein polyclonal antibodies (American Research Products, Inc., Belmont, MA, USA, 1:400)] and secondary antibodies [FITC-conjugated goat anti-mouse immunoglobulin polyclonal antibody (1:100) and goat TRITC-conjugated goat anti-guinea pig immunoglobulin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100)]. For the double immunostaining, sperm preparations were treated with both of primary antibodies and subsequently with both of secondary antibodies.

IVF tests

Collection and culture of oocytes for *in vitro* maturation, sperm preparation and insemination, and culture for early development of fertilized eggs were performed according to the methods of the chapter 2.

Statistical analyses

Differences in the obtained results between two groups were analyzed using the two-tailed unpaired *t*-tests after arcsine transformation (McDonald, 2014) with Ekuseru-Toukei 2010 that was an add-in software for the Microsoft Excel 2010 Japanese version.

3-3. Results and discussion

Detection of the tyrosine-phosphorylation of SPACA1 proteins in unfrozen spermatozoa of Japanese black bulls

To confirm the existence of the tyrosine-phosphorylated form of SPACA1 proteins in the unfrozen spermatozoa of Japanese Black bulls, freshly ejaculated or cauda epididymal spermatozoa were used for the immunoprecipitation-Western blotting techniques and the double immunostaining with the anti-phosphotyrosine antibody and anti-SPACA1 protein antibody. As shown in Fig. 7, the 42 kDa protein which was immunoprecipitated from the sperm extracts with an anti-phosphotyrosine antibody was specifically recognized by the anti-SPACA1 protein antibody. In the acrosomal principal segment of double-immunostained spermatozoa, moreover, detection patterns of tyrosine-phosphorylated proteins were in accord with those of SPACA1 proteins (Fig. 8). These results indicate that at least an aliquot of the SPACA1 proteins is a tyrosine-phosphorylated form with the molecular mass of 42 kDa.

Relationship between SPACA1 protein distribution of frozen-thawed spermatozoa and results of IVF tests using them

Indirect immunofluorescence of spermatozoa with the anti-SPACA1 protein antibody revealed four detection patterns of the antigens in the principal and the equatorial segments of the acrosome (Fig. 9). Specifically, the acrosomal equatorial segment was strongly stained in almost all of spermatozoa. However, immunofluorescence levels in the acrosomal principal segment were largely varied among spermatozoa and could be classified into four categories; ++: strong and wide, +: intermediate or faint and slightly thin, ±: faint and thin, −: almost no immunofluorescence. In addition, spermatozoa with uniform or irregular patterns of SPACA1 protein staining were classified into abnormal ones. In this study, the spermatozoa with the acrosomes of categories "++ and +" were considered as those with normal distribution of SPACA1 proteins.

I compared the percentages of frozen-thawed spermatozoa with normal distribution of SPACA1 proteins between frozen-thawed sperm samples with the results of the IVF tests (Fig. 10). The IVF data were those obtained in the chapter 2 (Fig. 6). Immunofluorescence levels in the head of SPACA1 proteins were largely varied among 5 bulls (bulls A1-A5). These bulls were classified into 2 groups according to the IVF results. As shown in Fig. 10, the percentages of frozen-thawed spermatozoa with normal distribution of SPACA1 proteins were significantly higher in the sperm samples with good IVF results than those with poor results. Thus, it is likely that the SPACA1 distribution is related with the penetrability of frozen-thawed spermatozoa into oocytes *in vitro*.

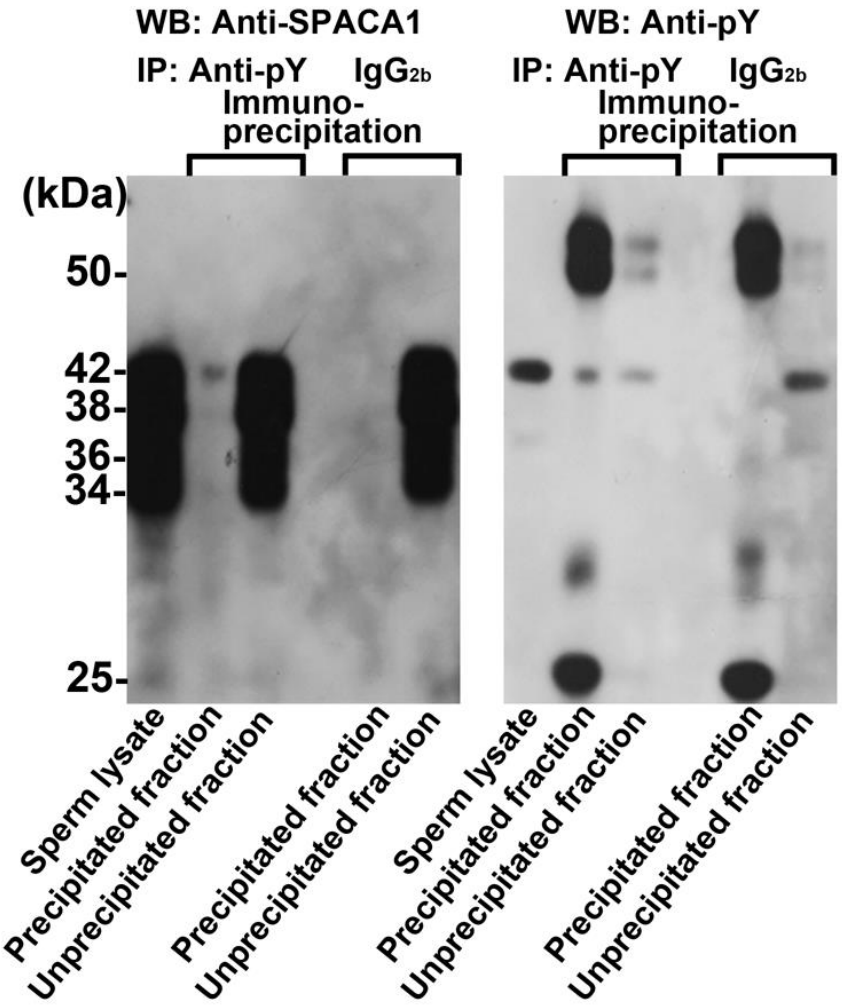

Fig. 7. Immunoprecipitation of a pY form of SPACA1 proteins in the lysates from freshly ejaculated spermatozoa of Japanese Black bulls.

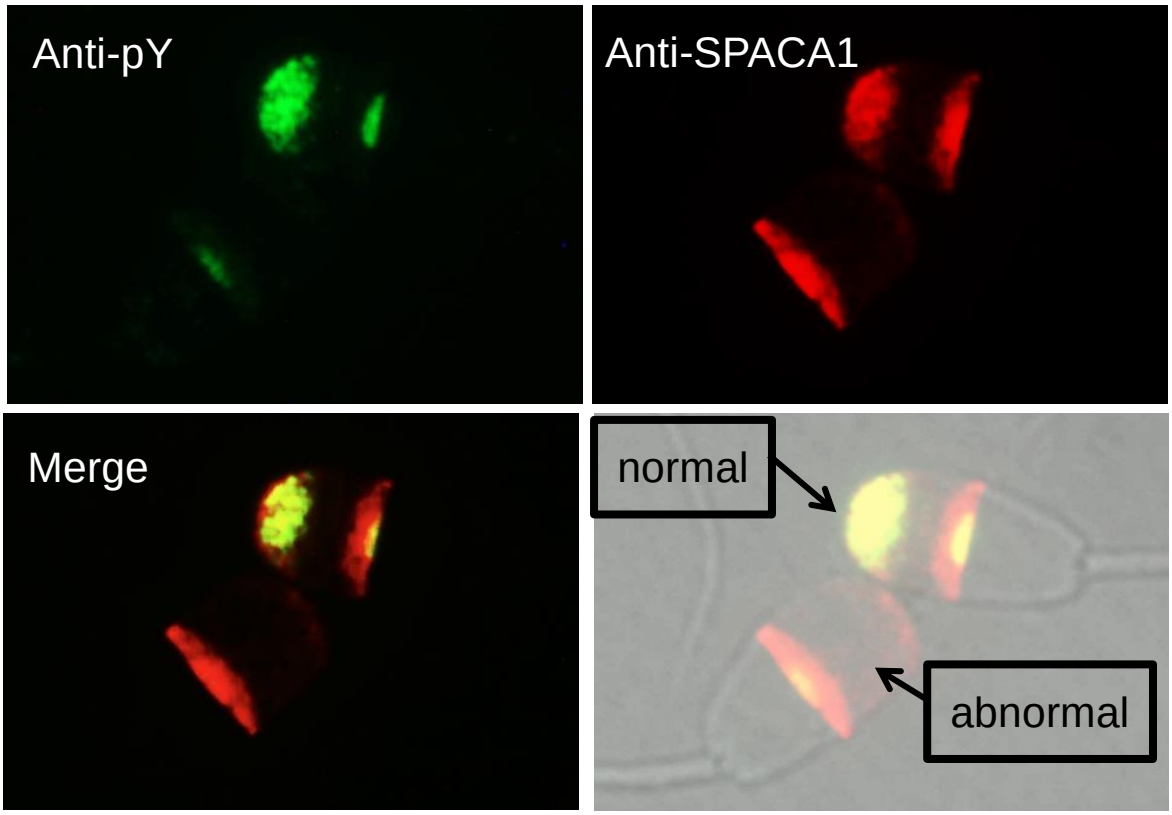

Fig. 8. Typical double immunostaining patterns of acrosomal pY proteins and SPACA1 proteins in cauda epididymal spermatozoa of Japanese black bulls.
Upper and lower spermatozoa exhibited normal and abnormal distribution patterns of these proteins in the principal segment of the acrosomes, respectively.

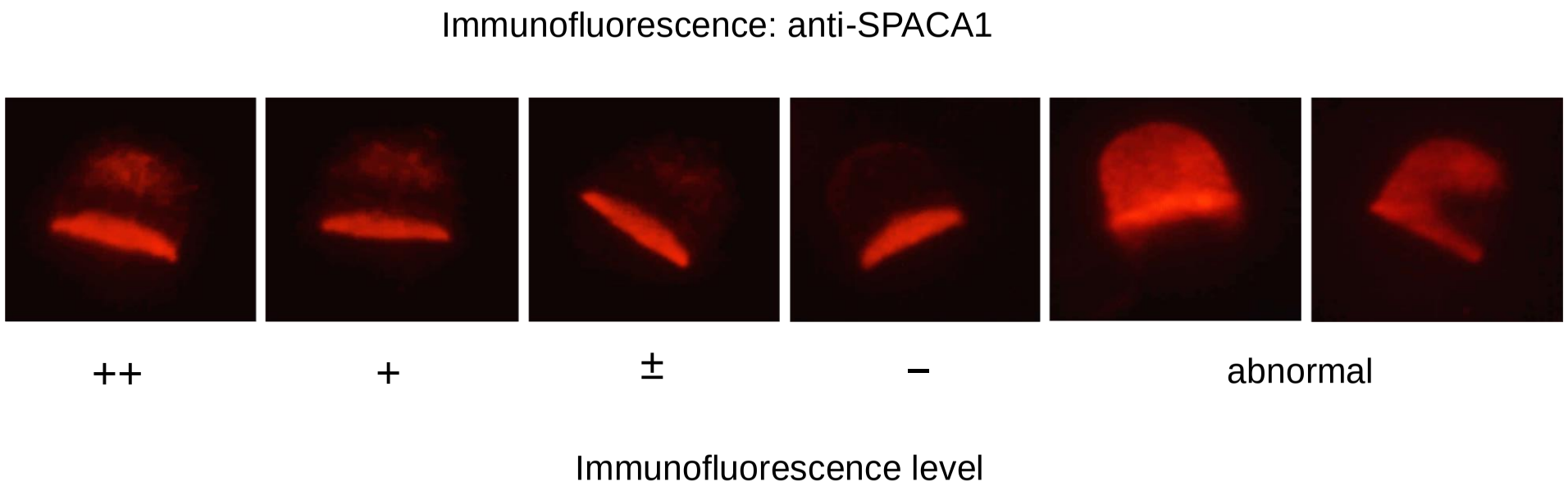

Fig. 9. Immunostaing patterns of SPACA1 proteins in the spermatozoa of Japanese Black bulls.

The acrosomal equatorial segment was strongly stained in almost all of the spermatozoa. However, immunofluorescence levels in the principal segment were largely varied among spermatozoa and could be classified into four categories; ++: strong and wide, +: intermediate or faint and slightly thin, ±: faint and thin, −: almost no immunofluorescence.

In addition, spermatozoa with uniform or irregular patterns of SPACA1 protein staining were classified into abnormal ones.

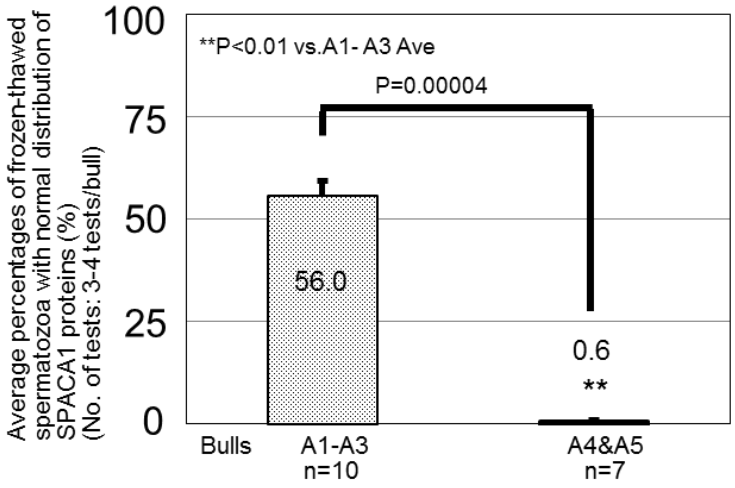

Fig. 10. Comparison of distributive normality of SPACA1 proteins in the frozen-thawed spermatozoa of Japanese Black bulls with different results of the IVF tests.
Values are mean ± SEM.

CHAPTER 4

Effects of acrosomal conditions of ejaculated spermatozoa on the results of *in vitro* fertilization in the human

4-1. Introduction

In an ordinary married couple with their intention to produce babies, if the wife should not become pregnant within one year, they would be identified as an infertile couple. The clinical treatments to overcome the infertility are currently classified into two categories. The first category includes the general treatments such as medical counseling and artificial insemination by husband (AIH). The second category is an advanced medical care including IVF and ICSI (Hoshi *et al.*, 1994; Yanagimachi, 2012). Nowadays, approximately 4% of babies are born from the infertile couples who undergo the advanced medical care in Japan (Saito, 2014). However, these clinical treatments are not always ideal for the infertile couples. In each of these clinical treatments, female patients are requested to receive processing for SOV by hormonal treatments and collection of matured oocytes. The hormonal treatments are occasionally accompanied by the side effects such as ovarian hyper stimulation syndrome (Haning *et al.*, 1979; Haning *et al.*, 1984; Blankstein *et al.*, 1987) and may afflict the patients mentally, physically and monetarily (Volgsten *et al.*, 2008). To reduce the burden on the couples, thus, it is necessary to select the most proper clinical treatments on the basis of medical pre-examination with molecular biomarkers in the gametes which can predict effectiveness of conventional IVF and ICSI of the infertile couples (Aitken *et al.*, 2013; Tavares *et al.*, 2013; Simon *et al.*, 2014; Tandara *et al.*, 2014).

The aim of this chapter was to find molecular biomarkers in the gametes which can predict effectiveness of conventional IVF of the infertile couples. In the chapter 2, I demonstrated that the assessment of acrosomal conditions in bull frozen-thawed spermatozoa by the immunodetection of acrosomal tyrosine-phosphorylated proteins is valid for the prediction of the results of AI. In the chapter 3, moreover, I identified at least aliquots of these phosphoproteins as SPACA1 proteins.

However, I found that it was very difficult to detect the acrosomal tyrosine-phosphorylated proteins of human ejaculated spermatozoa in my preliminary experiments (unpublished data). Thus, I focused on the SPACA1 protein as a candidate of the above-mentioned molecular biomarker. In the experiments of this chapter, I first attempted to examine the acrosomal conditions of human ejaculated spermatozoa by the lectin staining and indirect immunofluorescence of SPACA1 proteins. Next, I investigated individual differences in the distribution of SPACA1 proteins among infertile patients and examined possible their impact on the outcomes of conventional IVF.

4-2. Materials and methods

Ethic statement and patient background

All procedures of the sample collection and experiments were approved by the ethics committees of Shiga University of Medical Science (Permission numbers: #24-4) and the Graduate School of Agricultural Science, Kobe University (Permission number: #1). The patients were 9 infertile couples who visited the Department of Obstetrics and Gynecology of Shiga University of Medical Science Hospital (Otsu, Japan) for the purpose of undergoing the examinations and clinical treatments for the conventional IVF during the period from June 2012 to January 2013 (husband, 28-42 years old, wife, 31-39 years old). All of them agreed to the proposal of the participation in this study and signed consent forms permitting use of their gametes for research. In addition, conventional IVF treatments were made twice for the patients b and f. Seven male healthy volunteers (33-54 years old), each of whom has 2-4 biological children [the period since the birth of the last child in each volunteer was 0.5-17 years (0.5, 0.5, 2, 2, 4, 7 and 17 years)], made kind cooperation for this study in July 2015. In October 2015, furthermore, eight infertile male volunteers (34-46 years old) kindly offered additional samples to examine relationship between acrosome morphology and distribution of SPACA1 proteins.

Reagents and preparation of sperm samples

All reagents were purchased from Wako unless otherwise specifies. Nineteen and seven semen samples were collected from male patients of the above-mentioned infertile couples and male fertile volunteers, respectively, by masturbation after 3-5 days of abstinence, liquefied and then mixed with pureCeption® 80% (vol/vol Origio Japan K.K., Yokohama, Japan). The spermatozoa were separated from the supernatant (at 500 g for 20 min) and washed twice with 4 ml of Universal IVF Medium (Origio) (300 g for 4 min for first washing and at 200 g for 3 min for second washing). Subsequently, 0.5 ml of Universal IVF Medium was layered over the resultant sperm pellets in order

to recover motile spermatozoa (swim-up method).

Semen characteristics (semen volume, concentration, motility and morphology of spermatozoa, and leukocyte concentration) were routinely examined for both fresh ejaculates and sperm samples purified collected by the swim-up method according to the WHO criteria (World Health Organization, 2010). In addition, sperm morphology was assessed by the Diff Quick staining (Sysmex Corporation, Kobe, Japan). Briefly, 5-μl drop of the sperm sample was smeared, air-dried and stained with the kit. More than 100 spermatozoa were observed under the microscope with bright field illumination according to the criteria of morphological normality of sperm head (Moska *et al.*, 2011).

In eleven ejaculates collected from the patients between June 2012 and January 2013 (Table 1), the volume of ejaculate and sperm concentration were 2.0-4.1 ml (average 2.9 ml) and 46.0-218.0 x 10^6 cells/ml (average 128.1 x 10^6 cells/ml), respectively. The percentages of motile spermatozoa and morphologically abnormal spermatozoa were 27.7-69.1% (average 52.3%) and 60.9-98.1% (average 78.4%), respectively. All of the samples were contaminated with leukocytes [at the concentrations of 0.1-3.1 x 10^6/ml (average 0.8 x 10^6/ml)]. These results on the general characteristics were not significantly different from those obtained in the samples from healthy fertile volunteers (Table 2). In addition, these male patients did not show any severe symptoms of infertility such as azoospermia, oligospermia, asthenospermia and globozoospermia. After collection by swim-up method, almost all of the recovered spermatozoa of patients (Table 3) and healthy fertile volunteers (Table 4) exhibited progressive or circus movement with intensive flagellar beating and were free from leukocytes. The percentages of morphologically abnormal spermatozoa were significantly higher in the patients (79.0-94.0%, average 87.8%, Table 3) than the healthy fertile volunteers (52.8-76.6%, average 67.1%, Table 4). The recovered spermatozoa of the patients and volunteers were pre-incubated at 37°C in air [5% (vol/vol) O_2, 5% (vol/vol) CO_2] for 3 h in order to induce the capacitation in Universal IVF Medium and then used for insemination to oocytes.

Procedures for conventional IVF

Ovarian stimulation was performed using standard gonadotropin releasing hormone (GnRH) agonist/follicle stimulating hormone (FSH) protocol or antagonist/FSH protocol. Follicle maturation was triggered by 10,000 IU of human chorionic gonadotropin (hCG) injection or 300 μg of buserelin acetate nasal spray when the second leading follicles reached 18 mm in diameter. Ovums were retrieved transvaginally under ultrasound guidance 35 h after hCG administration and morphologically evaluated under the microscope by the clinical embryologists (professional technicians with discerning eyes for human germ cells and embryos).

Morphological normal ova were selected with great care and then inseminated with 2.0×10^6 spermatozoa pre-incubated for the induction of capacitation in Universal IVF Medium (Day 0). Successful fertilization was determined by the presence of two pronuclei visible at 19 h after insemination. They were cultured in the Complete Early Cleavage Medium® (ECM, Irvine Scientific-USA, Santa Ana, CA, USA) until 72 h after insemination. We performed the embryonic morphological observation on Day 2 or Day 3 and moved the cleaved embryo to the MultiBlast Medium® (Irvine Scientific-USA) at 72 h after insemination. In addition, morphological assessment of embryos to examine the successful development to the blastocyst stage by microscopy three times at 116 h, 120 h and 140 h after insemination (on Day 5 and Day 6) using Gardner system embryo grading (Gardner and Scoolcraft, 1999).

In this paper, the clinical data obtained according to this standard grading of human blastocysts were summarized as the rates of embryos showing normal development to blastocysts in order to recognize their relationship with the SPACA1 indexes at a glance.

Indirect immunofluorescence

Indirect immunofluorescence was performed with minor modifications as described previously (Harayama, 2003; Harayama *et al.*, 2010). In brief, all procedures were undertaken at room temperature except the treatment with the primary antibody. Aliquots of the patients' and

volunteers' samples after the above-mentioned pre-incubation were gently smeared on a glass slide and fixed in methanol for 10 min. The slides were gently rinsed with PBS twice, blocked with 5% BSA-PBS for 60 min and then treated with the primary antibody for 180 min at room temperature or overnight at 4°C. After rinsing twice with PBS, the slides were treated with the secondary antibody in PBS-BSA for 90 min. After being rinsed twice with PBS, the slides were covered with VECTASHIELD Mounting Medium and then coverslips. For each preparation, 100 spermatozoa were observed with a differential interference microscope equipped with epifluorescence (mirror unit U-MWIG: excitation filter BP 520-550, dichroic mirror DM 565LP, and emission filter 580LP, Olympus Optical Company Ltd, Tokyo, Japan). The antibodies used in this study were mouse anti-phosphotyrosine monoclonal antibody (1:1,000), guinea pig anti-SPACA1 protein polyclonal antibody (1:400), FITC-conjugated goat anti-mouse immunoglobulin polyclonal antibody protein (Dako, 1:100) and TRITC-conjugated goat anti-guinea pig immunoglobulin polyclonal antibody (Santa Cruz, 1:100).

Assessment of acrosomal normality in spermatozoa by FITC-PNA/PI staining

Eight infertile male volunteers semen samples were collected by masturbation after 3-5 days of abstinence, liquefied and then mixed with pureCeption® 80%. The spermatozoa were separated from the supernatant (at 500 g for 20 min) and washed twice with 4 ml of Universal IVF Medium (300 g for 4 min for first washing and at 200 g for 3 min for second washing). The FITC-PNA/PI staining was performed as described in the chapter 2. More than 100 spermatozoa were observed under the microscope with bright field illumination according to the criteria of morphological normality of sperm head (Moska *et al.*, 2011).

Statistical analyses

Spearman rank-order correlation coefficients (McDonald, 2014) between the obtained results were computed with Ekuseru-Toukei 2010 that was an add-in software for the Microsoft Excel 2010

Japanese version. Parameters of general characteristics of the semen and spermatozoa were analyzed between the patients and healthy fertile volunteers by two-tailed *t*-tests (McDonald, 2014) with the same computer software.

Table 1. Characteristics of fresh ejaculates which were collected from patients for conventional IVF treatments [A]

Identifying marks of IVF patients	Volume of ejaculate (ml)	Sperm concentration ($\times 10^6$/ml)	Sperm motility (%)	Morphologically abnormal sperm (%)	Leukocyte concentration ($\times 10^6$/ml)
a	2.2	126.5	40.7	94.5	3.1
b1[B]	2.5	158.0	43.0	65.8	0.6
b2[B]	3.9	188.0	69.1	84.0	0.1
c	2.0	139.0	64.7	81.3	0.9
d	3.2	218.0	62.8	60.9	0.2
e	2.8	46.0	58.7	65.2	0.4
f1[B]	2.8	145.0	67.6	72.4	0.4
f2[B]	3.4	95.0	61.1	63.2	0.7
g	3.4	106.5	27.7	98.1	0.9
h	4.1	82.5	31.5	96.4	0.8
i	2.0	105.0	48.5	80.9	0.4
Average	2.9	128.1	52.3	78.4	0.8

[A] Fresh ejaculates were colleted from the patients by masturbation.

[B] The conventional IVF treatments were made twice for patient b (b1 and b2, the first and second treatments, respectiverly) and patient f (f1 and f2, the first and second treatments, respectively).

Table 2. Characteristics of fresh ejaculates which were collected from healthy fertile volunteers [A]

Identifying marks of fertile male volunteers	Volume of ejaculate (ml)	Sperm concentration ($\times 10^6$/ml)	Sperm motility (%)	Morphologically abnormal sperm (%)	Leukocyte concentration ($\times 10^6$/ml)
j	1.1	108.0	86.1	82.5	2.8
k	3.5	86.5	39.8	71.1	0.4
l	2.1	121.0	76.0	80.2	0.1
m	1.6	148.0	62.8	79.1	0.3
n	1.6	93.5	42.2	84.0	0.6
o	3.2	88.0	64.7	81.9	0.2
p	2.0	94.0	82.9	70.3	0.1
Average	2.2	105.6	64.9	78.4	0.6
Vs patients a-i	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05

[A] Fresh ejaculates were collected from fertile male volunteers by musterbation after 3-5 days of abstinence.

Each of fertile male volunteers has 2-4 biological children.

The periods since the last birth of the baby were between 0.5 and 17 years.

Table 3. Characteristics of ejaculated spermatozoa of IVF patients which were collected for conventional IVF treatments by swim-up method

Identifying marks of IVF patients	Volume of sperm suspension (ml)	Sperm concentration ($\times 10^6$/ml)	Sperm motility (%)	Morphologically abnormal sperm (%)	Leukocyte concentration ($\times 10^6$/ml)
a	0.5	20.0	99.5	79.0	0.0
b1[A]	0.5	40.0	100.0	91.0	0.0
b2[A]	0.5	20.1	99.5	93.0	0.0
c	0.5	10.0	100.0	94.0	0.0
d	0.5	15.0	100.0	84.0	0.0
e	0.5	6.0	96.7	93.0	0.0
f1[A]	0.5	60.1	99.8	89.0	0.0
f2[A]	0.5	63.0	96.8	89.0	0.0
g	0.5	10.0	100.0	93.0	0.0
h	0.5	20.0	95.0	80.0	0.0
i	0.5	18.0	100.0	80.9	0.0
Average	0.5	25.7	98.8	87.8	0.0

[A] The conventional IVF treatments were made twice for patient b (b1 and b2, the first and second treatments, respectively) and patients f (f1 and f2, the first and second treatments, respectively).

Table 4. Characteristics of ejaculated spermatozoa of healthy fertile volunteers collected by swim-up method

Identifying marks of fertile male volunteers	Volume of ejaculate (ml)	Sperm concentration ($\times 10^6$/ml)	Sperm motility (%)	Morphologically abnormal sperm (%)	Leukocyte concentration ($\times 10^6$/ml)
j	0.5	35.0	100	65.8	0.0
k	0.5	36.0	100	52.8	0.0
l	0.5	78.5	100	73.3	0.0
m	0.5	16.0	100	62.5	0.0
n	0.5	64.0	100	76.6	0.0
o	0.5	82.0	100	69.6	0.0
p	0.5	65.0	100	69.3	0.0
Average	0.5	53.8	100.0	67.1	0.0
Vs patients a-i	NA	$P<0.05$	$P<0.05$	$P<0.01$	NA

NA: Not analyzed

4-3. Results and discussion

Outcomes of conventional IVF

Table 5 shows early development of human embryos produced by the conventional IVF treatments using ejaculated spermatozoa and oocytes from nine infertile couples. Rates of embryos showing normal development to blastocysts (see typical examples of blastocysts in Fig. 11) were greatly varied among these treatments with the wide range from 0 to 57%, and relatively high rates were obtained in the patients a and b (b1 and b2). In the patients d, f2 and g, early development of embryos was relatively delayed during the first 48-h period after insemination, compared with patient a. In patients e, f1 and h, rates of embryos showing normal development to 4-8 cell stages during the first 48-h or 68-h period after insemination were relatively lower than in patients a and b (b1 and b2). In patients c and i, moreover, fertilization rates were likely lower than any other patients. These results suggest that low ability of embryos (*in vitro*-fertilized eggs) to form blastocysts in the patients c-i may be due mainly to unfertilization, unsuccessful fertilization, or defective development of early embryos either between 1 cell and 4 cell stages or between 4 cell and blastocysts stages.

Results of immunodetection of SPACA1 proteins

Indirect immunofluorescence of spermatozoa with the anti-SPACA1 protein antibody revealed three detection patterns of the antigens in the principal and the equatorial segments of the acrosome (Fig. 12 upper panel). Specifically, the acrosomal equatorial segment was strongly stained in almost all of the spermatozoa. However, immunofluorescence levels in the acrosomal principal segment were largely varied among spermatozoa and could be classified into three categories; (A) strong, (B) intermediate or faint, and (C) almost no immunofluorescence. Hao *et al.* (2002) also observed two kinds of immunodetection patterns of SPACA1 proteins in human ejaculated

spermatozoa, though there was not description on the individual differences in the percentages of detection patterns in their report. In this study, thus, we immunostained the balance of the sperm samples (surplus spermatozoa) prepared for the insemination to oocytes and compared the obtained results of the detection patterns of the SPACA1 proteins among 11 samples from 9 patients. As shown in Fig. 12 lower panel, there were large differences among the patients. To simplify the results, we calculated the SPACA1 indexes according to the following numerical formula.

SPACA1 indexes = [(% of A-pattern sperm) x 2] + [(% of B-pattern sperm) x 1]

The obtained SPACA1 indexes could be divided into three classes; high class [higher than 150 points; patients a, b (b1 and b2)], intermediate class (between 75 and 150 points; patients c, d and e) and low class [lower than 75; patients f (f1 and f2), g, h and i)]. In the samples classified into high class, almost spermatozoa exhibited the pattern A. By contrast, intermediate-class and low-class samples contained mainly B-pattern and C-pattern spermatozoa, respectively. In addition, the samples of seven healthy fertile volunteers (Fig. 13) were classified into the high [volunteers j (SPACA1 index: 151 points) and o (189 points)] and intermediate classes [volunteers k (122 points), l (104 points), m (115 points), n (144 points) and p (127 points)].

Additional samples from eight male patients were subjected to the assessment of acrosome morphology and distribution of SPACA1 proteins (Fig. 14 upper panel). As the percentages of spermatozoa with morphologically normal acrosomes (4-38%) were largely varied among the patients, these patients were divided into two groups (patients q-u and v-x). The average percentages of spermatozoa with morphologically normal acrosomes were significantly lower in the patients v-x (relatively acrosomal poor conditions) than patients q-u (relatively acrosomal good conditions). In the SPACA1 index, however, there was not significant difference between these two groups (Fig. 14 lower panel).

Relationship between outcomes of conventional IVF and results of the immunodetection of SPACA1 proteins

In patients a, b (b1 and b2), d, e, f (f1 and f2), g and h with relatively high fertilization rates (67-89%), high [40-57%; patients a and b (b1 and b2)], intermediate (22-25%; patients d and e) and low rates [0-18%; patients f (f1 and f2), g and h] of embryos showing normal development to blastocysts (Table 5) were obtained in the IVF treatments with the sperm samples which were classified into high [177-199 points; patients a and b (b1 and b2)], intermediate (101-102 points; patients d and e) and low classes [13-39 points; patients f (f1 and f2), g and h] of SPACA1 indexes (Fig. 12 lower panel), respectively. Thus, we examined the relationship between outcomes of conventional IVF and results of the immunodetection of SPACA1 proteins. As shown in Fig. 15A, for all patients (patients a-i), a significant positive correlation was observed between "rates of embryos showing normal development to blastocyst" and "SPACA1 index" ($r=0.829$, $P=0.00162$). Moreover, when the results of patients c and i (lower fertilization, Table 1) were excluded from the statistical analyses, the correlation coefficient was enhanced to 0.983 ($P=0.000002$, Fig. 15B). In addition, no significant correlation was obtained between "fertilization rates" and "SPACA1 indexes" (Fig. 16) and between "rates of embryos showing normal development to blastocyst" and "rates of morphologically abnormal spermatozoa" (Fig. 17). These results suggest possible linkage of distribution of SPACA1 proteins in the acrosomal principal segment with blastocyst-forming ability of embryos.

It is generally considered that a spermatozoon which fertilizes an oocyte in the ampulla oviduct (*in vivo* fertilization) is required to overcome the putative hardship produced by the uterus and then to accomplish timely the capacitation-associated changes in the oviduct (Holt and Fazeli, 2010, 2015; Aitken and Nixon, 2013; Kawano *et al.*, 2014). This indicates that the embryo (*in vivo*-fertilized egg), which is derived from normal oocyte and highly selected spermatozoon (probably with best quality), has the high ability to undergo early development to blastocysts. In IVF treatments, however, spermatozoa are able to skip the hardship produced by the uterus and various restrictions which are necessary for the fertilization with the oocyte *in vivo*. This suggests that embryos (*in vitro*-fertilized eggs) may be derived from morphologically normal oocytes and

spermatozoa with various qualities (namely, non-selected spermatozoa) in IVF treatments. Thus, it is possible that different qualities of the spermatozoa could have larger impacts on the ability of embryos (*in vitro*-fertilized eggs) to form blastocysts.

In this study, results of development of the embryos (*in vitro*-fertilized eggs) to blastocysts were largely varied among patients and significantly correlated with the SPACA1 indexes of the sperm samples (Fig. 15), though IVF rates were not associated with these indexes (Fig. 16). Before IVF treatments, the spermatozoa with high motility were collected by the swim-up method (Table 3) but had large variations in the SPACA1 indexes among the samples (Fig. 12). Thus, the obtained embryos (*in vitro*-fertilized eggs) were derived from morphologically normal oocytes and spermatozoa with various SPACA1 conditions. If the SPACA1 conditions are supposed to indicate sperm qualities, it is reasonable that results of development of embryos (*in vitro*-fertilized eggs) to blastocysts were associated with the SPACA1 indexes of the sperm samples. However, further experiments are necessary to disclose what detailed conditions of the spermatozoa are indicated by the SPACA1 indexes. As mentioned above, low ability of embryos (*in vitro*-fertilized eggs) to form blastocysts in patients c and i of this study may be due mainly to unfertilization, unsuccessful fertilization, or defective development of the embryos to 4-8 cell stages (Table 1). Meanwhile, it has been considered that the activation of paternal genome from the spermatozoon (embryonic genome activation, EGA) occurs in human embryos mainly between the 4 and 8cell stages (Niakan *et al.*, 2012). In our future researches, thus, it is preferable that we should examine relationship of the SPACA1 indexes with other acrosomal molecules associated with fertilization and egg activation [for instance, acrosomal serine proteases (Kawano *et al.*, 2010), IZUMO1 (Okabe, 2013) and phospholipase C-zeta (Swann and Lai, 2013) rather than EGA-associated factors (Niakan *et al.*, 2012)].

In conclusion, this study provides initial data to promote large-scaled clinical investigation to demonstrate that the SPACA1 indexes are valid as molecular biomarkers which can predict effectiveness of conventional IVF of infertile couples.

Table 5. Early development of human embryos produced by the conventional IVF treatments in this study

Identifying mark of IVF patients	No. of zygotes	No. of fertilized eggs[A]	Fertilization rates (%)	No. of embryos showing normal development to 4-8 cell stages [4 cell stage, 5-7 cell stage, 8 cell stage] (observation of embryos at the period after insemination)[B]	Rates of embryos showing normal development to 4-8 cell stages (%)	No. of embryos showing normal development to blastocysts[C]	Rates of embryos showing normal development to blastocysts (%)
a	19	14	74	14 [8, 4, 2] (48 h)	74	8	42
d	9	8	89	8 [8, 0, 0] (48 h)	89	2	22
e	8	7	88	2 [2, 0, 0] (48 h)	25	2	25
f2[D]	17	15	88	12 [11, 1, 0] (48 h)	71	2	12
g	6	4	67	3 [3, 0, 0] (48 h)	50	0	0
i	10	2	20	1 [1, 0, 0] (48 h)	10	0	0
b1[D]	7	6	86	5 [1, 0, 4] (68 h)	71	4	57
b2[D]	5	4	80	4 [0, 1, 3] (68 h)	80	2	40
c	10	2	20	2 [0, 1, 1] (68 h)	20	1	10
f1[D]	11	9	82	4 [1, 3, 0] (68 h)	36	2	18
h	9	6	67	3 [0, 3, 0] (68 h)	33	1	11

[A] Successful fertilization was determined by the presence of two pronuclei visible at 19 h after insemination.

[B] Total embryos which were developed to 4-8 cell stage at 48 or 68 h after insemination.

[C] Total embryos which were developed to the blastocyst stage until 140 h after insemination.

[D] The conventional IVF treatments were made twice for patient b (b1 and b2, the first and second treatments, respectively) and patient f (f1 and f2, the first and second treatments, respectively).

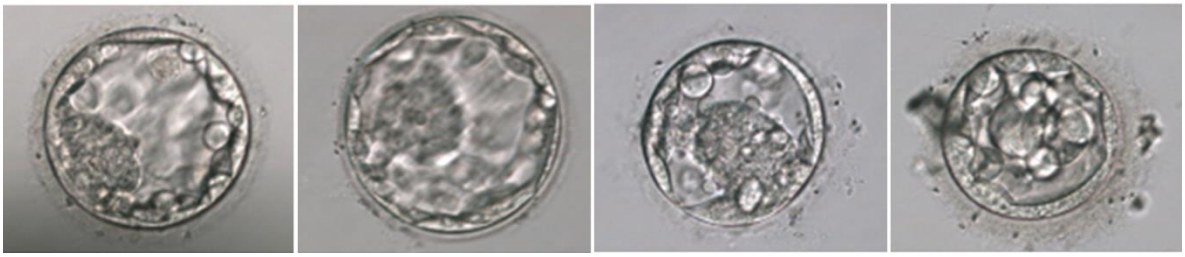

Fig. 11. Typical examples of blastocysts obtained in this study.
Morphological assessment of blastocysts was done using Gardner system embryo grading.

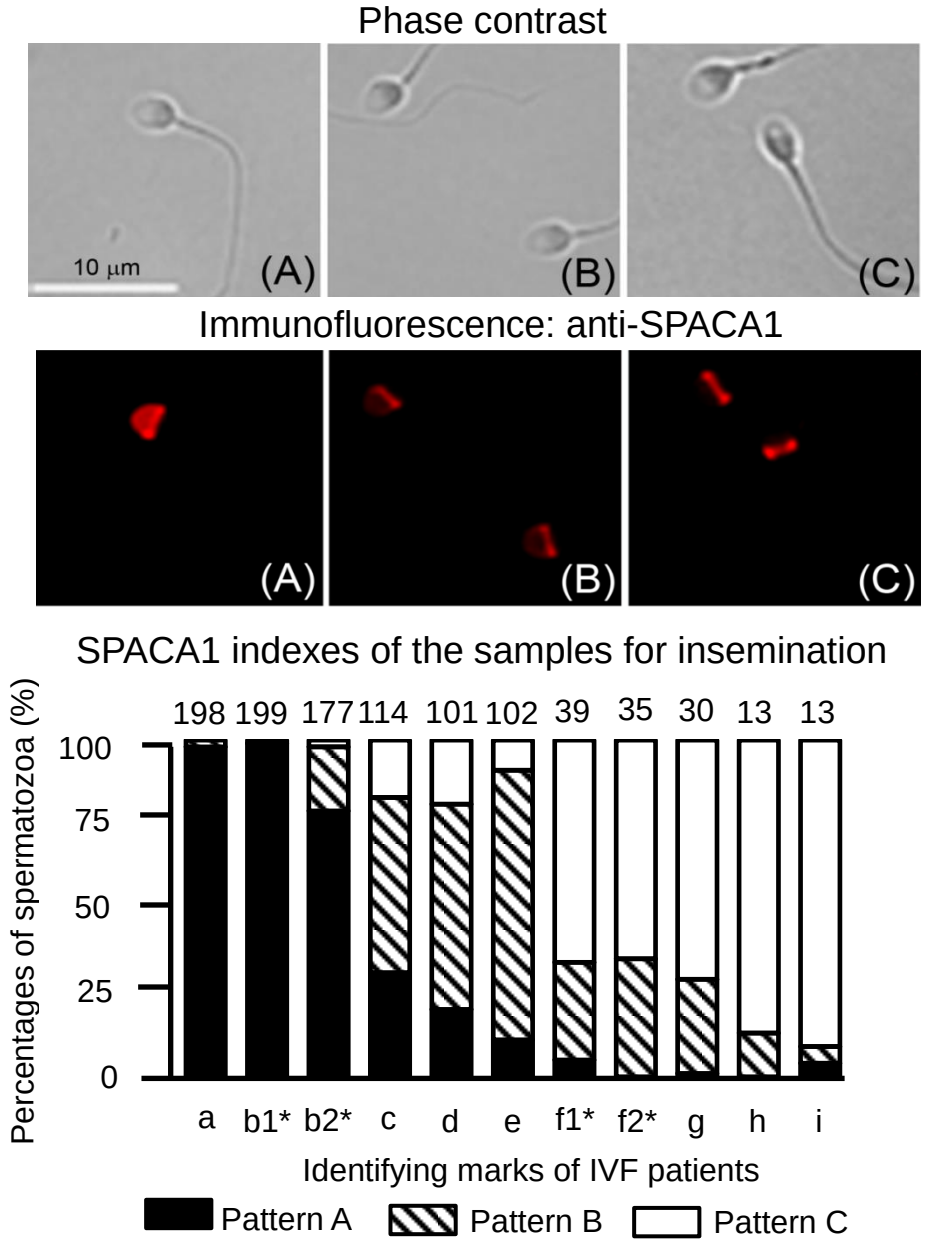

Fig. 12. Immunodetection patterns of SPACA1 proteins in human sperm samples prepared for insemination to oocytes.

In the upper panel, photographs of A, B and C indicate typical representatives of different detection patterns of SPACA1 proteins in the acrosomal region. In all spermatozoa, strong immunofluorescence was observed in the equatorial segment. However, immunofluorescence levels in the acrosomal principal piece were largely varied among spermatozoa and could be classified into three categories; (A) strong, (B) intermediate or faint, and (C) almost no immunofluorescence. A graph of the lower panel shows individual differences in the results of SPACA1 detection patterns among patients a-i. The SPACA1 indexes were calculated according to the following numerical formula {[(% of A-pattern sperm) x 2] + [(% of B-pattern sperm) x 1]}. *The conventional IVF treatments were made twice for patient b (b1 and b2, the first and second treatments, respectively) and patient f (f1 and f2, the first and second treatments, respectively). For each preparation, 100 spermatozoa were observed with a differential interference microscope equipped with epifluorescence.

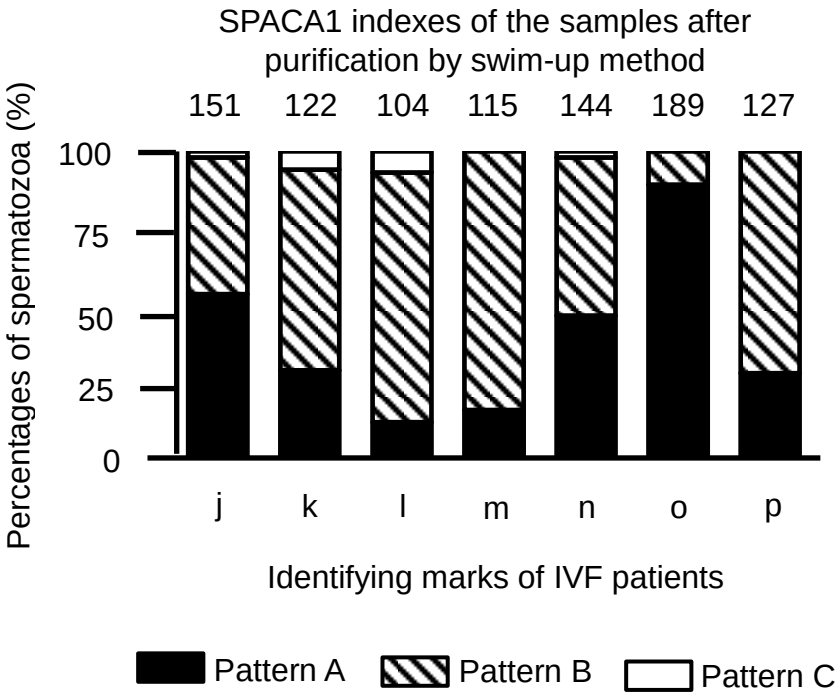

Fig. 13. Immunodetection patterns of SPACA1 proteins in sperm samples from healthy fertile volunteers.

The spermatozoa from healthy fertile volunteers, which were washed, collected by the swim-up method and then pre-incubated to induce capacitation according to the same procedures for infertile patients, were used for the immunostaining.

Details were described in the legend of Fig. 12.

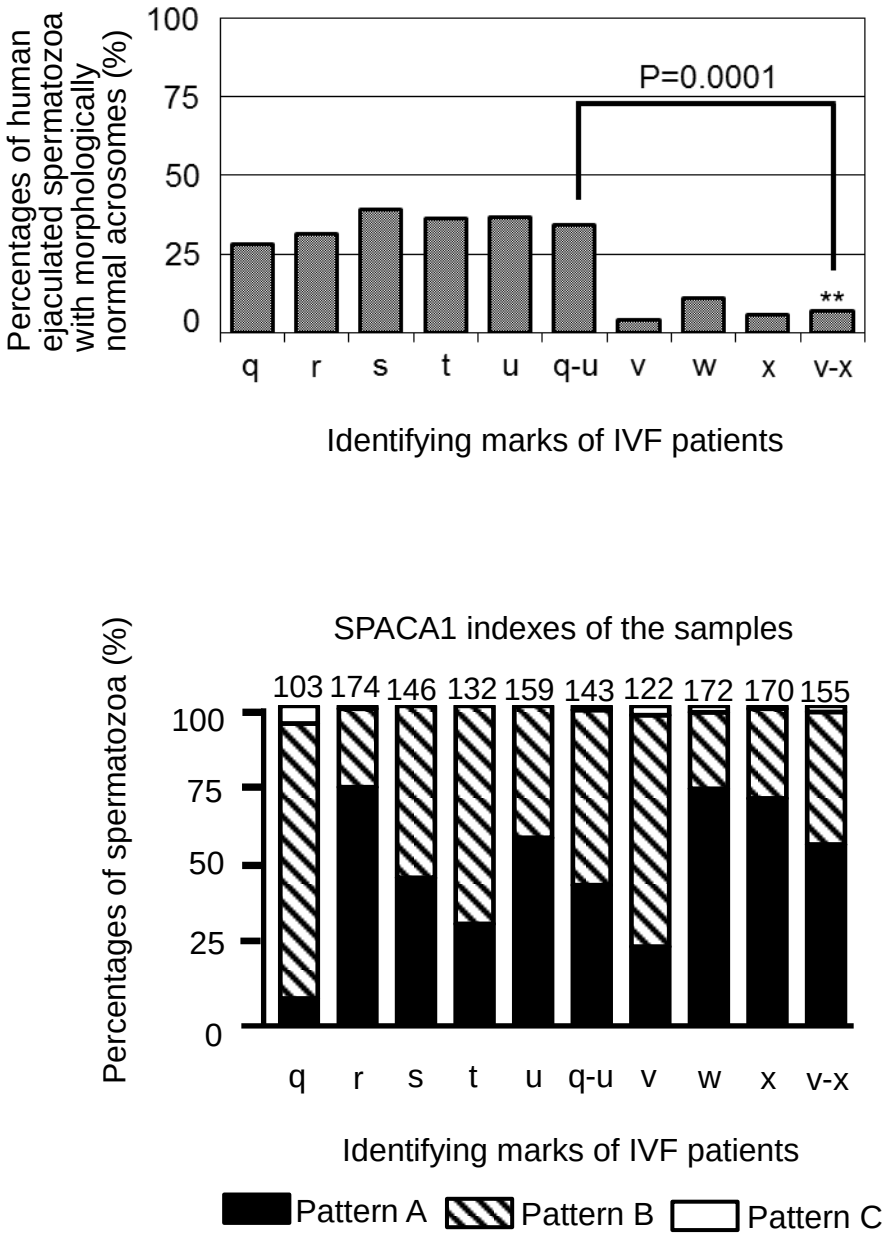

Fig. 14. Immunodetection patterns of SPACA1 proteins and acrosomal conditions in sperm samples from infertile male volunteers.

The spermatozoa from infertile male volunteers were used for FITC-PNA/PI and indirect immunofluorescence.
Details were described in the legend of Fig.12.

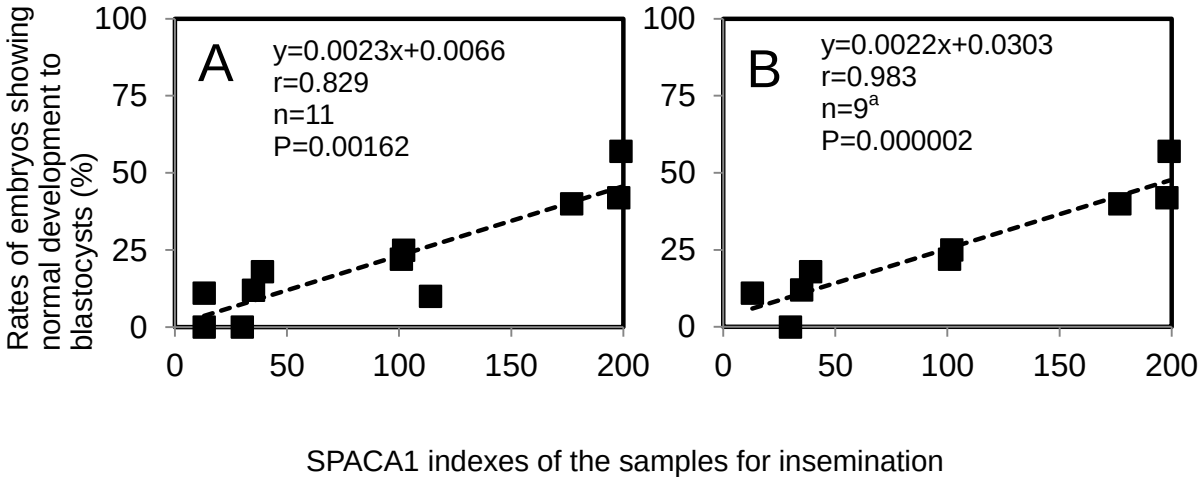

Fig. 15. Relationship between SPACA1 indexes and rates of embryos showing normal development to blastocysts in conventional IVF treatments.

[a] Data of the patients c and i with relatively lower fertilization rates (see Table 5) were excluded from the panel B.

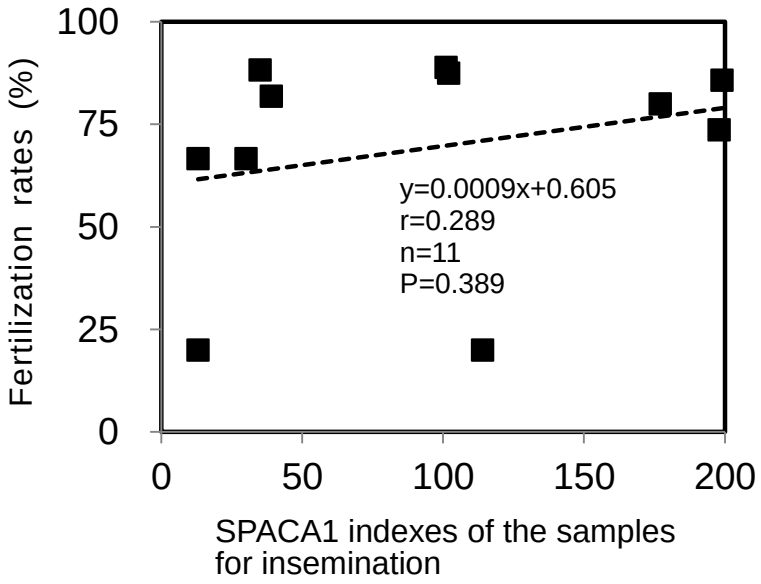

Fig. 16. Relationship between SPACA1 indexes and fertilization rates of conventional IVF treatments.

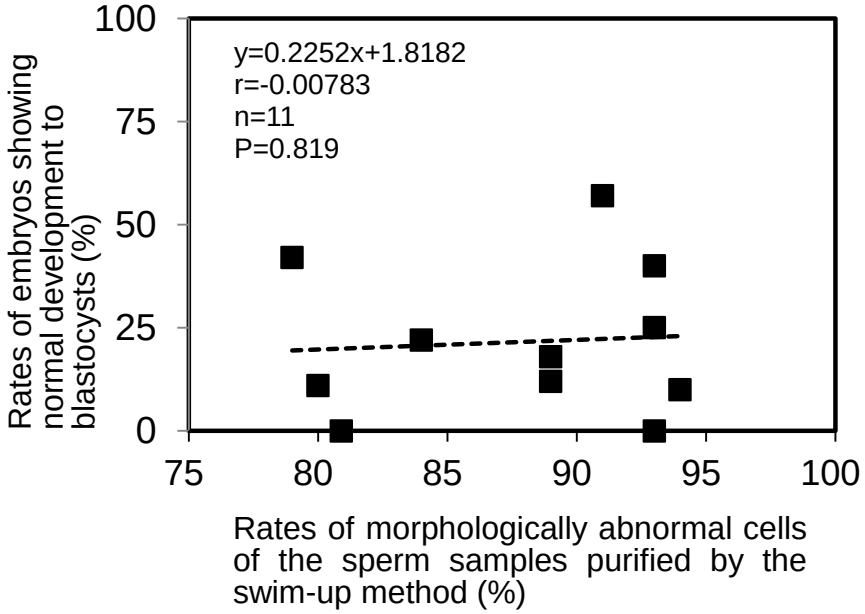

Fig. 17. Relationship between rates of morphologically abnormal spermatozoa and rates of embryos showing normal development to blastocysts in conventional IVF treatments.

CHAPTER 5

General summary

Artificial insemination (AI) is an indispensable reproductive technique for wide use of the spermatozoa collected from high-performance sires. Currently, almost all of the bovine reproduction are made by the AI using frozen-thawed spermatozoa in Japan. On the other hand, *in vitro* fertilization (IVF) has currently been well-established as a research tool for fertilization and early embryo development in mice and a biotechnological method to produce early embryos for embryo transfer (ET) in the cattle. The improvement of IVF makes it possible to produce early embryos efficiently and consequently to provide a number of calves for the beef industry. In the human, moreover, the conventional IVF techniques which were originally devised for the laboratory animal and livestock were applied as the clinical treatments for infertile couples.

As stated above, AI and conventional IVF are widely spread as reproductive biotechnologies through farms and medical clinics, respectively. However, there are recently increasing problems concerning these biotechnologies. Specifically, in the AI program of the cattle, the conception rates are decreasing in Japan. In the AI program, several samples from each lot of the frozen sperm straws were used for the examination of motility and then remaining frozen sperm straws of the qualified lots are always used for AI. However, there are large variations in the results of AI among bulls. Especially, AI using frozen-thawed spermatozoa with high motility from a certain bull results in relatively low conception rates. In human IVF, similarly, motility is considered as the most important parameter of sperm characteristics. Indeed, the spermatozoa are usually used for the IVF treatments after the swim-up method to recover highly motile ones. However, there are also large variations in the results of IVF even if highly motile spermatozoa are used for all treatments. In summary, it should be noted that the sperm motility examination (in other words, the examination of sperm flagellar functions) is not always valid for the estimation of the results of cattle AI and human IVF.

Functions of the acrosomal region are very important for the achievement of sperm fertilization with oocytes. However, the exact examination of the acrosomal conditions is scarcely adopted as the method of sperm assessment in bovine AI program and human conventional IVF treatments. The aim of this study was to investigate whether the exact examination of the acrosomal conditions is valid for the prediction of the results of bovine AI program and human conventional IVF treatments.

The purposes of the second chapter were to examine the relationship between male AI subfertility and acrosomal poor conditions of frozen-thawed spermatozoa and to identify possible male dysfunctions causing low conception rates in the AI using frozen spermatozoa with acrosomal poor conditions in Japanese Black bulls. I investigated individual differences among bulls in the results concerning (1) acrosomal conditions of frozen-thawed spermatozoa as assessed by not merely peanut agglutinin (PNA)-lectin staining but also immunostaining of acrosomal tyrosine-phosphorylated proteins, (2) routine AI using frozen-thawed spermatozoa as assessed by pregnancy diagnosis, (3) *in vivo* fertilization of frozen-thawed spermatozoa and early development of fertilized eggs as assessed by superovulation (SOV) /AI-embryo collection (EC) tests and (4) IVF of frozen-thawed spermatozoa with oocytes. The frozen-thawed spermatozoa were washed and then used for fluorescein isothiocyanate (FITC)-conjugated PNA/propidium iodide (PI) staining and indirect immunofluorescence of acrosomal tyrosine-phosphorylated proteins. In the routine AI tests, cows which were at the natural estrus or treated with $PGF_{2\alpha}$ analog were used for single AI with frozen-thawed spermatozoa. In the SOV/AI-EC tests, cows which received CIDR with GnRH analog were used for two AIs with frozen-thawed spermatozoa. In the IVF tests, oocyte-granulosa cell complexes (OGCs) were collected from 3 to 5 mm follicles, cultured in the maturation medium and inseminated with frozen-thawed spermatozoa. The percentages of frozen-thawed spermatozoa with normal acrosomal conditions assessed by the above-mentioned staining techniques were significantly correlated with the conception rates of routine AI, rates of transferable embryos in SOV/AI-EC tests and *in vitro* fertilization rates. These are consistent with the suggestions that

distribution of acrosomal tyrosine-phosphorylated proteins as well as acrosomal morphology of frozen-thawed spermatozoa are AI fertility-associated markers which are valid for the prediction of the AI results. And low conception rates in the AI using frozen-thawed spermatozoa with acrosomal poor conditions results from reproductive dysfunctions in the processes between sperm insemination into females and early embryo development, probably failed fertilization of frozen-thawed spermatozoa with oocytes.

In the third chapter, I confirmed the existence of the tyrosine-phosphorylated form of sperm acrosome associated 1 (SPACA1) proteins in unfrozen spermatozoa of Japanese Black bulls by the immunoprecipitation-Western blotting techniques, investigated the acrosomal distribution patterns of these proteins in the unfrozen spermatozoa by the double immunostaining with the anti-phosphotyrosine antibody and anti-SPACA1 protein antibody, and then compared distributive normality of SPACA1 proteins in the frozen-thawed spermatozoa with different results of the IVF tests. The frozen-thawed spermatozoa were washed and subjected to indirect immunofluorescence of acrosomal SPACA1 proteins. In the IVF tests, OGCs were collected from 3 to 5 mm follicles, cultured in the maturation medium and inseminated with frozen-thawed spermatozoa. The obtained results demonstrated that 42 kDa tyrosine phosphorylated protein was distributed over the anterior acrosome. Indirect immunofluorescence of spermatozoa with the anti-SPACA1 protein antibody revealed four detection patterns of the antigens in the principal and the equatorial segments of the acrosome. Immunofluorescence levels of SPACA1 proteins in the head were largely varied among bulls. Moreover, the SPACA1 distribution is related with the penetrability of frozen spermatozoa into oocytes *in vitro*.

The purposes of the forth chapter were to show the existence of individual differences in the sperm acrosomal conditions by the lectin-staining and indirect immunofluorescence of SPACA1 proteins among male patients of infertile couples and discussed possible their impacts on outcomes of conventional IVF. The spermatozoa were collected from male patients of infertile couples and volunteers, washed by centrifugation, collected by the swim-up method, and then used for clinical

treatments of conventional IVF. The ejaculated spermatozoa were washed and then used for FITC-PNA/PI staining and indirect immunofluorescence of SPACA1 proteins. In the clinical IVF treatments, fertilization rates and blastocyst development rates were evaluated. The immunocytochemical observations revealed that the SPACA1 proteins were localized definitely in the acrosomal equatorial segment and variedly in the acrosomal principal segment. Specifically, the detection patterns of SPACA1 proteins in the acrosomal principal segment could be classified into three categories; (A) strong, (B) intermediate or faint, and (C) almost no immunofluorescence. The SPACA1 indexes were largely different among male patients with the wide range. The SPACA1 indexes were significantly correlated with the rates of embryos (*in vitro* fertilized eggs) showing normal development to blastocysts, though they were barely associated with fertilization rates. These results are consistent with the suggestion that individual differences in the distribution of SPACA1 proteins among male patients of infertile couples may have impacts on the outcomes of conventional IVF.

In conclusion, this study proved that investigations of reproductive dysfunctions causing male AI subfertility would be contributory to recovery of high conception rates in the AI program of Japanese Black cattle. In addition, this study demonstrated that percentages of frozen-thawed spermatozoa with normal distribution of SPACA1 proteins were significantly higher in the sperm samples with good IVF results than those with poor results. In addition, these results suggest that SPACA1 indexes are valid as molecular biomarkers which can predict effectiveness of conventional IVF of infertile couples. This study provides the exact examination of the acrosomal conditions is valid for the prediction of the results of bovine AI program and human conventional IVF treatments.

REFERENCES

Agarwal A, Sharma R, Durairajanayagam D, Ayaz A, Cui Z, Willard B, Gopalan B, Sabanegh E. (2015) Major protein alterations in spermatozoa from infertile men with unilateral varicocele. *Reprod Biol Endocrinol* 8, 1-22.

Aitken RJ, Bronson R, Smith TB, De Iuliis GN. (2013) The source and significance of DNA damage in human spermatozoa; a commentary on diagnostic strategies and straw man fallacies. *Mol Hum Reprod* 19, 475-485.

Aitken RJ, Nixon B. (2013) Sperm capacitation: a distant landscape glimpsed but unexplored. *Mol Hum Reprod* 19, 785-793.

Arai M, Minami K, Ogura Y, Sakase M, Fukushima M, Harayama H. (2014) Effects of distribution of tyrosine-phosphorylated proteins on the acrosomal stability in Japanese Black bull spermatozoa. *J Reprod Dev* 60 Suppl, j111 (abstract P-24, in Japanese).

Austin CR. (1951) Observation on the penetration of the sperm in the mammalian egg. *Aust J Sci Res B* 4, 581-596.

Austin CR. (1952) The capacitation of the mammalian sperm. *Nature* 170, 326.

Baracaldo, MI, Martinez, MF, Adams, GP, Mapletoft, RJ. (2000) Superovulatory response following transvaginal follicle ablation in cattle. *Theriogenology* 53, 1239-1250.

Barbat A, Le M é,zec P, Ducrocq V, Mattalia S, Fritz S, Boichard D, Ponsart C, Humblot P. (2010) Female fertility in French daily breeds: current situation and strategies for improvement. *J Reprod Dev* 56 Suppl, S15-21.

Blankstein J, Shalev J, Saadon T, Kukia EE, Rabinovici J, Pariente C, Lunenfeld B, Serr DM, Mashiach S. (1987) Ovarian hyperstimulation syndrome: prediction by number and size of preovulatory follicles. *Fertil Steril* 47, 597-602.

Bonvadi MR, Madaen SK, Saghafi M. (2013) Effects of varicocelectomy on anti-sperm antibody in

patients with varicocele. *J Reprod Infertil* 14, 73-78.

Brackett, BG, Bousquet, D, Boice, ML, Donawick, WJ, Evans, JF, Dressel, MA. (1982) Normal development following *in vitro* fertilization in the cow. *Biol Reprod* 27, 147-158.

Buckler HN, Evans CA, Mamtora H, Burger HG, Anderson DC. (1991) Gonadotropin, steroid and inhibin levels in women with incipient ovarian failure during anovukatory and ovukatory rebound cycles. *J Clin Endocrionol Metab* 72, 116-124.

Chang MC. (1951) Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature* 168, 697-698.

Check JH. (2010) Antisperm antibodies and human reproduction. *Clin Exp Obstet Gynecol* 3, 169-174.

Chen XF, Wang HX, Liu YD, Sun K, Zhou LX, Huang YR, Li Z, Ping P. (2014) Clinical features and therapeutic strategies of obstructive azoospermia in patients treated by bilateral inguinal hernia repair in childhood. *Asian J Androl* 5, 745-748.

Chulkwunvwew CF, Awonuga DO, Ogo CN, Nwadike V, Chukwunvere KE. (2015) Patterns of seminal fluid analysis in male partners of infertile couples attending gynecology clinic at federal medical center, Abeokuta. *Niger J Med* 2, 131-136.

Cristser JK, Rowe RF Del Campo MR, Ginter OJ. (1980) Embryo transfer in cattle: Factors affecting superovulatory response, number of transferable embryos, and length of post-treatment estrous cycles. *Theriogenology* 13, 398-406.

Dan JC. (1952) Studies on the acrosome I. reaction to egg-water and other stimuli. *Biol Bull* 103, 54-66.

Dochi O, Kabeya S, Koyama H. (2010) Facors affecting reproductive performance in high milk-producing Holstein cows. *J Reprod Dev* 56 Suppl, S15-21.

Eddy EM. (2006) The Spermatozoon. In: Knobil and Neill's Physiology of Reproduction (3rd Edition) (ed JD Neill), pp. 3-54. Elsevier Academic Press, St. Louis.

Ellis DJ, Shadan S, James PS, Henderson RM, Edwardson JM, Hutchings A, Jones R. (2002) Post-testicular development of a novel membrane substructure within the equatorial segment of ram, bull, bore, and goat spermatozoa as viewed by atomic force microscopy. *J Struct Biol* 138, 187-198.

Flamenbaum I, Galon N. (2010) Management of heat stress to improve fertility in daily cows in Israel. *J Reprod Dev* 56 Suppl, S36-41.

Fujihara Y, Satouh Y, Inoue N, Isotani A, Ikawa M, Okabe M. (2012) SPACA1-deficient male mice are infertile with abnormally shaped sperm heads reminiscent of globozoospermia. *Development* 139, 3583-3589.

Gardner DK, Scoolcraft WB. (1999) *In vitro* culture of human blastocysts. In: Towards reproductive certainty: infertility and genetics beyond (eds R Jansen R & D Mortimer), pp. 377-388, Parthenon Press, Carnforth.

Graham JK, Moce E. (2005) Fertility evaluation of frozen/ thawed semen. *Theriogenology* 64, 492-504.

Hanada A, Shiotani Y, Suzuki T. (1986) A delivery example by nonsurgical transplant of cow embryos provided by *in vitro* fertilization. *Proceedings of 78th Japanese Society of Animal Scienc*, 18 (in Japanese).

Haning RV JR, Levin RM, Behrman HR, Kase NG, Speroff L. (1979) Plasma estradiol window and urinary estriol glucuronide determination for monitoring menotropin induction of ovulation. *Obstet Gynecol* 54, 442-447.

Haning RV JR, Boehnlein LM, Carlson IH, Kuzuma DL, Zweibel WJ. (1984) Diagnosis-specific serum 17, β-estradiol (E_2) upper limits for treatment with menotropins using a ^{125}I direct E_2 assay. *Fertil Steril* 42, 822-889.

Hao Z, Wolkowicz MJ, Shetty J, Klotz K, Bolling L, Sen B, Westbrook VA, Coonrod S, Flickinger CJ, Herr JC. (2002) SAMP32, a testis-specific, isoantigenic sperm acrosomal

membrane-associated protein. *Biol Reprod* 66, 735-744.

Hashizume R. (2000) Livestock artificial insemination workshop text, pp. 290-293. Japan livestock artificial insemination association, Tokyo (in Japanese)

Harayama H. (2003) Viability and protein phosphorylation patterns of boar spermatozoa agglutinated by treatment with a cell-permeable cyclic adenosine 3', 5'-monophosphate analog. *J Androl* 24, 831-842.

Harayama H, Nishijima K, Murase T, Sakase M, Fukushima M. (2010) Relationship of protein tyrosine phosphorylation state with tolerance to frozen storage and the potential to undergo cyclic AMP-dependent hyperactivation in the spermatozoa of Japanese Black bulls. *Mol Reprod Dev* 77, 910-921.

Holt WV, Fazeli A. (2010) The oviduct as a complex mediator of mammalian sperm function and selection. *Mol Reprod Dev* 77, 934-943.

Holt WV, Fazeli A. (2015) Do sperm possess a molecular passport? Mechanistic insights into sperm selection in the female reproductive tract. *Mol Hum Reprod* 21, 491-501.

Hoshi K, Yanagida K, Aita T, Yoshimatu N, Sato A. (1988) Changes in the motility pattern of human spermatozoa during *in vitro* incubation. *Tohoku J Exp Med* 1, 47-56.

Hoshi K, Yanagida K, Yazawa H, Katavose H, Sato A. (1994) Pregnancy and delivery after intracytoplasmic injection of an immobilized, killed spermatozoon into an oocyte. *J Assist Reprod Genet* 11, 325-326.

Imai K, Matoba S, Dochi O, Shimohira I. (2002) Different factors effects development competence and cryotolerance *in vitro* produced bovine embryo. *J Vet Med Sci* 10, 877-891.

Inoue N, Satouh Y, Ikawa M, Okabe M, Yanagimachi R. (2011) Acrosome-reacted mouse spermatozoa recovered from the perivitelline space can fertilize other eggs. *Proc Natl Acad Sci USA* 108, 20008-20011.

Iritani A, Niwa K. (1977) Capacitation of bull sepermatozoa and fertilization *in vitro* of cattle follicular oocytes matured in culture. *J Reprod Fert* 50, 119-121.

Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satou Y, Baba SA, Chiba K, Hirohashi N. (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proc Natl Acad Sci USA* 108, 4892-4896.

Jones R, James PS, Oxley D, Coadwell J, Suzuki-Toyota F, Howes EA. (2008) The equatorial subsegment in mammalian spermatozoa is enriched in tyrosine phosphorylated proteins. *Biol Reprod* 79, 421-434.

Kadokawa H, Sakatani M, Hansen PJ. (2012) Perspectives on improvement of reproduction in cattle during heat stress in a future Japan. *Anim Sci J* 83, 439-445.

Karoui S, Díaz C, González-Marín C, Amenabar ME, Serrano M, Ugarte E, Gosálvez J, Roy R, López-Fernández C, Carabaño MJ. (2012) Is sperm DNA fragmentation a good marker for field AI bull fertility? *J Anim Sci* 90, 2437-2449.

Kawano N, Araki N, Yoshida K, Hibino T, Ohnami N, Makino M, Kanai S, Hasuwa H, Yoshida M, Miyado K, Umezawa A. (2014) Seminal vesicle protein SVS2 is required for sperm survival in the uterus. *Proc Natl Acad Sci USA* 111, 4145-4150.

Kawano N, Kang W, Yamashita M, Koga Y, Yamazaki T, Hata T, Miyado K, Baba T. (2010) Mice lacking two sperm serine proteases, ACR and PRSS21, are subfertile, but the mutant sperm are infertile *in vitro*. *Biol Reprod* 83, 359-369.

Kawate N, Sakase M, Watanabe K, Fukushima M, Noda M, Takeda K, Ueno S, Inaba T, Kida K, Tamada H, Sawada T. (2007) Ovsynch plus CIDR protocol for timed embryo transfer in suckled postpartum Japanese Black beef cows. *J Reprod Dev* 53, 811-817.

Kuroda K, Fukushima M, Harayama H. (2007) Premature capacitation of frozen-thawed spermatozoa from subfertile Japanese Black cattle. *J Reprod Dev* 53, 1079-1086.

Loret De Mola JR, Garside WT, Bucci J, Tureck RW, Hevner S. (1997) Analysis of the human zona pellucida during culture: correlation with diagnosis and the preovulatory hormonal environment. *J Assist Reprod Genet* 6, 332-336.

McDonald JH. (2014) Handbook of Biological Statistics (3rd Edition), Sparky House Publishing,

Baltimore.

Meza-Vázquez HE, Martínez-Cornelio A, Espinoza-Guerrero X, Cárdenas-Rodríguez E, Maldonado-Alcaraz E, Serrano-Brambila E. (2008) Ejaculatory duct obstruction. *Cir Cir* 4, 349-353.

Mizuno Y, Isono A, Kojima A, Arai MM, Noda T, Sakase M, Fukushima M, Harayama H. (2015) Distinct segment-specific functions of calyculin A-sensitive protein phosphatases in the regulation of cAMP- triggered events in ejaculated bull spermatozoa. *Mol Reprod Dev* 3, 232-250.

Moska N, Murray Elaine, Wakefield P, Matson P. (2011) The staining pattern of human sperm with DiffQuik: relationship with sperm head morphology and a sperm chromatin structure assay (SCSA). *Reprod Biol* 11, 55-59.

Nabenishi H, Ohta H, Nishimoto T, Morita T, Ashizawa K, Tsuzuki Y. (2011) Effect of the temperature-humidity index on body temperature and conception rate of lactating dairy cows in southwestern Japan. *J Reprod Dev* 57, 450-456.

Niakan KK, Han J, Pedersen RA, Simon C, Pera RA. (2012) Human pre-implantation embryo development. *Development* 139, 829-841.

Okabe M. (2013) The cell biology of mammalian fertilization. *Development* 140, 4471-4479.

Polge C. (1952) Fertilization capacity of bull spermatozoa after freezing at 79 degrees C. *Nature* 169, 626-627.

Robertson I, Nelson RE. (1998) Certification and identification of the embryo. In: Manual of the International Embryo Transfer Society (3rd Edition), (eds DA Stringfellow & SM Seidel), pp.103-134, Savoy, IL.

Saito H. (2014) Report of registration, investigation committee in Ethical Review Board in 2013 *Japan Sciety of Obstetrics and Gynecology Magagine* 66, 2445-2481 (in Japanese).

Simon L, Liu L, Murphy K, Ge S, Hotaling J, Aston KI, Emery B, Carrell DT. (2014) Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples

undergoing assisted reproduction treatment. *Hum Reprod* 29, 904-917.

Soules MR, McLachlan RI, Ek M, Dahl KD, Cohen NL, Bremner WJ. (1989) Luteal phase deficiency: characterization of reproductive hormones over the menstrual cycle. *J Clin Endocrinol Metab* 69, 804-812.

Steptoe PC, Edwards RG. (1978) Birth after the reimplantation of a human embryo. *Lancet* 312, 366.

Sugie T. (1965) Succcessful transfer of a fertilized bovine egg by non-surgical techniques. *J Reprod Fertil* 10, 197-201.

Swann K, Lai FA. (2013) PLCζ and the initiation of Ca^{2+} oscillations in fertilizing mammalian eggs. *Cell Calcium* 53, 55-62.

Suzuki M, Hoshi K, Hoshiai H, Saitou A, Momono K, Mori R, Kyono K, Tsuiki A, Imaizumi H, Nagaike F, Uehara S, Naganuma T, Hirose H. (1983) One case of uterine tube-related infertility that succeeded for the fertilization and implantation by i*n vitro* fertilization-embryo transfer. *Japanese Journal of Fertility and Sterility*, 28. 439-443 (in Japanese).

Tandara M, Bajić A, Tandara L, Bilić-Zulle L, Šunj M, Kozina V, Goluža T, Jukić M. (2014) Sperm DNA integrity testing: big halo is a good predictor of embryo quality and pregnancy after conventional IVF. *Andrology* 2, 678-686.

Tani M, Hayashida T, Tomokawa K, Mito Y, Funakoshi D, Tani C, Sakatani M, Takahashi M, Kitahara G, Kamimura S. (2010) Effect of embryo transfer following artificial insemination (ETFAI) on reproductive performance in dairy cows in South- Western Japan. *J Vet Med Sci* 5, 627-629.

Tavares RS, Silva AF, Lourenço B, Almeida-Santos T, Sousa AP, Ramalho-Santos J. (2013) Evaluation of human sperm chromatin status after selection using a modified Diff-Quik stain indicates embryo quality and pregnancy outcomes following *in vitro* fertilization. *Andrology* 1, 830-877.

Toma SK, Stovall DW, Hammond MG. (1992) The effect of laparoscopic ablation or danocrine on pregnancy rates in patients with stage I or II endometriosis undergoing donor insemination.

Obstet Gynecol 80, 253-256.

Volgsten H, Skoog A, Ekselius L, Lundkvist Ő, Poromaa IS. (2008) Prevalence of psychiatric disorders in infertile women and men undergoing *in vitro* fertilization treatment. *Hum Reprod* 9, 2056-2063.

Weström L. (1980) Incidence, prevalence, and trends of acute pelvic inflammatory disease and its cosequences in industrialised countried. *Am J Obstet Gynecol* 138, 880-892.

World Health Organization. (2010) WHO laboratory manual for the examination and processing of human semen (5th Edition). WHO press, Geneva.

Yanagimachi R, Chang MC. (1963) Fertilization of hamster eggs in vitro. *Nature* 200, 281-282.

Yanagimachi R. (1970) The movement of golden hamster spermatozoa before and after capacitation. *J Reprod Fertili* 1, 193-196.

Yanagimachi R. (1994) Mammalian fertilization. In: The Physiology of Reproduction (2nd Edition), (eds E Knobil & JD Neill), pp. 189-317, Raven Press, New York.

Yanagimachi R. (2012) Fertilization studies and assisted fertilization in mammals: their development and future. *J Reprod Dev* 58, 25-32.

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