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= Research Note =

Time of insemination affects the sex ratio of Japanese Black cow offspring

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ABSTRACT

This study was conducted to assess whether varying the time of artificial insemination (AI) after detection of estrus influences the sex ratio of Japanese Black cows offspring. Data were collected from 1,829 inseminated cows, of which 753 gave birth to calves, over a period of two and a half years. The animals were divided in two groups: the early AI group (3 h after estrus detection) and late AI group (27 h after estrus detection). All cows were palpated 3 h after estrus detection. If the dominant follicle was large, the cows were inseminated (early AI). The cows with a small dominant follicle were palpated again 27 h after estrus detection and inseminated (late AI group). Although no difference was observed in the calving rates between the two groups, the proportion of male calves in the early AI group was significantly higher ($P < 0.05$) than that in the late AI group (56.9% vs. 41.1%, respectively). Our results indicate that delayed AI decreases the proportion of male calves.

Key words: Artificial insemination, estrus, insemination time, offspring, sex ratio

INTRODUCTION

Varying the timing of artificial insemination (AI) might influence the outcome of sex ratio of cattle offspring. If so, this could be economically beneficial and used for livestock production. Multiple efforts have been made to influence the sex of calves by varying the time of insemination [5,10]. Several studies have reported that early inseminations after the onset of estrus can result in a higher proportion of female calves, whereas delayed insemination result in a higher proportion of male calves. This effect on the sex ratio might be due to differences in the timing of sperm capacitation and survival time of the X and Y chromosome-bearing spermatozoa in the female reproductive tract [5,13]. Some studies provide other explanations to account for the effects of insemination time on the sex ratio of offspring. Differences between the results of these studies might exist because of other factors such as genotype, climate, quality of semen, parity number, postpartum time, and region [3,5,10]. Pursley *et al.* [8] reported that both early and late inseminations resulted in a higher percentage of females. Moreover, a large study in dairy cattle showed that there were no effects of parity, herd, or time of insemination on sex ratio [3]. Numerous factors have been suggested to be associated with the variation in the sex ratio of offspring, but these conflicting reports make it difficult to assess which factors might contribute to an altered sex ratio in cattle. In this study, data from one herd were evaluated, and the effect of time of insemination on the sex ratio of Japanese Black cow offspring was assessed.

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Table 1. Variation of the calving rates and the proportion of males calves according to the interval between the detection of estrus and artificial insemination (AI)

Interval between estrus and AI	Cows calved/total inseminated cows (%)	Calved males/total calved animals (%)
3 h	573/1,389 (41.3)	326/573 (56.9)[a]
27 h	180/440 (40.9)	74/180 (41.1)[b]
Total	753/1,829 (41.2)	400/753 (53.1)

[a,b] Values with different superscripts in the same column are significantly different ($P < 0.05$).

MATERIALS AND METHODS

Animals and AI

Data were collected from a herd consisting of approximately 350 Japanese Black cows over a period of two and a half years (between 2011 and 2013). Parity of the animals varied between one and eight. The cows were housed in a free stall barn with a sawdust floor and were fed a diet of hay and concentrates according to their production level [1].

Freeze–thawed semen derived from 25 Japanese Black bulls with proven fertility was used for insemination. Estrus detection was conducted daily from 11:00 to 11:30. The cows were classified into insemination groups based on the status of the dominant follicle, which was examined by rectal palpation before insemination, at 3 h after the detection of estrus. Based on the status of follicular development, if the dominant follicle was large and expected to ovulate, the cows were immediately inseminated (early AI group). However, if the dominant follicle was small and not expected to ovulate, the insemination was postponed. Cows were examined again 27 h after estrus detection. If the dominant follicle developed and the cow was expected to ovulate, insemination was performed (late AI group). However, if the dominant follicle did not develop and the cow was not expected to ovulate, or had already ovulated, the cow was excluded from the study. Estrus detection, all rectal palpations, and AIs were performed by one experienced technician, and no hormonal treatment was given to the cows, either before or after insemination. Ovulation was confirmed by rectal palpation 24 h after AI.

Statistical analysis

The total number of inseminated cows was 1,829 and the number of cows that delivered calves was 753. Calving rates and proportion of male calves were evaluated in two groups: the early insemination group (3 h after the detection of estrus) and the late insemination group (27 h after the detection of estrus). Data were then submitted to analysis of variance (ANOVA) using the general linear model (GLM) procedure of SAS (SAS for Windows, version 9.1, SAS Institute Japan, Tokyo, Japan). The statistical model included the type of insemination, season, sire, time of insemination and the two-way interactions. The differences with a probability value (P) of 0.05 or less were considered significant.

RESULTS AND DISCUSSION

Table 1 summarizes the number of inseminations, calving results and the proportion of male calves in the early and late insemination groups. No significant time × season and time × sire interactions were observed in calving rates and proportion of male calves. There was no significant difference between the calving rates obtained for the two groups. However, the proportion of male calves in the early insemination group was significantly higher ($P < 0.05$) than that in the late insemination group (56.9% vs 41.1%, respectively).

Many studies have assessed the sex ratio of calves with respect to the timing of AI during visually observed estrus, mounting behavior and intravaginal conductivity [5,10,12]. Controlling the sex ratio by varying the time of AI with respect to the onset of the estrus can be an effective technique in animal husbandry because of the low associated costs. The variations observed in the sex ratios of offspring because of varying the timing of AI are thought to have arisen owing to differences in the motility, capacitation time, and survival time of the X and Y chromosome-bearing spermatozoa [5,13]. In cows, estrus lasts for a period of 18 h on average and ovulation follows 10–12 h later; thus, the total time from the onset of estrus to ovulation is 30–32 h [5,9]. The recommended period for AI of cows is between 12 and 18 h after the onset of estrus [4,6]. It has been reported that late AI decreased fertility, and the highest conception rates for AI occurred between 4 and 12 h after the onset of

standing activity [2]. In this study, no difference was observed between the early (3 h) and late (27 h) insemination groups regarding the calving rates of inseminated cows, although these rates were lower than those reported by a previous study carried out in Japanese Black cows [11]. Moreover, the present data showed that delayed AI decreased the proportion of male calves. Contradictory to these results, it has been demonstrated that AI immediately after the detection of estrus resulted in more female calves, whereas AI performed later resulted in more male calves [5]. Another study reported that both early and late inseminations resulted in a higher percentage of females [8]. Rorie *et al*. [10] suggested that insemination at approximately 20 h or 10 h before expected ovulation did not alter the sex ratio of calves. During the present study, insemination was postponed if at the time of rectal examination, the follicle was not expected to ovulate, and insemination was then performed at 27 h. All dominant follicles ovulated within 24h after AI in each groups. Therefore, dominant follicle ovulated 3 to 27 h after estrus detection in early insemination group, and ovulated 27 to 51 h after estrus detection in late insemination group. This indicates that estrus period was long and ovulation was delayed in late insemination group. In humans, Martin [6] suggested that shorter follicular phases are associated with shorter periods of penetrable mucus and higher proportions of Y spermatozoa reaching the uterus. Conversely, longer phases are associated with longer periods of penetrable mucus and lower proportions of Y spermatozoa in the uterus. A similar phenomenon may be expected cows.

The disparity between results obtained from various studies is thought to have arisen due to methodological differences, especially the method of estrus detection, the use of different AI protocols, and the variability between males and ejaculates [5]. In conclusion, our results indicate that lasting estrus and delayed AI biased the sex ratio toward more females in Japanese Black cow offspring.

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= Mini Review =

Critical roles of seminal plasma on sperm migration in the female reproductive tract

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ABSTRACT

In mammals, ejaculated sperm have to travel a long distance through the female reproductive tract, from the vagina to the oviduct. A sperm's journey often exceeds the sperm's length by 1000-fold, and includes varied hurdles. However, our understanding of the state of the female reproductive tract after copulation has been highly limited due to its complexities. Gene-engineered animal are at last being used to reveal the *in vivo* mechanisms that sperm require the help by the seminal plasma, including a huge number of factors. One of them is assumed to function as a sperm guardian against the enemy hidden inside the female reproductive tract, efficiently achieving sperm guidance to oocytes. This review focuses on the regulatory mechanisms of the seminal plasma, so as to provide more insights into sperm's journey through the female reproductive tract.

Key words: seminal plasma, sperm migration, sperm selection, female reproductive tract

INTRODUCTION

In mammals, the female reproductive tract is a complex organ system that requires proper function and coordination in order to reproduce life within a body. The female reproductive tract is divided into three main parts: the vagina, the uterus, and the oviduct [49]. These organs have distinctive roles in reproduction: the vagina as the passage between outside of the body and the uterus, the uterus as the site of the development of the fetus, and the oviduct as the place of fertilization. Although internal fertilization is an effective and reasonable tool for achieving high fecundity, transmission of pathogens from semen during sexual behavior between unspecified partners is a critical problem for female health. On the other hand, the maternal immune system is able to strictly exclude xenoantigens to protect the mother and her fetus, while this system allows allogeneic sperm and semiallogeneic fetuses for the purpose of reproduction, leading to biological diversity [55]. The incidence of these adverse events suggests that the balance between exclusion and tolerance in the female reproductive tract is important for successful reproduction via internal fertilization.

Successful internal fertilization of a female requires only two components from a male: sperm and seminal plasma. The seminal plasma consists of secretions from several accessory sex glands (the prostate, seminal vesicles, epididymis, and bulbourethral glands), which show nutritive and protective effects for ejaculated sperm [32,60]. On the other hand, it has been reported that

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seminal plasma from rabbits contains factor(s) bringing great detriment to sperm fertility *in vitro* [7] a phenomenon known as sperm "decapacitation" [4]. The seminal plasma of other species decreased the fertility of rabbit sperm in the same way as rabbit seminal plasma [7], but the decapacitation factor(s) conserved among species have remained obscure. Moreover, this inhibitory effect of seminal plasma *in vitro* is not observed within female reproductive tracts. Even today, the physiological importance of seminal plasma in the female reproductive tract remains unclear.

The time periods of sperm's journey from ejaculation to arrival at the oviduct have been reported in various species [20,35,51]. Because of its occurrence inside of female bodies, it is difficult to accurately determine the period of this journey within the female reproductive tract. Overstreet and Cooper (1978) reported that rabbit sperm were found at the ampulla and fimbria of oviducts within 1–15 minutes after copulation, yet almost all of the sperm were dead; motile sperm that had migrated from the uterus into the oviduct were first detected at 90 minutes after copulation [42]. This famous article implies that rabbit sperm is physically able to reach the oviduct within 15 minutes, but another 75 minutes is needed for vigorous sperm capable of fertilization to arrive at the oviduct; raising the possibility that the sperm are selected by unknown processes in the female reproductive tract. In this review, we discuss the regulatory mechanisms of sperm migration, survival, and selection within the female reproductive tract.

PENETRATION OF EJACULATED SPERM INTO FEMALE REPRODUCTIVE TRACT

At copulation, human semen ejaculated into the vagina is spontaneously coagulated into a semisolid gelatinous mass, which then liquefies within 5–20 minutes [1,30]. The ejaculated sperm is first immobilized by semen coagulum, and then the liquefaction of the semen leads to the ejaculated sperm swimming toward the uterine cervix [44]. In rodents, the ejaculated semen separates in two phases: the solid phase, which forms the copulatory plug that fills the vagina, and the liquid phase, within which enriched sperm are deposited directly into the uterus [28]. These semen coagulations, found commonly in primates and rodents, are caused by a conserved system including transglutaminase IV (Tgm4) secreted from the prostate (the coagulating gland in rodents) [11], and its substrates, identified as the seminal vesicle proteins semenogelin I/II (SemgI/II) in primates and its mouse homolog seminal vesicle secretion 2 (SVS2) [33,43,44,57]. It is well known that *SEMG1/2* and *Svs2* genes are expected to evolve rapidly in their accumulation of amino acid replacement [25,33]. The molecular evolution of SemgII in primates correlates to the hardness of semen coagulum, and to the degree of female promiscuity [14]. This evidence proposes the possibility that the seminal protein adapts to the copulating style of the species in order to maximize fecundity. Recently, it was reported that the lack of Tgm4 or SVS2 causes a failure of copulatory plug formation, a reduction of sperm number in the uterus, and subfertility in mice [11,26]. This evidence indicates that semen coagulation is important for maintaining sperm number in female mice. That mouse seminal vesicle protein SVS2 is homologous with SemgI/II in primates [33] raises the possibility that Semg I/II also regulate the maintenance of sperm number in female primates. Although the precise biological function of coagulum in primates is still unclear, there is a high possibility that physical properties of semen adapt to the copulating style and morphology of the female reproductive tract in order to prevent from backflow of the semen.

SPERM TRANSPORT BY UTERINE PERISTALSIS

The famous inducers of uterine contraction in labor and delivery are two representative hormone groups, prostaglandins and oxytocin, which are dramatically increased and function in the uterine decidua during the late pregnancy term [16,22,41,56]. For effective parturition, the number of oxytocin receptors is significantly increased about 100-fold in uterine smooth muscle cells [17]. One of these hormones, prostaglandin F2α, is secreted from human endometrial epithelial cells even in the menstrual cycle [46]. The receptor of prostaglandin F2α, prostaglandin F receptor (FP), is also predominantly expressed in the human endometrial epithelium, and more highly expressed during the proliferative stage of the menstrual cycle [5,36].

In some vaginal semen depositors, such as primates, cows, and ewes, it is reasonable to assume that uterine contraction helps the sperm migration from the uterine cervix to the oviduct because a large portion of the seminal

plasma flows away from the vaginal vault. It was reported that the uterine smooth muscle moves and contracts strongly during the estrus period in humans [29,34], cows and ewes [21]. In domesticated cats, the patterns of the uterine contraction during estrus involve both directions, ascending and descending, resulting in uterine contents that flow back and forth [8]. Sperm migration throughout the uterus is improved by prostaglandins in rabbits [48], suggesting that the uterine contraction during estrus is elicited by prostaglandins or oxytocin.

Prostaglandins were first discovered and isolated from human semen in the 1930s, and named for the discovery source, the prostate gland [15]. Today, it is well known that large amounts of prostaglandins in the seminal plasma are produced mainly in the seminal vesicle [12]. In uterine semen depositors, such as rodents, pigs, and horses, prostaglandins in the seminal plasma directly change the uterine contraction. In swine, the contractive movements begin to be observed in the corpus uterus after direct administration of prostaglandins [38]. In mated female rats, uterine contractions are weakened by a treatment of indomethacin, an inhibitor of the production of prostaglandins [9]. Besides uterine contractions, many studies focused on the phenotypes of mice lacking an enzyme involved in prostaglandin biosynthesis, cyclooxygenase-2 [13,58], because prostaglandins play myriad roles as local mediators of inflammation and as modulators of physiologic functions [39]. Shortage of prostaglandins is implicated to associate with female infertility by causing multiple failures in ovulation, fertilization, and implantation [31,58]. Although the lack of endogenous prostaglandins in the female reproductive tract has been demonstrated in many reports, there are very few studies on the phenotype of prostaglandins in the semen; except for the sentence that "*Ptgs2*-deficient male mice are fertile" [58]. Further work needs to be done to determine whether prostaglandins of the seminal plasma have a role in sperm transport throughout the uterus.

SPERM SELECTION IN THE UTERUS

In some vaginal semen depositors, such as primates and cows, the restriction of sperm entry and sperm selection, such as the rapid selection of sperm with high DNA integrity, largely occurs at the uterine cervix between the vagina and the uterine body [54]. The cervix opens only during the estrus period and produces mucus whose biological function is determined by its macromolecular architecture [49,61]. The cervical mucus consisting of glycoproteins is highly hydrated at estrus, which leads to low viscosity and allows sperm migration into the uterine body [37].

In the cynomolgus monkey, a single epididymis-derived protein, beta-defensin 126 (DEFB126), coats the sperm surface during epididymal maturation, and then is detached from the sperm surface during *in vitro* capacitation [62]. DEFB126 is crucial for sperm to penetrate and move efficiently in the peri-ovulatory cervical mucus [54]. *In vitro* assays reveal that if DEFB126 has a highly negative charge it changes sperm capability for penetrating the cervical mucus. A common mutation of *DEFB126* in humans significantly reduces sperm penetration of even a viscous hyaluronic acid gel, used experimentally instead of the cervical mucus [53]. In a prospective cohort study, husbands with the *DEFB126 del/del* genotype actually showed a statistically significant decrease in fertility compared to those with *DEFB126 wt/wt* or *wt/del* genotypes [53]. In total, these evidences suggest the the mechanism that the uterine cervix selects mature sperm in epididymides, and consequently eliminates immature sperm.

In uterine semen depositors, such as rodents, pigs and horses, the seminal plasma directly enters the uterus and remains there, which induces more remarkable reactions in the uterus compared with those in the vaginal semen depositors. As described above, mouse SVS2 secreted from the seminal vesicles coagulates the semen (or forms a copulatory plug) in order to prevent the sperm from leaking out of the uterus [26]. On the other hand, SVS2 is partially degraded and enters the uterus along with ejaculated sperm as liquid semen [28]. To determine whether SVS2 functions as the decapacitation factor in female reproduction tracts, we produced mice lacking the *Svs2* gene. *Svs2*$^{-/-}$ male mice displayed strongly reduced fertility in natural mating, because of the ectopic acrosome reaction of the uterine sperm [26]. In the presence of SVS2, most of the uterine sperm did not alter acrosome intactness, but about 70% of the uterine sperm without SVS2 induced an acrosome reaction. This result brought us the idea that the ectopic acrosome reaction in the uterus might occur due to the induced deficiency in the decapacitation factor SVS2. Contrary to our idea, analyses of immunohistochemical distribution of the sperm-specific protein IZUMO-1 and observation of sperm configuration using transmission electron microscopy revealed that the

ectopic acrosome reaction of the uterine sperm does not occur; instead, what happens is simply sperm death caused by membrane disruption [26]. Importantly, this spermicidal effect was observed under *in vitro* culture conditions by the addition of uterine fluid collected from female mice, but not by the addition of oviductal fluid. These findings imply that the uterus selects the sperm by its status: sperm coated with SVS2, or those without.

The SVS2 receptor on the sperm membrane is identified as ganglioside GM1, and their interaction is regulated by an electrostatic difference [27]. For sperm selection by the uterus, there are at least two results of the binding of SVS2 to the sperm surface: one is the exclusion of the capacitated sperm, induced by detaching SVS2; and another is a stealth capability against uterine spermicide(s), engendered by coating with SVS2. Recently, it was reported that SVS2 maintains cholesterol level in the sperm membrane [2]. In the absence of SVS2, the cholesterol level in the sperm membrane is significantly decreased in the uterus. It is well known that the decrease of membrane cholesterol induces sperm capacitation [10]. Based on these evidences, it remains a possibility that the ejaculated sperm from *Svs2*$^{-/-}$ male mice are capacitated in the uterus, and then damaged by uterine spermicide(s). In artificial insemination of hamster sperm into the uterus, capacitated sperm neither enter the oviduct well, nor survive in the uterus well, compared with the incapacitated sperm [45]. On the other hand, in our report about the phenotypes of *Svs2*$^{-/-}$ male mice [26], transmission electron microscopic analysis demonstrated that a “thick wall” surrounds the uterine sperm from *Svs2*$^{+/+}$ male mice. This wall consists basically of seminal vesicle proteins including SVS2 and SVS4, suggesting a possibility that the wall physically blocks the interaction between uterine spermicide(s) and the sperm membrane.

SPERM TRANSPORT BY UTEROTUBAL JUNCTION BETWEEN UTERUS AND OVIDUCT

In uterine semen depositors, not only ejaculated sperm but also seminal plasma and pathogens enter the lumen of the uterus. Seminal plasma proteins are found abundantly in the lumen of the uterus, but never found in the oviduct [2,6,28]. Because the oviduct, the place wherein sperm fertilizes the oocyte, opens into the abdominal cavity, it is assumed that the uterotubal junction (UTJ) between uterus and oviduct functions as a barrier that only fertile sperm can pass through. Although anatomical morphology of the UTJ differs remarkably among species, there is a common characteristic that the lumen of UTJ is drastically narrower than that of the uterus, and filled with acidic mucus [24,49,50]. Although the barrier mechanism remains unclear, neither immotile, capacitated sperm nor pathogens can pass through the UTJ [3,45,47]. DEFB126 is required for ejaculated sperm to pass through the cervical mucus in vaginal semen depositors such as humans and monkeys [53,54,62]. The similar systems involving DEFB126 probably exist in the passage of sperm through the UTJ mucus. Several studies, using genetically modified mouse models, provide a hint to reveal the molecular mechanism of passage through the UTJ. Sperm without A Disintegrin And Metalloprotease 3 (ADAM3) cannot migrate to the oviduct, even if they are motile and morphologically normal [18,19,23,40,52,59]. ADAM3, as well as chaperone proteins and serine proteases such as calmegin, calsperin, protein disulfide isomerase homolog, and Prss37, is primarily present on the sperm head. Further studies on ADAM3 are needed to elucidate the mechanism of sperm transport through the UTJ.

CONCLUSION

Internal fertilization is indispensable to natural reproduction in mammals. To overcome infertility in humans, and the severely increasing issue of infertility in domestic animals, full understanding of this phenomenon is an attractive and extremely urgent theme for us. To unveil the overall configuration of internal fertilization, we wish to create a novel research field, traversing beyond given fields such as endocrinology, anatomy, cell biology, and immunology. It should not be forgotten that full competence of internal fertilization requires unique extracellular factors secreted from male genital organs. Sperms’ journey within the female reproductive tract is difficult to understand, and the production of genetically manipulated animals is only a royal road to reveal its molecular mechanisms. Among experimental subjects to detect phenotypes of gene-engineered animals, the judgement of fertility is relatively easier than the exploring phenotypes based on further internal mechanisms. Since it is burdensome to determine the cause of infertility in mice, classical methods are still needed on the research field. Today, the development and applications of CRISPR/Cas9 for genome engineering is

accelerating the discovery of novel factors, and presumably factors concerning internal fertilization in animals other than mice. Basic science targeted at internal fertilization in primates will shed light on the human reproduction system, leading, in the near future, to the identification of causes underlying human infertility.

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= Original Article =

Tyrosine phosphorylation of the flagellar aldose reductase is involved in the boar sperm capacitation

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ABSTRACT

In mammals, only the sperm that have undergone stepwise activation, including maturation in the epididymis and capacitation in the tubal isthmus, are able to initiate both hyperactivated motility and acrosome reaction to fertilize an ovum. Although the mechanisms in capacitation remain unclear, the tyrosine phosphorylation of functional proteins and the production of reactive oxygen species (ROS), are thought to be particularly important. Comprehensive analyses of the proteins that become tyrosine-phosphorylated during the capacitation process have identified a number of proteins, including aldose reductase (AR). Subsequent functional analysis showed that AR in the particulate fraction of the porcine sperm plays a central role in capacitation. In the present study, after fractionating sperm into cytosolic, membrane, flagellar, and head fractions, we scrutinized the localization of AR to be tyrosine-phosphorylated during capacitation and whether its activity was increased. It was found that the AR localized to the flagellum was tyrosine-phosphorylated, followed by the stimulation of its activity. Furthermore, NADPH levels in the sperm was found to be decreased during capacitation, while ROS levels increased. AR is the rate-limiting enzyme of the polyol pathway and uses NADPH as a coenzyme to convert glucose into sorbitol. These results suggest that the activation of flagellar AR by tyrosine phosphorylation reduces the amount of NADPH in sperm. This impairs the action of the glutathione cycle, which is important for its ROS elimination mechanism, leading to an increase in ROS levels. The present study suggests that sperm have a physiologically relevant ROS production system that is regulated by flagellar AR activities.

Key words: polyol pathway, NADPH, reactive oxygen species, sperm maturation

INTRODUCTION

When mammalian sperm are produced in the testes, they are immature and lack both forward motility and fertilizing capacity. While sperm are transported through ductus epididymidis, factors derived from its epithelium are provided to the immature sperm, either directly or via vesicles called epididymosomes [24], which confer the capabilities of flagellar motility and fertilizing ability on sperm. However, two more activation processes must be completed for the sperm to exert forward motility and fertility. The first activation process occurs at ejaculation to induce the forward motility. When the sperm leave the

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low bicarbonate concentration (2 mM) of the secretions in the cauda epididymis and are exposed to high bicarbonate concentration (20 mM) of the seminal vesicle secretions, a bicarbonate sensitive adenylate cyclase is activated. This promotes a cAMP-dependent phosphorylation cascade in the sperm, which is thought to act as a trigger for the initiation of forward movement [19,20,25]. The second activation is capacitation, which occurs in the isthmus of the uterine tube and sperm motility is further stimulated, so called hyperactivation [1,31]. Only sperm that have undergone the process of capacitation can perform the acrosome reaction at the ovum and may potentially fertilize it. The induction of capacitation has been shown to involve complicated reactions, including the detachment of cholesterol from the sperm cell membrane, influx of intracellular HCO_3^- and Ca^{2+} [2,29], an increase in pH inside the sperm [33], an increase in cAMP levels [6,21], reactive oxygen species (ROS) production [9,22,23], and phosphorylation of protein tyrosine residues [5,12,14,28]. A large number of studies have implicated both the ROS production and the tyrosine phosphorylation of functional proteins, in particular, as key factors in capacitation [11,12,13,16,28,32].

We have identified several kinds of proteins that are specifically tyrosine-phosphorylated during capacitation, including AR, and a functional analysis of these proteins has been performed. Although the protein kinase C (PKC)-dependent phosphorylation of AR has been reported [27], we were the first to discover its tyrosine phosphorylation during capacitation in porcine sperm. In addition, it was determined that AR is not a protein expressed by the sperm themselves but taken up into sperm from Sertoli cells during sperm formation and from the epididymal epithelial cells during the maturation process. Moreover, it was found that AR in the particulate fraction are activated by the tyrosine phosphorylation that accompanies capacitation and regulates both ROS levels and capacitation [8]. However, the location of the AR performing these reactions within the sperm remains unclear.

The present study was performed with the aim of determining the localization of AR which was tyrosine-phosphorylated during capacitation in porcine sperm. The mechanisms how tyrosine phosphorylation of AR regulates ROS levels and capacitation were also discussed.

MATERIALS AND METHODS

Animals

All animal experiments performed in the present study were approved by the Animal Experiment Committee of the University of Tsukuba and the Animal Research Committee of the Ibaraki Prefectural University of Health Sciences. Fresh porcine testes and epididymes were purchased from the local slaughter house.

Preparation of cauda epididymal sperm and induction of capacitation

Spermatozoa were collected from the porcine cauda epididymis by perfusing through the ductus epididymidis with air and washed at 20 ºC with Non-cap medium (4.8 mM KCl, 1.2 mM KH_2PO_4, 95 mM NaCl, 2 mM sodium pyruvate, 5.56 mM glucose, pH 7.4) by centrifugation at 400 × *g* for 5 min. The sperm pellets were washed twice with Non-cap medium by the centrifugation as above. When capacitation was induced, Cap medium (2 mM $CaCl_2$, 0.4% BSA, 25 mM $NaHCO_3$ in Non-cap medium) was used for washing the sperm pellets. Only the samples with over 95% of the sperm showing progressive motility were used for the experiments. Sperm capacitation was induced according to the method described in the previous paper [8]. The washed sperm were incubated either in 15 ml of Non-cap medium or in Cap medium (5×10^6 cells/ml) at 37 ºC for 3 h in a 5% CO_2 atmosphere. Capacitated sperm were assessed on the basis of the changes in the chlortetracycline fluorescence staining [34] and the percentage of sperm with hyperactivated motion in all of the motile sperm were calculated by using a computer-assisted sperm analysis (CASA) system (HTM-CEROS; Hamilton Thorne Research, Beverly, MA, USA).

Isolation of sperm fractions and extraction of sperm protein

Fractionation of sperm was performed according to the methods of Okamura & Sugita. [18] with slight modifications. Sperm were separated from incubation buffer by layering 10 ml of semen on 20 ml of 1.3 M sucrose in 0.15 M NaCl and centrifuged at 12,100 × *g* for 70 min at 4 ºC. The sperm pellet was suspended in PBS and washed twice by centrifugation at 12,100 × *g* for 5 min. After washed sperm were resuspended in 10 ml of 5 mM HEPES, pH 7.0, and homogenized by sonication with ULTRA S homogenizer (TAITEC, Saitama, Japan) at the

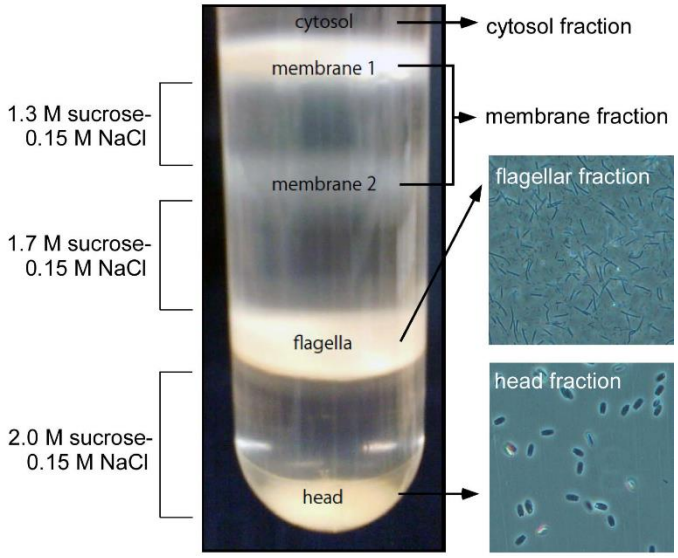

Figure 1. Sperm fractionation by sucrose density-gradient centrifugation. The preparation of cytosol, membrane, flagellar and head fraction from the porcine sperm were determined with described in 'Materials and Methods' section.

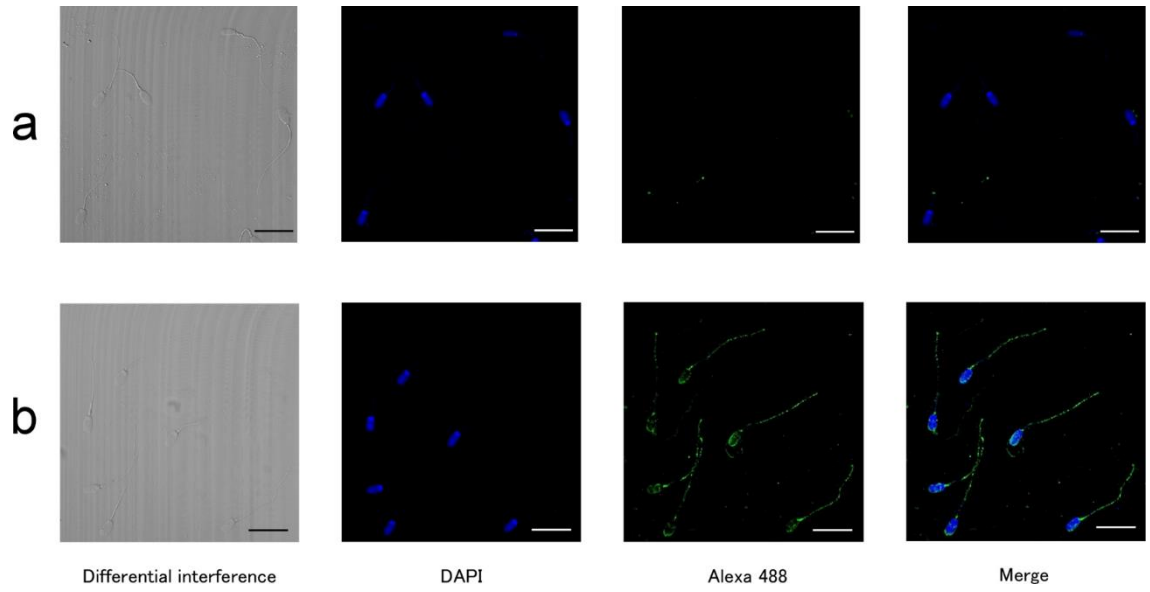

Figure 2. Immunofluorescent staining of AR in porcine cauda epididymal sperm. Localization of AR in the porcine cauda epididymal sperm was analyzed with anti-AR antisera (b). Anti-AR antisera neutralized with antigen were used as a negative control (a). The nuclei were counterstained with DAPI. Bars = 20 μm.

maximum output. Ten milliliter of sperm homogenate were layered on discontinuous gradients consisting of 8 ml of 1.3 M sucrose–0.15 M NaCl, 10 ml of 1.7 M sucrose–0.15 M NaCl, 10 ml of 2.0 M sucrose–0.15 M NaCl. The solutions were centrifuged at 100,000 × *g* for 3 h at 4 ºC in Beckman SW 28 rotor. The cytosol fractions were collected from the top layer of each gradient and membrane fractions (plasma membranes and outer acrosomal membranes) were collected from the sample, 1.3 M sucrose and the 1.3 M–1.7M sucrose interface. Flagellar fractions were collected from 1.7 M–2.0 M sucrose interface and head fractions were collected from the precipitates (Fig. 1). Subsequently, membrane, flagellar and head fractions were respectively washed with PBS by centrifugation, resuspended in the extraction buffer (1% Trition-X 100, 20 mM Tris-HCl, 2.5% protease inhibitor cocktail, 0.25% phosphatase inhibitor cocktail, pH 7.4), and homogenized by sonication with ULTRA S homogenizer at the maximum output. After centrifuged at 105,000 × *g* for 30 min at 4 ºC, each supernatant was collected and stored at –20 ºC until use.

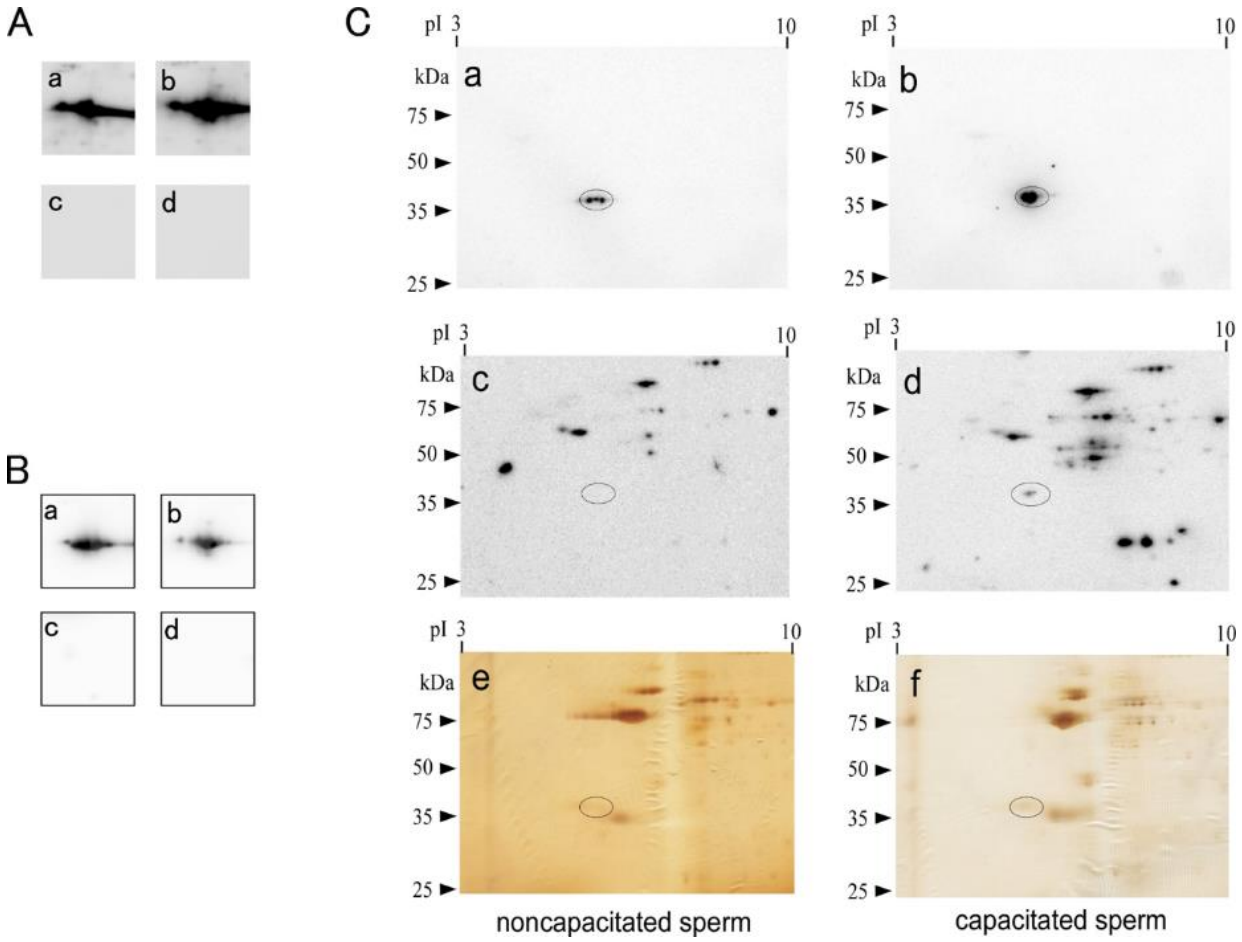

Figure 3. Tyrosine phosphorylation of AR in cytosol, membrane and flagellar fraction of sperm during capacitation. Protein extract of cytosol (A), membrane (B) and flagellar (C) fraction was separated by 2-DE. Western blotting analyses either on AR in the noncapacitated sperm (a) or capacitated sperm (b) or on tyrosine phosphorylated proteins in the noncapacitated sperm (c) or capacitated sperm (d) were done as described in Materials and Methods. Silver staining in the noncapacitated sperm (e) or capacitated sperm (f).

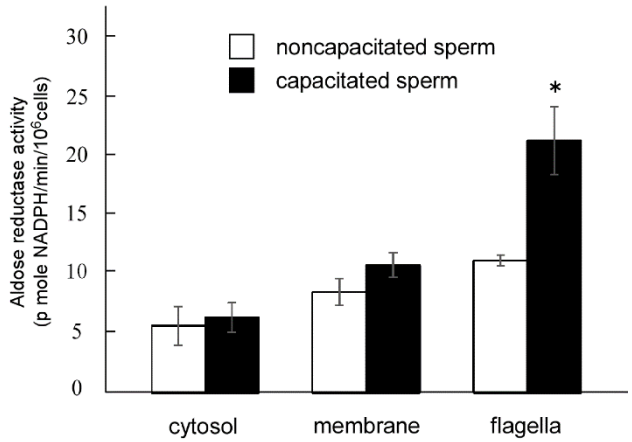

Figure 4. Changes in AR activities of the sperm fractions through capacitation. The AR activities of cytosol fraction, membrane fraction and flagellar fraction from the noncapacitated sperm (open bar) or capacitated sperm (closed bar) were determined as described in 'Materials and Methods' section. Data are expressed as mean ± S.E.M. from three determinations. *P < 0.05.

Immunocytochemical analysis of porcine sperm

The capacitated and noncapacitated sperm (5.0×10^7 cells) were washed twice with PBS by centrifugation at 400 × g for 5 min. Sperm were fixed on MAS coat slide glass with 4% paraformaldehyde for 60 min at room temperature. After washing with PBS, sperm were incubated with 3% H_2O_2 in PBS at room temperature for

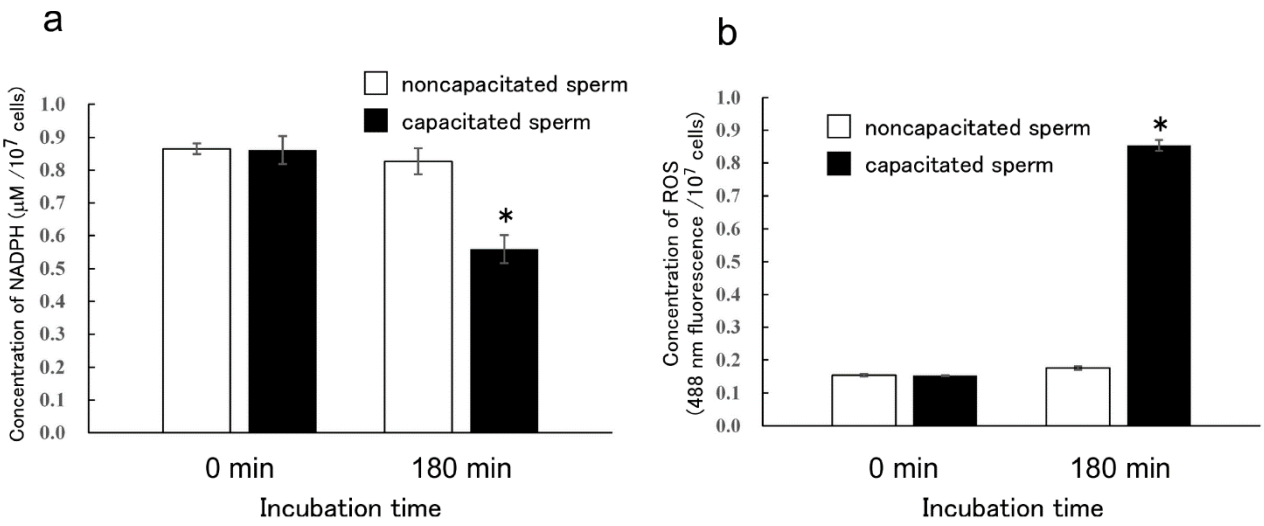

Figure 5. The time-dependent changes in NADPH and ROS levels of porcine sperm during capacitation. The concentration of NADPH (a) and ROS (b) either in noncapacitated sperm (open bar) or in capacitated sperm (closed bar) were determined as described in 'Materials and Methods' section. Data are expressed as mean ± S.E.M. from three determinations. *P < 0.05.

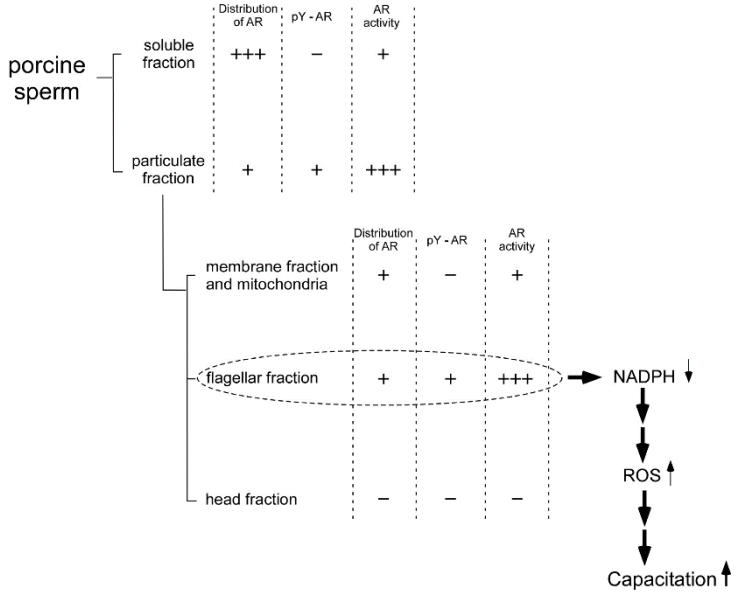

Figure 6. Tyrosine phosphorylation and enzyme activity of the flagellar aldose reductase is involved in the boar sperm capacitation. pY : tyrosine phosphorylation

30 min. The samples were washed with PBS and then blocked with PBS containing 3% BSA for 60 min at 37 ºC. The samples were incubated with anti-AR antisera in PBS at 4 ºC overnight. After washing three times with PBS, the samples were incubated with Goat Anti-Rabbit IgG H&L (Alexa Fluor 488, Abcam plc, Cambridge, UK) for 60 min at 37 ºC. After washing with PBS, nucleus were counterstained with DAPI (NucBlue Fixed Cell Stain ReadyProbesTM reagent, Life technologies). After washing with PBS, the sections were covered with cover glass and observed with a confocal laser scanning microscope (LSM-780, ZEISS, Oberchoken, Germany).

Determination of AR Activity

AR activity was determined according to the method of Katoh et al. [8]. Two hundred microliters of each samples were incubated with 0.15 mM NADPH and 10 mM DL-glyceraldehyde in 120 mM phosphate buffer (pH 6.2) at 20 ºC for 10 min, and the absorbance at 340 nm was monitored.

Determination of ROS and NADPH concentrations

The concentrations of ROS in the sperm were determined according to the method of Katoh et al. [8]. H_2DCFDA (200 μM) was added to the incubation mixture

for the induction of capacitation (2.5×10^8 cells in 5 ml of Cap medium) at 150 min. The mixture was further incubated for 30 min and sperm were collected by centrifugation at 400 × *g* for 5 min. The basal levels of ROS in sperm were determined with Non-Cap medium instead of Cap medium. The sperm pellet was suspended in PBS and washed twice by centrifugation at 400 × *g* for 5 min. The fluorescence of sperm at 530 nm in response to 488 nm excitation was observed under the fluorescence microscope and evaluated using a microplate reader (Varioskan; Thermo Fisher, Yokohama, Japan). The concentration of NADPH in sperm were determined by use of EnzyChrom™ NADP/NADPH Assay Kit. Sperm (1.25×10^8 cells) were collected from the incubation mixture for induction of capacitation at 0, 180 min and washed twice with PBS by centrifugation at 400 × *g* for 5 min. The sperm pellets were incubated with 100 µl of NADPH Extraction Buffer at 60 ºC for 10 min and then 20 µl of Assay Buffer and 100 µl of NADP Extraction Buffer were added. The mixture was briefly vortexed and centrifuged at 12,100 × *g* for 15 min at 4 ºC. Forty microliters of supernatants (2.3×10^7 cells) or 10 µM NADP Standard were incubated with 80 µl aliquots of Working reagent (60 µl of Assay Buffer, 10 µl of 1 M Glucose, 14 µl of formazan [MMT] solution, 1 µl of Enzyme Mix) at room temperature for 30 min and the absorbance at 565 nm was determined.

Statistical Analysis

Data were expressed as the means ± SEMs of at least 3 experiments. Statistical significance was calculated by two-way ANOVA. The difference between two means was determined by t-test, and differences among more than two means were determined by Tukey's test. The general linear models of Statistical Analysis System (SAS Inc., Cary, NC, USA) were used for these analyses. Differences were considered significant at $P < 0.05$.

RESULTS

It is known that AR is principally a soluble and cytosolic protein. In sperm, it has been reported that soluble AR mainly exists in the acrosomes. We have previously discovered that soluble AR is less active than that of the particulate fractions which is activated tyrosine phosphorylation during capacitation. On the other hand, the soluble AR is not phosphorylated at tyrosine residue [8].

Thus, in the present study, the particulate fraction was further fractionated into membrane, flagellar, and head fractions, and both the distribution of AR among those fractions and the levels of the tyrosine phosphorylation were determined by the immunohistochemical analyses, the two-dimensional gel electrophoresis and western blotting. However most AR was found in the cytosol, small amount was also found to localize in the flagellum (Fig. 2). Upon fractionating sperm by sucrose gradient centrifugation AR was found to be present at levels of 70% in the cytosol fraction, 20% in the membrane fraction, and 10% in the flagellar fraction, but was not present in the head fraction (data not shown). In contrast to the AR in the cytosol and membrane fractions, which was not tyrosine-phosphorylated (Fig. 3A, B), AR in the flagella was tyrosine-phosphorylated during capacitation (Fig. 3C). Next, enzyme activity was measured for the cytosol, membrane, and flagellar fractions where AR was localized. In the flagellar fraction, the activity of capacitated sperm was markedly higher than that of noncapacitated sperm. The activity of AR in the cytosol and membrane fractions was the same before and after capacitation and was lower than that in both the noncapacitated and capacitated sperm flagellar fractions (Fig. 4). With the results shown in Fig. 3, these findings strongly imply that increases in AR activity of the particulate fraction during capacitation are due to the tyrosine phosphorylation and activation of AR in the flagella.

AR is the rate-limiting enzyme of the polyol pathway, which produces fructose, a primary energy source for sperm, from glucose, using NADPH as a coenzyme to convert glucose into sorbitol. Consequently, an increase in AR activity by tyrosine phosphorylation is expected to lead to an increased consumption of NADPH to form $NADP^+$. Accordingly, we measured the NADPH levels in Non-cap and Cap sperm and determined that NADPH levels did not change in Non-cap sperm. However, in Cap sperm, NADPH levels were reduced by approximately 35% during capacitation (Fig. 5a). Moreover, although no changes in ROS levels were found in Non-cap sperm, ROS levels increased approximately 5.5-fold in Cap sperm during capacitation (Fig. 5b).

DISCUSSION

It is assumed that proteins expressed and taken up by sperm in specific regions of the epididymis are important in the sperm maturation process. Some of the proteins

produced by the epithelial cells of the ductus epididymidis are released into the lumen of the tubes in vesicles called epididymosomes, and sperm are known to take up these vesicles. The inclusion of AR in epididymosomes has been reported by Sullivan et al. [24] and, combined with our previous research [8], it strongly suggests that AR is incorporated into sperm from the epididymosomes and plays important role in the functional maturation of sperm in the epididymis.

In the present study, it was shown for the first time that the activity of AR in the flagellum is increased by tyrosine phosphorylation, which also leads to a reduction in NADPH levels in the sperm and an increase in ROS levels. From these results, we surmise that AR regulates capacitation via the mechanism shown in Fig. 6. First, AR is tyrosine-phosphorylated, and then the enhancement of its activities promote the utilization of glucose via the polyol pathway. This leads to a reduction in NADPH levels in sperm, impairing the action of the glutathione cycle, which is important for its ROS elimination mechanism, and thus leading to an increase in ROS levels. On the other hand, the promotion of the polyol pathway results in fructose accumulation that causes increased production of advanced glycation end products and also leads to ROS production. It has been found that GSH levels throughout the sperm are reduced by approximately 10% owing to capacitation [8]. Because tyrosine-phosphorylated AR is detected only in the flagellum, it seems likely that GSH levels would be even further reduced when only the flagellum is observed. Given a previous suggestion [7] that glutathione (GSH) levels are reduced to around 30% by freezing and thawing in a capacitation-like reaction called cryocapacitation, the increase in ROS levels found in the present study seems highly likely to derive from a reduction in GSH levels in the sperm. As long as ROS is regulated to an appropriate concentration, it may be an important regulatory factor of various sperm functions including capacitation [16,17]. We have reported that a membrane-permeable AR specific inhibitor, Alrestatin, inhibits the increase in the protein tyrosine phosphorylation, the decrease in the GSH levels, the elevation of ROS levels and the induction of both hyperactivated motility and induction of pattern B of chlortetracycline (CTC) fluorescence staining, all of which occur during capacitation [8]. So, AR is strongly suggested to act as the central role of the regulation of capacitation.

There are proteins, some of them tyrosine-phosphorylated proteins, whose localization in the sperm changes upon capacitation [3,30]. AR is known to be dominantly present in the soluble fraction in sperm. However, its specific activity there is lower than that of AR in the insoluble fractions, such as the flagellar fraction. It was found that this was due to that the flagellar AR was tyrosine-phosphorylated whereas soluble one was not. In the present study, no clear changes in the localization of AR were found to result during capacitation. Whether AR localized to the juxtamembrane region is tyrosine-phosphorylated during capacitation or changes in location from the cytoplasm to the membrane occur as a result of tyrosine phosphorylation will be the subject of future research. In this connection, it is interesting that AR is transferred to the mitochondria due to phosphorylation via protein kinase C stimulation [27].

The tyrosine kinase that phosphorylates AR remains to be identified. The involvement of the SRC family in capacitation in human and rats [4,10,15,16] as well as the involvement of a 34–45 kDa tyrosine kinase in capacitation of boar sperm has been reported. These tyrosine kinases are present in the acrosome, equatorial segment, midpiece, and main flagellar component of the sperm, suggesting that they are intimately involved in the acrosome reaction and hyperactivation [26]. Whether they participate in the phosphorylation of AR is a question of profound interest.

The present analysis of AR, a protein that has been identified to be tyrosine-phosphorylated during capacitation, suggests that sperm have a physiologically relevant ROS-production system that is regulated by intracellular NADPH levels. To date, the production of ROS in the sperm has been assumed to originate mostly in the mitochondria, but the results of the present study can be interpreted as revealing the existence of a new ROS-production system. Although it is clear that sperm functions, including capacitation, are regulated by ROS, the species of ROS involved and their roles await identification. A clear picture of the ROS-mediated system regulating sperm function could lead to the effective control of sperm fertility for infertility treatment, contraception, breeding, and preservation of genetic resources.

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