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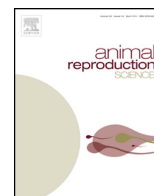

Changes in the distribution and molecular mass of boar sperm acrosome-associated 1 proteins during the acrosome reaction; their validity as indicators for occurrence of the true acrosome reaction

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ABSTRACT

The aims of this study were to investigate changes in the distribution and molecular mass of boar sperm acrosome-associated 1 (SPACA1) proteins during the acrosome reaction and to discuss validity of SPACA1 proteins as indicators for occurrence of the true acrosome reaction. Boar ejaculated spermatozoa were used for induction of the extracellular Ca^{2+}-dependent acrosome reaction (true acrosome reaction) or acrosomal damages (false acrosome reaction) and then subjected to double staining with the anti-SPACA1 protein antibody and FITC-PNA and Western blotting. Extracellular Ca^{2+}-dependently acrosome-reacted spermatozoa were characterized by appearance of SPACA1 proteins in the postacrosomal region (; these spermatozoa were classified into SP-3&AR pattern of double staining). However, SPACA1 proteins were not observed in the postacrosomal region of frozen-thawed spermatozoa with severely damaged acrosomes (; these spermatozoa were classified into SP-2&AR pattern). Moreover, the spermatozoa in which acrosomes were severely damaged by incubation with cyclodextrins and without $CaCl_2$ were classified into either SP-2&AR or SP-3&AR pattern. Although SPACA1 proteins were detected mainly as 36–42 kDa proteins in the spermatozoa with intact acrosomes, small types of SPACA1 proteins (15–28 kDa) increased in extracellular Ca^{2+}-dependently acrosome-reacted spermatozoa as well as frozen-thawed spermatozoa with damaged acrosomes. These results show the increase of boar spermatozoa classified into SP-3&AR pattern after incubation in the medium with $CaCl_2$ and without cyclodextrins indicates occurrence of the true acrosome reaction. Moreover, we suggest the increase of small types of SPACA1 proteins is a valid indicator for occurrence of the acrosomal disintegration arising from the true and false acrosome reactions.

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1. Introduction

Mammalian spermatozoa undergo a variety of physiological and biochemical modifications during their transit through female reproductive tract (Visconti, 2009; Aitken and Nixon, 2013; Harayama, 2013). These changes are collectively termed “capacitation” and regulated by the sperm

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interaction with female reproductive tract and its luminal fluid (Brüssow et al., 2014; Fujinoki and Takei, 2015; García-Vázquez et al., 2015; Tienthai, 2015; Kuo et al., 2016). Subsequently, fully capacitated spermatozoa are capable of undergoing hyperactivation in the flagella and acrosome reaction in the head (Harayama, 2013; Mizuno et al., 2015; Isono et al., 2016). The hyperactivation and acrosome reaction are indispensable for successful sperm fertilization with oocytes in the ampulla of oviducts and triggered by the rapid increases of intracellular Ca^{2+} level via the influx of extracellular Ca^{2+} in the flagellum and head, respectively (Visconti et al., 2011; Harayama et al., 2012; Kojima et al., 2015; Tomes, 2015; Lucchesi et al., 2016).

The sperm acrosome reaction is an extracellular Ca^{2+}-dependent exocytosis of acrosomal contents and morphologically characterized under the electron microscope by partial fusions between plasma membrane and outer acrosomal membrane at the multiple parts (Yanagimachi, 1994). In the experiments in which a number of samples are examined, however, the acrosome reaction is actually assessed by the observation of the stained spermatozoa (Talbot and Chacon, 1981; Mortimer et al., 1987; Didion et al., 1989; Larson and Miller, 1999) or the spermatozoa with exogenous fluorescent acrosomal proteins (Nakanishi et al., 1999) under the optical microscope or fluorescent microscope. The morphological changes during the acrosome reaction (which include shedding of the acrosome from the sperm head) are detectable as the acrosomal disintegration and loss under these microscopes.

The enzymatic contents released from the acrosome (for instance, acrosin, testisin and hyaluronidase) are involved in the digestion of cumulus oophorus and extracellular matrix of oocytes (Kimura et al., 2009; Kawano et al., 2010; Zhou et al., 2012). For several decades, it had been believed that the interaction between capacitated spermatozoa and zona pellucida was important for initiation of the acrosome reaction (Bleil and Wassarman, 1983). However, it was lately investigated that most of mouse capacitated spermatozoa began to undergo the acrosome reaction before their attachment to the zona pellucida (Jin et al., 2011), specifically in the upper isthmus of the oviduct (La Spina et al., 2016; Muro et al., 2016). Therefore, it is preferable to re-consider how mammalian spermatozoa initiate the acrosome reaction after ejaculation into female reproductive tract.

In the researches on the acrosome reaction of bull and boar spermatozoa, we previously used several kinds of staining techniques which evaluated solely acrosome morphology [*e.g.* Giemsa staining (Mizuno et al., 2015; Isono et al., 2016; Harayama et al., 2004b) and fluorescein isothiocyanate (FITC)-peanut agglutinin (PNA) staining (Fukuda et al., 2016)] or both acrosome morphology and sperm viability [*e.g.* triple staining with trypan blue, Bismarck brown and rose Bengal (Harayama et al., 1993)] under the optical microscope or fluorescent microscope. In these experiments, however, it was necessary for us to consider carefully the methodological limitation of the abovementioned staining techniques in the ability to distinguish the true acrosome reaction (extracellular Ca^{2+}-dependent acrosome reaction) and false acrosome reaction (acrosomal damages and loss). This demands that we should improve the staining techniques of the spermatozoa for the detection of the true acrosome reaction.

Sperm acrosome-associated 1 (SPACA1) proteins were discovered as membrane antigens of human spermatozoa and localized in the acrosomal principal and equatorial segments (Hao et al., 2002; Kishida et al., 2016). These acrosomal proteins might be involved in the sperm fusion with the oolemma, since the treatment with the anti-SPACA1 antibody prevented human spermatozoa from penetrating into zona-free hamster eggs (Hao et al., 2002). Distribution of SPACA1 proteins in the acrosomal principal segment are largely varied among spermatozoa from human (Kishida et al., 2016) and cattle (Harayama et al., 2010). Moreover, phenotypic analyses of *Spaca1*-disrupted mice demonstrated that Spaca1 proteins are indispensable for normal shaping of mouse sperm heads during the spermiogenesis (Fujihara et al., 2012). However, only limited reports were available on SPACA1 proteins in boar spermatozoa (Jones et al., 2008). The aims of this study were to investigate changes in the distribution and molecular mass of boar SPACA1 proteins during the acrosome reaction and to discuss validity of SPACA1 proteins as complementary indicators for occurrence of the true acrosome reaction (extracellular Ca^{2+}-dependent acrosome reaction) in boar spermatozoa.

2. Materials and methods

2.1. An animal use ethics statement

The Institutional Animal Care and Use Committee of Kobe University (Permission number: 24-03-10) approved the procedures of this study which were carried out according to the Guidelines of Animal Experiments.

2.2. Reagents and preparation of samples

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

Sperm-rich fractions from the ejaculates were obtained from mature boars (one Meishan pig and one Large White pig, >12 months old) by a manual method and then diluted with approximately two-fold volume of the tris-citric acid-glucose (TCG) solution (111.0 mM tris[hydroxymethyl]aminomethane, 34.7 mM citric acid and 185.0 mM glucose) (Kaneto et al., 2002). All of the samples were transported at 25–30 °C within 2 h to our laboratory. Samples from the Large White pig were kind gifts from General Technological Center of Hyogo Prefecture for Agriculture, Forest and Fishery. The spermatozoa were immediately washed in an isotonic Percoll [2 ml 90% (v/v) and 5 ml 45% (v/v), GE Healthcare UK Ltd., Buckinghamshire, UK] and then in a phosphate-buffered saline (PBS) containing 0.1% (w/v) polyvinyl alcohol (PVA, Sigma-Aldrich Co., St. Louis, MO, USA) (PVA-PBS) by centrifugation as described previously (Harayama et al., 2004a,b).

2.3. Sperm treatments

In this study, washed spermatozoa were subjected to three kinds of treatments which were for the induction

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