

HYALURONAN IN THE SOW'S OVIDUCT: ITS EFFECT ON BOAR SPERM MORPHOLOGY AND FUNCTION

Paisan Tienthai*

Abstract

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During sperm transport in the female pig, a proportion of the spermatozoa is arrested at the uterotubal junction and the isthmus, where an oviductal sperm reservoir is built up. The sperm reservoir maintains sperm viability and controls the release of fertilizing spermatozoa so that only a small subpopulation reaches the site of fertilization, thus diminishing the risk of polyspermy. *In vitro* research has focused on sperm binding as the main mechanism of sperm storage, sperm release and modulation of capacitation, but little attention has been paid to the sperm reservoir fluid, its composition and the control of capacitation *in vivo*. This review provides information about the characteristics of the oviductal sperm reservoir during the estrous cycle and its main luminal content which is composed of hyaluronan (HA) and includes the effects of HA on porcine spermatozoa. In inseminated sows, the spermatozoa are entrapped in a mucus-like oviductal fluid from the pre-ovulation UTJ and the adjacent isthmus. This fluid contains HA which is synthesized in the epithelium by HA synthase-3. The HA receptor “CD44” is particularly concentrated in the deep furrows of the sperm reservoir where most spermatozoa are trapped. Massive sperm capacitation does not occur in the sperm reservoir during pre-ovulation and peri-ovulation *in vivo*. However, the capacitation of spermatozoa in the sperm reservoir is increased post-ovulation, suggesting a role for HA in modulating sperm capacitation in pigs. The findings support the concept that the sperm reservoir keeps the potentially fertile spermatozoa viable and uncapacitated during their pre-ovulatory arrest and this data may help improve sperm preparation protocols for porcine *in vitro* fertilization (IVF) and the preservation of boar semen.

Keywords : Pig, oviduct, spermatozoa, hyaluronan

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บทคัดย่อ

ไพศาล เทียนไทย

ไฮยาโลโรแนนในท่อนำไข่สุกร: ผลกระทบต่อโครงสร้างและหน้าที่ของตัวอสุจิสุกร

ในระหว่างที่ตัวอสุจิของสุกรเคลื่อนที่เข้าไปในท่อนำไข่บริเวณคั่นของสุกรเพศเมีย ตัวอสุจิจำนวนหนึ่งไปรวมกันอยู่บริเวณรอยต่อระหว่างปีกมดลูกกับท่อนำไข่ซึ่งเป็นบริเวณที่กักเก็บตัวอสุจิ มีหน้าที่รักษาตัวอสุจิให้มีชีวิตรอดและควบคุมการปล่อยตัวอสุจิที่พร้อมสำหรับการปฏิสนธิในจำนวนที่เหมาะสมไปยังบริเวณที่มีการปฏิสนธิ เพื่อลดอุบัติการณ์การปฏิสนธิของตัวอสุจิหลายตัวต่อโอโอไซต์หนึ่งใบ การศึกษาวิจัยเรื่องท่อนำไข่ภายนอกร่างกายสัตว์ส่วนใหญ่มุ่งเน้นเกี่ยวกับการยึดเกาะของตัวอสุจิกับเยื่อท่อนำไข่ ซึ่งยึดถือเป็นกลไกหลักของการกักเก็บตัวอสุจิ การปล่อยตัวอสุจิ และการคาปาซิเตชันของตัวอสุจิ ขณะที่การวิจัยเกี่ยวกับองค์ประกอบของของเหลวที่ผลิตจากท่อนำไข่บริเวณที่กักเก็บตัวอสุจิรวมถึงการคาปาซิเตชันของตัวอสุจิในสัตว์มีชีวิตยังมีอยู่น้อยมาก ดังนั้นบทความปริทัศน์นี้จึงได้รวบรวมข้อมูลเกี่ยวกับลักษณะของบริเวณที่กักเก็บตัวอสุจิของสุกรที่เปลี่ยนแปลงในวงจรการเป็นสัด และของเหลวภายในซึ่งประกอบไปด้วยไฮยาโลโรแนนเป็นส่วนใหญ่ รวมทั้งผลกระทบของไฮยาโลโรแนนต่อตัวอสุจิของสุกร จากการศึกษาในแม่สุกรหลังการผสมพันธุ์ พบว่า ตัวอสุจิในบริเวณที่กักเก็บตัวอสุจิ ถูกดักจับไว้โดยของเหลวคล้ายเมือกเหนียวภายในส่วนรอยต่อของปีกมดลูกกับท่อนำไข่ส่วนอิสมัส ซึ่งของเหลวนี้ประกอบด้วยไฮยาโลโรแนนที่สังเคราะห์ได้จากเซลล์เยื่อท่อนำไข่ โดยไฮยาโลโรแนนชนิด-3 การปรากฏของ CD44 พบได้อย่างเด่นชัดที่เยื่อของบริเวณที่กักเก็บตัวอสุจิซึ่งจัดตัวเป็นร่องลึก การคาปาซิเตชันของตัวอสุจิส่วนใหญ่ในบริเวณนี้ยังไม่เกิดขึ้นในระยะก่อนตกไข่และระยะตกไข่ อย่างไรก็ตาม ตัวอสุจิในบริเวณนี้จะเกิดการคาปาซิเตชันเพิ่มขึ้นในระยะหลังตกไข่ บ่งชี้ว่า ไฮยาโลโรแนนเป็นสารที่ช่วยปรับเปลี่ยนโครงสร้างของตัวอสุจิเพื่อนำไปสู่จุดเริ่มของกระบวนการคาปาซิเตชันในสุกร รวมทั้งสนับสนุนว่าท่อนำไข่บริเวณที่กักเก็บตัวอสุจิรักษาการมีชีวิตรอดและคงสภาพของตัวอสุจิให้สมบูรณ์โดยควบคุมไม่ให้เกิดการคาปาซิเตชันในระยะก่อนตกไข่ ซึ่งข้อมูลเหล่านี้สามารถนำไปปรับปรุงขั้นตอนในการเตรียมตัวอสุจิของสุกรในกระบวนการปฏิสนธินอกอวัยวะและการเก็บรักษาน้ำเชื้อของพ่อสุกรได้เป็นอย่างดี

คำสำคัญ: สุกร ท่อนำไข่ ตัวอสุจิ ไฮยาโลโรแนน

Introduction

Porcine fertilization depends on several factors, including proper timing to allow mating or artificial insemination (AI) to be successful. In the latter, the prediction of the precise moment of ovulation and the use of semen with a defined quantity and quality of spermatozoa are of the utmost importance. Today, the pig AI industry has grown tremendously and the preservation of semen in proper extenders maintains sperm viability and fertilizing ability through the semen-processing procedures, until the dose is deposited in the female tract (Flowers and Esbenshade, 1993). The semen extenders should allow the processing of the desired number of spermatozoa towards reversible immobilization of the sperm function.

The *in vitro* production (maturation, fertilization and culture) of viable porcine embryos is possible and progressive, although with a much lower success rates than in cattle. The main problems are an insufficient cytoplasmic maturation of the porcine oocytes and a high proportion of polyspermic fertilization (Wang et al., 1991). A better proper type of *in vitro* fertilization (IVF) medium and certain modifications are essential to reduce polyspermy. Therefore, the studies on sperm function and their environment *in vivo*, particularly in the oviduct, may improve the understanding about the characteristics of spermatozoa, which can provide a more suitable IVF medium.

Billions of boar spermatozoa are delivered to the female during mating. Only a few hundred thousand are trapped for 36-40 hours in a sperm reservoir, at the uterotubal junction (UTJ) and the adjacent isthmus, awaiting ovulation and released in very small numbers towards the ampullary-isthmic junction (AIJ) to fertilize the oocytes (Hunter, 1981). The sperm reservoir has a very narrow lumen and contains mucous intraluminal fluid in which spermatozoa are trapped (Hunter et al., 1991; Suarez et al., 1997). Basically, most spermatozoa in the sperm reservoir maintain sperm viability, including the ability to fertilize, escaping the attacks of the female immune system (Rodriguez-Martinez et al., 1998).

The intraluminal fluid in the mammalian female genital tract, including that of the pig, contains substances that can affect sperm function, among these are glycosaminoglycans (GAGs). Hyaluronan (HA), the only one member of non-sulfated GAGs, has been related to the sperm capacitation by its interaction with the sperm plasma membrane, aiding the reduction of polyspermy and even the regulation of fertilization in several species (Rodriguez-Martinez et al., 1998). If HA and its receptor are present in the sperm reservoir of the female pig, these substances could be interesting clues to understanding some of the key processes during semen preservation, including their role in the preservation of sperm viability. The aims of the present review are to provide an overview of the pig sperm reservoir, with special reference to the HA and its effect on sperm function, particularly *in vivo* sperm capacitation.

General aspects of the spermatozoa

Spermatozoa are peculiar cells which undergo several important maturation steps throughout their life and are equipped with the ability to interact with the different environments through which they migrate (Bedford, 1974). However, these interactions modify the capability of the spermatozoa to accomplish their functions (de Lamirande et al., 1997). A mature spermatozoa has three specialized regions; the sperm head with the acrosome and the

DNA-containing, sperm nucleus, vital for zona pellucida penetration, completion of fertilization and zygote formation; the neck, which hosts the centriole required for mitosis of the zygote and the flagellum-cored tail, which displays a characteristic motility and nurtured by the energy provided by the mitochondria surrounding the mid-piece (Yanagimachi, 1994). The spermatozoon is covered by a dynamic plasma membrane responsible for all interactions with the surrounding environments. The structure of the sperm plasma membrane suffers modifications during sperm transport through the ductus epididymis, where sperm maturation occurs (Hammerstedt et al., 1982), but modifications also occur during the sperm transport through the female genital tract, including its interaction with the oocyte and its vestments (Flesh and Gadella, 2000). These modifications of the sperm plasma membrane are required for fulfillment of the sperm's function. Mammalian spermatozoa are unable to fertilize oocyte unless they reside in the female genital tract for a specific period of time and undergo capacitation, which primes them to elicit the acrosome reaction when reaching the zona pellucida of the ovum (Chang, 1951).

Characteristics and specific mechanisms of the oviductal sperm reservoir

The porcine sperm reservoir is localized in the UTJ and the adjacent isthmus (Viring et al., 1980), where most spermatozoa, present in the crypts of this region, show normal ultrastructure during the long pre-ovulation estrous period (Mburu et al., 1997). There are different reasons for this both before and after insemination. Firstly, the sperm reservoir is the most immediate portion of the oviduct they encounter (Lefebvre et al., 1995) and its narrow, tortuous lumen becomes even narrower during estrus (Flechon and Hunter, 1981), perhaps due to the edema of the lamina propria and promoted by the high levels of estrogens that dominate the proestrus to estrus period (Hunter, 2002). Although this mechanical action may arrest spermatozoa during the initial phase of transport, other mechanisms have been postulated for the establishment of the the sperm

reservoir. The membrane-bound localization of the enzyme carbonic anhydrase in the secretory cells of the sperm reservoir (Rodriguez-Martinez et al., 1991), which is similar to that in the cauda epididymis of the boar (Ekstedt et al., 1991), suggests that the medium in which spermatozoa bathe may have an electrolyte and acid-base status that could depress sperm motility, arresting the cells at this location (Harrison et al., 1996). There is also an increasing temperature gradient from the isthmus to the ampulla, which Hunter and Nichol (1996) related to a decrease in sperm motility, in this particular oviductal segment. In addition, binding of spermatozoa to the epithelium lining has been reported and associated with the formation of the sperm reservoir (Hunter et al., 1991). Finally, the presence of intraluminal mucus has been described in the isthmus of human (Jansen, 1978), rabbit (Jansen and Bajpai, 1982), bovine (Suarez et al., 1997) and porcine (Johansson et al., 2000; Tienthai et al., 2000) oviducts before ovulation, and therefore has to be included among the factors that could participate in the pre-ovulatory arrest of spermatozoa and the formation of the *in vivo* sperm reservoir (DeMott et al., 1995).

The prevailing conditions occurring in the sperm reservoir could suppress sperm metabolism and consequently motility (Smith, 1998), and lead to a certain degree of sperm quiescence, even in terms of the sperm's ability to survive in this location (Hunter, 2002). Spermatozoa incubated *in vitro* with oviductal epithelial cells have been shown to remain viable longer than when they are incubated in transport medium alone (Ellington et al., 1993), suggesting that the epithelium and the fluid it produces, preserves sperm viability. Co-incubation of spermatozoa with membrane vesicles, prepared from the apical membranes of oviductal epithelial cells, has been reported to maintain low intracellular Ca^{2+} levels and extended sperm viability (Murray and Smith, 1997). Oviductal fluid collected *in situ* (Nichol et al., 1997), or conditioned medium from cultured oviductal epithelium (Kim et al., 1997) has been seen to maintain sperm viability. Subsequently, the mechanisms that modulate

the survival of spermatozoa in the sperm reservoir may also arise from substances contained in the intraluminal fluid.

General properties of hyaluronan and its involvement in the reproductive system

The viscous, mucus-like material present in the lumen of the lower oviduct of various mammalian species consists of a variety of glycoproteins (Jansen and Bajpai, 1982; 1983; Suarez et al., 1997) and GAGs (Lee and Ax, 1984). There are two main groups of GAGs, the sulfate GAGs, comprising keratin sulfate, heparin, heparan sulfate, chondroitin/dermatan sulfate, and the non-sulfate GAG, i.e. hyaluronan (Goodman, 1997). Almost 70 years ago Meyer and Palmer (1934) described a high molecular weight polysaccharide that was called hyaluronic acid. Later, the polysaccharide was named hyaluronan (HA) since it is not acting as an acid at neutral pH. HA is synthesized in the interior of the plasma membrane by the addition of alternating N-acetylglucosamine and D-glucuronic acid groups, combined with $\beta(1-4)$ and $\beta(1-3)$ linkages (Fig. 1). HA is a large polymer with an average length of approximately 25,000 disaccharide units, with a relative molecular weight 4×10^6 kDa (Fessler and Fessler, 1966) and can form continuous networks and is therefore highly viscous (Toole, 2002). An example of this in a mammalian species is the HA-dominated cloud that is built by the mature cumulus-oocyte complexes (COCs) (Salustri et al., 1989). Basically, HA is synthesized by trans-membrane enzymes, so-called hyaluronan synthase (Weigel et al., 1997), and interacts with the surface of cells, via receptors, such as CD44, which are present on most epithelial cells (Alho and Underhill, 1989) and also with cumulus cells (Yokoo et al., 2002). HA has been localized in the female reproductive tract of rats (Laurent et al., 1995) and humans (Edelstam et al., 1991) and detected in the cervical mucus, uterine and oviductal secretions of ruminants (Lee and Ax, 1984).

Regulations and functions of hyaluronan in the oviductal sperm reservoir

Basically, the mammalian oviductal fluid is a complex mixture derived from blood plasma via selective transudation (Oliphant et al., 1978) and by secretion of specific products by the epithelium (Leese et al., 2001). The pig oviductal epithelium is composed of ciliated and non-ciliated secretory cells. The secretory cells, which are in relatively high concentration in the isthmus area, compared with the upper segments (Johansson et al., 2000), undergo a cycle of hypertrophy and atrophy throughout the estrous cycle (Iritani et al., 1974). The oviductal fluid is able to influence, both *in vitro* and *in vivo*, sperm survival (Zhu et al., 1994), sperm motility (Grippio et al., 1995) and sperm capacitation (Parrish et al., 1989), which supports previous suggestions about the role of the sperm reservoir-fluid in modulating sperm viability. Tienthai et al. (2000) reported that the pig oviductal fluid was slowly collected from late proestrus to metestrus and non-sulfated GAGs (HA) were quantified (Fig. 2). The relative concentrations of HA tended to be higher during standing estrus. HA was specifically present on the epithelial surface of the UTJ and the adjacent isthmus (Fig. 3), particularly before ovulation, and was detected by immunohistochemistry (Tienthai et al., 2000). Therefore, the existence of HA could be related, albeit circumstantially, to the presence of the mucus-like material reported by Johansson et al. (2000). In addition, porcine epithelium can synthesize HA via hyaluronan synthase-3 (Tienthai et al., 2003a). HA is highly hydrophilic and has the ability to form gels even at very low concentrations (Scott, 1999). Being strongly negatively charged, HA attracts cations, becomes osmotically active and captures water in high amounts, which in turn, increases its viscosity and resilience. Such conditions would be important for the arrest of spermatozoa while they colonize the sperm reservoir. The sperm reservoir could use the HA to build a mesh-like structure (Laurent and Fraser, 1992) or even form gel to prevent the uterine and ampullar fluids from passing through the sperm reservoir during the

pre-ovulatory interval, thus keeping spermatozoa isolated, as suggested by Hunter (2002). Furthermore, HA, owing to its highly conserved structure and absence of antigenicity, has been implicated in the masking of cells (Knudson and Knudson, 1999). It can be speculated that spermatozoa immersed in the HA-rich oviductal mucus of the sperm reservoir might escape recognition by the female immune system (Rodriguez-Martinez et al., 1998).

Around cells, HA usually interacts with the proteoglycans. This combination of GAGs builds a stable jelly in the extracellular matrix (Scott, 1999). Although HA exists in freely mobilized compartments, which could include the sperm reservoir luminal contents, it is usually firmly bound in specific associations with cells or binding proteins (Toole, 1999). HA interacts with the surfaces of cells via receptors (trans-membrane glycoproteins) which are present in most epithelial cells, including the oviduct of some species (Alho and Underhill, 1989). The main HA receptor (CD44) is conspicuously present on the membrane surface and the supranuclear domain (Fig. 3) of the porcine sperm reservoir epithelium (Tienthai et al., 2003b). CD44 may anchor the HA in the sperm reservoir and thus keep in place the viscous cloud of GAGs, in which spermatozoa are embedded in during pre-ovulation.

Boar spermatozoa and their interaction with hyaluronan in sperm reservoir

Sperm capacitation is a step-wise process, which occurs as a consequence of the re-organization and modification of sperm surface molecules, including the removal of cholesterol, and/or epididymal and seminal plasma proteins, adsorbed during ejaculation. These events are accompanied by lipid scrambling and regionalised lipid diffusion in the plasma membrane, which ends with the destabilisation of its architecture (Yanagimachi, 1994). The chemical modifications associated with capacitation occur without obvious morphological changes but some can be indirectly visualized by incubating sperm with the fluorescent antibiotic, chlortetracycline (CTC-assay) (Wang et al., 1995). The CTC-assay shows the displacement of

Ca²⁺-containing proteins in the sperm head plasma membrane, which occurs during the later part of capacitation and participates in the acrosome reaction (Abeydeera et al., 1997). The early stages of sperm capacitation can also be measured by loading spermatozoa with the fluorescent lipid dye Merocyanine-540. A higher degree of lipid disorder in the plasma membrane is indicative of the beginning of capacitation (Harrison et al., 1996).

Sperm capacitation is thought to occur in the sperm reservoir (Lefebvre and Suarez, 1996). Attachment to oviductal epithelial cells extends the life of spermatozoa and those spermatozoa that capacitate in the sperm reservoir are able to both release from the epithelial binding (Fazeli et al., 1999) and propel themselves by concomitantly developed, hyperactivated flagellar beating (Suarez, 1996). This release of spermatozoa from the pig sperm reservoir is thought to result from an endocrine signal (Hunter, 1995). Tienthai et al. (2004) indicated that a major sub-population of spermatozoa flushed from the sperm reservoir at various stages of standing estrus were viable and uncapacitated (69-73%). These findings are in agreement with previous work in which 63-74% of the spermatozoa flushed from the UTJ showed an intact plasma membrane (Mburu et al., 1996) and spermatozoa in the crypts of the UTJ revealed normal ultrastructure (Mburu et al., 1997). In any case, spermatozoa residing in the porcine pre-ovulatory sperm reservoir did not show, *in vivo*, the destabilizing changes in membrane lipids that define the first stages of capacitation (Harrison et al., 1993). In summary, the pig pre-ovulatory sperm reservoir does not promote sperm capacitation, but extends the viability and fertilizing capacity of the stored spermatozoa for most of the estrous period (Rodriguez-Martinez et al., 2001). Under conditions of routine IVF and at doses of 500 µg/ml, HA has been shown to elicit sperm capacitation of thawed-frozen boar spermatozoa but without any acrosome reaction (Suzuki et al., 2002). The addition of the same concentrations of HA to spermatozoa collected from the pig sperm reservoir and incubated in mBO medium with

bicarbonate, showed that sperm viability was maintained at the uncapacitated level (Tienthai et al., 2004). HA in the fluid and on the epithelial lining (Tienthai et al., 2000) may act as a cyto-protective reagent to preserve these spermatozoa until the surroundings change after ovulation.

Boar spermatozoa have been shown to progress sequentially from the sperm reservoir to the site of fertilization at the AIJ, in relation to the time of ovulation (Mburu et al., 1996; 1997). This progression is not immediate, although it may occur under the influence of ovarian hormones stimulating the oviductal epithelium and musculature (Hunter, 1995), for it does not result in a massive release and subsequent bulk migration of spermatozoa from the sperm reservoir. After ovulation, the luminal size of the pig sperm reservoir increases while the content of mucus-like material decreases, as described by Johansson et al. (2000). Also, the relative intensity of HA localization and its fluid level decrease postovulation compared with preovulation (Tienthai et al., 2000). Taken together, these changes may facilitate the progression of spermatozoa from the sperm reservoir. However, large numbers of spermatozoa are still found in the sperm reservoir even after ovulation, and most of them were uncapacitated (Tienthai et al., 2004). This indicates that sperm release from the sperm reservoir occurs in restricted numbers, which is necessary to avoid the occurrence of polyspermy. Spermatozoa in small numbers would leave the sperm reservoir either because the fluidity of the intra-luminal material increases and the lumen becomes wider or the contractility of the myosalpinx aids sperm progression (Fazeli et al., 1999), because capacitation allows their release from their binding to the epithelium.

Conclusion

HA is found in the oviductal fluid and on the epithelium, conspicuously in the deep furrows of the sperm reservoir during pre-ovulation and in relationship to stored spermatozoa. In addition, main HA receptor, CD44, is expressed by the oviductal epithelium and is particularly abundant in the sperm reservoir during pre-ovulation,

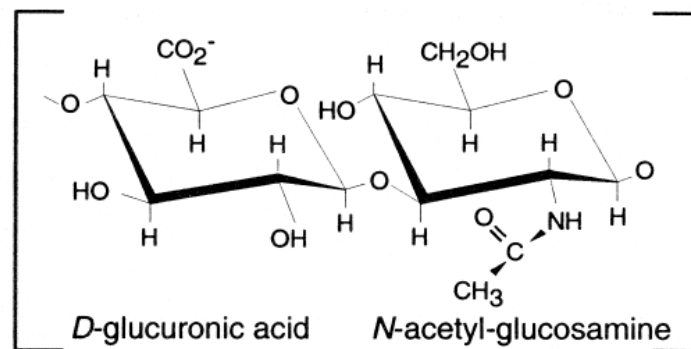


Figure 1 Structure of the repeating disaccharides of hyaluronan

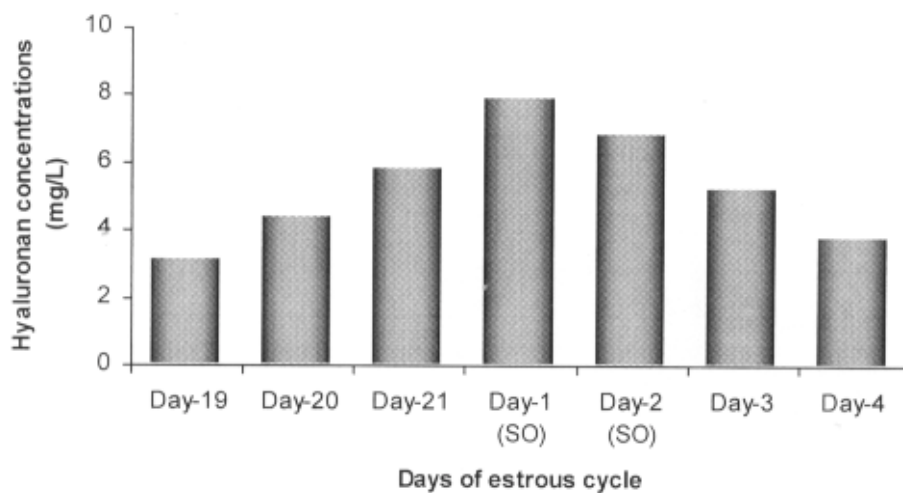


Figure 2 Relative mean daily concentrations of hyaluronan in porcine isthmic oviductal fluid collected via indwelling catheters between proestrus and metestrus (n=5 animals). SO: standing estrus.

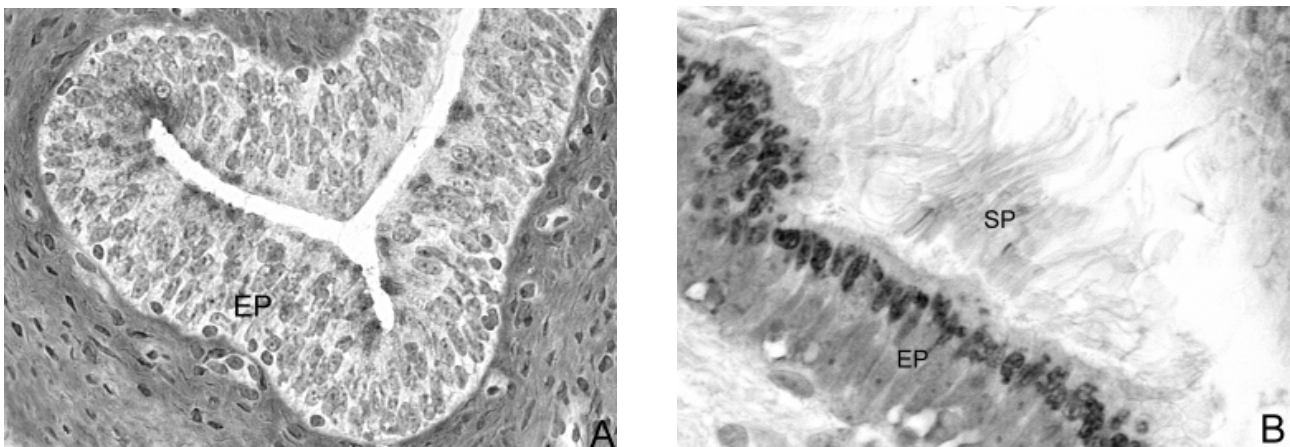


Figure 3 Histochemical staining for hyaluronan (A, x 20) and immunohistochemistry for CD44 (B, x 40) in sections of porcine UTJ during pre-ovulation. EP: epithelium, SP: spermatozoa

indicating that the HA-CD44 signaling pathway could play a role during sperm storage. The spermatozoa in the oviductal sperm reservoir *in vivo* are both viable and uncapacitated during pre- and peri-ovulation, supporting the role of HA in the sperm reservoir fluid in arresting sperm capacitation during pre- and peri-ovulatory periods.

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