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—Review—

The porcine sperm reservoir in relation to the function of hyaluronan

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Abstract. The oviduct plays a role in successful animal reproduction not only in spermatozoa and ova transport to the fertilization site but also by affording a microenvironment for fertilization and early embryonic development. The sperm reservoir (SR) is restricted in the uterotubal junction (UTJ) and caudal isthmus. Billions of porcine spermatozoa are distributed to the female reproductive tract during/after insemination, and small amounts of them are stored for about 36–40 hours in the SR, which maintains sperm viability in the pre-ovulation period through its surface epithelium and production of fluid. The SR regulates the release of spermatozoa so that only a small population moves towards the fertilization site (ampulla) to decrease polyspermy. This review attempts to provide information about the structure and function of the porcine SR, its intraluminal content (hyaluronan, HA), and the influences of HA on porcine spermatozoa *in vivo*. In pigs, the spermatozoa are stored in a mucous-like fluid within the UTJ and caudal isthmus in the pre-ovulation period. The oviduct fluid contains sulfated glycosaminoglycans (GAGs) and non-sulfated GAGs, i.e., HA. It is interesting to note that HA is synthesized by hyaluronan synthase-3 (HAS-3), and its receptor, CD44, is found in the epithelium of the porcine SR site. Additionally, sperm capacitation does not occur *in vivo* in the SR during the pre- and peri-ovulation periods, but spermatozoa in the SR will attempt to capacitate if exposed to bicarbonate. However, capacitation in the SR will rise in the post-ovulation period, indicating the role of HA in modulating sperm capacitation after ovulation. All data support the understanding that the porcine SR ensures the viability of fertile spermatozoa and maintains the non-capacitated status during the pre-ovulation period. This basic knowledge about the SR is believed to be useful to advance sperm preparation procedures for *in vitro* fertilization (IVF) and improve the preservation process of porcine semen.

Key words: CD44, Hyaluronan, Morphology, Oviduct, Pig, Sperm reservoir, Viability

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During fertilization in pigs, numerous factors such as the appropriate moment to permit mating and the exact time of ovulation, including the quality and quantity of spermatozoa, are essential to accomplish regulation. Nearly four decades ago, the elementary protocols for porcine artificial insemination (AI) were first implemented [1]. Still, at present, the procedures are being improved. The AI industry in swine farms has developed massively, and suitable extenders for boar semen preservation have been explored to obtain defined spermatozoa for traveling in the female reproductive tract [2]. *In vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of pig embryos were not accomplished until the 1990s, by which time these *in vitro* technologies were raised to a satisfactory level [3]. The biggest difficulties faced in this species are inadequate maturation of oocytes and a high percentage of polyspermy [4]. One solution among the various factors to attain an enhanced outcome is to scrutinize the benefits of supplementation of IVM/IVF media to increase the oocyte maturation rate and reduce the polyspermic fertilization rate, respectively. Consequently, studies involving sperm function and the surrounding microenvironment in the oviduct have contributed to distinguishing sperm, which should enable definite modifications to produce a better IVF medium [5]. During natural mating, billions of porcine spermatozoa are transported to the female, and a few hundred thousand are stored in a sperm reservoir (SR) for at least 36–40 h [6]. The uterotubal junction (UTJ) and posterior part of the isthmus have been confirmed to be the SR location in which the trapped spermatozoa await ovulation and are then unleashed in small amounts to the ampullary-isthmic junction (AIJ) for fertilization [7]. The morphology of the SR is depicted as a very narrow lumen with a sticky intraluminal fluid that is able to confine porcine spermatozoa [8]. Fundamentally, massive numbers of spermatozoa in the SR retain their viability and fertilizing ability as they stay away from assaults by female immune system cells [9]. Studies of the porcine oviduct confirmed that the oviduct fluid influences sperm functions in different manners [10, 11], and the main components of the fluid are glycosaminoglycans (GAGs). Hyaluronan (HA), a non-sulfated GAG, has been reported to modulate sperm capacitation-like alterations and reduce polyspermy by interaction with the sperm plasma membrane [12, 13]. Therefore, the appearance of HA and its association in the porcine SR could be evidence leading to realization of crucial requirements during sperm storage for the preservation of sperm viability and fertilizing ability. This review describes the general morphology and function of the porcine SR with a focus on the presence of HA, its receptors and synthesizing enzymes, including the effect of HA on boar spermatozoa.

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General Aspects and Definite Regulations of the Sperm Reservoir

The SR was first reported in hamsters and rabbits in 1963 [14], and almost two decades later, the specific morphology together with other evidence now suggest that the UTJ and caudal part of the isthmus represent the oviductal SR in pigs [6, 7]. These observations describe how massive numbers of spermatozoa stored in the porcine SR are trapped in the mucosal folds by chemotactic attraction and intraluminal secretion and defended from attack by polymorphonuclear leukocytes. Remarkably, the great amounts of spermatozoa with epithelial interaction, especially in the SR crypts, exhibit an intact plasma membrane (Fig. 1) during the pre-ovulation period of the estrous phase [15]. There have been various explanations for the mechanisms in the porcine SR before and after insemination. Firstly, the SR is the furthermost area of the oviduct that the spermatozoa confront [16], and its convoluted lumen becomes narrowest due to the subepithelial edema stimulated by a high estrogen level during the proestrus to estrus phases [7, 17]. These morphological changes could capture the massive numbers of spermatozoa during the early phase of sperm transport. Secondly, the expression of an enzyme, carbonic anhydrase, in the secretory cells, particularly in the deep grooves of the porcine SR [18], is similar to that in the boar cauda epididymis [19]. These findings imply that the secretory fluid in which spermatozoa is immersed may depict the suitable levels of electrolyte and acid-base status for slowing down sperm motility during storage in the SR [20]. Thirdly, the rise in the temperature gradient from the posterior isthmus to the ampulla is caused by the extent and activity of the vascular and lymphatic supplies in the pre-ovulation period and could also be associated with the reduction of sperm motility in the specific porcine SR [21]. Fourthly, the adherence of boar spermatozoa to the surface epithelium of the oviduct has been shown to be necessary for SR construction [22, 23]. Lastly, the intraluminal mucus-like fluid has been found in the oviduct in the estrus period in different species, such as the human [24], rabbit [25], cow [26] and pig [27, 28]. Therefore, these essential observations have to be incorporated into the factors that would contribute to detention of spermatozoa and establishment of an SR *in vivo* [29]. The principal circumstances taking place in the porcine SR could depress metabolism and subsequently motility during sperm storage [30], leading to a definite status of sperm quiescence for protecting viable spermatozoa and maintaining their capability [17]. In an *in vitro* experiment, spermatozoa attached to oviductal epithelial cells were revealed to remain viable for longer periods compared with a sperm population incubated in a culture medium alone [31], indicating that the oviduct epithelium and the secretory fluid it produces can preserve sperm viability. Furthermore, spermatozoa co-cultured with membrane secretory vesicles from the apical portions of oviduct epithelial cells have been shown to sustain intracellular Ca^{2+} at lower levels and thus prolong sperm viability [32]. Previous studies have also emphasized the achievement of *in situ* intraluminal fluid and cultured medium from oviductal epithelium in the reduction of polyspermic penetration [11, 33]. Consequently, certain mechanisms that modulate suitable sperm functions and maintain sperm survival in the porcine SR might come from component substances enclosed in the oviductal intraluminal fluid.

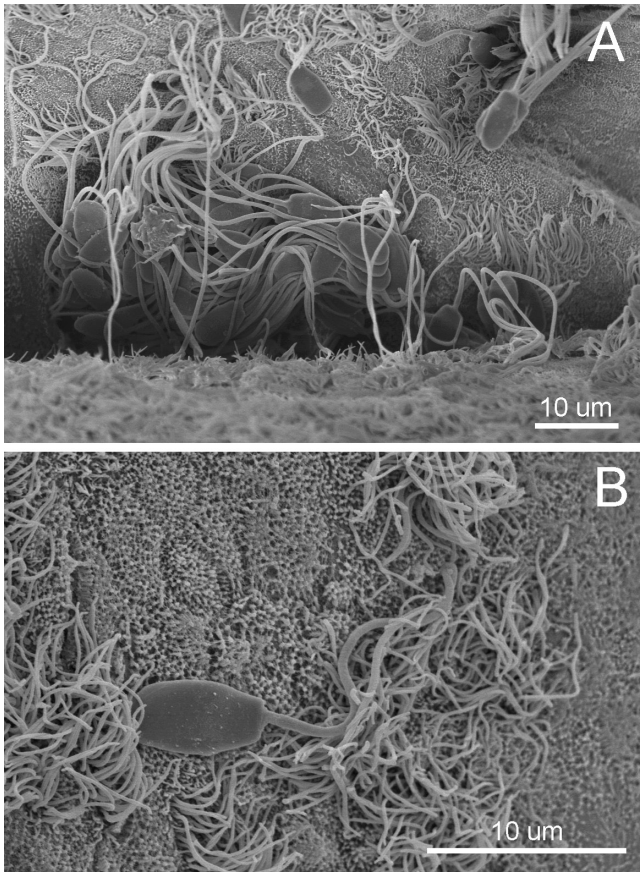

Fig. 1. Representative scanning electron micrographs of the porcine sperm reservoir (in the uterotubal junction or UTJ) during the pre-ovulatory period of the estrous cycle demonstrating (A) clusters of the sperm population in the profound furrow and (B) attachment of an intact spermatozoon (notice the morphology of the sperm head) to the microvilli and cilia on the lining epithelium.

Specific Attributes of Hyaluronan and Its Association in the Female Genital Organ

A mucus-like substance appears in the intraluminal secretion of mammal oviducts, particularly in the inferior segment, composed of a diversity of glycoproteins [26, 34] and GAGs [35]. There are two major classes of GAGs; one consists of sulfated GAGs, i.e. keratin sulfate, heparin, heparin sulfate, chondroitin sulfate and dermatan sulfate, and the other consists of non-sulfated GAG, i.e., HA [36]. Considering the fact that HA is present in porcine spermatozoa and oocytes, HA was selected as more important to describe than the other sulfated GAGs in this review. The properties of HA were described almost 80 years ago [37]; it is a constituent of the extracellular matrix, has a high molecular weight and is composed of disaccharide duplicates of *N*-acetylglucosamine and *D*-glucuronic acid. HA is an enormous polymer with approximately 25,000 disaccharide units and a molecular weight of about 4×10^6 kDa [38]. Generally, HA arises from salt formation and is present in high concentrations within continuous networks of various loose connective tissues [39].

Apart from the umbilical cord, vitreous humor and synovial fluid, HA is the main component of the cumulus-oocyte complex (COC) viscous cloud during cumulus expansion [40, 41]. Fundamentally, HA can be naturally synthesized by integral membrane enzymes with hyaluronan synthases recognized as HAS1, HAS2, and HAS3 [42, 43]. Interestingly, HA plays an assortment of vital roles in the extracellular matrix by adhering to cell surfaces and other elements by way of explicit and non-explicit interactions, i.e., HA receptors. Up till now, CD44 has been known as a multifunctional cell surface glycoprotein, and it is distinguished as the major transmembrane hyaluronan receptor appearing in most cell types [44, 45], including the cumulus cells of porcine COCs [46]. Moreover, several studies have indicated the appearance of HA in the female genital tract of various species, for example, rats [47], humans [48], horses [49] and cows [35]. These essential observations signify the involvement of HA in the regulations of spermatozoa, oocytes and early embryos during their journey in the female reproductive organ.

Mechanisms of Hyaluronan and Its Functions in the Porcine Sperm Reservoir

The luminal secretory fluid in the oviduct is a compound assortment originating from two major sources, i.e., selective transudation from blood plasma [50] and specific secretion from epithelial cells [51]. The porcine epithelial lining naturally consists of ciliated cells and secretory cells. The latter cells are present in greater number in the isthmic segment which is comparable to the ampulla or the infundibulum [27], and these cells undertake a series of proliferative and degenerative changes according to hormonal levels during the estrous cycle [52]. It is well known that the secretory intraluminal fluid affects sperm characteristics in different manners, for example, viability, motility and capacitation [53–55]. In this review, the collective oviductal fluid of pigs from proestrus to metestrus (Fig. 2) was measured for HA levels and found to a higher HA concentration at standing estrus (Fig. 3). HA was localized by histochemistry to the epithelium of the caudal isthmic portion and the UTJ, mostly in the pre-ovulatory period [28], suggesting that the mucus-like constituent found in various species would be involved in the presence of HA and implying the essential actions of HA in the SR. Furthermore, this research also proved that HA can be produced by porcine epithelial cells through an integral membrane protein, HAS3 [56]. As mentioned previously, the properties of HA are multipurpose, and they include such things as it being extremely hydrophilic, forming gel at low level of concentration and creating viscosity and elasticity [57]. There have been some reports that have implied that HA occupies the protective physiochemical actions that could afford additional protective influences on chondrocytes in articular cartilage [58]. Under these circumstances, HA might capture spermatozoa during their storage and inhibit the uterine and ampullary liquids flowing into the SR [59] during the pre-ovulation period, separating the sperm subpopulation from unsuitable surroundings [17]. It is believed that spermatozoa submerged in the HA-secretory mucus of the SR could antigenically disguise themselves from recognition by phagocytes of the female immune system [9]. Besides its structures and hygroscopic properties, HA is capable of interrelating with specific receptors, causing the stimulation of signaling cascades that affect

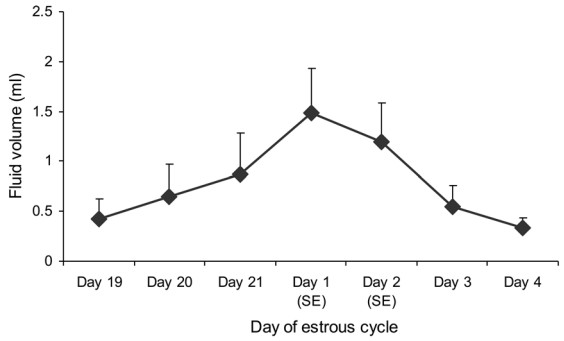

Fig. 2. Daily volumes (mean ± SEM) of sow intraluminal fluid collected from the isthmus via indwelling specific catheters during proestrus, estrus and metestrus (n = 5). SE = standing estrus.

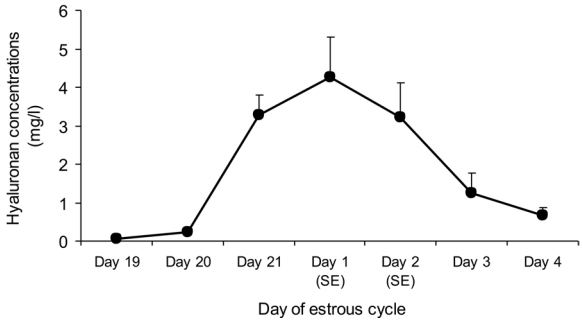

Fig. 3. Hyaluronan concentrations (mean ± SEM) in the intraluminal fluid of the non-inseminated sow isthmus analyzed using a Pharmacia hyaluronan test during proestrus, estrus and metestrus (n = 5). SE = standing estrus.

several cellular mechanisms [60]. The principal HA receptor, CD44 (expression of both protein and mRNA), is noticeably localized both on the membranous surface and in the supranuclear domain of the epithelial cells, especially in the porcine SR in the pre-ovulation period [61]. This could imply that the function of HA binding to CD44 on the membranous surface is accountable for cell-cell and, of course, cell-extracellular milieu [62] and restraint apoptosis [63]. However, the CD44 expression in the supranuclear region of most epithelial cells would indicate the participation of CD44 in indigenous HA uptake from and degradation of porcine oviduct fluid [35]. In porcine oocytes, the HA-CD44 interplay could influence fertility [64] and maturation during cumulus expansion [40]. Consequently, the appearance of CD44 in the efficient porcine SR suggests that the HA-CD44 signaling pathway might occur to reserve this location with the sticky cloud which the spermatozoa are bathed in to lengthen sperm viability, defend spermatozoa from the local female immune system and avoid the early stage of sperm capacitation [5] during the pre-ovulatory phase. In addition, the specific HA-CD44 pathway in the porcine SR could play an important role in oocyte maturation and other collaborations before fertilization [65] as well.

Relationships between Boar Spermatozoa and Hyaluronan in the Sperm Reservoir

Among several phenomena in the female reproductive tract after natural or artificial mating, sperm capacitation is one of the most essential incidents that facilitate successful fertilization. It is well known that sperm capacitation is a gradual step-wise procedure taking place when spermatozoa move along the female genital tube, and this specific mechanism involves sequential reorganization and adjustment of the sperm plasma membrane by exclusion of cholesterol and seminal plasma proteins [66]. These processes are complemented by the relocation of lipid diffusion in the plasma membrane and accomplished with the destabilization of its construction [67]. The molecular changes during capacitation appear without noticeable morphological alterations, but they can be visualized indirectly by examination with the chlortetracycline (CTC) assay [68]. It has been found that the CTC assay can detect the transposition of Ca^{2+} influx in the plasma membrane, which occurs during the latter step of capacitation and then takes part in the acrosome reaction [69]. However, the preliminary steps of sperm capacitation can be assessed by loading spermatozoa with the membrane fluorescent lipophilic dye Merocyanine 540, since an advanced level of lipid disorganization in the sperm plasma membrane is indicative of the initiation of capacitation [20]. Some studies have suggested that sperm capacitation could be induced and arise in the SR [70, 71]. In *in vitro* experiments, supplementation of epithelial cells from the oviduct prolongs sperm viability, and if the spermatozoa are persuaded to capacitate, they will be released from oviductal epithelial binding [72] by their concurrently improved hyperactivated movement [73, 74]. The regulation involved in sperm release from the porcine SR is believed to be due to the endocrine hint [75]. The data from the *in vivo* study specified that a principal subpopulation (73%) of boar spermatozoa collected from the SR in the pre-ovulation period was alive and displayed a non-capacitated pattern [76]. These observations correspond with previous research indicating that 63–70% of boar spermatozoa washed out from the SR exhibited an intact plasma membrane [77]. Furthermore, a group of spermatozoa stored in UTJ crypts revealed a completed plasma membrane under both scanning and transmission electron microscopy [15]. Thus, it can be concluded that the porcine SR at spontaneous standing estrus does not motivate sperm capacitation, whereas this particular location lengthens the viability and fertilizing capacity of the residing spermatozoa until the post-ovulation period [5, 75]. In addition, the HA supplementation (500 µl/ml) in the conditioned medium of IVF seemed to provoke capacitation-like changes in a CTC assay for porcine frozen-thawed spermatozoa not showing an acrosome reaction [13]. Later research added HA at a dose similar to that of spermatozoa flushed from the porcine SR, incubated the spermatozoa in modified Brackett-Oliphant (mBO) medium with bicarbonate ($NaHCO_3$) and performed Merocyanine 540 fluorescence staining, showing high percentages of non-capacitated spermatozoa [75]. Consequently, the HA present in the luminal fluid and on the oviductal epithelium [28] could serve as a defensive substance to preserve stored spermatozoa until occurrence of the modifications of the oviduct microenvironment due to the mechanisms of ovulation. It is important to note that porcine spermatozoa consecutively progress from the SR to AIJ for fertilization which is associated with the ovulation time [15, 76]. However, this advanced motility of boar spermatozoa is not instantaneous, even though it might happen under the influence of female hormones, promoting alterations of the oviduct epithelium and smooth muscle contraction [74]. As a result of the above, the gradual movement of small clusters and subsequent massive migration of spermatozoa from the SR was revealed. Earlier research suggested that the luminal diameter of the porcine SR enlarges in correlation with the amount of mucus-like substances reduced and liquefied [27]. Likewise, the relative concentration of HA both in intraluminal fluid and in the staining revealed by histochemistry tend to decline during the postovulatory stage compared with the preovulatory stage [28]. These changes in luminal size and reduction in the amount of mucus and HA might enable the progression of boar spermatozoa from the SR to their target. Nevertheless, this study also found that a bulky sperm subpopulation still existed in the SR in the post-ovulation period and that most spermatozoa showed a non-capacitated pattern [75]. Thus, this review was able to clarify the characteristics of spermatozoa released from the SR, which are released in limited numbers and how this phenomenon is required to prevent polyspermy. Besides the modifications of intraluminal substances from mucus-like substances to liquid, the boarder lumen and the smooth muscle contraction, restricted amounts of spermatozoa would abandon the SR because the mechanism of capacitation at the initiation stage permits their release from their binding to the oviductal epithelium [71].

Conclusion

HA, the non-sulfated GAG, is present in the intraluminal mucus-like component and on the epithelial surface; it is noticeably present in the grooves of the porcine SR in the pre-ovulation period, and the HA and specific morphological conditions in this site are appropriate for preservation spermatozoa. Additionally, the major HA receptor, CD44, is also localized by the oviductal epithelium, implying that the HA-CD44 signaling pathway might play a vital role during sperm storage. Finally, the spermatozoa collected *in vivo* from the SR are viable and non-capacitated in the pre- and peri-ovulatory phases, sustaining the HA function in the SR related to arresting sperm capacitation during these periods and then to initiate their capacitation after ovulation.

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