Seminal plasma proteins as markers of sperm fertility

Xavier Druart, Jessica P. Rickard, Guillaume Tsikis, Simon P. de Graaf

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Short title: Markers of sperm fertility

Authors: Xavier Druart[1]*, Jessica P. Rickard[2], Guillaume Tsikis[1] and Simon P. de Graaf[2]

Addresses: [1] Physiologie de la Reproduction et du Comportement, INRA, CNRS, IFCE, Université de Tours, 37380 Nouzilly, France

[2] The University of Sydney, Faculty of Science, School of Life and Environmental Sciences, NSW 2006, Australia

Correspondence to: xavier.druart@inra.fr (X. Druart)

Abstract

During ejaculation and deposition in the female genital tract, spermatozoa are exposed to seminal plasma, a mix of secretions primarily from the accessory sex glands. Proteins, which make up the largest contribution to seminal plasma by weight, have been the focus of much interest, in particular the identification of specific proteins both in the plasma and/or found bound to the sperm surface post ejaculation. Global proteomic studies of seminal plasma originating from a range of species over the last 15 years have revealed their hitherto unknown diversity and complexity. Seminal plasma is generally known to aid sperm survival and fertility in a range of species and studies have begun to reveal its link with sperm function and identification, as markers of fertility. This review summarizes recent data on proteins found on the sperm surface that originate from seminal plasma and have subsequently been shown to correlate with fertility, with a focus on the pig.

Keywords: Seminal plasma, Proteome, Sperm, Preservation, Fertility.

1. Introduction

From the testis to the cauda epididymis, spermatozoa undergo modifications that lead to the acquisition of fertilizing ability. During ejaculation and deposition into the female genital tract, spermatozoa are exposed to seminal plasma, a mix of secretions primarily from the prostate, ampulla, seminal vesicles and bulbo-urethral glands [1]. Predominantly made up of proteins (by weight), seminal plasma also contains a range of inorganic ions, salts, sugars, citric acid, prostaglandins and electrolytes [2]. The multi-organ origin of seminal plasma gives some indication of its complexity, but there are notable exceptions like dogs and camelids, which are devoid of seminal vesicles and produce seminal plasma containing far fewer proteins [3]. On the other hand, the seminal plasma of pigs mainly consists of seminal vesicle secretions [4] and their unique bulbourethral glands produce a gelatinous plug which signifies the end of ejaculation [5]. In part, it is this unique anatomy and the differing contributions from each accessory gland that results in ejaculates with seminal plasma content unique to each individual species [6]. This difference in composition also likely contributes to a variance in the function of seminal plasma between species.

The exposure of spermatozoa to seminal plasma is known to cause major remodeling of the sperm membrane [7] and the binding of individual proteins to the membrane itself. In general, under natural conditions, seminal plasma has been reported to act as a beneficial medium to spermatozoa, providing energy for metabolism and motility, buffering against pH changes, regulation and control of capacitation [8], establishing sperm reservoirs [9], providing protection from the female's immune system [10] and aiding transport through the female tract [1], all of which are essential for fertilization. However, in the boar, seminal plasma has been shown also to be detrimental to the survival of spermatozoa during freezing and incubation post thaw [11]. Exposure of boar spermatozoa to proteins from the non sperm-rich fractions of the

ejaculate are usually avoided when boar ejaculates are processed in vitro [11, 12]. This variation in function is thought to be related to the individual proteins per species, which may be found either bound to the sperm membrane or free in the serum.

Determining the cause of this variation in function has led to a global interest in identifying the individual proteins within the seminal plasma of each species that may contribute to its reported beneficial or negative effects, and whether any of these proteins may be markers of fertility. The identification of these proteins of interest as fertility markers holds incredible potential, not only in helping to understand the true role or function of seminal plasma, but also from an applied perspective for industry. Seminal plasma collected from males could be screened to assess their reproductive potential and suitability for cryopreservation. These markers could also be isolated and purified to be used in extenders to help improve the survival of spermatozoa post thaw and in the tract post artificial insemination (AI). Males with high or low fertility could also be identified prior to use, lifting the general success of AI and the genetic benefits of this technology in the pig industry.

This review will summarise the recent data on seminal plasma markers of fertility that have been identified on the sperm surface of the ram, bull and stallion and compare these to markers which have been identified in the boar. The proteome of the boar ejaculate will be closely examined, and the predicted role of these proteins will be explored to assess any similarities or differences between species.

2. The boar ejaculate

The boar ejaculate is of large volume (up to 500mL) with a relatively low sperm concentration of approximately 500 million/mL. It is also ejaculated in sequential fractions: first a sperm-rich fraction, followed by a second fraction made up predominately of seminal plasma but a

lower sperm concentration (post sperm-rich fraction) and finally the production of a gelatinous plug, signifying the end of the ejaculate [5]. The initial 10 mL of the sperm-rich fraction contains little seminal plasma, a higher sperm concentration and is considered the sperm-peak fraction of the ejaculate [13]. In routine procedures of AI, the gel fraction is discarded from the ejaculate by filtration through gauze and either the sperm-rich fraction or, increasingly, the whole ejaculate (sperm-rich plus post sperm-rich fraction) is processed for use.

The post sperm-rich fraction is predominately made up of secretions from the seminal vesicles and bulbourethral glands which, under natural conditions, progressively dilute the prostate-dominated fluid of the sperm rich fraction and is characterized by a high prevalence of spermadhesins and fibronectin type proteins. The spermadhesins are a family of low molecular weight proteins (15 kDa) that include 5 members, AQN-1, AQN-3, AWN, PSP-I and PSP-II, which account for at least 45 % of the total amount of pig seminal proteins [14]. In order to identify pig seminal proteins outside the spermadhesin family, different chromatographic or electrophoretic strategies of protein/peptide separation were performed to identify 39 [14] and 82 proteins [6] within boar seminal plasma. More recently, the combination of complementary chromatography with high resolution mass spectrometry has allowed the identification of a total of 1723 proteins, 1602 of these quantified [15]. Out of these proteins, 58 or 5% were found to be linked to reproductive processes, with 39 of them belonging to sperm function, showing the diversity of the function of seminal plasma proteins within the boar ejaculate.

Given the fractionation and therefore complexity of the boar ejaculate, studies have applied these methods in an effort to quantify the proteomic changes that occur to the sperm membrane both following ejaculation (i.e. comparing epididymal and ejaculated spermatozoa) as well as comparing the proteins expressed between the individual fractions of the ejaculate. The interaction of seminal plasma with epididymal spermatozoa during ejaculation leads to the binding of seminal proteins to the sperm surface. Two recent studies provided an in-depth

proteomic analysis of ejaculated boar spermatozoa with more than 1700 proteins identified [16, 17]. In order to identify those of seminal origin, the proteomes from epididymal and ejaculated spermatozoa were compared and a differential expression of 32 proteins among 1602 quantified proteins was revealed [18]. Most of these differential proteins originate from the seminal plasma [19] confirming that the modification of the sperm proteome during ejaculation mainly originates from the binding of seminal proteins to the sperm surface.

Perez-Patino et al. also performed an extended proteome analysis of the three seminal fractions (sperm-peak, sperm- rich and post sperm-rich) using iTRAQ based 2DLC-MS/MS to assess how the proteome of the boar ejaculate changed between the fractions [17, 19]. It revealed differences in composition, mainly between the sperm-rich and the post sperm-rich fractions. Notably, 25 proteins were overexpressed in spermatozoa of the post sperm rich fraction, some of these included the 5 spermadhesins (AQN1, AQN3, PSP-I, PSP-II and AWN), a binder of sperm protein (BSP), pB1 and a cell adhesion glycoprotein, fibronectin 1 (FN1)[17].

The proteome of fresh and frozen-thawed boar spermatozoa has also been compared. The freezing ability of spermatozoa from the sperm-rich fraction was superior to those from the whole ejaculate [20] suggesting that spermatozoa from the post sperm-rich fraction have a reduced cryosurvival compared to those from the sperm-rich fraction. Overall, the process of cooling and freezing caused an increase in the abundance of 35 proteins (some of these including ACE, TP1, SOD1 and ODF2) and a decrease in the abundance of 6 proteins [21]. Of the proteins that decreased, two were present in high amounts on ejaculated spermatozoa and belong to the Spermadhesins protein family (AWN, PSP-I) [21]. Given the higher binding of seminal proteins to spermatozoa collected from post sperm-rich fractions, it could be hypothesized that an elevated binding or extended exposure of seminal plasma to this sperm type could reduce the cryo-survival of pig spermatozoa [18].

158 The identification of these proteins on the surface membrane of different sperm types,
159 such as epididymal or ejaculated, individual fractions of the boar ejaculate or spermatozoa
160 which is fresh or frozen-thawed, offers clues to the potential role or function of these proteins
161 and highlights them as potential markers of fertility.
162
163 **3. Proteomic markers of boar sperm fertility**
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165 Following the identification of these proteins on the sperm surface, extensive literature has
166 focused on investigating their influence on sperm function and fertility. The proteins on the
167 surface of ejaculated spermatozoa could play a role in aiding survival through the female tract
168 or managing steps related to final maturation processes, such as the capacitation and/or
169 acrosome reaction.
170 Indeed, many of the proteins identified by Perez-Patino et al. have been referred to as
171 low weight glycoproteins and reported to be involved in sperm motility, capacitation, the
172 acrosome reaction, zona pellucida binding processes and modulating the female uterine
173 environment for later embryo development [15, 18, 22-27]. One of the key processes for sperm
174 survival in the tract is the delay of capacitation until the opportune time when they are
175 approaching the oocyte. Spermadhesins, AWN [28] and PSP-I [29] have been both thought to
176 contribute to the inhibition of premature capacitation within the uterus of the sow. Interestingly,
177 PSP-I has been also associated with a negative relationship to fertility. Novak et al. [25] found
178 a negative relationship between the concentration of PSP-I in the sperm-rich fraction and
179 farrowing rate. These authors also noted that the concentration of PSP-I was higher in the post
180 sperm rich compared to the sperm rich fraction, which is consistent with previous studies [19,
181 26]. Finally, PSP-I and PSP-II are reported to be mediators of the immune response within the
182 uterus and were reported to recruit a significantly higher number of immune cells to the lumen

183 within 10 min of exposure [30]. This would result in a faster rate of potential phagocytosis and
184 removal of cells from the tract. Together these findings suggest that the Spermadhesins are key
185 to the function and fertility of boar spermatozoa, in particular the concentration of PSP-I.

186 Given lower amounts of the protein were found in the sperm-rich fraction, perhaps this
187 is a natural way of ensuring spermatozoa are not exposed to higher levels and are ejaculated
188 first as part of the sperm-rich fraction. Considering the issue with low-dose AI volumes, perhaps
189 the concentration of PSP-I should be monitored in case these spermatozoa (if mixed with the
190 entire ejaculated before freezing) are more chemo-attractive to cells of the female immune
191 system. Other proteins linked to a negative relationship with boar fertility are AQN-3 and
192 SPMI, a seminal plasma sperm motility inhibitor which exhibits high homology to AQN-3 [27].
193 Here, authors compared the proteomes of intact and capacitated spermatozoa hypothesizing that
194 the capacitation reaction, an initial and essential factor necessary for successful fertilization,
195 would better reveal potential fertility markers. Spermatozoa were collected from boars that
196 sired high (average litter size 12.8) and low (average litter size 10.19) litter sizes, and the
197 proteomic changes between the two groups were assessed following in vitro capacitation.
198 AQN-3 and SPMI were more abundant in capacitated spermatozoa of low-litter-size boars and
199 were therefore negatively linked to fertility. However, glutathione peroxidase (GPX5) is
200 consistently considered a positive marker of boar sperm fertility, being identified as an
201 antioxidant enzyme which may protect sperm from oxidative damage. This protein was also
202 positively associated with farrowing rate and fertility index or pregnancy [25].

203 Perhaps the most important proteins of interest are the ones that are inadvertently
204 altered, modified or even removed during in vitro processing, in particular during the
205 cryopreservation of spermatozoa. Protocols related to the collection, extension, freezing and
206 thawing of spermatozoa in a wide range of species are constantly assessed in order to improve
207 success rates similar to that of fresh or natural mating. If markers could be identified which

208 could predict the success of freezing or improve the fertility associated with frozen-thawed boar
209 semen, then these markers could hold incredible promise for the pig industry.

210 The amount of lactadherin in pig seminal plasma was inversely correlated with sperm
211 tail defects and positively correlated with sperm motility [14]. However, when the protein
212 profiles of sperm membrane from boars with different post-thaw semen motility were
213 compared, lactadherin was found to be negatively associated with sperm resistance to
214 cryopreservation [31]. The authors of this study suggested that this contradiction between
215 improvement of motility of fresh compared with a decrease for frozen-thawed spermatozoa may
216 be explained by the fact that the long term exposure of spermatozoa to this protein during semen
217 manipulation prior to cryopreservation might exert a deleterious effect on their survival during
218 cryopreservation procedures [31], similar to what was seen in the expression of PSP-I above.
219 This suggests that if the concentration of lactadherin was monitored prior to freezing, survival
220 rates post-thaw might be improved. Another protein of interest, identified as differing between
221 fresh and frozen-thawed boar spermatozoa is Angiotensin-converting enzyme (ACE). ACE
222 increased in abundance on the surface of frozen-thawed boar spermatozoa [21] and has been
223 linked to premature capacitation, with its concentration inversely proportional to their
224 fertilization ability [32]. The management and removal of reactive oxygen species (ROS)
225 during freezing is paramount for the survival of spermatozoa post thaw. Therefore, the presence
226 of antioxidants like superoxide dismutase (SOD1) and glutationise perioxidase (GPX5), which
227 were identified as highly abundant on the surface of frozen-thawed boar spermatozoa [21], are
228 important for the metabolism of toxic compounds reducing the threat of lipid peroxidation. As
229 such, if the concentration of some of these proteins or markers of fertility could be determined
230 prior to freezing, this could determine whether negative markers like ACE or lactadherin should
231 be reduced or whether the sample requires supplementary levels of antioxidants to help protect
232 spermatozoa from the attack of free-radicals.

4. Contrast with other mammalian species

Comparing identified fertility markers between species can help the understanding of protein function and their potential role in aiding or inhibiting sperm fertility.

Analysis of ram seminal plasma by GeLC-MS/MS has allowed the identification of more than 700 proteins, showing a high abundance of Binder of Sperm Proteins (BSP1, BSP5), members of the spermadhesin family (SPADH1, SPADH2,bodhesin2) and newly identified proteins like liver enriched gene 1 (LEG1/C6orf58) with unknown reproductive function [33]. The comparison of the proteomes of epididymal and ejaculated ram spermatozoa revealed moderate changes induced by seminal plasma interaction, such as binding of BSPs, LEG1 and EDIL3 (epidermal growth factor-like repeats and discoidin I-like domains 3) [34]. Investigation of proteomic markers of sperm freezing resilience in ram seminal plasma showed that several negative seminal markers could be found, such as zinc alpha glycoprotein (ZAG) [35]. A previous study aiming to identify markers of liquid preservation in ram semen also identified the ZAG as a negative marker [33]. In humans, ZAG is secreted by the prostate [36], binds to the surface of spermatozoa at ejaculation and stimulates sperm motility through the cyclic AMP pathway [37]. The amount of ZAG in ram seminal plasma was also found to be positively associated with ram sperm motility [38]. This activation of sperm motility and the identification as a negative marker of preservation could appear contradictory. But when the effect of recombinant ZAG was tested on ram spermatozoa, a stimulatory effect prior to liquid storage and a detrimental effect post storage were found [33]. This biphasic effect is in line with the hypothesis that the interaction of seminal plasma with spermatozoa could be beneficial on the short-term period of normal reproductive physiology, but detrimental in the long-term condition of preservation. This appears also to be the case in the pig, with exposure to boar seminal

plasma, in particular with the spermadhesin, PSP-I [25] . Clearly the effect of seminal plasma proteins on ram sperm function and (theoretically) fertility is a careful balance between concentration and exposure. This delicate balance may also help explain the variability in seminal plasma seen not only between species but also individual males. It would be interesting to determine whether there are environmental factors that could alter the expression of proteins within a male ejaculate, therefore influencing sperm quality and freezing ability. It is worth noting that, to date, no studies correlating seminal plasma or sperm proteins with fertility have been undertaken in the ram; all studies mentioned above are limited to correlations with functional traits expressed in vitro.

In the bull, many studies have identified proteic markers of bull fertility by the quantification of sperm proteins [39-51] and seminal plasma proteins [39, 52-56]. BSPs are more often found to show a positive or negative relation with fertility in bovine either in seminal plasma or on the sperm surface. The discrepancy of positive or negative correlation of BSPs with fertility could be linked to the net amount of BSPs on the sperm surface. It could be hypothesized that low to moderate levels of BSPs are positively linked to fertility, whereas high levels of BSPs induce negative correlations. The negative association of BSP amounts in seminal plasma with frozen semen fertility is in accordance with experimental studies. These again suggest that prolonged exposure of sperm to seminal plasma BSPs during preservation/freezing is detrimental, as seen in studies in bull and ram sperm [57]. High amounts of BSPs in the seminal plasma, and especially BSP1, could induce damage to the spermatozoa during the preservation and freezing process [7, 58, 59]. SPADH1 (Acidic seminal fluid protein, spermadhesin-1) is a major 13 kDa protein isolated from bull seminal plasma and is characterized as a growth factor [60], mainly secreted by seminal vesicles [61], and to a much lesser extent by the epididymis [62, 63]. SPADH1 belongs to the spermadhesin family but binds loosely to the bull sperm surface and is lost after capacitation [64]. Bull SPADH1 may play a

283 role in the regulation of sperm metabolism and the protection of sperm membranes from
284 oxidative damage [65]. Indeed the amount of SPADH1 in bull seminal plasma was positively
285 associated with the freezing ability of spermatozoa [66] and the fertility of frozen semen [44].
286 Another parented protein, SPADH2 (spermadhesin Z13) was also positively associated with
287 bull fertility [42].

288 In the stallion, identified seminal plasma proteins have been classified into three groups:
289 Fn-2 type proteins (HSP1), cysteine-rich secretory proteins (HSP3, CRISP) and spermadhesins
290 (HSP7) [67-71]. Sequence comparison with Binder of Sperm Proteins confirmed HSP1 and
291 HSP2 as horse members of the BSPs family and are now identified as BSP1 and BSP2 [72]. A
292 2D-LC MS/MS analysis of equine seminal plasma allowed the identification of 59 proteins,
293 with Kallikrein E1 reported as one the major equine quantitative seminal proteins along with
294 BSPs and CRISP3. The stallion sperm proteome was investigated by recent mass spectrometry
295 methods and 975 proteins were identified [73]. Several seminal plasma proteins such as
296 kallikrein, clusterin and BSP1 were found to be negatively associated with fertility [74, 75]

297 Despite the unique seminal plasma proteomes which are known between species, there
298 are clear similarities in terms of the families of proteins which regularly appear in all species.
299 Binder of sperm proteins and spermadhesins appear to be homologous to all species and play
300 pivotal roles in the maturation, metabolism and survival of spermatozoa during freezing. Table
301 1 summarises some of the above-mentioned proteins which have been previously linked to
302 sperm function, preservation and/or fertility in the boar, ram, bull and stallion.

303

304 **5. Conclusion and future directions**

305

306 Global proteomic studies of seminal plasma in a range of species over the last 15 years have
307 revealed their hitherto unknown diversity and complexity. A huge number of proteomic datasets

308 are now available in the field of applied animal andrology, allowing scientists to explore the
309 contribution accessory sex glands make to sperm function, fertility and transit through the
310 female. The boar ejaculate is unique in the way it creates several subpopulations of
311 spermatozoa, with seemingly different function and tolerance to in vitro manipulation. These
312 phenotypically different sperm types within the ejaculate make the boar an excellent species to
313 not only identify sperm markers of fertility, but also compare the function of these markers
314 across other species, which have a more homologous ejaculate like the bull or ram. It would
315 be interesting to see how the same proteins behave in different species during supplementary
316 studies, whether they have similar effects or are species specific. Similarly, do these proteins
317 work independently or do they work in concert with others to ensure sperm survive the tract
318 and are capable of fertilization?

319 This review has summarized the potential markers for the in vitro estimation of domestic
320 mammalian fertility, as well as those that could be used as additives to ameliorate damage
321 caused by low temperature storage. However, before these protein markers can be applied in
322 industry, it would be beneficial to conduct further studies to confirm their influence on sperm
323 function. Proteins of interest would need to be isolated, separated and purified in an efficient
324 way in order to perform multiple supplementary studies in a range of sperm types. Technical
325 difficulties remain in how to purify proteins of interest for further biological testing, let alone a
326 means to create the quantities required for trials to demonstrate in vivo fertility following AI
327 with supplemented semen. Nevertheless, with improved efficiencies in recombinant protein
328 technologies, particularly through the addition of post translational modification, components
329 of seminal plasma beneficial for sperm storage and fertility may one day comprise additives to
330 semen diluents in many species. Separately, those proteins consistently demonstrated to
331 strongly correlate with field fertility will likely slowly make their way into the routine breeding
332 soundness assessments undertaken in the animal industries.

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573 **Table 1:** Protein markers of sperm function, preservation and/or fertility identified within the

574 seminal plasma of the boar, ram, bull and stallion.

Species	Protein name	Role	References
Boar	PSP-I, II	Capacitation, farrowing rate, immune	[25, 29, 30]
	AWN	Capacitation	[25, 28]
	AQN-1, 3	Motility, litter size	[25, 27]
	GPX-5	Preservation, farrowing rate	[25]
	Lactadherin	Motility, preservation	[31]
	ACE	Preservation, capacitation	[21]
	FURIN	Farrowing rate	[15]
	SPAM1	Farrowing rate	[15]
	Nexin-1	Litter size	[15]
	CAT	Litter size	[15]
Ram	BSP1, 5	Preservation, capacitation	[76, 77]
	BDH2	Motility	[38]
	AZGP1	Motility preservation	[33]
	ARSA	Motility	[38]
	SPADH1, 2	Preservation	[33]
	VCP	Preservation	[35]
	ENO1	Preservation	[35]
Bull	BSP1, 3, 5	Capacitation, sperm reservoir, preservation	[78]
	aSFP	Preservation, sperm binding	[66]
	OPN	Capacitation, fertility associated	[56]
	CLU	Maturation	[66, 79]
	L-PGDS	Preservation	[54, 66, 78]
	Spermadhesin Z13	Motility, fertility associated	[56, 66]
Stallion	BSP1, 2	Preservation	[67, 75]
	CRISP 3	Preservation, fertility associated	[74, 75]
	KLK3	Preservation, fertility associated	[74, 75]
	CLU	Fertility associated	[74, 75]

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